Original Article

Deregulation of Selected MicroRNAs in Sinonasal Squamous Cell Carcinoma: Searching for Potential Prognostic Biomarkers

(biomarker / epigenetics / head and neck cancer / microRNA / sinonasal squamous cell carcinoma)

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Abstract. Sinonasal carcinomas are head and neck tumours arising from the nasal cavity and paranasal sinuses characterized by unfavourable outcome, difficult treatment, diagnosis and prognosis. MicroRNAs are key molecules in the regulation of development and progression of cancer and their expression profiles could be used as prognostic biomarkers, to predict the patients' survival and response to treatment. In this study, we used quantitative real-time PCR with TaqMan® Advanced miRNA Assays to investigate the relative expression values of selected microRNAs in a unique set of formalin-fixed paraffin-embedded tissue samples obtained from 46 patients with sinonasal squamous cell carcinoma. Our results showed statistically significant up-regulation of three mature microRNAs: miR-9-5p (fold change: 6.80), miR-9-3p (fold change: 3.07) and let-7d (fold change: 3.93) in sinonasal carcinoma patients. Kaplan-Meier survival analysis and logrank test identified association between higher expression of miR-9-5p and longer survival of the patients (P = 0.0264). Lower expression of let-7d was detected in the patients with impaired survival, and higher expression of miR-137 was linked to shorter survival of the patients. We also identified several correlations between expression of the studied microRNAs and recorded clinicopathological data. Higher expression of miR-137 and lower expression of let-7d correlated with local recurrence (P = 0.045 and P = 0.025); lower expression of miR-9-5p and higher expression of miR-155-5p correlated with regional recurrence (P = 0.045 and P = 0.036). Higher expression of miR-9-3p correlated with occupational risk (P = 0.031), presence of vascular invasion (P = 0.013) and perineural invasion (P = 0.031). Higher expression of miR-155-5p was present in the samples originating from maxillary sinus (P = 0.011), cN1-3 classified tumours (P = 0.009) and G2-3 classified tumours (P = 0.017). In conclusion, our study supports the hypothesis of future prospect to use expression of miRNAs as prognostic biomarkers of squamous cell sinonasal carcinoma. In particular, miR-9-5p and miR-9-3p seem to be important members of the sinonasal cancer pathogenesis.

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Abbreviations: ANOVA – one-way analysis of variance, CDDP – cisplatin, EMT – epithelial-mesenchymal transition, FFPE – formalin-fixed paraffin-embedded, HNSCC – head and neck squamous cell carcinoma, HPV – human papilloma virus, miRNA – microRNA, PCR – polymerase chain reaction, RISC – RNA-induced silencing complex, RT-PCR – reverse transcription polymerase chain reaction, SCC – squamous cell carcinoma, SEM – standard error of the mean, SNC – sinonasal carcinoma, SSCC – sinonasal squamous cell carcinoma, WHO – World Health Organization.

Introduction

Malignant tumours arising from the nasal cavity and paranasal sinuses (SNC) are rare types of cancer, repre-

senting 3 % to 5 % of head and neck area malignancies and 1 % of all tumours. As well as in other types of head and neck cancer, squamous cell carcinoma (SCC) represents the most common subtype followed by intestinal and non-intestinal type adenocarcinoma (Kawaguchi et al., 2017). In 2015 (last statistical data available), the incidence of SNC in the Czech Republic was 0.69/100,000 with mortality rate 0.44/100,000 (Dušek et al., 2005). Sinonasal cancer usually manifests in advanced age (mean approx. 62 years) and its occurrence is significantly higher in men than in women (Dutta et al., 2015). The disease management of tumours arising from complex sinonasal area is challenging and even though aggressive combined treatment is commonly used, the overall survival remains poor. Only about 50 % of patients survive for five years (Dutta et al., 2015; Cracchiolo et al., 2018). Symptoms such as rhinorrhea, epistaxis, epiphora and nasal obstruction appear in early stages of the disease. Advanced lesions can cause blurred vision, diplopia or proptosis (Barnes et al., 2005).

SNC development is linked to risk factors including smoking (Mannetje et al., 1999), professional exposure to cancerogenous substances (mainly wood dust and leather dust) (Pérez-Escuredo et al., 2012; Mensi et al., 2013; Comiati et al., 2017) and high-risk HPV infection (mostly HPV-16), as approximately 20–30 % of SNC harbour transcriptionally active HPV infection. On the other hand, it has been demonstrated that patients with HPV-positive status have more favourable prognosis compared to HPV-negative tumours (Laco et al., 2015; Kilic et al., 2017).

