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MicroRNA expression correlates with disease recurrence and overall survival in oral squamous cell carcinoma

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ABSTRACT

Objectives: Locoregional disease recurrence and metastatic events are the leading causes of death and the most important prognostic factors in patients with head and neck squamous cell carcinoma (HNSCC). A major goal of oncology is the identification of clinical and molecular parameters to evaluate the individual risk of recurrence. MicroRNAs (miRNAs) have been shown to correlate well with tumor size and differentiation. Therefore, they are candidate biomarkers for estimating clinical outcomes.
Materials and methods: In this study, the expression levels of distinct miRNAs extracted from formalin-fixed, paraffin-embedded (FFPE) samples of oral squamous cell carcinoma were compared.
Results: Statistical analysis revealed significant correlations between distinct miRNAs and disease recurrence (miR-99*, miR-194*; $p < 0.05$) and overall survival (miR-99*; $p < 0.05$). The results were then validated via data from The Cancer Genome Atlas (TCGA).
Conclusions: Our data show that miR-99* and miR-194* can possibly serve as biomarkers for clinical outcome in HNSCC. These findings may help to identify high-risk patients, who could profit from a more individualized treatment and follow-up.

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is one of the six most common malignancies worldwide, with SCC of the oral cavity (OSCC) being a prevalent example. Despite developments in diagnosis and treatment via surgery and radio-chemotherapy, patient survival has not changed significantly in recent times (Chan et al., 2002; Siegel et al., 2013; Friemel et al., 2016; McGuire, 2016). Local recurrence and development of metastases remain the leading causes of death for patients suffering from OSCC. The extent of the primary tumor, the existence of distant metastases, and local neck node status are the most important prognostic factors regarding long-term outcome (Snow et al., 1982; Leemans et al., 1994; Grimm, 2012; D'Cruz et al., 2015).

Recent decades have seen valuable insights into altered cellular signaling and potential therapeutic targets. There have been great advances in the identification of oncogenes and important signaling pathways, such as the epidermal growth factor receptor (EGFR) and PI3K-AKT-mTOR (Lui et al., 2013; Freudlsperger et al., 2015; Horn et al., 2015). Targeted therapy via combinations of classical chemotherapeutic agents (e.g. platin with tyrosine kinase inhibitors or monoclonal antibodies) has been shown to increase the therapeutic response of carcinoma cell lines in several studies (Hartmann et al., 2015; Brands et al., 2016). Additional insights into the molecular landscape are therefore necessary to ensure optimal long-term success.

miRNAs are small (19–25 nucleotides), non-protein-coding RNA molecules, which have been studied since their discovery in 1993

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(Lee et al., 1993). General mechanisms of miRNA biosynthesis, post-transcriptional processing, and gene regulation via post-translational silencing have been explored *in vitro* in various studies (Ambros, 2004; Chekulaeva and Filipowicz, 2009). Their main function is post-translational gene modification via different base-pairing mechanisms (Bartel, 2009); nevertheless, their role in tumor formation and progression remains unclear and partly contradictory, because gene regulation does not work in a monocausal manner. Furthermore, the numerous interactions between different miRNAs make analysis more complicated (Lee et al., 2003, 2004; Guo et al., 2012; Wu et al., 2013). miRNAs are estimated to regulate up to 40% of the human genome, and they been shown to influence therapy response and outcome in different types of cancer (White et al., 2011; Kong et al., 2014; Nawaz et al., 2016). In the field of HNSCC, there is contradictory literature regarding the relationship between miRNA expression and disease progression or clinical outcome. While some studies have not been able to find any correlations between expression levels and outcome, Ries et al. extracted different miRNAs from whole blood samples of patients with oral squamous cell carcinoma and were able to define cut-off points to determine disease recurrence (Ries et al., 2014, 2017; Lamperska et al., 2016).

Recently, we have been able to show that miRNAs can be extracted and analyzed from formalin-fixed, paraffin-embedded (FFPE) samples of oral squamous cell carcinoma after storage times of up to 14 years. Several miRNAs displayed different expression levels in cancer and in the surrounding, non-tumorous tissue of the same patient. We have also managed to find significant correlations between specific miRNAs and clinical and pathological data, such as T-stage and tumor differentiation (Moratin et al., 2016).

