Evaluation pattern within tumor microenvironment and consequent gene expression in oral cancer

Hodnocení nádorového mikroprostředí a následné exprese genů u karcinomu ústní dutiny

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Summary

Background: Oral squamous cell carcinoma (OSCC) is one of the most common cancers in the head and neck squamous cell cancer group. The increasing frequency of oral carcinomas and their late-stage appearance is a major worldwide health concern. MicroRNAs (miRNAs) appear to play an important role in cancer growth and progression, according to growing data, whereas no information is available regarding *miR-7113-3p* and *miR-6721-5p* involvement in OSCC. In this article, the expression of *MAP2K1*, *miR-7113-3p*, and *miR-6721-5p* was examined for possible biological functions in the advancement of oral squamous cell carcinoma. ***Material and methods:*** We used quantitative real-time PCR (to examine the mRNA expression of *MAP2K1*, *miR-7113-3p*, and *miR-6721-5p* in fresh frozen OSCC tissues and adjacent normal fresh frozen tissues from 30 patients, and we investigated their relationship with clinical parameters. ***Results:*** *MAP2K1* expression was found to be dramatically increased in tumor tissues than in normal tissues, whereas miR7113-3p and *miR-6721-5p* expression was significantly decreased. Furthermore, a statistical correlation of P = 0.04 was also observed between increased *MAP2K1* expression and perineural invasion. Additionally, we noted that the downregulation of *miR-7113-3p* appears to correlate positively with overexpression of *MAP2K1* (P = 0.0218), and a negative correlation was observed between downregulation of *miR-6721-5p* and overexpression of *MAP2K1* (P = 0.7771). ***Conclusion:*** Based on these findings, *miR-7113-3p* and *miR-6721-5p* might be prospective biomarkers for OSCC patients, and could be utilized to detect OSCC at an early stage for future diagnosis. *MAP2K1* overexpression has been linked to the development of OSCC and perineural invasion.

Key words

OSCC – *MAP2K1* target gene – *miR-7113-3p* – *miR-6721-5p* – quantitative real-time PCR

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Souhrn

Východiska: Dlaždicobuněčný karcinom ústní dutiny (oral squamous cell carcinoma – OSCC) je jedným z nejběžnějších nádorů ze skupin dlaždicobuněčných karcinomů hlavy a krku. Zvyšující se výskyt karcinomů ústní dutiny a jejich zjištění v pokročilých stadiích je celosvětovým zdravotním problémem. Stále více údajů svědčí o tom, že při růstu a progresi zhoubných nádorů hrají důležitou roli microRNA (miRNAs), zatímco o významu *miR-7113-3p* and *miR-6721-5p* v OSCC nejsou k dispozici žádné informace. Tento článek pojednává o zkoumání exprese *MAP2K1*, *miR-7113-3p* a *miR-6721-5p* pro možné biologické funkce při rozvoji dlaždicobuněčného karcinomu ústní dutiny. ***Materiál a metody:*** Pomocí kvantitativní polymerázové řetězové reakce v reálném čase jsme stanovili expresi mRNA u *MAP2K1*, *miR-7113-3p* a *miR-6721-5p* v čerstvě zmražených tkáních OSCC a v čerstvě zmražených přilehlých normálních tkáních 30 pacientů a zkoumali jsme jejich vztah ke klinickým parametrům. ***Výsledky:*** Exprese *MAP2K1* v nádorové tkáni byla oproti normálním tkáním významně vyšší, zatímco exprese *miR-7113-3p* a *miR-6721-5p* byla významně nižší. Také byla pozorována statistická korelace p = 0,04 mezi zvýšenou expresí *MAP2K1* a perineurální invazí. Navíc jsme zaznamenali, že mezi down-regulací *miR-7113-3p* a zvýšenou expresí *MAP2K1* je pozitivní korelace (p = 0,0218) a mezi down-regulací *miR-6721-5p* a zvýšenou expresí *MAP2K1 je* negativní korelace (p = 0,7771). ***Závěr:*** Z těchto nálezů vyplývá, že u pacientů s OSCC mohou *miR-7113-3p* a *miR-6721-5p* sloužit jako prospektivní biomarkery, které by v budoucnu mohly být využívány k detekci OSCC v časném stadiu. Zvýšená exprese *MAP2K1* je spojena s rozvojem OSCC a perineurální invazí.

