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ORYGINAL ARTICLE

Association between clinical stage of oral cancer and expression of immunohistochemical markers

Authors' Contribution:
A–Study Design
B–Data Collection
C–Statistical Analysis
D–Data Interpretation
E–Manuscript Preparation
F–Literature Search
G–Funds Collection

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ABSTRACT: Aim: The aim of this study is to demonstrate the possible correlation between the expression of examined protein markers - p53, EGFR, PCNA, p44/42 in the mass of the tumor and the clinical stage of disease. Material: 48 patients of the Department and Clinic of Oral and Maxillofacial Surgery, Lublin diagnosed with oral cancer. The control group consisted of 10 patients diagnosed with leukoplakia lesions in the oral cavity. The methods: Immunohistochemical analysis using the detection system DAKO K5007 Cat - Dako REAL ™ Detection System, Peroxidase DAB +, Rabbit / Mouse. Results: Based upon the statistical results, significant correlation between p53 protein and tumor staging; however, a correlation between the level of expression of EGFR, p44/42, PCNA and staging was not likewise revealed. Conclusions: Looking for oral squamous cell carcinoma markers remains an actual issue. Identification of specific markers of oral cancer could be used in screening the population, determining prognosis and response to treatment.

KEYWORDS: oral cancer, neoplasm markers, squamous cell carcinoma, proliferation markers

INTRODUCTION

Oral cancer is currently an important medical problem worldwide, including Poland. Cancer remains the leading cause of death among women and is the second most common cause of death among men, despite the introduction of new diagnostic techniques and new treatments that selectively target cancer cells [1]. Cancers of the head and neck account for 2% to 5% of all cancers. Of patients with head and neck cancers, about 30% have oral cancer, which typically is the squamous cell carcinoma (95%). Other cancers of the oral cavity include cancers of the salivary glands, sarcomas, and melanomas. Oral cancer is most commonly found in the tongue and the floor of the mouth. In Poland, risk factors for oral squamous cell carcinoma include smoking, alcohol, mechanical irritation, age, and infections with human papilloma viruses (HPV) [2].

Different head and neck cancers have different prognoses. For instance, high 5-year survival rate in patients who undergoes surgery for cancer of the lower lip is typical, whereas the prognosis for patients with cancers of the oral cavity is much worse. This difference in prognosis is because cancers of the lower lip are detected and treated earlier than cancers of the oral cavity. Thus, early diagnosis is a key to survival.

Immunohistochemistry has been used to detect potential markers of head and neck cancer, which might help in diagnosis and prognosis [3,4], but, to date, there is a lack of clinically useful markers of oral cancer. Such markers, measured in the blood or in tumor tissue, could help in the screening of healthy and high-risk populations for oral cancer, as well as in the prediction of prognosis and monitoring of treatment.

Markers of cancer include proteins, lipids, carbohydrates, or hormones, which are secreted by cancer cells into the bodily fluids or which can be found on the cell surface; moreover, potential markers of cancer can be produced by non-malignant cells in response to neighboring cancer cells [3]. Molecular prognostic markers are classified into three groups depending on:

- the biology of cancer,
- the size and stage of tumor,
- the response of the organism to cancer-related changes.

Markers of the first group are expressed on primary tumor cells or metastatic cells. These markers help ascertain tumor aggressiveness and its response to particular treatments. Markers of the remaining two groups are measured in serum, and they help estimate disease severity; moreover, some of these markers activate the body's defense mechanisms against cancer.

Another group of tumor markers are molecular markers of carcinogenesis. The rapid and uncontrolled growth of cancer cells depends on many factors and is related to the gene changes associated with the cell cycle. Typically, cancer starts with a single mutation in a somatic cell, which then leads to further proliferation. DNA mutations associated with cancer interfere with cellular growth, differentiation, proliferation, aging, and death. Some cancers are hereditary because cancer mutations can be inherited [5, 6]. Many molecular changes, mainly mutations, may activate proto-oncogenes, which are then referred to as oncogenes. About 150 genes are known to be associated with carcinogenesis. Most of these genes encode proteins regulating the cell cycle, differentiation, and apoptosis. One such gene is the p53 gene, which lies on chromosome 17 p13. Under normal conditions, the gene encodes the p53 protein, which is a tumor suppressor molecule regulating the cell cycle. Mutations of the p53 gene cause an over-expression of the inactive form of the protein. The p53 protein blocks cell cycle phases G1 / S or G2 / M to allow repair mechanisms to remove DNA damage before restarting the mitotic cycle. When the damage cannot be repaired, the p53 protein activates apoptosis and cell

Tab. I. Oral cavity cancer staging.

