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Impact of epidermal growth factor receptor gene rs1468727 polymorphism on survival of the patients with oral squamous cell carcinoma

Утицај полиморфизма гена за рецептор епидермалног фактора раста *rs*1468727 на преживљавање болесника са оралним планоцелуларним карциномом

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SUMMARY

Introduction/Objective Genetic aberrations and environmental factors are known to play an important role in oral squamous cell carcinoma (OSCC). The aim of the study was to clarify the association of epidermal growth factor receptor (*EGFR)* gene polymorphism rs1468727 with overall survival (OS) in patients with OSCC.

Methods The study comprised a total of sixty-one patients diagnosed with OSCC. The follow-up period for each patient was 3 years from the date of surgery and during that period their genotypes for rs1468727 polymorphism of the *EGFR* gene were identified using Real-Time PCR. Binary logistic regression was used to investigate the influence of various variables on survival. Additionally, the Chi-square test of independence and Man–Whitney U test were done to examine the interplay between two categorical variables and two independent samples.

Results Two variables demonstrated a statistically significant influence on OS: the TNM Classification (TNM) stage and *EGFR* genotype. At the end of the follow-up period, 39 patients survived, with a noteworthy observation that more than half of the survivors had the *EGFR* rs1468727 CC genotype. The distribution of CC and CT genotypes was equal (chi-square = 0.397, df = 2 p = 0.820) among patients who deceased indicating that no statistically significant correlations were found between OS and demographic or tumor-related characteristics.

Conclusion *EGFR* rs1468727 homozygote (genotype CC) and TNM stage showed statistically significant influence on OS in the follow-up period. This study highlights the potential significance of homozygote *EGFR* rs1468727 CC in assessing the prognosis and treatment outcomes of patients undergoing surgery for OSCC.

Keywords: oral squamous cell carcinoma; epidermal growth factor receptor; polymorphisms

САЖЕТАК

Увод/Циљ Познато је да генетичке аберације заједно са факторима средине играју важну улогу у настанку оралног планоцелуларног карцинома (ОПК). Циљ истаживања је да се разјасни потенцијални утицај полиморфизма гена за рецептор епидермалног фактора раста (*EGFR*) *rs*1468727 на укупно преживљавање (УП) код пацијената са ОПК.

Методе Шездесет и један ОПК пацијент је био укључен у студију. Период праћења за сваког пацијента био је три године од датума операције. Генотип сваког болесника за *rs*1468727 полиморфизам гена *EGFR* детектован је коришћењем *PCR* методе у реалном времену. Бинарна логистичка регресија је кориштена како би се истражило која варијабла утиче на преживљавањем**.** Тест независности χ^2 и Ман–Витнијев *U* тест су кориштени за испитивање односа категоричких варијабли.

Резултати Две варијабле су показале статистички значајан утицај на УП: стадијум класификације малигних тумора (*TNM*) и *EGFR* генотип. Три године након операције (праћења) међу 39 преживелих болесника више од половине имало је генотип *EGFR rs*1468727 *CC*. Међу болесницима који нису преживели, дистрибуција *CC* и *CT* генотипова је била једнака (χ^2 = 0,397, *df* = 2 *p* = 0,820). Нису идентификоване статистички значајне корелације између УП и демографских или туморских карактеристика.

Закључак *EGFR rs*1468727 хомозигот (генотип *CC*) и *TNM* стадијум су показали статистички значајан утицај на УП у периоду праћења пацијената. Ова студија наглашава могући значај разматрања генетских фактора, као што је хомозиготни *EGFR rs*1468727 генотип *CC*, приликом процене прогнозе и исхода лечења пацијената који су били подвргнути операцији у циљу лечења ОПК.

Кључне речи: орални планоцелуларни карцином; рецептор епидермалног фактора раста; полиморфизми

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is a malignant head and neck tumor that affects the oral cavity, posing high morbidity and mortality risk [1]. OSCC is one of the most prevalent types of malignancies. It accounts for approximately 90% of all oral cavity cancers, signifying

its prevalence and clinical relevance in the field of oncology [2,3]. In Serbia, malignant tumors of the oral cavity account for approximately 1.1% of all malignant neoplasms [2].

