**An integrated approach to the morphological diagnosis of different types of pleomorphic adenomas of the salivary gland: long-term research results**

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**Abstract**

**Introduction.** Morphological research methods play an important role in the diagnosis of pleomorphic adenoma in the preoperative and postoperative periods. Pleomorphic adenoma is histologically extremely heterogenous and has a complex structure, which sometimes causes certain difficulties and misdiagnosis for pathologists in the morphological diagnosis of this tumor and its histological variant determination. Pathologists emphasize that pleomorphic adenoma may be confused with myoepithelioma, adenoid cystic carcinoma, mucoepidermoid carcinoma, basal cell adenoma because of its varied histopathological presentation. The results of our own long-term research allow us to identify certain morphological features of various pleomorphic adenomas and, on their basis, to formulate an integrated approach to the morphological diagnosis of these tumor variants.

**The aim** is to describe an integrated approach to the morphological diagnosis of different types of pleomorphic adenomas of the salivary gland.

 **Materials and methods.** Surgical and biopsy material from 30 patients with pleomorphic adenomas of epithelial, mixed and mesenchymal variants was studied using histological, immunohistochemical, genetic, morphometric and statistical methods.

 **Results.** The results of research allowed us to identify methods for determination the pleomorphic adenomas types. The first method requires an immunohistochemical reaction with a monoclonal antibody to human papillomavirus type 16, followed by counting the percentage of positively stained cells in the tumor. Thus, the mesenchymal variant of the tumor is diagnosed when the percentage of positively stained cells is < 40%. In the mixed variant, this indicator is ≥ 40%, but ≤ 70%, and in epithelial variant – > 70%. The second method was based on the multivariate discriminant analysis. Three formulae were derived to determine the tumor types (Fmesenchymal = - 41.03 + 4.96Х1 + 1.11Х2, Fepithelial = - 22.27 + 3.46Х1 + 0.85Х2, Fmixed = - 122.25 + 5.63Х1 + 3.2Х2, here Х1 - number of vessels, Х2 – specific volume of parenchyma).

**Conclusions**. The authors identified several methods for determining the histological variants of pleomorphic adenomas. These methods will improve the morphological diagnosis of pleomorphic adenomas variants in the preoperative and postoperative periods.

**Key words:** diagnosis, morphology, pleomorphic adenomas types, salivary gland.

**Introduction.** Salivary gland neoplasms are a heterogeneous group of tumors, accounting for only 3 to 10% of all head and neck neoplastic processes [1, 2].

Pleomorphic adenoma is the most common benign tumor of the salivary glands with an incidence rate between 4.2-4.9/100,000 person-years [3]. The frequency of pleomorphic adenoma among all salivary gland tumors ranges from 32.6 to 78.6% [4].

Pleomorphic adenoma is more often localized in parotid glands (85%), minor salivary glands (10%) and submandibular glands (5%). It can also be located in the lip, cheek, tongue, floor of the mouth, etc. This tumor definitely shows a female predilection with male-female ratio of 8:13 (5). Pleomorphic adenoma may be diagnosed at any age, with a maximum incidence in the 4th life decade [1].

The histogenesis of pleomorphic adenoma continues to be a controversial subject. Thus, while some authors suggest that the two tumor components (parenchyma and stroma) originate from different sources, mesenchymal and epithelial, others support the unicellular origin of this tumor [1]. Some scientists explain the development of pleomorphic adenoma by the presence of reserve cells in the tumor that can transform into different directions [2].

The risk of malignant transformation of pleomorphic adenomas is rare. Only 3% recur at 12.5-year follow-up, of which 6% seem to show malignant transformation (carcinoma ex pleomorphic adenoma) [6].

The diagnosis of pleomorphic adenoma is crucial in the choice of the tactics of patient treatment, preventing the development of complications and recurrences. The diagnosis cannot be established only on clinical history and simple physical examination, and requires complementary diagnostic methods [7].

Morphological research methods play an important role in the diagnosis of pleomorphic adenoma in the preoperative and postoperative periods [8]. Pleomorphic adenoma is histologically extremely heterogenous and has a complex structure [9], which sometimes causes certain difficulties and misdiagnosis for pathologists in the morphological diagnosis of this tumor and its histological variant determination. Pathologists emphasize that pleomorphic adenoma may be confused with myoepithelioma, adenoid cystic carcinoma, mucoepidermoid carcinoma, basal cell adenoma because of its varied histopathological presentation [5]. The results of our own long-term research [10-15] allow us to identify certain morphological features of various pleomorphic adenomas and, on their basis, to formulate an integrated approach to the morphological diagnosis of these tumor variants. The aim of the study is to describe an integrated approach to the morphological diagnosis of different types of pleomorphic adenomas of the salivary gland.