Klíčová slova

dlaždicobuněčný karcinom ústní dutiny – cílový gen *MAP2K1* – *miR-7113-3p* – *miR-6721-5p* – kvantitativní PCR v reálném čase

Introduction

The OSCC is one of the deadliest head and neck tumors, since it has a high risk of recurrence and invasion [1]. The global incidence of oral cancer has held the sixth rank among all human malignancies, and according to the literatures, its mortality rate is so high [2]. Regardless of therapy advances, OSCC has a poor prognosis, and its diagnosis and prediction remain challenging using current biomarkers [3]. Determining genetic pathways that contribute to the pathogenesis of OSCC may aid in the development of therapeutic and diagnostic targets, which both have received insufficient experimental consideration [4]. A study published in 2017 by a group of Chinese researchers found that levels of component proteins of the MAPK signaling pathway tend to be higher in patients suffering oral cancer [5]. *MAP2K1*, a gene related with the MAPK signaling pathway, is overexpressed in numerous cancers and may be linked to a prognostic biomarker of head and neck squamous cell cancer (HNSCC) [6]. Because of the oncogenic and tumor suppressive functions of microRNAs (miRNAs), they can be used as potentially diagnostic and prognostic biomarkers for a wide range of types of cancer [7]. The miRNAs are short, non-coding RNA molecules of 15–22 nucleotides that modulate gene expression by silencing the target mRNA. The miRNA family plays an important regulatory role in variety fundamental biological processes such as cell division, growth, and apoptosis [8]. In recent years, many researchers have conducted extensive studies on the abnormal expression of miRNAs in various disorders, including cancer. In a majority of cases, their expression is repressed compared with normal tissues. The first study to suggest a correlation between miRNAs and cancer was the detection of *miR-15a* and *miR-16-1* which were frequently deleted in genomic regions in chronic lymphocytic leukemia, between exon 2 and exon 5 of *Leu2* gene [9]. DNA methylation is a major regulator of miRNAs expression in OSCC, as it is in many other cancers. MiRNAs exhibit distinct expression patterns because tumor cells express themselves differently than normal cells. This vast spectrum of alterations in miRNA expression has also been noted between oral cancer cells and normal cells. In light of these findings, miRNAs may be beneficial as biomarkers for early-stage diagnosis of oral cancer, as well as in the introduction of cancer treatments and therapies based on miRNAs [10].

It has been confirmed that *hsa-miR-7113-5p* targets WNT10B in a previous study. According to microarray studies, this miRNA was downregulated in post-traumatic stress disorder [11]. Furthermore, research discovered that miR-6721 is linked to aberrant expression in patients with low cell-free DNA (cfDNA) fetal fractions [12]. Interestingly, alterations in the expression of *miR-7113-3p* and *miR-6721-5p*, as well as their correlation with the target gene *MAP2K1* in OSCC, have not yet to be examined in any research. In order to select *miR-7113-3p* and *miR-6721-5p*, we consulted the bioinformatics databases Mirwalk [13] and miRDB [14]. Therefore, the objective of the present study was to examine the changes in the expression levels of *miR--7113-3p*, *miR-6721-5p*, and *MAP2K1* in tumor and adjacent normal tissues from OSCC cells. *miR-7113-3p* and *miR-6721-5p* were selected as they were novel and no previous studies existed on the area this paper aimed at, to the best of our knowledge. In the final analysis, we determined that *MAP2K1* gene may be involved in OSCC malignancy progression by assessing the association of *MAP2K1* with clinical and pathologic features.

