

**UNIVERSITY OF MEDICINE AND PHARMACY CRAIOVA
DOCTORAL SCHOOL**

**PhD THESIS
-SUMMARY-**

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***STUDY OF ANGIOGENESIS IN ACINAR CELLS
CARCINOMAS OF THE SALIVARY GLANDS***

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INTRODUCTION

Salivary gland carcinomas are heterogeneous tumors characterized by local-regional invasion and distant metastasis. Acinar cell carcinoma (ACC) is a malignant tumor of a lower level that is clinically 1% of all salivary gland neoplasms, 5 to 11% of malignant tumors of the salivary gland and about 12.5% of the parotid gland carcinomas. [Eveson JW et al. 1985; HT Hoffman et al., 1999].

Angiogenesis is a prerequisite both for initiating the tumor process and for invasion and metastasis, and vascular endothelial growth factor (VEGF) is considered a prime mediator of this process. The relationship between VEGF and clinical outcome in malignancies from different locations were examined, giving inconclusive results. The proposed study aims to establish the degree to which angiogenesis contributes to the emergence and development of salivary gland carcinoma, particularly in acinar cell carcinoma and implicitly its prognostic value in the progression of this disease.

CHAPTER IV

MATERIAL AND METHOD

RESEARCHED MATERIAL

The study is analytical, retrospective and prospective, in which we compared the clinical, morphological and bio molecular features of acinar cell carcinomas of the salivary gland, diagnosed in the Pathology Laboratory of Clinical Emergency Hospital in Craiova, during 2000-2011. The group studied consisted of 12 patients hospitalized in the Clinic of Oral-Maxillofacial Surgery of the Clinical Emergency Hospital No.1 in Craiova with a diagnosis of ACC.

METHODS USED IN RESEARCH

For **clinical study** we investigated statistical retrospective and current observation sheets of patients diagnosed with acinar cell carcinoma.

For retrospective **histopathological study** we used paraffin blocks in the Pathology Laboratory of Craiova SCJU of which were performed serial sections stained with hematoxylin-eosin and PAS (Schiff periodic acid). For prospective study, of biological material obtained from surgical intervention were chosen three areas of interest in each tumor, including safety margins of the lesion.

In the **immunohistochemical study** we used paraffin blocks of which were performed sections required to classical histopathological processing with the usual stains. Immunohistochemical study was one with enzymatic detection using as a working technique the LSAB (Labelled Streptavidin-Biotin² System). The kit used was manufactured by Dako

Company, Redox, Romania - Code K0675. The result of these immunohistochemical reactions is to visualize antigens investigated using DAB chromogen by their brown coloring.

To highlight aspects of angiogenesis in acinar cells carcinomas of salivary gland were used also double sequential immunohistochemical reactions. Thus in a first reaction was visualized angiogenic target (CD105, VEGF, VEGFR1 and VEGFR1) and in the second part of immunostaining protocol we used primary antibody developed in the serum of an animal species different from that used in the production of primary antibodies used in the first reaction (alpha-amylase). As a working system in the second part of the protocol for double immunostaining we used the ABC-AP kit (alkaline phosphatase) (1:1:100, Dako) and for viewing fast red chromogen (Dako), which marked the antigenic target red. Lamellae were counterstained with hematoxylin eosin and were mounted with an aqueous medium based on glycerol (Dako)

CHAPTER V

CLINICAL AND EPIDEMIOLOGICAL STUDY OF ACINAR CELLS CARCINOMAS OF SALIVARY GLANDS

V.A. RESULTS

Clinical trial included a total of 12 cases diagnosed with acinar cell carcinoma between 2000 and 2011. For this time the distribution was random.

By studying the data in observation sheets we could do a statistical analysis, thus cases diagnosed with ACC had maximum incidence between 50-60 years in the females (F/M = 1.4/1), localized almost exclusively in the parotid gland (83.33% of cases). The analysis of clinical data shows that the presence of a parotid tumor formation was the most common reason for initial presentation. Most often they were presented as solitary nodules, painless, mobile, with a slow evolution without affecting the integrity of the skin or oral mucosa and no signs of peripheral facial paresis. Imaging investigations do not bring specific information to this pathology, but rather suggestive of a benign character tumor. Initial therapy for all cases was surgery.

