Evaluation of CK19 and VEGF gene expression, ki67 antigen, p53 protein, C-erb-B2, and their relationship in oral squamous cell carcinoma

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ABSTRACT

Oral squamous cell carcinoma (OSCC) is one of the ten most common malignant tumors globally. This study aimed to evaluate the expression changes of Cytokeratin 19 (CK19), vascular endothelial growth factor (VEGF), P53, ki67, and c-ert-B2 in OSCC patients. For this purpose, 30 patients were selected as the case group and 30 healthy individuals as the control group. The expression of CK19 and VEGF genes in their blood serum was measured. Also, the expression of ki67, P53, and c-ert-B2 markers in squamous cell carcinoma was evaluated using immunohistochemistry. T-test was used to analyze the data. The results showed that the presence of CK19 marker in people with OSCC was positive in 17 out of 30 patients and VEGF marker in 23 out of 30 patients. The mean of ki67 positive, P53 positive, and Cerb-B2 positive cells were 399.4, 221.4, and 26.8, respectively. The correlation test between the indices showed a statistical correlation between the incidence of ki67 and P53 ($r = 91.5\%$ and $p = 0.02$). While statistical correlation was not seen between the incidence of ki67 and Cerb-B2 index ($r = -1.7\%$ and $p = 0.97$) and P53 and C-erb-B2 index ($r = -13\%$ and $p = 0.8$) ($p < 0.05$). In general, the expression of VEGF and CK19 genes is higher in patients with OSCC than in healthy individuals. Therefore, examining the expression level of these two biomarkers in the blood of OSCC patients can be considered as a diagnostic screening method in the early stages of the disease. The immunohistochemical study of squamous cell carcinoma can also be used as a diagnostic screening test in the early stages of the disease.

Introduction

Oral cancer, often referred to as oral squamous cell carcinoma, is the most commonly squamous cell carcinoma of the head and neck (1). Recently, the mortality rate due to this cancer has been increasing (2). In old age, factors that increase the disease, carcinogens such as cigarettes, alcohol, and tobacco have been reported with increasing levels of DNA damage and the like (3). Epithelial cell carcinoma is the most common oral cancer of epithelial origin found in the oral cavity. One of the most common methods used to diagnose cancer in laboratory methods is tumor markers (4). Examining several biomarkers together can provide more accurate and reliable results for the diagnosis of cancers (5).

Cytokeratin 19 is a member of a large family of intermediate filamentous proteins consisting of more than 20 polypeptides, divided into acidic (CK9-CK23)

and primary (CK1-CK8) (6). CK 19 plays a crucial role in maintaining the entire epithelial cell and in the cell cycle response to stress and apoptosis (7).

Vascular endothelial growth factor (VEGF) is a homodimeric glycoprotein. As a cytokine, it is a significant multifunctional process in the inflammatory and wound healing process that binds to the VEGF receptor at the endothelial cell surface and is a potential inducer of angiogenesis (8).

The p53 is a tumor suppressor gene that is the most common cause of genetic alterations in human tumors (9). The product of this gene is a nuclear protein involved in cell cycle control, programmed cell death or apoptosis, and the elimination of gene stability (10). The protein product of the p53 mutant gene has a longer half-life and can be detected by immunohistochemistry. The occurrence of oncogenes has also been reported as a causative agent of

squamous cell carcinoma (11). Among them, the role of C-erb-B as a cell membrane protein associated with epidermal growth factors that act as an oncogene at an increased incidence has recently been considered. An increase in C-erb-B2 has been reported in samples taken from dysplastic tissue (12).

Changes in the p53 gene have been reported in head and neck cancers so that the expression of p53 has been shown in dysplastic and squamous cell carcinoma specimens (13). Some studies also show an increase in p53-positive cells from hyperplastic lesions to dysplastic and carcinoma (13, 14).