The purpose of this study was to correlate specific miRNA expressions and clinical outcome in a cohort of 43 patients, in terms of disease recurrence and overall survival, as well as to identify markers for risk stratification. Additionally, the observed results were validated using data from the *Cancer Genome Atlas*.

2. Material and methods

Thirty miRNAs were selected for further investigation using Affymetrix Genechip Arrays® (Affymetrix, Inc., Santa Clara, CA, USA), which were performed for five tumor cell lines and two non-tumorous tissue cell lines (Table 1). The miRNAs that demonstrated the most distinct expression differences between the different cell lines were selected for further investigation (Moratin et al., 2016).

2.1. Tissue samples

Formalin-fixed, paraffin-embedded (FFPE) samples from 43 patients with oral squamous cell carcinoma (OSCC), who were treated surgically in Würzburg between 2000 and 2003, were used for further analysis. The corresponding hematoxylin- and eosin-stained slide of each sample was reviewed by a member of the Institute of Pathology at the University Hospital of Würzburg, and thresholds for tumor and adjacent control tissue were marked to guide microdissection. Afterwards, the samples were cut into 10-μm-thick sections using a microtome (CUT4060, Weinkauf, Medizin und Umwelttechnik, Forchheim, Germany). In this way, 86 tissue samples were produced for further RNA isolation and extraction procedures.

2.2. RNA isolation

RNA isolation and processing were performed using a modification of the procedure proposed by Xi et al (Xi et al., 2007; Moratin et al., 2016). The tissue samples were placed in a 1.5-mL nuclease-free microcentrifuge tube. 1 mL of xylene was added to the tubes for deparaffinization, which was followed by vortexing for 5 minutes at room temperature. The samples were then stored at 60 °C for 3 minutes before they were centrifuged at 14,000 rpm for 7 minutes. Supernatants were then removed, 1 mL of ethanol (96%) was added for washing, and the tubes were then centrifuged at 14,000 rpm for another 7 minutes at room temperature, followed by the removal of the supernatants. The washing procedure was repeated twice. Eventually, the samples were air-dried, and 360 μL of digestion buffer (30 mM Tris-HCL, 20 mM EDTA, 1% SDS) and 40 mL of Proteinase K® (Qiagen N.V., Venlo, Netherlands) were added. This solution was then stored at 56 °C for 3 hours. Afterwards, 800 μL of TRIzol LS® (Life Technologies, Carlsbad, California) was added, followed by vortexing for 5 minutes. 100 microliters of 1-bromo-3-chloropropane (BCP) was added to the solution for phase separation, followed by vortexing for 2 minutes. Next, the samples were stored at room temperature for 3 minutes and centrifuged at 14,000 rpm at 4 °C for 7 minutes. Then, the upper phase was extracted and transferred to a new tube. An equal volume of isopropanol was added to each RNA solution. After mixing, the solution was stored at −20 °C for 1 hour. The tubes were then centrifuged at 14,000 rpm for 7 minutes at 4 °C, followed by the removal of the supernatants and another washing procedure using ethanol (96%). After another centrifugation at 14,000 rpm for 15 minutes at 4 °C, the supernatants were removed, and the pellets were left to air-dry for 5 minutes. Then, 20 microliters of nuclease-free water was added for storage. After the extraction procedure, spectrophotometric measurement was performed to evaluate the concentration and purity of the isolated RNA (Nanodrop ND-1000, Nanodrop Technologies Inc., Wilmington, DE, USA).

Table 1
List of the 30 miRNAs used for the analysis.

