

miR-186, miR-3651 and miR-494: Potential biomarkers for oral squamous cell carcinoma extracted from whole blood

JUTTA RIES¹, ELEFThERIOS VAIRAKTARIS², ABBAS AGAIMY³, RITA KINTOPP¹,
CHRISTOPH BARAN¹, FRIEDRICH W. NEUKAM¹ and EMEKA NKENKE¹

¹Department of Oral and Maxillofacial Surgery, Erlangen University Hospital, Erlangen, Germany;

²Department of Oral and Maxillofacial Surgery, University of Athens, Athens, Greece;

³Institute of Pathology, Erlangen University Hospital, Erlangen, Germany

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Abstract. microRNAs (miRNAs) are aberrantly expressed in the whole blood of patients suffering from different types of cancer. Collection of whole blood samples is a minimally invasive procedure. To date, little is known concerning the altered miRNA expression in patients suffering from oral squamous cell carcinoma (OSCC). The present study aimed to evaluate the difference in miRNA expression in whole blood samples in OSCC patients as compared to healthy volunteers who served as controls. In 20 blood samples from patients and healthy volunteers, the expression patterns of 1,205 human miRNAs were examined by miRNA microarray in order to identify those with the most pronounced differential expression. The results were verified by quantitative RT-PCR (RT-qPCR) for miR-186, miR-3651 and miR-494 using 57 samples of patients and 33 samples of healthy volunteers. Receiver operating characteristic (ROC) curves and the highest Youden index were calculated in order to assess cut-off points (COPs) that allowed the distinguishing of blood samples of OSCC patients from those of healthy volunteers. Significantly different expression rates were found for miR-186 ($p=0.01$), miR-3651 ($p=0.0001$) and miR-494 ($p=0.004$) between the OSCC patients and healthy controls. In the OSCC patients, there was a 2-fold upregulation for miR-494 and miR-3651 and a 2-fold downregulation for miR-186. Based on the determined COPs, significant correlations between miR-3651 overexpression and lymph node status ($p=0.04$), tumor grade ($p=0.02$) and clinical stage ($p=0.04$) were indicated. Aberrant expression levels of miR-186, miR-494 and miR-3651 in whole blood samples of OSCC patients may provide the possibility to establish a minimally invasive screening method for OSCC.

Introduction

microRNAs (miRNAs) are ~22 nt long, non-coding, single-stranded RNAs that regulate gene expression. They are essential regulators of cellular processes such as proliferation, apoptosis, differentiation and motility (1). miRNAs are aberrantly expressed in a number of different tumor tissues including oral squamous cell carcinoma (OSCC) (2-7). Alterations in miRNA expression levels have been shown to be associated with tumor progression, recurrence, development of metastases and chemoresistance (2,8-11).

miRNAs circulate in blood and are stable in this environment. Studies regarding different tumor entities have shown altered miRNA expression patterns in the blood of affected patients when compared to healthy volunteers (12-16).

These differential miRNA expression profiles in blood have also been observed when head and neck cancer patients were compared to healthy controls (17-21). Unfortunately, head and neck squamous cell carcinoma (HNSCC) comprises a heterogeneous group of tumors that are located in the oral cavity, the nasal cavity, nasopharynx, pharynx and larynx. Based on tumor localization, the biology of SCC can vary significantly. Consequently, expression patterns of miRNAs also differ in relation to tumor region (7). To date, only a few studies on a small number of circulating miRNAs have been performed exclusively in OSCC patients (17-20,22,23). Hence, the first step in identifying more miRNA candidates is expression analysis by miRNA microarrays which allows investigation of a large number of different miRNAs simultaneously. A further shortcoming of previous studies is that the selection of analyzed miRNAs was based on miRNA expression in cancer tissue samples. However, it is well known that miRNAs that exhibit altered expression levels in the blood of cancer patients are not necessarily identical to miRNAs that are differentially expressed in cancer tissue samples (17,18,23,24). Furthermore, only one of the blood components, either plasma or serum, was obtained for the investigations, although miRNA profiling based on whole blood appears to be the most reliable (25).

The present study aimed to evaluate the difference in miRNA expression patterns in whole blood samples of OSCC patients compared to healthy volunteers who served as controls in order to evaluate the usefulness of these biomarkers for

Correspondence to: Dr Jutta Ries, Department of Oral and Maxillofacial Surgery, Erlangen University Hospital, Glueckstrasse 11, D-91054 Erlangen, Germany
E-mail: jutta.ries@uk-erlangen.de

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detection of OSCC using a minimally invasive method based on whole blood samples.

