UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA
DOCTORAL SCHOOL

DOCTORAL THESIS

ABSTRACT

CONTRIBUTIONS TO THE HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY OF PLEOMORPHIC ADENOMAS OF MAJOR SALIVARY GLANDS

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Key words: pleomorphic adenoma, epithelial-mesenchymal transition

INTRODUCTION

Salivary glands represent the place of some tumoral localisations relatively rare as compared to other frequent localisations, affecting 3-4 people of 100.000 inhabitants. The pleomorphic adenoma is the most frequent salivary gland tumour [Ellis, Auclair, 1996; Eveson et al., 2005]. The etiology of parotid gland tumours is not completely known yet, but the main risk factor is represented by ionizing radiations [Ellis GL, Auclair PL, 2008; Pinkston JW, Cole P, 1999]. Also, as contributing factors may be mentioned: smoking, genetic predisposition, long use of mobile phones, viral infections and exposure to toxic substances such as nickel, lead, asbestos.

Starting from these aspects which the specialty literature signals, we have proposed this study, to contribute to a better understanding of etiopathogenic aspects of this affection, because, despite many studies carried out on this theme, there are still many unknown.

To that effect it has been carried out a histopathological and immunohistochemical study, on cases selected from the casuistry of the Pathological Anatomy Laboratory of Slatina County Clinical Hospital during 2010 – 2013.

STAGE OF KNOWLEDGE

CHAPTER 1
HISTOGENESIS AND HISTOPHYSIOLOGY OF SALIVARY GLANDS

Salivary glands are divided into two categories: major salivary glands and minor salivary glands (or small salivary glands). Major salivary glands are represented by three pairs of glands: the parotid gland, the submandibular gland and the sublingual gland.

Salivary glands have a tubulo-acinar structure, their morpho-functional unit being represented by lobules. Each lobule is composed of acini and ducts. Major salivary glands are composed of two major components: stroma and parenchyma. Stroma is represented by the conjunctive septums which begin from it and through which get in at the level of glands the vessels and nerves. The parenchyma is composed of two components: a secretory component (acini) and an excretory component (ducts). The secretory component is represented by acini of serous, mucous and mixed type. The excretory component, as compared to the localization, is

represented by two main types of channels and ducts, intralobular ducts and extralobular ducts [Crăițoiu Ș, Florescu M, Crăițoiu M, 1999].

The parotid gland represents the biggest salivary gland, having a craniocaudal size of about 5,8 cm and a dorsal-ventral size of about 3,4 cm. The average weight of the parotid gland is of 14g. The parotid gland has an irregular form, of feather, unilobular. The parotidian region, also known as retromandibular region, is an equal area, located behind the branch of the mandible, which contains the parotid gland [Crăițoiu Ș, Florescu M, Crăițoiu M, 1999].

The roles of salivary glands are fulfilled mainly by the roles of saliva in the organism. Besides their digestive role, the saliva also fulfils an essential role in maintaining normal trophicity of tissues in the oral cavity and in maintaining the structure and stability of teeth in the alveoli. The saliva fulfils multiple roles such as: digestive, protective, excretory, endocrine, hydroelectrolitical, in thermal regulation, in speaking.

CHAPTER 2
TUMOUR PATHOLOGY OF MAJOR SALIVARY GLANDS

Etiology of malign tumours of salivary glands is not enough known yet. Two theories are discussed: the „bicellular theory" and the „multicellular theory".

The „bicellular" theory asserts that the development of tumours is carried out based on one cell type of the two undifferentiated stem cells: the cell stock of excretory ducts or the cell stock of intercalated ducts. The „multicellular" theory asserts that each type of tumour is associated to a cell with specific differentiation, having its origin in the salivary glandular unity.

Recent studies suggest that „bicellular" theory of stem cells seems to be the base of the etiology of salivary gland tumours, explaining more logically why tumours like pleomorphic adenoma or Warthin tumour contain types of multiple distinct cells [Straif K et al, 1999; Stenner M, Klussmann JP, 2009].

The classification of salivary gland tumours is made in: benign tumours and malign tumours.

Pleomorphic adenoma, also known as mixed tumour, presents epithelial and mesenchymal histological structure. Pleomorphic adenoma has a frequency of 70% of major salivary gland tumours, being preponderantly located at the level of the parotid gland in the ratio of 52-84%, at

the level of submandibular glands in the ratio of 7-17% and at the level of other minor salivary glands in the ratio of 3-8%. The category of age preponderantly affected is comprised between 30 and 60 years, more often being affected the women than men [Spiro RH, 1986; Pinkston JA, Cole P, 1999; Everson JW, Cawson RA, 1985].

