Expression of PCNA, CD-31 and HER-2 in Serbian patients with oral squamous cell carcinoma

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Abstract: Several studies have investigated the expression of tumor markers, including p53, HER-2, PCNA, EGFR, VEGFR CD-31 and Bcl-2 in patients with oral squamous carcinoma (OSC). This study aimed to determine the expression of proliferating cell nuclear antigen (PCNA), endothelial functions of platelet/endothelial cell adhesion molecule-1 (CD31) and human epidermal growth factor receptor-2 (HER-2) according to OSC stage. The prospective study included 62 patients diagnosed with OSC stages II and III. Surgical specimens were obtained from tumor and peritumoral tissues. We determined the pathohistological degree of tumor differentiation and the immunohistochemical expression of PCNA, CD-31 and HER-2 for each specimen. Immunohistochemical analysis of the expression of PCNA in tumor cells demonstrated poor staining of immunoreactive tumor cells in 23 patients (10 in stage II, 7 in stage IIIa and 6 in stage IIIb). Moderately expressed PCNA-immunoreactivity in the tumor cells in 17 patients (7 in stage II, 6 in stage IIIa and 4 in stage III), and extremely strong PCNA-immunoreactive staining in tumor cells of 10 patients with IIIb stage, was observed. These results suggest that PCNA expression combined with pathohistological findings could possess a prognostic value in determining the survival rates for patients with oral squamous cell carcinoma.

Keywords: oral carcinoma; squamous cell carcinoma; tumor markers; PCNA, CD-31, HER-2

INTRODUCTION

Cancer is a multifactorial disease caused by a series of genetic alterations that lead to cellular proliferation and differentiation [1]. Cellular proliferation is an important indicator of the biological aggressiveness of cancer. Oral squamous cell carcinoma (OSC) is the sixth most common malignancy and is a major cause of morbidity and mortality [2]. Ninety percent of all oral malignancies (lip, oral cavity) are squamous cell carcinomas (SCC) with an estimated 354864 new cancer cases of lip and oral cavity in 2018 worldwide, while the number of new cases in Serbia was 726 [3].

Prognosis and survival rate are low despite the advance in OSC diagnosis and treatment recorded in recent years [4-6]. Evaluation of molecular changes in cancer and their extrapolation on clinical parameters might be beneficial in future diagnostic and treatment approaches [7-9]. The expression of different tumor markers in patients with OSC, including tumor protein 53 (p53), human epidermal growth factor receptor-2 (HER-2), proliferating cell nuclear antigen (PCNA), epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), endothelial functions of platelet/endothelial cell adhesion molecule-1 (CD-31) and B-cell leukemia/lymphoma 2 (Bcl-2), has been explored [10-13]. The presence of tumor cells in tissue at the tumor margins is a common finding in high-grade OSC and can be a direct or indirect indicator of disease progression during five-year survival rate monitoring [14].

Biological markers provide information about the differentiation and proliferation of tumor cells and carcinoma progression and therefore play a significant role in disease prognosis. The nuclear antigen of PCNA is an acidic nuclear protein that fluctuates during the cell cycle and is connected with cell proliferation [15]. PCNA is important for DNA synthesis and repair, cell proliferation and cycle progression. The levels of PCNA are low and undetectable in quiescent cells, but the production of this protein occurs before DNA replication. PCNA increases at G1/S phases, decreases at phase G2 of the cell cycle, and hence it is a sensitive index of proliferation [16,17]. Increased PCNA expression follows tissue progression from a normal epithelium to hyperplasia and dysplasia and premalignant and malignant lesion of the oral cavity [18,19].

Increased expression of different vascular markers such as VEGF, von Willebrand factor (VWF), CD-31, CD-34 and endoglin (CD105) in OSC was found to accompany the progression of histological abnormality [20]. CD-31 and CD-34 were shown to be more sensitive than factor VWF for evaluating tumor blood vessels, but no significant relationship between CD-31 and OSC stages was detected [20].

The expression of HER-2 in OSC was seen to range between 2.5-88% [21]. HER-2 belongs to the HER gene family that regulates cell growth, survival, differentiation and migration. HER-2 expression and gene amplification were rarely detectable in OSC and are apparently unrelated to tumor phenotype or survival rates of patients with OSC [22].