Material and methods

Cell collection

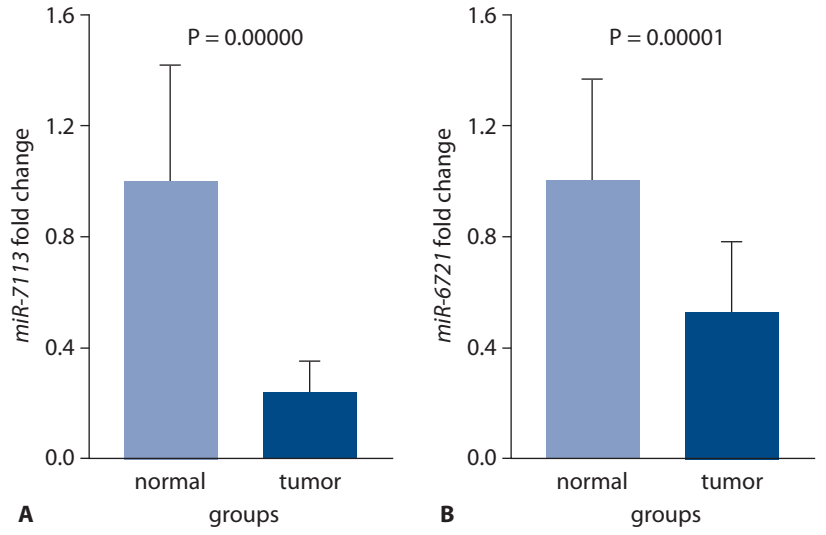
The study utilized 30 pairs of tumor and adjacent normal cell line collection. The samples were available at the Tumor Bank of Cancer Institute approved by an orthodontic specialist and a pathologist. We immediately preserved fresh tissue samples in liquid nitrogen and stored them at –80°C until RNA extraction.

RNA extraction and quantitative real-time PCR

After following the manufacturer's instructions, TRIZOL reagent was used to extract RNA (Invitrogen, Sigma, USA). Electrophoresis in 1.5% agarose gel and a Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) were used to confirm the qual-

Tab. 1. Real-time quantitative polymerase chain reaction primers.

Genes 5'-3' primer sequence
MAP2K1
F: GGTGTTCAAGGTCTCCCACAAG
R: CCACGATGTACGGAGAGTTGCA
miR-6721-5p
F: CGGGCTGGGCAGGGGCTTATT
R: CGCAGGGTCCGAGGTATTC
miR-7113-3p
F: TCCAGGGAGACAGTGTGTGA
R: CCAGTGCAGGGTCCGAGGTA
ACTB
F: GATCAAGATCATTGCTCCTCCTG
R: CTAGAAGCATTTGCGGTGGAC
U6
F: CTCGCTTCGGCAGCACA
R: AGAGCAGGGTCCGAGGT



Graph 1. Downregulated expressions of *miR-7113-3p* (A) and *miR-6721-5p* (B) in oral squamous cell carcinoma. Data are shown as means ± SD of three separate experiments. The transcript levels were normalized to *U6* expression. (P < 0.050; N = 30).

