ANALYSES OF NUMERICAL ABERRATIONS OF CHROMOSOME 17 AND TP53 GENE DELETION/AMPLIFICATION IN HUMAN ORAL SQUAMOUS CELL CARCINOMA USING DUAL-COLOR FLUORESCENCE IN SITU HYBRIDIZATION

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Abstract: In Romania, oral and facial cancers represent approximately 5% of all cancers. Deactivation and unregulated expression of oncogenes and tumor suppressor genes may be involved in the pathogenesis of oral squamous cell carcinoma. The genomic change results in numerical and structural chromosomal alterations, particularly in chromosomes 3, 9, 11 and 17. The aim of our study was to identify numerical aberrations of chromosome 17, deletion or amplification of p53 gene and to reveal correlations between abnormalities of chromosome 17and of p53 gene with TNM status and grading in 15 subjects with oral squamous cell carcinoma. 80 % of cases presented chromosome 17 polysomy and only 20% of cases had chromosome 17 monosomy. 46.6 % of samples revealed p53 gene amplification and 33.3 % of them p53 deletion. Polysomy of chromosome 17 was also detected in tumor-adjacent epithelia. The degree of the cytogenetic abnormality did not correlate with the stage of the disease, the histological differentiation of oral squamous cell carcinoma and lymph node metastasis. Molecular cytogenetic techniques, using fluorescence in situ hybridization with chromosome-specific DNA probes, facilitate the confirmation of presumed chromosomal aberrations with high sensitivity and specificity.

Keywords: oral squamous cell carcinoma, FISH, Chromosome 17, p53 gene

INTRODUCTION

In Romania, oral and facial cancer represents approximately 5% of all cancers. Although the percentages seem relatively small, however, the disease has a serious impact on affected individuals. The etiology appears to be multifactorial, strongly related to lifestyle, although other factors, such as infective agents, also are implicated. Clarifying the biological processes driving human tumorigenesis may help researchers to identify and develop future preventive and therapeutic strategies.

Lip cancer dominates tumor pathology, followed by cancer of the tongue, floor of the mouth and cheek mucosa. More than 95% of the carcinomas of the oral cavity are of epithelial squamous type [6].

The development of oral squamous cell carcinoma (OSCC) depends on both environmental and genetic factors. Most oral cancer subjects have had prolonged exposure to tobacco and alcohol, but these carcinogens cannot fully account for the development of cancers in these individuals [4]. More recent studies have shown that tobacco causes damage of the cell DNA and alcohol reduces the effectiveness of mechanisms for DNA repairing that would be needed after aggressive agents caused damages [16].

The accumulation of genetic abnormalities in carcinogenesis is divided in the following phases: initiation, promotion, conversion, progression [1]. Cytogenetic analysis has now detected an accumulation of genetic lesions in oral cancers. Numerical changes in chromosomes 7 and 17 have been described and these might be associated with an upregulation of EGFR and p53 genes, and could contribute to critical events in laryngeal carcinogenesis [5].

Chromosome 17 has a high content of guanine and cytosine, regions rich in genes, a high amount of short interspersed elements and a lack of long interspersed elements.

Deactivation and unregulated expression of oncogenes and tumor suppressor genes may be involved in the pathogenesis of oral squamous cell carcinoma [14]. Molecular cytogenetic techniques, using fluorescence in situ hybridization with chromosome-specific DNA probes, facilitate the confirmation of presumed chromosomal aberrations with high sensitivity and specificity.

The acquisition of genetic instability is an essential step during carcinogenesis [11]. In most tumors, including OSCC, such a genomic change results in numerical and structural chromosomal alteration. A high frequency of chromosome 17 abnormalities has been reported in some human cancers such as breast carcinoma [13], colon carcinoma [10] and bladder carcinoma [7]. Different studies revealed that cells with polysomy 17 are significantly increased in squamous cell carcinoma, thus, chromosome 17 abnormality seems to be correlated with carcinogenesis of OSCC [12].

