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ORIGINAL ARTICLE

Impact of caspase 3 expression in meningiomas based on tissue microarray analysis

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Summary

Purpose: *Concerning primary central nervous syste[illegible] plasms, meningiomas demonstrate the most commo[illegible] in adults worldwide. Deregulation of apoptotic pathwa[illegible] tumours - including meningiomas - is correlated to ch[illegible] oresistance and poor prognosis. Caspas[illegible] proteins acting as strong apoptotic d[illegible] purpose was to correlate caspase 3 ex[illegible] pathological features.*

Methods: *Fifty meningiom[illegible] comprising a broad spect[illegible] (meningotheliomatous, psammom[illegible] angiomatous, micr[illegible] immunohistochemi[illegible] cores by using anti-ca[illegible] analysis was al[illegible]*

Results: *[illegible]pression of caspase 3 protein was observed [illegible]7/50 (34%) cases, whereas the rest of them (33/50-/66%) a[illegible]ted moderate to low levels of the molecule. Caspase 3 [illegible]erall expression was significantly correlated to grade of the examined tumors and to mitotic index (p=0.002, p=0.001, respectively). Interestingly, caspase 3 expression status was associated also with the histotype of the examined meningiomas (p=0.016).*

Conclusions: *Caspase 3 aberrant expression is observed in meningiomas associated with their differentiation grade, mitotic activity and partially with specific histotypes. Agents that could enhance caspase 3 expression - also modifying its apoptotic activity - represent a very promising aspect in oncology treatment regimens.*

Key words: *meningioma, apoptosis, caspase, immunohistochemistry*

Introduction

Meningiomas represent the second most common brain tumours and the most common intracranial primary central nervous system (CNS) tumours in adults. Recurrence of these tumours - especially in higher grade meningiomas - is correlated with an aggressive biological behaviour affecting the response rates to surgery/radiation applied therapeutic regimens [1]. Meningiomas' histologic substrate is the arachnoid cap cells of the meninges on the periphery of the brain. Histopathologically, meningiomas comprise a broad spectrum of histopathologic subtypes (meningotheliomatous, psammomatus, transitional, fibrous, angiomatous, atypical and anaplastic) [2]. Brain tissue invasion is the

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most critical histopathologic evidence of aggressive biological behavior of the tumor. Furthermore, meningiomas' extra-cranial metastatic potential is low, and its metastatic activity and penetration is extremely rare. Significant series of meningiomas and detected gross chromosomal and specific gene aberrations (rearrangements/intra- or inter- translocations, gains, frame-shift deletions/insertions, point-driver mutations, or in-frame fusions) also show the significance of the grades of differentiation (Grade I-III) [3-6]. Numerical imbalances affect also other chromosomes besides chromosome 22. Fragment deletions have been detected on chromosome 1p and 2q33-q35. Regional amplifications occur on chromosome 6p21-p22 and on chromosomes 13q33, 17 and 19. In conjunction to chromosomal and gene instability described above, meningiomas are characterized by a broad spectrum of somatic single nucleotide variants, demonstrating specific single nucleotide polymorphism [7]. Interestingly, there is limited evidence of viral implication in the development of meningiomas, including human cytomegalovirus (HCMV), Epstein-Barr virus (EBV), HSV 6/7, Human Papilloma Virus (HPV) and Hepatitis B Virus (HBV) [8].

In cancer tissues, programmed cell dea... inhibited due to a deregulation in the expres...on of apo- and anti-apoptotic proteins. This gen... imbalance drives the cancer cell to i...mortaliz... tion which reflects the aberrant tiss... pr...eration. For this reason, caspases and th... other ...optotic mitochondria or not - depende... m...

considered as important targets for specific targeted therapeutic strategies enhancing the apoptotic levels of tumor cells [9, 10]. In the current study, we explored the role of caspase 3 expression in meningiomas and its potential impact on meningioma pathologic features.

Methods

Study group

For the purposes of our study, 50 ...rival, ...malin-fixed and paraffin-embedded meni...oma tissu... ...ecimens were selected. According to pat...gy classifi...ion histotypes, 12 meningotheli...tous, ...amm...atus, 6 transitional, 5 fibrous, 2 ...giomatous, ...cystic, 5 atypical, 5 anaplastic, 1 par...ry were ...cognized. Concerning the corresp... ...atients, 39 (78%) were female, and the r... (11;22 ...ale. T... ethics committee consented t... use of th... ...es in the 1st Dept of Pathology ... School U...ersity of Athens for research purposes, a...ding to World Medical Association De...ration of He...ki. The tissue samples were fixed ...% neutral-buffe...d formalin. Hematoxylin and eos... (H&E)-stained slides of the cor¬responding samples were revie...d for confirmation of histopathologic diag...es. All l...ons were classified according to the histo... and grading criteria of World Health Organization (WHO) including also conventional mitotic ...s (mitoses per high power fields-HPF) [11,12].