Materials and methods

Patients and sample collection. The present study was approved by the Ethics Committee of the University of Erlangen-Nuremberg, Erlangen, Germany. Whole blood samples of 57 OSCC patients (test group) and 33 healthy volunteers (control group) were collected. Patient informed consent was obtained. Patients were included in the present study if OSCC occurred for the first time and whole blood samples were able to be collected before surgical removal of the tumor or radiotherapy and/or chemotherapy. Healthy volunteers were selected based on the absence of general disease and acute or chronic inflammation.

After the collection of whole blood, tissue samples of the OSCC (test group) and normal oral mucosa (control group) were harvested. All tissue samples were examined by two experienced pathologists.

Age and gender of the patients were compiled. Grading (G1-G3), staging (I-IV) and TNM classification of the OSCC cases were carried out according to the guidelines of the World Health Organization and the International Union Against Cancer (26). Subsequently, tumors were grouped as early (including stage I and II) and late (including stage III and IV) clinical stages and as N=0 and N>0 in order to indicate cases with negative and positive lymph node status, respectively. Furthermore, subgroups were established based on tumor size dividing the samples into small (T1/T2) and large (T3/T4) malignancies.

Sampling of whole blood and miRNA isolation. Two samples consisting of 2.5 ml of whole blood of the OSCC patients and healthy volunteers were collected in a PAXgene Blood RNA Tube (PreAnalytiX GmbH, Hombrechtikon, Switzerland) before tumor removal. The samples were carefully inverted, incubated at room temperature for 2 h and stored at -80°C until miRNA isolation.

Whole RNA was extracted using the PAXgene Blood miRNA Kit (PreAnalytiX GmbH). RNA concentration was measured with a NanoDrop spectrometer (PEQLAB, Erlangen, Germany). The integrity and size distribution of total RNA were checked using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) and the RNA 6000 Nano kit (Agilent Technologies, Waldbronn, Germany). Subsequently, the RNA samples were stored at -80°C.

Gene expression analysis by miRNA microarray. Twenty blood samples of the test and the control group were analyzed. miRNA microarrays were performed on the GeniomH real-time analyzer (GRTA; Fehit GmbH, Heidelberg, Germany) using Agilent's SurePrint G3 human v16 miRNA Array Kit, 8x60K (release 16.0) microarrays (Agilent Technologies, Inc.) which were updated from the Sanger miRBase 16.0. The miRNA microarrays included 1,205 human and 144 human viral miRNAs. The study was focused on the miRNA expression differences between whole blood samples of the patients compared to those of the normal controls. By comparison of the normalized and background subtracted median intensity

values which were received after microarray analyses using the Geniom Wizard Software, miRNAs showing a different behavior between the two groups were identified. To this end, the following different statistical measures were applied: parametric t-test (unpaired, two-tailed), Wilcoxon Mann-Whitney test (WMW, unpaired, two-tailed), a linear model with p-values computed by an empirical Bayes approach (Limma), the area under the receiver operator characteristic curve (AUC) and determination of the fold-change quotients. All p-values were adjusted for multiple testing by Benjamini-Hochberg (27) adjustment.

Expression signals showing at least a 2-fold difference in abundance between the tumor and normal samples and p-values of Limma <0.05 were identified as differentially regulated. AUC values can range from 0 to 1. A value of 0.5 indicated equal distribution among the healthy and diseased subjects and indicated that the intensity values generated by RNA from the blood of patients and healthy subjects could not be used to make a distinction between the groups. An AUC value >0.5 indicated higher expression intensities of the respective miRNA in OSCC samples (upregulated miRNA) whereas an AUC <0.5 indicated higher expression values of the miRNA in the controls (downregulated miRNA). An AUC of 1 and 0 corresponded to a perfect separation.

Real-time quantitative reverse transcription-PCR (RT-qPCR) analysis. miR-186, miR-494 and miR-3651 which were identified to be differentially expressed by miRNA microarray analysis between the groups were analyzed in the test group (n=57) as well as in the control group (n=33) by RT-qPCR. These analyses were conducted using 500 ng of total RNA. In the first step, miRNA was reverse transcribed using the miScript II RT Kit according to the manufacturer's recommendations (Qiagen, Hilden, Germany). Detection of amplification was performed on 2.5 ng of cDNA on an ABI 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using the miScript SYBR-Green PCR Kit and miRNA-specific quantitative RT-PCR primer sets for the miRNA of interest (Qiagen). The features of the miRNAs are summarized in Table I.