ity and quantity of extracted RNAs, respectively (the light absorption ratio of 260–280 nm in pure RNA is around 1.9–2.0 and it has a 28S to 18S bond strength of 2 : 1). Total extracted RNA was reverse transcribed using BioFACT's cDNA Synthesis kit to synthesize complementary DNA (cDNA) (Daejeon, South Korea), according to the manufacturer's protocol. Additionally, cDNA for miRNAs was synthesized using appropriate stem-loop RT primers and the MiR-Amp kit (Pars Genome, Iran). The SYBR Green RT-PCR Kit (BioFact, Daejeon, South Korea) was used to conduct the quantitative real-time PCR analysis on a Roche ExicyclerTM 96 thermocycler. The following thermal cycling profile was used for quantitative real-time PCR on miRNAs and *MAP2K1*: 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s for denaturation, 60 °C for 30 s for annealing, and 72 °C for 20 s for elongation. By employing *ACTB* as a housekeeping gene, the expression of *MAP2K1* was normalized. In addition, the expression of miR-7173-3p and *miR-6721-5p* was standardized using *U6* as an endogenous control. Following completion of the preceding stages, the received information was checked for Melting curve and the obtained diagrams were examined for dimer formation. The findings of the melting curve of these samples revealed that the miRNAs product was proprietary and had their own TM, as well as a single peak, thus confirming the correctness of the primers and the accuracy of real-time PCR. Finally, the CT number was calculated using the provided data. Primers were designed using Oligo Analyzer and the Primer3plus program, evaluated for optimal properties through the BLAST program, and synthesized by BIONEER (Daejeon, South Korea). A summary of the primer sequences can be found in Tab. 1.

Statistical analysis

The results were provided as the mean ± standard error of the mean of three identical experiments carried out in triplicate. GraphPad Prism software 9.0.0 (GraphPad Software, Inc., San Diego, CA, USA) and SPSS software were used to analyze the data (version 21.0; SPSS, Inc., Chicago, IL, USA). To determine the normal distribution of sample data, the one-sample Kolmogorov-Smirnov test was performed. The independent-sample Kruskal-Wallis tests were used to evaluate the association between *MAP2K1* levels and clinicopathological features in OSCC patients. Furthermore, the one-way analysis of variance (ANOVA) was employed to compare the levels of *MAP2K1* expression in different tumor sizes and clinical stages. The correlation between *miR-7113-3p*, *miR-6721-5p*, and *MAP2K1* expression was investigated applying Pearson's correlation and regression analysis. Gene expression differences were calculated using Genex6 software. In order to analyze the relationship between the levels of variables and disease probability, the odds ratio method was employed. This parameter was calculated using logistic regression in SPSS software. Finally, the diagnostic value was evaluated using the receiver operating characteristic (ROC) curve. A P-value ≤ 0.050 was regarded as statistically significant.

Results

miR-7113-3p and *miR-6721-5p* expression was downregulated in OSCC

The expression patterns of *miR-7113-3p* and *miR-6721-5p* were examined in 30 paired OSCC tissues and adjacent normal oral tissues using quantitative real-time PCR. *MiR-7113-3p* and *miR-6721-5p* expression levels were both reduced, (4.24-folds and 1.85-folds, respectively) in OSCC tissues compared to normal tissues (P = 0.00000 and P = 0.00001, respectively) (Graph 1).

MAP2K1 gene expression was upregulated in OSCC

In this investigation, quantitative real-time PCR was used to evaluate the expression of *MAP2K1* as a possible target for *miR-7113-3p* and *miR-6721-5p* in 30 paired OSCC tissues and adjacent normal oral tissues. Mirwalk and miRDB algorithms were used to discover potential co-targets of *miR-7113-3p* and *miR-6721-5p* in OSCC. Following that, online bioinformatics databases confirmed that *MAP2K1* might be an acceptable direct target for the corresponding miRNAs. *MAP2K1* expression was observed to be considerably higher (3.087-folds) in tumor tissues compared to adjacent normal oral tissues (P = 0.00000) (Graph 2).