Primer No.	miRNA	Primer No.	miRNA
1	miR-589	16	miR-3654
2	miR-194*	17	miR-4458
3	miR-194	18	miR-3156
4	miR-184	19	miR-4449
5	miR-224	20	miR-4786-5p
6	miR-193a-5p	21	miR-663b
7	miR-125b-2*	22	miR-210
8	miR-146a	23	miR-3176
9	miR-105*	24	miR-212
10	miR-105	25	miR-3687
11	miR-767-5p	26	miR-342
12	miR-141	27	miR-99*
13	miR-200a	28	miR-224*
14	miR-200b*	29	miR-34c
15	miR-200c	30	mirR205*

2.3. miRNA — reverse transcription and semi-quantitative, real-time PCR

For relative quantification, complementary DNA (cDNA) was synthesized from the isolated miRNAs using a miScript II RT Kit® (Qiagen, Venlo, Netherlands), according to the manufacturer's protocol. The process included adding a HiFlex-Buffer, a miScript Nucleic Acids Mix, a miScript Reverse Transcription Mix, and nuclease-free water to the RNA template. After incubation at 37 °C for 1 hour, the solution was deactivated at 95 °C for 5 minutes. After synthesis, the cDNA was stored at −20 °C until further use. miRNA expression profiles were then evaluated using semi-quantitative, real-time PCR. For this purpose, a Qiagen® miScript® SYBR® Green PCR Kit (Qiagen N.V., Venlo, Netherlands) and a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Life Science, Hercules, California, USA) were used. Primers were provided by Qiagen. Each of the 86 samples required PCR measurements, resulting in two measurements per patient.

2.4. Statistical analysis

Clinical follow-up was assessed until April 2017, resulting in a maximum follow-up time of 17 years. For correlation analysis, time until disease recurrence and death was evaluated. Statistical analysis was performed using SPSS Statistics® (IBM, Armonk, NY, USA). The ΔΔCq-Method proposed by Schmittgen et al. was used for data normalization (Schmittgen and Livak, 2008), with actin β serving as the control gene. To determine the correlation between miRNA expression profiles and patient data, Kendall's tau was used as the correlation coefficient. Comparisons between groups were analyzed using two-tailed Student's t-tests for paired samples. The influence of miRNA expression on disease recurrence and patient survival was investigated via a log-rank test, and hazard ratios were calculated using Cox regression analysis. p-values < 0.05 were regarded as statistically significant.

3. Results

3.1. Patients and follow-up time

43 patients were included in the study: 30 males (70%) and 13 females (30%). The mean age was 56 ± 9.8 years, and the ages ranged from 37 to 77. The mean time from diagnosis to death was 4.1 ± 3.8 years, and the mean follow-up time for all patients was 4.39 ± 4.82 years. In total, 35 patients (81.4%) died during the follow-up period. Table 2 shows the clinical and pathological characteristics of the examined patient cohort. In 14 cases (32.6%), locoregional recurrence was observed in our patient collective during the observation period. The mean time from the diagnosis to a local recurrence was 2.4 ± 2.6 years. Six of these cases (42.9%) occurred within the first 3 years after diagnosis. At the end of data collection, 30 patients (69.8%) had died and 13 patients (30.2%) were alive without disease recurrence. There was a significant trend towards better disease-free survival for female patients ($p = 0.05$), which may be due to the significant cumulation of larger tumors in the male patients ($p = 0.02$). Table 2 displays relevant clinical and pathological features of the investigated cohort.

3.2. Expression levels of selected miRNAs

14 miRNAs displayed statistically significant expression differences between the tumor tissue and adjacent control tissue, with 13 miRNAs displaying higher expression in tumor tissue (Table 3). Only miR-99* was significantly over-expressed in the adjacent control tissue compared with the corresponding tumor (Figs. 1 and 2).

3.3. Correlation of microRNA expression with events of disease recurrence

Correlation analysis revealed a significant association of low expression levels of miR-99* and miR-194* with events of disease recurrence ($p < 0.05$; Table 4).

3.4. Survival analysis in relation to microRNA expression

Uni- and multivariate analysis of survival was performed to estimate the prognostic value of several sequences. In our cohort, only miR-99* expression had a significant impact on overall and progression-free survival (Fig. 3). Table 5 shows the results for miR-99* and several relevant covariates, with their corresponding hazard ratios, and confirms miR-99* as an independent prognostic factor regarding overall survival in OSCC.

Table 2
Clinical and pathological patient characteristics (TNM stage according to UICC TNM Classification (6th ed.), 2002).