Uncontrolled cell proliferation is one of the symptoms of malignancy (15). This cell proliferation can be detected by staining nuclear antigens associated with cell growth and division and examining it immunohistochemically using a light microscope. The ki67 is an antigen associated with a non-histone nuclear protein expressed in stages S, M, G1, G2 of the cell cycle (16). Thus, it will be a good indicator for cell proliferation and mitosis. A significant correlation was reported in the occurrence of ki67 and p53 so that this has been attributed to the proliferative activity of cells (17).

Since the role of c-erb-B2 and its relationship with ki67 and p53 markers in squamous cell carcinoma of the mouth is not well known and there are not many findings from their relationship, so we decided to Evaluate p53, ki67, C- erb-B2, CK19, VEGF, and their relationship between these markers in squamous cell carcinoma.

Materials and methods

Thirty patients who had OSCC referred to the Cancer Institute voluntarily participated in the study based on the diagnosis of a specialist, and 30 healthy people voluntarily participated in the study as a control group. Case and control groups in this study were considered the same in terms of age and sex.

Evaluation of CK19 and VEGF gene expression

10 ml of peripheral blood was taken from all participants in the present study and RNA extraction was performed using RNeasy Midi Kit (Qiagen Cat no.75144), and the total mRNA was prepared according to the kit protocol. The leukocytes were degraded by the solution in the kit and then homogenized, the precipitates and ethanol were transferred to RNeasyMidicolumn to bind RNA to the column, and finally, the column was washed and RNA was extracted. Complementary DNA (cDNA) was performed using the Viva 2-steps RT-PCR Kit (Cat no RTPL12). This kit is suitable for the synthesis of cDNA strands in a two-step method. Oligo dt was performed with a random hexamer. The quality and quantity of RNA and cDNA at the end of each step were evaluated and controlled using a Nanodrop device. The specific primers for each marker are listed in Table 1 (18). The GAPDH gene was used as internal control.

Table 1. Characteristics and sequences of studied primers; GAPDH (A), CK-19 (B), and VEGF (C), Forward (F), Reverse (R)

Gene		Primer Sequence (5'→-3')	Product Length	Annealing Temp.
A	F	GTAACCCGTTG AACCCCATT	152 bp	56 °C
	R	CCATCCAATCG GTAGTAGCG		
B	F	TCCGAACCAAG TTTGAGAC	222 bp	56 °C
	R	AATCCACCTCC ACACTGA		
C	F	AAGGAGGAGG GCAGAATCAT	248 bp	56 °C
	R	ATCTGCATGGT GATGTTGGA		

For real-time RT-PCR, at this stage, HotTaq EvaGreen qPCR Mix (Cat No.BT11101) was performed with QIAGEN Rotor-Gene. Real-time RT-PCR reaction components including 4µl Mastermix, primer F and R 1µl each, DNA 2µl, and distilled water was added to have a final volume of 20µl, respectively. Temperature and time conditions: initial denaturation at 95°C for 15 minutes, denaturation at 95°C for 15 seconds, binding of primers at 56°C for 60 seconds and multiplication at temperature, respectively. The extension condition was 72°C for 25 seconds (for 40 cycles).

Evaluation of ki67 antigen, p53 protein, and C-erb-B2

First, a cancerous tissue sample with sufficient volume, without necrotic areas and bleeding was obtained from all 30 patients participating in this study. Four sections were prepared from the selected samples (one 5micron section for hematoxylin-eosin staining and the other 3 sections for

immunohistochemical staining). Staining samples by immunohistochemistry method was performed as follows:

First, a 2-micron section of the sample was prepared and then treated with polyclonal antibodies P53P clone Do-7, C-erb-B2, and ki67 Antigen (Dako, Denmark). After being placed in Citrate/HCl Buffer 10mmd with a pH of 6.0, they were placed in the microwave for 10 minutes. They were then cooled to room temperature and washed with PBS. They incubated again with the mentioned antibodies for 1 hour and washed with PBS. They were incubated with Biotinylated antibodies for 30 minutes and washed with PBS. They were exposed to 3,3-Diamino Benzidine 3,3 DAB (Hydrochlorid) chromogen and stained with Ethyl-green. After dehumidification, they were cleared with Xylol. Finally, they were mounted with Entellan glue. All slides were viewed with an Olympus light microscope and magnified 10 and 40. The following method was used to determine the classification index. By counting 3 epithelial cells, the number of stained cells in this set was counted and Labelling Index (LI) was obtained using the following formula for each of the indicators:

$$Labelling\ Index\ (LI) = \frac{Number\ of\ stained\ cells}{1000\ epithelial\ cells}$$

