

Immunohistochemical Identification of Cytokeratins in Salivary Gland Tumors

ALINA IACOB¹, ANCA SIN², SIMONA MOCAN³, ALINA ORMENISAN^{1*}, RALUCA MONICA COMANEANU⁴, VIOLETA HANCU⁴, EMOKE FULOP², MARIANA TILINCA²

¹Targu Mures University of Medicine and Pharmacy, Faculty of Dental Medicine, 38 Gh. Marinescu Str., 540139, Targu Mures, Romania

²Targu Mures University of Medicine and Pharmacy, Faculty of Medicine, 38 Gh. Marinescu Str., 540139, Targu Mures, Romania

³Emergency County Hospital Targu Mures, Department of Pathology, 50 Dr. Gh. Marinescu Str., 540136, Targu Mures, Romania

⁴Titu Maiorescu University of Bucharest, Faculty of Dental Medicine 67A Gh. Petrescu Str., 031593, Bucharest, Romania

Immunohistochemistry is an important resource in the diagnosis of tumors by defining the borderline in poorly differentiated tumors. The aim of our study was the histopathological diagnosis of malignant salivary gland tumors by hematoxylin and eosin (HE) staining techniques, and to identify cytokeratins (CK) as epithelial markers in order to distinguish the role of the myoepithelial component in tumor development. The accuracy of immunohistochemical diagnostic is based on strict adherence to a laboratory techniques of great value, which tends to be introduced into routine practice. Immunohistochemical methods is an integral part of histopathological diagnosis. Immunohistochemistry major contribution to the histopathological diagnosis is to identify the origin of tumor tissue causing undifferentiated approach and subsequent therapeutic conduct.

Keywords: immunohistochemistry, cytokeratins, salivary gland tumors

Immunohistochemistry (IHC) uses a step-by-step approach from a set of generic markers containing cytokeratins (for epithelial differentiation), melanocytic markers, CD45 (leukocyte common antigen for hematopoietic differentiation), and vimentin (for mesenchymal differentiation) [1]. The advantage of this method is the high sensitivity and specificity, and the classical correlation with morphological parameters. It can be used retrospectively and adapted to examine tissues both by optical microscopy and electron microscopy. The importance of this method has been recognized and improved over the time by precise determination of the chemicals used, their dosage and time of action, thus allowing deployment of chemical reactions under optimum conditions for viewing normal and pathological cellular and tissue structure for accurate diagnosis. Detecting intermediate filaments as markers plays a crucial role in the diagnosis of salivary gland tumors [2]. From this point of view, cytokeratins are important diagnostic markers in epithelial neoplastic processes. Immunohistochemical examination of salivary gland tumors implies complex rigorous processing of the biological sample collected surgically. This requires prior preparation of biological fragments in the laboratory, following the appropriate steps for the microscopic examination protocol. IHC is a new method that has no diagnostic value on its own, but it completes histopathological examination [3].

Salivary gland tumors are relatively rare with an incidence of about 2.5-3 cases per 100,000 inhabitants in the US, and represents approximately 3% of head and neck tumors. Most salivary gland tumors are related to the parotid gland (75-80%), of which only 20% are malignant [4, 5]. However, these tumors are important to identify because some may have an extremely aggressive development. Salivary gland tumors are classified according to the 2005 WHO criteria which divided these tumors into tumors of epithelial origin, 10 benign and 24 malignant types, and a significantly lower number of tumor

of other origins, mesenchymal or lymphoid [6]. Malignant salivary gland tumors, although a smaller proportion in the overall incidence of tumors, are very different and sometimes difficult to diagnose based only on immunohistochemical investigations, thus, they require histopathological investigations [3, 4, 7].

Experimental part

We conducted a retrospective clinical study at the Oral and Maxillofacial Surgery Clinic of Targu Mureș between May 2012 - February 2014. The study involved 118 patients hospitalized for various tumor formations (head and neck tumors, bone or soft tissue tumor lesions, lymph nodes, congenital tumors, salivary gland tumors, etc.). Of these patients 69 (58.47%) were male and 49 (41.52%) female, aged 18-92 years. Informed consent was obtained in accordance with the guidelines of the Ethics Committee of the University of Medicine and Pharmacy of Targu Mures (No. 30/ 26.06.2012). The following inclusion criteria were used: age over 18 years, and the presence of a tumor formation in the main salivary glands (parotid and/ or submandibular). We excluded patients who did not receive treatment or surgical biopsy, and who had no histopathological results in order to perform immunohistochemical tests, as well as patients with surgical contraindications or for biopsy sampling. Patients received surgical treatment, and the specimens were examined histopathologically.

