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ORIGINAL ARTICLE

Immunohistochemical expression of caspases 9 and 3 in adenoid cystic carcinoma of salivary glands and association with clinicopathological parameters

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Summary

Purpose: *Adenoid cystic carcinoma (ACC) is one of the most common malignant salivary gland tumors. It is characterized by a high rate of recurrence, perineural invasion and development of distant metastases many years after removal of the primary tumor. Disorders of the induction of apoptosis and its cascade reactions where caspases are involved may be significant in the pathogenesis of this tumor.*

Methods: *The immunohistochemical expression of caspase 9 and caspase 3 was analyzed by tissue microarray (TMA) in 50 cases of ACC in relation with different clinicopathological parameters (gender, age, localization, histological type and overall survival).*

Results: *Caspase 9 was expressed in the cytoplasm and nuclei of ACC tumor cells with varying degrees of staining intensity (1+, 6%; 2+, 54%, 3+, 40%). Comparison of caspase 9 expression in tumor cells with clinicopathological parameters (gender, age, localization, histological type and overall survival) showed no statistically significant difference except that the expression was more pronounced in females. Caspase 3 was expressed in the cytoplasm of tumor cells with varying degrees of staining intensity (1+, 22%; 2+, 36%; 3+, 42%). No correlation between the expression of caspase 3 and clinicopathological parameters was noticed.*

Conclusions: *The expression of caspases 9 and 3 in ACC of the salivary glands can contribute in the better characterization of molecules involved in apoptosis of tumor cells.*

Key words: *adenoid cystic carcinoma, apoptosis, caspase 3 and 9, prognosis, survival*

Introduction

ACC is one of the most common malignant salivary gland tumors. It accounts for 4-10% of all epithelial tumors in salivary glands, being a dominant histological type of malignancy in minor and submandibular salivary glands. It is a tumor with poor long-term prognosis, which is characterized by a high rate of recurrence, perineural invasion and development of distant metastases many years after removal of the primary tumor. In relation to the histological type, it can be divided into cribriform, tubular and cystic ACC [1-6]. No standard treatment for ACC has been established yet. Some studies have shown that surgical treatment and postoperative radiotherapy are important to control the disease, while others were unable to confirm the connection between postoperative radiotherapy and chemotherapy and better prognosis for this tumor [7].

Apoptosis, or programmed cell death, has an important role in embryonic development, immune system function and the maintenance of tissue homeostasis [8]. Deregulation of apoptosis

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is one of the most important factors in the pathogenesis of cancer and its biological behavior [9]. Different studies have shown that chemotherapeutic effects depend on the induction of apoptosis and disruption of apoptotic signaling cascade may therefore be an important cause of resistance to chemotherapy [10,11].

There are two major pathways for the initiation of apoptosis: extrinsic and intrinsic. The extrinsic pathway starts by activating receptors on the cell surface leading to activation of caspase 8 which further activates caspase 3. The intrinsic pathway, or mitochondrial, is activated by the release of the cytochrome C from mitochondria which, by making a complex with the Araf-1, activates caspase 9 via a complex known as apoptosome. The activated caspase 9 leads to activation of caspase 3. Caspase 3, both in extrinsic and intrinsic pathways, is responsible for the nuclear changes in apoptosis [12].

Caspases play a central role in the activation and propagation of apoptotic signaling and they are divided into two groups: initiator (caspases 2,8,9 and 10) and effector (caspase 3,6 and 7) [13,14]. Detection of the expression of caspases 9 and 3 in relation with different parameters has been carried out in many human malignancies: esophageal squamous cell carcinoma, diffuse large B–cell lymphomas, Hodgkin's lymphoma, squamous cell carcinoma of the head and neck, prostate cancer, colon carcinoma, meningiomas, adenocarcinoma of the breast, carcinoma of the thyroid gland, lymphoblastic leukemia, etc [9-13,15-21].

In this study we evaluated the immunohistochemical expression of caspases 9 and 3 in ACC of salivary glands and their relation with several clinicopathological parameters.