OSCC is localized in various regions of the oral mucosa, including buccal mucosa, mobile tongue, gingiva, and mucosae of the floor of the mouth. Clinically, it can manifest as ulceration, infiltration or vegetation with leukoplakia or erythroplakia being precancers important for its development [4].

Numerous risk factors, including cigarette smoking, alcohol consumption, poor dental hygiene, persistent irritability, and genetic abnormalities, have been linked to OSCC [4]. The synergistic consumption of alcohol and cigarettes showed increased odds of the occurrence of OSCC [4]. Recent research indicates a higher prevalence of OSCC in males compared to females, and older adults are thought to be at the highest risk of developing OSCC [5,6].

Management of the OSCC involves a multidisciplinary team approach. Surgery presents the cornerstone in OSCC treatment in combination with adjuvant radiotherapy and chemoradiation for high-risk patients, while systemic therapy can be used in neoadjuvant settings for advanced-stage disease or as a palliative setting [7,8].

The complex behavior of malignant neoplasm is closely linked to genetic instability [9,10]. OSCC has been linked to abnormalities in a number of oncoproteins, including EGFR, K-ras, c-myc, FGF3, and cyclin D1 [11].

The *EGFR* gene encodes a transmembrane glycoprotein EGFR, belonging to ErbB (epidermal growth factor receptor) family [12]. Upon ligand binding, receptor autophosphorylation follows, triggering a chain of intracellular signaling events [12]. The EGFR signaling pathway is frequently dysregulated in cancer cells, promoting their proliferation, resistance to apoptosis, enhancing capacity for metastasis, and facilitating angiogenesis [12].

Neoplastic cells must evade the effective cell cycle checkpoint regulatory system. The most frequent genetic change observed in all human malignancies is the inactivation of *p53*, leading to persistent cell proliferation and suppression of apoptotic signaling [11]. The *CDKN2A* gene is the second most frequently mutated gene in OSCC. During the G1 to S phase transition of the cell cycle, the *CDKN2A* gene encodes a protein called p16 which promotes cell cycle progression [13].

Single nucleotide polymorphisms (SNPs) within the *EGFR* gene have been identified as potential factors influencing the clinical outcomes and survival of cancer patients. SNPs can

impact *EGFR* gene expression, protein levels, and signaling, thereby affecting the response to treatment and overall prognosis. Extensive research has been conducted to investigate the predictive and prognostic utility of *EGFR* SNPs, with a particular focus on small-molecule tyrosine kinase inhibitors (TKIs) and anti-EGFR monoclonal antibodies (mAbs). SNPs can influence the efficacy of TKIs and mAbs by altering the binding affinity of the inhibitors to EGFR, modulating downstream signaling pathways, or influencing the expression levels of EGFR itself [14].

The objective of this study was to determine the association between *EGFR* rs1468727 gene polymorphism, TNM stage, demographic factors and tumor characteristics with OS in patients diagnosed with OSCC.

METHODS

The tissue samples were collected from 61 patients between 2014 and 2018 by maxillofacial surgeons at the Clinic for Maxillofacial Surgery in the Clinical Center of Vojvodina, Serbia. Each tissue block, originating from the central part of the tumor from OSCC patients, was paraffin-embedded. Before surgery, all patients underwent biopsy to confirm the presence of OSCC. As part of the preoperative preparation, a computerized tomography (CT) examination of the head, neck and chest was performed, and the stage of the disease was determined by the TNM classification based on clinical examination and CT diagnostics as well as clinical parameters of tumor dimensions [15].

The inclusion criteria were: newly pathohistological diagnosed patients of any sex with untreated resectable OSCC, aged 18 or older, with no radiologically diagnosed distant metastasis.

The exclusion criteria were: patients with a history of a prior malignancy other than basal cell carcinoma of the skin, with recurrent oral carcinoma, a history of therapeutic irradiation, with autoimmune disease or HIV infection, as well as those with distant metastasis.