The development and progression of human tumors often involves inactivation of tumor suppressor gene function. Observations that specific chromosome deletions were correlated with distinct groups of cancer suggested that some tumors may share common defective tumor suppressor genes.

The TP 53 gene, located on the short arm of chromosome 17p13, consists of 11 exons coding for a nuclear phosphoprotein, which can bind to specific DNA sequences acting as a transcription factor. Gain or loss of chromosome 17 were detected in many cancers, including OSCC [4]. Many recent studies have focused on the TP53 tumour suppressor gene, analysing its gene status and protein status. Dysfunction in the p53 tumor suppressor gene is involved in the etiopathogeny of OSCC. *TP53* mutations are frequently found in tobacco-associated cancers. The exact role of the *TP53* genetic alterations in different stages of the tumorigenic process is not completely established.

The p53 gene in her anti-oncogenic role works through multiple pathways. The p53 gene has the

capacity to induce repair of the damaged DNA by activating repair proteins and by stopping the cell cycle at the G/S regulation point, arresting growth of the cells. Another anti-cancer role is completed by initiating apoptosis of a cell with irreparable DNA damage.

The aim of our study was to identify numerical aberrations of chromosome 17, deletion or amplification of p53 gene and reveal possible correlations between the numerical aberrations of chromosome 17, deletion or amplification of the p53 gene, TNM status and grading in 15 subjects with OSCC.

MATERIALS AND METHODS

This study was performed retrospectively on 15 subjects diagnosed with oral squamous cell carcinoma. Tumor samples were processed by usual techniques for inclusion in paraffin in the pathology lab of UMF Timisoara. For each subject, 5 µm sections from the paraffin blocks were stained with hematoxiline-eosine 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.

Additional sections were prepared for FISH technique evaluation. Samples from the 15 neoplasms and adjacent seemingly normal epithelium were mechanically and enzimatically disaggregated with 0.2% collagenase in order to obtain interphase nuclei. Dual-color FISH assay was performed using direct fluorescent labeling probes for the chromosome 17 centromere and TP53 gene (17p13.1). FISH analysis was done with commercially available probe from Vysis LSI TP53 Spectrum Orange/ CEP 17 Spectrum Green Probe according manufacturer's protocol Abbott/Vysis with small adjustments.

The slides were denatured for 5 min in 70% formamide/2 × SSC at 73°C. Then the slides were dehydrated by immersion in 70% cold ethanol solution for 5 minutes, steps that were followed by immersion in 80% and 100% cold ethanol. The slides were then air-dried and on each plate 10 µl of the probe LSI TP53/CEP 17 was added in the selected hybridization area. The smears were covered with a 22x22 mm coverslip, sealed and incubated for 16 hours in a humid chamber at 37°C in order to hybridize. Then, two washes were performed post hybridization, using washing solutions: 0.4 × SSC/0.3% NP40 at 73°C for 2 minutes, and 2 × SSC/0.1% NP40 for 2 minutes. The slides were then air-dried and 4',6-Diamidino-2-phenylindole (DAPI II) was added for counterstaining.

We analyzed the slides using a Zeiss Axio Imager M1 epifluorescence microscope (Zeiss, Jena, Germany) equipped with filter sets for DAPI, SpectrumOrange and SpectrumGreen and a triple filter (simultaneous DAPI / Orange / Green) at a magnification of ×1,000. Images were captured using MetaSystems digital camera and analyzed using Isis version 5.2, MetaSystems software for quantitative analysis of samples generated by FISH technique (Altlussheim, Germany). For each subject hybridized signals were counted in 200 interphase nuclei.

Using in situ hybridization we analyzed the numerical aberrations of chromosome 17 and p53 gene deletions/amplification in 15 paraffin embedded OSCC samples. The aberrations of chromosome 17 and p53 abnormalities were correlated with TNM staging and grading.

RESULTS

In situ hybridization for revealing the numerical aberrations of chromosome 17 and p53 gene deletion/amplification in 15 paraffin embedded OSCC samples was performed.

