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TP53 AND *C-MYC* CO-ALTERATIONS – A HALLMARK OF ORAL CANCER PROGRESSION

SIMULTANA ALTERACIJA *TP53* I *C-MYC* GENA – OBELEŽJE PROGRESIJE ORALNIH KARCINOMA

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Summary

Background: Head and neck squamous cell carcinoma, including oral cancer, is the sixth most common cancer worldwide. Despite advances in surgery and treatment, the 5-year survival rate has not improved significantly. Therefore, reliable molecular markers for oral cancer progression are badly needed.

Methods: We conducted a copy number analysis to estimate amplification status of *c-myc*, *cycD1* and *EGFR* oncogenes, mutational PCR-SSCP analysis to determine activation of *H-ras* oncogene and inactivation of *TP53* tumour suppressor gene and methylation specific PCR analysis to evaluate hypermethylation of *p16* and *MGMT* genes.

Results: *c-myc* oncogene was amplified in 56.7%, *cycD1* in 20% and *EGFR* in 16.7% of Oral Squamous Cell Carcinoma (OSCC) cases while *H-ras* was activated in 33.3% of samples. Amplification of *c-myc* was significantly associated with the tumour grade 2. Interestingly, *EGFR* and *H-ras* alterations were mutually exclusive. *p16* and *MGMT* were inactivated by hypermethylation in 30% and 13.3% of

Kratak sadržaj

Uvod: Skvamocelularni karcinomi glave i vrata (HNSCC) uključujući i skvamocelularni karcinom usne duplje (OSCC) ubrajaju se u šest najčešćih tipova humanih maligniteta. Uprkos značajnim napredcima u hirurškom i terapijskom tretmanu, stopa petogodišnjeg preživljavanja kod ovog tipa maligniteta nije značajnije popravljena. Upravo zato, definisanje pouzdanih molekularnih markera progresije kod OSSC predstavlja apsolutni prioritetet.

Metode: Amplifikacioni status *c-myc*, *cycD1* i *EGFR* gena određen je pomoću eseja za detekciju broja genskih kopija, aktivacija *H-ras* onkogena i inaktivacija *TP53* tumor supresora određena je PCR-SSCP mutacionom analizom, a hipermetilacija promotora *p16* i *MGMT* gena je ispitana metil specifičnim PCR-om (MSP).

Rezultati: Amplifikacija *c-myc* onkogena detektovana je kod 56,7%, *cycD1* onkogena kod 20%, a *EGFR* onkogena kod 16,7% analiziranih oralnih skvamocelularnih carcinoma. Istovremeno, mutaciona aktivacija *H-ras* onkogena detektovana je kod 33,3% ispitanih uzoraka. Amplifikovani

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List of Abbrevations: HNSCC, Head and Neck Squamous Cell Carcinoma; OSCC, Oral Squamous Cell Carcinoma; EGFR, epidermal growth factor receptor; MGMT, O^6-methylguanine DNA methyltransferase; cycD1, cyclin D1; PCR, Polymerase Chain Reaction; SSCP, Single Strand Conformation Polymorphism.

cases. Co-alteration of *cycD1* and *p16* were not observed in any of the analyzed samples. *TP53* was inactivated in 56.7% of samples and was significantly associated with progression of OSCC, grade 2 and stage 2. Moreover, *TP53* and *c-myc* oncogene were simultaneously altered in grade 2 OSCC.

Conclusions: The most promising marker of OSCC progression remains the *TP53* tumour suppressor, which is the most frequently mutated gene in oral cancers. Since there is synergism between *TP53* and *c-myc*, it seems that co-alteration of these two genes could be also a good marker of OSCC progression from grade1 to grade 2 tumours.

Keywords: *c-myc,* oncogenes, oral squamous cell carcinoma, *TP53,* tumour progression, tumour suppressors

c-myc, statisticki značajno korelira sa gradusom 2 OSCC. Posebno intrigantan je bio nalaz po kom se onkogene aktivacije u *EGFR* i *H-ras* genu medjusobno isključuju. Hipermetilacija promotora *p16* gena detektovana je kod 30%, a *MGMT* kod 13,3% analiziranih uzoraka. Ko-alteracije *cycD1* i *p16* gena nisu zapažene ni u jednom od analiziranih uzoraka. Inaktivacija *TP53* gena detektovana je kod 56,7% uzoraka i utvrđeno je da statistički značajno korelira sa gradusom 2 i statusom 2 OSCC. Pored ovoga, utvrđeno je da statistički značajan broj uzoraka gradusa 2, sa aktiviranim *TP53* genom ima istovremeno aktiviran i *c-myc* onkogen.