Tissue microarrays construction (TMA)

Areas of interest were identified on H&E-stained slides by a conventional microscope (Olympus BX- 50). The corresponding paraffin blocks were obtained for the

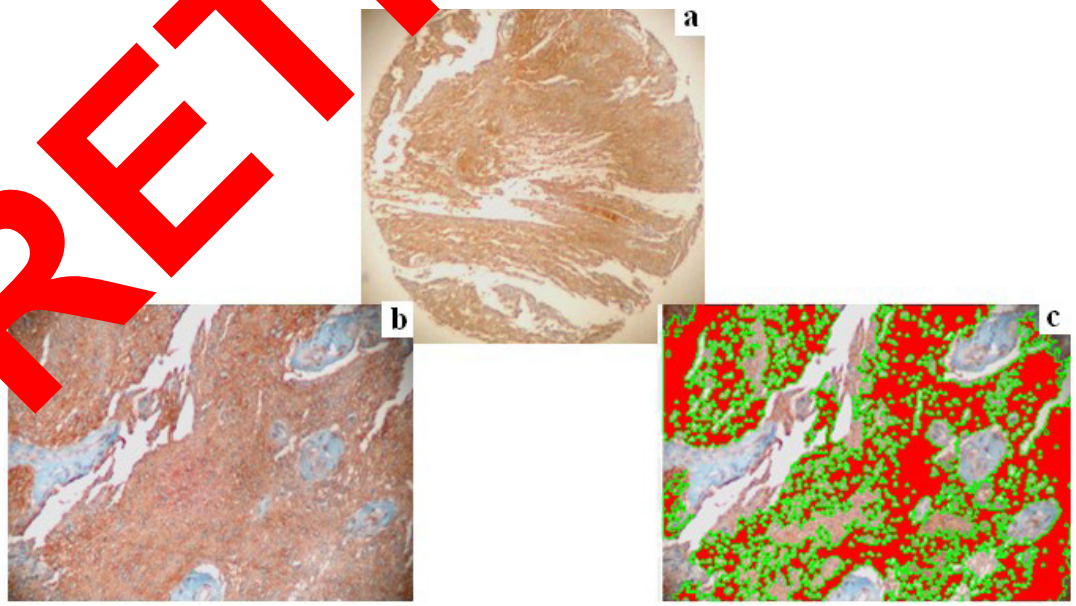

Figure 1. Caspase 3 high expression in a case of meningioma (transitional histo-type). **A:** A Tissue core immunostained by caspase 3 (original magnification 40x). **B:** Caspase 3 diffuse cytoplasmic and sub-membranous staining pattern (original magnification 100x). **C:** Digitized evaluation of caspase 3. Red/green areas represent different levels of protein expression as staining intensity values.

construction of one TMA block. Using TMArrayer- 100 (Chemicon International, USA). 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-caspase 3 antibody (monoclonal, clone 3CSP03-Neomarkers/LabVision, Fremont, CA, USA) at dilution of 1: 50 was applied in the corresponding cases. IHC for the antigen was carried out on a 4μm tissue microarray section. The TMA slide was initially deparaffinized in xylene and rehydrated via graded ethanol. Then the slide was immunostained for the marker according to the EN Vision+ (Dako, Denmark) assay using an automated staining system (I 6000 - Biogenex, USA) and according to the manufacturer's instructions. This specific assay is based on a soluble, dextran-polymer system preventing endogenous biotin reaction and increasing the quality of stained slides. Briefly, after peroxidase blocking, the sections were incubated with the primary antibody for 35 min at room temperature and then incubated with Horseradish peroxidase labelled polymer-HRP LP for 30 min. The antigen-antibody reaction was visualized using 3-3, diaminobenzidine tetrahydrochloride (DAB) as a chromogen substrate. Finally, the TMA section was slightly counterstained with hematoxylin for 30 sec, dehydrated and mounted. For negative control, the primary antibody was omitted in other slides. Cytoplasmic predominantly and sub-membranous staining was considered acceptable for caspase 3 (Figure 1b).