The biopsies collected from the 118 patients were sent for processing, examination, and interpretation to the Pathology Department of the Clinical Emergency County Hospital of Targu Mures. All the biopsies were embedded in paraffin and examined histologically using HE staining. For all the cases diagnosed as malignant tumors, we performed immunohistochemical studies that focused on the expression of immunohistochemical markers of the cytokeratins CK 5, CK 6, CK 7, CK 20, AE1/ AE3, and pancytokeratin (Pan-CK).

* email: ormenisanalina@yahoo.fr

Histopathological technique used in the study group in order to select cases of malignancy for subsequent immunohistochemical evaluation

The study of cell structure, regardless of origin, requires a special cytology technique necessary to maintain structural integrity in conditions as close as possible to those existing in the living body. We used the method of fine slicing in order to obtain thin and transparent preparations. The main stages were: harvesting, fixing, washing, embedding, sectioning, staining, mounting, and labeling of the samples. For this purpose, the collected biological material was treated promptly with 10% formalin for 24 h, which has the ability to preserve the cellular structure indefinitely, by interrupting vital cellular processes and preventing autolysis. Washing with running water stopped fixation and artifacts were removed. These can occur due to coagulation proteins following fixation. Paraffin embedding consisted of tissue embedding in laboratory paraffin melted at +56°C. The paraffin solidified at room temperature to form a common block of the material to be studied. Since mass inclusion is anhydrous, the biological material is dehydrated by passing it in three successive 5-min baths of alcohol in increasing concentrations 70, 90, 100%. Subsequently, alcohol removal was performed by immersing the dried tissue in an embedding mass of xylene. Sectioning of the paraffin block was performed using Sakura Accu-Cut SRM microtome, yielding 0.3-0.5 μm thick sections. These sections were placed on glass slides previously treated with a thin layer of Meyer albumin. Sections placed on slides were stored in a thermostat at 57°C for 24 h. HE staining was used to visualize cell structures. Paraffin sections were placed in three baths of xylene for deparaffination for 10-15 min. Hydrated sections were deparaffinized by three successive 5 min baths of ethanol of decreasing concentrations 100, 90, 70%. Unstained sections were placed in aqueous hematoxylin solution for 1-2 min. Later they were stained with eosin solution for 2-3 min, after which staining was ceased by washing with distilled water. A dehydration step followed, the stained sections were immersed in three successive baths of ethanol of increasing concentrations 70, 96, 100% for five min. To remove the alcohol, slides were washed in three successive baths of xylene. Mounting of the stained sections was done using Canada balsam by covering them with a slip. Optical microscopy was used to examine the specimens.

Immunohistochemical technique to identify cytokeratins in malignant tumors confirmed by histopathology

The paraffin blocks were sectioned into 0.3-0.5 μm thick slices with a microtome on silanized slides. These sections were subjected to successive procedures auxiliary to the immunohistochemical reaction. Thus, sections were incubated in the thermostat at 57°C overnight and subsequently deparaffinized by three consecutive 5-minute baths of xylene. The sections were rehydrated by two 5-min baths in 96% alcohol allowing the penetration of the antibodies in the tissue antigens. Due to the fact that the enzymatic activity is typically present in erythrocytes, granulocytes, DAB chromogen (3,3'-diaminobenzidine dihydrochloride) reacts with the enzyme to form a brown precipitate which hampers interpretation of the immunohistochemical reactions. We inhibited the endogenous peroxidase by 3% hydrogen peroxide for 15 min. Unmasking antigens provides better accessibility and reduces the possibility of antigen nonspecific background

staining. Antigen unmasking was performed by moist heat. The slides were placed in special boxes which can hold the unmasking buffer, these slide boxes were placed in a thermostat with moist heat which maintains a temperature of 99-100°C in order to achieve a stable temperature of 98°C inside the boxes. After exposure in the buffer for 20 min, the slides were removed and allowed to cool for 20 min at room temperature. Washing was performed with PBS buffer solution in two baths of 5 min. Blocking non-specific reactivity was performed with the Large Ultra V Block for 5 min, followed by washing with PBS buffer for 5 min.