V.B. DISCUSSIONS

From this study it appears that ACC represents 3.68% of all salivary gland tumors, a slightly higher percentage of those cited by some authors according to whom ACC represents 1% of all salivary gland neoplasms.

The result of the investigation of cases of ACC indicates an average of 45.75 years for the incidence of ACC and a ratio of women to men of 1.4 to 1. The proportion is similar to female-male incidence rate of 1.36 to 1 mentioned by other studies; data from the literature have shown a prevalence of ACC between the ages of 40 and 49 years.

According to the results, this type of salivary tumor develops mainly in the parotid gland, almost 80% of ACC occurring at this level. The study Casuistry confirms specialized studies in terms of the percentage of cases of ACC located in the minor salivary glands (16.8% in this study).

The reason for presenting of the patients in the study was the presence of a painless tumor formation parotid localized, i.e. upper lip, jugal mucosa evolving for several years. And in other studies was seldom reported the presence of pain; have been cases evolving for decades before presentation.

Many authors consider that, in ACC, the clinical pathological characteristics may be important to predict the outcome of patients, especially tumor size, presence of pain, compromised surgical margins and involvement of the deep lobe of the parotid gland.

Authors known in the ACC study estimate that, despite being a low-grade neoplasm, death rates due to this tumor is between 1.3% and 26%, with 8.3% to of 45% local recurrence, affecting regional lymph nodes 3.8 % -16%, and distant metastases of 2.6% - 14%.

CHAPTER VI

HISTOPATHOLOGICAL STUDY OF ACINAR CELLS TUMORS OF SALIVARY GLANDS

VI.A.RESULTS

Regarding pathological parameters we looked histological patterns, involving surgical margins, presence of perineural and/or vascular invasion.

We did histopathological diagnosis following diagnostic criteria established by WHO (2005). Thus we investigated tumors that were classified into one of the following: solid (4 cases), microcystic (1 case), papillary cystic (2 cases), mixt - solid + microcystic (2 cases), mixt with more than two patterns (3 cases).

Tumor growth pattern most frequently encountered was the solid type present in all investigated cases, but as proportion prevailing in only 4 cases (33.33%).

In terms of cytological differentiation of acinar type is most characteristic cell type, without being the dominant one.

Regarding *other morphological aspects*, in most cases, tumors infiltrated normal adjacent tissues. Stroma ranged from delicate fiber vascular tissue to dense collagen tissue. In two cases we observed in the stroma a marked presence of a lymphoid infiltrate, including the presence of germ centers, while hemorrhage and hemosiderin deposits were typically present in the stroma of these tumors.

There were no cases of perineural or vascular invasion, but lymphatic dissemination was observed in three cases.

VI.B.DISCUSSIONS

Most studies indicate laminar pattern of solid and microcystic type as major histomorphological patterns for ACC.

Sometimes a mixture of several *patterns of tumor growth* is a common aspect as observed in our study. In 42% of cases investigated we observed a mixture of two or more growth patterns with solid/lobular and microcystic patterns as the most commonly associated. The second most frequent pattern of growth was the solid one(33.33%), followed by papillary-cystic (16.66%) and microcystic (8.33%).

Although acinar cell type characteristic is serous, in the composition of this type of tumor are found, as indicated by well-known authors in the field, other *cytological differentiation* types: intercalated duct cell, vacuolar cells, clear cell, nonspecific glandular cells, oncocytic and even neuroendocrine cells. Usually these cells are found in various proportions in the composition of this type of tumor, but the dominant populations are the acinar and intercalated duct cells, as indeed we have found in the present study.

The ACC study found that almost 67% of diagnosed cases showed infiltration of normal adjacent tissues, dissemination in local regional lymph nodes in 25% of patients and none of the cases of perineural or vascular invasion associated. Despite general agreement that it is a tumor with low malignancy, there are authors who believe that most cases of ACC are infiltrative and are sometimes associated with recurrence and metastasis.