MicroRNAs (miRNAs) are short (~22 nucleotides) non-coding RNA molecules that are involved in gene expression regulation and were discovered during the experiments with *Caenorhabditis elegans* in 1993 (Lee et al., 1993). The primary function of miRNAs is to repress translation via RNA interference as part of the RNA-induced silencing complex (RISC). Until now, 2,588 mature miRNAs have been identified in humans (miRBase), which is not yet a final number (Di Leva et al., 2014; Kozomara and Griffiths-Jones, 2014).

MicroRNAs are important molecules in the development and progression of cancer. They can be both up- or down-regulated in a variety of cancer types and reflect the clinicopathological features of the tumour. MiRNA expression profiles can be potentially used as prognostic biomarkers, to predict the patients' survival and response to the treatment (Paczkowska et al., 2017). They can be successfully detected in easily accessible biofluids such as serum, plasma and urine (Ferracin and Negrini, 2015).

In the present study, we used quantitative real-time PCR to investigate the relative expression values of miR-9-5p, miR-9-3p, miR-137, miR-143-3p, miR-155-5p, miR-223-3p and let-7d in a unique set of formalin-fixed, paraffin-embedded (FFPE) tissue samples obtained from patients with sinonasal squamous cell carcinoma (SSCC). Although RNA isolated from FFPE samples is fragmented and degraded, it is still suitable for miRNA expression analysis. Due to their small size, miRNAs (< 25 nt) are less susceptible to the degradation processes (Liu and Xu, 2011).

Material and Methods

Patients and samples

A total of 63 FFPE tissue samples from SSCC patients and normal sinonasal tissue were analysed: 46 were cancer cases and 17 were control samples. Only tumours primarily originating from the nasal cavity, maxillary sinuses and ethmoid complex were included, while no tumours were found in the frontal or sphenoid sinuses. The samples used as controls were eight mucosal specimens from the nasal cavity and nine from the maxillary sinus, which were obtained from patients treated for a non-malignant diagnosis such as chronic rhinitis and sinusitis (10 women and 7 men with median age of 54).

Paraffin samples for this study were obtained from the Departments of Pathology of three University Hospitals in the Czech Republic (Hradec Králové, Prague, Olomouc) and all malignancies were diagnosed between August 1995 and August 2014. Carcinoma cases were classified according to the current World Health Organization (WHO) classification (El-Naggar et al., 2017) The study was approved by the Ethics Committee of University Hospital Hradec Králové (201511 S27P).

The tumour types included exclusively squamous cell carcinoma (conventional, verrucous, papillary, basaloid, spindle cell, acantholytic, and adenosquamous). Vascular invasion, perineural spread, status of resection margins (in the case of radical surgery), and microscopic findings in the surrounding mucosa were described.

Data such as gender, age at the time of diagnosis, smoking history (non-smoker vs. ex-smoker vs. current smoker), occupation (high- vs. low-risk), tumour localization (nasal cavity, maxillary sinus and ethmoid complex), laterality and clinical/pathological TNM were recorded for every patient. During the follow-up (until February 2016) period, the local recurrence, regional recurrence, distant recurrence, death, and tumour-related death staging were documented (Table 1). The patients were treated with radical surgery, radiotherapy, and chemotherapy in various combinations.

HPV status was analysed using HPV DNA *in situ* hybridization (ISH), HPV E6/E7 mRNA *in situ* hybridization, HPV DNA polymerase chain reaction (PCR) and typing, and HPV E6/E7 mRNA reverse transcription and polymerase chain reaction (RT-PCR). For the purpose of statistical analysis, a case was considered HPV-positive if it was positive for HPV DNA ISH/PCR and/or HPV E6/E7 mRNA ISH/PCR. Detailed HPV analysis was performed as described (Laco et al., 2015).

RNA isolation

Two to four 5-μm-thick sections were cut from FFPE tissue samples and deparaffinized using xylene and ethanol. Total RNA including miRNAs was isolated from the FFPE tissue samples using an RNeasy FFPE Kit

Table 1. Clinicopathological data of sinonasal cancer patients

Clinicopathological characteristics[a]		SCC
		No. of patients
Gender (46)	Male	31
	Female	15
Age (46)	≤ 50	5
	> 50	41
Smoking status (39)	Smoker	22
	Non-smoker	17
Occupation (39)	High risk	3
	Low risk	36
Localization (45)	Nasal cavity	21
	Maxillary sinus	22
	Ethmoid complex	2
c/pT (37)	T1, T2	10
	T3, T4	27
c/pN (45)	N0	36
	N1, N2, N3	9
cM (45)	M0	44
	M1	1
Resection margin (46)	Positive	38
	Negative	8
Grading (43)	G1	38
	G2, G3	5
Vascular invasion (46)	Yes	6
	No	40
Perineural invasion (46)	Yes	1
	No	45
Local recurrence (41)	Yes	15
	No	26
Regional recurrence (40)	Yes	3
	No	37
Distant recurrence (39)	Yes	3
	No	36
HPV status (46)	Negative	30
	Positive	16

[a]Due to a few missing clinical data, sums do not always add up to the total number of patients.

