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Assessment of p53 and HER-2/neu genes status and protein products in oral squamous cell carcinomas

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Abstract

Identification of the genes involved in tumor initiation and progression has led to development of new markers and generated targets for new drugs. This study aimed to evaluate p53 and HER-2/neu genes status of and their protein products in oral cancer patients. Tumor specimens from 116 cases diagnosed with oral squamous cell carcinoma were analyzed. P53 and HER-2/neu immunoreactivity were studied. FISH analysis was performed to elucidate p53 and HER-2/neu gene status. Male cases represented 84% of the group. The majority of cases were between 51–60 years and moderately differentiated oral carcinoma had an incidence of 58.6%. Thirty-four cases showed p53 overexpression, negative immunoreaction was observed in 16.37% of cases. 66.38% of cases had p53 deletion, with an increased rate observed in neoplasms of the tongue. Immunohistochemical analysis of HER-2/neu receptor protein revealed that 76.72% were negative, 5.17% had weak immunostaining, 14.65% had +2 score, the others had +3 score. 24.1% of cases were analyzed using FISH technique, of which 25% were without amplification, but with polysomy for chromosome 17. 18.1% of total cases were amplified, with the rate HER-2/neu:CEP17 higher than 2. Of the 77 cases with a single p53 allele, 20 associated HER-2/neu amplification, 31 had positive anti-HER-2/neu immunoreaction, but did not have HER-2/neu:CEP17 rate >2. There was a significant association between HER-2/neu amplification and deletion of a p53 allele. These results could justify more extensive research to assess p53 and HER-2/neu gene status as significant prognostic factors in oral cancers.

Keywords: oral squamous cell carcinoma, p53 gene, HER-2/neu gene, FISH technique, immunohistochemistry.

Introduction

Oral cancer is the eighth most common cancer worldwide compared to the total malignancies. In Romania, oro-maxillo-facial cancer represents approximately 5% of all cancers with serious impact on patients because the high rate of recurrences or metastases within two years and a five-year survival rate of 50%, lower than for other cancers [1, 2]. Often found in older people, is also observed in young adults [3], the ratio men/women is 2/1 [4].

Molecular mechanisms in oral cancer are still unclear [5, 6], but it seems that gene alterations are being influenced by exposure to environmental agents, including cigarette smoke, alcohol, and viruses such as HPV [7]. Previous studies [8] have demonstrated the complexity of karyotypes, associating gene amplification, having as consequences overproduction of their protein products and increase of the number of target molecules for the mutations with malignant potential [9, 10]. Identification of molecular markers with prognostic value for survival becomes very important for therapy management. Molecular staging can assess the likelihood of recurrence and may be useful in correlating the phenotypic characteristics of neoplasm with the treatment optimal scheme.

The study aims to evaluate the status of p53 and HER-2/neu genes and their protein products in oral cancer patients.

Materials and Methods

In this retrospective study, a group of tumor specimens from 116 cases diagnosed with oral squamous cell carcinoma was considered. The histopathological material was represented by the paraffin blocks corresponding to the selected cases existing in the Laboratory of Pathology, Municipal Hospital of Timişoara, Romania. All necessary data and information on histological type of tumor, the staging, grading and the rest of the characteristics of the cases were taken from electronic records of the hospital. Four μm sections from the paraffin blocks were stained with Hematoxylin–Eosin for the establishment of the histopathological type and differentiation stage, based on the *WHO International Classification of Diseases for Oncology* (1990). Clinical staging was performed using the TNM Staging.

For FISH technique evaluation, samples from the neoplasms and adjacent seemingly normal epithelium were disaggregated with 0.2% collagenase. FISH analysis was performed with commercially available probe from Vysis

LSI TP53 SpectrumOrange/CEP17 SpectrumGreen Probe according to the manufacturer's protocol Abbott–Vysis with small adjustments.

We analyzed the slides using a Zeiss Axio Imager M1 epifluorescence microscope (Zeiss, Germany) equipped with filter sets for DAPI, SpectrumOrange and SpectrumGreen and a triple filter (simultaneous DAPI/Orange/Green) at a magnification of ×100. Images were captured using MetaSystems digital camera and analyzed using Isis version 5.2 MetaSystems software (Altlussheim, Germany). For each case, hybridized signals were counted in 200 interphase nuclei.

