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Differential challenges in salivary gland neoplasms

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Abstract: Salivary gland tumors are neoplasms characterized by a high level of pleomorphism and histological overlap. One tumor may contain several cell types; therefore, it is necessary to include immunohistochemical staining, as well as morphometric analysis of tumor cells as prerequisites for an appropriate diagnosis. Our research included 120 tumors, such as pleomorphic adenoma, Warthin tumor, basal cell adenoma, myoepithelioma, adenoid cystic carcinoma, mucoepidermoid carcinoma, salivary duct carcinoma, polymorphous low-grade carcinoma and myoepithelial carcinoma. The aim of the study was to differentiate benign and malignant tumors based on the characteristics of nuclei. The expression of Ki67 and the morphometric nuclear parameters – area, perimeter, Feret diameter, integrated optical density, circularity, and roundness, were analyzed. It was observed that the Ki67 proliferative index was statistically significantly higher in malignant tumors ($P<0.001$). Adenoid cystic carcinoma exhibited the highest value, whereas the lowest value was exhibited in basal cell adenoma. Morphometric analysis showed statistically significantly increased values of integrated optical density ($P<0.001$) and nuclear size parameters ($P<0.05$) in malignant tumors. The determination of the Ki67 proliferative index and morphometric analysis of the integrated optical density and area can differentiate benign from malignant tumors with high precision. The presented values suggest the obtained results as cut-off values.

Keywords: salivary gland; neoplasm; morphometry; immunohistochemistry; Ki67

INTRODUCTION

Salivary gland tumors are extremely rare neoplasms. Given their pathological traits, these tumors represent a great diagnostic challenge. The incidence of these tumors ranges from 3% to 6% of all tumors of the neck and head region, with an annual incidence of 0.05 to 2 newly discovered tumors in 100000 individuals [1,2]. The parotid gland is the most common site of salivary gland tumors, with an incidence of 80-85%, and almost 75% of these tumors are benign neoplasms. The second most frequent is the submandibular gland with 10% incidence, but half of the tumors at this localization are malignant. The sublingual gland has an incidence of 1%, however, about 80% of tumors are malignant. Minor salivary glands are places with the highest incidence of malignant tumors [3,4]. Epidemiological data show a different incidence in different ethnic groups and parts of the world, which further complicates the global incidence of these tumors [5,6]. Studies have shown that 9% of malignant salivary gland tumors include lymphomas and metastatic tumors, which should be taken into consideration when it comes to differential diagnosis [7].

Pleomorphic adenoma, mucoepidermoid carcinoma and acinar cell carcinoma comprise 90% of all epithelial tumors of salivary glands. Regardless of age,

sex or the biological behavior of tumors, pleomorphic adenoma is most common among all patients, with an incidence of 50%. Warthin tumor is the second most frequent among benign tumors, whereas mucoepidermoid carcinoma is most frequent among malignant tumors. The majority of canalicular adenomas and polymorphous low-grade adenocarcinomas originate in minor salivary glands. Warthin tumor is most commonly found in the parotid gland or the periparotid lymph node [7-9].

In their own study, Dardick and Nostrand state that the relationship between the normal structure of a salivary gland and the histological image of the tumor itself can help us understand the morphological classification. Still, one should bear in mind that the similarity does not necessarily mean the tumor has originated from the structure it imitates [10]. However, Dardick himself suggested a morphogenetic approach to examine the changed differentiation of stem cells, which is the consequence of altered gene expression [11,12].

The basic marker in the differentiation of benign and malignant tumors is Ki67, more precisely, the calculation of the proliferative index Ki67. Cell proliferation is considered one of the fundamental biological processes in oncogenesis. Ki67 is present in all active phases of the cell cycle, whereas it is absent in G0. Its expression increases with the progression of the cell cycle and peaks during the G2 and M phases. The proliferative activity, expressed as a percentage, was shown to be an essential prognostic and predictive factor in many tumor types [13]. Ki67 is a large protein of about 396 kDa whose percentage value was compared with the differentiation, as well as the prognosis of tumors of many organs, but not so much with salivary gland tumors. Furthermore, Ki67 is significant in ribosome synthesis during cell division, which has been proven in the correlation of Ki67 expression and protein synthesis, with the ribosome function. [13,14].