Digital image analysis assay (DIA)

Caspase 3 protein expression le[illegible] were [illegible]uated quantitatively by calculating the c[illegible]sponding [illegible]ning intensity levels (densitometry evalu[illegible]n) in the s[illegible]ned cells (malignant). We perfor[illegible] DIA u[illegible] a sen[illegible]uto-mated system (hardware: [illegible]croscope CX[illegible]ympus, Melville, NY, USA, Digi[illegible]camer[illegible]ony, Tok[illegible], Jp; Windows XP/NIS-Elements [illegible] AR v3[illegible] Nikon Corp, Tokyo, Japan). Ar[illegible] of inte[illegible] per ti[illegible]e section were identified (5 o[illegible]l fields at [illegible]agnification) and filed in a di[illegible] base as sna[illegible]ots. Measurements were performed by [illegible]lementing a specific macro (cytoplas[illegible]membrano[illegible]nd sub-membranous expression [illegible] tumor cells, acc[illegible]ding to the manufacturer's da[illegible]heet. Based on an algorithm, normal tissue sections (co[illegible]ol) were m[illegible]sured independently and compared to the [illegible]respondi[illegible] values in malignant tissue sections.

Table 1. Clinicopathologic parameters and total Caspa[illegible]3 ex[illegible] results

Clinicopathologic parameters	*Caspase 3*			*p value*
	M[illegible]gioma[illegible] *[illegible]50)* *n*	*OE* *17/50 (34%)* *n (%)*	*MLE* *33/50 (66%)* *n (%)*	
Gender				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
[illegible]	36 (72)	7/50 (14)	29/50 (58)	
[illegible]	8 (16)	5/50 (10)	3/50 (6)	
[illegible]	6 (12)	5/50 (10)	1/50 (2)	
Histotyp[illegible]				0.016
atypica[illegible]	5/50 (10)	3/50 (6)	2/50 (4)	
anaplastic	5/50 (10)	5/50 (10)	0/50 (0)	
papillary	1/50 (0.5)	0/50 (0)	1/50 (2)	
meningotheliomatous	12/50 (24)	1/50 (2)	11/50 (22)	
psammomatous	12/50 (24)	4/50 (8)	8/50 (16)	
transitional	6/50 (12)	2/50 (4)	4/50 (8)	
fibrous	5/50 (10)	2/50 (4)	3/50 (6)	
angiomatous	2/50(4)	0/50 (0)	2/50 (4)	
microcystic	2/50(4)	0/50 06)	2/50 (4)	

OE: overexpression (high expression) staining intensity values ≤ 130 at stained cells
MLE: moderate-low expression staining intensity values > 130 at ≤ 160 at stained cells

A broad spectrum of continuous grey scale values (0-255) at the RedGreenBlue (RGB) analysis was available for discriminating different protein expression levels (Figure 1c). Immunostaining intensity values decreasing to 0 represented a progressive overexpression of the marker, whereas values increasing to 255 showed a progressive loss of its staining intensity. Total results and DIA values are shown in Table 1.

Statistics

For statistical analyses, descriptive and inferential techniques were applied. Quantitative variables were presented as mean ± standard deviation, while qualitative variables were presented in frequency tables. Due to the small number of subjects in each group, to evaluate the relationship between qualitative and quantitative variables, the nonparametric Mann-Whitney and Kruskall-Wallis tests were applied. To evaluate the relationship between independent qualitative variables, where appropriate, control x^2 for linear trend and control Fisher test were applied. Statistical significance was evaluated in pairs and differences < 0.05 were considered statistically significant. Total IHC results and differences (p values) are described in Table 1.

Results

According to digital expression analysis examined immunostained meningioma tissue microarray cores demonstrated different expression levels of caspase 3. Overexpression of caspase protein was observed in 17/50 (34% case) whereas the rest of them (33/50-/66%) demonstrated moderate to low levels of the molecule. Overall expression was significantly correlated to the grade of the examined tumors and to mitotic index (p=0.002, p=0.001 respectively). Interestingly, caspase 3 expression status was significantly associated also with the histotypes of the examined meningiomas (p=0.01). Especially, caspase 3 overall expression demonstrated significant variations comparing subgroup of histotypes including the first (psammomatic, microcystic, meningoepithelial, angiomatous, transitional psammomatus, and fibrous meningiomas) in the second the histotypes were (anaplastic and atypical (p=0.005). No statistical significance was shown correlating caspase 3 to gender of the examined patients (p=0.382).