Zaključak: *TP53*, najčešće mutirani gen u oralnim karcinomima, ostaje za sada i najpouzdaniji marker progresije kod OSCC. Obzirom na detektovani sinergizam između *TP53* i *c-myc* gena, možemo reći da su istovremene promene u ova dva gena još pouzdaniji pokazatelj progresije OSSC iz gradusa 1 u gradus 2.

Ključne reči: *c-myc,* onkogeni, progresija tumora, *TP53,* tumor supresori, skvamocelularni karcinom usne duplje

Introduction

Head and neck squamous cell carcinoma (HNSCC), including oral cancer, is the sixth most common cancer, with an annual incidence of more than 500,000 cases worldwide (1). Oral squamous cell carcinoma (OSCC), which arises from the oral mucosa lining, is the most frequent malignant neoplasm of the head and neck region and accounts for over 90% of these tumours (2). This aggressive malignancy is associated with high mortality and with severe physical, emotional, and psychosocial damage among the rare long-term survivors. Despite advances in surgery and treatment, the 5-year survival rate for patients diagnosed with oral squamous cell carcinomas has not improved significantly and remains at about 50% (3, 4). The prognosis of OSCC is influenced by many factors, such as performance status, TNM staging and pathological grading of differentiation. However, these factors are not sufficient in predicting the outcome. This motivates the search for factors with prognostic relevance in order to better tailor the individual management of OSCC patients. Recent research has focused on identification of molecular prognostic markers that could stage patients in more meaningful prognostic groups.

Oral carcinogenesis is a highly complex multifactorial multi-step process that is driven by the accumulation of multiple genetic and epigenetic alterations. Majority of them represent the activation of oncogenes and the inactivation of tumour suppressor genes, meaning that numerous oncogenes and tumour suppressor genes are implicated in oral carcinogenesis. The most thoroughly studied oncogenes implicated in the pathogenesis and prognosis of OSCC are epidermal growth factor receptor (*EGFR*), and members of gene families – *ras*, *myc* and cyclins. EGFR, the biological receptor of EGF and TGF-α, is frequently overexpressed in oral cancer and was shown to result from *EGFR* gene amplification in 30% of oral cancers (5). Similarly, *c-myc* oncogene, a transcription factor that regulates important processes such as stimulation of proliferation and inhibition of differentiation is found to be amplified in 20 to 40% of oral cancers (6, 7). Some studies showed that members of the *ras* oncogene family are overexpressed in oral cancer, too. However, *H-ras* is usually activated by point mutations in codons 12, 13 and 61. The frequency of *H-ras* mutations in OSCC varies and ranges from 5% in Western countries (8) up to 35% in Asian population (9). Finally, cyclins are essential in controlling the cell cycle. Among cyclins, *cyclin D1* appears to be the most strongly implicated in human oncogenesis since its overexpression plays an essential role in the transition between G1 and S phase in proliferating cells (10). Amplification of *cyclin D1* gene was reported in 25–70% of oral cancers (11).

Among tumour suppressors the most widely studied genes in OSCC pathogenesis are *TP53* and *p16*. Protein products of these genes control the cell cycle and are involved in the inhibition of cell proliferation. Structural and/or functional alterations in these genes produce uncontrolled cell proliferation. Therefore, it is not surprising that reported frequencies of *TP53* alteration ranges from 20–90% in HNSCC, depending on the methodologies, type of tumour material and heterogeneity of tumour site examined (12).

However, the available evidence about the molecular markers still remains inconclusive. Accordingly, there is a continuous need to define the most important biological markers for OSCC progression and invasiveness.

