UNIVERSITY OF MEDICINE AND PHARMACY

CRAIOVA

FACULTY OF MEDICINE

MD THESIS

ANGIOGENESIS AND LYMPHANGIOGENSIS IN ORAL SQUAMOUS CARCINOMAS

A HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY

ABSTRACT

MD COORDINATOR:
Professor MD Laurențiu MOGOANTĂ

MD-Title Candidate,
ADINA MAGDALENA BUNGET

CRAIOVA, 2013

INTRODUCTION

The oral cavity cancer is the 6th cause of cancer worldidely. Only in the USA alone there are diagnosed over 21,500 new cases of oral carcinomas every year and 6.000 Americans die annualy because of this illness (Landis and colleagues 1999). The incidence of oral carcinoma var iesworldwidely, rating over 40 cases in 100,000 inhabitants in some parts of France, South Asia, Hungary and Singapore.

The oral cancer etiology is multifactorial and still incompletely known. The main etiopathological factors are considered to be cigarrette smoke exposure and alcohol intake.

Chronic exposure to carcinogens determines genetical abnormalities at the level of oral mucous cells. When these genetical abnormalities determine protooncogene activation and tumoral supressor gene inactivation, the cells alter their growth and multiplication rythm. These cellular populations have a marked tendency to accumulate additional genetical abnormalities, due to their genomic instability. The genomic instability is the result of rapid cellular cycles and of the inadequate control of cellular proliferation due to a low capacity of readjusting defects. In these cellular populations, the accumulation ratio of acquired genetical abnormalities logarithmically increases with time (Califano and colleagues 1996).

Regarding the relation between alcohol intake and oral cancer onset, in 1988 the International Agency for Research on Cancer (IARC) showed that alcoholic drinks are carcinogenic for humans (Room R, Rehm J, 2010; Rehm J, Baliunas D, Borges GLG, et al. 2010). The mechanisms by which alcohol intake exerts its carcinogenic effect have not been exactly defined yet. Most often there is mentioned the genotoxic effect of acetaldehyde resulted during alcohol metabolism (Seitz HK, Stickel F, 2007; Gyamfi MA, Wan YJY, 2010). The alcohol may be an important factor in cancer onset, either by increasing the expression of certain oncogenes, or by reducing the cell capacity of DNA readjusting and thus increasing the probabilty of oncogene mutations onset.

In the last 20 years, in a great number of head and throat squamous carcinomas, there was determined the existence of genetical material of the Human Papilloma Virus (HPV) (Steinberg, 1999). Generally, in the case of squamous cell carcionmas the percentage of cancers containing the HPV genetical material seem to vary, depending on the anatomical localization at the level of the aerodigestive tract. According to the same author (Steinberg

1999), the tumoral localization with the highest HPV infection frequency is the tonsil (74%), followed, in a decreasing order, by the tongue (22%) and the hard pallate (5%).

CHAPTER I. Development of Oral Cavity

In the third week of intrauterine life, between the frontal prominence and the first branchial arch, on the ventral side, there appears a large, slightly deep, ectodermal cavity, called anterior aditus, deeply prolonging onto the pharyngeal membrane that separates it from the cephalic intestine. The anterior aditus cavity becomes more and more prominent and, thus, there forms the stomodeum or primitive mouth [2]. Step by step, the buccopharyngeal membrane perforates itself and dissapears, throuigh a necrosis process that starts at the beggining of the 4th week, when the stomodeum opens into the pharynx. By developing the hard pallate and soft pallate, the stomodeum will be separated into the nasal and buccal cavities.

Before the hard pallate formation in the primitive mouth, there appear the adenohypophysis and the pharyngeal bursa of Luschka.

Around the primitive buccal foramen, out of the mesoderm there develops some prominences that will give birth to the facial buds. Facial buds develop based on certain induction mechanisms, which also determine the development of cerebral vesicles. The facial region differenciates based on the frontal bud and the branchial arches during two important stages: budding stage and coalescence stage. Within the budding stage, out of the first brachial arch there emerge the superior maxillary bud and the inferior maxillary bud. Within the coalescence stage, the mandibular buds, arising from the 1st brachial arch, diagonally grow and develop, inward and foreward, joining on the median line. Through the two buds joining, right and left, there arises the mandibular arch. In the anterior and superior regions, the mandibular buds give birth to the inferior lip.

The nasal buds join and form the intermaxillary or incisive part that will subsequently form the superior arcade, by cleaving with the maxillary buds.

The superior maxillary buds laterally cleave with the mandibular buds and form the cheek regions and the buccal commissures.

