Int. J. Oral Maxillofac. Surg. 2018; xxx: xxx–xxx
https://doi.org/10.1016/j.ijom.2018.01.020, available online at https://www.sciencedirect.com

International Journal of Oral & Maxillofacial Surgery

Research Paper
Head and Neck Oncology

Genetic and epigenetic alterations in the tumour, tumour margins, and normal buccal mucosa of patients with oral cancer

N. Eljabo[1], N. Nikolic[1], J. Carkic[1], D. Jelovac[2], M. Lazarevic[2], N. Tanic[3], J. Milasin[1]

[1]Institute of Human Genetics, School of Dental Medicine, University of Belgrade, Belgrade, Serbia; [2]Clinic for Maxillofacial Surgery, School of Dental Medicine, University of Belgrade, Belgrade, Serbia; [3]Laboratory for Radiobiology and Molecular Genetics, Institute of Nuclear Sciences "Vinca", University of Belgrade, Belgrade, Serbia

N. Eljabo, N. Nikolic, J. Carkic, D. Jelovac, M. Lazarevic, N. Tanic, J. Milasin: Genetic and epigenetic alterations in the tumour, tumour margins, and normal buccal mucosa of patients with oral cancer. Int. J. Oral Maxillofac. Surg. 2018; xxx: xxx–xxx.

Abstract. Despite adequate surgical resection, oral squamous cell carcinoma (OSCC) shows a high rate of recurrence and metastasis, which could be explained by the presence of molecular alterations in seemingly normal tumour margins and the entire oral mucosa. The aims of this study were (1) to assess the presence of gene amplification (c-Myc and HER2) and promoter methylation (p14 and p16) in the tumours, tumour margins, and unaffected oral mucosa of 40 OSCC patients, and (2) to evaluate the possibility of using these alterations as prognostic markers. c-Myc and HER2 genes were quantified by means of real-time PCR (qPCR), and p14 and p16 methylation status was determined by methylation-specific PCR (MSP PCR). All tissues examined exhibited molecular alterations in various proportions. Tumour tissues, as expected, showed the highest prevalence of alterations, while oral mucosa showed the lowest. Multiple alterations (co-alterations) in tumours and tumour margins were significantly more frequent than in unaffected oral mucosa ($P < 0.001$ and $P = 0.027$, respectively). HER2 amplification in margin tissue ($P < 0.001$) and swabs ($P = 0.013$), as well as the existence of three co-alterations in margins ($P = 0.001$) and macroscopically unaffected oral mucosa ($P < 0.001$) were correlated with shorter disease-specific survival.

Key words: gene amplification; promoter hypermethylation; OSCC; tumour margin; field cancerization.

Accepted for publication 26 January 2018

Squamous cell carcinoma (SCC) is one of the most common malignancies in the oral cavity[1]. The early stages of the disease, which have a good prognosis, are usually asymptomatic. Thus most cases are diagnosed at an advanced stage[2], characterized by a poor prognosis and low survival. Despite advances in diagnosis and therapy, the incidence of recurrence, local and distant metastasis, and mortality rates remain high[1].

Oral squamous cell carcinoma (OSCC) arises as a result of the accumulation of

0901-5027/000001+07

genetic and epigenetic alterations, which lead to the activation of proto-oncogenes and inactivation of multiple tumour suppressor genes[3,4]. Gene amplification is a frequent mechanism of proto-oncogene activation resulting in gene overexpression; it also represents a common manifestation of genome instability in tumour cells[5]. Oncogene amplification plays a critical role in the development of several human cancers including OSCC. It is associated with aggressive tumour behaviour, metastasis, resistance to chemotherapy, etc., and thus may be used as an indicator of a poor prognosis[6].

The HER2 gene (also known as ERBB2 and c-erbB2) encodes an epidermal growth factor receptor (EGFR) family protein with tyrosine kinase activity that possesses a strong cell proliferation enhancement potential[7]. The c-Myc gene product is an important transcriptional factor involved in control of the cell cycle, and its overexpression can induce cell cycle progression and cellular growth and differentiation[8].

Gene promoter hypermethylation, one of the most studied epigenetic modifications, usually results in transcriptional inactivation. DNA methylation changes targeting tumour suppressor genes (TSG) were identified as the first epigenetic alterations in cancer and have been related to the early stages of carcinogenesis[9,10]. It has also been shown that the methylation of several TSGs plays a significant role in the development and progression of OSCC. More specifically, $P14^{ARF}$ and $P16^{INK44a}$ epigenetic silencing has been observed in OSCC tissues and precancerous oral lesions[11,12].

