DOCTORAL THESIS

SUMMARY

The histopathological and immunohistochemical study of oral squamous carcinomas

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INTRODUCTION

Oral cancer accounts for approximately 3% of all human cancers and is currently a major health problem worldwide, both in frequency of lesions and their incidence in different age groups as well as by many risk factors involved. Over 90% of malignant tumors with oral location are squamous cell carcinomas (OSC), estimating over 125,000 annual deaths worldwide related to this tumor.

Oral carcinogenesis is a multistage process including precursor lesions and invasive and metastatic lesions, and relatively easy access to explore clinicopathologic oral squamous carcinoma tumors designate as an ideal model to investigate the cancerous process.

Clinical staging using TNM classification has long been used as a standard tool for planning treatment and estimating prognosis. However, this prognostic method does not provide sufficient predictive information about optimal treatment that would be beneficial for each patient. OSC pathogenesis involving multiple molecular pathways and therefore the molecular changes underlying tumor progression is obscure and is the subject of numerous studies. An important aspect of the OSC is the different biological behavior for patients with similar clinicopathologic features, some tumors having a better prognosis than others. Identification of molecular changes responsible for this process may contribute to a better understanding of tumor behavior. In recent years there have been identified several biomarkers that can provide useful prognostic information in the management of head and neck squamous carcinomas.

This study aims to evaluate the expression for several markers in order to identify their role in oral carcinogenesis, also following the possible correlations between these parameters and clinico-morphological analysis and selection of those with statistical significance. Thus, we followed for two categories lesion, dysplasia and carcinomas, the expression of p53, cyclin D1, p16, Ki67, EGFR, Her2/neu and CD44.

The study included a total of 224 OSCs that were analyzed clinically and epidemiologically. Subsequently, histopathological analysis followed histological variety, degree of differentiation, inflammatory response, pattern of invasion, presence of vascular and perineural invasion, metastatic adenopathy and status of safety margins.

Immunohistochemical analysis was performed on a lot of 44 selected cases and evaluated the immunoeexpression in oral squamous carcinoma of proteins involved in cell cycle (p53, cyclin D1, p16), cell proliferation (Ki67), tumor growth (EGFR, Her2/neu ) and tumor- peritumoral matrix relationship (CD44). Also the immunoeexpression of analyzed markers was evaluated and compared with clinico-pathological parameters, in this sense using statistical analysis (chi square, ANOVA).
Keywords: oral squamous cell carcinoma, dysplasia, degree of differentiation, invasion, immunohistochemical expression, prognosis.

I. CURRENT STAGE OF KNOWLEDGE

Chapter I- “Epidemiological considerations and risk factors in oral squamous carcinomas”- describes the incidence of oral squamous cell carcinoma in the world in relation to age, sex and oral location and depending on tumoral pathology in general. There are also analyzed the main risk factors involved in the occurrence of tumors in the oral cavity and squamous cell carcinomas in particular.

Chapter II- “Oral carcinogenesis: hypothesis, mechanisms”- investigate current hypotheses on the initiation and development of oral squamous cell carcinomas and systematizes genetic and proteomic changes in oral carcinogenesis. In this sense refers to genetic alterations that initiate the cancer, cell cycle alterations that trigger and maintain cell proliferation and disruption of mechanisms that ensure cellular homeostasis and provide tumor progression and metastasis.

Chapter III- “Prognostic factors in oral squamous carcinomas”- analyze clinical factors (age, sex, area of origin, location, tumor size, social status), histopathological factors (tumor stage and grade, pattern of invasion, histological type, margins of resection, presence of vascular invasion and metastases) and molecular factors (alteration of suppressor genes and activation of tumor oncogenes) on which can be appreciated oral squamous carcinoma prognosis.

Chapter IV- “The classification of oral tumors”- presents the last classification by 2005 of the Working Group for head and neck tumors within the World Health Organization.
II. PERSONAL CONTRIBUTIONS

The study proposes an evaluation of carcinogenesis process for squamous carcinomas developed in the oral mucosa and their precursor lesions, using traditional methods of investigation as well as modern techniques such as immunohistochemistry.

Identification of the complex mechanisms acting at the molecular level and the interactions between them provides valuable information on tumor initiation and development, and predictions on prognosis. In this study are evaluated markers involved in different stages of carcinogenesis and aims to identify possible prognostic and therapeutic targets. Research in this direction is very actual and achieving the required results can contribute to improve the quality of life and life expectancy of patients.