Results evaluation

In the presence of a single green signal, we considered the cell monosomic for chromosome 17. If there were more green signals, the cell was considered polysomic for chromosome 17. The presence of two orange signals was correlated with the existence of both p53 alleles. Orange signal loss was correlated with p53 monoallelic gene deletion. The existence of a number of signals higher or lower than 2, reveals cell aneuploidy.

FISH analysis was performed to elucidate the HER-2/neu gene status in those cases classified as HER-2/neu positive with the immunohistochemical technique. It was used in the context of dual-color-FISH, HER-2/neu DNA probe kit from Abbott–Vysis Company, which contained locus specific probes for HER-2/neu (SpectrumOrange) and centromeric probes region for chromosome 17 (SpectrumGreen). Counting was performed using criteria proposed by Hopman AH *et al.* [11] only distinct isolated nuclei were counted.

We studied p53 immunoreactivity using anti-p53 monoclonal antibody, clone DO7, IgG2b isotype (DAKO®, Glostrup, Denmark). Interpretation of results required p53 reaction control, for positive control, we included a carcinoma known to have high p53 expression and for negative control a buffer replaced the primary antibody on normal and tumor tissue samples. Polyclonal antibody against human c-erbB-2 oncoprotein (DAKO®, Carpinteria, USA) was used to assess immunoreactivity of oral tumor tissue for HER-2/neu, to detect cases showing over-expression of the transmembrane receptor protein. For positive control, we included in the study a strong positive breast carcinoma and for negative control, a buffer replaced the antibody. FISH and IHC tests were conducted separately from each other, without any clinical information.

Statistical analysis was performed using specialized computer programs: SPSS 10 and Epi Info™ 3.5.1, software designed for database management and statistical processing of data from public health domain. Statistical comparison was made with *chi*-square test and Student's *t*-test.

Results

Specimens from male cases represented 84% of the group and specimens from female cases, 16% of the group. The distribution of the group according to the age (Figure 1) reveals that the majority of affected individuals were between 51 and 60 years, followed by those between 41 and 50 years. The average age of female cases was 69.4±9.3 years, and of the male cases 56.45±9.04 years.

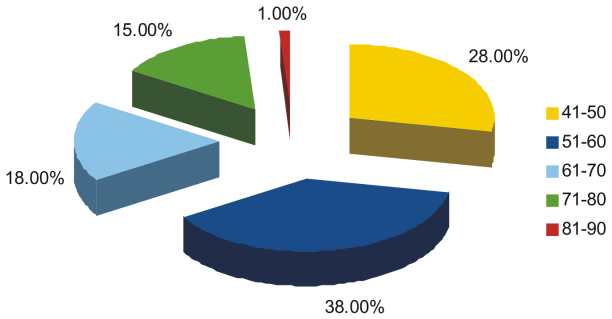

Figure 1 – ***Distribution of the group according to age intervals.***

Peak of incidence in terms of location was observed in cancer of the lower lip (22.4%), followed in frequency by tongue carcinoma (19.82%) and mouth floor carcinoma (18.96%). The rarest form was carcinoma of the upper lip (1.72%).

Morphological assessment was completed by estimating the degree of histopathological tumor differentiation. Moderately differentiated oral carcinoma (G2) in this study had a peak incidence of 58.6%, followed by well-differentiated carcinomas (G1), which represented 21.55%. The rarest form was the poorly differentiated one, which represented 19.82% of the study group. In the male group, moderately differentiated carcinoma was predominant, with an incidence of 53.44%. In the female group, well-differentiated G1 carcinoma was the predominant type, representing 42.1% from the group of females and 6.89% of the entire group. The rarest histological form in males was the well-differentiated one (17.53%), while in females, the non-keratinizing one (26.32%). Well-differentiated carcinomas were prevalent in the group with the age range 51–60 years. A relatively high proportion of moderately differentiated cases were noted between 41–60 years. Poorly differentiated epidermoid carcinomas showed a high incidence in cases aged 51 and 60 years.