Microscopically, the pleomorphic adenoma is characterised by the concomitant presence of glandular epithelial cells and of mesenchymal cells. The epithelial cells have different forms, cubic, round, fusiform, polyedric, without mitoses, being grouped into glanduliform cavities. The myxomatous stroma has different aspects: hyaline, pseudocartilaginous, mucoid, myxomatous, in few cases being osteoid [Morinaga S et al, 1987; Stennert E et al, 2011].

Clinically, the pleomorphic adenoma initially appears under the shape of small nodes, clearly delimited, solitary or multiple, usually elastic, mobile on profound planes not painful spontaneously and on palpation.

From the **histological** point of view at the level of a pleomorphic adenoma of the parotid gland may be met glandular epithelial typical features as well as cell monstrosities which may present unspecific mitoses of glandular cells, things which lead to the idea of a possible malignant feature. Upon the microscopic analysis the pleomorphic adenoma is characterized by a morphologic multitude. It may be noticed epithelial and myoepithelial cells, it may prevail epithelial cells, moment in which it takes the name of cellular pleomorphic adenoma, it may be noticed a lipomatous differentiation or a bone metaplasia, and the mesenchymal tissue may be chondroid, mucoid or myxoid [Stennert E et al, 2011; Erlandson et al, 1984; Seifert G, Sobin LH, 1991] .

PERSONAL CONTRIBUTIONS

CHAPTER 3
HISTOPATHOLOGICAL STUDY OF PLEOMORPHIC ADENOMA OF SALIVARY GLANDS

The histopathological material originated from the casuistry of the Pathological Anatomy Laboratory of the Emergency Clinical Hospital No. 1 of Craiova and was represented by the archived paraffin blocks. We mention that the ***histopathological study*** investigated the main microscopic morphological features of pleomorphic adenoma of salivary gland. In the **morphological study** we used the classical histological technique by paraffin inclusion.

As **staining methods** we used:

❖ Hematoxylin-Eosin (H.E.) for the diagnostic re-evaluation in compliance with the classification criteria of mammary gland tumours established by WHO (2003);

❖ Masson's trichrome aniline blue staining to appreciate the degree of tumour fibrosis;

❖ Alcian Blue-Periodic Acid Schiff (AA-PAS) to appreciate the profile of mucins (neutral versus acid) secreted by tumour cells.

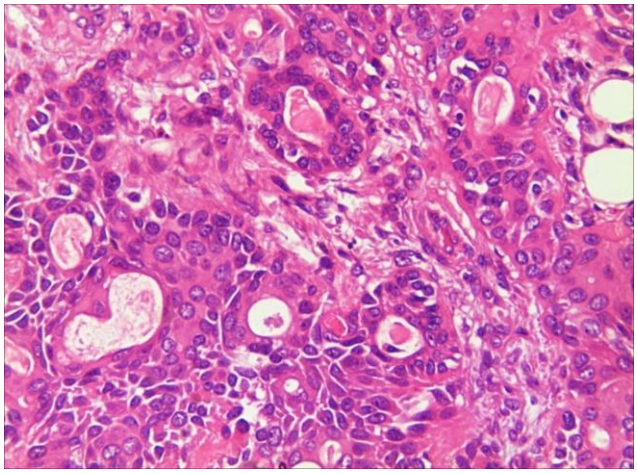

Fig. 3.1. Typical pleomorphic adenoma with predominance of epithelial component – tubular pattern - microcystic, tubular luminal epithelium. Col. H.E, X200

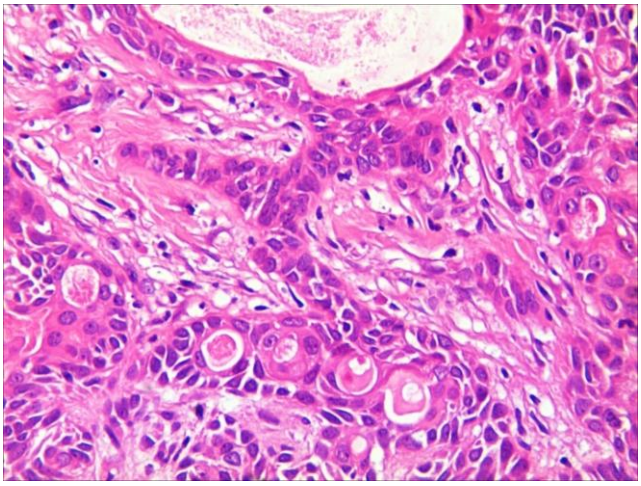

Fig. 3.2. Typical pleomorphic adenoma with predominance of epithelial component – oncocitary differentiation of tubular luminal epithelium. Col. H.E, X200