Discussion

Apoptosis represents the genetically programmed cell death mediated by a complex of proteins which influence positively or negatively intrinsic and extrinsic pathways [13]. Two main pathways are involved in the previously described apoptotic procedure: intrinsic and extrinsic, respectively. In both of them several proteins are characterized as inducers or inhibitors of apoptosis. The first uses mitochondrial proteins with prominent the cytochrome c from the inter-membrane space of the organelle. Its activity in the cytoplasm activates caspases (especially caspase-9) complex under the control of p53 and Bcl-2 (B-cell lymphoma-2) proteins [14]. Caspases are significant proteins acting as strong apoptotic death promoters. Caspases (cysteine-aspartic proteases) represent a family of enzymes that influence several functions crucial for cell homeostasis such as inflammatie pyroptosis (a distinct aspect of programmed cell death mediated by microbial infection that triggers an immune response), necroptosis, tissue differentiation and development in the embryonic early stages of life [15]. They also act as tumor suppressor genes, whereas their role in the ageing process is under investigation. Approximately 15 protease proteins have been identified and cloned implicating eight chromosomes (1, 2, 4, 7, 10, 11, 16, and 19). The corresponding protein products are initially inactive (pro-caspases) enzymes. Their dimerization or oligomerization create the final functional heterotetramer domain due to a cleavage process which develops an active heterodimer complex consisting of two subunits small and a large one. According to their implication in the apoptotic pathways, caspases are characterized as initiators and executioners, respectively. In the first group inserted have been caspase-2,-8,-9, and -10, whereas caspase-3,-6, and-7 belong to the second category [16].

In the current study we analyzed by IHC a significant number of meningioma tissue cores (TMA) including a variety of histologic subtypes and grades. Caspase 3 expression was observed in high, moderate, and low levels associated with their differentiation grade, mitotic activity, and partially with specific histotypes. Similar protein and gene expression analyses focused on apoptotic pathways including c-FLIP, XIAP, Bcl-2, caspase 3, 8 and 9, cytochrome c, APAF 1 and Smac/DIABLO molecules detected low protein levels regarding caspases. A study group observed a potential blocking of these apoptotic inducers mediated by c-FLIP inhibition [17]. Similarly, overexpression of tumor necrosis factor-related apoptosis-inducing ligand R2 (TRAIL-R2) combined with low levels of caspase 8 has been also detected in a series of meningiomas [18]. In contrast to caspase 8, caspase 3 has been found to be overexpressed more frequently in meningiomas. A combined calpain and caspase-3 upregulation has been detected in a series of brain tumors including transitional meningiomas [19]. It is also important to be mentioned that survivin, an inhibitor of apoptosis that binds to caspases-3 and -7 external domains is overexpressed in men-

ingiomas, downregulating their function [20]. Another molecule which interacts with caspase-3 is the midkine, a heparin-binding growth factor that acts as an inducer for growth, survival, migration, and differentiation of various cells. A study group co-analyzed their expression showing that midkine reduced active caspase-3 levels, negatively affecting the response rates to camptothecin-mediated apoptotic cell death in meningioma cells in *in vitro* cultures, leading to increased chemotherapeutic regimens resistance [21]. Based on this increased need for enhancing apoptotic rates in meningiomas, novel studies have focused on specific agents. One of them is fenretinide. This is a synthetic retinoid promoting apoptosis in tumor cell cultures in several malignancies. A study group reported significant levels of caspase activation in meningiomas mediated by fenretinide. Interestingly, the agent provided apoptosis in all three grades of meningioma primary cells cultures [22]. Additionally, valproic acid (VPA), a commonly used anti-epileptic drug, seems to induce apoptosis by increasing cleaved caspase-3 and PARP apoptotic molecules in meningiomas stem cells cultures providing also elevated radiosensitivity [23].

In conclusion, caspase 3 expression demonstrates different expression patterns in meningiomas associated with their differentiation grade, mitotic activity, and also partially with specific histotypes. Caspase 3 should be a potent target for novel therapeutic strategies in meningiomas based on agents that could enhance caspase-mediated apoptotic death and response rates to specific chemoradiation regimens.

Conflict of interest

The authors declare no conflict of interests.

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