Normalization and relative quantification of whole blood miRNA expression. The values of RT-qPCR analyses were normalized by the ΔCT method. For that purpose the primer sets RNU6-2 (U6 snRNA, RNA U6 small nuclear 2) and SNORD44 (small nucleolar RNA, C/D box 44), which are known to be stably expressed in whole blood across normal and cancer patients, were taken as internal controls (Qiagen) (Table I). The mean value of both controls was applied as the normalization value. Relative quantification of differences in expression ($RQ = 2^{-\Delta\Delta CT}$) between the two groups was carried out by the $\Delta\Delta CT$ method using Microsoft Excel® 2003 for Windows (Microsoft Corporation, Redmond, WA, USA).

Statistical analyses of RT-qPCR. For statistical evaluation of the RT-qPCR analysis, the program IBM® SPSS Statistics 19 (Chicago, IL, USA) was applied. In addition, relative expression (RQ) of the examined genes between the two groups was determined by the $\Delta\Delta CT$ method taking into account the mean

Table I. List of miRNAs, snRNAs (endogenous controls) and the miScript Primer assay (Qiagen) used in the RT-qPCR analyses.

Sanger ID	Sanger accession mature miRNA	Sequence	Ref. no. (Qiagen)
miRNAs			
hsa-miR-186-5p	MIMAT0000456	CAAAGAAUUCUCCUUUUGGGCU	MS 00008883
hsa-miR-3651	MIMAT0018071	CAUAGCCCGGUCGCGUGGUACAUGA	MS 00023121
hsa-miR-494-5p	MIMAT0026607	AGGUUGUCCGUGUUGUCUUCUC	MSC 0002535
Ref. seq.			
Endogenous controls			
RNU6-2	NR_002752		MS 00033740
SNORD44	NR_002750		MS 00007518

Table II. Demographic characteristics of the test group (OSCC patients) and the control group (healthy volunteers).

Characteristic	Test group	Control group	Total
No. of cases	57	33	90
Mean age \pm SD (years)	64.4 \pm 11.01	60.5 \pm 20.68	60.5 \pm 15.29
Age range (years)	35-93	15-88	15-93
Gender n (%)			
Male	39 (68.4)	23 (69.7)	62 (68.9)
Female	18 (31.6)	10 (30.3)	28 (31.1)

OSCC, oral squamous cell carcinoma; SD, standard deviation.

values of all Δ CT within a group. Two-fold changes in miRNA expression rates ($2 \leq RQ \leq 0.5$) between the two groups were defined as statistically relevant.

The mean value of duplicate Δ CT values of each sample was used for the data results. Expression data were controlled for normal distribution by Shapiro-Wilk test. Data derived from RT-qPCR and presented as Δ CT values were expressed as the median (ME), the interquartile range (IQR), standard deviation (SD) and range. Graphical diagrams are plotted as Box-Whisker plots which represent the median, the interquartile range, and minimum and maximum values of determined miRNA expression. Statistical relevance of the apparent expression between the two groups was analyzed by Mann-Whitney U test. A p-value ≤ 0.05 was considered to indicate a statistically significant result.

Furthermore the expression profile of each differentially expressed miRNA was used for creation of receiver operator characteristic (ROC) curves. This method displays the discriminatory accuracy of the marker for distinguishing between two groups. It is a plot of the sensitivity (true-positive rate) vs. 1-specificity (false-positive rate) over all possible threshold values of the marker. The area under the ROC curve (AUC) value of miRNAs defines the usefulness of an miRNA with respect to its ability to separate the two different groups of blood donors.

Additionally, by using the ROC curve, the highest Youdan index was calculated. This value is associated with the critical expression point or the optimal threshold value, respectively, cut-off point (COP) for the biological marker. The COP indicates which value of increased or decreased expression is relevant for the discrimination between malignant and normal samples and allows assigning a particular sample to a certain group (28).

Based on these COPs, the two groups were divided into two subgroups which showed an expression rate over or under the COP. Afterwards, associations between altered miRNA expression and malignancy, clinical features and histopathological parameters were calculated by the Chi-square test.