Variable		n	%
Gender	Female	13	30.2
	Male	30	69.8
Clinical outcome	Locoregional recurrence	14	32.6
	No recurrence	29	67.4
	Death during follow-up	30	69.8
	Alive at the end of follow-up	13	30.2
pT	1	13	30.2
	2	16	37.2
	3	5	11.6
	4	9	21.0
pN	0	15	34.9
	1	8	18.6
	2a	2	4.7
	2b	10	23.2
	2c	3	7.0
	3	5	11.6
pM	0	40	92.9
	1	3	7.1
Grading	1	3	7.0
	2	25	58.1
	3	15	34.9
Localization	Tongue	14	32.5
	Alveolar process/mandible	7	16.3
	Maxilla/hard palate	7	16.3
	Soft palate	7	16.3
	Floor of the mouth	8	18.6

3.5. Validation via miRNA expression data from The Cancer Genome Atlas (TCGA)

To validate the results of our survival analysis, miRNA data sets for different cohorts of HNSCC patients were assessed via The Cancer Genome Atlas (TCGA) (Goswami and Nakshatri, 2012; Antonov et al., 2013; Wong et al., 2018). While there were no data available for miR-99*, a significant association of miR-99 — the leading strand of miR-99* — with patient survival could be confirmed. In all cases, up-regulation of miR-99 was significantly correlated with better overall survival ($p < 0.01$). Moreover, the analysis revealed a significant positive impact of elevated levels of miR-99 on overall survival in other tumor entities, such as kidney renal papillary cell carcinoma, hepatocellular carcinoma, and lung adenocarcinoma ($p < 0.01$). Furthermore, several sequences that failed to show significance in the survival analysis of the internal cohort, displayed significant impact on survival in the larger TCGA cohort (e.g. miR-200b, miR-200c, miR-193a; $p < 0.05$).

4. Discussion

Local disease recurrence and the occurrence of metastases are the most important prognostic factors regarding the clinical outcome for patients suffering from HNSCC. Despite different therapy strategies, survival rates have remained around 50% in recent years (Siegel et al., 2013). Specific biomarkers therefore need to be identified to further individualize HNSCC treatment and follow-up.

Our aim was to identify miRNA expression profiles to predict the risk of recurrence and survival in 43 patients with oral squamous cell carcinoma. To achieve this goal, we analyzed the expression patterns of 30 pre-selected miRNAs in tumors and control tissue from FFPE samples. We have recently been able to successfully demonstrate the feasibility of extracting miRNAs from FFPE samples after storage times of more than 14 years. Moreover, significant correlations between expression data and clinicopathological data,

Table 3
Mean expression levels (ΔCq-values) of 14 miRNAs in OSCC, with significant differences in tumorous and adjacent control tissue (positive differences indicate a higher expression in the tumor).

Sequence		Mean	SD	Difference		*p*
				Mean	SD	
miR-99*	Tumor	−3.278	1.473	−1.265	1.977	<0.01
	Control	−2.014	1.552			
miR-105*	Tumor	−8.095	1.316	0.700	1.890	0.02
	Control	−8.795	2.198			
miR-105	Tumor	−7.066	1.808	0.629	1.490	<0.01
	Control	−7.696	1.419			
miR-141	Tumor	1.148	2.179	0.955	2.380	0.01
	Control	0.193	2.819			
miR-200a	Tumor	0.459	2.206	0.800	2.489	0.04
	Control	−0.340	2.917			
miR-200b*	Tumor	−1.468	1.731	0.863	2.126	0.01
	Control	−2.331	2.358			
miR-200c	Tumor	4.903	1.828	1.377	2.424	<0.01
	Control	3.526	2.737			
mirR205*	Tumor	−1.272	1.887	1.516	2.141	<0.01
	Control	−2.789	2.723			
miR-210	Tumor	1.535	1.774	1.371	2.228	<0.01
	Control	0.164	2.548			
miR-224	Tumor	1.909	1.747	1.437	2.138	<0.01
	Control	0.472	2.360			
miR-224*	Tumor	−1.846	1.622	0.454	1.400	0.04
	Control	−2.300	1.686			
miR-663b	Tumor	−0.913	1.882	1.112	2.499	<0.01
	Control	−2.025	2.861			
miR-767-5p	Tumor	−6.100	1.724	0.855	1.769	<0.01
	Control	−6.955	2.142			
miR-3687	Tumor	2.659	1.292	0.585	1.808	0.04
	Control	2.073	2.178			

such as tumor size, T-stage, differentiation grade, age, and the gender of patients, could be observed (Moratin et al., 2016).