The brachial arches are mesencymatous items demarcated by the ectoderm to the exterior and by the endoderm to the interior, and joined through ecto- and endodermal interbrachial notches. There are 4 pairs of well-represented brachial arches and two rudimentary ones. The first four arches lead to the formation of soft and hard parts of the throat and head, while the other two belong to the superior thorax.

Starting from the 5th week there forms the tongue, by the emergence of a mucous membranary sac that gradually fills with mandibular mass. The material comes from the mandibular buds, with the involvement of other brachial arches. The tongue body or the oral part is situated anteriorly to the lingual V, containing the gustative papillae, and plays a part in the process of mastication. It has an ectodermic origin, it will be formed of three lingual primordials belonging to the first branchial arch: a pair that represents the lateral lingual tubercles and a median swallowing situated between the other two, namely the uneven tubercle. The tongue root or the pharyngeal part contains glands and the lymphoid tissue, playing a part in deglutition. The two parts of the tongue are demarcated by the endodermic terminal notch that will from from the hypobranchial copula, emerging from the IInd branchial arch with the contribution of the IIIrd and IVth arches.

In the second month of intraurterine life there arises a proliferation of the embrionary oral epithelium of ectodermic origin, they extend towards the interior and forming thicknesses that deepen within the subadjacent mesencyme and they will generate:

- labial or vestibular blade, which will form the oral vestibule by resorbtion,

- primary dental blade, which deepens in the future maxillary mesenchyme, giving birth to dental buds.

Out of the primitive dental blade or primitve dental canal, towards the deep side, there leave 20 prominencies called the dental buds of primitive or temporary teeth. Starting from the 20th week, out of the primary dental blade, through its proliferation, there arises the secundary dental blade. This will determine the formation of permanent teeh buds that replace the temporary teeth. The dental buds, both the ones of the temporary teeth and those of the permanent teeth, emerge after certain epithelial and conjunctive cellular transformation.

CHAPTER II. Anatomy and Histology of Oral Cavity

The oral cavity is a complex, morphofunctional unit, made up of histologically and functionally different structures, which lead to a multitude of physiological acts at this level. The six walls of the oral cavity are constituted of osseous, rigid structures and of cutaneous, mucous, musculoaponevrotic structures that are mobile and allow alterations of the form and volume of oral cavity in relation to the functional necessities.

The oral cavity is divided into two compartments, separated by the alveolar and dental arcades:

- the buccal vestibule is demarcated anteriorly by the lips and cheeks and posteriorly by the dental and alveolar arcades. At the meeting point between the labio-jugal wall and the alveolar processes there form the vestibular sac bottoms, one for each maxillary;

- the oral cavity itslef, situated behind the alveolar and dental arcades.

The oral cavity is constituted of hard structures, represented by the teeth, the alveolar bone, and of soft structures, forming the oral mucosa. It is a pink, conjunctive epithelial membrane that lines the oral cavity interiorly and continues to the pharyngeal mucosa posteriorly.

Histologically, the oral mucosa is made up of a covering epithelium and a chorion, separated by a continuous basal membrane. The oral mucosa is a choriopapillary mucosa due to the fact that the chorion sends projections called papillae to the epithelium, while the epithelium, in its turn, sends certain epithelial apices among the papillae.

The epithelium covering the oral mucosa is a stratified, pavement-like epithelium, without any keratinization, presenting certain areas with otrho- or parakeratinization. Together with the epithelial cells (keratinocytes), in the basal and spinal strata, there may also be found other types of migrated cells: melanocytes, Langerhans cells, Merkel cells.

The chorion of the oral mucosa, also called the lamina propria, is mostly represented by a loose conjunctive tissue. It presents a variable structure and thickness, according to the topographical area of its location.

The vascularization of the oral cavity is very rich, being represented by branches of the internal carotid artery, which forms two arterial networks: one within the deep chorion and the other within the superficial chorion.

The innervation of the oral mucosa is well-represented both by nervous fibers belonging to the somatic nervous system, and by nervous fibers belonging to the vegetative nervous system. The nervous structures are represented by sensitive nervous fibers, branches of the trigemen nerve, and by the Meissner, Golgi, Rufini sensitive corpuscles, which ensure a tactile, thermic and painful sensitivity.