Evaluation of cases according to tumor size and histological grading revealed that in the present study 48.27% of patients were in T1 stage. From the total group of 116 cases, 48 males were in T1 stage compared to eight female cases of the same stage. A total of 16 (13.79%) cases with stage T1 had G1 well-differentiated carcinomas, 34 (29.31%) moderately differentiated carcinoma – G2, and six (5.17%) undifferentiated carcinoma – G3. Of male cases with T1, 12 (10.34%) were G1, 32 (27.59%) had moderate degree of histological differentiation – G2, and four (3.45%) had poorly differentiated carcinomas. Of the eight T1 female cases, four (3.45%) were graded G1, two (1.72%) were classified as G2 and two cases were considered to be poorly differentiated – G3. Nine (7.76%) of the 50 patients with T2 stage showed well-differentiated carcinomas – G1, 33 (28.45%) moderately differentiated carcinomas – G2 and eight (6.89%) – G3, undifferentiated carcinomas. Of the nine G1 patients, five cases were males and four females. T3 incidence was 6.03%. None of the six cases classified as T3 was G1 – well-differentiated carcinoma. A single male had moderately differentiated form (0.86%). Four male cases and one female were classified as poorly differentiated carcinomas (3.45%).

T4 incidence was 2.58%. Three male and one female cases were classified as T4 (2.59%). None of the cases was classified as well or moderately differentiated.

Regarding the distribution of the group according to the lymphonodular invasion, 79 cases were without invasion and 37 had positive lymph nodes. Of the positive cases, stage N1 was the most common and N3 status had the lowest incidence.

Regarding metastases, it was found an increased frequency (84.48%) of cases without metastasis (M0). The incidence of distant metastasis was quite low (15.51%).

Results of immunohistochemical analysis with anti-p53 antibody

Immunohistochemical (IHC) classification of the degree of protein expression was obtained considering as p53-negative (-) cases without immunostaining in tumor nuclei, p53 weakly positive (+) cases with reaction in less than 25% of the cells, moderately positive (++) cases with reaction in more than 25% but less than 50% of the nuclei, p53 overexpression (+++) cases with more than 50% of nuclei with immunoreaction. Of the 116 cases, we found a positive reaction in 97 tissue specimens from cases of both sexes. Thirty-four (29.3%) cases showed p53 overexpression (Figure 2). Negative immunoreaction was observed in 16.37% of cases.

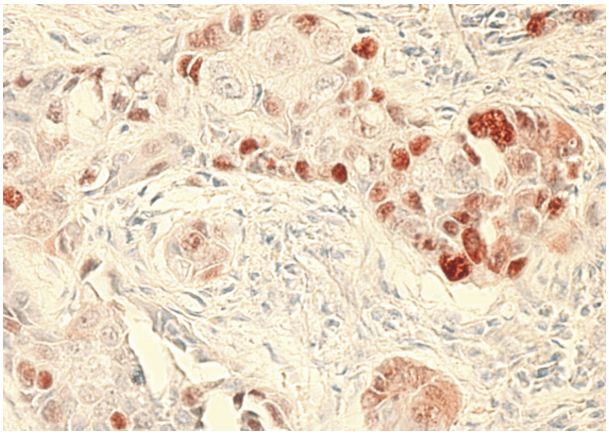

Figure 2 – ***Oral squamous cell carcinoma, keratinized, with intensely positive anti-p53+++ immunoreaction. ABC method, visualization DAB, ×200.***

Results of FISH technique for evaluation of p53 gene status

Seventy-seven (66.38%) cases had p53 deletion (Figure 3), of which 62 (53.45%) cases were males and 15 (12.93%) were females. In 35 males, representing 30.17%, and four females, representing 3.45%, we could observe the presence of both p53 alleles (Figure 4). In these cases were observed two signals from probes SpectrumOrange LSI p53 and two signals for the centromeric probe for chromosome 17, SpectrumGreen. A single case with disomy for p53 gene (both p53 alleles) showed polysomy of chromosome 17.

Among the cases with p53 deletion, 15 were G1, 43 were G2 and 19 were G3. An increased rate of anti-oncogene deletion was observed in neoplasms of the tongue (11 males, five females) followed by those located at inferior lip level (14 males, a case of female gender).