The aim of this study is to provide additional information regarding molecular markers with the greatest potential for predicting clinical outcome in patients with OSCC. These include regulators of the cell cycle: *p16*, *TP53* and *cyclin D1*, DNA repair gene *MGM*T and genes of the commonly activated signaling pathway in tumorigenesis, the *EGFR/H-ras/c-myc* pathway. In addition, we also describe a modification of a method for copy number analyses which was used to investigate amplification of *c-myc* and *cycD1* oncogenes.

Materials and Methods

Tissue samples and DNA Extraction

Genomic DNA used in this study was isolated from 30 fresh frozen, surgically resected SCC of oral cavity collected at the Clinic of Maxillofacial Surgery, School of Dentistry, University of Belgrade. For each obtained tumour sample written consent and approval were acquired according to the ethical standards laid down in the1964 Declaration of Helsinki, the International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS), Geneva 1993, and the Guidelines for Good Clinical Practice CPMP/ICH/135/95), September 1997.

Genomic DNA was extracted from thirty frozen tumour tissue samples and five blood control samples according to the standard phenol/chloroform extraction procedure described by Sambrook and colleagues (13). The quality of the extracted DNA was verified by agarose gel electrophoresis and the concentrations were assessed spectrophotometrically. Isolated DNA was stored at +4 °C until further analyses.

Copy number analysis by quantitative real time PCR

In order to estimate amplification status of the two most often amplified oncogenes in human tumours including OSCC, *c-myc* and *cycD1*, a TaqMan based assay was created. We used *RNase-P* gene as the internal control (reference gene). The rationale for this was that *RNase-P* gene is a single copy gene and is therefore most suitable for normalization in copy number quantification analysis.

Our assay included forward and reverse primers for *c-myc* and *cycD1* oncogenes as well as highly specific 6-Fam-TAMRA labeled probes for them. For the analysis of *cycD1*, the following probe and primers were used: fluorescent TaqMan Probe 6-FAM-5′-CAGCCTTGTTGTTTACGGCCTCTTTGAG-3′-TAMRA; primer *cycD1*_F 5′-GGACAACGGGCGGATAGAG-3′; and primer *cycD1*_R 5′-CACAGTCA TCCCAGGGTTTAACA-3′. For the analysis of *c-myc*, the following probe and primers were used: TaqMan Probe 6-FAM-5′-AGAAGCCGCTCCACATACAGTCCTGG-3′-TAMRA; primers *c-myc*_F 5′-GGACGACGAGACCTTCATCAA-3′; and *c-myc*_R 5′-CCAGCTTCTCTGAGACGAGCTT-3′. In addition, TaqMan® Copy Number Reference Assay, *RNase P*, was used for internal control (accession # 4316831, Applied Biosystems).

The experiments were set in 96-well plastic plates, with samples prepared in duplicates, in total reaction volume of 20 μL, with primers /probe ratio 3:1 (0.1 μmol/L probe : 0.3 μmol/L primers), 1x TaqMan Master Mix and 150 ng of tested DNA per reaction. Each plate contained at least two controls, DNAs isolated from the blood of healthy individuals. This control samples were used as calibrators. PCR reactions were carried out in the ABI Prism 7500 Sequence Detection System at 50 °C for 2 minutes, 95 °C for 10 minutes, followed by 40 cycles at 95 °C for 15 seconds, and 60 °C for 1 minute. The experimental threshold was calculated based on the mean baseline fluorescence signal from cycle 3 to 15 plus 10 standard deviations. The point at which the amplification plot crosses this threshold is defined as Ct, which represents the cycle number at this point and is inversely proportional to the number of target copies present in the initial sample. A mean value of each Ct duplicate was used for further calculation. Each run included a no-template control, as well. The obtained results were analyzed by RQ Study Add ON software for 7500 v 1.3 SDS instrument with a confidence level of 95% ($p \leq 0.05$).