ORAL CAVITY	STAGING					
	I	II	III	IVA	IVB	IVC
mouth floor		2	7	8		1
mandible gingiva	1		2	5		
tongue	1		3	1		
retromolar space	1		1	1	2	
lower lip	3			1		
hard palate	1	2				
chick			2	1		
maxillary sinus			1	1		
upper lip			1			
total	7	4	17	18	2	1

Tab. II. Expression of examined markers, signed in visual scoring in cancer tissue. (0 is no staining, 1 is weak staining, 2 is moderate staining and 3 is strong staining).

SCORE	MARKERS IN 48 SAMPLES OF CANCER TISSUE			
	EGFR	P53	P44/42	PCNA
0	15	21	10	10
1	14	16	23	13
2	12	8	14	14
3	7	3	1	11

Tab. III. Expression of examined markers, signed in visual scoring in leukoplaka tissue. (0 is no staining, 1 is weak staining, 2 is moderate staining and 3 is strong staining).

SCORE	MARKERS IN 10 SAMPLES OF LEUKOPLAKIA TISSUE			
	EGFR	P53	P44/42	PCNA
0	0	0	1	0
1	0	3	2	0
2	10	7	7	9
3	0	0	0	1

Tab. IV. Dependence between examined markers expression and tumor staging.

	STAGING X P53		
CHI^2	CHI^2	DF	P
	30,92336	df=15	p=,00900
	STAGING X EGFR		
CHI^2	CHI^2	DF	P
	16,11748	df=15	p=,37430
	STAGING X PCNA		
CHI^2	CHI^2	DF	P
	10,98378	df=15	p=,75374
	STAGING X P44/42		
CHI^2	CHI^2	DF	P
	12,40798	df=15	p=,64792

Tab. V. Correlation between expression of examined markers in tumor tissue.

EGFR	RANG SPEARMAN CORRELATION P <,05000			
	EGFR	P53	P44/42	PCNA
EGFR	1,000000	0,426148	0,246712	0,246877
p53	0,426148	1,000000	0,447636	0,345342
p44/42	0,246712	0,447636	1,000000	0,338019
pcNA	0,246877	0,345342	0,338019	1,000000

Tab. VI. Correlation between expression of examined markers in leukoplakia.

EGFR	RANG SPEARMAN CORRELATION P <,05000			
	EGFR	P53	P44/42	PCNA
EGFR	1,000000			
p53		1,000000	-0,509175	0,000000
p44/42		-0,509175	1,000000	0,215166
pcNA		0,000000	0,215166	1,000000

death. Mutations of the p53 gene cause an uncontrolled p53 protein expression and thus impair apoptosis. The presence of p53 in histologically revised mucosa and precancerous changes indicates an increased risk of malignant transformation, and overexpression of nuclear p53 is associated with an increased risk of disease progression [7]. If detected in the suprabasal layers of the epithelium derived from precancerous lesions, p53 predicts malignant transformation better than the degree of dysplasia. Mutations in the p53 gene are likely implicated in the carcinogenesis of the head and neck cancers, but their prognostic importance is still unclear [8].

Markers of the squamous cell carcinoma of the oral cavity or pharynx remain to be discovered. Although the squamous cell carcinoma antigen (SCC-Ag) is detected in squamous cell carcinoma tissue, its expression can be increased in patients with diseases other than cancer, for instance, psoriasis. Potential markers of head and neck cancers include substances synthesized in response cancer-related changes, including increased cell proliferation. These substances include proliferating cell nuclear antigen (PCNA), Ki-67, p44/42 MAPK, and EGFR. Proliferation of tumor cells can be estimated with markers such as PCNA or argyrophylic nucleolar organizer regions (AgNORs) [9]. PCNA and Ki-67 are detected in precancerous tissue and squamous cell carcinomas of the oral cavity, and an increased expression of these antigens is associated with a greater degree of epithelial dysplasia.