The lips are musculo-cutaneous formations that constitute those crinckles that form the buccal foramen. They present an external part, covered by skin, and an internal part, covered by the oral mucosa. Between the two layers, there may be found the orbicularis oris muscles that shape the lips, blood vessels, nerves and the sallivary glands. The red of the lips represents the tresspassing surface between the lips and the oral mucosa. Within the lip structure, first of all there may be found the orbicularis oris muscle, constituted of striated muscular fibers, which form a central skeleton. The red of the lips covers the free margin of

the lips, having a thinner epithelium than in other regions, and a very well vascularized conjunctive tissue, free of salivary glands. The presence of blood vessels near the epithelium confer the red aspect of this region.

The tongue is a musculo-epithelial organ, playing multiple physiological parts in sucking, phonation, deglutition and receptor for gustative sensitivity. It is made up of muscles constituting the central structure, lingual mucosa that lines the external surface, lymphoid formations that make up the lingual amigdala, vessels and nerves.

The teeth belong to the dental apparatus, being implanted in the dental arcades of the maxillae. Anatomically speaking, the teeth are constituted of three regions: the crown (the visible region within the oral cavity), the root (found within the alveolar bone) and the tooth parcel (the tresspassing area between the coronary and the root).

CHAPTER III. Malignant Lesions of Oral Cavity

The oral cavity cancer seems to be one of the most frequent neoplasias. The incidence of this illness varies depending on the region, from 3-4% to approximately 40%. The etiopathology of oral cavity cancer is heterogenous and incompletely known. Clinical and statistical studies showed that this illness is correlated to smoking and alcohol intake. In the United States, 90% of the risk for developing oral cavity cancer is directly attributed to smoking (Shopland and colleagues, 1991). The relative risk for developing oral cancer in heavy smokers is 7 times higher than in non-smokers. The risk for heavy drinkers is 6 times higher than in those who do not drink alcohol drinks. The risk for patients abusing both of ciggarettes and of alcohol is 38 times higher than in abstinent persons de (Blot şi colab. 1988).

Tobacco contains over 30 known carginogenic agents. Their majority are polycyclic aromated hydrocarbons and nitrosamines (International Agency for Research on Cancer 1986). High tar intake was associated with oral cavity and pharyngeal cancer in a dosis depending manner.

Alcoholic drinks intake also increases the risk for oral cancer and other types of head and throat cancer. Chronic drinkers present a cancer risk 10 times higher than abstinent or occasional drinkers.

A poor oral hygene is also associated with oral cavity cancer, without being established any direct causability relation. Chronic gum inflammation was met in cancer patients more frequently (Maier H et al, 1993).

Chronic infection with the Human Papilloma Virus (HPV) seems to be an important risk factor, especially for tongue and oropharyngeal cancer (Kreimer AR, et al, 2005). The

Herpes simplex virus was associated with oral cavity cancer. A stronger association was observed in the case of a HSV-1 infection history (Schildt EB, 1998).

Also, worldwide mortality varies depending on the region and country, on the social and economical status, on cancer diagnose programs and on health education. A 5-year survival rate for oral cavity and pharyngeal cancer is of 46% worldwidely, but it differs in developed countries (59%) and in developing ones (39%).

Premalignant lesions comprise a number of alterations of the oral cavity mucosa, with a varied morphology that, under certain circumstances, presents a risk for malignant transformation. These lesions may exist over a longer or shorter period of time before the onset of a malignant lesion at mucosa level. The most precancerous lesions of oral mucosa are represented by: leukoplakia, dysplasia, Bowen disease, Querat erythroplasia, exfoliate actinic cheilitis, oral florid papillomatosis, chronic candidiasis, lichen planus, submucosa fibrosis and xeroderma pigmentosum.

CHAPTER IV. Study Objectives

In the present MD thesis we proposed the following objectives:

- the assessment of tumoral lesions at oral cavity level in the persons admitted to a specialty healthcare service, namely the Clinic of Oral and Maxillofacial Surgery over a 5-year period, establishing the percentage of every tumoral lesion and especially of the squamous cell carcinoma;
- the assessment of risk factors involved in oral carcinomatosis;
- the assessment of social environment, sex and onset age of oral tumoral lesions;
- a histopathological study on oral squamous cell carcinoma;
- a immunohistochemical study on squamous cell carcinoma, by evidentiating the angiogenesis and tumoral lymphangiogenesis, the participation of macrophages and mastocytes within the angiogenesis processes and their correlation with the cellular proliferation factors.

CHAPTER V. Clinical and Statistical Study of Oral Cavity Tumors

Our study assessed the tumoral lesions at oral cavity level, in the persons admitted to the Clinic of Oral and Maxillofacial Surgery between 2008 and 2012, who were subjected to radical surgeries. The study comprised 143 patients, aged between 1 and 82 years old. By assessing the correlation between the social environment and tumoral lesions, we established that 64% came from the rural area and only 36% came from the urban area.