Differential PCR

Amplification status of *EGFR* oncogene was determined by duplex PCR reaction that engaged two pairs of primers, one for the target gene (*EGFR*) and the other for the reference gene (*β-actin*). For differential PCR analysis, a 110-bp fragment of the *EGFR* gene was co-amplified with a 168-bp fragment of *β-actin* (*ACTB*) gene. The primer sequences were as follows: *EGFR*_F 5′-AGCCATGCCCGCATTAGC TC-3′ and *EGFR*_R 5′-AACCCTTCAACGTAAGGAAA-3′ for *EGFR*, and *ACTB*_F 5′-CTCTTTTCTTTCCCGATAGGT-3′ and *ACTB*_R 5′-CTCCAGCTTCTCGTAGGGTC -3′ for the *ACTB*. D-PCR was performed in the total reaction volume of 25 μL with 150 ng of DNA, 1× PCR buffer (50 mmol/L KCl, 10 mmol/L Tris–HCl, pH 8.3, 0.01% gelatin), 1.5 mmol/L MgCl, 0.2 mmol/L each dNTP, 1 μmol/L each of four primers, 1U Taq-Polymerase. Thermal cycling included thirty repeats of denaturation at 95 °C/1 minute, annealing at 58 °C/1 minute and extension at 72 °C/1 minute, with initial denaturation (95 °C/10 minutes) before and final extension (72 °C/10 minutes) after the repeating temperature steps. Generated PCR products were applied to 9% polyacrylamide gel for electrophoresis, stained with silver-nitrate, photographed and analyzed by ImageQuant 5.2 by comparing the median pixel

intensity in a given/selected area of two bands (*EGFR* and *ACTB*) in the same lane (sample). When median pixel intensity of *EGFR* band was equal or higher then 25% of median pixel intensity of *ACTB*, it was interpreted as gene amplification.

PCR-SSCP analysis of TP53 and H-ras genes

The most frequently mutated exons of *TP53* tumour-suppressor gene (5–9) and sequences surrounding the hot-spot codons of *H-ras* oncogene (12,13 and 61) were amplified by PCR and subjected to further mutational screening by single strand conformation polymorphism (SSCP). Primers and PCR conditions are given in *Table I*, and conditions for SSCP mutational screening have been described earlier (14). In brief, amplicons were mixed with double volume of formamide containing 0.05% xylene cianol and 0.05% brom phenol blue, denatured at 95 °C for 5 minutes, cooled on ice for 1 minute and loaded on non-denaturating polyacrilamide gels (PAA). Concentration of PAA gels ranged from 7–12% depending on the amplicon length, and they were prepared with or without glycerol (10% in final) depending on the temperature at which electrophoresis was run. In order to avoid false positives or negatives, samples were examined under at least two different SSCP conditions, including different crosslink (%C=5.26; %C=3.6), temperature, ion charge, etc. Genomic DNA isolated from the blood of five healthy individuals was used as negative control.

Bisulfite modification of DNA and methylation-specific PCR

The methylation status of promoter region of *p16* and *MGMT* genes was determined by methylation-specific PCR (MSP). Extracted genomic DNA was modified by bisulfite treatment according to procedure described by Herman et al (15). In brief, 500ng of total genomic DNA was denatured with 3 mol/L NaOH and bisulfate modification done overnight (eg. eighteen hours) at 55 °C. Besides tumour specimens, totally methylated and unmethylated human DNAs were always included in the bisulfite treatment procedure as controls. To estimate methylation status of the promoter regions of *p16* and *MGMT* genes, a set of four primers per each gene was used: one pair for the detection of methylated CpG-cytosine and the other for the identification of unmethylated CpG-cytosine. Primers for *p16* were as follows: *p16*_MF 5'-TTATTAGAGGGTGGGGCGGATCGC-3' and *p16*_MR 5'-GACCCCGAACCGCGACCGTAA-3' for methylated CpG- cytosine producing an amplicon 150 bp long,

Table I Primers and PCR conditions for the amplification of *H-ras* and *TP53* genes.

