OBJECTIVE: To prove the origin of myoepithelial tumors using immunohistochemical analysis as well as to accurately differentiate benign from malignant tumors using the Ki-67 proliferative index and morphometric analysis of tumors' nuclei.

STUDY DESIGN: The study included 18 myoepitheliomas, 12 myoepithelial carcinomas, and 16 pleomorphic adenomas. Nine antibodies were used for the immunohistochemical analysis. A digital morphometric analysis was performed on tissue sections of a salivary gland tumor using ImageJ. Eight variables were analyzed. The Ki-67 labeling index was calculated from the ratio of the positive number of tumor cells to the total number of tumor cells counted per microscopic field.

RESULTS: The immunohistochemical analysis was essential in proving the origin of myoepithelial tumors. The value of the Ki-67 proliferative index is significantly higher in myoepithelial carcinomas as compared to myoepitheliomas (p<0.001) and pleomorphic adenomas (p<0.01). The following results were obtained using morphometric analysis. In the patients with myoepithelial carcinomas the values of nuclear size were significantly higher as compared to the patients with myoepitheliomas (p<0.05). In comparison to pleomorphic adenoma, the parameter values of area, perimeter, Feret diameter, and minimum Feret were higher (p<0.05), as well as the parameter value Integrated Optical Density (p<0.01). Compared to the patients with pleomorphic

Morphological, Immunohistochemical, and Morphometric Analysis of Myoepithelial Tumors of the Salivary Gland

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adenomas, the patients with myoepitheliomas expressed significantly higher Integrated Optical Density parameter values \((p<0.05)\).

CONCLUSION: We consider that it is necessary to do an antibody panel with the Ki-67 proliferative index calculation as well as to carry out the morphometric analysis of the nuclei of these cells. (Anal Quant Cytopathol Histpathol 2016;38:323–330)

Keywords: immunohistochemistry, morphometry, myoepithelial carcinoma, myoepithelioma, pleomorphic adenoma.

Tumors uncommonly arise in the salivary glands, and they comprise approximately 1% of all neoplasms in the whole body. They are known to have diverse histomorphological features in individual lesions, and there are a number of types and variants, in addition to histological patterns similar to those observed in different tumor entities. Therefore, these tumors may present a considerable diagnostic challenge.\(^1\)

Salivary gland neoplasms composed exclusively of myoepithelial cells (myoepitheliomas) are unusual and intriguing. Myoepitheliomas of salivary glands are extremely rare, comprising approximately only 1–1.5% of all salivary gland tumors.\(^2,3\) Their histopathologic features, immunohistochemical profile, and clinical behavior are not well characterized. A majority of the myoepitheliomas described in the literature have been benign, and the malignant counterpart (myoepithelial carcinoma) has been recognized recently.\(^4\)

Most myoepitheliomas occur in the parotid gland, and a few are located in the oral cavity.\(^5\) In 1943 Sheldon was the first to classify tumors as myoepitheliomas when he categorized 3 such tumors in a review of 57 mixed tumors of the salivary glands.\(^6,7\) A definition provided by the World Health Organization in 1991 distinguished a myoepithelioma from a pleomorphic adenoma, classifying it as an independent entity. These tumors exhibit a mixture of 4 cellular morphologies, but salivary gland tumors in which ducts comprise <5% of sections are classified as myoepitheliomas.\(^5,9\) Also, contrary to a pleomorphic adenoma, a myoepithelioma does not present a chondroid or an osteoid formation.\(^10\)

Myoepitheliomas are infrequent, encapsulated, and solid tumors of salivary glands with equivalent occurrence rates in males and females. Although these tumors are reported to develop at any time between the age of 6 and 81, the mean age for this tumor type is 40.\(^11-13\) Furthermore, the mean age of myoepithelial carcinoma patients is around 55 (range, 14–86), with the incidence being almost the same by gender.\(^5\) Histologically, myoepitheliomas are classified as epithelioid, spindle, plasmacytoid, stellate, and clear cell types, with epithelioid being the most common type, whereas the others are very rare.\(^14\) A myoepithelial carcinoma is an extremely rare salivary gland tumor described much later by Stromayer in 1975.\(^15\)

The differential diagnosis of these tumors is often quite difficult in the everyday practice of clinical pathologists. The histological image varies substantially, and it can often lead to some other tumor type. Considering the possibility of misdiagnosis, the aim and subject of our study was to prove the histogenesis of these tumors, as well as the differential diagnosis, by applying immunohistochemistry and morphometry.