All patients included in the study were also HPV-negative. The follow-up period for each patient was 3 years, measured from the date of surgery until the last consultation with the operator.

The Medical Faculty Ethics Committee of the University of Novi Sad approved this study, which was carried out in accordance with the Declaration of Helsinki. All patients signed informed consent and underwent standardized preoperative and operative surgical procedures.

Clinical data including age, gender, alcohol consumption, cigarette consumption, TNM stage and the survival rate during the follow-up period were determined for all patients. The pathohistological data were: tumor size, the depth of tumor invasion and the existence of lymph node metastases.

DNA isolation and rs1468727 *EGFR* polymorphism genotyping

By using QIAamp DNA FFPE Tissue Kit, (*Qiagen*), genomic DNA was extracted from tissue blocks

The *EGFR* polymorphism rs1468727 was genotyped using TaqMan SNP Assays MTO Human SM 10 (Applied Biosystems, Foster City, USA). PCR reaction contained 50 ng DNA, 1µl of assay and 12,5 µl of Taq DNA polymerase master mix (TaqMan) and water to reach the final volume of 25 µl.

PCR was carried out with the following temperature profile: initial denaturation step (95 °C for 5 min), followed by 30 cycles of denaturation (95°C for 1 min), annealing (69°C for 1 min) and extension step (72°C for 1 min), with the final extension step (72°C for 5 min). The assay was performed in a 96-well plate and the fluorescence was measured in the Applied Biosystems 7500 Fast Real-Time PCR System instrument. All necessary PCR control reactions were set up and performed in each run.

Summary statistics, including the mean, median, and standard deviation for numerical variables, and frequencies for categorical variables, were presented to provide an overview of the data.

We employed binary logistic regression to investigate the impact of various variables on survival outcomes. This modeling approach is well-suited for Bernoulli-distributed dependent variables, which take binary values (0 or 1) based on the presence or absence of a specific criterion, in our case, overall survival (OS). The results of the binary logistic regression analysis were reported in terms of coefficients (B), standard errors (S.E.), significance tests (Wald, degrees of freedom, p-values), and odds ratios.

An odds ratio greater than 1 indicates a positive association between independent and dependent variables, implying an increase in the likelihood of the outcome of the dependent variable with the predictor's presence. Conversely, an odds ratio below 1 describes a negative association.

We employed the Chi-square test of independence to assess relationships between categorical variables. It allowed us to explore potential dependencies between various categorical factors and the survival outcome.

We used the Mann–Whitney U test to compare numerical variables between two independent groups. This test was appropriate for our study as it does not assume a normal distribution of data and is robust against outliers.

We set the significance level at $p < 0.05$ for all statistical tests.

All statistical analyses were conducted using IBM SPSS Statistics, version 25. IBM SPSS.

RESULTS

In the 3-year follow-up period after surgery, a total of 22 patients deceased (36.1%). The analysis was conducted to determine the potential statically significant difference between survival outcomes and collected characteristics. Summary statistics of demographic data, patients and cancer characteristics are presented in Table 1.

At the end of follow-up period, 39 patients survived and more than half of them had genotype CC. An equal distribution between CC and CT genotypes (chi-square = 0.397, df = 2 p = 0.820) was observed among patients who deceased.

A binary regression model was used in this study to explain the survival of the patients by entering the following variables as independent ones: age, gender, alcohol consumption, cigarette consumption, presence of lymph node metastases, tumor size, depth of tumor invasion, as well as *EGFR* genotype.

The Omnibus test of model coefficients confirmed that the model fit the data significantly better than the model without any independent variables (chi-square = 14.276, df = 1, p = 0.032). The Nagelkerke R Square value amounted to 36%, while the overall classification percentage was 75.9%.

Forward Selection based on Likelihood Ratio was used to perform a stepwise selection method and chose statistically significant determinants of OS. According to the results, two variables significantly influenced OS: the TNM stage and *EGFR* CC genotype.