Primer	Gene	Length (bp)	Primer sequence	Temperature profile
H1S	exon 1	63	5'-GACGGAATATAAGCTGGTGG-3'	94 °C (85s) 60 °C (95s) 72 °C (72s)
H1A	*H-ras* gene		5'-TGGATGGTCAGCGCACTCTT-3'	40 cycles
H12,13-5'	exon 1	123	5'-ATGACGGAATATAAGCTGGT-3'	95 °C(60s) 56 °C(60s) 65 °C(60s)
H12,13-3'	*H-ras* gene		5'-ATATCTCCACTCGGACCGC-3'	35 cycles
H2S	exon 2	73	5'-AGACGTGCCTGTTGGACATC-3'	94 °C(95s) 60 °C(110s) 72 °C(80s)
H2A	*H-ras* gene		5'-CGCATGTACTGGTCCCGCAT-3'	40 cycles
H61-5'	exon 2	109	5'-AGGTGGTCATTGATGGGGAG-3'	95 °C(60s) 56 °C(60s) 65 °C(60s)
H61-3'	*H-ras* gene		5'-AGGAAGCCCTCCCCGGTGCG-3'	35 cycles
E5S	exon 5	269	5'-TGTTCACTTGTGCCCTGACT-3'	95 °C(60s) 60 °C (60s)
E5A	*p53* gene		5'-CAGCCCTGTCGTCTCTCCAG-3'	35 cycles
Ep5-1	exon 5	238	5'-CCTTCCTCTTCCTACAGTAC-3'	94 °C(15s) 55 °C(15s) 72 °C(90 °C)
Ep5-2	*p53* gene		5'-CCCAGCTGCTCACCATCGCT-3'	35 cycles
E6S	exon 6	181	5'-GCCTCTGATTCCTCACTGAT-3'	95 °C(60s) 60 °C(60s) 72 °C(30s)
E6A	*p53* gene		5'-TTAACCCCTCCTCCCAGAGA-3'	35 cycles
E7S	exon 7	171	5'-ACTGGCCTCATCTTGGGCCT-3'	95 °C(60s) 60 °C (60s)
E7A	*p53* gene		5'-TGTGCAGGGTGGCAAGTGGC-3'	35 cycles
Ep7-1	exon 7	138	5'-GTGTTATCTCCTAGGTTGGC-3'	94 °C(15s) 55 °C(15s) 72 °C(90 °C)
Ep7-2	*p53* gene		5'-AAGTGGCTCCTGACCTGGAG-3'	35 cycles
E8S	exon 8	229	5'-TAAATGGGACAGGTAGGACC-3'	95 °C(60s) 60 °C (60s)
E8A	*p53* gene		5'-TCCACCGCTTCTTGTCCTGC-3'	35 cycles
Ep8-1	exon 8	234	5'-CCTTACTGCCTCTTGCTTC-3'	94 °C(15s) 55 °C(15s) 72 °C(90 °C)
Ep8-2	*p53* gene		5'-TGAATCTGAGGCATAACTGC-3'	35 cycles
E9S	exon 9	210	5'-ACTAAGCGAGGTAAGCAAGC-3'	95 °C(60s) 60 °C (60s)
E9A	*p53* gene		5'-CTGGAAACTTTCCACTTGAT-3'	35 cycles

and *p16*_UF 5′-TTATTAGAGGGTGGGGTGGATTGT-3′ and *p16*_UR 5′-CAACCCCAAACCACAACCATAA-3′ for unmethylated CpG- cytosine producing an amplicon 151bp long. Primers for *MGMT* were: *MGMT*_MF 5′-TTTCGACGTTCGTAGGTTTTCGC-3′ and *MGMT*_MR 5′-GCACTCTTCCGAAAACGAAA-CG-3′ producing amplicons of 81bp, and *MGMT*_UF 5′-TTTGTGTTTGATGTTTGTAGGTTTTTGT-3′ and *MGMT*_UR 5′-AACTCCACACTCTTCCAAAAACAA-AAA-3′ producing amplicons of 93 bp. Methylation specific PCR either for *p16* or for *MGMT* gene, was set up in total reaction volume of 50 μL with the following final concentrations of reaction components: 1×PCR buffer, 6.7 mmol/L $MgCl_2$, 1.25 mmol/L dNTPs, 0.6–0.8 μmol/L each of primers, 0.4 μg/μL BSA, 5% DMSO and 50–100 ng of modified DNA. All reactions were hot started at 95 °C for 5 minutes before the 1.5 U of TaqDream Polymerase (Fermentas Life Sciences, Lithuania) was added. Reactions were carried out in GeneAmp^R PCR System 9700 (Applied Biosystems, Foster City, CA,USA) for 40 cycles with the temperature profiles that included: 45 seconds of denaturation at 95 °C, 45 seconds of annealing at the temperature that depended on used upper listed primers (65 °C/*p16M*; 60 °C/*p16U*; 57 °C/*MGMT*_*M* and *U*), and 1 minute of extension at 72 °C. Each MSP reaction was ended with the final extension at 72 °C for 5 minutes.