**Material and methods.** In this study we used surgical and biopsy material from 30 patients with pleomorphic adenomas of the salivary glands. The patients underwent treatment in Kyiv City Clinical Hospital No. 12 (Ukraine, Kiev) from 2018 to 2019. The Ethics and Bioethics Commission of Kharkiv National Medical University approved the study, all the participants signed an informed consent in accordance with data protection regulation and the Declaration of Helsinki.

Morphological study of biopsy and surgical material was carried out at the Alpern Department of General and Clinical Pathological Physiology of Kharkiv National Medical University (Kharkiv, Ukraine), Department of Pathologic and Topographic Anatomy of Shupyk National Healthcare University of Ukraine (Kyiv, Ukraine). Among all cases of pleomorphic adenomas, we found a mesenchymal variant in 15 cases, a mixed variant in 10 cases, and an epithelial variant in 5 cases.

Surgical and biopsy material was fixed in a 10% solution of neutral buffered formalin, carried out according to the generally accepted method and embedded in paraffin. Serial sections of 3-4 μm thick were made from paraffin blocks. Microspecimens stained with hematoxylin and eosin were studied, using an Olympus BX-41 microscope (Japan) with subsequent processing with the Olympus DP-soft version 3.1 software, which was used to conduct a morphometric study. By morphometry in the tumor tissue, the specific volumes (%) of the parenchyma and stroma, the number of vessels in the field of view of the microscope at × 100 magnification was counted.

Using mouse monoclonal antibody (MCA) to human papillomavirus (HPV) type 16 (clone CAMVIR-1, «Diagnostic BioSystems», USA), the authors have carried out an immunohistochemical study. Brown staining of cell nuclei characterized positive expression of the marker. Visualization was performed, using an EnVisionTM FLEX detection system (Dako, Denmark). Antigen was unmasked in citrate buffer pH 6.0 at 95 °C. Primary antibodies were incubated at room temperature for 30 min, secondary – for 20 min. Sections were counterstained with Gill hematoxylin. The authors assessed the immunohistochemical reaction by a semi-quantitative method, counting the percentage of positively stained cells in the field of view of a microscope × 400.

The expression of microRNA-34a, microRNA-29a was assessed by reverse transcription and quantitative polymerase chain reaction (PCR) in real time. Reverse transcription was performed using a set of TaqMan MicroRNA reagents (Applied Biosystems, USA) with a specific primer for microRNA and 10 ng of total RNA. Real-time qPCR microRNA assays TaqMan (Applied Biosystems, USA) were used: U6 snRNA, ID001973 (as endogenous control), hsa-microRNA-34a, hsa-microRNA-29a, ID000426, ID002447 (Applied Biosystems, USA). Temperature cycles were as follows: initial denaturation step 95°C 10 min; 50 cycles of 95°C – 15 s and 60°C – 60 s. The level of microRNA expression was normalized to U6 snRNA and was presented in relative units (RU). Amplification was performed using real-time PCR 7500Fast (Applied Biosystems, USA).

The authors used the following methods of descriptive statistics in the study: means, errors of the mean, confidence interval (СI). The nonparametric Mann-Whitney U test was used to compare the means in the groups [16, 17]. Differences were considered significant at p<0.05. Methods of multivariate discriminant analysis were used to predict the type of the tumor [18]. In all calculations, we used Microsoft Excel. Statistical analysis was performed using IBM SPSS software Statistics 28 (license No. Z125-3301-14).

**Results.** The results of long-term research allowed us to identify an integrated approach to the morphological diagnosis of different types of pleomorphic adenomas of the salivary gland, which gives us several methods of their determination.

The first method requires an immunohistochemical reaction with a MCA to HPV type 16, followed by counting the percentage of positively stained cells in the tumor tissue. This MCA was expressed by the epithelial cells of the nests and strands, solid, trabecular, cystic, glandular, ductal and tubular structures; some myoepithelial cells; fibroblast cells, immune cells, vascular endotheliocytes, cellular elements of myxoid and mucoid zones. The percentage of positively stained cells significantly (p<0.05) varied in different pleomorphic adenomas (Table 1).

Table 1 shows the values of CI for the means, allowing us to determine the histological variants of pleomorphic adenomas. Thus, the mesenchymal variant of the tumor is diagnosed in cases when the percentage of positively stained cells is < 40%. In the mixed variant of the tumor, this indicator is ≥ 40%, but ≤ 70%, and in epithelial variant – > 70%.