While tumors of this localization are quite rare, they are often difficult to diagnose as the histological image can be very similar among tumors, regardless of their biological behavior. Further problems arise when it comes to small incision biopsies, i.e. when the whole tumor is not included. Even though immunohistochemical analysis is a method of choice, the expression of the same antibodies is often found in many tumors, implying that additional diagnostic methods are required. A well-established diagnosis is imperative for every doctor given that further treatment depends on it. Thus, as salivary gland tumors have a broad histological presentation that often overlaps, irrespective of dissimilar biological characteristics, they represent a diagnostic challenge. We determined the values of the Ki67 proliferative index and employed morphometric analysis to separate benign from malignant tumors with high precision. In a limited number of cases it is possible to determine the tumor type by analyzing preparations. The first step involves the determination of the Ki67 proliferative index level as well as biological characteristics of the tumor. A low proliferative index is associated with benign neoplasms, i.e. neoplasms with a good prognosis. Another method includes the analysis of the characteristics of nuclei using morphometry. The analysis of the shape and size of nuclei is of great help in the determination of benign and malignant neoplasms. [15]

MATERIALS AND METHODS

Study design

The study was conducted at the Institute of Pathology, Faculty of Medicine, Niš. The gender ratio varied depending on the tumor, but there was a slight predominance of women, especially in the case of malignant tumors. The age of the patients ranged from 12 to 75. Malignancy in children was not registered. After surgical intervention, the tissue was fixed in 10% formalin for at least 24 h, as recommended by the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) [16]. The study included 120 representative salivary gland tumors, 60 benign and 60 malignant. The operative material consisted of 100 samples out of a total of 120, with the remaining 20 being biopsies. The benign tumor group included pleomorphic adenoma (tumor mixtus), Warthin tumor, basal cell adenoma and myoepithelioma, and the malignant tumor group was comprised of adenoid cystic carcinoma, mucoepidermoid carcinoma, salivary duct carcinoma, polymorphous low-grade carcinoma and myoepithelial carcinoma.

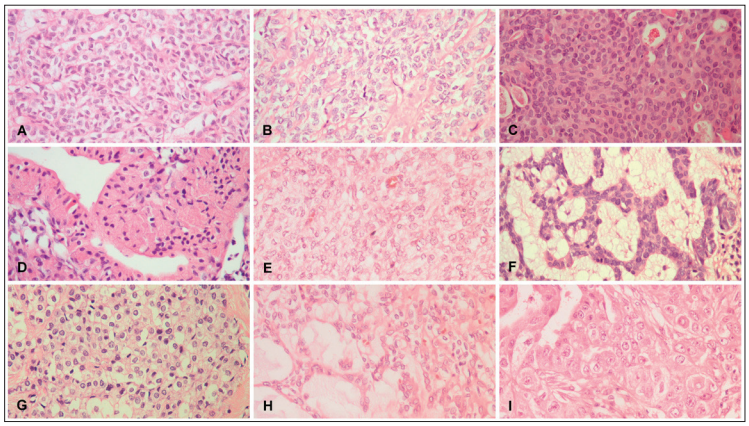

Fig. 1. Histological presentation of benign and malignant salivary gland tumors (H&E, magnification ×40). **A** – Myoepithelioma; **B** – Tumor mixtus; **C** – Basal cell adenoma; **D** – Warthin tumor; **E** – Myoepithelial carcinoma; **F** – Adenoid cystic carcinoma; **G** – Mucoepidermoid carcinoma; **H** – Polymorphous low-grade adenocarcinoma; **I** - Salivary duct carcinoma.