CHAPTER VII

PECULIARITIES OF ANGIOGENESIS IN ACINAR CELLS CARCINOMAS OF SALIVARY GLANDS

VII.A RESULTS

The study aimed to investigate angiogenesis in adenocarcinomas with acinar cells by assessing microvascular density CD105+, the expression of VEGF and its receptors VEGFR1 and VEGFR2, and to establish a correlation between these angiogenic parameters and major clinical and pathological variables of these patients.

Regarding the *expression of CD105 and microvascular density assessment (MVD)*, vascular endothelial cells positive for CD 105 were clearly identified by their brown DAB staining. In normal residual parenchyma of salivary glands from tumor resection margins, microvessel rarely expressed CD 105, and staining was weak and pale. We also observed a moderate staining for CD105 in skeletal muscle cells in cases with muscle invasion. CD105 stained intratumoral and peritumoral vessels intensively.

The density of microvessels in tumor specimens ranged from seven to 41 (median being 20).

Regarding *the expression of VEGF* in residual normal parenchyma of salivary gland responsiveness to VEGF was limited to the ductal epithelial cells and myoepithelial cells. Acinar reactivity to VEGF was observed mainly in serous acini. The most obvious, however, reactivity with VEGF was observed in the cytoplasm of tumor cells.

VEGFR1 expression in residual normal parenchyma of salivary gland responsiveness to VEGF was limited to the ductal epithelial cells and myoepithelial cells. We saw no reactivity for VEGFR1 in acinar level, but a weak reaction was recorded in the vascular endothelial cells.

Regarding *the expression of VEGFR2* in the tumor specimens, for VEGFR2 reactivity was lower than that for VEGFR1 and was observed in only 50% of the ACC investigated cases.

VII.B DISCUSSIONS

In the present study, the reaction for CD105 has identified a process of active angiogenesis in all cases of ACC investigated. We have also established a significant difference between MVD-CD105 values for different patterns of tumor growth. In the literature it is known that CD105 promoter is predominantly active in proliferating endothelial cells, and this molecule is currently under evaluation as an ideal target for anti-angiogenic therapies, aimed at preventing the development of a neovascularization. Therefore, of this therapy could benefit mainly patients with salivary gland tumors with high MVD, which express CD105.

Vascular endothelial growth factor (VEGF) is the main factor in promoting angiogenesis and expression may therefore be an indicator for the angiogenic potential and biological aggressiveness of the tumor. Our study has demonstrated that VEGF reactivity is mainly located in the cytoplasm of tumor cells with the highest intensity in the intercalary and unspecified glandular cell type, as well as microcystic and solid variants of ACC. The assumption that VEGF and angiogenesis may be used to prevent or treat salivary cancer metastasis is partially supported by the studies performed.

There are authors who argue that the biological activity of VEGF depend on its reaction with specific receptors VEGFR1 and VEGFR2.

CHAPTER VIII

CONCLUSIONS

Selected cases of ACC study during 2000-2011, admitted at OMF Surgery Clinic allowed the following observations:

- Acinar cell carcinoma is a rare tumor of the salivary glands, representing less than 4% of salivary gland tumors admitted the said time period.