Comparing p53 gene status and protein expression, it was noticed that 12 (10.34%) cases with deletion of the gene were IHC negative (-) and 65 (55.84%) cases with deletion of the gene were IHC positive. Among the cases with p53 deletion and IHC positive, 22 (18.97%) showed protein overexpression (+++).

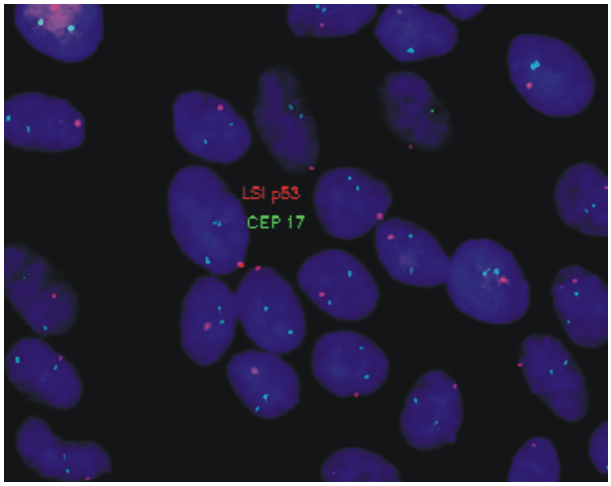

Figure 3 – ***FISH analysis using Vysis LSI TP53 SpectrumOrange/CEP17 SpectrumGreen Probe. Note one signal for the orange fluorochrome showing p53 deletion.***

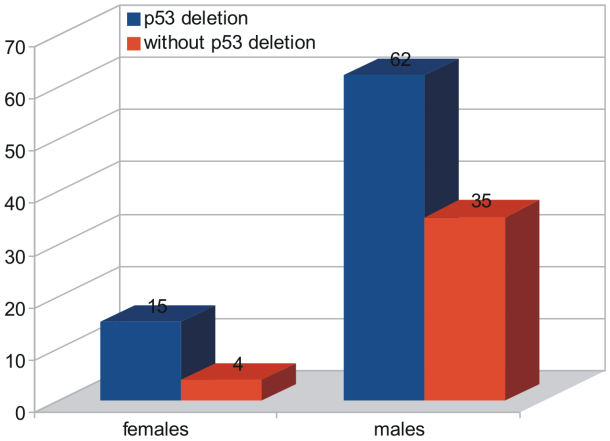

Figure 4 – ***Gender-based distribution of the group according to p53 gene deletion.***

Results of immunohistochemical analysis with anti-HER-2/neu antibody

The results of immunohistochemical analysis of HER-2/neu receptor protein revealed that 89 (76.72%) cases were IHC negative, six (5.17%) cases had weak immunostaining (+1), 17 (14.65%) cases had +2 score and four cases had +3 score. A comparison between immunohistochemical expression of HER-2/neu protein and p53 protein is presented in Table 1.

Table 1 – ***Correlation between anti-HER-2/neu immuno-reactivity and p53 immunoexpression***

Protein expression	HER-2/neu negative	Score +1	Score +2	Score +3
P53-	15 (12.93%)	0 (0.00%)	4 (3.45%)	0 (0.00%)
P53+	24 (20.69%)	2 (1.72%)	4 (3.45%)	1 (0.86%)
P53++	26 (22.41%)	2 (1.72%)	2 (1.72%)	2 (1.72%)
P53+++	23 (19.83%)	2 (1.72%)	8 (6.90%)	1 (0.86%)

Results of FISH technique for evaluation of HER-2/neu gene amplification

FISH analysis was performed to evaluate HER-2/neu gene status in cases classified as HER-2/neu positive with the immunohistochemical technique. Twenty-eight (24.1%) cases were analyzed using this technique.