The oral tumoral lesions were mainly diagnosed in males: 96 were men (69%), while 45 (31%) were women.

Related to the entire group, a percentage of 27.2%, namely 39 patients had various vices, being either smokers (33%), or alcohol consumers (10%), the majority having both vices (57%). The ages of the patients were between 1 and 87 years old, with a mean value of 63±14.45 years old. The great majority of patients was over 50 years old (86.71%). Regarding dental hygene, the study showed that this was absent in a number of 76 patients (53), compared to a satisfactory dental hygene in 47%.

The localization of tumoral lesions was: at lower lip level (n=52, 36.4%), at mandible level (n=36, 25.2%), hard pallate (n=18, 12.6%), tongue (n=12, 8.4%), buccal commissure and superior lip (8 cases each, 5.6% each), tongue line and superior maxilla (4 cases each, 2.8% each) and at mandible arch level (3 cases, 2%).

The macroscopic aspects of oral cancers were extremely diverse; there predominated the infiltrative lesions, and the ulcerovegetative lesions as well. Most of the tumors were diagonsed in the IIIrd and IVth stages. The microscopic study revealed that out of 143 tumoral lesions, 18 were benign tumors, while 125 were malignant tumors. By far, of the malignant tumors, the main histological type of the studied tumors was the squamous carcinoma (n=115, 92%). Of these, the well-differenciated type of squamous carcinoma was found in 61 cases (53.05%), moderately differenciated squamous carcinomas in 36 cases (31.30%) and poorly differenciated squamous carcinomas in 18 cases (15.65%).

CHAPTER VI. Histopathological Study on Oral Squamous Cell Carcinoma

The histopathological study comprised 115 cases of oral squamous cell carcinomas, representing over 92% of the total of malignant tumoral lesions at oral cavity level. At the oral cavity periphery there were identified dysplastic lesions, both in the oral carcinomas and lip carcinomas, which confirms the hypothesis that neoplasic lesions are preceeded by dysplastic lesions. In serious dysplastic lesions, the covering epithelium histoarchitecture was completely affected, the whole epithelium being formed of cells presenting various atypias due to the loss of cellular polarity.

Out of 115 patients, with squamous cell carcinoma idenitified within our study, well-differentiated squamous carcinomas were represented by 61 cases (53.05%), moderately differenciated squamous carcinomas were diagnosed in 36 cases, representing 31.30%, while poorly differentiated squamous carcinomas were identified in 18 cases, representing 15.65% of the total number of squamous cell carcinomas.

Well-differentiated squamous carcinomas were made up of squamous cells islands, with various shapes and sizes, with keratin pearls inside, as a result of a "neoplastic maturation" process. Moderately differentiated carcinomas were made up of cordons or islands of atypical, neoplastic, ovalary, oblong, round epithelial cells infiltrating within the tumoral fibrous stroma, while poorly differentiated cell carcinomas appeared as cellular cordons, islands or cells with an epithelial aspect, having various shapes and sizes, with no similarity to the origin epithelium.

The tumoral stroma was made up of a young conjunctive tissue, rich in fibroblasts, congested blood vessels, angiogenesis vessels and inflammatory type cells, more numerous within the tumoral proliferation areas.

CHAPTER VII. Immunohistochemical Study on Oral Carcinomas.

Out of a total number of 115 oral squamous cell carcinomas histopathologically diagnosed, we selected 43 for the immunohistochemical study, leaving aside the old biological material that could be considered an artefact. In order to maintain the ratio of histopathologically diagnosed biological material, we selected 24 well-differentiated oral carcinomas, 15 moderately differentiated oral carcinomas and 4 poorly differentiated ones.

For the immunohistochemical study we used the following antibodies:

- PCNA, (Dako), IgG2a k, PC10 clone (cellular proliferation marker);
- p53 (Dako), IgG2b k, DO-7 clone (proto-oncogene);
- Ki-67 (Dako), IgG1k, MIB-1 clone (cellular proliferation marker);
- CD34 (Dako), IgG1 (vascular endothelium);
- CD68 (Dako), IgG1, KP1 clone (macrophages);
- D2-40 (Abcam), IgG1, M2A clone (lymphatic endothelium);
- Mast Cell Tryptase (Dako), IgG1, AA1 clone, M7052 (mastocytes).