The odds ratio, often denoted as Exp (B) in logistic regression output, provided insight into the relationship between the independent variables and the likelihood of the dependent

variable outcome. A person with genotype CC is more likely to survive (Table 2). The odds ratio of 3.118 suggests that, while keeping other variables constant, each unit increase in the TNM stage results in approximately 3 118 times higher odds of not surviving. This indicates a positive association between the TNM stage and the likelihood of not surviving, implying that higher TNM Stage is associated with higher odds of not surviving (Table 2).

The odds ratio of 0.061 indicated a statistically significant association between the genotype *EGFR* rs1468727 CC and lower odds of not surviving. Specifically, individuals with genotype *EGFR* rs1468727 CC had approximately 0.061 times lower odds of mortality compared to individuals with the reference genotype. This indicates a negative association between genotype *EGFR* rs1468727 CC and the likelihood of death, implying that having genotype *EGFR* rs1468727 CC decreases the likelihood of not surviving.

DISCUSSION

EGFR-mediated signaling pathways play a crucial role in facilitating tumor cell growth and survival, providing tumor cells with significant advantages that lead to uncontrolled proliferation. Consequently, this unregulated cell division results in increasing the number of cancerous cells and acceleration of tumor growth [16].

Several mechanisms have been proposed to explain how the *EGFR* SNPs might affect survival or treatment outcomes in cancer patients: the *EGFR* SNPs can influence the expression level of the *EGFR* gene, which could potentially impact the responsiveness of cancer cells to certain treatments or influence disease progression [17]. The *EGFR* rs1468727 SNP might interact with other genetic factors to collectively influence survival outcomes [18]. It is extremely challenging to isolate the specific effect of a single SNP on a given phenotype when other related SNPs may do the same through linkage disequilibrium and other mechanisms [19]. Certain EGFR genotypes could potentially influence drug efficacy, toxicity, or overall treatment response [17, 20].

EGFR signaling interacts with numerous other pathways involved in cell proliferation, apoptosis, angiogenesis, and DNA repair. Consequently, the *EGFR* polymorphisms may influence the activity of these pathways indirectly, thus potentially affecting survival outcomes [16].

In this study, we examined the associations between demographic characteristics, *EGFR* SNP rs1468727 and tumor-associated characteristics with survival in OSCC patients. The

results showed that TNM stage and *EGFR* CC genotype had a statistically significant influence on OS. In contrast, no statistically significant correlations were identified between OS and each of the following variables: age, sex, alcohol and cigarette consumption, presence of metastases, tumor size and PH depth of tumor invasion.

Our data indicated that individuals with *EGFR* rs1468727 CC genotype and OSCC were more likely to survive. Su et al. [21] reported the predictive significance of *EGF* and *EGFR* polymorphisms in a group of locally progressed head and neck squamous cell carcinoma (HNSCC) patients undergoing post-operative chemotherapy-radiotherapy (CT-RT). Additionally, Saravani et al. [22] evaluated the potential impact of three polymorphisms: rs2227983, rs2227984, and rs2293347 in OSCC patients in southeast Iran. Their study showed that the *EGFR* G>A (rs2227983) polymorphism contribute to OSCC susceptibility. Specifically, patients with the *EGFR* R521K G/G (11.1%) and G/A (15.9%) genotypes exhibited poorer 5-year OS rates compared to those with the A/A (62.5%) genotype. The prognostic value of the R521K polymorphism was further investigated in the study by Bandrés et al. [23]. The R497K variant was associated with a poorer prognosis than the other variants. Patients with the R521K polymorphism and the G/G genotype in exon 13 had the highest chance of disease-related mortality.

In contrast, the (CA)n polymorphism in intron 1 was not associated with OS in the same patient group. No other references regarding the connection between *EGFR* rs1468727 CC genotype and OSCC were found. Nonetheless, Li et al. [18] discovered that the overall survival in Chinese population of patients with glioma and *EGFR* rs1468727 CC genotype was much shorter, indicating different effect in different tumor types.