**Table 1:** The percentage of positively stained cells in pleomorphic adenomas of various histological variants in an immunohistochemical reaction with a MCA to HPV type 16.

|  |  |  |
| --- | --- | --- |
| **Pleomorphic adenomas variants** | **M±m** | **СI (95%)** |
| **Mesenchymal** | 27.2±0.69  | (25.72; 28.63) |
| **Epithelial** | 75.8±0.75 *1* | (73.71; 77.89) |
| **Mixed** | 56.9±1.70 *2,3* | (53.06; 60.74) |

*1* – significant (p<0.05) differences of indicators in mesenchymal and epithelial variants of the tumor; *2* – significant (p<0.05) differences of indicators in epithelial and mixed variants of the tumor; *3* – significant (p<0.05) differences of indicators in mesenchymal and mixed variants of the tumor.

The second method for determining the histological variants of pleomorphic adenomas was not as simple as the first one, based on the multivariate discriminant analysis. The purpose of the latter was to determine two discriminant functions (canonical roots), which divided all objects into three groups depending on the histological variant of the tumor. These two discriminant functions are two hyperplanes, which divide the entire n-dimensional space of indicators into three regions corresponding to three tumor variants.

The authors determined the indicators for discriminant functions by the method of sequential selection of 5 parameters (specific volume of the parenchyma and stroma; number of vessels in the microscope field of view × 100; expression level of microRNA-34a and microRNA-29a (Table 2)) (10, 11). We obtained these parameters by the morphometric study. The values of the specific volume of parenchyma, specific volume of stroma, expression level of microRNA-34a, expression level of microRNA-29a were significantly (p<0.05) different in pleomorphic adenomas types. The absolute number of vessels was large (p<0.05) in the mesenchymal and mixed variants compared with the epithelial variant. The absolute number of vessels did not differ (p>0.05) in mesenchymal and mixed variants of the tumor (Table 2).

**Table 2:** Morphometric parameters of pleomorphic adenomas variants.

|  |  |
| --- | --- |
| **Name of parameter** | **Pleomorphic adenomas variants** |
| **Mesenchymal** | **Epithelial** | **Mixed** |
| **Specific volume of parenchyma, %** | 15.50±4.44 | 87.86±2.16 *1* | 53.67±1.49 *2,3* |
| **Specific volume of stroma, %** | 84.50±4.41 | 12.14±2.18 *1* | 46.33±1.48 *2,3* |
| **Number of vessels, absolute quantity** | 12.83±1.02 | 8.86±1.02 *1* | 12.50±0.84 *2* |
| **Expression level of microRNA-34a, RU** | 630.29±98.51 | 838.62±82.23 *1* | 305.59±57.18 *2,3* |
| **Expression level of microRNA-29a, RU** | 120.62±24.47 | 78.40±19.73 *1* | 22.23±3.55 *2,3* |

*1* – significant differences (p<0.05) of indicators in mesenchymal and epithelial variants of the tumor; *2* – significant differences (p<0.05) of indicators in epithelial and mixed variants of the tumor; *3* – significant differences (p<0.05) of indicators in mesenchymal and mixed variants of the tumor.

The authors took into account statistical significance of each parameter and its redundancy for classification. Thus, the discriminant functions contained only two parameters: "specific volume of parenchyma" and "number of vessels".

The statistical significance and quality of the resulting discriminant model were evaluated based on Wilks’ *Λ*-statistics and amounted to *Λ*=0.026 at F (4.52) = 67.5 (p<0.000).

The contribution of each parameter to the discriminant function was evaluated using the factor structure matrix (Table 3). Table 3 shows that the highest correlation with the first discriminant function (canonical root) was the indicator "number of vessels", and with the second – the indicator "specific volume of parenchyma".

**Table 3:** Factor structure matrix.

|  |  |  |
| --- | --- | --- |
| **Name of parameter** | **Canonical root 1** | **Canonical root 2** |
| **Specific volume of parenchyma** | -0,063 | -0,998 |
| **Number of vessels** | -0,978 | 0,205 |

Thus, three formulae were derived to determine the histological variants of pleomorphic adenomas (F*mesenchymal*, F*epithelial* and F*mixed*). We can determine the type of tumor with the highest value according to the classification function:

F*mesenchymal* = - 41.03 + 4.96*Х1* + 1.11*Х2*,

F*epithelial*= - 22.27 + 3.46*Х1* + 0.85*Х2,*

F*mixed*= - 122.25 + 5.63*Х1* + 3.2*Х2,*

here *Х1* - number of vessels, *Х2* – specific volume of parenchyma.

The accuracy of determining the histological variant of the tumor using classification functions was assessed based on aposteriori classification. This model showed 100% accuracy (Table 4).