In order to avoid counting errors, each section was counted twice and cell count was checked by two pathologists.

Statistical Analysis

The comparison of means between the two groups was performed using a T-test and the percentage of positive markers in the two groups was compared by SPSS software (version 20, SPSS Inc., Chicago, IL). Statistical difference in the level of P <0.050 was considered significant.

Results and discussion

The study population consisted of two groups of patients with OSCC (case group) and healthy individuals (control group) who according to the estimated sample size of the study were 30 people in each group. Comparison of these two groups did not show a significant difference between the mean age of the control group (48.14 ± 10.94 years and an age

range of 24-69 years) and a case group (46.44 + 11.35 and an age range of 25-71) (P = 0.323). In this experiment, 15 males and 15 females were present in the control group and 18 males and 12 females in the case group, which did not have a statistically significant difference between the two groups (P = 0.067).

Real-time RT-PCR results for the VEGF marker were positive in the 23 case group; In other words, in 23 cases in the case group, this biomarker was expressed based on the Real-time RT-PCR method, and in the control group, 10 out of 30 people were positive. Statistical comparison of the positive rate of this marker between the case group and the control group, which was performed using the Two-sample binomial test, showed a statistically significant difference between the two groups (P = 0.022). Also, the CK19 marker was positive in 17 patients in the case group and six individuals in the control group. Statistical comparison of the positive rate of this marker between the case group and the control group, which was performed using the Two-sample binomial test, showed a statistically significant difference (P = 0.015) (Fig 1).
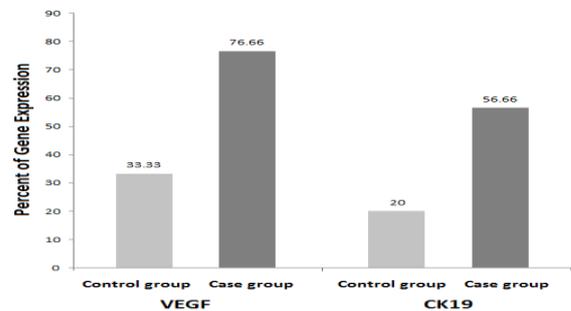
Figure 1. Percent of gene expression for VEGF and CK19 in control and case groups

Differences in marker expression were assessed by $2^{-\Delta\Delta ct}$ for CK19-mRNA and VEGF-mRNA (Fig 2). Thus, about the VEGF marker, the number of initial copies of this marker in the case group was 2.55 times more than the control group and about CK19 the number of initial copies of this marker in the case group was 1.35 times more than the control group.
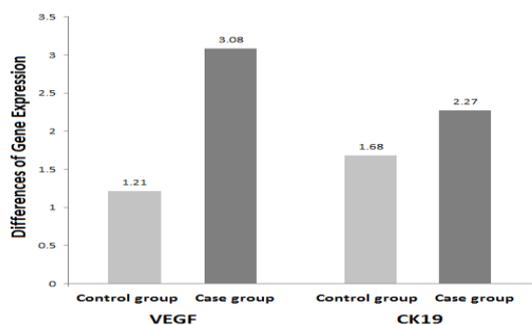
Figure 2. Differences of gene expression for VEGF and CK19 in control and case groups