Results

Clinical and histopathological parameters of the study participants. Whole blood samples of 57 OSCC patients (test group) and 33 healthy volunteers (control group) were collected. Demographic characteristics of all participants are documented in Table II. Histopathological parameters of all patients with OSCC are shown in Table III.

Gene expression analysis by miRNA microarrays. Expression patterns of 1,205 miRNAs were determined by miRNA microarray in the test and the control group. In the hierarchical cluster analysis, clusters of upregulated and downregulated miRNAs in the blood of OSCC patients vs. healthy volunteers were recognized which clearly separated the two groups (data not shown). By determination of fold changes and statistical evaluation by t-tests and empirical Bayes approaches (Limma) followed by Benjamini-Hochberg adjustment, the 30 most prominently deregulated miRNAs were identified. Out of these, three candidates were chosen for further analyses. As shown in Table IV, miR-186 was downregulated ~2-fold in the test group, whereas the level of miR-494 was 4.7-fold and miR-3651 was 2.5-fold higher in the blood of OSCC patients. The Limma p-values revealed that these changes were statistically significant. The usefulness of the miRNAs with respect to their ability to separate the two different groups of blood donors was also demonstrated by the AUC values lying ~0.8 and under 0.2 (miR-186), respectively.

Table III. Association between the miRNA expression rates and the clinical and histopathological parameters of all OSCC patients.

Variables	No. of cases	Total no. (%) of positive cases for each miRNA		
		hsa-miR-186	hsa-miR-3651	hsa-miR-494
Total no.	57	35 (61.4)	48 (84.2)	32 (56.1)
Tumor size (T)				
1	17	13 (76.5)	14 (82.4)	10 (58.8)
2	18	10 (55.6)	14 (77.8)	9 (50.0)
3	6	3 (50)	6 (100)	4 (66.7)
4	16	9 (56.3)	14 (87.5)	9 (56.3)
T1/T2	35	23 (65.7)	28 (80.0)	19 (54.3)
T3/T4	22	12 (54.5)	20 (90.9)	13 (59.1)
Lymph node status				
N0	33	20 (60.6)	25 (75.8)	18 (54.5)
N ⁺	24	15 (62.5)	23 (95.8)	14 (58.3)
			p=0.04^a	
Tumor grade				
G1	8	4 (50.0)	4 (50.0)	5 (62.5)
G2	36	24 (66.7)	32 (88.9)	19 (52.8)
G3	13	7 (53.8)	12 (92.3)	8 (61.5)
			p=0.016^a	
Clinical stage				
Early (I+II)	26	17 (65.4)	19 (73.1)	15 (57.7)
Late (II+IV)	31	18 (58.1)	29 (93.5)	17 (54.8)
			p=0.035^a	

Based on their Δ CT value, the cases were defined as positive and negative. Only significant p-values are indicated. ^aStatistical analysis was carried out by the Chi-square test. miRNA, microRNA; OSCC, oral squamous cell carcinoma.

Table IV. Aberrant expression of the examined miRNAs as determined by miRNA microarray analysis.

miRNA	Median C	Median T	FC	Limma raw p	Limma adjusted p	AUC	Regulation
hsa-miR-186	8.98	8.17	0.45	0.000150	0.012	0.18	Down
hsa-miR-3651	4.60	5.53	2.53	0.000262	0.02	0.80	Up
hsa-miR-494	4.99	6.53	4.66	0.000379	0.02	0.82	Up

miRNA, microRNA; FC, fold-change, tumor vs. normal samples. The statistical significance was determined by an empirical Bayes approach (Limma raw p) and adjusted for multiple testing by Benjamini-Hochberg adjustment (Limma adjusted p). AUC, area under the curve.

RT-qPCR screening for miRNA expression differences. Data were derived from RT-qPCR and are presented as Δ CT values. The results are graphically plotted as Box-Whisker plots which represent the median (ME), the interquartile range (IQR), standard deviation (SD) and range as well as the minimum and maximum Δ CT values (Fig. 1). Using Mann-Whitney U test, a significantly differential expression was determined between the test group and the control group for miR-186, miR-3651 and miR-494 with higher Δ CT values standing for lower miRNA expression. miR-186 was significantly reduced in the patient group. It was downregulated 2-fold and the p-value for

differential expression was 0.01. The expression of the two other miRNAs was significantly increased. The fold-change for miR-3651 was 2-fold whereas this value amounted to 2.3 for miR-494. The p-values were 0.0001 and 0.004, respectively (Fig. 1, Table V).