PCNA is a nuclear protein whose expression is linked to the S phase of the cell cycle. PCNA is a useful proliferative marker, and its expression increases in late G1 and S phases of the cell cycle, and then decreases from the G2 / M to the G1 phase. High proliferative activity in combination with p53 overexpression predicts a poor prognosis in patients with leukoplakia [5].

The mitogen activated protein kinases (MAP kinase) are a family of serine/threonine-specific protein kinases. Initially, they were described as proteins activated by cell stimulation or growth factors. The MAP kinases can be classified into one of the three following families: extracellular signal-regulated kinases (ERK), C-Jun N-terminal stress--activated protein kinases (JNK/SPAK), and p38 kinases. The ERK kinase has two homologous forms, ERK1 (P44) and ERK2 (p42) [10].

The epidermal growth factor receptor (EGFR) is an important member of the family of the membrane-bound tyrosine kinase receptors activated in tumor cells of epithelial origin. This receptor

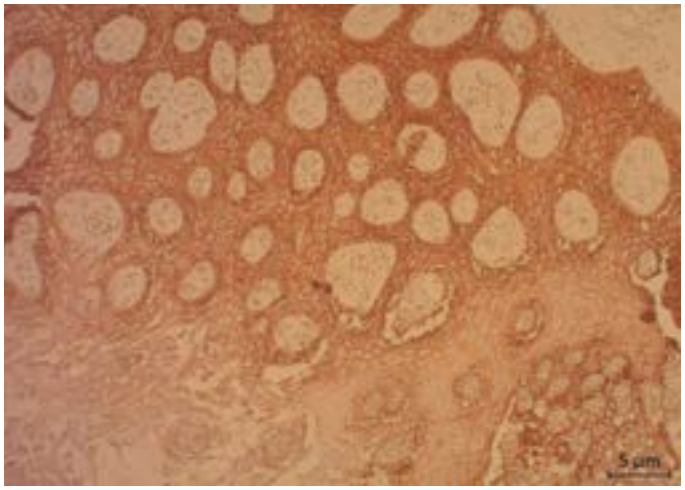

Fig. 1. EGFR staining in leukoplakia lesion. Moderate (2) staining in visual scoring.

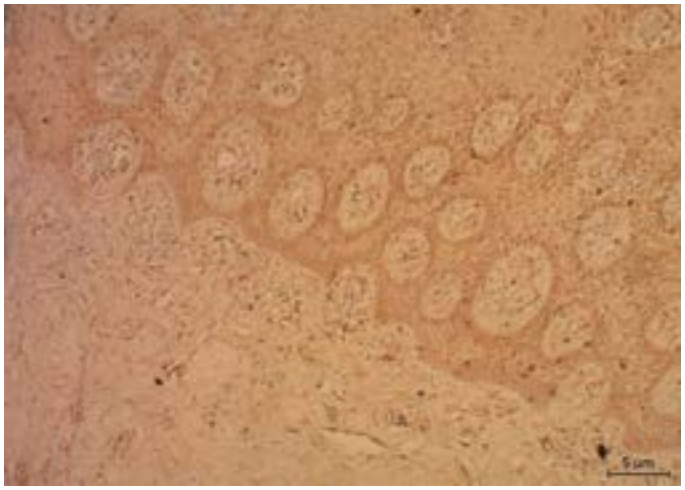

Fig. 2. p44/42 staining in leukoplakia lesion. Weak (1) staining in visual scoring.

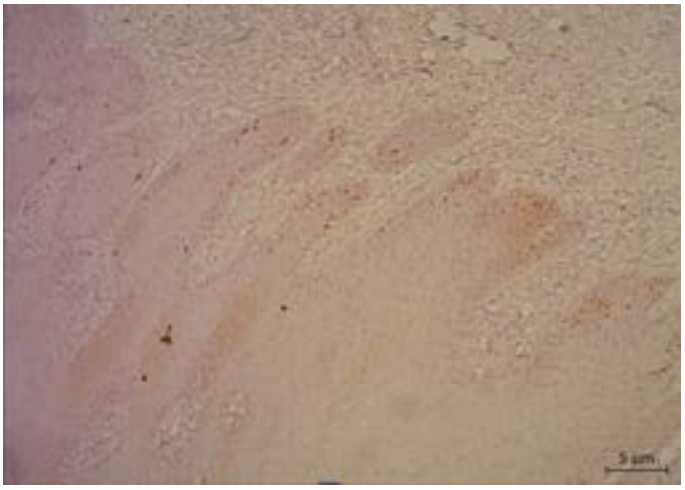

Fig. 3. p53 staining in leukoplakia lesion. Weak (1) staining in visual scoring.