Table 1. Clinicopathological characteristics of ACC and expression of caspase 9 and 3

Number of patients	*N (%)*	*p value*
Sex		p=0.090
Male	19 (38.0)	
Female	31 (62.0)	
Age years, mean±SD (median, range)	58.00±12.79 (59.0; 29-78)	
Tumor localization		p=0.000
Parotid salivary glands	4 (8.0)	
Submandibular salivary glands	9 (18.0)	
Minor salivary gland palate duri	29 (58.0)	
Other minor salivary glands	8 (16.0)	
Histology		p=0.726
Cribriform	14 (28.0)	
Tubular	18 (36.0)	
Solid	18 (36.0)	
Caspase 9		p=0.000
+	3 (6.0)	
++	18 (54.0)	
+++	21 (40.0)	
Caspase 3		p=0.206
+	11 (22.0)	
++	18 (36.0)	
+++	21 (42.0)	
Survival		p=0.090
Alive	19 (38.0)	
Dead	31 (62.0)	

Methods

A retrospective study was performed involving 50 patients diagnosed with ACC between 1998-2008 at the Institute of Pathology, School of Dentistry, Belgrade, Serbia. All the patient records from the Clinic of Maxillofacial Surgery, School of Dentistry, University of Belgrade, were reviewed. The clinical characteristics of the patients are outlined in Table 1.

Construction of tissue microarrays

In each case, three selected tissue cylinders (''cores'') with a diameter of 1.2 mm were punched from the tumor areas of a ''donor'' tissue block and placed in three recipient paraffin blocks (TMA blocks), each containing 62 cores. To ensure the quality, reproducibility and homogeneous staining of the slide, controls were included (human cervical carcinoma for caspase 9 and tonsilar tissue for caspase 3). Negative controls were performed by omitting of the perimary antibody).

Immunohistochemistry

Five-micrometer cut sections from TMA blocks were deparaffinized, rehydrated, placed in 3% H_2O_2 for 10 min to block endogenous peroxidase activity, and washed with tap water. Then, they were processed with 0.01 citrate buffer (pH 6.0) and treated in a microwave oven for 20 min at 600 W and placed in a bath of tap water for 20 min, then in distilled water and in TBS buffer (pH 7.6) for 5 min, and placed in diluted goat serum for 10 min. Afterwards the tissue sections were incubated for 1 h with the following rabbit monoclonal primary antibody (Anti-Caspase 9 antibody [E23], dilution 1:100, Abcam Inc., MA, USA) and mouse monoclonal antibody (CPP3, dilution 1:100, , Leica Biosystems,

Table 2. Caspase 9 expression in relation to clinicopathological characteristics of patients with ACC

Characteristics	*Caspase 9*			*p value*
	+ *N (%)*	*++* *N (%)*	*+++* *N (%)*	
Sex				p=0.000*
Male	0 (0)	17 (89.5)	2 (10.5)	
Female	3 (9.7)	10 (32.3)	18 (58.1)	
Age, years, mean±SD (median, range)	53.67±4.51 (54; 49-58)	58.81±13.16 (60; 31-78)	57.55±10.23 (57.5; 29-75)	p=0.794**
Tumor localization				p=0.564*
Parotid salivary glands	0 (0)	3 (75)	1 (25)	
Submandibular salivary glands	0 (0)	3 (33.3)	6 (66.7)	
Minor salivary glands palate duri	2 (6.9)	16 (55.2)	11 (37.9)	
Other minor salivary glands	1 (12.5)	5 (62.5)	2 (25)	
Histology				p=0.308*
Cribriform	1 (7.1)	5 (35.7)	8 (57.1)	
Tubular	2 (11.1)	11 (61.1)	5 (27.8)	
Solid	0 (0)	11 (61.1)	7 (38.9)	
Survival				p=0.707*
Alive	1 (5.3)	9 (47.4)	18 (58.1)	
Dead	2 (6.5)	9 (47.4)	11 (35.5)	

*x^2 test; **one way ANOVA

Newcastle, UK). Streptavidin-biotin method using DAKO's LSAB+ kit (DAKO, Denmark) was applied, with diaminobenzidine (DAB) as the chromogen solution and Mayer's hematoxylin for the counterstain.