There is ongoing discussion about the potential use of miRNAs as biomarkers in different types of malignoma, including HNSCC (Ries et al., 2014; Chen et al., 2017; Qu et al., 2017; Todeschini et al., 2017). Ries et al. were able to identify altered concentrations of several miRNAs in whole blood samples from patients with OSCC, and correlations between these miRNAs and disease recurrence

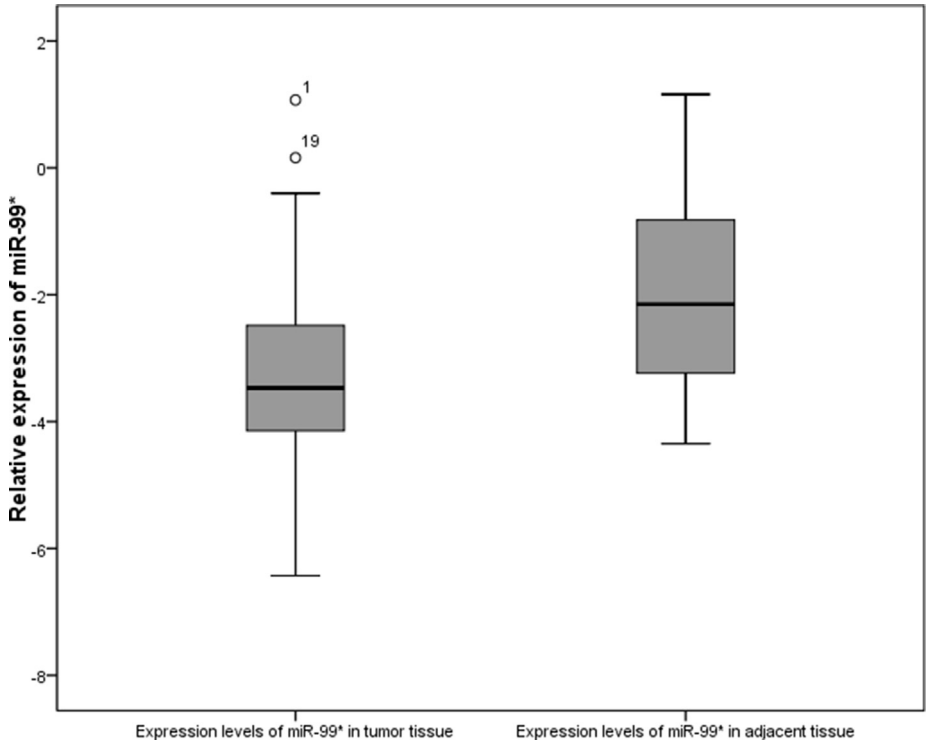

Fig. 1. Boxplots depicting expression levels of miR-99* in tumor tissue and samples of adjacent tissue. miR-99* levels were significantly higher in adjacent tissue compared with tumor tissue samples ($p < 0.01$).