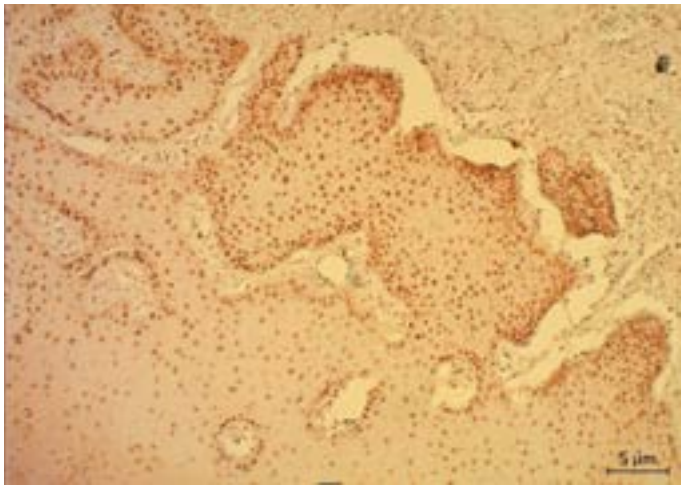

Fig. 4. PCNA staining in leukoplakia lesion. Moderate (2) staining in visual scoring.

regulates cellular growth, proliferation, apoptosis, differentiation, migration, and secretion of certain proteins. Activation of EGFR is implicated in the progression of cancer, including metastasis [11,12].

Markers of the head and neck cancer could expedite diagnosis and help assess treatment outcomes and detect recurrence on follow-up [3].

MATERIALS AND METHODS

We included 48 patients with oral cancer, diagnosed in 2002-2006 in the Department of Maxillofacial Surgery, Medical University of Lublin. We also included 10 controls with homogeneous leukoplakic lesions (precancerous condition) in the oral cavity.

We analyzed the clinical cancer stage at first admission to the Department of Maxillofacial Surgery, Medical University of Lublin for the assessment of expression of selected protein markers. According to the guidelines of the American Cancer Society from April 2009, the clinical cancer stage was ascertained retrospectively based on the TNM classification, clinical data, and ancillary data from the patients' medical records [13].

Immunohistochemistry was performed in the Department of Pathology, Medical University of Lublin in paraffin-embedded biopsy samples from the Department of Oral and Maxillofacial Surgery, Medical University of Lublin.

IMMUNOHISTOCHEMISTRY

First, 3-µm thick paraffin sections were placed onto microscopy slides (Super Frost Plus®, Thermo Scientific). Then, the slides were incubated for 12 hours in a thermostat at 58 degrees Celsius. The next day, the sections were placed in serial dilutions of xylene, to remove the paraffin, and then in a graded alcohol series (97%, 80%, 75%) to eliminate xylene.

For sections stained for p44/p42, PCNA, and p53, antigen heat--induced epitope retrieval (HIER) was used. These sections were placed in 0.01 M citrate buffer (pH 6.0) and heated thrice for 5 minutes each time. Then, the slides were left in the buffer at room temperature for 20 minutes.

For sections stained for the EGFR, antigen retrieval unmasking by proteolytic enzymes was used. These sections were placed in a concentrated solution of proteinase K (1 drop per 2 ml of buffer) and then underwent enzymatic digestion with TBS for 4 min at room temperature.

After initial preparation, all slides were transferred to the TBS buffer and then incubated for 5 min with a 3% solution of hydrogen peroxide to block endogenous tissue peroxidase.