All immunostained sections were independently evaluated by two pathologists (BD and SG). The results of immunohistochemical staining were scored by a semiquantitive technique: expression of Caspases 9 and 3 was scored into 3 grades according to the intensity of the staining: 1+,2+ and 3+. The staining pattern of the biopsies was defined as follows: 1+ weak or negative, 2+moderate, 3+ strong.

Statistics

Statistical analyses were performed using SPSS software v. 22.0 (SPSS Inc., Chicago, ILL, USA). Descriptive data for all groups and variables were expressed as mean ± SD for continuous variables, or percent of a group for discrete variables (categorical data). Categorical data were analyzed using the Pearson's chi-square test. Normal distribution was tested using the Kolgomorov-Smirnov test and normal distribution of continuous data was tested with one way ANOVA test. Kappa coefficient of agreement was used for the evaluation of correspondence between two pathologists. Overall survival rates were calculated from the day of diagnosis by the Kaplan-Meier method, and differences were evaluated by the log-rank test. All reported p values were two-sided. Differences were considered significant when p value was <0.05.

Results

Analyzed were biopsies of 50 patients with ACC (19 ; 38% male and 31 ; 62% female). The median age of the patients was 58 years (range 28 -78). The tumor was most often localized in the minor salivary glands of the hard palate (58%), and least frequently present in the parotid salivary glands (8%). Concerning the histological type, tubular and solid forms occurred in 18 (36%) cases each, while 14 (28%) cases showed a cribriform image. At the end of follow-up (the shortest being 8 months and the longest 158), 19 (38%) patients were alive and 31 (62%) deceased. The mean overall survival or ACC was 91 months.

The index of evaluation reliability was satisfactory (k=0.741 for caspase 9; k=0.846 for caspase 3), i.e. almost perfect consistency in the evaluation of the tested preparations.

The immunoreactivity of caspase 9 in ACC tumor cells had variable staining intensity (p=0.000) (Table 1). The greatest number of patients, 27 (54%), had 2+ intensity expression, 20 (40%) had 3+, while only 3 (6%) cases showed 1+ positivity (Figure 1). The antibody specific for caspase 9 (anticaspase 9 antibody [E25]) stained the cytoplasm and nuclei of tumor cells.

In relation to gender, differences in the lev-

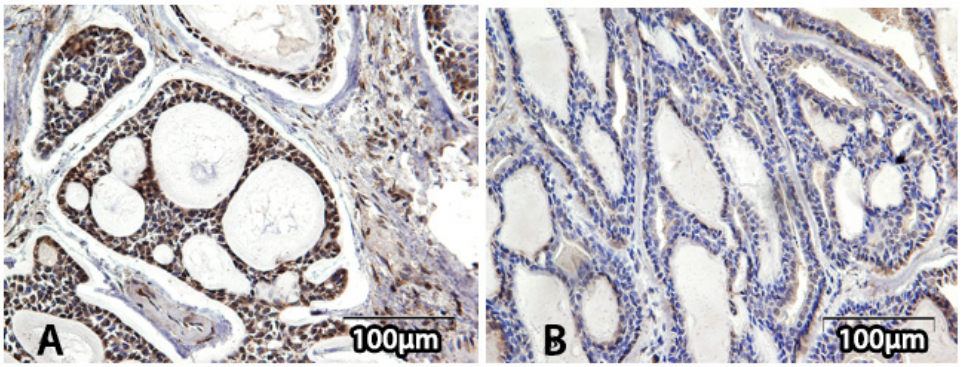

Figure 1. Caspase 9 immunostaining in ACC (x200). **A:** strong expression, **B:** moderate expression.