HER-2/neu:CEP17 rate and chromosome 17 polysomy

HER-2/neu:CEP17 rate was determined using the ratio between HER-2/neu and CEP17 in 60 nuclei. The total number of HER-2/neu signals was divided by the total number of CEP17 signals. A rate of ≥2 was achieved, according to the directions of the Abbott–Vysis Company for gene amplification. According to the described guidelines, in this study we obtained the following results: of the 28 cases analyzed by FISH, seven cases (representing 25% of those IHC positive and 6.03% of total) were without amplification, but with polysomy for chromosome 17 (Figure 5).

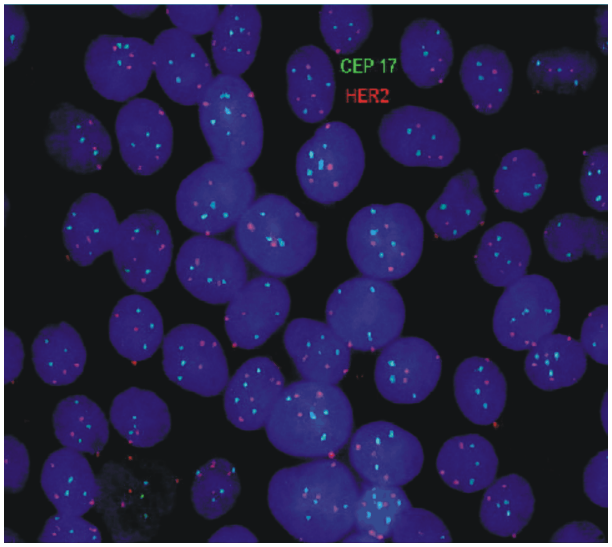

Figure 5 – ***FISH analysis using Vysis HER-2/neu SpectrumOrange/CEP17 SpectrumGreen Probe, reveals chromosome 17 polysomy.***

Two of these cases had a HercepTest score +2 and five cases had a +1 HercepTest score. For chromosome 17 polysomy, the evaluation criterion was a number greater than two CEP17 signals/tumor nucleus. Twenty-one cases (representing 75% of IHC positive and 18.1% of total) were amplified, with the rate HER-2/neu:CEP17 higher than 2 (Figure 6). Amplified cases had HercepTest scores of +1, +2, and +3, of which 16 cases were +2 (Figure 7), one case +1, four cases +3. HER-2/neu:CEP17 rate in cases that were not amplified ranged between 0.5 and 1.7 and in cases with amplification, the rate ranged between 2.1 and 5.4. The study revealed an average rate of 3.05 HER-2/neu:CEP17/tumor nucleus ± 1.51.

Regarding HER-2/neu gene amplification and histological grade of differentiation, in the studied group we noted that most cases with amplification had associated in the tumor phenotype a moderate degree of histological differentiation, while the majority of cases without amplification were G1. Among cases with G3, 4.31% showed amplification, of which four (3.45%) cases were males and one female. Of 21 cases with HER-2/neu gene amplification, 10 (8.62%) cases were diagnosed with stage IV. Among the cases with early stage disease, four cases were associated with HER-2/neu gene amplification.

Cases without distant metastases were associated with a higher proportion of HER-2/neu amplification. Among cases without amplification, six (5.17%) were not associated with distant metastases.

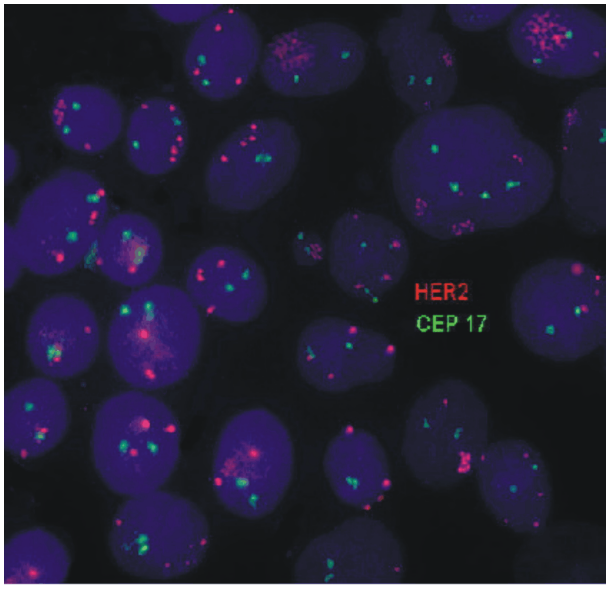

Figure 6 – ***FISH analysis using Vysis HER-2/neu SpectrumOrange/CEP17 SpectrumGreen Probe. Note two green signals specific for chromosome 17 and more red signals showing HER-2/neu amplification.***

