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miR-146a expression and the *miR-146a* gene variant rs2910164 as diagnostic and prognostic biomarkers of oral cancer

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Abstract: This study aimed to analyze the diagnostic and prognostic significance of miR-146a-5p in oral cancer and the association of the rs2910164 gene variant with miR-146a-5p expression. Thirty-five oral cancer patients participated in the study. Expression of miR-146a-5p in oral cancer, adjacent non-cancerous tissue, and the rs2910164 genotype variant were examined by real-time PCR. No difference was observed in miR-146a-5p expression between cancerous and non-cancerous tissues (P=0.272). It was concluded that miR-146a-5p is not a good diagnostic biomarker. There was no difference in miR-146a-5p expression depending on the rs2910164 genotype. The rs2910164 variant was associated with tumor histological grade (P=0.037), stage (P=0.036), and lymph node status (P=0.025). There was a difference in survival between oral cancer patients with high and low miR-146a-5p expression (P=0.026), but not between patients with the GG and GC genotypes (P=0.400). Oral cancer patients with the wild-type GG genotype and high miR-146a-5p expression had significantly shorter survival when compared to patients with miR-146a-5p expression below the optimal cut-off and the wild-type genotype (P=0.035). A high miR-146a-5p level could be considered a potential biomarker of poor survival in patients with oral cancer, especially in those with the wild-type GG genotype of the rs2910164 variant.

Keywords: oral cancer, miR-146a-5p, rs2910164, biomarker

INTRODUCTION

The search for molecular biomarkers that can distinguish cancerous from non-cancerous tissue and predict disease outcomes is a crucial objective in modern molecular genetics and cancer pathology. Oral cancer is one of the most common cancers of the head and neck for which there are no clinical biomarkers [1]. The need to identify diagnostic molecular biomarkers arises from the fact that despite surgical treatment, recurrence occurs in up to 30% of patients with oral cancer [2]; therefore, the prognosis in patients with oral cancer is not favorable, which has led to an increasing number of studies seeking prognostic biomarkers.

Small non-coding RNA molecules known as microRNAs (miRNAs) have been extensively investigated as potential biomarkers in various human pathologies, including cancers. [3]. miR-146a is frequently studied for its dual role in various malignancies, functioning as both an oncogene and a tumor suppressor gene [4]. There are conflicting results regarding miR-146a expression in oral cancer [5-8]. Pleiotropic effects of miR-146a in oral cancer development have been suggested, including the promotion of proliferation and apoptosis [9], cancer stemness through Numb regulation [10] and the signal transducer CD 24-protein kinase B Akt (CD24-AKT)-β catenin axis [11]. Among target genes, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)1 was identified as a putative target of miR-146a-5p by the luciferase reporter assay [12]. miR-146a was observed to be involved in oral cancer progression and metastases by targeting the

huntingtin (*HTT*) gene [5]. Also, miR-146a promotes proliferation, invasion, and epithelial-mesenchymal transition in oral cancer cell lines [13]. An oncogenic role of miR-146a has been reported in oral cancer, confirmed by its gene targets, which include the protein numb homolog (NUMB), the TNF receptor-associated factor protein (TRAF6), and the interleukin-1 receptor-associated kinase (IRAK1), all of which are negatively regulated by miR-146a [14].

Changes in one nucleotide in miRNA genes can affect both the transcription and functionality of mature miRNA [15]. The rs2910164 variant in pre-miR-146a has been primarily studied in oral cancer through case-control studies, independent of mature miR-146a expression levels. The variant rs2910164 in the *miR-146a* gene was found to be associated with decreased oral cancer risk according to the results of a meta-analysis [16]. Conflicting results on the effect of rs2910164 on miR-146a expression can be found in the literature [17,18]. No studies have investigated the association between the *miR-146a* gene variant rs2910164 and miR-146a expression in oral cancer, underscoring the novelty and importance of the current study. The present study investigated the diagnostic and prognostic potential of miR-146a-5p expression in oral cancer patients, and the impact of the pre-miR-146a variant rs2910164 on mature miR-146a-5p expression in oral cancer was analyzed.

MATERIALS AND METHODS

Ethics statement

This study was approved by the Ethics Committee of the Faculty of Medicine, University of Belgrade, and the study was per the Declaration of Helsinki and in compliance with local regulatory requirements (Approval No.: 1550/VII -6). Informed consent was obtained from all subjects included in the study.

Study group and biological material

The study group included 35 patients with oral cancer. All participants were of the same) ethnicity (Serbian, Caucasian). The patients were treated at the Clinic for Otorhinolaryngology and Maxillofacial Surgery, University Clinical Centre of Serbia from January 2018 to November 2020. Patients provided written informed consent for the retrieval of biological samples of oral cancer, matched non-cancerous tissue (2 cm from the macroscopic tumor margins), and peripheral blood. A pathologist confirmed that all cases were oral squamous cell carcinomas. The collected tissues were placed in tubes containing RNAlater (Ambion, USA) and stored at -80°C. Peripheral vein blood was collected in EDTA tubes and stored at -20°C. The guidelines recommended in the Union for International Cancer Control Staging Manual, 8th ed. were used for tumor staging classification. The characteristics of the study group have been published previously [19]. Patient follow-up was a maximum of 38 months, with a median of 20 months.