(Qiagen, Hilden, Germany) according to the manufacturer's protocol. The extracted RNA was ultimately eluted in 30 µl of RNase-free water. Concentration and purity of the isolated RNA was determined by a NanoDrop ND 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) by measuring the optical density at 260 nm and 280 nm (A260/280 ratio). After isolation, the samples were immediately processed or stored at –80 °C.

Quantitative real-time PCR of microRNAs

The synthesis of cDNA was done using a TaqMan® Advanced miRNA cDNA Synthesis Kit with universal reverse transcription primers (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol with 8–10 ng of total RNA in the reaction. Real-time PCR was done with TaqMan® Fast Advanced Master Mix (Applied Biosystems) and specific TaqMan® Advanced miRNA Assays (Applied Biosystems) in Rotor-Gene Q (Qiagen). Assays for hsa-miR-9-5p, hsa-miR-9-3p, hsa-miR-137, hsa-miR-143-3p, hsa-miR-155-5p, hsa-miR-223-3p, hsa-let-7d and hsa-miR-361-5p (endogenous control) were used. All steps were performed following the manufacturer's protocol.

All reactions were performed in triplicates and the reaction volume was 10 µl with 2.5 µl of the sample. The reaction conditions were set according to the manufacturer's protocol and involved enzyme activation at 95 °C for 20 s followed by 40 cycles of denaturation at 95 °C for 3 s and annealing/extension at 60 °C for 30 s. Fluorescence data were analysed in the Rotor-Gene Q Series Software. Relative expression of each miRNA was determined using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008) with expression levels of miR-361 for data normalization. This workflow was chosen based on literature review and manufacturer's recommendation for endogenous controls listed in the user guide for TaqMan® Advanced miRNA Assays.

Statistical analysis

All statistical analyses were performed using STATISTICA (data analysis software system) version 13 (TIBCO Software Inc., Tulsa, OK). The miRNA expression values were log-transformed to normal distribution of data for parametric tests. Student's *t*-test was used to compare the level of expression of miRNA in tumour and non-tumour samples. The null hypothesis was based on the theory that there was no difference between the expression levels of the studied miRNAs between tumour samples and control samples.

One-way analysis of variance (ANOVA) and regression analysis were used to analyse the correlation between the expression level of miRNA and various clinicopathological features. The Kaplan Maier method and logrank test were used to determine the overall survival rate and significance. For this purpose, miRNA expression data were divided into quartiles, and patients with extreme deregulation of the tested miRNAs (top or bottom quartiles) were grouped together for Kaplan-Maier testing. All tests were two-tailed and $P < 0.05$ was considered statistically significant. All data were reported as mean ± standard error of the mean (SEM).

Results

MicroRNA relative expression levels in SSCC samples

In this study we analysed the expression levels of miR-9-5p, miR-9-3p, miR-137, miR-143-3p miR-155-5p, miR-223-3p and let-7d using TaqMan® Advanced miRNA Assays and the real-time PCR method in 46

Table 2. Relative expression of selected miRNAs in sinonasal squamous cell carcinoma

miRNA	P value	Fold change
Up-regulated (tumour versus control)		
miR-9-5p	**< 0.0001**[a]	**6.80**
miR-9-3p	**0.017**	**3.07**
miR-143-3p	0.17	1.46
miR-155-5p	0.27	1.46
let-7d	**< 0.0001**	**3.93**
Down-regulated (tumour versus control)		
miR-137	0.98	–1.04
miR-223-3p	0.18	–1.71

[a] Bold numbers show statistically significant results.

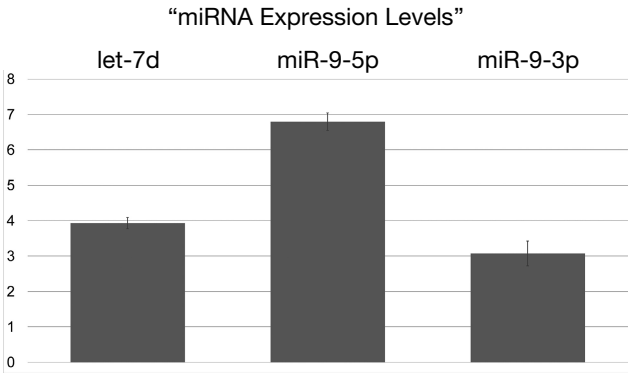

Fig. 1. Fold change of relative expression of deregulated miRNA in SSCC ($2^{\Delta\Delta Ct}$ method)
Error bars outline standard error of the mean.

SSCC and 17 control samples (Table 2). Real-time PCR data showed statistically significant up-regulation of three mature miRNAs (Fig. 1).