Immunohistochemical analysis of the Ki67 proliferative index

The Ki67 index was determined by analyzing and counting positive nuclei on 10 visible areas, at a magnification of ×40. The index was expressed in percentage as the ratio of positive tumor cells and negative cells. Micrographs were taken, the photos were transferred to a computer and the cells were counted using the ImageJ version 1.43u software pack. The proliferative index was expressed as a percentage, as the ratio of positive (stained) nuclei and the total number of tumor cells in one visual field.

Morphometric analysis

Morphometric analysis was performed in the software pack ImageJ version 1.43u (public domain software, Wayne Rasband, National Institutes of Health, Bethesda, Maryland, USA). One hundred random nuclei of tumor cells that did not overlap were analyzed at a magnification of ×40. Six nuclear parameters were analyzed as follows: area, perimeter (perim), circularity, roundness, the Feret diameter (Feret) and integrated optical density (IOD).

Data analysis

Having analyzed the tumor type as a dichotomous variable with the aim of determining independent variables that can play a statistically significant role in predicting malignant tumor types, univariate and multivariate logistic regression analyses were performed. The determination of the cut-off values of the examined parameters that differentiate between tumors based on their biological behavior into benign and malignant was performed using Receiver Operating Characteristics (ROC) analysis. The cut-off values were values with the highest sum of sensitivity and specificity.

RESULTS

As pointed out, tumors of this localization represent a diagnostic problem because of their histological presentation, which can be very confusing (Fig. 1). The basic presentation of benign and malignant tumors often overlaps on hematoxylin-eosin (H&E) preparations, which leaves room for misdiagnosis. A tumor mixtus can be confused with basal cell adenoma, low-grade polymorphic adenocarcinoma, or even malignant myoepithelioma, while Warthin tumor can have the presentation of salivary duct carcinoma; also, adenoid cystic carcinoma can be confused with mucoepidermoid carcinoma or low-grade polymorphic adenocarcinoma. Certainly, the tumor prognosis is very different, as well as the surgical and further oncological treatment. Therefore, a correct pathohistological diagnosis is the basis for the further course of treatment.

Proliferative Ki67 index in benign and malignant salivary gland tumors

The expression of Ki67 was analyzed in all 120 salivary gland tumors. The cut-off value in the differentiation of aggressivity and biological behavior was 5%. Intensive positive staining was considered a positive reaction, similar to the intensity of staining of the basal layer cells of the squamous epithelium. The value of the proliferative index was statistically significantly higher in malignant when compared to benign tumors. Fig. 2 shows the expression of Ki67 antibodies in all types of salivary gland tumors analyzed in this study. The highest proliferative index among all tumor types was

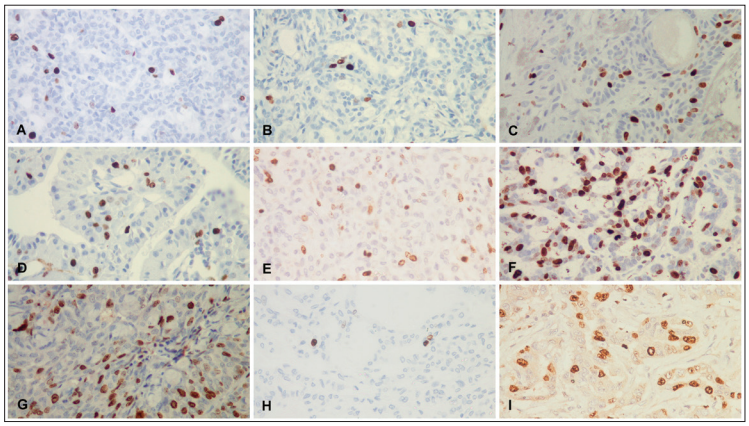

Fig. 2. Expression of **Ki67** in benign and malignant salivary gland tumors (magnification ×40). **A** – Myoepithelioma; **B** – Tumor mixtus; **C** – Basal cell adenoma; **D** – Warthin tumor; **E** – Myoepithelial carcinoma; **F** – Adenoid cystic carcinoma; **G** – Mucoepidermoid carcinoma; **H** – Polymorphous low-grade adenocarcinoma; **I** – Salivary duct carcinoma.