RNA isolation, reverse transcription, and relative expression of miR-146a-5p

Total RNA was isolated from tissue using the miRVana miRNA isolation kit (Invitrogen, USA). cDNA synthesis was performed using the TaqMan™ MicroRNA Reverse Transcription kit (Invitrogen, USA) under the following conditions: 30 min at 16°C, 30 min at 42°C, 5 min at 85°C and a hold at 4°C. Synthesized cDNA was stored at -20°C. A mix of cDNA, dH_2O, 2xTaqMan™ Universal PCR Master Mix NoAmpErase™ UNG, and TaqMan™ qRT-PCR assay ID000468 (Applied Biosystems, USA) was used for relative quantification of mature miR-146a-5p expression; an assay ID001093 was used for the endogenous control RNU6B. Amplification conditions on the QuantStudio 3 instrument were as follows: 5 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C. All reactions were performed in triplicate. The comparative ΔCt method was used to calculate relative gene expression. miR-146a-5p expression was reported as $2^{-\Delta Ct}$ and used for statistical analysis.

DNA isolation and genotyping of the rs2910164 variant in the *miR-146a* gene

DNA was isolated from peripheral blood and tumor tissue using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, USA). The isolated DNA was stored at -20°C until genotyping analysis. A reaction mix of TaqMan SNP Genotyping Assay (ID: C_15946974_10), 2xTaqMan™ Universal PCR Master Mix (Applied Biosystems, USA), dH_2O, and isolated

DNA was used to genotype the rs2910164 variant in the *miR-146a* gene. Allelic discrimination was performed using the QuantStudio 3 instrument. The temperature profile was as follows: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C.

Data analysis from the Cancer Genome Atlas

The expression level of miR-146a-5p was retrieved from the Cancer Genome Atlas (TCGA) database from HNSC – head and neck squamous cell carcinoma project data version 2016_01_28 (https://gdac.broadinstitute.org/). The TCGA-HNCS group included 532 samples (miRseq) from patients with head and neck cancer. After excluding samples related to the larynx, oropharynx, hypopharynx, and tonsils, 354 samples remained, of which 322 were oral cancer and 32 were normal samples. The oral cancer specimens exhibited malignancy at various anatomical sites, including the tongue, floor of the mouth, hard palate, alveolar ridge, and lips, from patients of diverse genetic backgrounds. After filtering for Caucasian individuals, 309 cases remained, including 279 oral cancer cases and 30 normal samples. Extracted data were used for further statistical analysis.

Statistical analysis

Statistical analysis was performed using the GraphPadPrism v.9 software. Categorical variables were analyzed using the χ^2 test or Fisher's exact test. Depending on the result of the Shapiro-Wilk test used to test the distribution of scaled data, appropriate parametric or non-parametric statistical tests were applied. When data were not normally distributed, the Wilcoxon rank sum test was used to compare miRNA expression levels between the groups. For unpaired cases, the T-test was used for normally distributed data, whereas the Mann-Whitney U test was used when data did not pass the normality test. Spearman's correlation test was used for correlation analysis when the data were not normally distributed. Receiver operating curve analysis (ROC) with the area under the curve (AUC) was used to estimate the diagnostic potential of the miR-146a-5p expression levels. The optimal cut-off values were determined using Cutoff Finder software [20]. The optimal cut-off value for discriminating oral cancer from non-cancerous tissue was determined based on ROC curve analysis and minimizing the Manhattan distance, which corresponds to the maximum Youden index (Youden index=sensitivity+specificity-1). The mixture model was used to dichotomize the relative expression of miR-146a-5p in oral cancer tissues as high- or low-expressed after overall survival was analyzed. The association of miR-146a-5p expression with overall survival was estimated by Kaplan-Meier survival analysis. The log-rank test was used to compare survival curves. The hazard ratio (HR) was calculated using Cox proportional regression. The post-hoc power of the study was calculated by G*Power 3.1 software [21,22]. For a P value less than 0.05, the results were considered significant.

RESULTS

Relative expression of miR-146a-5p and its diagnostic potential in oral cancer

Relative expression of miR-146a-5p did not differ between cancerous and adjacent non-cancerous tissues (P=0.272) (Fig. 1A and B). The mean relative expression of miR-146a-5p in cancerous tissues was 42.409±46.760 and in non-cancerous tissues, 34.782±41.734. The relative expression of miR-146a-5p correlated significantly between cancerous and non-cancerous tissues (Rho=0.695, P<0.0001) (Supplementary Fig. S1). The relative expression of miR-146a-5p cannot be used to sensitively distinguish cancerous from non-cancerous tissues (AUC=0.560, P=0.388) (Fig. 2).

miR-146a gene variant rs2910164 in oral cancer patients and its association with miR-146a-5p expression

Genotyping of the rs2910164 variant was performed in both peripheral blood and tumor tissue in oral cancer patients. The genotypes between these two analyzed samples were 100% concordant. In our study group, 20 patients (57%) had the wild-type GG genotype, 15 (43%) were heterozygous GC, while the mutated CC genotype was not recorded for the analyzed rs2910164 variant. Gene variant rs2910164 was in Hardy-Weinberg equilibrium (P=0.106).