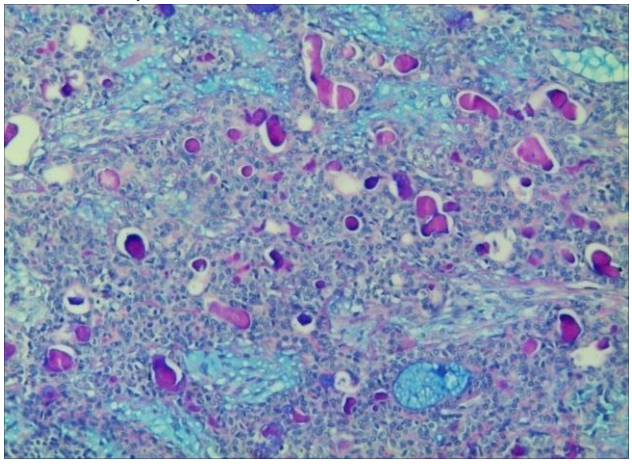

Fig. 3.3. Typical pleomorphic adenoma with predominance of epithelial component – predominant intraluminal material PAS + at the level of neoplastic tubular proliferations. col. AA-PAS,

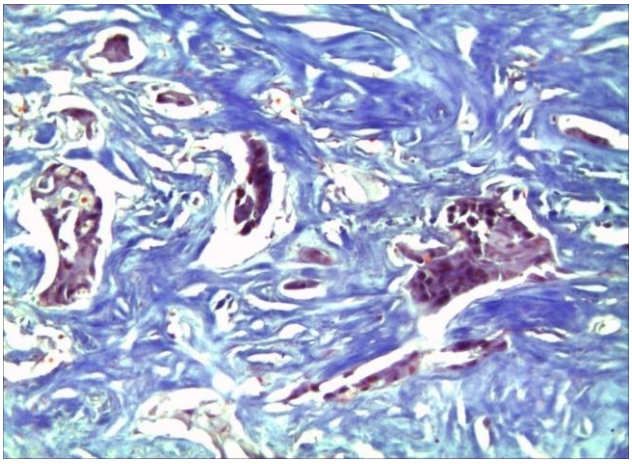

Fig. 3.4. Typical pleomorphic adenoma parenchyma/stroma balanced ratio – sclero-hyaline stromal areas. Col. Masson's trichromic, X100

The **ductal** neoplastic cell typically presented under the shape of a columnar or cuboidal cell, with pale eosinophil cytoplasm, finely granulary and with an ovalar nucleus centrally placed. These cells are placed on one single row, closely joined between them, delimitating the lumen of tubular, micro- and macrocystic, cribriform or pseudoangiomatous neoplastic proliferations (**Fig. 3.1.**). In another case, neoplastic epithelial component took the form of oncocitary morphology. Thus, we have noticed the presence of ductal structures of which lumen has been delimitated by high cylindrical cells, with nuclei placed in palisades in the centre of the cell or towards the lumen of cystic spaces, with granular fine cytoplasm and intensely eosinophil (**Fig. 3.2.**).

The lumens may be free or full with a poor eosinophil material similar to colloid, intensely positive PAS. This material is composed of neutral mucopolysacccharides being secreted by ductal luminal cells (**Fig. 3.3.**). Another aspect of the tumoral stroma met by us in 15 cases has been that **fibrous or fibro/ sclero-hyaline** represented by the presence of fascicules of collagen fibres of variable thicknesses (thicker under the sclero-hyaline form), placed among the epithelial proliferations, which often suffer hyaline dystrophy and even calcareous precipitations (**Fig. 3.4).**

The histopathological classification of pleomorphic adenomas depending on the stromal/parenchyma component.