The immunohistochemical reaction was carried out in successive steps, as follows: primary AC pipetting: we used antibodies provided by DAKO (table 1) chosen based on sensitivity, and reactivity, as described in the literature. The washing was done with PBS buffer for 5 min. We used the Ultra Vision LP Detection, System AP Polymer, a detection system with second-generation polymer, which gave a good view of the antigens investigated by noise reduction. Detection of antigens consisted of two sub-stages: application of Primary Antibody Enhancer for 20 min at room temperature, followed by washing with PBS buffer for 5 min and AP Polymer for 30 min at room temperature. Washing was performed with PBS buffer for 5 min. To visualize the reaction, we used a microscope with the aid of diaminobenzidine (DAB, Lab Vision), which stains the investigated structures dark brown. Nuclear counterstaining was done with hematoxylin. Subsequent dehydration was performed with successive baths in 70% alcohol, 96% alcohol, absolute alcohol for 5 min. This was followed by three successive xylene baths for 5 min. Canada balsam was used to mount the sections.

Controlling the immunohistochemical reaction

External and internal control of the reactions (positive and negative control). Although positive immunoreactions remove much of the subjectivity of the examiner, a strict quality assessment of the sample is required to exclude false negative and false positive reactions. Thus, the interpretation of the immune reactions was done comparatively, reporting the results of the external and internal positive control, as well as the negative control of the reaction.

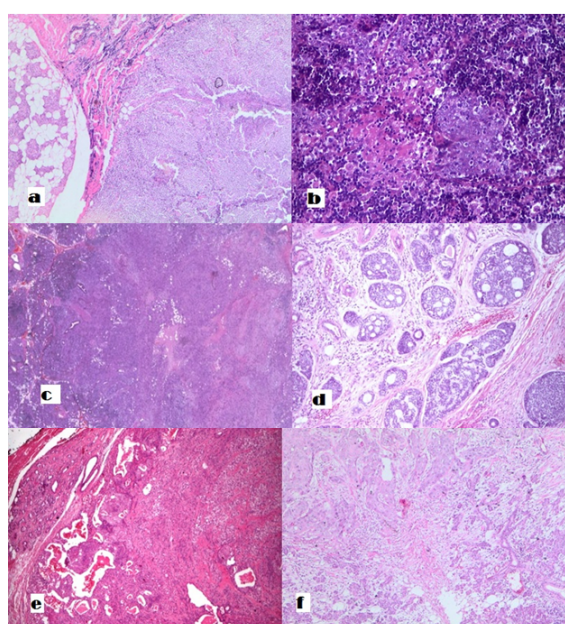
Negative control was the last section of the immunohistochemical panel, a section of a tumor for each group of studied tumors which underwent standard methodology with the exception of the primary antibody, replaced by negative control solution (solution used to dilute antibodies). Under normal conditions, the negative control does not present immunolabelling of any tissue component, which helped us in eliminating false positive reactions.

The external *positive control* was a histological section that indisputably contained the investigated antigen, previously revealed by the methodology used. This section had to present a diffuse and intense staining with a minimal background reaction. Apart from the examined slides, we included a representative one serving as positive control for each studied antigen (table 1).

To assess the positivity of immunohistochemical reactions, we considered positive reaction the immunolabelling according to the antigenic localization of each antibody (table 1). Lack of staining was considered negative reaction.

Name and features of the primary antibody	Dilution	Unmasking	Localization of the antigen	Positive control
Cytokeratin AE1/AE3 Dako	1:50	HpH	cytoplasmic	Epithelium
Cytokeratin 5/6 Clone D5/16B4 Dako	1:50	HpH	cytoplasmic	Squamous epithelium
Cytokeratin 7 Clone OV-TL12/30 Dako	1:100	HpH	cytoplasmic	Breast cancer
Cytokeratin 20 Clone KS 20.8 Dako	1:50	HpH	cytoplasmic	Digestive tract epithelium

Table 1
PRIMARY ANTIBODIES USED IN STUDY



Results and discussions

Based on the histopathological examination of the 118 patients included in the study, HE staining revealed 53 cases of salivary gland tumors. Of these, 39 (73.58%) cases were confirmed benign tumors, and 14 (26.42%) cases malignant tumors of the salivary glands. Histopathological examination identified 1 marginal zone lymphoma, 1 diffuse large B-cell lymphoma (fig. 1.a HE), 1 type of undifferentiated lymphoepithelial carcinoma (fig. 1.b HE), 1 myoepithelial carcinoma (fig. 1.c HE), 1 adenoid cystic carcinoma (fig. 1.d HE), 1 oncocytic carcinoma, 2 cases of mucoepidermoid carcinoma (fig. 1.e HE), and 6 metastases of squamous cell carcinoma (fig. 1.f HE).