No difference in miR-146a-5p expression was observed in oral cancer tissues between patients with the wild-type GG genotype and those with the heterozygous GC genotype for variant rs2910164 (P = 0.739,

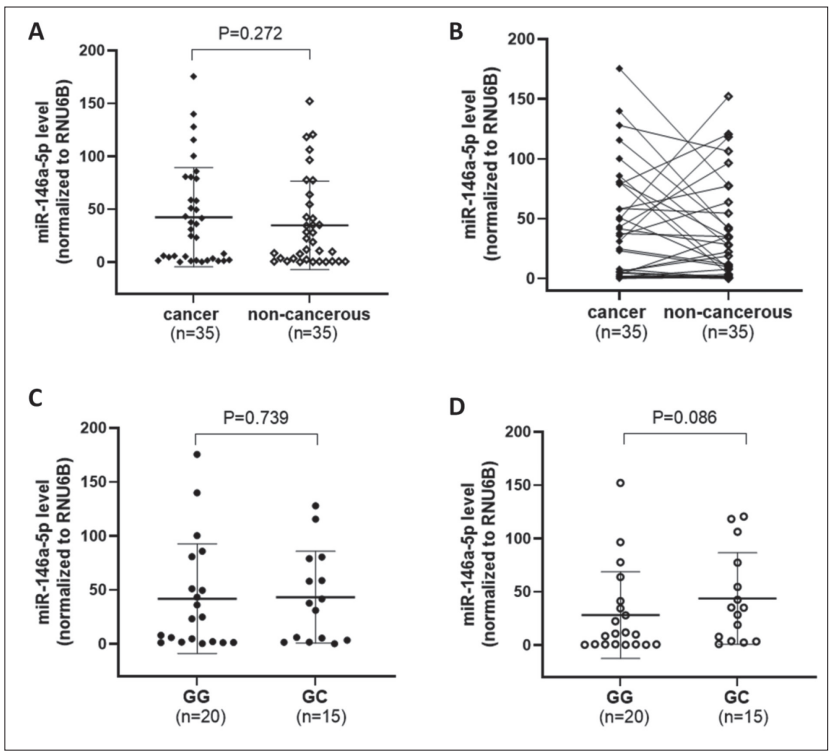

Fig. 1. miR-146a-5p expression: **A** – oral cancer and non-cancerous tissue; **B** – paired samples of oral cancer and adjacent non-cancerous tissue; **C** – oral cancer tissue depending on the genotype of rs2910164 variant; **D** – adjacent non-cancerous tissue depending on the genotype of rs2910164 variant. Relative expression of miR-146a-5p is normalized to RNU6B and reported as $2^{-\Delta Ct}$.

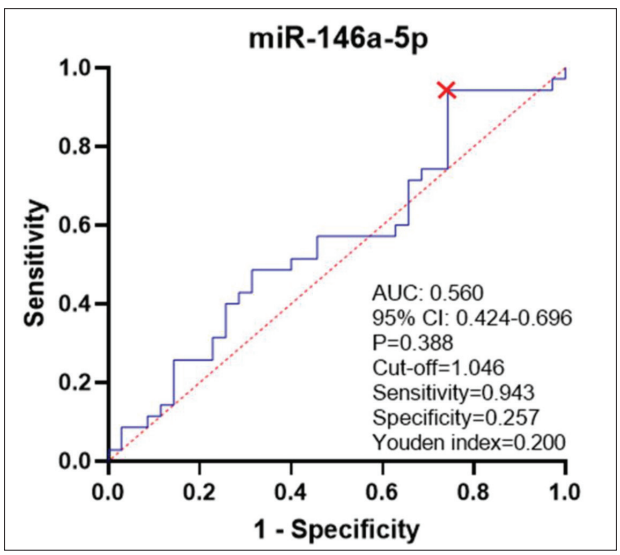

Fig. 2. ROC curve of miR-146a-5p relative expression for discrimination of oral cancer from non-cancerous tissue. The optimal cut-off value was derived using the Cutoff Finder software based on the ROC curve: minimizing the Manhattan distance – maximization of Youden index (sensitivity+specificity -1).

Mann-Whitney U test) (Fig. 1C). The mean miR-146a-5p expression in patients with the GG genotype was 41.810±50.720, whereas in GC it was 43.210±42.640. The rs2910164 variant was not associated with miR-146a-5p expression in tumor tissues when stratified as high and low expression (Table 1). When miR-146a-5p expression in non-cancerous tissues was compared between the GG and GC genotypes, no significant difference was observed (P=0.086) (Fig. 1D). The analyzed variant did not correlate with miR-146a-5p expression in cancerous (Rho=0.057, P=0.744) or in non-cancerous tissue (Rho=0.297, P=0.083).