Subtypes of pleomorphic adenoma	**Type I predominance of stromal component**	**Type II Balanced ratio**	**Type III predominance of epithelial component**	**Total no. of cases**
No. of cases	19	18	8	45
Percentages	42,2%	40%	17,8%	100%

Table no.3.1. Distribution of casuistry depending on the stromal component/tumoral parenchyma ratio

In the 19 cases of pleomorphic adenoma with predominance of stromal component, the epithelial component represented up to 20% of the tumour mass. The epithelial neoplastic proliferations had a trabecular, tubular and insular pattern, with small epithelial isles reduced to some groups of cells usually placed at the edge of the myxoid stromal component or inside it. In the 18 pleomorphic adenomas with stromal/parenchyma balanced ratio component was better represented, being up to 50% of the tumour mass. The dominant tumour pattern was the tubular and solid-insular one. In 8 cases of typical pleomorphic adenomas, the epithelial component was dominant, being 70-80% of the tumour mass. The epithelial patterns most frequently met have been the tubular and the insular ones. To these, on small areas, were added micro and macrocystic, cribriform or pseudoangiomatous types of patterns.

CHAPTER 4
IMMUNOHISTOCHEMICAL STUDY OF THE EPITHELIAL-MESENCHYMAL TRANSITION PROCESS IN PLEOMORPHIC ADENOMAS OF SALIVARY GLANDS

In the ***immunohistochemical study,*** the researched material was represented by 15 cases of pleomorphic adenomas of salivary gland representative for the group of 45 cases which constituted the object of the histopathological study.

In the immunohistochemical study of the 15 cases of pleomorphic adenomas of salivary gland we have used concentrated antibodies developed in mouse or rabbit directed against people, of which main features are given in the table below (**Table 4.1.**).

Table no. 4.1. Antibodies used in the study of pleomorphic adenomas of salivary gland

Antibody	Clone	Antigenic exposure	Dilution	Positive control
E-cadherin	Monoclonal mouse-NCH-38	citrate pH 6	1:50	Invasive ductal mammary carcinoma
Cytokeratin 18 (CK18)	Monoclonal mouse-DC 10	Proteinase K	1:30	Gastric mucous
Vimentin	Monoclonal rabbit-SP20	citrate pH 6	1:200	Tegument
Smooth muscle actin	Polyclonal rabbit	citrate pH 6	1:100	Mammary gland
P63	Monoclonal mouse-4A4-	citrate pH 6	1:50	Mammary gland
S100	Polyclonal rabbit	citrate pH 6	1:400	Tegument
GDF5	Polyclonal rabbit	citrate pH 6	1:100	Gastric mucous
Aggrecan	Monoclonal mouse-4F4	citrate pH 6	1:100	Bronchia
BMP6	Monoclonal mouse-Morph-6.1	citrate pH 6	1:50	Gastric mucous

Algorithm of immunohistochemical diagnosis

The antibodies used in this study especially „focused" on the epithelial-mesenchymal transition process in pleomorphic adenoamas of salivary gland. Thus some of them addressed to:

►double reactions of type *vimentin+CK18* focused on the epithelial-mesenchymal transition process;

►double reactions of *E-cadherin + Smooth muscle actin* focused on the epithelial-mesenchymal transition process;

►double reactions of type *GDF5+p63, Aggrecan+ Smooth muscle actin and BMP6+S100* focused on the myoepithelial-mesenchymal transition process.

►simple reactions *GDF5*, *Aggrecan* and *BMP6* followed the reactivity of various tumoral components of pleomorphic adenomas of salivary gland to these markers, with their possible implications in the epithelial-mesenchymal transition process.

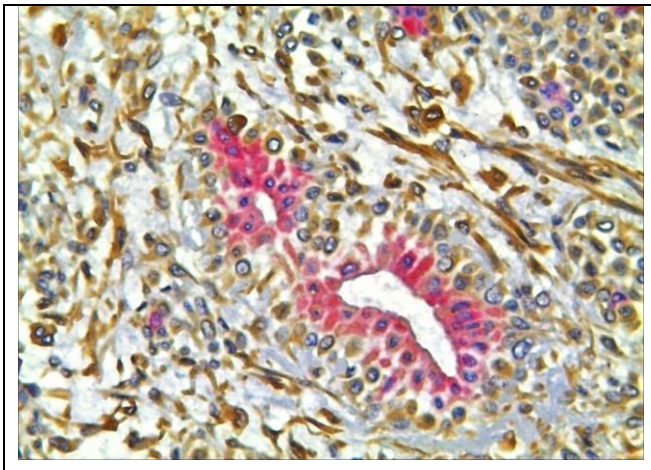

Fig. 4.1. Typical pleomorphic adenoma parenchyma/stroma balanced ratio - tubulo-cystic proliferative areas. Reactivity for CK18 (red) predominant of luminal cells. Col. IHC Vimentin/CK18, X200