Correlation between *MAP2K1* expression and *miR-7113-3p, miR-6721-5p* in OSCC patients

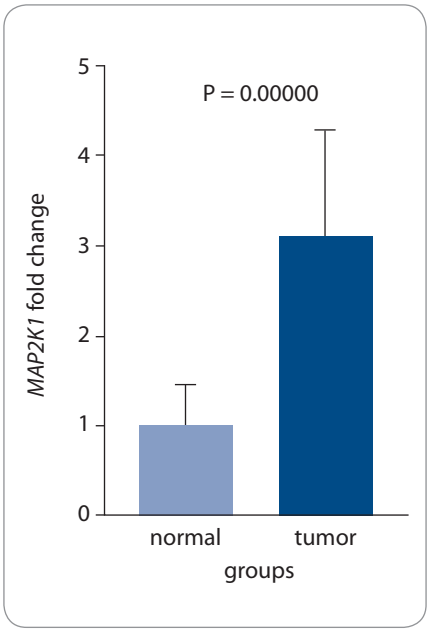
The Pearson's correlation analysis was used to examine the connection between *miR-7113-3p* and *miR-6721-5p* levels and *MAP2K1* expression in OSCC. We discovered an inverse and significant correlation between *miR-7113-3p* downregulation and *MAP2K1* target gene overexpression in OSCC (r = −0.295, P = 0.021). A direct and nonsignificant correlation was also identified between *miR-6721-5p* downregulation and *MAP2K1* overexpression (r = 0.037, P = 0.777) (Graph 3).

Potential diagnostic values of *MAP2K1* in OSCC

Based on ROC curve analysis, *MAP2K1* was evaluated for its potential to diagnose OSCC. The area under the curve (AUC) of *MAP2K1* was 0.9466 (95% CI = 0.8934–0.9999; P = 0.00000). The best cutting point is indicated by the threshold. This cutting point's sensitivities and specificities are also provided. To choose the best cut point, a value of J or the Youden index is employed (J = 0.8333). The optimal *MAP2K1* cutting point is $\Delta_{ct=6.8125}$, with a sensitivity of 0.8667 and a specificity of 0.9667 (Graph 4).

Potential diagnostic values of *miR-7113-3p* and *miR-6721-5p* in OSCC

Furthermore, the potential diagnostic value of *miR-7113-3p* and *miR-6721-5p* for OSCC was assessed by ROC curve analysis. According to the following tables, the value of AUC for *miR-7113-3p* is 0.9666 (95% CI = 0.9284–1; P = 0.00000), and for *miR-6721-5p*, the AUC is equal to 0.8261 (95% CI = 0.7155–0.9367; P = 0.00000) (Graph 5, 6).



Graph 2. Quantitative real time polymerase chain reaction analysis of *MAP2K1* expression in oral squamous cell cancer tissues and adjacent normal tissues (N = 30). The transcript levels were normalized to *ACTB* expression. The data are presented as means ± SD (P < 0.050).

Association between *MAP2K1* expression and clinicopathological features

The association between *MAP2K1* expression levels and some other clinicopathological parameters was investigated in Tab. 2 to gain a better awareness of its possible function in the development of oral cancer. It was found that *MAP2K1* expression was remarkably associated with tumor PNI (P = 0.041). According to the presence of PNI in 30 patients, 37% were positive (N = 11), and 63% were negative (N = 19). *MAP2K1* expression was increased in all patients, although considering the small number of patients with PNI, the presence of PNI was significantly associated with *MAP2K1* upregulation (P < 0.05) (Tab. 2).

Tab. 2. Clinicopathological characteristics and *MAP2K1* expression.

Clinicopathological characteristic	Total cases (n=30)	P-value
tumor size (cm)		
< 2	5	0.904
2–5	14	
> 5	11	
pathological grading		
I	17	0.865
II	13	
clinical stage		
I	3	
II	3	
III	7	0.139
IV	17	
lymph node metastasis		
yes	5	0.601
no	24	
unknown	1	
depth invasion		
yes	9	0.865
no	21	
necrosis presence		
yes	7	0.345
no	23	
clinical metastasis		
yes	1	0.614
no	29	