MiR-9-5p was up-regulated 6.80 fold ($P < 0.0001$) in comparison to the control samples and the up-regulation was detected in 45 of 46 cases (Fig. 2). MiR-9-3p was upregulated 3.07 fold ($P = 0.017$) and it was upregulated in 35 of 44 cases (we were unable to measure the relative expression of two samples) (Fig. 2). Finally, let-7d was upregulated by 3.93 in SSCC samples in comparison to the control tissue ($P < 0.0001$) and 43 of 46 tumour samples were deregulated (Fig. 2).

MicroRNA and patients' survival

The follow-up for the group of patients ranged from two to 111 months (median 23 months). For the survival analysis, we used Kaplan-Meier analysis accompanied by logrank test to obtain the P value. The patients were divided into two categories for each miRNA based on their expression levels. The results suggested that patients with high expression of miR-9-5p (group of patients with expression levels in the top quartile) had better chance of survival than the patients with lower expression of miR-9-5p ($P = 0.0264$) (Fig. 3).

Our data subsequently showed that patients with low expression of let-7d had impaired survival in comparison to patients with higher expression of the same miRNA (based on a lower quartile value). These results were statistically significant ($P = 0.0417$) (Fig. 4.). On the other hand, patients with higher expression of miR-137 had significantly impaired survival interval in comparison to the patients with lower expression ($P = 0.0278$). The groups were divided based on the 3rd quartile value (Fig. 5).

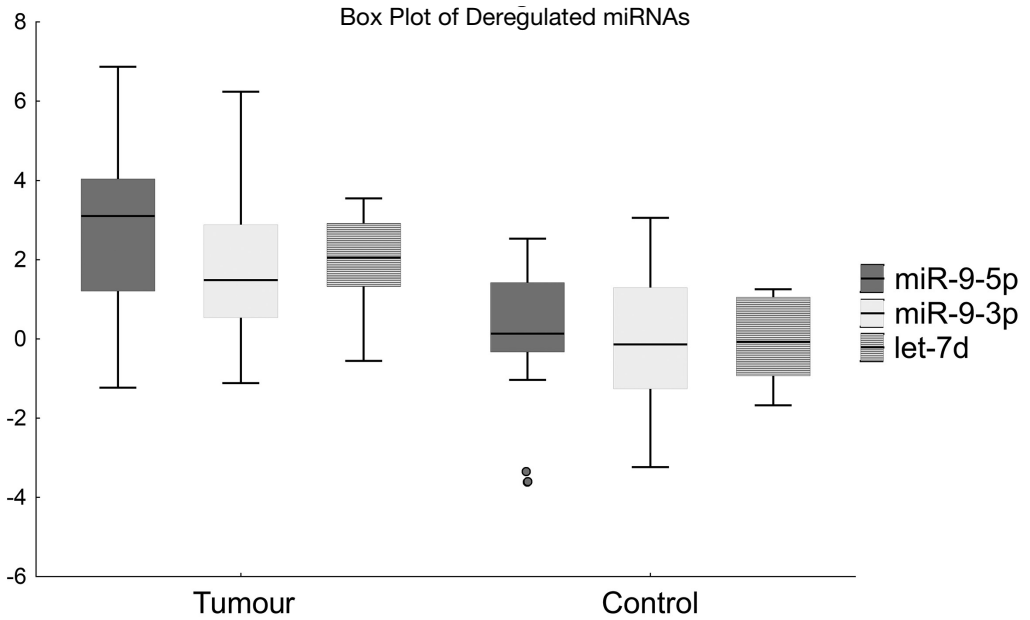

Fig. 2. Box plots of miR-9-5p, miR-9-3p and let-7d relative expression in SSCC samples (compared to control samples) based on -ΔΔCt values. Median values are outlined in the centre with whiskers showing non-outlier range and outliers. Extremes are presented as dots.