Statistical analysis

Significant differences between the data sets were determined by STATISTICA 6.0 software (StatSoft, Inc., Tulsa, USA). The correlations between clinicopathological parameters and alterations of *TP53*, *MGMT*, *p16*, *c-myc*, *cycD1* and *EGFR* genes were evaluated using Fisher exact test. Statistical differences were considered significant when P was < 0.05 (*) and $P<0.01$ (**).

Results

Amplification of c-myc, cycD1 and EGFR oncogenes

We determined amplification status of *c-myc* and *cycD1* oncogenes by Quantitative Real Time PCR using modification of a method described by Akervall and colleagues (16). Namely, we used *RNase P* as a reference gene for the normalization and performed quantification by $2^{-\Delta\Delta Ct}$ method (17). DNA from healthy individuals was used as a calibrator. We interpreted results of quantification > 2 as an abnormality, signifying oncogene amplification. Obtained results are summarized in *Table II*. Specifically, *c-myc* oncogene was amplified in 17 patients out of 30 (56.7%) while *cycD1* was amplified in 6 patients or 20% of cases.

Unlike *c-myc* and *cycD1*, amplification of *EGFR* oncogene was assessed by differential PCR (*Figure 1*) and was found to be amplified in only 5 patients (16.7%).

Further, we analyzed relationship between altered oncogenes and histopathological characteristics (*Table II*) of patients. Amplification of *c-myc* showed to be significantly associated with the grade 2 of the disease (*Table III*). Alterations of *cycD1* and *EGFR* have not revealed any association.

Mutational status of H-ras oncogene and TP53 tumour suppressor gene

H-ras oncogene is usually activated by point mutations in codons 12, 13 and 61. Therefore, we analyzed mutational status of *H-ras* oncogene by PCR-SSCP and obtained that it was activated in 33.3% of patients (*Table II*). Analysis of relationship with histopathological characteristics did not reveal any significant association.

On the other hand, mutational analysis of *TP53* revealed that it was inactivated in 17 out of 30 (56.7%) patients (*Table III*). Moreover, inactivation of *TP53* was significantly associated with progression of tumour, grade 2 (*Table IV*) and stage 2 (*Table V*).

Methylation status of p16 and MGMT tumour suppressor genes

Methylation status of *p16* and *MGMT* was assessed by MSP-PCR (*Figure 2*). Obtained results revealed that *p16* was hypermethylated in 9 patients (30%) and *MGMT* only in 4 out of 30 (13.3%). No correlation was found with histopathological characteristics of the patients.

Relationship between alterations of analyzed genes

In order to reveal possible association among studied genes, we further analyzed whether there were co-alterations between any of them during the evolution of tumour. The only significant association we found was the one between *c-myc* oncogene and *TP53* tumour suppressor gene in patients with grade 2 OSCC. Namely, Fisher exact test revealed that frequency of *TP53* mutations was significantly higher in grade 2 tumours with amplified *c-myc* (*Table VI*). In other words, *c-myc* oncogene and *TP53* tumour suppressor gene were simultaneously altered in grade 2 tumours of oral cavity. All remaining combinations revealed no co-alterations of any significance. However, it is interesting to notice that *EGFR* was never amplified in samples that have had mutated *H-ras*. Similarly, amplification of *cycD1* and inactivation of *p16* have not been detected in the same patient. In other words, alterations in genes from these two sets are mutually exclusive.

Table II Molecular and histopathological characteristics of patients.