The 14 cases of malignant tumors of the salivary glands which were subjected to IHC to determine cytokeratins

Fig.1. Results of histopathological examination. a. diffuse large B-cell lymphoma 2x, HE, b. lymphoepithelial carcinoma 4x, HE, c. myoepithelial carcinoma 2x, HE, d. adenoid cystic carcinoma 4x, HE, e. mucoepidermoid carcinoma 2x, HE, f. squamous cell carcinoma 4x, HE

Tumor (histological diagnosis)	No. of cases	Immunohistochemical markers	
		Positive reactions	Negative reactions
Marginal zone lymphoma	1	-	CK AE1/AE3
Diffuse large B-cell lymphoma	1	-	CK AE1/AE3
Lymphoepithelial undifferentiated carcinoma	1	CK AE1/AE3	CK 5/6, CK 7
Myoepithelial carcinoma	1	CK 5/6 and CK 7	-
Adenoid cystic carcinoma	1	CK 7	-
Oncocytic carcinoma	1	CK AE1/AE3 and CK 7	CK20
Mucoepidermoid carcinoma	2	CK AE1/AE3, CK 7, CK 20, CK 5/6	-
Metastasis of squamous cell carcinoma	6	CK AE1/AE3, CK 5/6	-

Table 2
RESULTS OF HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL EXAMINATIONS IN THE STUDIED GROUP

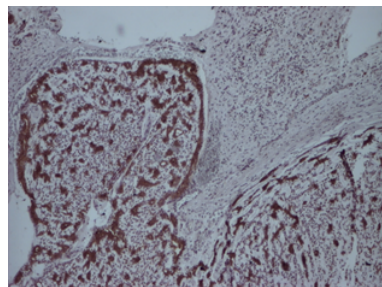


Fig. 2. Positive reaction for CK7 (a) and CK 5/6 (b) in the cytoplasm of tumor cells

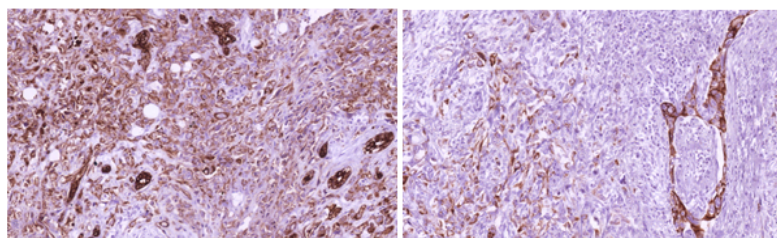


Fig. 3. Positive reaction for CK 7 in adenoid cystic carcinoma

CK 5, CK 6, CK 7, CK 20, AE1/ AE3, Pan-CK yielded the results presented in table 2.

The lymphoepithelial undifferentiated carcinoma located in the parotid gland presented, in terms of IHC, tumor cells positive for CK AE1/ AE3, and negative for CK 5/6 and 7 (with positive endogenous control on the slide).

In the case of the myoepithelial carcinoma of the parotid gland, IHC tests confirmed the myoepithelial origin of the tumor. The tumor cells were positive for CK 5/6 and CK 7 (fig. 2).

The location of the mucoepidermoid carcinoma in the submandibular gland and in the parotid gland showed tumor cells with intense and diffuse positive expression for Pan-CK, CK 7, focal to CK 20, CK 5/6.

In terms of IHC, the adenoid cystic carcinoma presented CK 7, which was positive in the ductal epithelial component (fig. 3).

The oncocytic carcinoma showed tumor cells with intense and diffuse positive expression for CK AE1/ AE3 and CK 7, and negative for CK 20.

The rare case of marginal zone lymphoma located within the parotid gland showed tumor cells negative for CK AE1/ AE3.

In another case of diffuse large B-cell lymphoma, the cells were negative for CK AE1/ AE3.