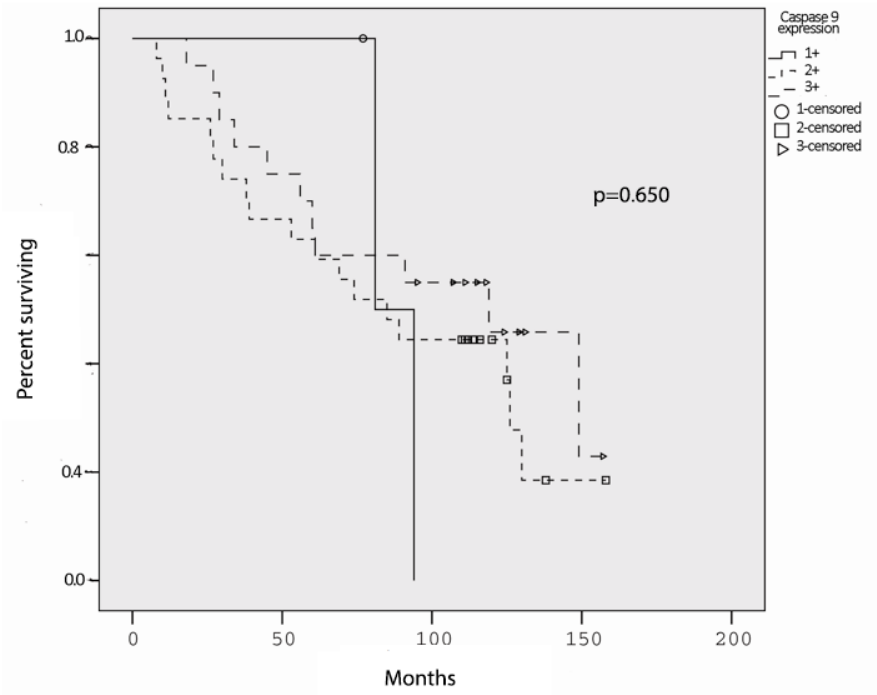

Figure 2. Overall survival and caspase 9 expression.

el of expression of caspase 9 in tumor cells were significantly more pronounced in the female population (x^2, p=0.000; Table 2). The vast majority of the male patients (89.5%) had 2+ expression of caspase 9, whereas in females the majority (58.1%) had 3+ expression of caspase 9.

No statistically significant correlation was found between expression of caspase 9 and tumor localization. A high level of expression (3+) was registered in the ACC submandibular salivary glands, while in other glands moderate (2+) positivity was noticed. Also, no statistically significant difference was registered between survival and different levels of expression of caspase 9 (log rank, p=0.650). Of the total number of patients, 27 (54%) had 2+ expression of caspase 9, and survival was 85.2% after 1 year, 63.0% after 5, 44.4% after 10, and 18.5% after 13 years. The mean overall survival for this group of patients was 85 months (95% CI 51.08 -118.92) (Figure 2) . As for other clinicopathological parameters, such as patient age, histological type etc, no statistically significant difference in relation to the expression of caspase 9 was registered (Table 2).

In contrast to caspase 9, caspase 3 was only expressed in the cytoplasm of ACC tumor cells. Most of the patients, (21; 42%) showed strong expression (3+), 18 (36%) had moderate expression (2+), and low positivity (1+), was noticed in 11 (22%) patients (Figure 3). The correlation between the different levels of the expression of caspase 3 were not statistically significant (p=0.206) (Table 1).