Patients	*c-myc* amp	*Hras* mut	*cycD1* amp	*EGFR* amp	*TP53* mut	*p16* met	*MGMT* met	Grade	pT stage
M1	1	1	0	0	1	1	0	G2	T3
M2	0	1	0	0	0	0	0	G1	T1
M3	1	1	1	0	1	0	0	G2	T2
M4	0	1	1	0	0	0	0	G1	T1
M5	1	0	0	0	1	1	1	G1	T2
M6	1	0	0	0	0	1	0	G1	T2
M7	1	0	0	0	0	1	0	G1	T2
M8	1	1	0	0	1	0	0	G2	T2
M9	0	0	0	0	1	0	0	G2	T2
M10	1	1	0	0	0	0	0	G1	T2
M11	1	0	0	0	1	1	0	G2	T2
M12	1	0	0	0	0	0	1	G1	T1
M13	0	0	0	0	1	1	0	G2	T2
M14	0	1	0	0	0	1	0	G1	T1
M15	1	1	0	0	1	0	1	G2	T3
M16	1	0	0	1	1	0	0	G2	T2
M17	0	0	1	0	1	0	0	G1	T2
M18	1	0	0	1	1	1	0	G2	T1
M19	1	0	1	1	1	0	0	G2	T2
M20	0	0	0	1	1	0	0	G1	T1
M21	0	0	0	0	0	1	0	G2	T2
M25	1	1	0	0	0	0	0	G2	T1
M26	1	0	0	0	1	0	0	G2	T1
M27	0	0	0	0	1	0	0	G2	T2
M28	1	1	0	0	1	0	1	G2	T4
M29	1	0	1	0	1	0	0	G2	T1
M30	0	0	0	1	0	0	0	G1	T1
M31	0	0	0	0	0	0	0	G1	T1
M32	0	0	0	0	0	0	0	G1	T1
M33	0	0	1	0	0	0	0	G1	T1

1 – gene alteration is present; 0 – gene is wt.

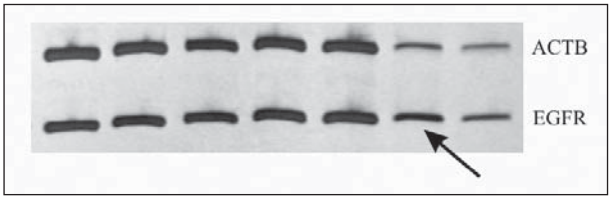

Figure 1 Differential PCR for determination of *EGFR* amplification status. Arrow indicates a sample with amplified *EGFR* gene.

Table III Association between *c-myc* amplification and tumour grade.

Variables	Amplification of *c-myc* oncogene		
	YES N[a] (%)	NO N (%)	p value
G1	5 (16.7)	9 (30.0)	
G2	12 (40.0)	4 (13.3)	p = 0.0355*

[a]N – number of patients per group; *indicates statistically significant values, p≤0.05.

Table IV Association between mutational status of p53 and tumour grade.

Variables	Mutated *TP53*		
	YES N[a] (%)	NO N (%)	p value
G1	3 (10.0)	11 (36.7)	
G2	14 (46.7)	2 (6.6)	p = 0.0004*

[a]N – number of patients per group; *indicates statistically significant values, p≤0.01.

Table V Association between mutational status of p53 and tumour stage.

Variables	Mutated *TP53*		
	YES N[a] (%)	NO N (%)	p value
T1	4 (14.3)	9 (32.1)	
T2	11 (39.3)	4 (14.3)	p = 0.0298*

[a]N – number of patients per group; *indicates statistically significant values, p≤0.05.

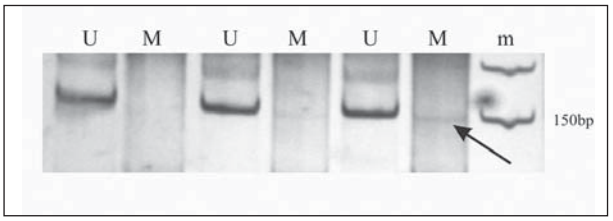

Figure 2 Methylation specific PCR (MSP) for determination of methylation status of promoter region of *p16* tumour suppressor gene. U – unmethylated; M – methylated; m – length marker. Arrow indicates a sample with hypermethylated promoter of *p16* gene.

Table VI Association between alterations of *c-myc* and *p53* genes and tumour grade.

Variables	Amplified *c-myc* oncogene		
	TP53 mutated Na (%)	*TP53* wt N (%)	p value
G1	1	4	
G2	11	1	p = 0.0099*

[a]N – number of patients per group; *indicates statistically significant values, p≤0.05.