Most of these studies aimed to clarify the molecular origin of OSC and to determine the biomarkers for early detection, prognosis, prediction of outcome and therefore more efficient treatment regimens of patients with OSC [7,8,23,24]. The present study aimed to determine the expression of PCNA, CD-31 and HER-2 in patients with OSC according to histopathologic and clinical tumor stage.

MATERIALS AND METHODS

Study design

The study was designed as a prospective study of a series of cases. The principles of ICH Good Clinical Practice were strictly followed, and approval from the Ethics Committee of the Military Medical Academy was obtained.

The study population included 62 patients with OSC stages II and III according to TNM (T-tumor, N-lymph node, M-metastasis) classification [25,26]. All patients underwent the same type of surgery at the Clinic for Maxillofacial Surgery of Military Medical Academy in the period between 1995 and 2015. Stage II (T1N0M0) includes tumors between 2 and 4 cm in size, with no metastasis detected in distant organs and lymph nodes. Stage IIIa (T3, N0, M0) includes tumors larger than 4 cm. In stage IIIb (T1-3, N1, M0) tumor size can range between 2 and more than 4 cm, with metastasis detected in the ipsilateral lymph node with a size up to 3 cm [25,26].

After tumor excision, histological analysis of the specimens was performed in the Institute for Pathology and Forensic Medicine of the Military Medical Academy. After standard morphological diagnosis based on tissue sections fixed in formalin and embedding in paraffin, immunohistochemical (IHC) analysis was performed (in the Laboratory for Immunohistochemistry and Electron Microscopy of the Institute for Medical Research, Military Medical Academy).

Histopathological analysis

For histopathological analysis, tumor tissue sections were fixed in 5% buffered neutral formalin and processed in a V.I.P. Sakura apparatus (Sakura, Netherlands) for automatic fixation, dehydration and embedding tissue, and were then embedded in paraffin. Prepared tissue blocks were sliced (thickness 5-7 μm). The tissue sections were mounted on separate adherent chips (SuperFrost, Thermo Fisher Scientific Gerhard Menzel B.V. and Co., Germany) and dried at 56°C for 1 h.

The histological grade of the tumor, the degree of infiltration of the peritumoral tissue as well as perivascular, perineural and lymphatic infiltration were determined using an Olympus AX70 (Olympus Optical co. GMBH., Germany) microscope. The degree of infiltration of connective, adipose, muscle and bone tissues was scored as follows: 0 – no infiltration; 1 – infiltration is present at 0.5 cm from the edge of the tumor; 2 – presence of infiltration at 0.5-1 cm from the edge of the tumor; 3 – infiltration of tissue at >1cm from the edge of the tumor.

Immunohistochemical analysis

Marking of tumor cells in the peritumoral tissue was performed with monoclonal and polyclonal anti-human antibodies (Dako Cytomation, Denmark). Visualization of the marker was carried out using highly sensitive and specific methods, IHC and labelling with the streptavidin-biotin complex method (Universal LSAB™+ Kit HRP). We determined the presence and number of tumor cells in the peritumoral tissue by semiquantitative analysis of specimens using PCNA, HER-2 and CD-31 tumor markers (Supplementary Fig. S1). The findings were defined as the level of expression of a particular tumor marker (PCNA, HER-2 and CD-31) in the peritumoral tissue specimen. The level of expression was scored based on the intensity of staining and the number of immunoreactive cells, as follows: 0 – no immunoreactive cells; 1 – poor staining, rare positive cells; 2 – moderate staining, small number of positive cells; 3 – intense staining, numerous positive cells.

Statistical analysis

For statistical analysis, we used the commercial statistical program SPSS for Windows, ver. 18.0. To test the normality of the distribution of the statistical characteristics, we used the Kolmogorov-Smirnov test. To test the intergroup differences, we used the Kruskal-Wallis test and analysis of variance (ANOVA), depending on the type of variables. All categorical variables were presented as the percentage frequency of certain categories. For categorical variables, the statistical significance of differences was examined using the chi-square test.