The staining of the ki67 index in different parts of the tumor indicated the brown staining of the cell nucleus so that staining with the ki67 antibody created a uniform brown color in the nucleus (Fig 3A). All sections were stained with ki67 antibody. The mean of ki67 positive cells was 399.4 (ranging from 357 to 448 positive cells). The staining of the P53 index showed the brown cell nuclei in a background of blue cytoplasm with a mean of P53 positive cells of 6.221 (with a range from zero to 739 positive cells) (Fig 3B). The C-erb-B2 staining showed scattered brown cells with a mean of 26.8 positive C-erb-B2 cells (ranging from 8 to 76 positive cells) (Fig 3C).
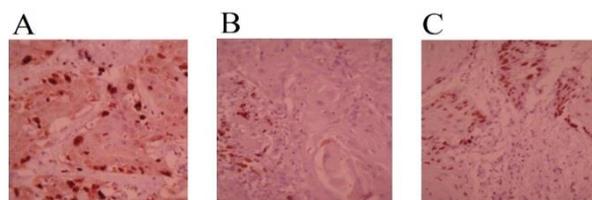
Figure 3. Immuno-histochemical staining in oral squamous cell carcinoma; A: Ki67, B: p53, and C: C-erb-B2

The correlation test between the indices showed a statistical correlation between the incidence of ki67 and P53 (r = 91.5% and p = 0.02). While statistical correlation was not seen between the incidence of ki67 and Cerb-B2 index (r = -1.7% and p = 0.97) and P53 and C-erb-B2 index (r = -13% and p = 0.8) (p <0.05).

Oral cancer is one of the most common cancers globally and one of the ten most common causes of death (2). More than 90% of oral cancers are squamous cell carcinomas, and about 9% of cancers include salivary gland carcinomas, sarcomas, and lymphomas (1). Most oral cancers are diagnosed at an advanced stage (19). These lesions are detected when

they lead to clinical symptoms due to high progress, leading to a poor prognosis for oral cancer in most parts of the world. Recently, the role of biomarkers in the early detection of cancer has received particular attention (4). Examining several biomarkers together can provide more accurate and reliable results for the diagnosis of cancers (3, 19).

The present study, to investigate the changes in CK19-mRNA and VEGF-mRNA expression in the peripheral blood of patients with oral squamous cell carcinoma and compared with healthy individuals, showed that the expression of this biomarker in patients with cancer is higher than healthy individuals.

CK19 is a large family of intermediate filamentous proteins and belongs to the creatine family (20). VEGF is also a cell-produced signal protein that stimulates tissue and blood vessels (21). Payehghadr *et al*. compared 50 samples of squamous cell carcinoma of the mouth and 50 healthy samples. In this study, serum VEGF levels in patients were higher than in healthy individuals (22). In this study, the expression of Carcinoembryonic CK19-mRNA and antigen CEA-mRNA was observed to increase the expression of these biomarkers in oral squamous cell carcinoma.

Another study showed that VEGF expression by real-time PCR in patients is higher than in healthy individuals, which is consistent with the present study's findings (21). In another study by Guo *et al*. to study the expression of CK20, CK19, and CEA-mRNA in peripheral blood in patients with lung cancer by nested RT-PCR, it was found that CEA-mRNA expression was 48.2%, CK20 expression was 41.0%, and CK19 was 73.5% of the samples had at least one positive marker and this was related entirely to metastasis (23).

Also, the results of this study showed positive staining of PS3 ki67 and Cerb-B2 markers in squamous cell carcinoma. There was also a statistical correlation between the incidence of ki67 and P53; while there was no statistical correlation between the incidence of ki67 and Cerb-B2 and P53 and C-erb-B2. Recent studies in cell biology have shown the precise mechanism of the cell cycle system, which has been shown to play an essential role in the development of many cancers (24). As a tumor suppressor gene, p53 is one of the critical proteins that regulate the cell cycle (25). In many studies, the association between

the incidence of p53 and its changes has been accepted as a genetic change known in early-stage head and neck cancers. Various studies have shown an increase in P53 cells from hyperplastic lesions to dysplastic and carcinoma (25, 26). One of the crucial functions of the P53 wild type is to induce apoptosis or cell cycle arrest. Therefore, the loss of apoptotic function of P53 may be one of the reasons for the continued growth of tumors. The P53 mutation can induce other parts of tumor progression (26). Eighty-eight percent of oral cancer samples show an increase in the expression of the P53 gene (9).