Microscopic features

Histopathological techniques are typically used to identify normal and pathological tissue structures. Histopathology is considered the gold standard in histopathological diagnosis because there are criteria that allow microscopic diagnosis of malignant or benign tumor. HE staining is used in this sense. Staining is considered correct if the expected results are obtained: nuclei are stained blue-violet because they contain nucleic acids, and the cytoplasm is red because of eosin. Amid the red cytoplasm cytoplasmic proteins can be distinguished which act as a base, releasing $-NH_3$ groups to which the stain attaches. Thus, fine granular proteins stained blue with hematoxylin can be identified. HE staining enables overall assessment of the main cellular compounds, as well as estimating parameters of cellular activities. The morphology of malignant tumor cells is different from that of the normal ones. The nucleus of tumor cells is larger; the nuclear membrane displays irregularities, the chromatin is more visible, hyperchromatically; the nucleus-cytoplasm ratio is greater; the nucleoli are larger and more numerous. The number of cells in mitosis is often higher in the cancer cell population, frequently more than 20 mitoses per 1000 cells, while benign or normal cells have a ratio of 1-1000. Abnormal mitoses, the feature of *giant cells* with polymorphic traits or multiple nuclei, are more commonly found in malignant tissue. The presence of normal tissue invasion by neoplasm is a definite feature of malignancy indicating increased likelihood of metastasis [8]. Histopathological diagnosis of salivary gland tumors is done by assessing the increase of the tumor margins, histological

architecture, structure and cell differentiation along with the components of tumor stroma. These issues must be combined with clinical data.

Immunohistochemistry is a relatively new, ground-breaking technique, conceived and perfected by Albert Coons in 1941, and it brings additional data to correctly identify cell differentiation and classification in difficult cases, particularly in malignant cells, with outstanding prognostic and therapeutic implications for the patient (C, E). IHC is an analytical method of molecular immunology that is applied on tissue sections. It is based on antigen-antibody reaction; the antigens are in the biological material, while the antibodies used can be mono- or polyclonal [9]. Viewing the different antigens can be done on tissue, cell, and subcellular paraffin samples. The most important condition for the success of IHC reactions consists in the correct fixation of the biopsy, so that epitopes or antigenic sites are not destroyed or inactivated. Optimal fixation lasts 24 h; fixation exceeding 48 h inactivates most epitopes causing false negative reactions. The optimum solution for fixation is 10% buffered formalin also used for histological processing.

IHC and molecular pathology are used in cancer diagnosis for a detailed classification of tumors. This approach has made it possible to identify numerous constituents with antigenic properties of the cells and intercellular matrix of particular importance in the histological diagnosis of tumors and other pathological processes. Mono- and polyclonal antibodies can help identify: the structural proteins in the cells, the cell cycle regulatory proteins, oncoproteins, substances in the intercellular matrix, viral and microbial antigens, etc. The most frequently examined structural proteins are those related to intermediate filaments of the cytoskeleton in the histopathological and differential diagnosis of tumors. Five types of filamental proteins can be distinguished: cytokeratins in epithelial cells, vimentin in the cells of mesenchymal origin, desmin in smooth and striated muscle cells, glial fibrillary acidic protein, and the neurofilament protein in nerve cells. In addition to the filamental proteins, there are also many other antigens/markers that can be identified with specific antibodies, such as membrane proteins, cytoplasmic and nuclear proteins, secretion products, and cellular receptors. Emphasizing them has diagnostic or prognostic significance also in neoplastic processes. The expression of listed cell antigens is the result of morphological and functional differentiation of normal or tumor cells. The most important and frequently used epithelial markers are the anti-cytokeratin antibodies.

Cytokeratins are fibrous structural proteins that are components of the intermediate filaments of the epithelial cell cytoskeleton and in cell junction structure, desmosomes and hemidesmosomes. Cytokeratins are represented by 20 different polypeptides, which are usually