recorded in adenoid cystic carcinoma and its value was statistically significantly higher compared to mucoepidermoid carcinoma (P<0.05), myoepithelial carcinoma (P<0.01) and all other tumors (P<0.001). The lowest value of the proliferative index was recorded in myoepithelioma, similar to polymorphous low-grade adenocarcinoma (Table 1). The values of the proliferative index in all malignant tumors, with the exception of polymorphous low-grade adenocarcinoma, were higher than the values in all benign tumors.

Table 1. Proliferative index values regarding tumor type

Tumor type***	X ±	SD	(Me)	Min -	Max
Benign*					
Myoepithelioma	2.48 ±	1.05	(2.40)	1.30 -	5.30
Tumor mixtus	5.97 ±	4.05 ^{ah*g**}	(4.60)	1.20 -	13.00
Basal cell adenoma	3.85 ±	3.71	(2.34)	0.76 -	12.00
Warthin tumor	4.78 ±	1.65 ^{ah**}	(5.18)	2.05 -	6.98
Malignant***					
Myoepithelial carcinoma	17.48 ±	10.24^{acdi***b**}	(16.65)	3.40 -	37.40
Adenoid cystic carcinoma	**32.87**±	**18.86**^{abcdh***e**g*}	**(31.63)**	**3.60** -	**72.30**
Mucoepidermoid carcinoma	18.29 ±	9.76^{acdh***b**}	(19.53)	7.96	39.55
Polymorphus low-grade adenocarcinoma	2.57 ±	1.32	(2.78)	1.18 -	4.78
Salivary duct carcinoma	28.69 ±	10.17^{abcdh***eg*}	(27.56)	15.52 -	43.39

*p<0.05, ** p<0.01, ***p<0.001 (Kruskal Wallis test, Mann-Whitney U Test)
*p<0.05, ** p<0.01, ***p<0.001 (Kruskal Wallis test, Mann-Whitney U Test)
a – vs myoepithelioma, b – vs tumor mixtus, c – vs basal cell adenoma, d – vs Warthin tumor, e – vs myoepithelial carcinoma, f – vs adenoid cystic carcinoma, g – vs mucoepidermoid carcinoma h – vs polymorphous low-grade adenocarcinoma, i – vs salivary duct carcinoma. X – mean value; SD – standard deviation; Me – mediana; Min – minimum; Max – maximum

Morphometric analysis of benign and malignant salivary gland tumors

The values of the nucleus area, perimeter and the IOD are statistically significantly higher in malignant than in benign tumors. In the benign tumor group, the mean value±SD for the area was 44.19±8.34, whereas in the malignant tumor group it was 51.43±20.20. Using a Student's independent t-test, a statistically significant difference was obtained of P<0.05. By analyzing the obtained values for the perimeter, the mean value in the benign tumor group was 25.15± 2.29, and 26.75±5.08 in the malignant tumor group. A statistically significant difference was found in the malignant tumor group of P<0.05. By measuring the IOD, a group of malignant tumors was separated with high certainty (P<0.001). The mean value for benign tumors was 11.93±5.20, and for the malignant tumor group, 16.87±7.45. The circularity values, as well as the values for the Feret diameter and roundness were higher in the malignant than in the benign tumors; however, the difference was not statistically significant.

Results of univariate and multivariate logistic regression

Univariate and multivariate logistic regression analyses were used to determine whether malignant tumors could be predicted based on the values of their morphometric parameters. The results of the univariate logistic regression analysis revealed that the following morphometric parameters were statistically significantly higher in malignant tumors: area, perimeter (P<0.05) and IOD (P<0.001). An increase in the area for a single unit of measurement increased the possibility of a malignant tumor by 3.3% (IP 0-6%), an increase in the perimeter increased the possibility by 10.9% (IP 0-22%), whereas an increase in the IOD increased the possibility by 14.4% (IP 6-23%) (Table 2).

