



A Pilot Study Reveals the Potential of miR-31-3p and miR-196a-5p as Non-Invasive Biomarkers in Advanced Laryngeal Cancer

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Abstract

Introduction: Recently, miRNAs have become popular molecules used as non-invasive biomarkers in cancer diseases.

Aim: The aim of the study was to explore the expression of four miRNAs isoforms: miR-31-3p, miR-196a-5p, miR-210-3p and miR-424-5p in plasma and tissue samples from patients with advanced laryngeal squamous cell carcinoma (LSCC) and healthy controls.

Materials and methods: Fresh-frozen tumour and normal laryngeal tissue as well as plasma samples were obtained from 22 patients diagnosed with advanced LSCC. The control group included plasma samples from 21 cancer-free volunteers. Total RNA (including miRNAs) extraction, reverse transcription and real time qPCR were the laboratory techniques used in the study. The obtained results were analyzed using SPSS software v. 23.

Results: We found that miR-31-3p, miR-196a-5p, and miR-210-3p levels were significantly elevated in laryngeal tumour tissue, but only the levels of miR-31-3p and miR-196a-5p were significantly upregulated in the plasma LSCC target group. Positive correlation was obtained for miR-31-3p ($r_s=0.443$, $p=0.039$) and miR-196a-5p ($r_s=0.548$; $p=0.008$) between plasma and adjacent tumour tissue LSCC samples. ROC analyses were used to evaluate the discriminative power of both miRNAs alone and in combination. The combination of miR-31-3p and miR-196a-5p showed best results with AUC=0.978 (95% CI: 0.945–1.000, $p<0.001$) with 100% sensitivity and 81% specificity at cut-off: RQ=2.99.

Conclusions: Based on this miR-31-3p and miR-196a-5p are proposed as potential biomarkers for validation in larger LSCC group and could be included in a non-invasive miRNAs set for detection of advanced LSCC.

Keywords

laryngeal squamous cell carcinoma, LSCC, miRNAs, non-invasive biomarker, plasma

INTRODUCTION

In recent years, micro RNAs (miRNAs) have become popular targets of investigation, not only in tissue samples, but also as non-invasive markers in various body fluids such as plasma, serum, saliva.¹ Some of the advantages of the circulating non-invasive markers are their easy accessibility using non-invasive or minimally invasive methods and their high stability in body fluids.²

There are a great many studies showing that it is possible to use miRNAs as biomarkers in body fluids which determine their power of non-invasive biomarkers. It is suggested that miRNAs could be an ideal class of blood-based biomarkers for cancer diagnostics because miRNA expression is frequently deregulated in cancer because expression patterns of miRNAs in human cancer appear to be tissue-specific, and miRNAs are highly stable in peripheral plasma due to their small size and relative resistance to nucleases.^{3,4} The presence of circulating miRNAs in body fluids such as plasma and serum was investigated for the first time by Lawrie et al.⁵ It was shown in their study that the serum expression levels of miR-21 had the potential of being a diagnostic marker in diffuse B-cell lymphoma.⁵ Since then, the circulating miRNAs have been reported with aberrant expressions in blood plasma or serum in various cancer diseases such as papillary thyroid cancer, prostate and colon cancer.⁵⁻⁷

Studies reveal the potential of these molecules as circulating biomarkers in laryngeal squamous cell carcinoma (LSCC) and they are an interesting target to be further explored. The study of Wang et al.⁸ analyzed in a large LSCC group (n=280) and a control group (n=560) the levels of miR-155 expression in tissue and plasma. The authors reported strong correlation between tissue and circulating miR-155 expression and great accuracy of miR-155 expression levels for detection of early laryngeal cancer.⁸ Another study, Yilmaz et al.⁹, using miRNA microarray profiling and validation with RT-qPCR, demonstrated that miR-221 levels were significantly elevated in plasma samples from a pre-operative LSCC patients whereas in plasma samples obtained from the same LSCC patients after surgery and from control group, miR-221 was with normal expression. The study of Ayaz et al.¹⁰ reported for the first time that levels of miR-331-3p, miR-603, miR-1303, miR-660-5p and miR-212-3p were detected in LSCC plasma samples in comparison to healthy controls without any detected disease, which they suggested as potential circulating miRNAs biomarkers for early LSCC. Studies published after Ayaz et al.¹⁰ showed that miR-331-3p, miR-603, miR-660-5p, and miR-212-3p were also found in plasma samples obtained from other cancer and rheumatoid diseases¹¹⁻¹⁴, which suggests that the previously chosen miRNAs by Ayaz et al. were not tissue and disease specific and could not be used as biomarkers only for laryngeal malignancy.