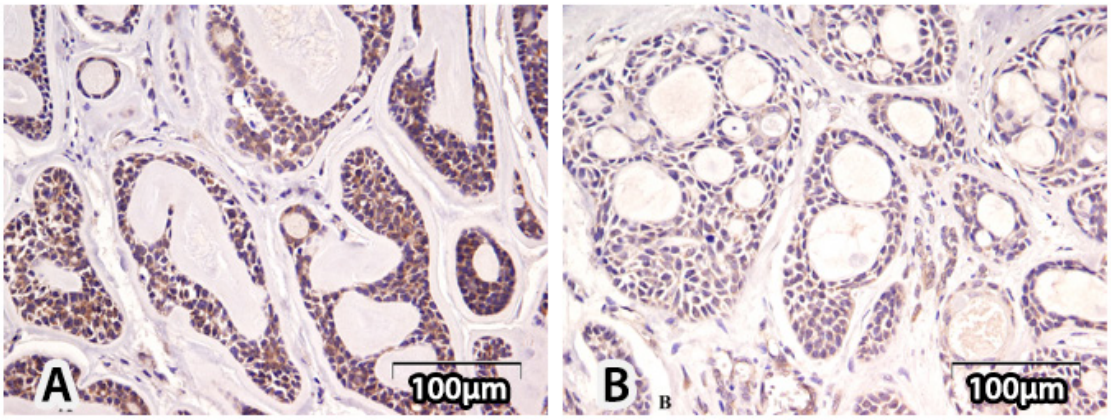

Figure 3. Caspase 3 immunostaining in ACC (x200). **A:** strong expression, **B:** moderate expression.

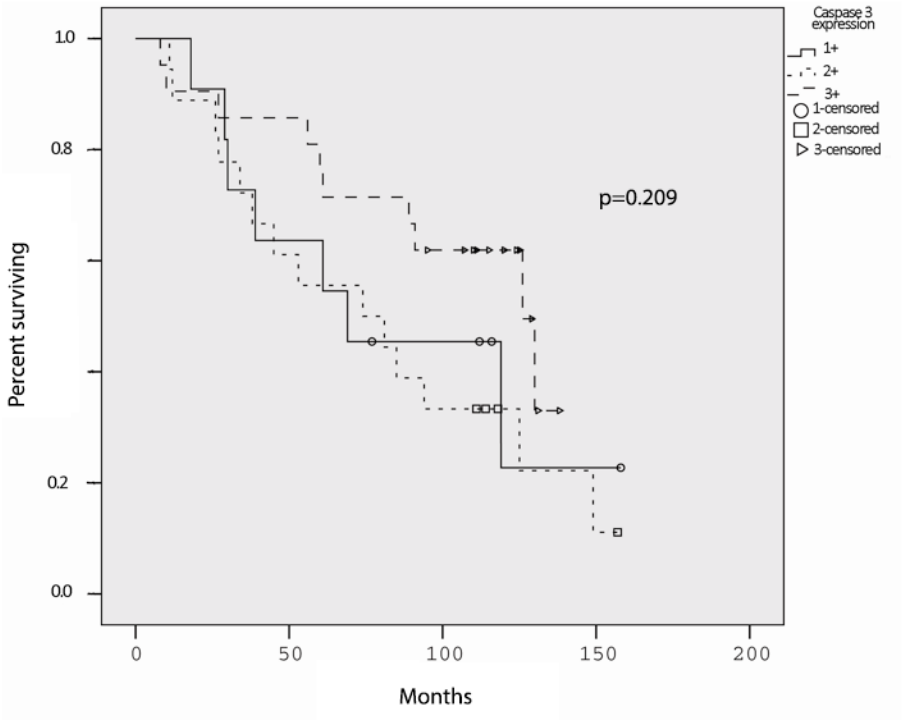

Figure 4. Overall survival and caspase 3 expression.

In this study there was no statistical connection between immunohistochemical expression of caspase 3 and clinical parameters such as gender (p=0.354), patient age (p=0.412), tumor location (p=0.532) and histological tumor type (p=0.265) (Table 3). In relation to gender, 31.6% of the males were 3+ positive and 36.8% were 2+ positive, while in females 3+ prevailed (48.4%), followed by 2+ (35.5%). The histological tumor types (solid and cribriform) had almost uniform expression of caspase 3, while most patients (55.6%) with tubular tumor had 3+.

No statistically significant differences in survival were found in relation to the different levels of expression of caspase 3. Of the total number of patients, 21 (42%) had 3+ expression of caspase 3, with 1-year survival 90.5%, 5-year survival 76.2%, 10-year survival 61.9%, and at the end of follow-up there were no living patients in this group. The mean survival for this group of patients was 126 months (95% CI 89.1-162.9; Figure 4).