expressed in pairs. Depending on their nature and molecular weight, cytokeratins are of two types: acid cytokeratins, or type 1, with a molecular weight of 36-64 kD, CK9, CK10, CK12, CK13, CK14, CK16, CK17, CK18, CK19, and CK20, and basic or neutral cytokeratins, type 2, with a molecular weight of 52-67 kD, CK1, CK2, CK3, CK4, CK5, CK6, CK7, and CK8. The expression of these cytokeratins is specific to a type of epithelium, which enables detection of various tumor originating cells. Low molecular weight cytokeratins (alkaline 7 and 8, acid 17, 18, 19, 20) are expressed by most epithelial tumors [10]. Their expression is different in stratified squamous epithelia depending on the layers and keratinized or nonkeratinized type of epithelium. Complex epithelia, as are the salivary glands, express a combination of cytokeratin. The main types and subtypes of cytokeratins and anti-cytokeratin antibodies are: CK 6 (GM 56 kD) present in hair follicle epithelium and suprabasal cells of stratified epithelia. It is expressed in approximately 75% of epidermoid carcinomas of the head and neck, but the expression is dependent on the degree of differentiation of the tumor. CK 7 (GM 54 kD) can be found in many epithelia including ductal, glandular, and transitional ones. Anti-cytokeratin 7 antibodies do not react with squamous epithelium. CK 7 is positive in simple epithelia: lung, cervix, breast, bile ducts, urothelium, kidney, and mesothelioma. CK 7 is negative in gastrointestinal epithelium, hepatocytes, and squamous epithelia. CK 20 (GM 46 kD) is positive for intestinal epithelium, gastric cells, foveolar cells, urothelial umbrella cells, and Merkel cells. It is present in adenocarcinomas of the digestive tract, gallbladder and pancreas, mucinous ovarian tumors, transitional carcinoma, and Merkel cell carcinoma. It is not obvious in epidermoid carcinomas, adenocarcinomas of the breast, lung and endometrial carcinomas, nonmucinous ovarian tumors, and small cell lung carcinoma. CK AE1 and AE3 are a combination of several types of cytokeratin comprising types 1, 2, 3, 4, 5, 6, 8, 10, 11, 15, 16, and 19. AE1 and AE3 antibodies are powerful markers of the epidermis, nonkeratinized stratified epithelia, digestive epithelium, urothelium, mesothelium, the epithelium of glandular excretory ducts, mammary gland epithelium. Pan-cytokeratin is a combination of types 5, 6, 8, and 18. It can identify all epithelia and all epithelial tumors and is used for differential diagnosis of anaplastic tumors [11, 12].

Adenoid cystic carcinoma is a malignant epithelial biphasic tumor composed of duct and modified myoepithelial cells. The tumor shows expression for CK7 markers [13].

Mucoepidermoid carcinoma is a malignant epithelial tumor composed of mucous, epidermoid, intermediate, columnar, clear, and oncocytic cells. It is positive for CK5, CK6, CK7, CK8, CK14, CK18, CK19, and negative for CK20 [13].

Myoepithelial carcinoma is a malignant tumor of the salivary glands with tumor cells exclusively presenting myoepithelial differentiation. Tumor cells can be varied and may appear as fusiform, stellate, epithelioid, plasmacytoid, and clear cells. Immunoreactivity for both CK and at least for one myoepithelial marker is mandatory in the diagnosis of myoepithelial carcinoma. Expression of CK AE1/ AE3 is important for the diagnostic certainty [13].

Oncocytic carcinoma is a rare malignant entity of the salivary glands, located mostly in the parotid gland, which appears as a painless, firm, fast growing mass. It can be uni- or multilocular, unencapsulated, with necrotic areas [4]. Immunohistochemically, tumor cells can present positivity for AE1/ AE3, CK7 [14].

Lymphoepithelial carcinoma represents less than 0.5% of malignant tumors of the salivary gland and it is a variant of anaplastic carcinoma with dense lymphoid stroma [8, 15, 16]. Undifferentiated lymphoepithelial carcinoma should be differentiated from malignant lymphoma, differentiation which is made on the basis of immunohistochemical reactions. Undifferentiated carcinomas are immunopositive for Pan-CK, and negative for leukocyte common antigen, while malignant lymphoma presents the opposite immunostaining results [3].

In pathology, as well as in the case of salivary gland tumors, IHC allows for a molecular typifying of the cells comprised by the tumor because these cells are similar and are indistinguishable by standard histological examination. Thus, we can distinguish between tumors with epithelial, acinar, ductal, myoepithelial, basal or mixed (most often) differentiation. Although many specific antibodies are used in current practice to differentiate primarily between epithelial cells, but also myoepithelial and basal ones, special importance is held by CK, these allowing for the identification of epithelial tumors as has been evidenced in this study.

The chemical and biochemical principles which apply for the laboratory processing of any tissue or cytological specimen must be specifically adapted to anatomical location. [17]

Salivary gland carcinomas, although rare, because of the aggression that they may develop and because of their location, imply a major responsibility of the entire medical team responsible for the high quality of the histopathological and immunohistochemical examination. Immunohistochemical diagnostic accuracy is based on strict adherence to a laboratory technique of great value which tends to be introduced into routine practice.

Conclusions

Cytokeratins are important epithelial diagnostic markers in neoplastic processes of the salivary glands that allow immunohistochemical identification of the tissue origin of undifferentiated tumors, thus directing approach and course of treatment. Immunohistochemical methods are an integral part of histopathological diagnosis of particular importance both in basic research and in clinical practice, with a major impact in diagnostic certainty as our study reveals.

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