Association of miR-146a-5p expression and the rs2910164 variant with demographic and clinicopathological characteristics of the study group

miR-146a-5p expression in oral cancer tissue was classified into low- and high-expressed groups based on the optimal cut-off value determined by the Cutoff Finder using a mixture model method [20]. Fifteen (42.9%) patients had low-expressed, and 20 (57.1%) had high-expressed miR-146a-5p. Age was associated with miR-146a-5p expression (P=0.025). There was no association between the relative expression of miR-146a-5p and the clinicopathological characteristics of the study group (Table 1). Oral cancer patients with the wild-type GG genotype were more likely to have moderately differentiated (P=0.037), advanced III and IV disease stages (P=0.036), and positive lymph nodes (P=0.025).

miR-146a-5p expression and rs2910164 variant association with survival of oral cancer patients

There was a significant difference in the survival of patients with high and low miR-146a-5p expression in cancerous tissue according to the Kaplan-Meier survival curves (P=0.026) (Fig. 3A). There was no difference in overall survival between patients with the wild-type and heterozygous rs2910164 variant genotypes (P=0.400) (Fig. 3B). miR-146a-5p expression stratified by the rs2910164 variant genotype was associated with

Table 1. Association of relative expression of miR-146a-5p in oral cancer tissue and the rs2910164 gene variant with demographic and clinicopathological characteristics of oral cancer patients.

Demographic and clinicopathological characteristics		Relative expression of miR-146a-5p in tumor			Gene variant rs2910164		
		low (n=15)	high (n=20)	P	GG (n=20)	GC (n=15)	P
Sex	male	12	14	0.503	14	12	0.503
	female	3	6		6	3	
Age (years, median)	<59	11	7	**0.025**	11	7	0.625
	>59	4	13		9	8	
Smoking habits	nonsmoker	4	6	0.829	7	3	0.331
	smoker + ex-smoker	11	14		13	12	
Alcohol consumption	no intake	3	7	0.331	7	3	0.331
	moderate + high	12	13		13	12	
Oral hygiene	good	4	11	0.094	8	7	0.693
	poor	11	9		12	8	
Location of primary tumor	tongue	11	14	0.184	17	8	0.071
	hard palate	3	1		2	2	
	the floor of the mouth	1	5		1	5	
Histological gradus	well differentiated	2	8	0.213	7	3	**0.037**
	moderately differentiated	9	9		12	6	
	poorly differentiated	4	3		1	6	
Stage	I+II	7	7	0.486	5	9	**0.036**
	III+IV	8	13		15	6	
Tumor size	≤2cm	1	1	0.501	0	2	0.082
	2-4cm	8	7		7	8	
	>4cm	6	12		13	5	
Nodal status	N-	9	9	0.380	7	11	**0.025**
	N+	6	11		13	4	
Recurrences	no	11	15	0.911	15	11	0.911
	yes	4	5		5	4	
rs2910164 genotype	*wild-type* GG	9	11	0.767	-	-	-
	heterozygous GC	6	9		-	-	

High and low expression levels refer to higher or lower than the optimal cut-off (8.864) determined by the Cutoff Finder using a mixture model in oral cancer tissue. P values were determined by the χ^2 or Fisher's exact test as appropriate. Significant P values are indicated in bold. Abbreviations: n – number of cases; TNM – tumor node metastasis.

overall survival. Oral cancer patients with the wild-type GG genotype and high miR-146a-5p expression had significantly shorter survival compared to patients with miR-146a-5p expression below the optimal cut-off and wild-type genotype (P=0.035) (Fig. 3C). Stratified by the heterozygous GC genotype, there was no difference in overall survival between patients with high and low miR-146a-5p expression (P=0.277) (Fig. 3D). ROC analysis showed that miR-146a-5p expression in both tumor and non-cancerous tissues cannot be used to predict lethal outcomes in oral cancer patients (respectively: AUC=0.436, P=0.571 and AUC=0.466, P=0.763). The Cox hazard ratio analysis showed that miR-146a-5p expression and the rs2910164 variant are not independent prognostic predictors (Table 2).

TCGA analysis of miR-146a-5p expression data and survival

Since the -5p form of miR-146a is the dominant form, data on the miR-146a-5p level (RPM log2) were retrieved from the TCGA database. There was no significant difference in miR-146a-5p expression between oral cancerous and non-cancerous samples (P=0.275) (Supplementary Fig. S2A). When considering only matched samples from the same patients (n=28 matched samples), no significant difference in miR-146a-5p level was observed between oral cancer and matched normal samples (P=0.144) (Supplementary Fig. S2B). According to ROC analysis, miR-146a-5p expression is not a good biomarker for distinguishing between