In order to confirm the statistical relevance of the markers, a ROC curve was established and AUC was determined. All markers yielded a significant AUC value. The upregulated miRNAs yielded an AUC of 0.82 and 0.72, respectively. The AUC value of the downregulated miR-186 was 0.69. Thus, this analysis confirmed that all three miRNAs were of significant

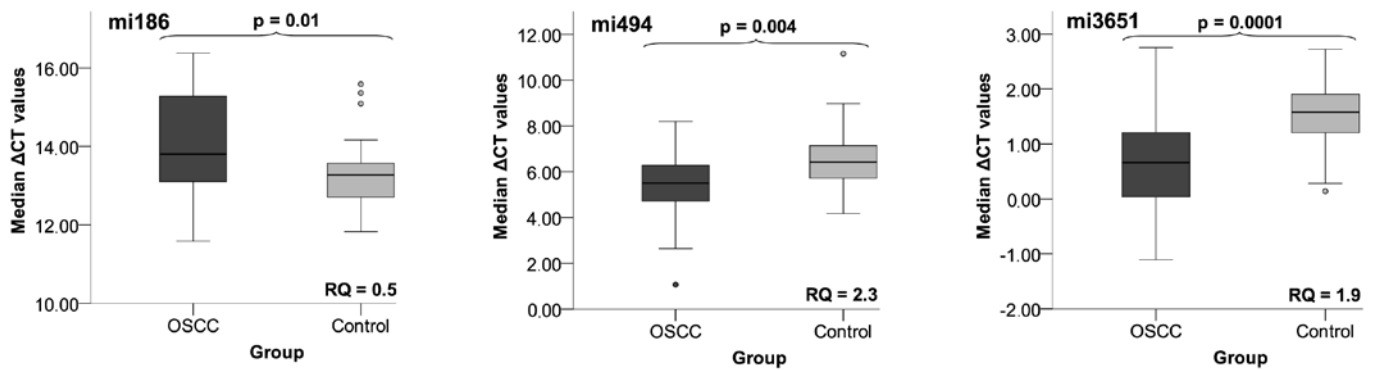


Figure 1. Box-Whisker plots of the median expression rates of the miRNAs in whole blood of patients and volunteers derived from RT-qPCR and expressed as median Δ CT values. Higher Δ CT values stand for lower miRNA expression. The median, the interquartile range and the standard deviation are provided. Fold-change of miRNA expression (RQ) was determined by the $\Delta\Delta$ CT method comparing the average Δ CT values of the two groups. The expression was statistically relevant in the OSCC patients as compared to the healthy volunteers. Statistical analysis was carried out by the Mann-Whitney U test. miRNA, microRNA; RT-qPCR, quantitative RT-PCR.

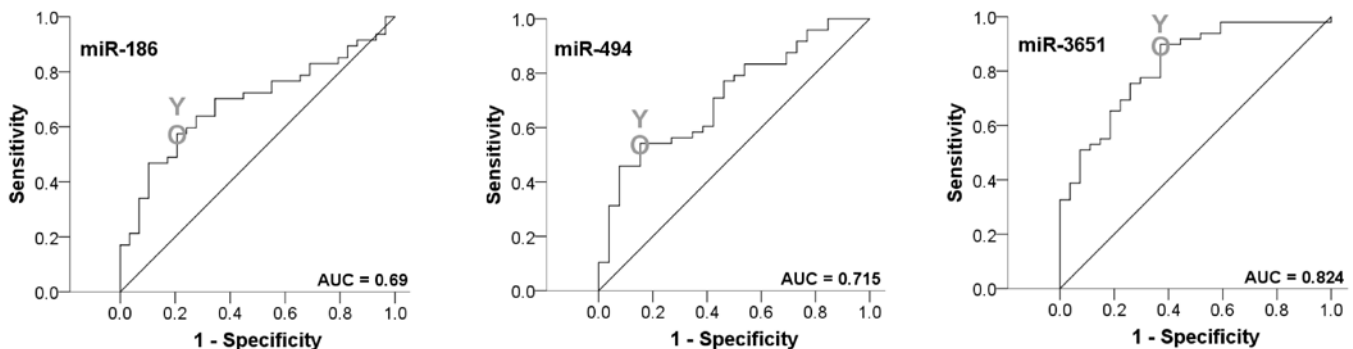


Figure 2. ROC curve for miRNAs based on the RT-qPCR data. The diagram is a plot of the sensitivity (true-positive rate) vs. 1-specificity (false-positive rate) over all possible Δ CT values. The AUC values indicate that the two groups may be distinguished by expression analysis of the markers. The circle shows the point of the highest Youden (Y) indices which are associated with the COP. The determined values of AUC, Y and COP for the examined miRNAs are listed in Table V. ROC, receiver operating characteristic; miRNA, microRNA; RT-qPCR, quantitative RT-PCR; AUC, area under the curve; COP, cut-off point.