RESULTS

Sociodemographic characteristics

The present study enrolled 62 patients, 28 patients with stage II and 34 with stage III (Fig. 1) cancer. The age of the patients was 56.19 ± 1.41 years (range 39-85 years) (ANOVA, p>0.05). Tumor size less than 2 cm (T1 stage) was found in 38.71% of patients, while tumors larger than 4 cm (T3 stage) were found in 50% of patients.

Immunohistochemical analysis according to tumor stage

Tumor marker (PCNA, CD-31 and HER-2) expression corresponding to tumor size was significant only for PCNA (chi-square test, p=0.002) (Table 1).
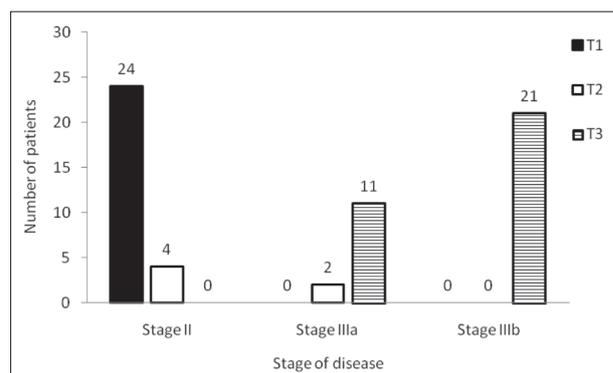

Fig. 1. Distribution of patients with squamous cell carcinoma according to the clinical stage (II, IIIa and IIIb) and tumor size (T1 – <2cm, T2 – 2-4 cm; T3 – >4 cm). Data are presented as absolute numbers of patients. The total number of patients was 28 patients with stage II, and 34 with stage III. Tumor size was <2cm (T1 stage) and was found in 24 or 38.71% patients; tumors larger than 4 cm (T3 stage) were found in 32 or 50% of patients.

Immunohistochemical analysis of PCNA expression in tumor cells demonstrated poor staining of immunoreactive tumor cells in 23 (37%) patients, of which 10 (36%) were in stage II, 7 (54%) in stage IIIa and 6 (29%) in the stage IIIb. Moderately expressed PCNA-immunoreactivity in tumor cells was detected in 17 patients (7 or 25% of the patients in stage II, 6 or 46% in stage IIIa, and 4 or 19% in stage IIIb); extremely strong PCNA-immunoreactive staining was noted in tumor cells of 10 or 48% of the patients with stage IIIb

Table 1. Expression of PCNA, HER-2 and CD-31 in peritumoral tissues according to tumor size.

Marker	Expression	Tumor size			p
		T1	T2	T3	
PCNA	0	9	2	1	0.002*
	1	10	-	13	
	2	5	4	8	
	3	-	1	9	
HER-2	0	24	7	26	0.066
	1	-	-	5	
	2	-	-	-	
	3	-	-	-	
CD-31	0	22	4	25	0.103
	1	2	3	6	
	2	-	-	-	
	3	-	0	-	

Expression: 0 – no immunoreactive cells; 1 – poor staining, rare positive cells; 2 – moderate staining, a small number of positive cells; 3 – intense staining, a greater number of positive cells.
Tumor size: T1 – tumor <2 cm; T2 – tumor 2-4 cm; T3 – tumor >4 cm.
*p<0.05 (chi-square test).

Table 2. Expression of PCNA, HER-2 and CD-31 in peritumoral tissue cell according to clinical tumor stage.

Marker	Expression	Stage II	Stage III		p
			IIIa	IIIb	
PCNA	0	11	-	1	0.001*
	1	10	7	6	
	2	7	6	4	
	3	-	-	10	
HER-2	0	28	13	16	0.005*
	1	-	-	5	
	2	-	-	-	
	3	-	-	-	
CD-31	0	24	13	14	0.038*
	1	4	-	7	
	2	-	-	-	
	3	-	-	-	

Level of expression: 0 – no immunoreactive cells; 1 – poor staining, rare positive cells; 2 – moderate staining, a small number of positive cells; 3 – intense staining, numerous positive cells.
*p<0.05 (chi-square test).

stage of the disease. Differences in the level of PCNA expression in peritumoral tissue cells, depending on the stage of the disease, were statistically significant (Kruskal-Wallis test; p<0.001).