On the contrary, our research suggested that individuals with *EGFR* rs1468727 CC genotype were more likely to survive. The conflicting result could be attributed to variations in the sample size, difference in tumor type or geographical locations.

Metadata analysis conducted by de Morais et al. [24] involved a review of 14746 papers and focused on eleven relevant studies, which matched the criteria, to identify clinical and pathologic factors related to the prognosis of OSCC in young patients. The analysis included a total of 2 317 patients with OSCC, with men comprising the majority of the sample. Regarding the tumor-node-metastasis stage, the majority of research indicated that cases were typically detected in their early stages (I and II). The studies also revealed considerable variation in locoregional recurrence rates and histologic grade of malignancy. Regional lymph node

metastases decreased both the overall and individual survival rates which is consistent with our findings.

Kaminagakura et al. [25] reported that younger patients had a greater relapse rate (p = .02), but there was no difference in OS (p = .86) that was statistically significant. The clinical stage of the tumors in the younger patients was less advanced, and there was an increased utilization of surgery, radiation, and chemotherapy, leading to improved overall survival.

This study emphasized the significance of early detection and vigorous treatment of oral squamous cell carcinoma [25]. Zhang et al. discovered that there was no statistically significant difference between the youngest and oldest patient groups in both disease-free survival (DFS) or disease-specific survival (DSS) (P = 0.605 and P = 0.520) [26]. Costa et al. [27] discovered a higher incidence of OSCC among men, Caucasians, smokers, and alcohol consumers. In our study, the mean age of patients was 65.4 ± 10.1 with the majority of 47 (77%) patients being male.

Tsou et al. presented molecular evidence demonstrating how acrolein-containing cigarette smoke contributed to EGFR amplification and activation of downstream signaling in OSCC [28]. Shahsavari et al. showed that the age, gender, grade, and stage of OSCC patients did not exhibit any statistically significant relationships with EGFR expression (P > 0.05). However, in the group of esophageal squamous-cell carcinoma (ESCC) patients, there was a statistically significant connection between EGFR expression and stage (P = 0.006) [29].

The study by Costa et al. [27] found no associations between EGFR expression and alcohol or tobacco use. Similarly, our study did not discover any statistically significant correlations between age, sex, alcohol and cigarette consumption, genotype and OS of the patients. Costa et al. [27] reported that the disease development and survival rates were adversely impacted by tumors with positive margins, larger size, and stronger EGFR expression and our data indicated that the TNM stage of illness and *EGFR* genotype impacted the survival of the patients.

Bandres et al. demonstrated that *EGFR* genotypes might be useful indicators in predicting the survival of OSCC patients with metastatic or recurrent disease. In addition, their research indicated that *EGFR* polymorphisms could be advantageous for EGFR-targeted antibody therapy [23]. EGFR, a cell-surface receptor and a druggable kinase, can be targeted by drugs to modulate its activity [20]. In a subset of malignant neoplasms, the *EGFR* gene is abnormally amplified, rearranged, and mutated, contributing to the development and progression of cancer.

As a result, targeting the abnormal EGFR has become a major focus in signal blockade strategies for treating various cancers, including OSCC. Onda et al. conducted flow cytometry analysis to evaluate the expression levels of EGFR in OSCC cell lines, revealing high expression levels in all tested OSCC cell lines. This finding suggests that EGFR may have a significant role in the development and progression of OSCC.

By targeting abnormal EGFR, researchers aim to inhibit its activity and disrupt the signaling pathways that promote cancer growth [30].

Despite these insights, our data cannot conclusively highlight the significance of *EGFR* rs1468727 gene variants.

CONCLUSION

According to the results, two variables had a statistically significant influence on OS: the TNM stage and *EGFR* rs1468727 CC genotype. Higher TNM stage was associated with a decreased likelihood of survival, while individuals with *EGFR* rs1468727 CC genotype were more likely to survive. This study underscores the potential significance of genetic factors, particularly homozygote *EGFR* rs1468727 CC, in assessing the prognosis and treatment outcomes in OSCC. Ongoing research of genetic factors and OSCC is crucial to uncover novel avenues for medical care and improve patient outcomes.