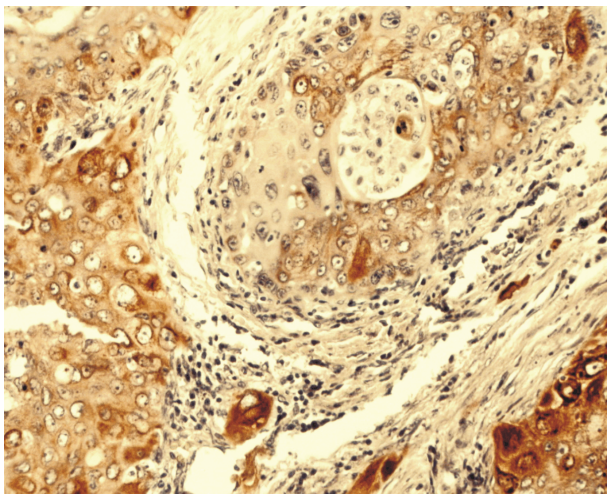

Figure 7 – ***Oral epidermoid carcinoma, with moderate membrane immunoreaction anti-HER-2/neu +2. ABC method, visualization DAB, ×200.***

HER-2/neu gene and protein expression status

Out of the 28 oral squamous cell carcinoma (OSCC) cases with positive immunoreactivity anti-HER-2/neu, one case (0.86%) with +1 score presented of HER-2/neu amplification and 16 cases with moderate immunostaining, +2, showed amplification. In four cases (3.45% of the study group) with protein overexpression, all showed gene amplification (Figure 8).

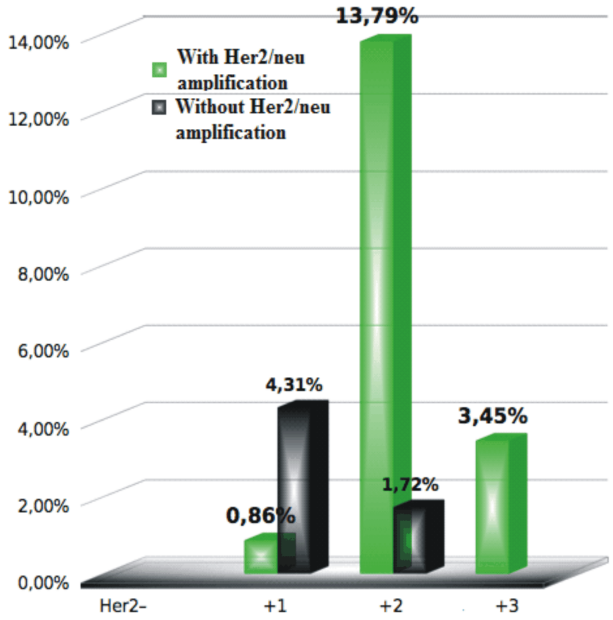

Figure 8 – ***Distribution of the group according to the immunohistochemical expression of HER-2/neu protein and gene status.***

Comparison between p53 gene status and HER-2/neu gene status

Of the 77 cases which showed a single p53 allele, 20 cases associated HER-2/neu amplification, 31 had positive anti-HER-2/neu immunoreaction, but did not have HER-2/neu:CEP17 rate >2 (Table 2).

Table 2 – ***Correlation between p53 gene status and HER-2/neu:CEP17 rate***

Gender	P53 monoallelic deletion in cases with HER-2/neu amplification	P53 monoallelic deletion in cases without HER-2/neu amplification
Male	16 (13.79%)	29 (25.00%)
Female	4 (3.45%)	2 (1.72%)
Total	20 (17.20%)	31 (26.72%)

Some cases showed both HER-2/neu amplification and p53 deletion, 16 (13.79%) were males and four (3.45%) were females. Of the cases with positive HER-2/neu expression, p53 deletion, but without HER-2/neu amplification, 29 (25%) were males and two (1.72%) were females. There is a significant association between HER-2/neu amplification and deletion of a p53 allele (p<0.001, α=0.001), gamma coefficient = 0.365 indicates a direct and weak correlation, p53 deletion is not a risk factor for HER-2/neu amplification (RR=0.33<1) and the 95% confidence interval is (0.22, 0.49).