Table 2. OR values for assessing the impact of statistically significant morphological parameters of interest for the differentiation of malignant tumors, the results of the univariate logistic regression analysis.

	OR	95.0% IP		P
		Lower	Upper	
Area	1.033	1.00	1.06	* 0.0215
Perim	1.109	1.00	1.22	* 0.0402
IOD	1.144	1.06	1.23	*** 0.0003

*P<0.05, ***P<0.001; OR – odd ratios; IP – increases the possibility; Perim – perimeter; IOD – integrated optical density.

Table 3. OR values for assessing the impact of statistically significant morphological parameters for the differentiation of malignant tumors; results of multivariate logistic regression analysis.

	OR	95.0% IP		P
		Lower	Upper	
IOD	1.144	1.063	1.231	***0.0003
Constant	0.17			0.0013

*** P<0.001; OR – odd ratios; IP – increases the possibility; IOD – integrated optical density

Table 4. OR values for the assessment of the proliferative index impact on the possibility of a malignant tumor, the results of the univariate logistic regression analysis.

	OR	95.0% IP		P
		Lower	Upper	
Proliferative index	1.266	1.147	1.398	*** 0.0000

*** P<0.001; OR – odd ratios; IP – increases the possibility.

Important predictor variables from univariate analysis were included in the model of the multivariate logistic regression (Table 3). Using the backward conditional method in the third, final step, the multivariate logistic regression analysis chose the IOD (P<0.001) from the starting model with three predictor variables as the only significant predictor for malignant tumors. A unit increase of the IOD values increased the possibility of malignant tumors by 14.4% (IP 6.3-23.1%). A change in individual IOD values caused an 18.3% change in the biological behavior of the tumor (R2 (0.183)).

Univariate logistic regression analysis determined that the proliferative index (P<0.001) has a statistically significant impact on the likelihood of a malignant tumor as well. An increase in the proliferative index for a single unit of measurement increased the risk of a malignant tumor by 26.6% (IP 14.7-39.8%) (Table 4).

Assessment of the classification characteristics of the proliferative index and IOD based on ROC curve analysis

Specificity begins to decrease considerably when sensitivity exceeds 68.97%. The area under the curve (AUC) amounted to 0.863 with a standard estimation error of 0.036. Therefore, the statistical significance was P<0.001 and the 95% confidence interval ranging from 0.792 to 0.934 pointed to a high degree of reliability.

ROC curve analysis defines sensitivity and specificity for each value of the proliferative index. When a proliferative index value of 13.43 is set as the cut-off value, the sum of the sensitivity and specificity was the highest, i.e. the sensitivity of the method was 68.97%, whereas the specificity was 100% (Fig. 3A). Specificity begins to decrease considerably when sensitivity exceeds 59.68%. The AUC was 0.713, the standard estimation error 0.048, the statistical significance P<0.001, the 95% confidence interval from 0.619 to 0.807, all pointing to a relative degree of reliability in drawing a conclusion.

ROC curve analysis defines the sensitivity and specificity for each IOD value. When the IOD value of 14.97, for which the sum of sensitivity and specificity is the highest, was set as the cut-off value, the sensitivity of the method was 59.68%, whereas the specificity was 81.48% (Fig. 3B). The obtained values for the Feret diameter, perimeter, circularity and roundness were not statistically significant, indicating insufficient reliability in drawing a conclusion.