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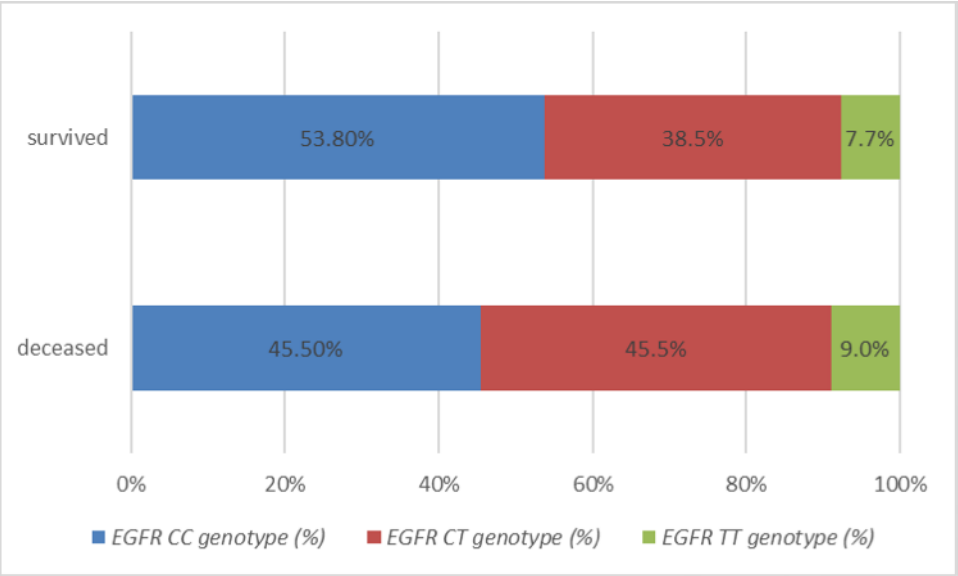
Table 1. Summary statistics for analyzed variables in the total group of patients; results are presented for the total sample of patients and are further stratified into two groups based on survival outcomes: survived and deceased patients

Characteristics	Total mean ± SD or n (%)	Survived mean ± SD or n (%)	Deceased mean ± SD or n (%)	Survival differences p-values
Demographic characteristics				
Age	65.4 ± 10.1	65.5 ± 8.9	65.3 ± 12.3	0.636
Males	47 (77)	29 (74.4)	18 (81.8)	0.728
Alcohol consumers	40 (65.6)	27 (69.2)	13 (59.1)	0.603
Cigarette consumers	50 (82.0)	35 (89.7)	15 (68.2)	0.079
Tumor-associated characteristics				
With lymph node metastases	25 (41)	13 (33.3)	12 (54.5)	0.108
Largest tumor dimension (cm)	1.3 ± 0.5	1.2 ± 0.5	1.5 ± 0.5	0.027
PH depth of tumor invasion (mm)	8.9 ± 5. 7	7.7 ± 5. 8	10.8 ± 5.17	0.016
TNM stage				0.052
I	5 (8.2)	4 (10.3)	1 (4.5)	
II	17 (27.9)	13 (33.3)	4 (18.2)	
III	19 (31.1)	13 (33.3)	6 (27.3)	
IVa	16 (26.2)	13 (33.3)	7 (31.8)	
IVb	4 (6.6)	9 (23.1)	4 (18.2)	

Figure 1. Comparative overview of *EGFR* rs1468727 genotype frequencies in groups of survived and deceased patients*



*Distribution of CC, CT, and TT genotype frequencies in survived and deceased patients

Table 2. Logistic regression results

	B	S.E.	Wald	Df	Sig.	Exp (B)
TNM Stage	1.137	.360	10.006	1	0.002	3.118
rs1468727 *EGFR* Genotype CC	-2.794	1.438	3.773	1	0.050	0.061
Constant	-5.502	1.701	10.468	1	0.001	0.004

B – coefficients; S.E. – standard errors; Df – degrees of freedom; Sig. – tests for significance