In the group of tumors with Hercep score 2+, 16 cases had HER-2/neu gene amplification, considering the HER-2/neu:CEP17 ratio, which represented 13.79%.

Discussion

Oral squamous cell carcinoma represents about 90% of all mouth cancers [1]. The highest rates of oral cancer are found in developing countries such as South-East Asia [12]. There are large variations of the incidence, according to geographical area, increased frequency of OSCC is registered in India, Australia, France, Brazil and South Africa. The majority of the cases were aged between 50–60 years at diagnosis, in concordance with other studies [13], but other authors [14] describe the most common cases aged between 40 and 50 years. Other studies revealed that neoplasms occurred earlier. The authors correlate malignant transformation with diets that are low in vegetables and fruits and with excessive smoking and consumption of alcohol. Alcohol together with tobacco, represent best-recognized risk factors for oral and pharyngeal cancers [15]. Some studies claim that genomic changes accumulated over time, due to the action of exogenous and endogenous factors are responsible for the occurrence of oral cancer [16].

Moderately differentiated oral carcinoma (G2) in this study had a peak incidence of about 58%, followed by well-differentiated carcinomas (G1), data that are concordant with some studies [17], but other authors reported as dominant G1 [12]. Data regarding recurrence were not available, thus it is possible that the proveniences of the tissue specimens might had been from the secondary tumors, which probably have a lower degree of differentiation than the primary tumor.

In our study, about 48% of patients were in T1 stage and 43% in T2 stage. These results are discordant with other studies [17, 18] that described a lower incidence for T2 stage, perhaps because they included in their study cases from developed countries where regular dental check was performed, and neoplasms have been detected in early stages. T3 incidence was 6.03%, which is consistent with literature data [19, 20].

Classical and molecular cytogenetic analyses together with molecular analyses have revealed consistent genetic abnormalities associated with the development and/or progression of OSSC. Specific genetic changes take place during head and neck tumorigenesis, such as loss of chromosomal segments or/and gains of different chromosomal regions. The extensive genomic imbalance was demonstrated by different studies [10, 21].

Some authors have concluded that p53 gene mutation has an unfavorable independent prognostic role for survival and may be responsible for secondary tumors. Other authors have not found any notable prognostic role of this gene in oral carcinogenesis. A retrospective study of 56 patients with a follow-up period of 45 months showed that p53 mutation revealed by the genome analysis is a marker of relapse and predicts a descendant trend for overall survival [22, 23].

Studies showed that cases with tumors, showing overexpression of HER-2/neu, have a shorter duration of disease-free survival than those whose tumors do not have HER-2/neu overexpression [24].

In our study, we could not establish any statistically significant correlation of early stages of disease and p53 protein overexpression (p=0.629, α=0.05), the only significant correlation in value, was the predilect occurrence of overexpression in stage IV (RR=1.11>1). Confidence interval for the obtained values was 95% and the relative risk (0.73–1.70). Value of relative risk indicate the association of p53 protein overexpression with advanced stages of the disease, which causes interpretation of increased levels of p53 protein in tumor tissue as a negative prognostic factor. Monoallelic p53 gene deletion was found in all stages of disease, including stage I, suggesting that

p53 deletion is an early event in oral carcinogenesis. We noticed that neither the site of origin of primary tumors, nor histological grade of differentiation had shown any correlation with p53 overexpression, observation that is consistent with some studies [25], but in contradiction with other previous studies, which found that high p53 protein levels are associated with increasing histological severity [26, 27]. P53 overexpression was a significant factor in univariate analysis, although, it has lost its usefulness in multivariate analysis. This may be due to the relative small size of the group and/or lack of information on the survival rate.