The LSI TP53 SO/CEP17 SG probe hybridizes to chromosome 17. The Spectrum Orange TP53 probe hybridizes to 17p13.1. The probe is 172 kb in size and covers the entire TP53 gene, called p53.

The CEP 17 is labeled in Spectrum Green and hybridizes to the 17p11.1-q11.1 region of chromosome 17. All the subjects presented numerical alterations of chromosome 17 and of the TP53 gene. 200 nuclei were scored under x100 magnification, using an oil immersion objective and the fluorescent microscope for each defined histological area from the tumor and tumor-adjacent epithelia, each nucleus being assessed for the chromosome copy number.

The numerical aberrations of chromosome 17 varied from individual to individual. Counting was performed using criteria proposed by Hopman et al. (1994) [11]. Specifically, only distinct isolated nuclei were counted, fluorescent signals were scored as true hybridization events only if they were approximately the same size and intensity as those in adjacent cells, and paired signals were scored as single events. We interpreted as monosomy 17 if the mean number of signals in analyzed cells for each subject was lower than two. Chromosome polysomy was defined as the fraction of the cells demonstrating three or more signals in each nucleus. We found 12 subjects (80 %) which presented chromosome 17 polysomy and only 3 subjects (20%) with chromosome 17 monosomy (Fig. 1 & Fig. 2) were detected. Of the 12 subjects with polysomy, seven (46.6%) presented chromosome 17 trisomy (Fig. 3) and five subjects presented tetrasomy (Fig. 4).

Seven individuals (46.6 %), which presented p53 gene amplification (Fig. 3, Fig. 4) and 5 subjects (33.3 %) with p53 deletion (Fig. 1, Fig. 2) were detected. The polysomy of chromosome 17 in the tumor cells was higher than in the tumor-adjacent epithelia ($P < .001$).

The aberrations of chromosome 17 and p53 deletions were correlated with age, gender, localization, grading and TNM staging (Table 1).

The degree of the cytogenetic abnormality did not correlate with the stage of the disease, the histological differentiation of OSCC and lymph node metastasis, but because of the reduced number of subjects, no ferm conclusions could be drawn.

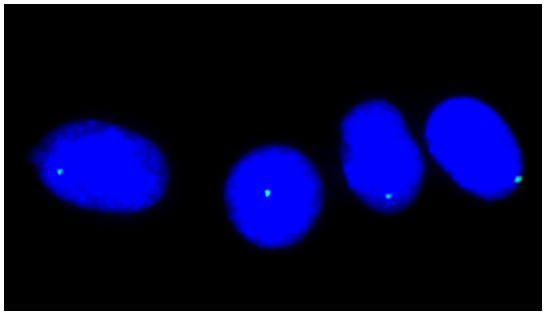

Figure 1. FISH analysis using Vysis LSI TP53 Spectrum Orange/ CEP 17 Spectrum Green Probe. Note only one specific green signal showing monosomy 17 and no red signals, showing p53 deletion.

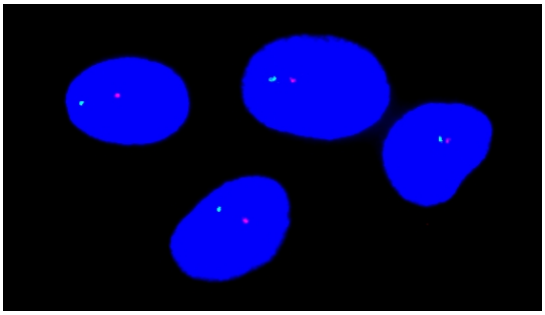

Figure 2. FISH analysis using Vysis LSI TP53 Spectrum Orange/ CEP 17 Spectrum Green Probe. Note one signal for each fluorochrome showing monosomy 17 and p53 deletion.