Table 3. Caspase 3 expression in relation to clinicopathological characteristics of patients with ACC

Characteristics	*Caspase 3*			*p value*
	+ *N (%)*	*++* *N (%)*	*+++* *N (%)*	
Sex				p=0.412**
Male	6 (31.6)	7 (36.8)	6 (31.6)	
Female	5 (16.1)	11 (35.5)	15 (48.4)	
Age, years, mean±SD (median, range)	53.91±15.90 (54; 29-75)	60.50±13.18 (60; 29-78)	58.00±10.56 (57.5; 39-78)	
Tumor localization				p=0.532*
Parotid salivary glands	0 (0)	2 (50)	2 (50)	
Submandibular salivary glands	2 (22.2)	2 (22.2)	5 (55.6)	
Minor salivary gland palate duri	6 (20.7)	10 (34.5)	13 (44.8)	
Other minor salivary glands	3 (37.5)	4 (50)	1 (12.5)	
Histology				p=0.265*
Cribriform	5 (35.7)	4 (28.6)	5(35.7)	
Tubular	1 (5.6)	7 (38.9)	10 (55.6)	
Solid	5 (27.8)	7 (38.9)	6 (33.3)	
Survival				p=0.153*
Alive	4 (21.1)	4 (21.1)	11 (57.9)	
Dead	7 (22.6)	14 (45.2)	10 (32.3)	

*x^2 test; **one way ANOVA

Discussion

ACC is one of the most common malignancy of the salivary glands. It is an indolent tumor but with a persistent and recurrent growth, which gives late metastases. In relation to gender it is most often diagnosed in females, mainly in the 5th and 6th decades of life [22]. In our study of 50 patients, females clearly prevailed (68%) and their mean age was 58 years. The oldest patient was 78 and the youngest 29 years. This disease is very rare in persons under the age of 20 years [23-25]. In this study, ACC was mostly localized in the minor salivary glands of the palate (58%). This coincides with the results of other authors [25], although other authors reported that it occurs more often in the parotid and submandibular glands [26]. Histological characteristics of this tumor include the presence of myoepithelial and ductal cells that can make cribriform, tubular and solid forms [25]. In this study, cribriform and solid forms were found in 18 (36%) patients, while 14 (28%) cases exhibited a tubular form. Other studies have shown a positive correlation between clinicopathological parameters and survival of patients with ACC. Correlated parameters are age, tumor localization, higher grade, histological type, perineural invasion, positive surgical margins, nodal disease and distant metastases [27,28]. Our results indicated that clinicopathological parameters (gender, age, histological type, localization) had no significant impact on overall survival, the mean value of which was 91 months (95% CI 46.71-135.29).

In the pathogenetic mechanisms of many diseases, from neurodegenerative disorders to malignant tumors, those of great importance are connected with disturbances in the apoptotic pathways [29]. Apoptosis has been studied in many tumors, but the results of numerous studies are not consistent. With the intrinsic pathway of apoptosis, in the mitochondria, tumor processes can regulate the expression of Bcl-2 family (an inhibitor of apoptosis), or decrease the expression of caspases (caspases 8,9,3) [30]. Caspase 9 is especially significant in the intrinsic pathway of apoptosis.

In our work, the immunohistochemical expression of caspase 9 in ACC cells was studied and evaluated. The intensity of staining the tumor cells was 2+ in the majority of cases (54%) and positivity was present in the cytoplasm and nucleus of tumor cells. A similar staining was found in Ewing's sarcoma tumor cells [31]. But unlike our study, the intensity of staining was greatest (66%) with 1+, while reactivity 0 and +2 was not proven in this tumor. Contrary to this work and our results, the expression of caspase 9 in prostate

cancer cells was observed only in the cytoplasm of tumor cells and was expressed almost equally in both the normal prostate tissue and the intraepithelial lesions and cancer [13]. In patients with colorectal cancer, a high expression of caspase 9 was demonstrated (100% positive cells) [15].