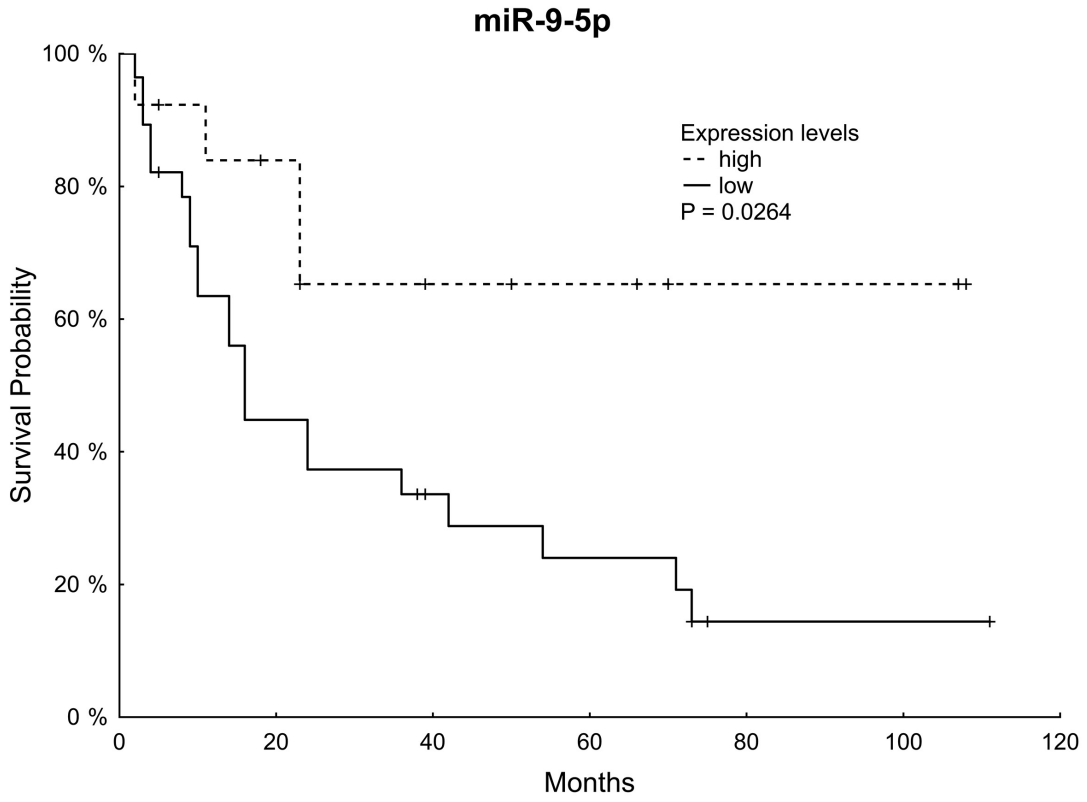

Fig. 3. Kaplan-Maier survival plot for miR-9-5p. Higher expression of miR-9-5p was detected for the patients with longer survival interval with statistical significance (P = 0.0264). Vertical hatch marks show censored data.

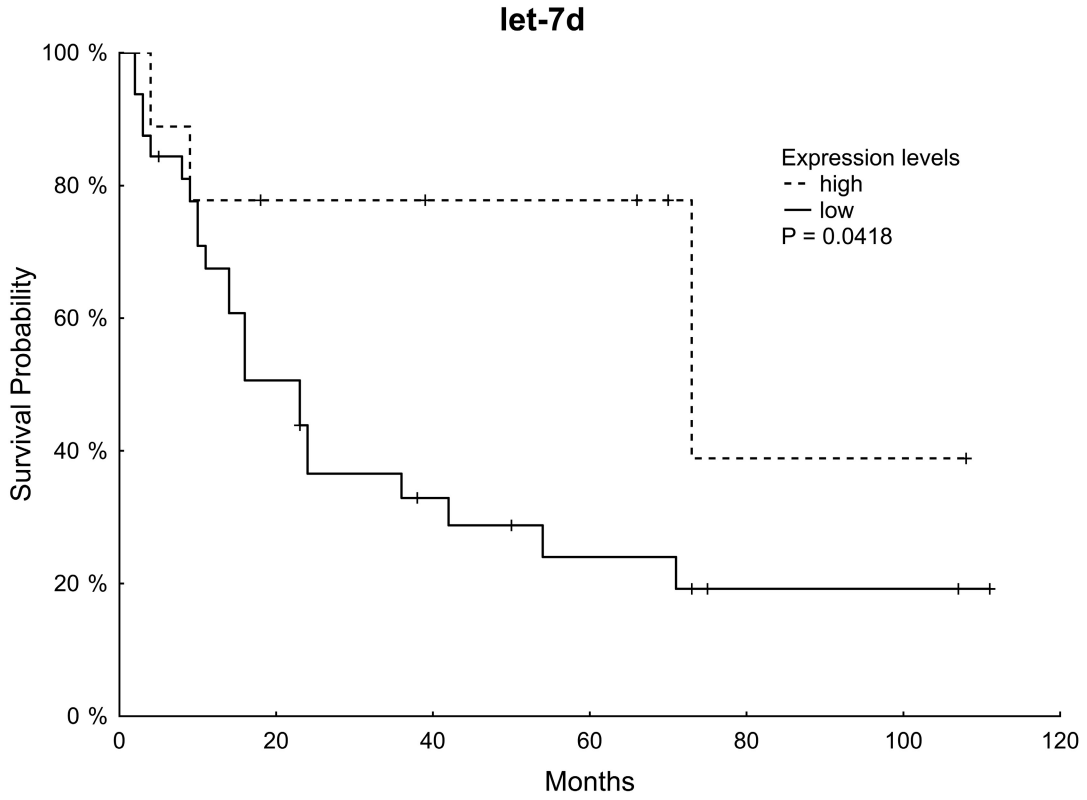

Fig. 4. Kaplan-Maier survival plot for let-7d. Lower expression of let-7d was detected for the patients with shorter survival interval. The results are statistically significant with P value 0.0417. Vertical hatch marks show censored data.