Identification of HER-2/neu in tumor cells has become important because the amplification of this gene has a predictive role for response to Herceptin (Trastuzumab), monoclonal antibody capable of identifying molecules expressed on the cell surface and causes the death of these cells. Herceptin can induce remission in cases diagnosed with aggressive tumors, where primary and secondary chemotherapy failed.

Samples collected from the patients with oral carcinoma were examined simultaneously for HER-2/neu gene amplification using FISH, and HER-2/neu protein over-expression using IHC. Both FISH and IHC tests were conducted separately from each other without taking into account clinical information. Both are important techniques in detecting existing changes in the structure and function of HER-2/neu gene. However, IHC is essentially a qualitative analysis whose utility is further compromised by: variable sensitivities of the antibodies, non-standardizing of staining protocols, and lack of uniform criteria for positivity. More importantly, IHC is subject to antigenic changes resulting from the attachment and integration of tissues in paraffin. FISH is a quantitative evaluation method, which essentially avoids many of these problems, but has the disadvantage of being a laborious and expensive procedure [28, 29].

Knowing the level of polyploidy is essential for determining HER-2/neu status. FISH and IHC results are easier to interpret, if known the number of chromosomes 17/ neoplasic cell. By knowing this parameter, it is much easier to specify the differential diagnosis between an actual gene amplification and HER-2/neu signal multi-plication, which is due to polyploidy of chromosome 17. In the group of carcinomas with polysomy were taken into account all cases that showed at least 3.0 signals from chromosome 17, thus, an average number of CEP17 signals greater than 2.0. We considered this value as a reference one, because in diploid cell tissue sections, the average signal number from chromosome 17 can vary from 1.5 to 2 due to nuclear truncation, when sectioning the paraffin block [30].

Polysomy is an expression of chromosomal instability in malignant cells. Losses or gains of small chromosomal regions represent the changes in gene function, *e.g.*, deletions leading to decrease in performance of tumor suppressor genes, while gene material gains reveal a strong gene activation. A high rate of amplification (three times more gene signals than reference signals) usually reveals oncogene activation and overexpression. Tumors with strong HER-2/neu overexpression of the HER-2/neu receptor protein present gene amplification in over 90% of cases [28, 31].

In a study performed by Scheer M *et al.*, FISH and IHC techniques were performed to determine the status of HER-2/neu gene and protein in 94 oral carcinomas, the authors obtaining gene amplification in a number of 14 (33%) cases out of the 42 IHC positive. Comparing the results obtained in this study with those of the mentioned author, the percentage difference between the amounts of amplified tissue specimens was noticed; in our study, we obtained gene amplification in 18.1% of cases. The results of IHC techniques were different, Scheer M *et al.* reporting positive IHC in only 11.2% of cases, while in this study positive membrane immunoreaction could be detected in 24.4% of cases. Of the 28 cases investigated with FISH method, seven were without gene amplification. This may be due to gene transcriptional and post-trans-criptional activation [29]. Another study analyzed the relationship between salivary levels and EGFR and HER-2/neu immunoexpression in tumor samples, concluding that low levels of EGF in saliva may suggest a higher susceptibility for OSCC development [32].

Conclusions

We detected p53 protein overexpression in 29.3% of OSCC cases, noting heterogeneity of staining intensity and patterns. Values of relative risk indicate association between p53 protein overexpression and advanced stages of the disease, which leads to interpreting increased levels of p53 protein in tumor tissue as a negative prognostic factor. FISH analysis revealed that p53 monoallelic deletion was the predominant cytogenetic abnormality, present in 66.38% of cases. Twenty-five percent of the IHC positive cases were non-amplified for HER-2/neu gene, but had polysomy for chromosome 17, the remaining cases had amplification. Limits in determining the prognostic significance and clinical implication of these data are due to small sample size. These results could justify a more extensive research to assess p53 and HER-2/neu gene status in this type of cancer and may be useful for other studies attempting to determine new gene and protein markers as possible molecular targets for therapy of malignant tumors of the oral mucosa. Correlation of morphological aspects with the immunohistochemical and molecular ones will allow increased diagnosis accuracy, prognostic and predictive value of biomarkers in order to achieve individualized tumor therapy for patients diagnosed with OSSC.

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