Discussion

HNSCC is a serious public health issue globally, with a high fatality rate. The most frequent kind of HNSCC is OSCC, which remains a concern for head and neck specialists despite major advances in diagnostic techniques and treatments [15]. Oral cancer is a multifactorial disease caused by a combination of genetic abnormalities and environmental factors, the most important of which are tobacco and alcohol use [16]. Epigenetic alterations, such as DNA methylation, histone modifications, and noncoding RNA modifications (miRNAs), have been shown to play an important regulatory role in the development and

progression of oral cancer [17]. MiRNAs seem to be essential in the epigenetic regulation of cellular processes such as cell cycle regulation, differentiation, apoptosis, and migration. MiRNA dysregulation leads to tumor-related events throughout cancer development [18]. In this way, miRNAs can control gene expression that is involved in cancer biology by acting as oncogenes or tumor suppressors [19].

In numerous recent studies, it has been shown that miRNAs expression is altered in oral squamous cell carcinoma, and some miRNAs are shown to function as tumor suppressors or tumor promoters during tumorigenesis. Tumor suppressor miRNAs like *miR-26-a, miR-99a-5p, miR-375,* and *miR-139-5p* were discovered to be downregulated in oral cancer and inhibit oncogenes, whereas oncomiRs like *miR-21, miR-31, miR-93, miR-211,* and *miR-373* were observed to be up-regulated in oral cancer and inhibit tumor suppressors [20]. Furthermore, it has been demonstrated in a study examining the expression of numerous miRNAs that miR-31 may be an ideal candidate for clinical application in oral cancer due to its high sensitivity in tissue, saliva, and plasma [21].

There have been some important candidate miRNAs implicated in progression of oral cancer as earlier studies demonstrated. Downregulation of miR-125a, miR-184, and miR-16 as well as upregulation of miR-96 were noted in both oral tumors and surgical margins, suggesting combinatorial regulation of these miRNAs and target transcription factors contributes to oral tumorigenesis and is useful in detecting minimal residual disease after surgery [22].

While *miR-7113-3p* and *miR-6721-5p* have frequently been reported to contribute in a variety of cancers, no study has evaluated their expression in OSCC. For instance, miR-7113 was upregulated by AnAc in MDA-MB-231 cells and targets host gene *NDUFS8* to cause breast cancer [23]. According to Guo's research, hsa-*miR-7113-3p* participates in the LINC00973-miRNA-mRNA cRNA network, which is enhanced in non-small-cell lung cancer tissues [24]. According to the findings, the circ 0034467_ *miR-6721-5p* - SLC19A1 regulatory network may serve as a key regulator in prostate cancer [25]. Additionally, one study demonstrated that *miR-6721-5p* was downregulated by *HOXC6*, another gene related to cancer progression [26]. Based on these results, *miR-7113-3p* and *miR-6721-5p* could represent potential biomarkers in OSCC and different cancers by exerting oncogenic or tumor- suppressive functions. It is, however, necessary to conduct more research to verify these findings.

The current study aimed to discover new diagnostic or prognostic biomarkers for OSCC. *MiR-7113-3p* and *miR--6721-5p* are significantly downregulated in OSCC tissues compared to normal tissues, according to our analyses. The role of *MAP2K1* in tumorigenesis and cancer progression has been noted previously as a candidate for further studies. Activated *MAP2K1* promotes cancer cell proliferation and confers drug resistance. The results of Zhe Jin's study suggested that blocking *MAP2K1* and *miR-330-3p* also inhibited the ability of HepG2 cells to migrate. In this study, *miR-330-3p* suppressed migration of liver cancer cells by interacting with *MAP2K1* [27]. In addition, You et al. observed *MAP2K1* overexpression in non-small cell lung cancer, and discovered that *miR-449a* regulated *MAP2K1* expression by directly targeting its 3'UTR [28]. *MAP2K1* mutations have been identified at a lower frequency in several cancers, including lung adenocarcinoma, melanoma and gastric cancer. About 1% of HNSCC cases exhibit *MAP2K1* mutations, the same as lung cancer [29]. *MAP2K1* has been shown to regulate tumorigenic development in OSCC. It is primarily responsible for cancer proliferation, chemoresistance, invasion, and migration in oral cancer [30]. Further studies identified that *MAP2K1* activation increased CD44 expression and promoter activity, whereas CD44 attenuation reduced both *in vitro* migration and *in vivo* oral tumor formation [31]. According to another study, *MAP2K1* activation frequently occurs in oral malignancies and is linked to tumor cell proliferation, migration, and invasion by regulating antiapoptotic and proliferative pathways [32]. These findings confirmed what we had discovered.