Table V. Statistical results of the comparison between the patient vs. the control group for the investigated miRNAs based on their Δ CT values.

miRNA	FC	AUC	Y-index	COP (Δ CT)	P-value ^a
hsa-miR-186-5p	-2.1	0.69	0.368	13.64	0.01
hsa-miR-3651	1.95	0.824	0.528	1.479	0.0001
hsa-miR-494-5p	2.3	0.715	0.388	5.608	0.004

miRNA, microRNA; FC, fold-change tumor (test group) vs. normal samples (control group). AUC, area under the curve; Y, Youden; COP, cut-off point expressed as the Δ CT value. ^aStatistical association was determined by the Mann-Whitney U test.

diagnostic value for discrimination between healthy volunteers and OSCC patients (Fig. 2, Table V).

The highest Youden indices were 0.388 for miR-494, 0.528 for miR-3651 and 0.368 for miR-186 (Fig. 2, Table V). The optimal threshold values (COPs) expressed in Δ CT standards for distinguishing the patients from the healthy

control were 5.61 for miR-494, 1.48 for miR-3651 and 13.64 for miR-186. For the miRNAs miR-494 and miR-3651, a Δ CT under the COP (upregulated) was considered to be positive for malignancy corresponding to an increased level of the marker in whole blood. For miR-186, a Δ CT value over the COP was positive for the OSCC test group (downregulated). Using the determined COPs, the two groups were divided into positive and negative lesions in order to confirm whether the parameters allowed the detection of malignancy in a certain sample.

The statistical evaluation by the Chi-square test revealed that the changes in the expression rates of the miRNAs were statistically relevantly associated with malignancy. The results are illustrated in Fig. 3 and summarized in Table VI. Of the OSCC patients, 61.4% (35/57) exhibited decreased levels of miR-186; 84.2% (48/57) and 56.1% (32/57) showed increased values of miR-3651 and miR-494, respectively. In contrast, only 24.2% of the whole blood samples from the control group showed decreased levels of miR-186. Positive levels for miR-3651 and miR-494 were found in the blood of healthy persons in 33.3% and 18.2% of the samples, respectively. The correlation between malignancy and the detection of altered expression rates were significant for all investigated miRNAs ($p < 0.01$). Thus, aberrant expression of all miRNAs was

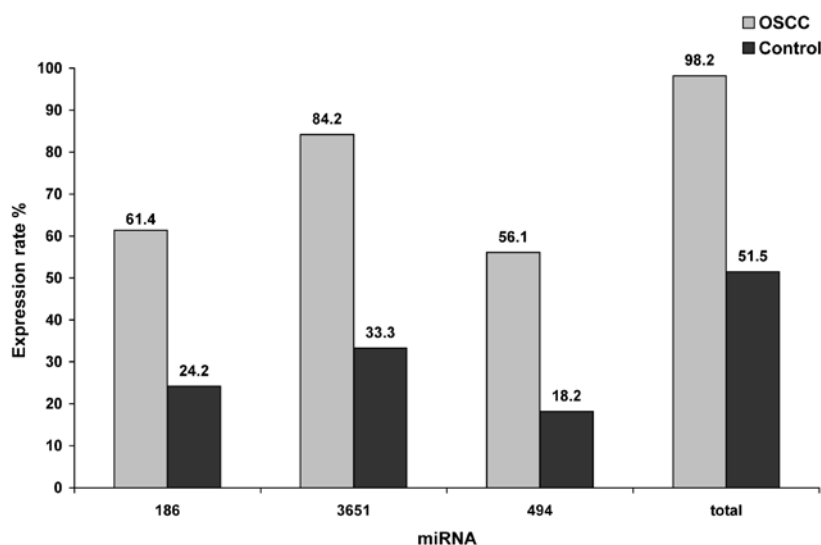


Figure 3. Division of the test and control group into positive and negative subgroups based on the ascertained COPs of single miRNAs expressed as Δ CT values. The specimens were positively (malignant) judged if the values lied below (for miR-494, miR-3651) or above (miR-186) the COP. Altered expression levels in the blood samples between OSCC patients and healthy volunteers appeared to be significant. Therefore, the COP may be a parameter allowing the allocation of a sample to a group and the proof of malignancy. COPs, cut-off points; OSCC, oral squamous cell carcinoma.