Specific objectives of the study include:
- Expanding knowledge about the clinical histopathological and immunohistochemical factors, involved in oral carcinogenesis, in order to deepen the mechanisms of initiation and its progression;
- To identify and define the clinical parameters that characterize oral squamous cell carcinomas and their precursor lesions, for their early diagnosis;
- To identify and define the morphological parameters that characterize oral squamous cell carcinomas, in order to apply early and differentiated therapies;
- Identification of mechanisms and markers involved in invasion and aggressivity of oral squamous cell carcinoma;
- Identification of the most specific markers for prognosis in invasive and metastasing carcinomas.

CHAPTER IV- „Material and Methods”- provides information about the material studied and methods used in research.

INVESTIGATED MATERIAL
This study was performed over a period of 5 years (2007-2011), including a total of 224 cases of OSC. The studied material was human material from patients hospitalized in the Clinic of Oro-Maxillo-Facial Surgery of the Emergency County Hospital Craiova. Human used material was represented by biopsy fragments or surgical excision pieces.

METHODS
From the laboratory pathological registers or directly, we obtained data regarding macroscopic aspects (shape, size, number, consistency) and histopathological diagnosis for the cases studied retrospectively.

In a first phase I followed to obtain epidemiological and clinical data on patients' sex and age, area of origin, risk factors, time since the onset of illness, symptoms, tumor location, size and their shape, presence of satellite adenopathy.

Pieces were processed by the classical technique with paraffin embedding and and Hematoxylin–Eosin stain, using also special stains such as Giemsa, which allow a good classification of lesions according to histological appearance.

Histopathological analysis was performed on a sample of 224 CSOs and included the following assessment criteria: histological variety, degree of differentiation, the extent of inflammatory response, pattern of tumor invasion, presence of metastatic adenopathy, presence of...
perineural and vessels invasion and presence of residual malignant cells at the safety surgical margins.

**Immunohistochemical technique** was performed for a group of 44 selected cases. Working systems used were the CSA II biotin-free catalyzed Amplification System (code K1497) for EGFR and LSAB™ + Kits, Universal (code K0679) for the other antibodies, for visualisation using DAB (diaminobenzidine).

The panel of antibodies used is presented below:

<table>
<thead>
<tr>
<th>ANTIBODY</th>
<th>CLONE</th>
<th>DILUTION</th>
<th>RETRIEVAL</th>
<th>INCUBATION</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>p16</strong></td>
<td>E6H4™, CINtec® Histology Kit ready to use</td>
<td>citrate buffer, pH 6</td>
<td>30 min RT</td>
<td></td>
</tr>
<tr>
<td><strong>p53</strong></td>
<td>DO-7, Dako</td>
<td>1:50</td>
<td>5 cycles MW in citrate buffer, pH 6</td>
<td>30 min RT</td>
</tr>
<tr>
<td><strong>cyclin D1</strong></td>
<td>EP12, Dako</td>
<td>1:100</td>
<td>7 cycles MW in Tris-EDTA buffer, pH 9</td>
<td>30 min RT</td>
</tr>
<tr>
<td><strong>ki67</strong></td>
<td>MIB1, Dako</td>
<td>1:50</td>
<td>5 cycles MW in tampon citrat, pH 6</td>
<td>30 min RT</td>
</tr>
<tr>
<td><strong>EGFR</strong></td>
<td>E30, Dako</td>
<td>1:1000</td>
<td>-</td>
<td>30 min RT</td>
</tr>
<tr>
<td><strong>Her2/neu</strong></td>
<td>polyclonal, Dako</td>
<td>1:250</td>
<td>7 cycles MW in citrate buffer, pH 6</td>
<td>30 min RT</td>
</tr>
<tr>
<td><strong>CD44</strong></td>
<td>DF1485, Dako</td>
<td>1:50</td>
<td>7 cycles MW in citrate buffer, pH 6</td>
<td>30 min RT</td>
</tr>
</tbody>
</table>

The panel of used antibodies

For the interpretation of obtained reactions we used a semiquantitative quantification that take into account the percentage of labeled cells and staining intensity, and for Her2/neu was made according to the criteria of the American Society of Clinical Oncology / College of American Pathologists (ASCO / CAP) for breast carcinoma.