- In our experience ACC developed especially in the fifth decade of life, and predominantly sex affected are females (58.33% or cases women / men = 1.4 cases).
- Lesion Topography of cases studied confirm the literature. The most common location was the parotid (80%) ACC developing almost exclusively at this level, most affected in the present study if the right parotid (60% of cases with parotid localization).
- Most cases of ACC studied were presented as solitary nodular formations, moving on over and underlying plans, with an average size of 2.5 cm. None of ACC cases taken from the study shows no facial paresis.
- Imaging investigations have not proven the efficiency in the orientation towards the malignant nature of acinar cell carcinoma, as of our results it is clear that CCA shows benign features on US and CT.
- Local clinical examination and imaging investigations could not determine changes that are characteristic of this type of tumor and may for just suggest a diagnosis. Only histopathology determines the diagnosis of certainty.
- About two-thirds of the cases were diagnosed stage I and II, while lymphatic dissemination was observed only in three cases, confirming the malignant nature of the neoplastic process.
- Superficial or total parotidectomy with facial nerve conservation as initial therapy seems to be the right attitude with regard to the absence of recurrence and metastasis in immediate postoperative period.
- Macroscopic appearance of operative parts is not suggestive to the malignant nature of acinar cell carcinoma because most tumors are unique lumps, well defined, even encapsulated.
- Postoperatively, patients may experience transient facial paresis, but this morbidity is insignificant compared to the overall benefit to reduce the risk of relapse and morbidity, respectively its associated mortality.
- Although considered a tumor with low malignancy grade, all cases of ACC taken in the study, confirmed by histopathology were oriented towards oncology department for therapy.
- It requires careful long-term monitoring of cases of ACC considering the warnings of authors in the field on the possible occurrence of relapses and metastases within 20 to 30 years postoperatively.
- This type of tumor was characterized by a certain degree of structural pleomorphism due to the many growth patterns (solid, microcystic, papillary-cystic and follicular) and the types of cytological differences that enter in the composition of this tumor can pose real problems of differential diagnosis.
- The most common type of growth pattern was the solid one, otherwise present in all cases investigated, but that prevailed in only 33.33% of cases.

However, in 42% of cases investigated we have highlighted the association of two or more patterns of tumor growth.

- Although serous acinar type is most characteristic histopathologic appearance of this type of salivary gland tumor, most common cytological differences in our study were the nonspecific glandular and intercalary duct-like type.
- For proper appreciation of this and that the percentage differentiation of acinar type, the best proved to be PAS-D stain, which allowed identification of zymogen-like granulations in the cytoplasm of neoplastic cells.
- The most frequent type of stroma was the fibrous one, but in two cases we noticed a significant association of lymphoid component, which presented even an appearance of lymphoid follicles with distinct germinal center.
- In 66.66% of cases investigated we recorded the presence of tumor invasion into adjacent tissues, but we did not note the existence of vascular or perineural invasion in any of the cases investigated.
- In 25% of cases we noticed this dissemination in lateral-cervical lymph nodes, and two thirds of the cases investigated were staged in stages I and II pTNM of disease. Such a profile would correspond to a low-grade malignant tumor.
- Acinar cell carcinoma is considered a low-grade malignancy without predictors of relapse or metastasis local or regional well defined.
- Our investigation certifies the existence of active angiogenesis in ACC, which is dependent on histologic variant, the higher scores of MVD-CD105 being recorded in CCA with solid pattern and particularly in areas associated inflammatory infiltrate.
- The reactivity of tumor cells for CD105 was one low and varied with cytological differentiation subtypes that go into these tumors. Highest immunoreactivity was recorded mainly in intercalary type tumor cells.
- VEGF Immunoreactivity in ACC has been shown to vary with the histopathological subtype and subtype of cell cytology differentiation. Thus maximum reactivity was found in microcystic and solid variants, respectively in tumor cells of intercalary type and nonspecific glandular type.
- VEGF immunoreactivity score was statistically correlated with pTNM stage, IHC 2 score being prevalent in stages II and III, this proving the prognostic value of this marker in evaluating this type of salivary gland tumor.
- Maximum intensity of VEGF immunomarker was recorded in the vessels of tumor stroma, being the growth factor most actively involved in angiogenesis of these tumors.
- In terms of VEGF receptor expression in ACC tumors we have shown that their reactivity was exceeded by that for VEGF and tumor reactivity for

VEGFR2 was lower compared with that in VEGFR1. In general, VEGFR2 immunoreactivity followed the reactivity tumor trend for VEGF and VEGFR1.

- Tumor Cytological Subtypes such as intercalary ductal cells type or nonspecific glandular type seemed to be the most reactive to these receptors. Co-expression of VEGF with its receptors at this level suggests the intervention of autocrine control loops that seem to be responsible for tumor progression.
- Greater immunoreactivity of vascular endothelial cells compared for VEGFR2 compared to VEGFR1 proves the key role played by this receptor in the angiogenesis of ACC.
- All of these markers may be important therapeutic targets especially in patients in advanced stages of the disease which leads us to assume that research of angiogenesis, of angiogenesis inhibitors may represent in the future the key to success in the treatment of this pathology.