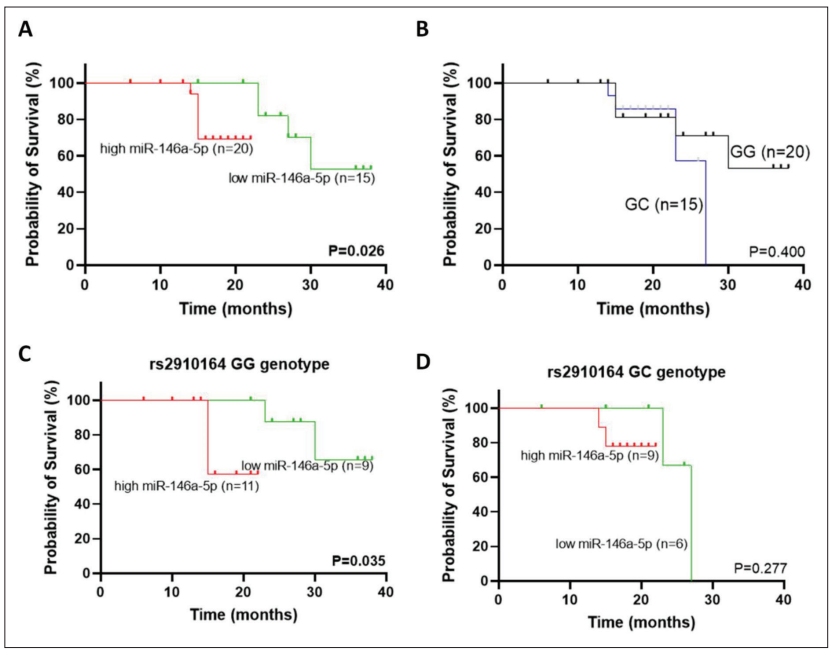

Fig. 3. Kaplan-Meier survival curve plots of oral cancer patients depending on **A** – miR-146a-5p relative expression; **B** – genotype of gene variant rs2910164; **C** – miR-146a-5p relative expression stratified by the wild-type GG genotype; **D** – heterozygous GC genotype of gene variant rs2910164. High and low miR-146a-5p expression refers to values above or below the optimal cut-off value of 8.864, which was determined using the Cutoff Finder software with a mixture model.

Table 2. Hazard ratio Cox-regression analysis of miR-146a-5p expression and rs2910164 variant

Hazard ratio Cox-regression analysis		
Variable	**HR (95% CI)**	**P**
miR-146a-5p (low vs. high)	0.017 (0.000-22.530)	0.266
TCGA miR-146a-5p (low vs. high)	1.509 (1.010-2.254)	0.044
miR-146a rs2910164 (GG vs. CC)	1.791 (0.426-7.525)	0.426

HR (95% CI) – Hazard ratio with a 95% confidence interval; TCGA – The Cancer Genome Atlas
High and low expression levels refer to higher or lower than the optimal cut-off value (8.864) derived by the Cutoff Finder software using the mixture model in oral cancer tissue or median (7.315) of relative gene expression for TCGA data.

oral cancer and the surrounding non-cancerous tissues (AUC=0.588, P=0.109) (Supplementary Fig. S3).

Extracted TCGA data were used for survival analysis. TCGA patients were stratified into high- or low-expressed groups using the median of miR-146a-5p level. There was a significant difference in survival of TCGA oral cancer patients (P=0.043) (Supplementary Fig. S4). Cox hazard ratio analysis indicated that patients with low miR-146a-5p expression had a higher hazard risk (HR=1.509, 95% CI: 1.010-2.254, P=0.044). Analysis of the TCGA dataset showed that the stage and nodal status were associated with miR-146a-5p level (P=0.001 and P=0.003, respectively) (Supplementary Table S1).

DISCUSSION

miR-146a is a microRNA that has been extensively researched in cancer, demonstrating dual roles as both an oncogene and a tumor suppressor. However, there is insufficient data to establish its clear role in oral cancer [4]. In most studies, the rs2910164 variant was investigated as a potential risk factor for susceptibility to oral cancer, independent of gene expression [16].

Although the expression of miR-146a-5p was higher in oral cancer tissues than in adjacent noncancer tissues, the difference was not significant, which was in line with the findings of TCGA data analysis. Based on our results, it is unclear whether miR-146a-5p acts as an oncogene or tumor suppressor miRNA in oral cancer. Studies have reported increased miR-146a expression in oral cancer cell lines [9,12,14] and oral cancer tissues [5-7], confirming its oncogenic role. In contrast, miR-146a expression was found to be decreased in histologically advanced oral cancer, indicating its tumor-suppressive role [8]. Moreover, both oncogenic and tumor suppressive roles of miR-146a have been reported in oral cancer [4]. Inconsistencies between studies may result from differences in the size of the study groups, the anatomical locations of cancer in the oral cavity, clinicopathological characteristics of the study group, and other factors. It is worth noting that classifying miRNAs as either oncogenic or tumor-suppressive remains a matter of debate, as their roles can vary depending on tissue type, context, and tumor stage [23, 24]. Increased miR-146a expression was also observed in the regeneration region after surgical excision and in non-cancerous mucosa [25]. This may be explained by the cancerization field proposed by Slaughter [26], or by the increased proliferation of tissues during the regeneration phase. This could also account for the strong correlation of miR-146a expression between oral cancer and adjacent non-cancerous tissue observed in our study.