It is important to note that some samples show an increase in P53 but no mutation in the gene. One of the functions of P53 is to stop the cell cycle following DNA damage (9). In this case, the DNA is allowed to repair. In the present study, the expression of p53 was observed in half of the samples. This finding is a confirmation of the other conclusions. However, a point mutation in the p53 gene appears to play an essential role in developing squamous cell carcinomas of the head and neck (24).

Cell proliferation is one of the indicators of differentiation between benign and malignant tumors (27). Increased incidence of ki67 has been shown to be a good indicator of cell proliferative activity in malignant and precancerous oral lesions. Cell proliferation and DNA synthesis are essential factors in determining the prognosis of cancers (27). Since altered P53 expression is one of the genetic events involved in the pathogenesis of squamous cell carcinoma, its comparison with the ki67 proliferation index could indicate cell proliferation in squamous cell carcinoma (26). The ki67 reacts with the nucleus of proliferating cells at all stages of the cell cycle, except for phase G0 (28). Recent studies have shown a significant association between ki67 LI and increased malignancy, decreased survival, and metastasis (29). The expression of p53 and ki67 has been associated with cell proliferation activity and tumor progression. In addition, the absence of p53 expression inactive cell proliferation has shown promising results in radiotherapy treatment (30). Batinac *et al.* consider the incidence of p53 and ki67 to reflect the malignancy of skin neoplasms, including squamous cell carcinoma and basal cell carcinoma (31).

Despite these studies, Sommer and Olofsson could not find an association between the incidence of p53 and ki67 and PCNA about tumor size, degree of differentiation, and recurrence (32). In this study, we investigated the relationship between p53 and ki67, and we found a correlation between the incidence of p53 and ki67 (r = 91.5% and p = 0.02). According to the increasing findings of scientific reports, the increase in p53 incidence can be considered an indicator of cell immaturity or their proliferative activity. Since the present study was performed only histopathologically, this finding should be tested with clinical indicators.

The other part of this study was dedicated to the study of the C-erb-B2 index. C-erb-B2 acts as an oncogene at the time of change and has been reported in a range of carcinomas. In our study, C-erb-B2 was evident in all samples, and the mean of positive C-erb-B2 cells was 26.8±27.22. Statistical analysis of indices in this study showed that between the incidence of ki67 and C-erb-B2 (r = -1.7 and p = 0.97) and there was no statistical correlation between P53 and C-erb-B2 (r = -1.3 and p = 0.8). There is little research on the role of C-erb-B2 in oral carcinomas. Although Kristensen *et al.* reported the incidence of C-erb-B2 in cervical squamous cell carcinoma and Vora *et al.* in squamous cell carcinoma of the tongue (33, 34), Khademi *et al.* study found no association between histological grading and lymph node involvement with C-ert-B2 is shown (35).

According to the findings of this study and other studies, it seems that the incidence of ki67 and p53 and their relationship with each other indicate the degree of malignancy (36, 37). This is associated with the histological degree of malignancy even in the invasive regions of the umbilical cord. Although the function of C-erb-B2 as an anti-apoptotic agent has been linked to disease prognosis in several clinicopathological studies, the present study at the histopathological level did not show such an association in the incidence of ki67, p53, and C-erb-B2. It can indicate the degree of differentiation of squamous cell carcinoma in this study and is related to the type of study. Therefore, to study the prognostic role of this index, clinical-pathological studies should be considered simultaneously with considering the degrees of carcinoma. The expression of VEGF and CK19 genes are higher in patients with squamous cell

carcinoma of the mouth than in healthy individuals. Therefore, examining the expression level of these two markers in the blood of patients can be considered a potential biomarker in the diagnosis of OSCC.

Acknowledgments

Interest conflict

The authors declare no conflict of interest.

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