Materials and Methods

Tumor Localization

Our analysis included 18 myoepitheliomas, 12 myoepithelial carcinomas, and 16 pleomorphic adenomas at the Pathology Department, Faculty of Medicine, University of Nis. The frequency between gender was even. The patients were between 48 and 70 years of age, and their characteristics are shown in Table I. Six out of a total of 30 myoepithelial tumors were localized on the hard palate, whereas the others were localized in large salivary glands—4 in the submandibular gland and 20 in the parotid gland. All analyzed pleomorphic adenomas were localized in the parotid gland.

Immunohistochemical Analysis

First, hematoxylin-eosin–stained slides of available paraffin blocks were reviewed, and then cases with a certain diagnosis and adequate cellular tissue were selected for immunohistochemical staining.

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissue sections.
using Dako-Autostainer Link 48 (Dako, Burlington, Ontario, Canada). Color was developed by EnVision Flex Target Retrieval Solutions (Dako) using diaminobenzidine (DAB) as the chromogen. Detection Kit (Dako) was used in accordance with the manufacturer’s instructions.

The following antibodies were applied: anti-Ki67 (MiB-1, ready to use; DAKO, Glostrup, Denmark), anti-Calponin (CALP, 1:50; DAKO), anti-carcinoma embryonic antigen (CEA) (II-7, ready to use; DAKO), anti–glial fibrillary acidic protein (GFAP) (6f2, ready to use; DAKO), anti-cytokeratin 5/6 (CK5/6) (D5/16B4, ready to use; DAKO), anti-vimentin (V9, ready to use; DAKO), anti-cytokeratin AE1/AE3 (CK-AE1/AE3) (AE1/AE3, ready to use; DAKO), anti-Wilms tumor protein (WT1) (6f-H2, ready to use; DAKO), anti-S-100 protein (S-100, ready to use; DAKO), and anti-cytokeratin 14 (CK14) (LL002, 1:20; Novocastra Laboratories, Newcastle, UK).

Morphometric Analysis
A digital morphometric analysis was performed on tissue sections of the salivary gland tumor using Image J version 1.43u (public domain software, Wayne Rasband, National Institutes of Health, Bethesda, Maryland). It included a high-resolution color digital camera (Nikon, DS-Fi1, Tokyo, Japan) transferring the image from the microscope (Nikon, ECLIPSE 50i) to a PC-compatible computer. Binary images were manually edited using a computer mouse. The analysis was randomly performed on 100 well-preserved tumor cells, on hematoxylin–eosin–stained sections without overlapping, at magnification ×400. Eight nuclear variables were estimated: area, perimeter, circularity, Feret diameter, Integrated Optical Density (IntDent), Feret angle, minimum Feret (MinFeret), and roundness. The measured results of the selected objects were automatically transferred and logged in previously defined tables.

Ki-67 Proliferative Index
The Ki-67 labeling index was calculated from the ratio of the number of tumor cells stained by Ki-67 to the total number of tumor cells counted per microscopic field.

Statistical Analysis
Continuous variables were described by means, standard deviations, and medians. The distributions of the continuous variables were tested for normality by the Shapiro-Wilk test. The differences between independent groups were analyzed by an unpaired t test in case of a normal distribution or by the Mann-Whitney U test if a distribution of data was not normal. The level of significance was set at 0.05. The calculations were carried out using the SPSS statistical package version 15.0.

Results
Microscopic Features
A myoepithelioma is made up entirely of modified myoepithelial cells in which the normal phenotypic expression of nonneoplastic myoepithelial cells has been changed. Parotid gland tumors are usually encapsulated, in contrast to those which arise in other major glands, such as the submandibular or sublingual gland, or minor salivary glands, which either tend to lack a capsule or have only a partial capsule but are invariably well circumscribed. Myoepithelial cells exhibit 5 cell morphologies: epithelioid, spindle, plasmacytoid, stellate, and clear cells (Figure 1). A tumor may contain a combination of these subtypes.

Epithelioid. Epithelioid cells were the predominant cell type in 47% of the tumors (14 of 30). These polygonal cells had central nuclei with coarse chromatin, prominent nucleoli, and pale eosinophilic or amphophilic, sometimes focally clearing, cytoplasm. No true glands or lumina were seen in this type.