Discussion

In the present study we investigated the prognostic value of frequently altered oncogenes and tumour suppressor genes in 30 cases of Oral Squamous Cell Carcinoma. To that end, we conducted a copy number analysis to estimate amplification status of *c-myc*, *cycD1* and *EGFR* oncogenes, mutational analysis to determine activation of *H-ras* oncogene and inactivation of *TP53* tumour suppressor gene and methylation analysis to evaluate methylation status of *p16* tumour suppressor and *MGMT* DNA repair gene. Obtained alterations were correlated with each other and with histopathological parameters.

Copy number analysis revealed unusually high frequency of *c-myc* amplification (56.7%) in this cohort, which was considerably above previously reported 20 – 40% (6–8). Tumorigenicity of the c-myc protein is ascribed to the promotion of cell proliferation and inhibition of apoptosis (18, 19). Its overexpression in OSCC is the result of gene amplification and is usually associated with poorly differentiated tumours (20). Our results confirm this finding. Namely, amplification of *c-myc* oncogene was significantly associated with advanced tumours of grade 2 compared to grade 1, indicating its role in tumour progression. However, its correlation with survival of OSCC patients is not clear (21).

Contrary to *c-myc*, *EGFR* and *cycD1* oncogenes were amplified with relatively low frequency, 16.7% and 20.0% respectively, in this set of samples. At first sight, this was an unexpected result because both of them are frequently amplified in OSCC (5, 11). However, if we take into consideration mutational oncogenic activation of *H-ras*, which appeared at very high frequency for European population of 33.3%, the picture becomes more complex but clearer and even expected. Namely, *H-ras* is usually induced by *EGFR*, which mediates extracellular mitogenic stimuli. Downstream event of Ras activation may actually be induction of *cycD1* and promotion of cell replication (22). Ras activates *cycD1* promoter via an AP-1 like sequence (23) and achieves predominant CycD1 nuclear localization. As a consequence, the cells may acquire ability for non-adherent growth (24) or independent cell cycle progression (25). Thus, activation of either of these oncogenes could have the same tumorigenic consequence, that is continuous cell replication, since they act in the same signaling pathway. Therefore, oncogenic activation of all of them at the same time could not be justified from biological point of view and, maybe, we should consider them as a package. In favor of this is our finding that *EGFR* and *H-ras* were never altered in the same tumour sample. According to our results, alterations in these two oncogenes are mutually exclusive. That could be the explanation for such a low frequency of *EGFR* amplification. Lower frequency of *cycD1* amplification (20%) then reported by other authors (11) could be the consequence of another, additional finding. Specifically, we have not observed co-alteration of *cycD1* and *p16* in any of the analyzed samples. This is important because *cyclin D1* activation and *p16* inactivation have the same effect, *i.e.*, an increase in *pRb* phosphorylation and progression from G1 to S phase in the cell cycle (26). Hence, alteration of either of these genes will have the same outcome and there is no need for simultaneous changes in both. In other words, if *p16* is inactivated, oncogenic activation of *cycD1* is not necessary and would not be biologically rational.

One of the tumour suppressor genes most frequently involved in human OSCC is *TP53* (27). We found that it was mutated in 56.7% of tumour samples, which is within reported frequencies (12). Inactivation of *TP53* was significantly associated with more advanced primary tumours, grade 2 (p= 0.0004) and pT stage 2 (p=0.0298). Many reports support this result (12, 28), but there are many which show that inactivation of *TP53* is an early event (29, 30). Opinions about this issue are contradictory. Moreover, we showed co-alteration of *TP53* and *c-myc*, *i.e.*, inactivation of *TP53* significantly correlated with the amplification of *c-myc* in grade 2 tumours. The interactive involvement of *c-myc* and *TP53* with cell proliferation and apoptosis, when altered by mutational events, seems to be a key regulatory element of oncogenesis and co-alteration of these two important genes is considered to be a relatively early

event in OSCC progression, before significant invasion (reviewed by Papakosta et al (31)).

In conclusion, despite major advances in our understanding of the molecular pathology of OSCC, numerous gaps have still to be filled. The most promising marker remains the *TP53* tumour suppressor, which is the most frequently mutated gene in OSCC. Since there is synergism between *TP53* and *c-myc*, it seems that co-alteration of these two genes could be also a good marker of OSCC progression from grade1 to grade 2 tumours.

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Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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