The number of angiogenesis vessels was high both in the dysplastic processes and in well-differentiated or poorly differentiated carcinomas. We should mention that the number of angiogenesis vessels was significantly higher where the inflammatory-type cells were found in the tumoral stroma.

Regarding the lymphangiogenesis, in our study we observed that there is a high number of lymphatic vessels within the oral squamous cell carcinomas. We believe that these are new lymphogenesis vessels, which were the result of some lymphangiogenic stimuli, ellaborated by tumoral cells, either parenchymatous, stromal or inflammatory peritumoral ones.

In our study, the positive CD68 macrophages were highly idenitifed both in the stroma and in the tumoral parenchyma. The macrophages distribution was different in every case, even from one area to another within the same histopathological piece. Mastocytes were highly identified perivascularly and within the peritumoral inflammatory infiltrate. They played an active part in the process of angiogenesis.

P53 was highly positive in 86% of the squamous cell carcinomas included in the study, which indicates a deep alteration of the genetical material, while Ki67 was more highly positive in the poorly differentiated carcinomas.

PCNA was highly positive in all cases of the studied carcinomas.

Conclusions:

The squamous cell carcinomas represented 92% of the total number of malignant tumors diagnosed at oral cavity level in the patients admitted to the Clinic of Oral and Maxillofacial Surgery within the Clinical Emergency Hospital of Craiova, between 2008 and 2012.

The affection was found mainly in the male patients, the sex ratio being 2/1 in favour of the males. According to the social environment, 64% of the squamous carcinomas paients came from the rural area and only 36% came from the urban area.

The age of the patients with oral cavity tumors was between 1 and 87 years old, with a mean value of 63±14.45 years old. The great majority of patients were aged over 50 years old (86,71%).

The tumoral lesions were localized: at inferior lip level (36.4%), at mandible level (25.2%), hard pallate (12.6%), tongue (11.2%), buccal commissure (5.6%), superior lip (5.6%), superior maxilla (5.6%) and at mandible arch level (2%).

Out of 115 patients, well-differentiated squamous carcinomas were represented by 61 cases (53.05%), moderately differenciated squamous carcinomas were diagnosed in 36 cases (31.30%), while poorly differentiated squamous carcinomas were identified in 18 cases (15.65%).

The number of angiogenesis vessels and lymphogenesis, as well as their vascular extent, were high both in the dysplastic processes and in well-differentiated or poorly differentiated carcinomas.

P53 was highly positive in 86% of the squamous cell carcinomas included in the study, which indicates a deep alteration of the genetical material, while Ki67 was more highly positive in the poorly differenciated carcinomas.

References

1. Landis SH, Murray T, Bolden S, Wingo PA. Cancer statistics, 1999. CA Cancer J Clin 1999;49:8–31.
2. Shopland DR, Eyre HJ, Pechacek TF. Smoking-attributable cancer mortality in 1991: is lung cancer now the leading cause of death among smokers in the United States? J Natl Cancer Inst 1991;83:1142–1148.
3. Blot WJ, McLaughlin JK, Winn DM, et al. Smoking and drinking in relation to oral and pharyngeal cancer. Cancer Res 1988;48:3282–3287.
4. Califano J, van der Reit P, Westra W, et al. Genetic progression model for head and neck cancer: implications for field cancerization. Cancer Res 1996;56:2488–2492.
5. Room R, Rehm J. Alcohol and non-communicable diseases-cancer, heart disease and more. Addiction 2010;106:1-2.
6. Rehm J, Baliunas D, Borges GLG, Graham K, Irving H, Kehoe T, Parry CD, Patra J, Popova S, Poznyak V, Roerecke M, Room R, Samokhvalov AV, Taylor B. The relation between different dimensions of alcohol consumption and burden disease: an overview. Addiction 2010;105:817-843.
7. Seitz HK, Stickel F, Homann N. Pathogenetic mechanisms of upper aerodigestive tract cancer in the alcoholic. Int J Cancer 2004;108:483-487.
8. Seitz HK, Stickel F. Molecular mechanisms of alcohol-mediated carcinogenesis. Nat Rev Cancer 2007;7:599-612.
9. Gyamfi MA, Wan YJY. Pathogenesis of alcoholic liver disease: the role of nuclear receptors. Exp Biol Med 2010;235:547-560.
10. Steinberg BM. Viral etiology of head and neck cancer. In: Harrison LB, Sessions RB, Hong WK, editors. Head and neck cancer: a multidisciplinary approach. Philadelphia (PA): Lippincott-Raven; 1999. p. 35–47.