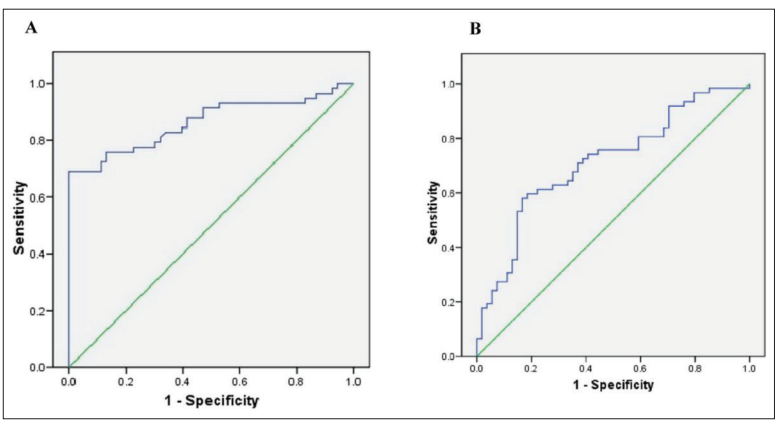

Fig. 3. **A** – ROC curve for the assessment of the classification characteristics of the proliferative index for the biological behavior of a tumor; **B** – ROC curve for the assessment of the classification characteristics of the IOD for the biological behavior of a tumor.

DISCUSSION

Salivary gland tumors are rare neoplasms that account for about 40 different tumor types. Considering their high variability in histological presentation and biological behavior, tumors of this localization pose a great challenge for both surgeons and pathologists in terms of diagnosis, prognosis and treatment. The evaluation of the mitotic index, primarily the Ki67 proliferative index, proved to be a crucial parameter for differential diagnosis, biological behavior and aggressiveness prognosis in many tumors. Our study encompassed 120 salivary gland tumors, both benign and malignant. We analyzed the proliferative index, and in correlation with other clinical-pathological characteristics, we obtained significant results for the differential diagnosis of these neoplasms.

For the purposes of our study, we used 5% as the cut-off value for the Ki67 proliferative index, but the existing literature includes studies conducted using higher values for tumor aggressiveness, such as >10% [17, 18]. Our results ranged from 0.76% to 13% in the benign tumor group. Recurrent cellular pleomorphic adenoma of the soft palate exhibited the highest value. In the malignant tumor group, the results ranged from 1.18% to 72.30%. Adenoid cystic carcinoma showed the highest proliferation, followed by salivary duct carcinoma, whereas polymorphous low-grade adenocarcinoma showed the lowest proliferation. These are very important and useful data for differential diagnosis, especially in cases of similar, almost overlapping histological presentations.

Similar to the results of our study, Shida et al. [19] obtained slightly higher values of the Ki67 proliferative index in the basal cell adenoma group. Our results match the results of Horii et al. [20], which showed the highest proliferative activity of pleomorphic adenoma in the benign tumor group. On the other hand, Terada [21] reported data on the proliferation in Warthin tumor of about 3%, which does not correlate with our results. By comparing the expressions of p53 and Ki67, Saghravanian et al. [22] stated that they are of great importance for the differentiation of polymorphous low-grade adenocarcinoma and adenoid cystic carcinoma. In their study, in the adenoid cystic carcinoma group, 24% of tumor cell nuclei displayed Ki67 expression as opposed to only 3.88% in the polymorphous low-grade adenocarcinoma group; our results are in correlation with these findings. Hellquist et al. [18] showed that a proliferative index higher than 10% is an independent parameter compared to the aggressiveness of acinus carcinoma cells. Similar results were obtained in [23,24]. A high degree of proliferative activity in patients with myoepithelial carcinoma correlated with an unfavorable course of the disease [25]. Based on the large variability of the results obtained in our study, as well as in studies of other researchers, low proliferative index values are not necessarily associated with a low-grade tumor. The results of our study revealed that the value of the Ki67 proliferative index varied depending on the case and the histological type of the tumor. The proliferative index varied from 0.76% in basal cell adenoma to 72.30% in adenoid cystic carcinoma. We proved that the index was considerably higher in the malignant salivary gland tumor group.

Six nuclear parameters were analyzed using the software pack ImageJ as follows: area, perimeter, circularity, roundness, Feret diameter and the IOD of 60 benign and 60 malignant salivary gland tumors. Digital images were taken on previously checked preparations stained using the standard (H&E) method.