Three 5-minute rinses in the TBS buffer (Tween 20) were followed by incubation with the primary antibodies. The primary antibodies used were as follows:

- EGFR Ab-10, clone 111.6 (Thermo Scientific; dilution, 1:100; 1 hour at room temperature)
- Phospho p44/p42 MAPK (ERK1/ERK2; Cell Signaling Technology ®; dilution, 1:100; 24 hours at 4 degrees Celsius).
- p53 monoclonal mouse anti-human p53 protein (clone DO-7; DAKO dilution, 1:50; for half an hour at room temperature).
- PCNA - mouse monoclonal anti-proliferating cell nuclear antigen (clone PC10; dilution, 1:5000; half an hour at room temperature).

All slides were then washed three times for 5 min in the TBS buffer (Tween 20). We used the following detection system: DAKO K5007 Cat - Dako REAL ™ Detection System, Peroxidase DAB + Rabbit / Mouse.

The slides were transferred to distilled water, stained with Mayer hematoxylin, rinsed, and then treated with a series of alcohols and xylenes before cover glasses were mounted on the slides.

We performed visual scoring of the microscopy specimens. Moreover, the total percentage area stained at each intensity level was multiplied by weighted intensities (for no staining, 0; for weak staining, 1; for moderate staining, 2; for strong staining, 3).

STATISTICAL ANALYSIS

We used the Statistica 8 software (StatSoft Inc., USA). The Yate`s Chi-squared test with a fixed ratio was used. P <0.05 was considered statistically significant.

RESULTS

Forty-eight patients (34 men and 14 women) had squamous cell carcinoma in the oral cavity, in the following sites: floor of the mouth, 18; the lower gums, 8; the tongue, 5; the retromolar triangle, 5; the lower lip, 4; the hard palate, 3; the cheek, 2; the maxillary sinus, 2; the upper lip, 1.

DISCUSSION

Risk factors for malignant transformation have been studied extensively. New molecular, biochemical, and immunohistochemical methods could help in diagnosis, risk stratification, screening, and follow-up of patients with cancer. There are many clinically important markers of cancer, such CEA and Ca 19-9 in colorectal cancer, CA 15-3 in breast cancer, AFP in primary hepatocellular carcinoma, SCC-Ag in cervical cancer, CA 19-9 and CEA in gastric cancer, NSE, SCC-Ag, Cyfra 21-1, and CEA in lung cancer, beta HCG, AFP, and PLAP in embryonic testicular tumors, Ca-12, beta HCG, AFP, and CEA in ovarian tumors, and thyroglobulin and calcitonin in thyroid cancer. To date, however, there are no markers that could improve the diagnosis and treatment of patients with squamous cell carcinoma of the oral cavity. According to the American Cancer Research, in 2011, in the United States, 39,400 new cases of oral cancer were diagnosed, and 7900 deaths occurred due to squamous cell carcinoma of the oral cavity. Zatoński et al. showed that in the Central and Eastern Europe incidence and mortality of cancer differs significantly depending on sex, age, and staging. [14]. In 2007, in the province of Lublin, 170 new cases of oral cancer were recorded, and there were 80 deaths related to oral cancer.

In this study, we investigated whether markers of epithelial cell proliferation would be related to the progression of squamous cell carcinoma of the oral cavity, which could help in the diagnosis and treatment. In our study, leukoplakia, and not healthy oral mucous membrane, served as the control condition, because we wanted to examine expression of proliferation markers in relation to the progression of neoplasia.

In our study, the clinical cancer stage correlated significantly with the expression of the p53 protein. In contrast, the expressions of EGFR, PCNA, and ERK1 and ERK2 kinase - p44/p42 did not correlate with the clinical cancer stage. The expression of the p53 protein correlated positively with the expressions of the remaining markers of proliferation. We also found that the expression of proliferation markers was high in the control group (leukoplakia); however, among patients with leukoplakia, we did not observe the correlations found in patients with oral cancer. Our findings suggest that none of the examined markers can be ruled out as an objective indicator of cancer. Positive correlation between the expression of the p53 protein and disease progression may have a predictive value. An increased expression of this marker can be found in precancerous changes and in cancer. We think that this marker could be used in combination with other markers to determine disease severity and clinical and histopathological stage. Further research should concentrate on finding specific markers that could improve the diagnosis and treatment of patients with oral squamous cell carcinoma.

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