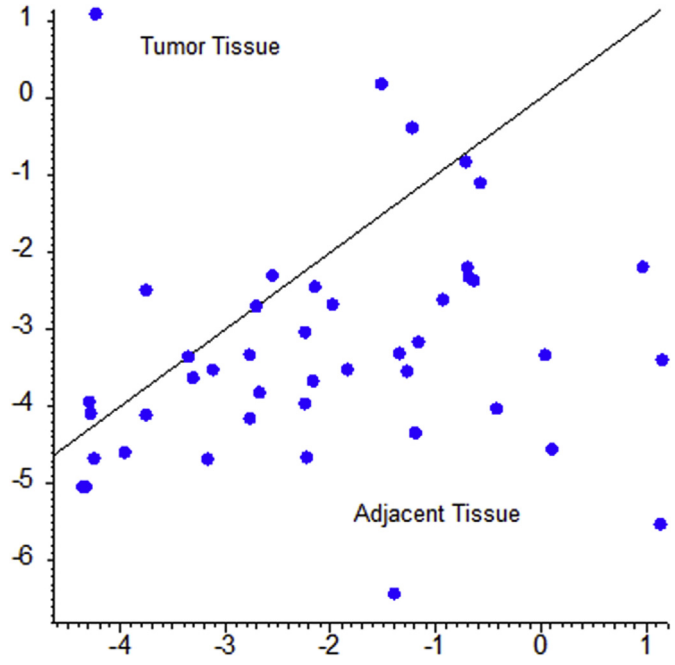

Fig. 2. Distribution of mean expression levels of miR-99*. Points above and below the diagonal indicate over- and under-expression in tumorous and adjacent tissue, respectively.

Table 4
Correlation analysis of expression levels of miR-99* and miR-194* with events of locoregional disease recurrence, using Kendall's Tau.

Tested sequence	*n*	Kendall's Tau	*p*-value
Locoregional recurrence			
miR-99*	43	−0.309	0.04*
miR-194*	43	−0.345	0.02*

(Ries et al., 2017). Our data demonstrate intra-individual expression differences for multiple miRNAs between cancerous and adjacent control tissue. These expression differences can potentially be explained by the cellular functions of the specific miRNAs. Moreover, similar expressions of miRNAs that indicate disease recurrence in tumors and non-tumorous tissue may help to establish screening techniques for peripheral blood and saliva samples.

Several tested microRNAs, such as the miR-200 family, have previously been described as tumor suppressors in various publications, which could be confirmed by significant under-expression in tumor tissue samples in our analysis (Zidar et al., 2011; Jabbari et al., 2014; Jamali et al., 2015; Chen and Zhang, 2017). This confirms the validity of our data. miR-200b, the leading strand of miR-200b*, has been shown to be associated with increased overall survival in an HNSCC patient cohort after radiotherapy in combination with 5-fluorouracil/mitomycin C (Hess et al., 2017). In our analysis, higher levels of miR200b* also demonstrated a trend towards better overall survival, although the results were not significant (HR: 0.544; $p = 0.13$). Nevertheless, the miR-200 family was confirmed to be associated with better survival in HNSCC patients via TCGA survival analysis.

While most investigated miRNAs did not show a significant impact on clinical outcome in terms of overall and progression-free survival, which may partly be due to the relatively small cohort, miR-99* and miR-194* correlated significantly with events of disease recurrence, and therefore may serve as biomarker candidates.

Higher expression of miR-99* was significantly correlated with better overall and progression-free survival. While the miR-99 family has been shown to function as a tumor suppressor in various types of malignoma, including prostate carcinoma, cervical carcinoma, breast cancer, and human glioma, our investigation was the first to demonstrate its significance in head and neck cancer (Sun et al., 2011; Lyng et al., 2012; Xin et al., 2013; Moratin et al., 2016; Zhang et al., 2016). Jing et al. could demonstrate progression of lung cancer via down-regulation of the mir-99 family, which resulted in activation of Erk1/2 and AKT, two pathways that are known to promote tumor progression in HNSCC as well (Freudlsperger et al., 2011; Chan et al., 2017; Jing et al., 2018). In our