Primary **statistical analysis** (mean, standard deviation) and comparison tests of the averages (ANOVA) were useful for assessing differences in the studied groups.

To assess the dependence of the two-factor classification chi-square test was used, the results with value less than 0.05 being considered significant.

For this purpose were used the module Data Analysys of Microsoft Exceel program and commands such Compare means, Descriptive Statistics and Crosstab within the module Analysis belonging to automatically SPSS 10software.
CHAPTER V- “Results” and CHAPTER VI- “Discussions” indicate the results obtained in the study, which are reported to recent data from literature.

In the clinico-epidemiological study are presented aspects of patients' gender and age, area of origin, risk factors, time since the onset of illness, symptoms, tumor location, size and shape, presence of satellite adenopathy. In this study lesions predominated in males (83.9%), with maximum incidence of diagnosis in the 7th decade of life (34.8%) in patients from urban areas (72.7%), most commonly being identified ulcerated tumors (37.9%), larger than 2cm (65.3%), with unilateral lymphadenopathy (46.1%) and localized to the lip (35.5%), followed by the lingual lesions (33%) . Also, smoking is indicated to be the main risk factor for oral squamous cell carcinomas and its association with alcohol, irritative factors or precancerous lesions were identified in 45.9% of cases. Most patients were presented to the doctor after a period of between 6-12 months after the disease debut, patients frequently accusing the pain, local discomfort or foreign body sensation. There were statistically significant differences (p <0.05) for the associated risk factors and the degree differentiation of tumor lesions according to their location.

Literature data indicate an average age of diagnosis of 64 years, 90% of patients having over 40 years (Jemal A, 2008). American Cancer Society estimated that in 2005 the oral cavity cancers represented approximately 1.9% and 0.9% of new cases of disease in men and women respectively (American Cancer Society, 2007). It is considered that diet rich in raw fruits, vegetables, bread, wheat and other cereals, fish, red wine which is specific to rural areas as compared with the rich in preservatives such as nitrites and nitrates, known to have an increased carcinogenic risk, specific for urban population, plays an important role in establishing rapport neoplasia identified between the two areas of patients origin (Bosetti C, 2003). Smoking and alcohol abuse are considered dominant risk factors with a strong synergism, its antecedents being found in 75% of patients with cancers of the oral cavity and in the oropharynx (Johnson N, 2001). It is estimated that 30-35% of tumors have lingual location, followed by gingival location in 20-25% cases and much lower percentage of the floor with 5-7%, soft palate with 4-6% and genian mucosa with only 2-3% (Wooglar JA, 1995). The aspects found about macroscopic development pattern and location of oral malignant tumors are similar to literature data (Reichart PA, 2000).

Tumour size include diameter, width and depth of the tumor. TNM system considers only the tumor diameter. However, many studies have confirmed that constantly evaluation of all these parameters seems to be the only independent prognostic factor negatively affecting metastases to lymph nodes, local recurrence and survival (Brown B, 1989, Jung J, 2009). Several studies have suggested a quick way classification of a node according to its macroscopic form, such as reniform shape indicate a reactive hyperplasia adenopathy, and round shape or lymph node clustering indicate the neoplastic infiltration (Soames JV, 2005).

Histopathological analysis followed histological variety, degree of differentiation, the extent of inflammatory infiltrate, pattern of tumor invasion, presence of metastatic adenopathy, presence of perineural invasion, vessels invasion, the presence of residual malignant cells at the safety surgical margins.