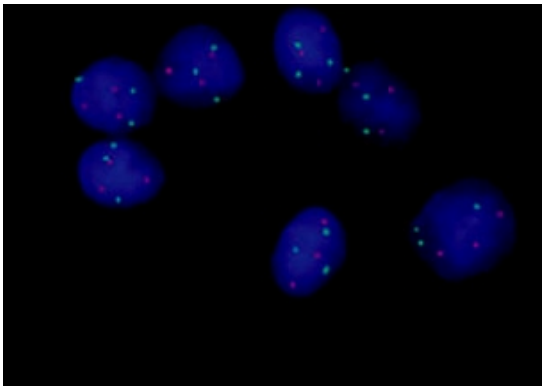

Figure 3. FISH analysis using Vysis LSI TP53 Spectrum Orange/ CEP 17 Spectrum Green Probe. Note three specific green signals showing trisomy 17 and three specific red signals showing p53 amplification.

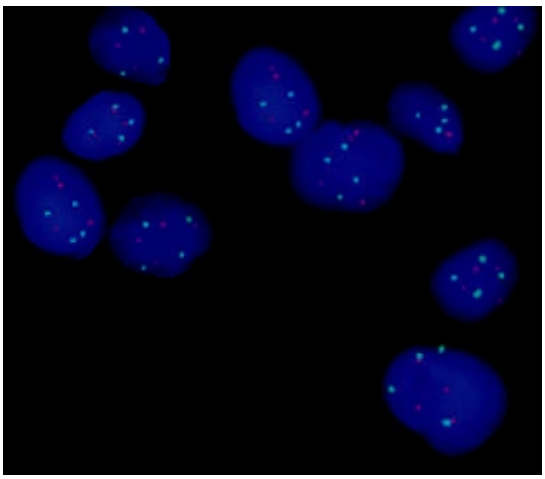

Figure 4. FISH analysis using Vysis LSI TP53 Spectrum Orange/ CEP 17 Spectrum Green Probe. Note four specific green signals showing tetrasomy 17 and four specific red signals showing p53 amplification.

DISCUSSION

Cytogenetic analysis of head and neck tumors has revealed extensive genetic heterogeneity and karyotype complexity [2]. An extensive amount of chromosomal abnormalities has been previously described also in head and neck squamous cell carcinoma (HNSCC). There are various methods, including classical and molecular cytogenetics (CGH and interphase FISH) [6] and loss of heterozygosity (LOH) that can be used to detect them [9]. All these molecular and cytogenetic techniques usually disclose similar results and indicate an extensive genomic imbalance.

Chromosome 17 abnormality has been shown to have a strong correlation with neoplastic development and progression [10]. FISH method can be used for evaluating the degree of genomic instability and aneuploidy, possible prognostic markers, in OSCC.

Genetic instability is putatively involved in the multistep process of carcinogenesis of most cancers. Current evidence suggests that this genomic instability occurs at two levels: the nucleotide level and the chromosome level [12]. Gains or losses of whole or large parts of human chromosomes in tumor cells are found in most cancers [4]. This has been proposed as a major driving force for determining the rate of accumulation of specific genetic hits in several human cancers [8]. In our study, a significant number of aneuploidies was detected in most of the neoplastic cells, and this was frequently represented by polysomy, rather than monosomy of chromosome 17.

The p53 gene is a tumor suppresor gene which induces a G1 arrest and is involved in DNA repair and apoptosis. Abnormalities in the p53 gene cause an inefficient checkpoint system for the repair and destruction of mutant cells. These result in an increased genomic instability. Point mutations are most frequent mutations that affect the wild-type p53, which thus becomes inactivated in human tumors. The mutant p53 has a very short half-life time and can be easily detected with immunochemistry. A significantly higher risk for p53 mutations was observed in smokers compared with non-smokers [15].