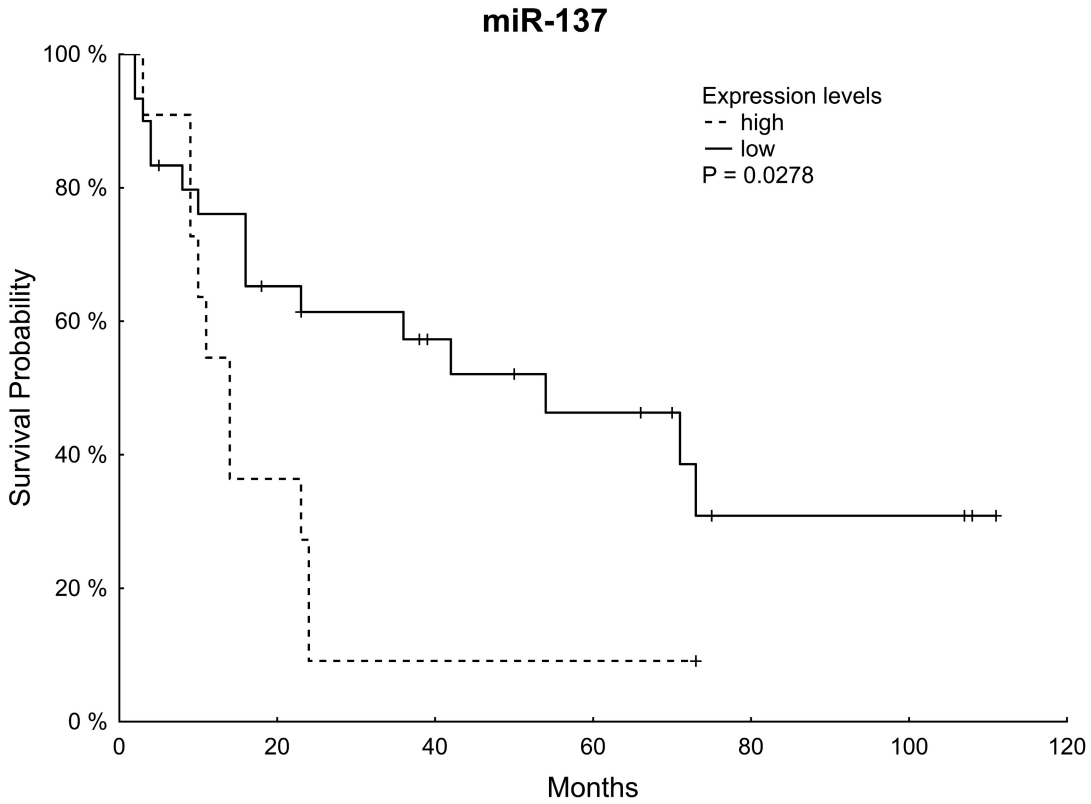

Fig. 5. Kaplan-Maier survival plot for miR-137. Higher expression of miR-137 was detected for the patients with shorter survival interval. The results are statistically significant (P = 0.0278). Vertical hatch marks show censored data.

Correlation with clinicopathological characteristics

Clinicopathological data of all the patients are summarized in Table 1. The age range of the patients at the time of diagnosis was between 27 and 82 years and median age was 65 years. During the follow-up period, 26/41 patients died, of whom 11/39 due to the tumour. Control samples (N = 17) were acquired from seven male and 10 female patients. The youngest patient was 24 years old and the oldest one 74 years old, while median age was 54 years.

We used statistical analysis to determine the relationships between the recorded clinicopathological characteristics with selected miRNA expression levels determined in SSCC samples. All statistically significant results and results approaching statistical significance are described in the following text (Table 3).

First, we determined the correlation between higher expression of miR-137 and the presence of local recurrence (P = 0.045) followed by lower expression of miR-9-5p and the presence of regional recurrence (P = 0.045) and higher expression of miR-155-5p and regional recurrence (P = 0.036). On the other hand, local recurrence also correlated with lower expression of let-7d (P = 0.025). Expression of miR-9-3p correlated with occupation, when patients with risky occupations had higher expression than patients without risk factors (P = 0.030). Relative expression of miR-155-5p correlated with localization (P = 0.011). Higher expression was found in the samples originating from maxillary sinus, whereas lower expression of miR-155-5p was present in samples from the nasal cavity – the same tendency was detected in control samples (P = 0.053). Higher expression of miR-155-5p also correlated with stages 1–3 of cN classification (P = 0.009) and higher graded (G2, G3) tumour samples (P = 0.017). Finally, the presence of vascular invasion was linked to higher expression of miR-9-3p similarly as the presence of perineural invasion (P = 0.013, P = 0.031, respectively).