Differences in the degree of expression of PCNA, CD-31 and HER-2, according to clinical tumor staging, was statistically significant for all investigated markers (Table 2). At stages IIIa and IIIb, 20 (59%) of the patients had a higher level of PCNA (expression levels 2 and 3) compared to patients in stage II. The expression of HER-2 and CD-31 were positive only in 10 patients with IIIb stage.

Significant differences were found in the level of expression of PCNA and CD-31 in metastatic lymph nodes (N1 and N2). PCNA had 1 and 2 levels of expression (Table 3). On the other hand, CD-31 expression level was 0 in most of the cases (Table 3).

DISCUSSION

We investigated the level of expression of certain markers in OSC specimens. We found that the levels of PCNA expression depended significantly on tumor size, TNM stage and metastatic lymph node presence.

OSC is the result of the final stage in the process of carcinogenesis, resulting from genetic and epigenetic

Table 3. Expression of PCNA, HER-2 and CD-31 in peritumoral tissue cell according to lymph node status.

Marker	Expression	N stage		p
		N1	N2	
PCNA	0	11	1	p=0.001*
	1	17	6	
	2	13	4	
	3	1	9	
HER-2	0	41	16	p=0.060
	1	1	4	
	2	-	-	
	3	-	-	
CD-31	0	38	13	p=0.036*
	1	4	7	
	2	-	-	
	3	-	-	

Level of expression: 0 – no immunoreactive cells; 1 – poor staining, rare positive cells; 2 – moderate staining, a small number of positive cells; 3 – intense staining, numerous positive cells.
*p<0.05 (Chi-square test).

defects, and potentiated by the action of cancerogenic substances. Two classes of genes have a key role in this process: proto-oncogenes and tumor suppressor genes, which are necessary for the control of proliferation, differentiation and cell death [27-29].

A set of proteins, including p53, Bcl-2, HER-2, PCNA, epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF), were analyzed and correlated with histopathologic parameters in studies that aimed to analyze the metastatic potential of tumors [12,30,31]. Numerous studies aimed to detect and identify the prognostic properties of the tumor markers for the growth of the OSC and to establish the correlation between the expression level of certain markers and disease prognosis [12,31-33]. Contemporary studies are investigating the role of early detection of premalignant and malignant lesions, early detection of local recurrence and distant metastasis, and early prevention of secondary tumors, as well as a selection of the most effective treatment for the patient [7,17,34-36].

Basic morphological features for estimation of the malignancy level include the histological grade, nuclear grade, mitotic index and nucleocytoplasmic ratio [37-40]. These parameters are significantly correlated with the degree of tumor infiltration of marginal resection tissue [41,42]. Studies have shown differences in the survival rate of patients with OSC [4-6]. Additionally, the low survival rate of patients diagnosed with OSC was correlated with increased expression of PCNA [43,44]. Thus, it was suggested that PCNA might be used as a biomarker for the initial assessment of OSC aggressiveness [43,44].

No specific antigenic properties were found in OSC. To date, the most studied genes and markers are the genes for p53, cyclin D1, transforming growth factor (TGF), EGF, VEGF, E-cadherins and collagen VII [10,45,46]. However, none of these markers has proved to be specific for the prognosis and diagnosis of OSC.

Previous studies found a positive correlation between PCNA proteins with tumor grade and the clinical stage of OSC [43,44]. Our results indicate that the expression of PCNA in OSC is in a high correlation with the histological grade of the tumor and the degree of infiltration of peritumoral tissue, which are indicators of poor prognosis and reduced survival rates

of patients with OSC. The prognostic value of PCNA could be significant for patients with OSC. The synthesis of PCNA protein correlates with cell proliferation as it is expressed in the nuclei of cells during DNA synthesis [43]. Tumor proliferation was identified as an important predictor of biologic behavior for various malignancies [47]. PCNA is one of the proteins important in cell proliferation, and antibodies to this protein help to assess the tumor proliferation activity of oral lesions. Varying degrees of atypia in OSC were analyzed employing PCNA immunoreactivity. Focal immunoreactivity of PCNA was noted in highly differentiated OSC and elevated cell proliferation, and aneuploidy was observed in the entire tumor in poorly differentiated OSC [48]. The intensity of PCNA immunoreactivity is in correlation with tumor cell differentiation, nuclear atypia and the patterns of invasive margins in the underlying connective tissue [44]. Increased expression of PCNA was indicative of poor differentiation, higher nuclear atypia and more invasive growth of tumor cells [44]. The presence of PCNA proteins in chronic and premalignant lesions, such as oral lichen planus and epithelial dysplasia. could be helpful in the evaluation of malignant potential [49].