Spindle. Spindle-cell morphologic characteristics were seen in 13% of the tumors (4 of 30). The cells were spindle-shaped with centrally placed, elongated, “cigar-shaped” nuclei with an intertwining fascicular pattern of tumor cell arrangement.

Plasmacytoid (hyaline). Four out of 30 tumors showed the predominant plasmacytoid cell morphologic characteristics (13%) imitating plasma cells. Plasmacytoid cell myoepitheliomas have round to ovoid cells with profuse eosinophilic cytoplasm and oddly located nuclei. The tumors showed a hyaline stroma.

Stellate. Six out of 30 tumors expressed unique cell morphologic characteristics (20%): the tumor cells were ovoid to short spindle with centrally placed nuclei, a moderate amount of cytoplasm, and unclear cell borders. The cells had a diffuse, sheet-like pattern arrangement.
Clear. Only 2 tumors were almost completely made up of epithelioid cells with profuse clear cytoplasm (7%). Due to the glycogen content, these polygonal tumor cells have clear cytoplasm. Sometimes the clear cells can exhibit a signet ring-like or lipoblast-like appearance.

Immunohistochemical Profile
The immunohistochemical analysis was performed in all cases, and the results of immunohistochemical staining are listed in Table II. The immunohistochemical profile of myoepithelial carcinomas did not show any predilection with respect to different cell types. By using immunohistochemistry we proved only the origin of tumor cells, which is significant in the differential diagnosis of all myoepithelial tumors. However, immunohistochemistry has no significance in the differentiation of benign from malignant tumors.

Ki-67 Proliferative Index
We compared the values of the Ki-67 proliferative...
index in benign and malignant myoepitheliomas and pleomorphic adenomas, considering the morphology and origin of these tumors. The value of the Ki-67 proliferative index is significantly higher in myoepithelial carcinomas, compared to myoepitheliomas (p<0.001) and pleomorphic adenomas (p<0.01). Statistically, this parameter value is also higher with respect to myoepitheliomas (p<0.5).

In general, the value of the proliferative index is highest in myoepithelial carcinomas (17.48±10.24%) and lowest in myoepitheliomas (2.48±1.05%). The proliferative index values in pleomorphic adenomas amount to 5.97±4.05%. The highest value of the Ki-67 proliferative index was recorded in patients with distant metastases in lymph nodes.

Morphometric Tumor Analysis

The research included 46 patients with a tumor, 18 (39%) of whom with the myoepithelioma type, 12 (26%) with the myoepithelial carcinoma type, and 16 (35%) with the pleomorphic adenoma type.

In the patients with myoepithelial carcinomas, the values of nuclear size (area, perimeter) were significantly higher as compared to those of the patients with myoepitheliomas (p<0.05). In comparison to pleomorphic adenoma, the parameter values of area, perimeter, Feret diameter, and MinFeret diameter were higher (p<0.05), as well as the parameter value IntDent (p<0.01).

Compared to the patients with pleomorphic adenomas, the patients with myoepitheliomas expressed significantly higher IntDent parameter values (p<0.05). The values obtained by the morphometric analysis of these tumors are shown in Table III.

### Differences in nuclear shape (circularity and roundness) were not statistically significant.

### Discussion

The majority of myoepithelial tumors are located in the parotid gland, whereas others arise in the submandibular gland or in the accessory glands of the oral cavity. The tumors are usually painless, and the duration of symptoms before the diagnosis may vary from months to years. Even though the clinical and biological behavior of these tumors is unknown, they are locally destructive, with metastases being uncommon. In our study of a total of 12 myoepithelial carcinomas, only 1 of them had metastases in the regional lymph nodes of the neck, whereas there were no other distant metastases. In a study that analyzed 48 patients with myoepithelial carcinomas, Kong et al found metastases in lymph nodes in 4 patients, which is consistent with our results.

When it comes to tumor architecture, different patterns may be seen—trabecular, nested, or solid. Moreover, an unusual reticular pattern variant has been reported by Dardick et al in which narrow, interconnected cords of tumor cells in a netlike fashion are surrounded by an abundant mucoid stroma or a loose vascularized stroma.