In the available literature there are very few works that show an association between the expression levels of caspase 9 and clinicopathological parameters in human tumors. However, in our study there was a positive correlation between the expression of caspase 9 in half of the patients. Statistically significant higher expression was found in female patients. In relation to other clinicopathological parameters (age, location, histological type) caspase 9 didn't show statistical significance. Also, no significant difference was found between different expressions of caspase 9 and overall survival (log rank, p=0.650). Caspase 9 was not a predictor of overall survival even in patients with tumors of the Ewing's sarcoma family [31], while in patients with colorectal cancer it was a significant predictor of survival [15].

Caspase 3 is an effector caspase participating in extrinsic and intrinsic pathways of apoptosis [32]. The expression of caspase 3 in the ACC of salivary glands in our study was expressed only in the cytoplasm of tumor cells. Localization of caspase 3 in the cytoplasm was shown in the oral granular cell tumor [33], and mucoepidermoid carcinoma of salivary glands [34]. Other studies generally indicate cytoplasmic and nuclear localization of caspase 3. An example of such a positivity was found in half of the cases of malignant peripheral nerve sheath tumors (MPNSTs), while the other half (neurofibroma cells) showed nuclear positivity [35]. Vranic in his publication reported that caspase 3 was predominantly expressed in cell nuclei of malignant and atypical meningiomas [36], in contrast to other authors who showed mainly cytoplasmic positivity in atypical meningiomas [16]. Exploring the importance of cellular localization of caspase 3, some authors have suggested that it may reflect the biological behavior in the studied tumors of the pancreas, because its cytoplasmic localization was more prevalent in invasive and the nuclear in non-invasive pancreatic tumors [37].

In our study cytoplasmic positivity of caspase 3 in ACC tumor cells was 2+ in the majority of the cases (42%) 36% was estimated as 3+, and 22% of the cases had either low positivity (1+) or lacked positivity completely. Data from the literature mainly show somewhat less expressed caspase 3 in various human tumors. One study showed only 5% of the cases with strong expression of caspase 3 in malignant and atypical meningiomas, whereas mild or moderate (35% and 31% of the cases, respectively) prevailed [36]. The expression of caspase 3 was determined in squamous cell carcinomas (SCCs) of the tongue, using the image analysis. No high expression of caspase 3 was shown in any case of SCCs, and weak positivity was present in the vast majority of the cases (90.5%) [38]. Hsia et al. evaluated the expression of caspase 3 in 40 cases of esophageal squamous cell carcinoma and proved that it was strongly pronounced in most cases [9]. Contrary to this study, in mucoepidermoid carcinoma of the salivary glands 59.1% of the total number of cases did not show positivity for caspase 3 [34].

Our study found no statistical significance between the expression levels of caspase 3 and clinicopathological parameters (gender, age, localization, histological type). Similar to our results, the authors who examined the expression of caspase 3 in meningiomas did not prove statistical significance in relation to age, gender and tumor localization [16,36]. Also, the expression of caspase 3 in papillary thyroid carcinoma did not show statistical significance in relation to gender and the age of the patients [19], and in esophageal squamous cell carcinoma the histological tumor type had no statistical significance in relation to its expression [9].

In most cases the expression of caspase 3 in the ACC of the salivary glands was assessed as moderate or strong, and did not have a statistical significance in relation to overall survival. Similar results were demonstrated in patients with atypical or malignant meningiomas [36] and patients with SCCs of the tongue [38]. However, our results showed that, although there was no statistical significance, the greater median survival was in the patients with strong (3+) expression of caspase 3 (mean 126 months; 95% CI 89.1-162.9), in comparison to patients with 2+ and 1+ expressions where it was 69 months (95% CI 11.46-126.54). In esophageal squamous cell carcinoma, Hsia et al. came to the conclusion that the expression of caspase 3 can be a significant prognostic factor of survival, because its reduced expression stimulates the growth of tumor cells and shortens the patient survival [9]. The lack of or reduced expression of caspase 3 was also associated with worse prognosis in patients with nasopharyngeal carcinoma [39] and glioma [40] as well. Contrary to these results, Cunha et al., showed that

the increased levels of cytoplasmic expression of caspase 3 were associated with more aggressive histological appearance of the examined tumors, and thus with worse prognosis [35].

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