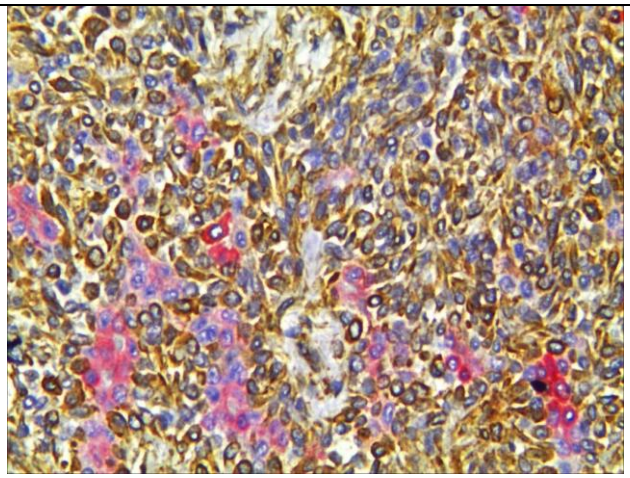

Fig. 4.2. Typical pleomorphic adenoma parenchyma/stroma balanced ratio – solid proliferative areas. Reactivity for CK18 (red) of some of abluminal cells. Col. IHC Vimentin/CK18, X100

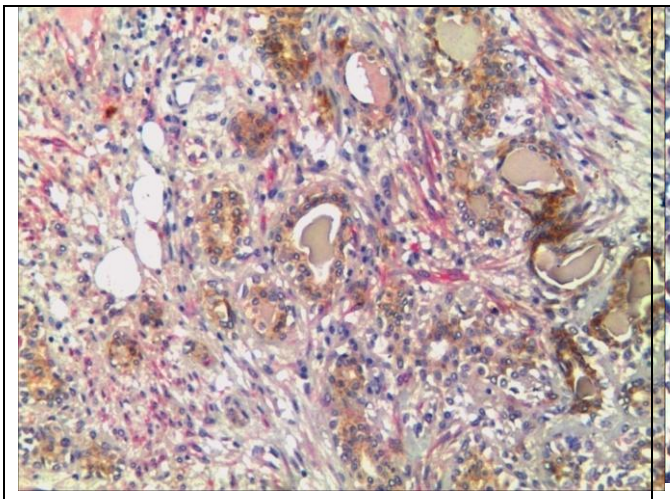

Fig. 4.3. Typical pleomorphic adenoma parenchyma/stroma balanced ratio - tubulo-cystic proliferative areas. Reactivity for E-cadherin (brown) predominant of luminal cells. Col. IHC E-cadherin/AMN, X100

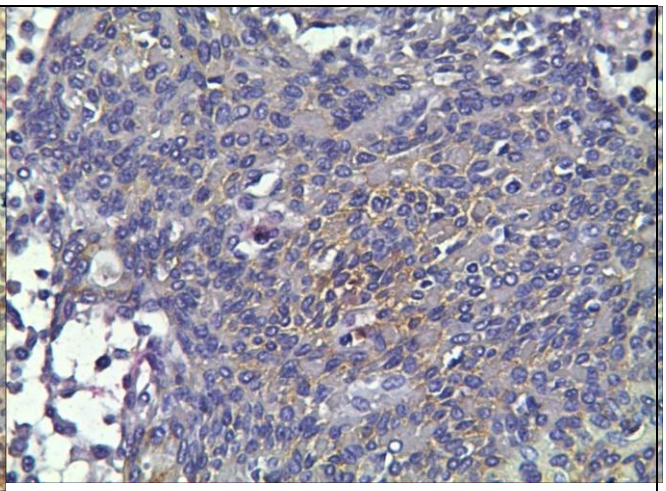

Fig. 4.4. Typical pleomorphic adenoma parenchyma/stroma balanced ratio – solid proliferative areas. Reactivity present for E-cadherin (brown) of abluminal cells in the centre of proliferations. Col. IHC E-cadherin/AMN, X200