A myxoid, mucoid, or hyalinized stroma, which tends to be scant in hypercellular tumors, may be seen among tumor cells. Only 2 out of 30 analyzed myoepithelial tumors had a hyalinized stroma. Even though chondromyxoid stromata, which are normally seen in pleomorphic adenomas, are not

### Table II Findings of the Immunohistochemical Analysis

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Myoepithelioma N=18 No. (%)</th>
<th>Myoepithelial carcinoma N=12 No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK AE1/AE3</td>
<td>18 (100)</td>
<td>12 (100)</td>
</tr>
<tr>
<td>Vimentin</td>
<td>18 (100)</td>
<td>12 (100)</td>
</tr>
<tr>
<td>Calponin</td>
<td>18 (100)</td>
<td>12 (100)</td>
</tr>
<tr>
<td>WT1</td>
<td>14 (78)</td>
<td>10 (83)</td>
</tr>
<tr>
<td>CEA</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>CK 5/6</td>
<td>16 (88)</td>
<td>12 (100)</td>
</tr>
<tr>
<td>CK 14</td>
<td>18 (100)</td>
<td>10 (83)</td>
</tr>
<tr>
<td>S-100</td>
<td>18 (100)</td>
<td>12 (100)</td>
</tr>
<tr>
<td>GFAP</td>
<td>4 (22)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

### Table III Morphometric Characteristics of Tumor Nuclei

<table>
<thead>
<tr>
<th></th>
<th>Myoepithelial carcinoma (n=12)</th>
<th>Myoepithelioma (n=18)</th>
<th>Pleomorphic adenoma (n=16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area</td>
<td>55.01±12.39</td>
<td>42.42±7.36**</td>
<td>35.82±3.57</td>
</tr>
<tr>
<td>Perimeter</td>
<td>28.85±3.43</td>
<td>25.59±2.47**</td>
<td>23.28±1.37</td>
</tr>
<tr>
<td>Circularity</td>
<td>0.82±0.04</td>
<td>0.80±0.04</td>
<td>0.83±0.02</td>
</tr>
<tr>
<td>Feret diameter</td>
<td>10.68±1.30</td>
<td>9.71±1.10**</td>
<td>8.66±0.49</td>
</tr>
<tr>
<td>IntDent</td>
<td>7.30±1.38</td>
<td>5.88±1.32**</td>
<td>4.53±0.38**</td>
</tr>
<tr>
<td>FeretAngle</td>
<td>95.01±14.40</td>
<td>88.36±18.25</td>
<td>79.77±20.82</td>
</tr>
<tr>
<td>MinFeret</td>
<td>7.14±0.93</td>
<td>6.07±0.50**</td>
<td>5.72±0.28</td>
</tr>
<tr>
<td>Roundness</td>
<td>0.71±0.04</td>
<td>0.66±0.07</td>
<td>0.69±0.04</td>
</tr>
</tbody>
</table>

*p<0.05.
**p<0.01.
commonly seen in myoepitheliomas, chondroid metaplasia has been reported in some cases of myoepitheliomas and myoepithelial carcinomas, along with squamous, adipocytic, and osseous metaplasia.\(^4,20\)

Sometimes, the morphologic similarity between myoepitheliomas and pleomorphic adenomas can create complications in differentiating between them (for example, a myoepithelial-rich pleomorphic adenoma versus a myoepithelioma). However, the differentiation is based on the total absence of ductal elements. On one hand, some authors allow 5–10% of ductal differentiation within myoepitheliomas,\(^11,21\) while there are other authors who favor the total absence of ductal elements as a requirement for the diagnosis of myoepitheliomas.\(^4,22\)

Many authors consider that the behavior of myoepitheliomas and pleomorphic adenomas is similar, while others argue that myoepitheliomas tend to exhibit a more aggressive growth pattern and a less predictable biologic behavior on long-term follow-up.