OSC with keratinization (50%) or without keratinizations (32.1%), moderately differentiated (41%), with lymphoid infiltrate at the interface of tumors- adjacent matrix (83.4%) and infiltrative pattern of invasion (43.7%) was the most commonly reported. Over the past two decades have been proposed several histologic parameters as prognostic factors including invasion pattern, degree of keratinization, nuclear pleomorphism, mitotic rate and lymphocytic inflammatory response (Bundegaard T, 2002). Despite the development of management overall survival of patients with OSC has not improved significantly in the past 20 years, the survival
rate at 5 years ranging between 45-50% (Bagan JV, 2007). The literature indicates that basaloid or sarcomatoid forms of carcinomas as more aggressive (Berthelet E, 1994), unlike verrucous carcinoma, a well differentiated and less aggressive tumor (Gnepp D, 1988). The keratinized or not appearance of these lesions is not a prognostic factor in itself, but illustrates the degree of differentiation, important being cytonuclear atypia and mitotic activity. Studies in the literature indicate low prognostic value of the degree of differentiation of oral squamous carcinomas because most are moderately differentiated (Jerjes W, 2010). Several studies have shown an inverse relationship between lymphocytic infiltration and metastatic potential in the lymph nodes (Hiratsuka H, 1997) as well as survival (Kurokawa H). The presence of an invasion front in the form of non-cohesive cords or islands or isolated neoplastic cells form may be associated with a poor prognosis, with an increased risk of metastasis, whereas tumors with a compact appearance of the invasion front, which grow push (a phenomenon called "pushing"), is associated with a much less aggressive and low risk of metastasis (Thompson DR, 2006).

The study indicated vascular invasion (18.3%), perineural invasion (52.6%) and the presence of lymph node metastasis (22.2%) in the case of poorly differentiated carcinomas. Most studies report statistical association between lymphovascular and perineural invasion with prognosis (Chen YW, 2008, Brown B, 1989).

In this study, oral squamous carcinomas in tumoral stage I and III were most frequently identified (59.8%). The increased incidence of cervical lymph node metastasis is the most important negative prognostic factor for OSC, at diagnosis more than 40% of patients already have metastases (Chen YW, 2008).

Safety margins invasion was identified in 42.5% of cases. The study reported statistically significant differences (p <0.05) of histological type, pattern of invasion, stage and depth of invasion, and perineural and vascular invasion with the degree of tumors differentiation.

**Immunohistochemical analysis** was performed on a group of 44 oral squamous cell carcinomas, for which are investigated the expression of p53, cyclin D1, p16, Ki67, EGFR, Her2/neu and CD44 in tumors and the dysplastic lesions identified in the safety surgical limits.

The immunopositive reactions for p53 was present in a proportion of 59% of cases, without obvious correlation with histological type and tumor differentiation grade. The analysis for Cyclin D1 immunostain reaction indicated positivity in 59% of the OSC, the most in more than 50% of tumor cells (29.5%) with moderate or high intensity and without correlation with tumor grade. Although were not defined any specific mutations of tumor progression, it was established an experimental model of the process. The first change is the p16 gene inactivation, which is associated with the transition from normal to hyperplasia / hyperkeratosis (Chin Su, 2005). In the next step would appear oncoprotein p53 mutations which are associated with progression to dysplasia lesions. Finally, Cyclin D1 amplification and overexpression is a late event that is associated with invasion.

Ki67 index analysis indicated inverse relationship with the degree of differentiation of malignancies, its values are higher when the degree of investigated OSC was lower. In literature it is reported that Ki67 proliferation index is significantly higher in tumors with low histological grade of differentiation (Vicente JC, 2002).

EGFR expression did not correlate with the degree of differentiation of the OSC, but was better expressed in well and moderately differentiated tumors, probably because this receptor linked to the degree of differentiation of neoplastic keratinocytes. The literature also confirms EGFR overexpression in three quarters of cases of analyzed OSC, with values up to 75% (Vosoughhosseini S, 2012). Information on positivity for EGFR and the degree of differentiation
of the OSC reported positivity in 50% of cases, more frequently in well-differentiated forms (Bernardes VF, 2010).

The analysis of Her2/neu expression in OSC indicated protein overexpression in a small number of tumors (11 cases), all poorly differentiated. The role of HER-2/neu in carcinomas of the head and neck carcinoma is not well defined. Overexpression has been observed in many cancers and is used in cancer therapy regimens. It turned out that HER-2/neu overexpression increases metastatic potential in several stages by promoting invasion and metastatic cascade (Khan AJ, 2002), suggesting that this gene may play an important role in carcinogenesis.

The immunoreactions for CD44 indicated a direct proportional relationship with the degree of tumor differentiation, well-differentiated forms revealing the most intense immunostain, moderately differentiated forms with an average immunostaining intensity, while in the poorly differentiated immunostaining was weak. The clinical significance of CD44 expression in squamous cell carcinomas of the head and neck is that of a tumor marker for diagnosis and prognosis (Assimakopoulos D, 2002). One such study on OSC and dysplastic lesions indicated that immunostain pattern and intensity varied depending on the degree of dysplasia and the degree of differentiation of the OSC (Bahar R, 1997).