**Table 4:** Matrix of aposteriori classification.

|  |  |
| --- | --- |
| **Pleomorphic adenomas variants** | **Rows: observed groups****Columns: stipulated groups** |
| **Percentage of faithful** | **Mesenchymal** | **Epithelial** | **Mixed** |
| **Mesenchymal** | 100% | 15 | 0 | 0 |
| **Epithelial** | 100% | 0 | 5 | 0 |
| **Mixed** | 100% | 0 | 0 | 10 |
| **Total** | 100% | 15 | 5 | 10 |

**Discussion.** The most common benign salivary gland tumor is pleomorphic adenoma [19]. In order to minimize the incidence of this neoplasm, the etiological factors causing it should be well known [20].

Pleomorphic adenoma is a polyetiological tumor. Viruses play a certain role in its development. The etiological role of HPV type 16 in the genesis of this tumor development is a debatable issue [21]. HPV type 16 is a high-risk oncogenic virus [22, 23].

A comprehensive immunohistochemical study with a MCA to HPV type 16 allowed us to reveal a causal relationship between the infection of a patient with HPV type 16 and development of pleomorphic adenoma of the salivary gland in him [12]. The MCA to HPV type 16 was expressed by the parenchyma and stroma cells of pleomorphic adenomas, consistent with the literature data [20]. Calculating the percentage of positively stained cells in various pleomorphic adenomas variants, we found that the maximum, moderate and minimum values, respectively, were in epithelial, mixed and mesenchymal tumor variants. Analyzing the mean CI of positively stained cells percentage, we identified the method for determining the histological variants of pleomorphic adenomas while morphologically studying the biopsy or surgical material.

In our previous studies [10] and literature data [1], it was noted that pleomorphic adenomas were characterized by a morphological heterogeneity, the presence of parenchymal (epithelial) and stromal (mesenchymal) components. Some scientists separate the myoepithelial cell component [5]. The ratio of parenchymal and stromal components can be different, which made it possible for us to distinguish mesenchymal, epithelial and mixed variants of this tumor during morphometric study. It was noted that the maximum, moderate and minimum values of the specific volume of parenchyma were in epithelial, mixed and mesenchymal tumor variants. The maximum, moderate and minimum values of the specific volume of stroma were in mesenchymal, mixed and epithelial tumor variants.

Vascularization has rarely been studied in pleomorphic adenomas. Some scientists have noted that pleomorphic adenoma is a poorly vascularized tumor [24].

Swelam W. et al. suggest that pleomorphic adenoma cells produce vascular endothelial growth factor (VEGF) in several functional forms for their own proliferation or differentiation, and that the VEGF expression is controlled by hypoxic circumstances of poorly vascularized pleomorphic adenomas [25].

There is no information in the literature about the vascularization features in different histological variants of pleomorphic adenomas. In our study, we noted that the mesenchymal and mixed variants were characterized by a large number of vessels compared to the epithelial variant.

Angiogenesis activation was found in carcinoma ex pleomorphic adenoma compared to pleomorphic adenoma. This process is necessary for tumor growth, invasion and metastasis [6, 24, 25].

The modern genetic direction in the tumors diagnosis is the study of microRNAs role. MicroRNAs are a group of endogenous 21-25 nucleotide noncoding RNAs which target gene coding in the posttranscriptional level. They are involved in various important biological processes such as development, differentiation, proliferation and apoptosis [26]. MicroRNAs appear to be new promising biomarkers for tumor diagnosis and prognosis [27]. Among all microRNAs, molecules responsible for apoptosis controlling (proapoptotic) (microRNA-29a, microRNA-34a) are of considerable interest.

In our study, we determined the expression level of microRNA-34a, microRNA-29a in various histological variants of pleomorphic adenomas. In all tumor variants, the expression of these microRNAs was increased compared to the physiological norm. We obtained the physiological norm of microRNA-34a, microRNA-29a expression level earlier and it amounted to 47.72±28.83 and 8.12±4.40, respectively [11].

Analyzing the expression level of microRNA-34a, we found that the maximum, moderate and minimum values were in epithelial, mesenchymal and mixed tumor variants, respectively. The maximum, moderate and minimum values of the expression level of microRNA-29a were in mesenchymal, epithelial and mixed tumor variants.

The statistical analysis of the above 5 indicators ("specific volume of parenchyma", "specific volume of stroma", "number of vessels", "expression level of microRNA-34a", "expression level of microRNA-29a") allowed us to identify two indicators ("number of vessels", "specific volume of parenchyma") which were used in determining the discriminant functions (formulas). These formulae help us to identify the histological variants of pleomorphic adenomas.

**Conclusions.** In consequence of a comprehensive study, the authors identified several methods for determining the histological variants of pleomorphic adenomas. These methods will improve the morphological diagnosis of pleomorphic adenomas variants in the preoperative and postoperative periods.

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**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

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