Currently available protocols for the treatment of OSCC include extended local excision with adjuvant radiotherapy and chemotherapy, with the aim of improving the prognosis for patients more likely to experience recurrence[13]. The accuracy of resection is based on the histological status of the tumour margins; however some OSCC patients with histologically tumour-free margins may have recurrences and experience treatment failure[14]. This indicates that histopathological assessment is not sufficient, i.e. that the histological status of the resection margins is not always a dependable predictor of recurrence. More than 20 years ago, Brennan et al. showed that P53 mutations may be present in so-called 'negative' margins[15]. Indeed, genetic alterations in tumour margins, associated with local recurrence, were later confirmed by several authors[16,17]. Similar to the alterations found in the margins in OSCC patients, subtle molecular changes of the entire oral mucosa, which still appears macroscopically and microscopically normal, cannot be ruled out, and this might also explain to some extent the propensity of OSCC to recurrence and second primary tumours.

The aim of this prospective study was to assess the mutational/epimutational status of four genes involved in cell cycle control in oral cancer. The incidence of HER2 and c-Myc amplification and p14 and p16 promoter methylation was determined in the tumours, histologically tumour-free margins, and normal mucosa of 40 OSCC patients.

Materials and methods

Patients and samples

The study was performed in accordance with the ethical principles governing medical research and human subjects as laid down in the Declaration of Helsinki (2002 version, http://www.who.int/bulletin/archives/79%284%29373.pdf) and with the approval of the Ethics Committee of the School of Dental Medicine. All study participants were informed of the procedures and signed a written consent form. The study included 40 patients (62% male, mean age 65.31 ± 10.50 years; 55% smokers, 45% alcohol consumers) with diagnosed OSCC, treated at the Clinic for Maxillofacial Surgery, School of Dental Medicine, University of Belgrade during the years 2014 to 2016.

The histopathological diagnosis of OSCC was established according to the World Health Organization (WHO) guidelines, and the tumour staging was performed using the TNM classification. All tumours were primary tumours with an infiltrative pattern of invasion. The locations of the tumours were as follows: lip ($n = 11$), mandibular mucosa ($n = 3$), buccal mucosa ($n = 1$), floor of the mouth ($n = 16$), anterior tongue ($n = 1$), and oropharynx ($n = 8$). Three samples were taken from each patient: tumour, tumour margin, and a buccal mucosa swab. Margin samples were taken at least 5 mm from the edges of the surgical defects after primary tumour excision, and the absence of neoplastic cells was confirmed histologically. Swab samples, considered an acceptable source for OSCC biomarker detection, were taken from the healthy buccal mucosa, 2 days after tumour resection and after careful mouth disinfection[18]. DNA was obtained from the frozen tumour and margin tissue by proteinase K digestion and phenol–chloroform extraction, and a commercial kit (Invitrogen, Carlsbad, CA, USA) was used for DNA extraction from swabs.

Real-time polymerase chain reaction

A quantitative real-time polymerase chain reaction (qPCR) and comparative cycle threshold (Ct) method of quantitation of HER2 and c-Myc were performed, as reported previously[19]. Maxima SYBR Green qPCR Master Mix (2X) (ThermoFisher Scientific, Waltham, MA, USA) was used following the manufacturer's instructions, and 20 ng of DNA was added as a template in each reaction. All real-time PCR experiments were performed in duplicate. Primer sequences and annealing temperatures are listed in Table 1. The Ct value was calculated for each sample and

Table 1. qPCR and MSP PCR primer sequences, product lengths, and annealing temperatures.

Primer	Primer sequence (5′–3′)	Length (bp)	Annealing temperature (°C)
HER2	CCTCTGACGTCCATCCT		
	ATCTTCTGCTGCCGTCGTT	98	55
c-Myc	GCTCCAAGACGTTGTGTGTTCG		
	GGAAGGACTATCCTGCTGCCAA	158	55
D2R	CCACTGAATCTGTCCTGGTATG		
	GTGTGGCATAGTAGTTGTAGTGG	112	55
p14 U1	TTTTTGGTGTTAAAGGGTGGTGTAGT		
p14 U2	CACAAAAACCCTCACTCACAACAA	132	53
p14 M1	GTGTTAAAGGGCGGCGTAGC		
p14 M2	AAAACCCTCACTCGCGACGA	122	53
p16 U1	TTATTAGAGGGTGGGGTGGATTGT		
p16 U2	CAACCCCAAACCACAACCATAA	151	60
p16 M1	TTATTAGAGGGTGGGGCGGATCGC		
p16 M2	GACCCCGAACCGCGACCGTAA	150	65
HPV16F	TCAAAAGCCACTGTGTCCTG		
HPV16R	CGTGTTCTTGATGATCTGCA	120	53

qPCR, quantitative real-time polymerase chain reaction; MSP PCR, methylation specific polymerase chain reaction; bp, base pairs.

Please cite this article in press as: Eljabo N, et al. Genetic and epigenetic alterations in the tumour, tumour margins, and normal buccal mucosa of patients with oral cancer, *Int J Oral Maxillofac Surg* (2018), https://doi.org/10.1016/j.ijom.2018.01.020