Our results suggest that miR-146a-5p is not suitable as a diagnostic marker because it lacks the ability to discriminate between cancerous and non-cancerous tissues. These data are confirmed by TCGA data analysis. The fact that miR-146a-5p expression correlates in both tumor and non-tumor tissues further supports the conclusion that miR-146a-5p expression is unsuitable as a diagnostic biomarker for oral cancer. To our knowledge, this is one of the first studies to report the diagnostic potential of miR-146a-5p.

The relative expression of miR-146a-5p in oral cancer and adjacent non-cancerous tissue did not differ between patients with wild-type GG and heterozygous GC genotypes of variant rs2910164. For more accurate results regarding the effects of the rs2910164 variant in the *miR-146a* gene on the development of oral cancer as well as on the expression level of miR-146a-5p, a larger sample of the population of the same ethnicity is needed to avoid the effects of population stratification due to different allele frequencies and potentially different effects. The lack of effect of rs2910164 on miR-146a-5p expression in our study could also be attributed to the absence of a somatic mutation in rs2910164 since the genotypes were consistent between blood and tumor tissue. Previous studies suggested that the rs2910164 variant affects the level of mature miR-146a. It has been reported that both pre-miR-146a and mature miR-146a are synthesized independently of the rs2910164 genotype, but that their expression level depends on the allele [9]. However, data are inconsistent between different studies and cancer types. Some data indicate an association of the C allele with increased miR-146a expression [17,27] but also decreased expression in different cancer types [18,28]. *In silico* analyses predict that the rs2910164 variant affects pre-miRNA thermodynamic stability and is associated with decreased efficiency of processing mature miR-146a [18]. Specifically, the rs2910164 variant can influence the selection of the mature microRNA strand for incorporation into the RNA-induced silencing complex (RISC). This, in turn, can affect the ratio of -5p and -3p strands in cancerous tissue and impact the selection of target genes by the -5p strand [18].

We analyzed the prognostic significance of both relative miR-146a-5p levels and the rs2910164 variant. There was a significant difference in overall survival among patients with oral cancer based on miR-146a-5p expression levels, but no difference was observed between patients with the wild-type and heterozygous variant rs2910164 genotypes. We observed a significant difference, but an opposite trend in overall survival among high and low miR-146a-5p expression in public TCGA data, with poor survival for low miR-146a-5p expression. Since the TCGA survival data showed an opposite trend to our results, this difference may be explained by the different cut-off value applied and a longer follow-up period. miR-146a-5p expression could be considered a potential survival biomarker in oral cancer patients. Hung et al. [14] also observed an increased expression of miR-146a in blood plasma before tumor resection compared to the period after resection, which implies that circulating miR-146a originates from the tumor. The authors also noted that a decrease in miR-146a expression levels tended to correlate with improved survival. The results of this study also imply a positive correlation between the miR-146a expression level and tumor burden, which could represent a potential prognostic biomarker for disease relapse. However, further studies are needed to investigate this potential in greater detail.

The choice of an optimal cut-off value is critical for identifying diagnostic, prognostic, and predictive biomarkers. A mixed model in Cutoff Finder software determined the optimal cut-off value for classifying miR-146a-5p expression in oral cancer tissue as high and low. The model used is based on the distribution of the potential biomarkers in the group of patients [20]. The cut-off value for classifying miR-146a-5p expression as either high or low may be a possible reason for inconsistent results in the literature. Poor survival was observed in patients with oral cancer who had decreased *HTT* gene expression, which is directly regulated by miR-146a, likely due to increased migration and invasion associated with elevated miR-146a expression [13]. Functional analysis showed that the knockdown of miR-146a inhibited cell migration and invasion. In addition, miR-146a is also known to regulate other genes, such as *IRAK1*, *TRAF6*, *NUMB* [14], and SRY-related HMG-box genes (*SOX*)*2* [8]. *SOX2* expression has been linked to poor prognosis in tongue cancer patients [29], and the suppression of *SOX2* expression can lead to epithelial-mesenchymal transition (EMT) [30]. Increased miR-146a expression also promotes EMT-like phenotypic features by downregulating the expression of epithelial markers such as E-cadherin and

upregulating the expression of mesenchymal molecular markers such as N-cadherin and vimentin, which are known to promote the process of EMT [31]. It is also interesting to note that the sialyltransferase ST8SIA4, a direct target gene of miR-146a, is downregulated in tumor cells and plays a role in EMT [13].

Post-hoc analysis revealed that the statistical power to distinguish miR-146a-5p expression between cancerous and non-cancerous tissues was 88.2% at a significance level of 0.05 and an effect size of 0.5. When evaluating the relative expression in oral cancer and adjacent non-cancerous tissue in relation to rs2910164 genotypes, the statistical power was found to be 40.3%. This indicates a relatively modest statistical power to detect differences in miR-146a-5p expression based on the genotype of the *miR-146a* gene variant rs2910164. The small sample size in our study group is a potential limitation that should be taken into account when interpreting the results. The data from the public TCGA base confirmed the diagnostic and prognostic importance of miR-146a-5p. Selecting the optimal cut-off value is also a critical issue. To classify miR-146a-5p expression as high or low, we employed CutOff Finder software along with a mixture model. When analyzing the TCGA data, the median was used as the cut-off due to unequal group stratification resulting from the mixture model, as this is a common method for determining cut-off values. This approach may have contributed to inconsistencies in survival rates based on miR-146a-5p expression between the TCGA cohort and our study group.