Discussion

Sinonasal cancers and other tumours arising from the anatomically complex area of the head and neck are serious malignant diseases with difficult treatment, poor prognosis and low survival rate (Kawaguchi et al., 2017). Similarly as molecular genetic alterations, the accumulation of aberrant epigenetic events (such as miRNAs that play a role in epigenetic determination of gene expression) can influence the head and neck cancer pathology (Boscolo-Rizzo et al., 2017). MicroRNAs are recognized as key molecules in cancer development and progression. They are established as promising prognostic cancer biomarkers because of their resistance to degradation in many tissue types (including FFPE tissues

Table 3. Regression coefficients of miRNA expression with clinicopathological data

		P value						
miRNA		miR-9-5p	miR-9-3p	miR-137-5p	miR-143-3p	miR-155-5p	miR-223-3p	let-7d
Occupation	High risk	0.88	**0.031**[a]	0.33	0.39	0.11	0.37	0.39
	Low risk							
Localization	Nasal cavity	0.99	0.92	0.78	0.11	**0.011**	0.40	0.078
	Maxillary sinus							
cN	cN0	0.74	0.74	0.81	0.31	**0.009**	0.55	0.68
	cN1-3							
Grade	G1	0.24	0.42	0.56	0.46	**0.017**	0.52	0.22
	G2-3							
Vascular invasion	Yes	0.90	**0.013**	0.90	0.60	0.71	0.65	0.36
	No							
Perineural invasion	Yes	0.74	**0.031**	0.30	0.32	0.45	0.46	0.90
	No							
Local recurrence	Yes	0.11	0.56	**0.045**	0.13	0.17	0.59	**0.025**
	No							
Regional recurrence	Yes	**0.045**	0.72	0.76	0.59	**0.036**	0.81	0.25
	No							

[a] Statistically significant ($P < 0.05$) results are marked in bold.

and body fluids). The amount of miRNA-focused research is nowadays growing rapidly (Ferracin and Negrini, 2015; Moody et al., 2017).

Up- or down-regulation of miRNAs usually occurs in the same direction across different cancer subtypes. For example, miR-21 is up-regulated in almost all human cancer types, and its oncogenic effect has been extensively described (Kalfert et al., 2015; Sannigrahi et al., 2017; Bahrami et al., 2018; Moridikia et al., 2018). Up-regulation of miR-21 in SSCC tissue was described in our previous study, where we also confirmed its validity as a prognostic biomarker of SSCC (Kovarikova et al., 2017). Another study dealing with miRNA expression in sinonasal carcinoma was performed by Ogawa et al. (2012), who found down-regulation of miR-34a in cisplatin (CDDP)-resistant cell lines of sinonasal squamous cell carcinoma, suggesting that miR-34a correlates with poor prognosis of sinonasal SCC patients with CDDP treatment.

In the current study, we investigated the relative expression of several selected miRNAs and the relationship between their expression and clinicopathological characteristics of the patients. Based on a review of literature dealing with deregulation of miRNA in various types of head and neck cancer, the following miRNAs were selected for inquiry: miR-9-5p, miR-9-3p, miR-137, miR-143-3p miR-155-5p, miR-223-3p, let-7d, with real-time PCR (TaqMan® Advanced miRNA approach) as our method of choice (the limited number of miRNAs we were able to investigate by the selected method might be a potential weakness of this study).

Our data shows that both 3' and 5' **miR-9** forms are abundantly expressed and up-regulated in SSCC samples in comparison to controls. In concordance with our results, Salazar et al. (2014) were able to find up-regulation of miR-9 in small amounts of saliva of HNSCC patients.

Among our selected miRNAs, up-regulation was most significant for the 5' form of miR-9 with 6.80 fold change. Form 3' of the same miRNA was significantly up-regulated as well, with fold change 3.07. More importantly, we detected a significantly longer survival interval in patients with higher expression of miR-9-5p ($P = 0.0264$). Citron et al. (2017) identified up-regulation of miR-9 in patients with HNSCC, and they also concluded that this particular miRNA is an important mediator of recurrence formation in HNSCC by regulating the EMT process. Similarly, Yu et al. (2012) reported that nicotine-treated cells had significantly higher expression of miR-9 leading to promotion of metastatic processes by E-cadherin repression. Their conclusions support our findings of correlation between vascular invasion and up-regulation of miR-9-3p ($P = 0.013$) and, consecutively, of correlation between perineural invasion and the same microRNA form ($P = 0.031$). We registered correlation between cM1 and higher expression of miR-9-3p, although it was not statistically significant ($P = 0.20$). However, these results are influenced by the fact that there was only one patient with cM1 recorded in our set with miR-9-3p expression of 4.63, while the mean expression of the rest of the samples was 1.6. Moreover, our results showed a relationship between the regional recurrence of SSCC recorded in our patients and relative expression of miR-9-3p ($P = 0.0045$).