**CHAPTER VII- „Conclusions”** indicates the study conclusions.

- Analysis of distribution according to age groups indicated that the tumors had an incidence progressively increased with each decade of life, more than three quarters being diagnosed (78.6%) between 50-79 years;
  - The incidence of lesions by gender revealed a net predominance in males that we found 188 cases (83.9%), most patients being from urban areas respectively 163 cases (72.7%);
  - I noticed that often the risk factors were associated with each other, mostly in alcohol and tobacco (45.9%) or alcohol, smoking, irritation factors and precancerous lesions (16.5%);
  - Most patients, respectively 86 cases (38.4%) were presented to the doctor after a period of between 6-12 months after the onset of disease;
  - As regards the tumor localization, in order of frequency they were located in the: lips (35.2%), tongue (33%), floor (16.9%), gingiva (8.4%) and palate (6.2%);
  - There were significant statistically differences (p <0.05) for risk factors associated and the degree of differentiation of tumor lesions according to tumors localization;
  - The analysis tumor size indicated that 72.4% of cases corresponded to T1/T2 categories;
  - The most common OSC histological varieties were the forms without keratinization (50%) and the keratinized ones (32.1%);
  - The analysis of differentiation in OSC indicated predominance of moderately differentiated forms in 92 cases (41%), followed in 75 cases (33.5%) of well differentiated OSC and 57 cases (25.5%) with poorly differentiated OSC;
  - The analysis of lymphoid infiltrate at the interface tumor / adjacent matrix indicated its presence in almost half of cases (102 cases or 45.5%) with medium intensity;
  - Classification of tumors regarding the pattern of tumor invasion revealed predominance of infiltrative pattern, respectively in 73 cases (43.7%);
  - Perineural and vascular space invasion was present predominantly in moderately and poorly differentiated OSC and in 23 cases (13.7%) was associated with metastatic adenopathy;
  - The analysis of the clinico-anatomical stage indicated that most cases corresponded to stage I and III, each with 50 cases;
  - Surgical safety limits invasion was identified in 42.5% of cases.
• Were found statistically significant differences (p < 0.05) of histological type, pattern of invasion, stage and depth of invasion, and perineural and vessels invasion with the degree of tumor differentiation;
  • The analyzed markers expression in both carcinomas and dysplasia indicates their intervention in oral carcinogenesis in its early stage, at least for some of these tumors;
    • The immunostain appreciation for p53 oncoprotein indicated p53 gene mutations involved in neoplastic transformation that can occur early in oral carcinogenesis;
    • The positivity for p53 may be an indicator of tumor progression, but can not be used as a factor for predicting malignant potential of oral squamous carcinomas;
    • The positivity for p16, used as a surrogate marker for identifying OSC HPV positive was low and inversely proportional to the expression of p53, suggesting that different mechanisms involved in oral carcinogenesis;
  • P16 and p53 positivity in dysplastic adjacent OSC areas indicate that these changes are the beginning of malignant transformation; also p53 and p16 positivity in both lesions, dysplasia and carcinomas, and increase the proportion of p53 positive cases from dysplasia to carcinoma suggests that p53 and p16 gene mutations are involved in neoplastic transformation and may occur early in oral carcinogenesis;
  • The analysis of Ki67 index indicated inverse relationship with the degree of tumor differentiation;
    • Overexpression of p53 and Ki-67 appear to be reliable indicators of tumor progression in OSC;
    • While p53 immunoexpression dominated the poorly differentiated OSC group, p16 and EGFR immunoexpression dominated the well differentiated OSC group;
    • The analysis of Her2/neu expression in OSC indicated the protein overexpression for a small number of tumors (11 cases), all poorly differentiated;
    • Statistical analysis indicated differences in Ki67 proliferation index, as well as HER2/neu and CD44 reactions depending on the degree of tumors differentiation;
    • There were statistical relations between p53 reactions and cyclin D1, respectively EGFR and also between HER2/neu and EGFR, indicating cooperation of growth factors involved in the regulation of cell cycle proteins in oral carcinogenesis;
    • Ki67 and CD44 are useful markers for assessing the degree and risk of progression of oral dysplastic lesions.