Monitoring the nuclear parameters of benign and malignant tumors was performed within, as well as between tumor groups. A statistically significant difference ($P<0.05$) was found for the parameters related to the size of the nuclei (area and perimeter) and this was in direct correlation with the nature and type of tumor. The Feret diameter was higher in the malignant tumor group, but there were no statistically significant differences. The values of the parameters associated with the shape of nuclei, i.e. circularity and roundness, were slightly higher in the malignant tumor group. Statistically, the values of the IOD considerably depended on the nature and type of tumor ($P<0.05$), with salivary duct carcinoma showing the highest value. Multivariate logistic regression analysis pointed out IOD as the most important predictor in the differentiation of benign from malignant salivary gland tumors ($P<0.001$).

The available literature offers a limited number of studies dealing with the morphometric characteristics of tumors. Using light microscopy, we observed some

of the characteristics of nuclei, whether they are hyperchromatic, enlarged or small, with even or serrated edges, whether atrophy can be detected, the appearance of chromatin. Differential diagnosis of salivary gland tumors is often quite difficult, but morphometry enables the quantification of a pathohistological finding, reducing the possibility for errors in setting the final diagnosis. The morphometric analysis of salivary gland tumors has been applied, but the number of studies that have compared the results of these analyses in different tumor types is limited. In previous studies, the authors mostly examined the nuclear area, circularity, the perimeter and IOD using different software packages [26,27]. The results for these parameters revealed their diagnostic significance in the differentiation of various benign and malignant tumors of the parotid gland [28]. A study of the differentiation of ductal and lobular breast carcinoma on cytological material emphasized the applicability of this method, not only in the differentiation but also in grading the carcinoma itself [29]. A study on the morphometric characteristics of oral mucosa cells in patients with diabetes mellitus type I showed that nuclear parameters were statistically higher in this group of patients [30]. The morphometric characteristics of benign and malignant tumors of the parotid gland revealed that that the nucleo-cytoplasmic ratio was considerably higher in the malignant tumor group. However, this method is much more difficult for routine use, indicating that nuclear characteristics were the most useful for the differentiation of tumors; these results revealed higher values of these parameters in malignant tumors [31]. The examination of characteristics of salivary duct carcinoma with squamous differentiation showed that the mean value of the nuclear area of this type of carcinoma is lower than the mean value of squamous cell carcinoma formed in the area of pleomorphic adenoma and higher than the nuclear area in middle and high-grade mucoepidermoid carcinoma; moreover, a statistically significant difference between the nuclear area of salivary duct carcinoma and other salivary gland carcinomas with squamous differentiation was recorded [32].

CONCLUSIONS

Based on the results of the conducted study, we assessed and compared the characteristics of benign and malignant salivary gland tumors on standard H&E preparations and analyzed the Ki67 proliferative index and morphometric parameters of nuclear characteristics, and found that the Ki67 proliferative index showed statistically significant differences between benign and malignant salivary gland tumors. In the malignant tumor group, the proliferative index ranged from 1.18-72.30%, while in the benign tumor group it ranged from 1.30-5.30%. The obtained results are in accordance with the biological behavior of tumors, local aggressiveness, as well as the rate of spreading metastases. The morphometric parameters associated with the size of tumor cell nuclei, i.e. the area and perimeter, were statistically higher in the malignant tumor group. The values of the IOD were statistically higher in the malignant tumor group. Once again, salivary duct carcinoma exhibited the highest values. Multivariate logistic regression analysis showed that this variable was the key predictor in the differentiation of malignant cell nuclei. The morphometric parameters of IOD and the size of nuclei pointed to statistically significantly higher values in the malignant tumor group.

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Author contributions: NZ, MK and MJM conceived and designed the study, all co-authors performed the experiments and collected the data, TD, AC, MS and NZ analyzed data and wrote the manuscript.

Conflict of interest disclosure: The authors declare that they have no conflict of interest.

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