Based on bioinformatics analysis, we identified *MAP2K1* as a direct target of *miR-7113-3p* and *miR-6721-5p*. We discovered a significant increase in *MAP2K1* gene expression in tumor tissues, particularly in comparison to adjacent normal tissues from OSCC patients, which supports previously reported results. Moreover, *miR-7113-3p* and *miR--6721-5p* expression levels were significantly decreased. In the current study, we correlated the expression level of *miR-7113-3p* and *miR-6721-5p* to *MAP2K1* mRNA and we observed a significant inverse correlation between *miR-7113-3p* downregulation and *MAP2K1* target gene overexpression in OSCC ($r = -0.295$, $P = 0.021$). There was also a non-significant association between *miR-6721-5p* downregulation and *MAP2K1* overexpression ($r = 0.037$, $P = 0.777$). PNI is a form of tumor progression in which cancer cells encroach along nerves [33]. PNI is well known to be associated with a poor outcome in cancers of the colorectal, pancreas, and salivary glands. PNI has been reported to occur in 2–82 % of oral squamous cell carcinoma. There is also a correlation between PNI and prognostic factors [34]. According to the present study, there was a significant association between *MAP2K1* overexpression and PNI status in OSCC tumors ($P = 0.041$) and no remarkable association was found between vascular and depth invasions with *MAP2K1* overexpression ($P = 0.627$ and $P = 0.865$ respectively). Additionally, we observed increased *MAP2K1* expression in tumors in late stages (grade II), but no significant correlation was found ($P = 0.139$). According to the results of the present study, the overexpression of *MAP2K1* is not correlated with necrosis presence ($P = 0.345$), clinical metastasis ($P = 0.614$), tumor size ($P = 0.904$), pathological grading ($P = 0.865$), smoking status ($P = 0.443$) and family history ($P = 0.456$). To confirm these results, better understand the connection between the *MAP2K1* gene and *miR-7113-3p* and *miR-6721-5p* expression in oral cancer malignancy, and modify the aggressive behavior of oral cancer cells in clinical trials, additional research on the expression of the *MAP2K1* protein is required.

Sample size, repetition cycles and multiple analyses to endorse the result were the limitations of our study.

Conclusion

The results of this study revealed the first evidence of evaluation of *miR-7113-3p* and *miR-6721-5p* expression in OSCC and showed increased expression of the *MAP2K1* gene and decreased expression of *miR-7113-3p* and *miR-6721-5p* in tumor tissues, compared to normal adjacent tissues. As potential diagnostic and prognostic biomarkers for OSCC patients, *miR-7113-3p* and *miR-6721-5p* have the potential to become powerful biomarkers in the near future, and they may even contribute to the early diagnosis and prognosis of this disease.