In clinical practice the histopathological diagnosis of salivary gland tumors is made carefully through the assessment of the growth pattern of tumor borders, histological architecture, cellular structure and differentiation, and components of tumor stromata, along with clinical information. Although the gold standard method used for diagnosing salivary gland tumors is still hematoxylin-eosin staining, immunohistochemistry can improve the accuracy of such an analysis, even though its role may be limited. Immunohistochemistry can be a useful tool for examining those subjects that cannot be assessed by histological examination, such as cell nature and differentiation status, cell proliferation, and tumor protein expression. We herein show the usefulness of immunohistochemistry in defining tumor histogenesis in general surgical pathology practice. As suggested in various studies, calponin represents one of the most specific tumor markers of myoepithelial origin, regardless of the fact that its positivity was also noted in ductal cells. Furthermore, S-100 and vimentin exhibit almost the same characteristics. In accordance with the stated results of previous studies, our results also show the absolute positivity of these markers in myoepitheliomas and myoepithelial carcinomas. On the other hand, CK5/6 and CK14, markers which are also positive in these tumors, show positivity in the basal layer of the squamous epithelium as well. Considering a diverse histological image of myoepithelial tumors, CK-AE1/AE3 is of great differential and diagnostic significance in comparison to tumors of mesenchymal origin.\(^1,23-25\) In their studies, Curran et al and Shah et al discussed the significance of glial fibrillary acidic protein (GFAP) antibodies in the differentiation of pleomorphic adenomas with regard to other salivary gland tumor types.\(^26,27\) Moreover, positivity was observed in tumors of epithelial origin, though weak and rare, which is also in accordance with our results. Langman et al published a study in 2011 in which they discussed the importance of the WT1 antibody as a reliable marker of neoplastic myoepithelial cells.\(^28\) The results of our study support this fact, given that WT1 showed a considerably high sensitivity in tumors. Given the presented results, it is necessary to apply the mentioned group of markers in terms of higher reliability and certainty in tumor cell origin.

Cell proliferation is one of the crucial biological mechanisms in oncogenesis, with Ki-67 being the most commonly used cell proliferation marker. This antigen exists in all active parts of a cell cycle (the G1, S, G2, and M phases) but is absent in the G0 phase. Its expression increases with the progression of a cell cycle and reaches its peak during the G2 and M phases.\(^29\)

In this study we evaluated the Ki-67 expression, a proliferative marker in benign and malignant myoepithelial tumors. The Ki-67 labeling index was increased in malignant myoepitheliomas in comparison to benign myoepitheliomas and pleomorphic adenomas, thus indicating a high proliferative activity. This finding was in agreement with previous studies.\(^30,31\) In our study we used a 5% cutoff value for the Ki-67 index, as in the study of Faur et al.\(^32\) However, other studies used higher values. Nagao et al, Norberg-Spaak et al, and Prado et al noted correlations between the Ki-67 index and tumoral grade and suggested that a Ki-67 index value higher than 10% is suggestive of an aggressive tumor.\(^33-35\)

A standard morphologic analysis is to some extent considered to be a subjective method, and therefore the morphometric analysis—a quantitative method with objective and reproducible results—could be a very useful supplement to a standard microscopy analysis.

The field of nuclear cell morphometry is an innovative area of study which has had little
evaluation so far in the area of myoepithelial tumors. In the literature there are only a few works on digital morphometric analysis of salivary gland tumors, with a small number of morphometric parameters used in those studies.\textsuperscript{36,37} In our opinion, the reason for this is the lack of its application in a routine clinical setting.

In general, the results of previous morphometric studies conducted on different benign and malignant tumors reveal significant differences mainly in the values of nuclear morphometric parameters.\textsuperscript{36-39} As expected, our study confirmed that morphological criteria of malignancy are primarily related to changes in the nucleus. The present series is small, but our results suggest that relatively simple morphometric parameters may readily distinguish benign from malignant mixed tumors. Such information should be of great use in guiding therapy in patients with mixed tumors of salivary glands.

It should be taken into consideration that the available literature provides us with very few works on morphometric characteristics of salivary gland tumors, with not a single work on myoepitheliomas. Regarding this fact, we suggest the following parameters in the differentiation of these tumors: area, perimeter, Feret diameter, Integrated Optical Density, Feret angle and minimum Feret. Apart from these parameters, we also examined circularity and roundness, but they were of no statistical significance.

It is essential to draw attention to the fact that some myoepithelial carcinomas can demonstrate a very bland cell morphology that may not agree with the clinical impression of malignancy and thus make identifying a myoepithelial carcinoma a difficult task histologically, particularly on limited biopsies. The observation of an infiltrative pattern, focal necrosis, and perineural invasion among other malignant indices may be the only clue to a malignant change in a seemingly bland myoepithelioma, a task that may be possible only on a careful evaluation of a resected tumor.

Considering all the mentioned facts on the nature of these tumors, morphology, characteristics, and behavior, in order to ensure an adequate diagnosis, we consider that it is necessary to examine cell characteristics in great detail, to do a necessary antibody panel with the Ki-67 proliferative index calculation, and to conduct the morphometric analysis of the nuclei of these cells.

References