Evidence suggests that mutation of p53 is an important late step in carcinoma [2]. ln oral squamous cell carcinoma, p53 abnormalities may result in increased genomic instability and contribute to carcinogenesis. It is estimated that in head and neck cancer, p53 mutations are present in 33% to 59% of tumors [10]. The mutations in the p53 gene and alterations in the p53 protein resulting in its accumulations in cells may play critical role in tumorigenesis [8]. Recent studies [1] found p53 to be overexpressed in 63% of oral carcinomas, with p53 mutations in 36% of individuals. Altered p53 expression in premalignant lesions is associated with increased chromosomal polysomy [1]. It has been reported that immunoreactivity for p53 protein can be detected in benign tumors and premalignant lesions, including dysplasia of the oral mucosa [3]. Different studies revealed that p53 alterations can occur early in carcinogenesis and the alterations are maintained upon progression to overt malignancy [7]. In the literature is

reported that all the patients who presented recurrence, metastasis, and death with tumor dissemination had p53 overexpression [3].

Other data from literature revealed that the frequency of cells with polysomy increased with histological progression. Within each histological grade, there was an intersubject variation of the chromosome polysomy levels, suggesting that the biological factors might influence the rate of accumulation of genetic hits. Genomic instability may also lead to chromosome non-disjunction and to the generation of cells with zero, one, two, and three or more chromosome copies [6].

Chromosome polysomy might be considered a quantitative marker of ongoing or accumulated genomic instability in tumors [13]. Although in our study there were only 2 subjects of OSCC showing an increased number of clones with monosomy 17, this seems to suggest that the loss of chromosome 17 may have occurred as an early event before its transformation to OSCC.

Table 1. Correlation between age, gender, TNM status, grading and cytogenetic findings.

No.	Age/Gender	Oscc localization	Grade	Tnm	Mean number of signals	
					CEP17	TP53
1	51/M	Tongue	G2	$T_2N_1M_0$	3	3
2	79/M	Lower lip	G3	$T3N_1M_1$	3	3
3	50/M	Tongue	G2	$T_2N_2M_0$	4	1
4	72/M	Floor of the mouth	G3	$T_2N_0M_0$	3	1
5	56/M	Lower Lip	G3	$T_1N_1M_0$	1	1
6	49/M	Floor of the mouth	G2	$T_2N_2M_0$	4	2
7	54/m	Lower lip	G2	$T_2N_2M_0$	3	3
8	69/F	Jugal mucosa	G2	$T_2N_1M_0$	4	3
9	81/F	Laryngeal	G2	$T_4N_1M_0$	3	3
10	52/M	Gingival	G2	$T_1N_0M_0$	4	4
11	60/F	Tongue	G3	$T_2N_0M_0$	3	2
12	51/M	Lower lip	G2	$T_2N_2M_0$	4	3
13	55/F	Laryngeal	G1	$T_1N_0M_0$	1	0
14	60/M	Lower lip	G2	$T_3N_2M_0$	3	2
15	81/M	Palatinal	G3	$T_3N_2M_0$	1	1

FISH analysis has enhanced the possibility to detect genomic changes and to diagnose complex karyotypes with marker chromosomes [5]. Standard FISH analysis requires the knowledge of the segments involved in the chromosome aberration. In our study, FISH analysis showed a high frequency (80%) of polysomy 17, regardless of the anatomical subsites (oral, laryngeal, and oropharyngeal tumors).

Our results as well as other previously reported findings suggest that losses and gain of genes mapped to chromosome 17 play a major role in the etiopathogeny of OSCC. The degree of numerical abnormality of chromosome 17 varied from subject to subject. This finding suggests that numerical chromosome 17 abnormality is involved in the process of carcinogenesis and development of oral malign neoplasm. Even if we found only 2 subjects with high level of monosomy 17, it might suggest that this phenomenon is an early event of oral cancer. Polysomy of chromosome 17 was also found in tumor-adjacent epithelia, in a lower rate than it occured in tumor cells. The finding of genotypic abnormalities such as polysomy of chromosome 17 in the tumor-adjacent epithelia supports the theory of field cancerization. Such genotypic parameters may provide a genetic basis for the development of an early recurrence or second primary tumors after therapeutic treatment of oral squamous cell carcinomas.

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