Based on the significant difference in the PCNA index between preinvasive and invasive squamous epithelial lesions of the cervix, Pahuja et al. [50] suggested that the PCNA index might have a predictive value for the prognosis of patients with squamous cell carcinoma. The PCNA index could be helpful in the identification of patients whose carcinoma's *in situ* lesion will progress and require treatment, and those whose lesion will remain static or regress [51]. PCNA in tissue sections can be used as an adjunct to diagnose preneoplastic and neoplastic lesions [52]. The use of a specific biomarker of dysplasia in conjunction with histological procedures could improve the accuracy, precision and sensitivity of potential screening program. Accordingly, PCNA could play an important role in the pathogenesis, progression and metastasis in patients with OSC [53,54].

The family of ErbB receptors includes four receptors known as HER-1, HER-2, HER-3 and HER-4. Ligand binding to the ErbB receptors (transmembrane tyrosine kinase receptors) leads to the transcription of genes responsible for the inhibition of apoptosis, cell growth, angiogenesis, cell adhesion, cell motility and

invasion, and enhancement of the malignant potential in epithelial tissues [55]. HER-2 has been reported to be overexpressed in many cancers and associated with poor prognosis. [56]. Immunohistochemical staining has been the most common method used to detect overexpression of ErbB receptors. Bernardes et al. [55] showed that 97.8% of tumor specimens were negative for HER-2 in OSC patients. Thus, HER-2 was not confirmed as a prognostic factor, with which our results are in agreement.

Neoangiogenesis is one of the essential events in neoplastic progression. Vascular marker CD-31 is an important marker used for quantifying microvessels and correlating with tumor growth and prognosis [57]. CD-31 expression in tumor blood vessels in OSC showed no significant relationship with OSC stage [20]. However, overexpression of CD-31 correlated with the progression of histological abnormality [20]. In our study, we found overexpression of CD-31 in 11 tumor specimens. Tumor angiogenesis and the density of newly formed vessels have a potential prognostic relevance in the assessment of OSC, with CD-34 possessing a higher correlation in the assessment of tumor vascularization than CD-31 [58,59].

The present study evaluated the immunohistochemical expression of PCNA, HER-a and CD-31 in tumor samples after OSC resections. Expression of positively stained PCNA cells increased with more advanced stages of the disease, i.e. from II to the IIIb TNM stage, suggesting an increase in the proliferative activity of tumor cells. Therefore, PCNA expression along with clinical characteristics might be useful in predicting the aggressiveness and recurrence rate of OSC. Large-scale multicentric studies are needed to define the economic feasibility for implementing this type of testing in all patients diagnosed with OSC because of the high costs of immunohistochemical analysis, especially in developing countries. The present study has some limitations as this was a single-center study that included a small sample of the patients and the expression of only three tumor markers, PCNA, HER-2 and CD-31.

CONCLUSIONS

Immunohistochemical analysis of PCNA expression in tumor cells demonstrated poor staining of immu-

noreactive tumor cells in 23 patients (10 in stage II, 7 in stage IIIa and 6 in stage IIIb), and moderate expression of PCNA-immunoreactivity in 17 patients (7 in stage II, 6 in stage IIIa and 4 in stage IIIb). Very strong PCNA-immunoreactive staining was observed in tumor cells in 10 patients with stage IIIb of the disease. Differences in the level of expression of PCNA in peritumoral tissue cells were statistically significant depending on the tumor stage. Further work is needed to fully elucidate the role and use of PCNA in early detection of premalignant and malignant lesions, local recurrence and distant metastasis, prevention of secondary tumors and choice of an appropriate therapeutic protocol. The presented results suggest that PCNA, in combination with histopathological parameters, could be an important prognostic factor.

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Supplementary Material

The Supplementary Material is available at: http://serbiosoc.org.rs/NewUploads/Uploads/Loncarevic%20et%20al_4455_Supplementary%20Material.pdf