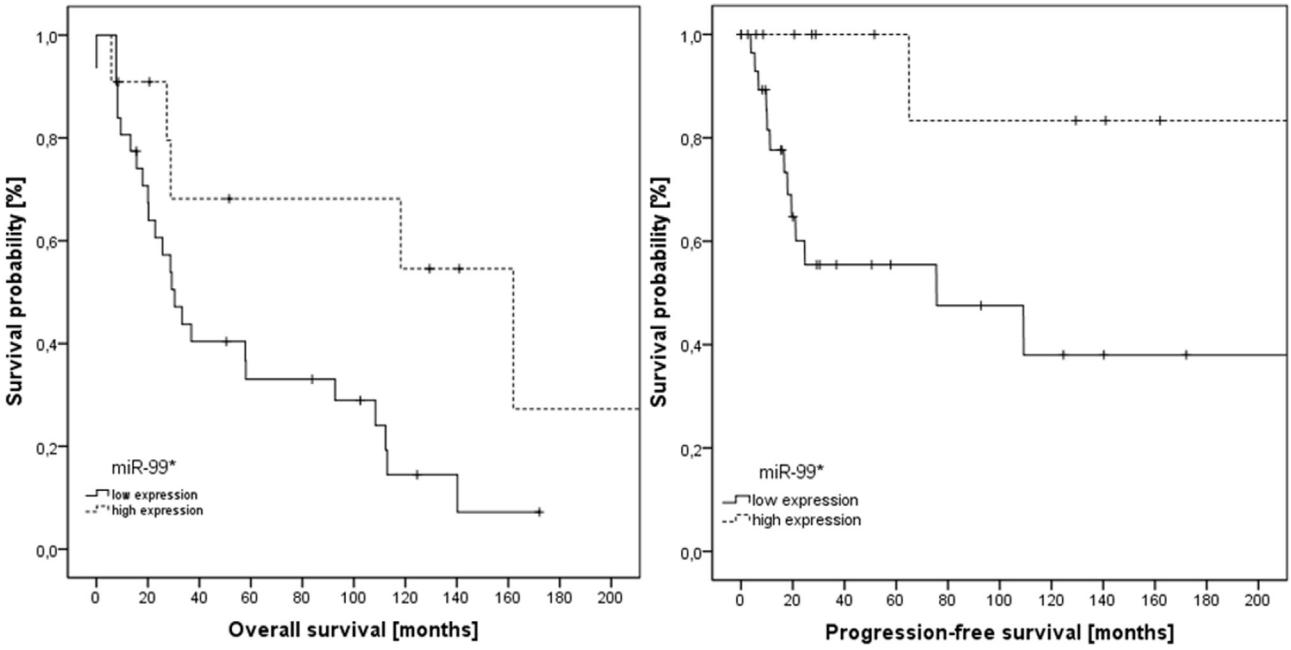

Fig. 3. Kaplan–Meier curves for overall and progression-free survival according to expression levels of miR-99*. Dashed lines represent high miRNA expression (log-rank test: $p < 0.05$).

Table 5
Results of uni- and multivariate analysis of overall survival in consideration of miRNA99* expression and relevant clinical covariables.

Characteristics	Univariate		Multivariate	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
miR-99*	0.354 (0.132–0.949)	0.04*	0.288 (0.086–0.966)	0.04*
N+	2.010 (0.834–4.842)	0.12	2.507 (0.914–6.874)	0.07
Disease recurrence	1.919 (0.907–4.058)	0.09	1.349 (0.570–3.192)	0.49
T-stage	1.223 (0.849–1.761)	0.28	1.128 (0.683–1.863)	0.64
G	1.508 (0.767–2.965)	0.36		
Age ≥ 65 years	1.166 (0.443–3.068)	0.77		

analysis, miR-99* was over-expressed in adjacent tissue compared with cancer tissue from the same patient ($p < 0.001$). The under-expression of miR-99* in cancer samples seems logical when its function as a tumor suppressor is assumed.

The over-expression of miR-193a-5p has been shown to suppress the metastasis of osteosarcoma, esophageal carcinoma, and non-small-cell lung cancer (Zhang et al., 2014; Yu et al., 2015; Lin et al., 2016; Pu et al., 2016). In line with these findings, miR-193a-5p was significantly associated with better overall survival in the TCGA cohort.

5. Conclusion

Locoregional disease recurrence and metastatic events remain the leading causes of early death in patients suffering from HNSCC. While standard follow-up procedures mainly include clinical and radiological examinations, there is still a lack of biomarkers for the detection of recurrence and the prediction of overall survival. Our analysis reveals promising candidates for the further investigation of miRNAs as possible biomarkers for risk assessment and detection of disease recurrence in HNSCC. In our analysis, miR-194* and miR-99* displayed a statistically significant correlation with local recurrence ($p < 0.05$). Moreover, expression levels of miR-99* and its leading strand miR-99 were significantly associated with worse overall and progression-free survival in our cohort and in the TCGA data. Follow-up studies in a prospective setting are warranted to evaluate clinical practicability and prognostic value.

Conflicts of interest

The authors declare that they have no conflicts of interest. This research did not receive any specific grants from funding agencies in the public, commercial, or not-for-profit sectors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcms.2019.01.015.

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