Interestingly, patients working in high-risk industries for developing SNC had significantly higher expression

of miR-9-3p. We have not found any correlation between miR-9 expression and the presence of HPV in our samples, even though Vojtechova et al. (2016) concluded that miR-9-5p is specific for HPV-positive tonsillar tumours.

Our findings of deregulated of miR-9-5p and miR-9-3p strongly support the argument that expression of various forms of one miRNA (especially 5' and 3' forms) can play different biological roles in cancer pathology. Methods with the ability to distinguish between expression of all the variants should be used to get more reliable results of miRNA expression experiments especially in tissue samples (Guo et al., 2016).

miR-137 has been reported to be down-regulated in HNSCC and to have a role in suppression of oncogenes. We have not found miR-137 to be deregulated in our set of samples, which is the same conclusion made by Sousa et al. (2016) in HNSCC. However, we were able to associate higher expression of miR-137 with impaired survival of the patients with SSCC ($P = 0.0278$) and we found a correlation between the local recurrence of sinonasal tumours and higher expression of miR-137. miR-137 has been previously described as a predictive marker of prostate cancer recurrence after radical prostatectomy (Pashaei et al., 2017).

miR-143-3p and miR-223-3p have been reported to be deregulated in various types of head and neck cancer (Lajer et al., 2012; Bufalino et al., 2015; Manikandan et al., 2015; Chen et al., 2016). It was proposed that miR-143 functions in the apoptosis, invasion and migration processes of head and neck cancer (Bufalino et al., 2015; Chen et al., 2016). Bozec et al. (2017) suggested angiogenic properties of miR-223-3p and they reported up-regulation of the miRNA in HNSCC. We have not found any significant deregulation of miR-143-3p or miR-223-3p in our SSCC samples in comparison to control samples, even though they have been abundantly expressed in the studied tissue.

miR-155-5p has been found to be deregulated in HNSCC (Sannigrahi et al., 2017). Its oncogenic role in development of various types of head and neck cancer such as nasopharyngeal carcinoma (Wang and Sun, 2016), laryngeal squamous cell carcinoma (Wang et al., 2016) and papillary thyroid carcinoma (Lee et al., 2015) and its relevance as prognostic biomarker (Lerner et al., 2016) and the ability to promote metastatic processes (Baba et al., 2016) has been reported. Hess et al. (2017) have shown miR-155 as a potential marker for oropharyngeal cancer, more specifically for resistance to cisplatin treatment, local recurrence, and surrogate marker for tumour-infiltrating lymphocytes.

Although we did not find any statistically significant differences in the expression of miR-155-5p between carcinoma samples and control samples ($P = 0.27$), we were able to identify several correlations between miR-155-5p expression and some clinicopathological data suggesting the idea of miR-155-5p relevance as a prognostic biomarker of HNSCC. First of all, higher expression was present in advanced stages of the disease (cN1-3 and grade G2-3) with P values equal to 0.009 and 0.017, respectively. The expression was also higher in tumours with regional recurrence ($P = 0.036$) and in tumours arising from the maxillary sinus ($P = 0.011$).

From our experiments, it seems that microRNA **let-7d** is up-regulated in SSCC samples in comparison to sinonasal control tissue ($P < 0.0001$). Members of the miRNA let-7 family are generally considered tumour suppressors, with down-regulation present in cancer tissue (Manikandan et al., 2016). However, up-regulation of the miRNA was found in the study done by Hilly et al. (2016) in more aggressive tumours of the oral tongue than in less aggressive tumours. According to our data analysis, we concluded that the patients with extremely high expression of let-7d had better chance of survival than the patients with lower up-regulation of the same miRNA ($P = 0.0418$). We were also able to find a correlation between lower up-regulation of let-7d and the presence of local recurrence in our patients ($P = 0.025$). It implies that lower levels of let-7d are associated with impaired survival in HNSCC (Childs et al., 2009).

In conclusion, our study supports the hypothesis of future prospect to use expression of miRNAs as prognostic biomarkers of squamous cell sinonasal carcinoma. Especially both forms (5' and 3') of miR-9 seem to be important members of SSCC pathogenesis. We observed not only up-regulation of miR-9-5p and miR-9-3p in tumour samples, but also association with high expression of miR-9-5p with longer survival interval of the patients and correlation of miR-9 expression with several clinicopathological characteristics.

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