Table VI. Association between apparent expression rates of the particular miRNAs and malignancy.

	Test group (n=57)	Control group (n=33)	P-value ^a
Sanger ID of miRNA	Positive cases, n (%)	Positive cases, n (%)	
hsa-miR-186-5p	35 (61.4)	8 (24.2)	0.001
hsa-miR-3651	48 (84.2)	11 (33.3)	0.0001
hsa-miR-494-5p	32 (56.1)	6 (18.2)	0.001
At least one	56 (98.2)	17 (51.5)	0.0001

Positivity of the tumor tissue of particular patients was ascertained using the determined COP of each respective miRNA. Test group, OSCC patients; control group, healthy volunteers. ^aStatistical relevance of the association was determined by the Chi-square test. miRNA, microRNA; COP, cut-off point; OSCC, oral squamous cell carcinoma.

statistically relevantly associated with malignancy and may indicate the existence of OSCC. Moreover 98.2% of the blood samples of patients suffering from OSCC exhibited altered expression of at least one of the examined miRNAs whereas only in 51.5% of the healthy volunteers such an altered abundance was evident. The association to malignancy was statistically relevant ($p=0.0001$, Table VI).

Statistical analysis of the comparison between altered miRNA values in whole blood of OSCC patients and clinical and histopathological parameters revealed that there was no statistically relevant association between the altered expression of any miRNA to tumor size. In addition, subdivision of the tumors into T1/T2 and T3/T4 categories of size yielded no statistically significant association between this parameter

and altered expression ($p>0.05$). Moreover, no significance was shown between the changes in expression rate of miR-186 and miR-494 and lymph node status, tumor grade and clinical stage. Only evaluated levels of miR-3651 were weakly statistically related to tumor grade ($p=0.043$), lymph node status ($p=0.035$) and clinical stage ($p=0.016$). The results of statistical assessment are summarized in Table III.

Discussion

miRNAs influence a number of cellular pathways and are responsible for malignant transformation and tumor progression of different cancer entities. A number of these pathways may also be important for the development and progression of oral squamous cell carcinoma (OSCC) (2,8,11,29). Since the discovery of stable miRNAs in whole blood, serum and plasma and their aberrant expression in tumor patients vs. healthy individuals, circulating miRNAs have been shown to be valuable biomarkers for diagnostic investigation and even for cancer management and direct monitoring of disease using a minimally invasive method (5,12,13,16,21). The present study aimed to evaluate the difference in miRNA expression in whole blood samples of OSCC patients compared to healthy volunteers who served as controls.

We demonstrated that the expression patterns of miR-186, miR-3651 and miR-494 were significantly altered in the whole blood of patients suffering from OSCC when compared to these patterns in healthy volunteers. Upregulation of miR-3651 and miR-494 and the downregulation of miR-186 were significantly correlated with the absence and presence of OSCC. It appears that these three miRNAs are relevant biomarkers for discrimination of OSCC patients from healthy individuals and may be useful for the establishment of a minimally invasive blood-based diagnostic method.

The relevance of miRNAs for the evaluation of prognosis of OSCC seems to be less pronounced. The changes in the expression of miR-494 and miR-186 were statistically