The results of our study demonstrate that the relative expression of miR-146a-5p can be used as a potential prognostic biomarker in patients with oral cancer. The level of miR-146a-5p can be used as an indicator of poor survival after surgical resection, especially in patients with the wild-type GG rs2910164 variant genotype. The translational potential and clinical relevance of using miR-146a-5p expression as a prognostic biomarker need to be validated in future prospective studies to confirm our initial observations.

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Author contributions: Conceptualization, KZ; methodology, MV and KZ; software, MV and KZ; validation, MV and KZ; formal analysis, MV and KZ; investigation, MV and KZ; resources, GS; data curation, KZ; writing – original draft preparation, MV and KZ; writing, review and editing, MV, GS and KZ; visualization, MV and KZ; supervision, KZ; project administration, GS and KZ; funding acquisition, GS and KZ. All authors have read and agreed to the published version of the manuscript.

Conflict of interest disclosure: The authors declare no conflicts of interest.

Data availability: Data underlying the reported findings have been provided as a raw dataset avaliable here: https://www.serbiosoc.org.rs/NewUploads/Uploads/Vukovic%20et%20al_Raw%20Dataset.pdf

The clinicopathological characteristics of the study group are not publicly available due to privacy reasons. The publicly available TCGA dataset was analyzed in this study. This data can be found here: http://firebrowse.org/?cohort=HNSC&download_dialog=true

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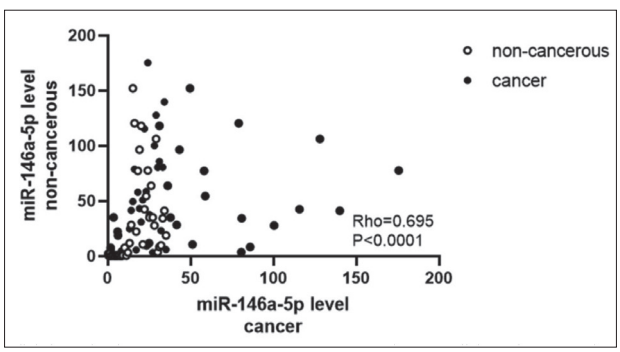
SUPPLEMENTARY MATERIAL

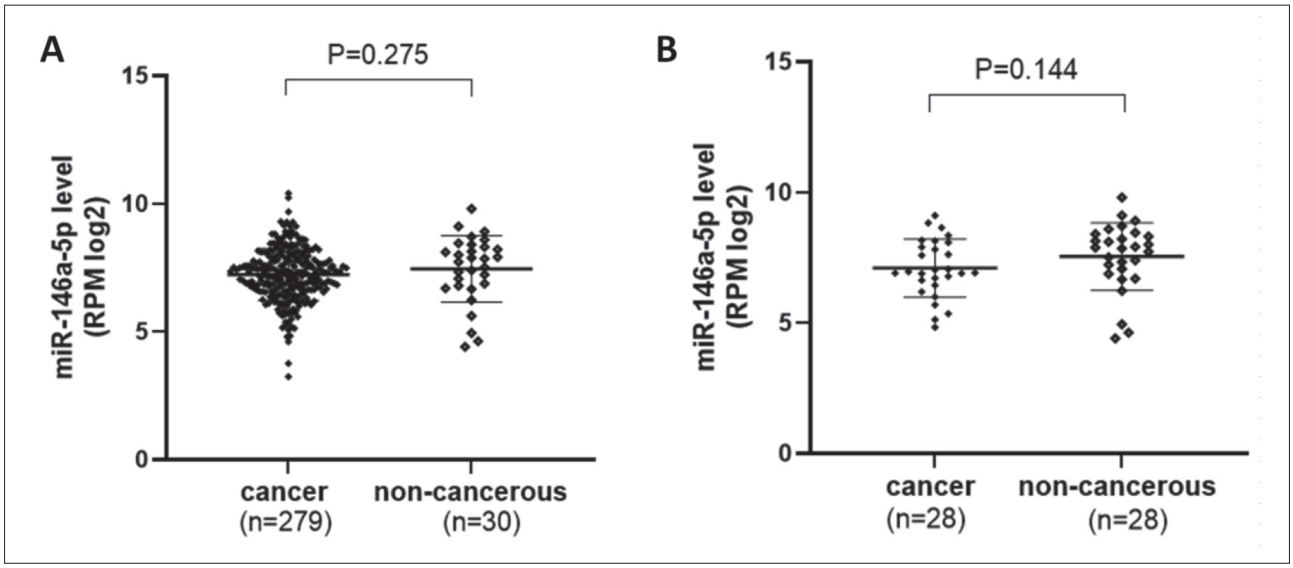
Supplementary Table S1. Association of miR-146a-5p expression in oral cancer with demographic and clinicopathological characteristics of oral cancer patients from TCGA database.