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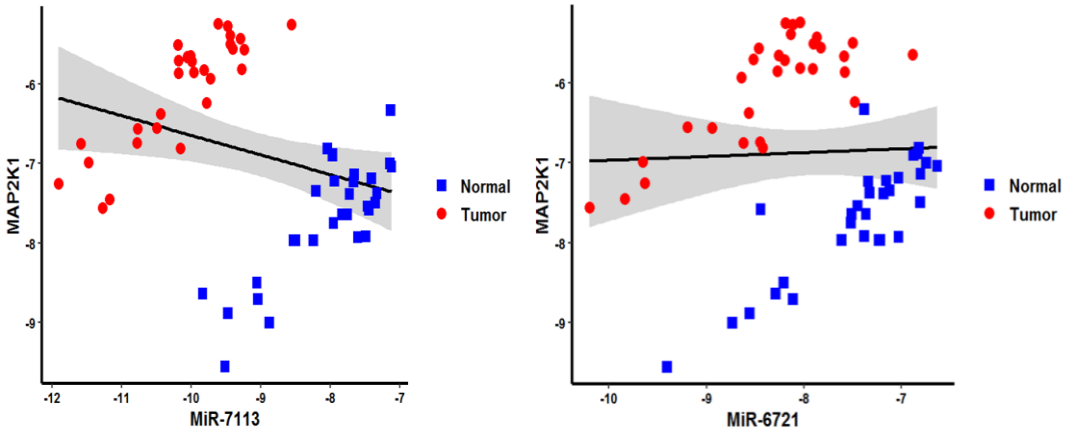
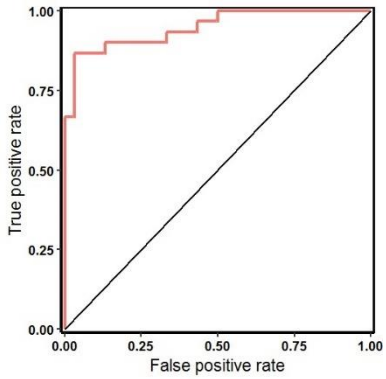
For Fig. 3–6, see the online version of the article at www.linkos.cz.



A) B)

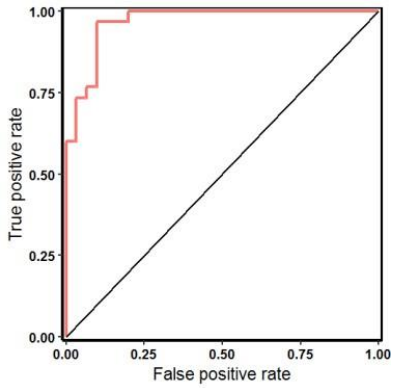
Graph 3. Pearson's correlation analysis between *MAP2K1* mRNA expression and *miR-7113-3p* (A) and *miR-6721-5p* (B) levels in oral squamous cell cancer patients. The data are presented as means ± SE. *MAP2K1* expression was significantly correlated with *miR-7113-3p*, whereas *MAP2K1* expression and *miR-6721-5p* was not significantly correlated (P = 0.021 and P = 0.777, respectively).



AUC	CI_lower	CI_high
0.946667	0.893397	0.999937

Threshold	Sensitivities	Specificities	J
6.8125	0.866667	0.966667	0.833333

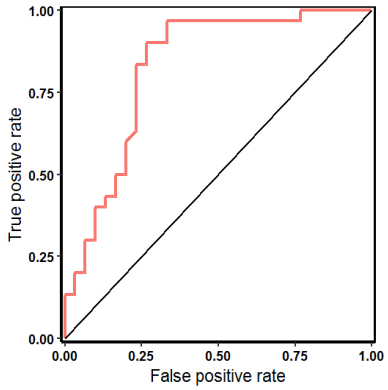
Graph 4. ROC curve analysis related to *MAP2K1* expression can distinguish patients with oral squamous cell cancer patients from healthy controls (P = 0.0000).



AU	CI_lower	CI_high
0.9666	0.92843	1

Threshold	Sensitivities	Specificities	J
9.1475	0.966667	0.9	0.866667

Graph 5. Analysis of the ROC curve for *miR-7113-3p* expression (P = 0.0000).



AUC	CI_lower	CI_high
0.826111	0.715481	0.936742

Threshold	Sensitivities	Specificities	J
7.465	0.966667	0.666667	0.633333

Graph 6. Analysis of the ROC curve for *miR-6721-5p* expression (P = 0.00001).