independent of the clinical stage of disease and TNM classification. Therefore, detection of these miRNAs in whole blood may only be an additional method for the identification of OSCC lesions. However, it is postulated that miR-186 and miR-494 are conserved across species and they have been reported to be disease-associated. Thus, miR-186 appears to be involved in inhibition of invasion when overexpressed in cancer lesions and has been shown to be an anti-invasion target for therapeutic development for non-small cell lung cancer (30). Furthermore, downregulation of miR-186 in esophageal cancer and lung adenocarcinoma was associated with poor prognosis independent of TNM stage by interference of the miRNA with cell cycle regulation (31,32). Moreover, miR-186 may induce cellular senescence and regulate the cell cycle and apoptotic response (33,34). Hence, on the basis of the identified functions, one may conclude that the altered expression of miR-186 may play a critical role in oral carcinogenesis and progression. However, all of the reported results are based on expression analyses in tissue samples, and the expression patterns of miRNA may not be reflected in blood (17,18,23,24). miR-494 has also been reported to be a tumor suppressor by inducing cell cycle arrest, cell senescence and apoptosis, and by suppressing cell proliferation. Moreover, miR-494 is downregulated in different types of cancer tissues (35-37). However, we found that this miRNA was increased in the blood of patients. This seems to be contradictory. However, the result may be due to selective exosome-mediated release of the miRNA into the extracellular environment. The exclusion of this miRNA from the cellular matrix leads to loss of its tumor-suppressive function and consequently to cancer cell phenotypes (19). On the other hand, increased levels of the human-specific miR-3651 showed a weak correlation with lymph node metastasis and clinical stage and strong association with more dedifferentiated tumors. Thus, this miRNA may be useful in prognostic applications. Yet, further studies including a larger number of patients and controls are urgently needed to confirm this hypothesis. Additionally, its overexpression may be involved in dedifferentiation and development of metastases. However, its function and its involvement in cancer development and progression have not been investigated to date. Thus the impact of this molecule in OSCC must be elucidated in the future.

Previously, a number of differentially expressed miRNAs in the blood of patients suffering from head and neck carcinomas including OSCC have been identified as potential blood biomarkers (19). Circulating miRNAs, miR-184, miR-31 and miR-24, have been intensely studied, and their power in the clinical monitoring of disease is suggested (17,18,20). Unfortunately, we did not find any deregulation of these miRNAs in our study. This may be due to several reasons. Firstly, in the previous studies, only a small number of patients were included. Secondly, only a small collection of circulating miRNAs which were preselected by their deregulation in cancer tissues was evaluated. However, miRNAs showing disease-associated expression changes in blood are not necessarily the same ones that are differentially expressed in cancer tissues. Consequently, aberrant expression may not be correlated (24). Additionally the differential miRNA expression was evaluated either in plasma or serum of OSCC patients and not in whole blood. However, differential miRNA expression was recently reported in patient-matched serum and plasma samples.

Furthermore, significant differences between cell-free and cellular blood miRNA profiles were shown (15,38). At present, isolation of miRNAs and miRNA-profiling based on whole blood appear to be the most reliable (25). Using this method, the major advantage appears to be the higher miRNA content and the increased chance for detection of the disease since not only tumor-secreted oncogenic miRNAs are measured, but also changes in the miRNA profile as a consequence of 'host-reaction' based upon the reaction of the immune system in response to cancer are determined (14,25,39). Therefore, the whole blood approach offers the potential to diagnose cancer at a very early stage when the concentration of tumor-secreted miRNAs is still low. Nevertheless, reports using serum or plasma for RT-qPCR-based miRNA profiling are also promising for OSCC. Thus, evaluated postsurgical concentrations of different miRNAs in plasma samples of OSCC patients have been associated with recurrence and can predict worse clinical outcome (17,18,20,22). Recently, one study showed that the markers may also be applied to risk assessment of precancerous lesions (19). Additionally, recent studies using various tumors may encourage further research on evaluation of blood miRNAs as biomarkers for OSCC management (40-42). Consequently, although the research of circulating miRNAs as blood-based biomarkers for OSCC is still in the beginning, this approach may allow the establishment of a minimally invasive, sensitive and specific method for OSCC detection, screening and monitoring. However, due to the relatively small number of samples used in this study, further validation using a larger cohort is needed to fully assess the utility of particular miRNAs as oral cancer biomarkers. Additionally, in the future their impact in clinical monitoring and prognosis must be evaluated by follow-up studies as described for other malignancies.

Although miRNA-based therapeutics have not yet reached clinical trials for cancer, due to their function as oncogenes and tumor-suppressor genes and their involvement in many cellular processes, miRNAs may be a valuable novel emerging class of targets for disease gene therapy as novel therapeutic targets. Furthermore, promising results in animal models of related diseases have shown their usefulness as potential therapeutic drugs for personalized treatment strategies (43-45).

In conclusion, the aberrant expression of miR-186, miR-494 and miR-3651 in the whole blood of OSCC patients identified in the present study may serve as the basis for establishing these miRNAs as minimally invasive biomarkers for the detection and monitoring of OSCC.

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