TCGA demographic and clinicopathological characteristics		Relative expression of miR-146a-5p in tumor (RPM log2)		
		low (n=140)	high (n=139)	P
Sex	male	92	93	0.833
	female	48	46	
Age (years, median)	<62	72	72	0.951
	>62	68	67	
Smoking habits	nonsmoker	38	40	0.818
	smoker+ex-smoker	98	97	
	NA	4	2	
Location of the primary tumor	tongue	65	67	0.670
	hard palate	2	4	
	the floor of the mouth	24	21	
	alevolar ridge	9	7	
	oral cavity + buccal mucosa	40	38	
	lip	0	2	
Histological gradus	well differentiated	20	25	0.103
	moderately differentiated	88	74	
	poorly differentiated	28	33	
	anaplastic	0	4	
	NA	4	3	
Stage	I+II	27	54	**0.001**
	III+IV	106	80	
	NA	7	5	
Tumor size	≤2cm	22	31	0.081
	2-4cm	36	45	
	>4cm	82	63	
Nodal status	N-	63	89	**0.003**
	N+	75	50	
	NA	2	-	

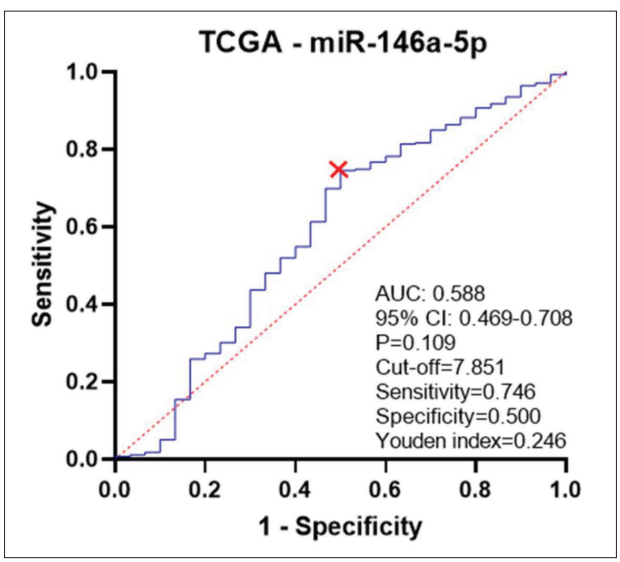
NA – not available; TCGA – The Cancer Genome Atlas; high and low miR-146a-5p levels refer to higher or lower than the median value (7.315) in oral cancer tissue.



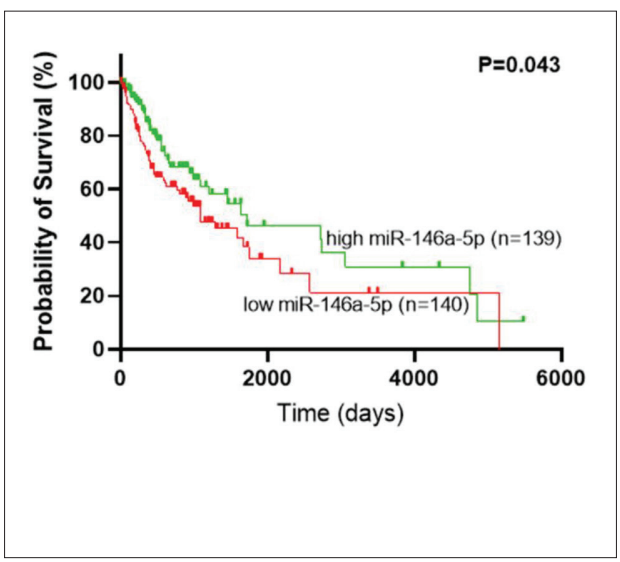
Supplementary Fig. S1. Correlation analysis between miR-146a-5p level in oral cancer and non-cancerous tissue. Relative expression of miR-146a-5p is normalized to RNU6B and reported as $2^{-\Delta Ct}$.



Supplementary Fig. S2. miR-146a-5p expression in the TCGA dataset. **A** – oral cancer and non-cancerous tissue; **B** – matched oral cancer and non-cancerous tissue of the same patients. TCGA – The Cancer Genome Atlas.



Supplementary Fig. S3. ROC curve of TCGA miR-146a-5p expression for discrimination of oral cancer from non-cancerous tissue. The optimal cut-off value was derived by the Cutoff Finder software using the method based on the ROC curve by minimizing the Manhattan distance – maximization of the Youden index (sensitivity + specificity -1). TCGA – The Cancer Genome Atlas.



Supplementary Fig. S4. Kaplan-Meier survival curve plots of TCGA oral cancer patients depending on the miR-146a-5p level. High and low miR-146a-5p levels refer to higher or lower than the median value (7.315) in oral cancer tissue. TCGA – The Cancer Genome Atlas.