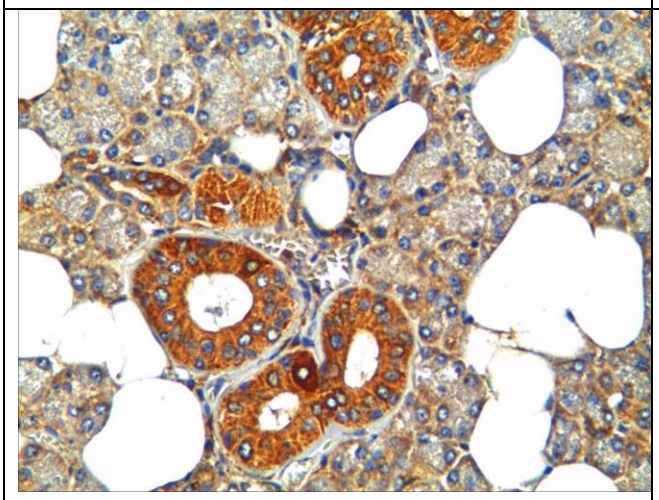

Fig. 4.5. Pleomorphic adenoma – residual glandular parenchyma. Cytoplasmic reactivity for GDF5 (brown) at the level of intralobular ductal units. Col. IHC GDF5, X200

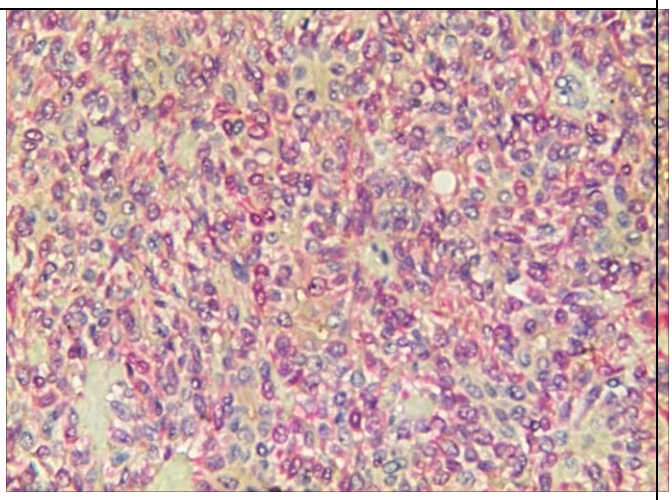

Fig. 4.59. Pleomorphic adenoma with predominance of parenchyma. Poor reactivity for BMP6 (brown) of neoplastic cells with fusiform and cuboidal morphology inside the solid proliferative areas, most of them being positive for protein S100 (red). Col. IHC BMP6/S100, X200

In the carried out study we noticed a variable immunoreactivity of these neoplastic cells especially for markers of type: alpha- smooth muscle actin (AMN), protein S-100 and P63. While

AMN and P63 were positive especially at the level of myoepithelial cells in epithelial proliferations, abluminal cells of proliferative ductal units and most of the cells in proliferation areas with solid pattern (cells with cuboidal, fusiform and plasmacytoid morphology), protein S-100 were present especially at the level of myxoid and chondroid stromal areas marking myoepithelial cells with star-shaped and plasmacytoid morphology as well as incomplete cells.

In the study carried out by us, the ductal **luminal** cells were positive especially for *CK18* and *E-cadherin*, certifying their epithelial origin. The reactivity for CK18 has also been noticed at the level of abluminal cells in the proximity of ductal luminal cells and more rarely at the level of some myoepithelial cells with cuboidal or plasmacytoid morphology in the composition of solid proliferative areas or myxoid areas.

Following the immunohistochemical doubles CK18/vimentin, we have noticed that some of the abluminal cells in the composition of ductal-like proliferative units and some of the myoepithelial cells with cuboidal or plasmacytoid morphology in solid proliferative areas co-expressed both markers. This fact could be the proof of the existence of a ***TEM*** type process through which luminal epithelial cells of ductal-like proliferative units would transdifferentiate in neoplastic cells of mesenchymal type towards the periphery of proliferative units.

GENERAL CONCLUSIONS

- Over the period 2010-2013 the pleomorphic adenoma represented the most frequent type of salivary gland tumour.
- Histopathologically, this type of tumour was characterised by a marked structural pleomorphism, given on the one hand by the multitude of cytological differentiations and of proliferation patterns, and on the other hand by the diversity of de stromal component.
- The cells in the myxoid areas in the immediate proximity of epithelial proliferative areas have a similar immunohistochemical profile of mixed epithelial-myoepithelial or epithelial-mesenchymal type. Some of neoplastic stromal cells with star-shaped or plasmacytoid morphology being at distance of epithelial proliferative areas have predominantly mixed epithelial-mesenchymal immunoprofile, and the rest of stromal cells in myxoid areas have a pure mesenchymal phenotype (expressing only vimentin and protein S100).
- The potential of epithelial-myoepithelial/mesenchymal transdifferentiation of luminal cells in the composition of proliferative units is proved by the immunohistochemical expression of these cells of pure mesenchymal protein cells of ă BMP6, aggrecan and GDF5 type.

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