Early detection is still challenging in laryngeal carcinoma. Tests, based on evaluation of circulating miRNAs in

plasma or serum, could offer important additional information to the currently used diagnostic instruments allowing screening and early detection of LSCC. Development of methods for early investigation of molecular markers acquires wide popularity as innovative and minimal invasive technique. Despite the great number of research studies in this area, there are still no validated non-invasive miRNA markers included broadly in the clinical practice, which could give additional information about the diagnosis of or predicting the disease.

In the present study, we investigated the expression levels of four known oncogenic miRNA isoforms (miR-31-3p, miR-196a-5p, miR-210-3p, and miR-424-5p), which are previously studied in laryngeal tissue or laryngeal cell line samples.¹⁵⁻¹⁸ Moreover, our team investigated the four miRNA isoforms in larger laryngeal tissue sample group.¹⁹

No published data are available for the potential of the chosen miRNA isoforms as circulating biomarkers in advanced LSCC plasma samples.

AIM

The aim of the current study was to evaluate the expression levels of the chosen miRNAs, the association between expression levels and clinicopathological characteristics, and to explore the possibility of using them as diagnostic biomarkers in advanced laryngeal cancer. This pilot project is done after selection of the set of non-coding RNAs based on literature search.

MATERIALS AND METHODS

Plasma samples

Twenty-two patients diagnosed with laryngeal cancer were enrolled in the present study, and 21 volunteers (without oncological disease) served as a control group. Patients and controls were recruited at the Ear, Nose and Throat Department in the Queen Joanna – ISUL University Hospital in Sofia between 2015 and 2016. Informed consent was obtained from each patient and volunteers and the Ethics Committee of Medical University of Sofia approved the current study with a protocol No. 8/22.04.2016. Tumour and normal laryngeal tissue samples were collected from each patient during surgery. Blood samples were taken from the LSCC patients and controls before surgery, and transported within one hour to the Molecular Medicine Center, Medical University of Sofia. The blood samples were centrifuged at 2000 rpm for 15 min and plasma samples were separated in cryo tubes and immediately stored at -80°C. During surgery, additional material for immunohistochemistry was collected that supported determination of clinicopathological characteristics of the included samples. None of the LSCC patients in this study had received chemotherapy or radiotherapy before surgery.

Isolation of total RNA from plasma samples and reverse transcription

Total RNA was isolated from frozen tissue and plasma samples using a kit for RNA extraction from tissue (miRNeasy Micro Kit, Qiagen, Hilden, Germany) and plasma (miRNeasy Serum/Plasma Kit, Qiagen, Hilden, Germany). The quantity of the total RNA samples was assessed by NanoDrop 2000 (ThermoFisher Scientific, Walmington, DE, USA). 250 ng total RNA of each sample were used to prepare cDNA, specific for miRNAs expressions by using miScript II RT Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Then, the RT samples were processed further or stored at -20°C until use.

Real-time quantitative polymerase chain reaction (RT-qPCR)

The expression of mature miRNAs was assayed using miScript SYBR Green PCR kit (Qiagen, Hilden, Germany) on a 7900HT Fast Real Time PCR System (Applied Biosystems, California, USA). The miScript Primer assays (Qiagen, Hilden, Germany) were used for miR-31-3p, miR-196a-5p, miR-210-3p, and miR-424-5p. Expression levels were normalized to the internal control RNU6B (Qiagen, Hilden, Germany). The relative quantification (RQ) of miRNAs in plasma and tumour LSCC samples was analyzed by the $2^{-\Delta\Delta\text{Ct}}$ method. An average dCt from non-oncological control group was used to calculate ddCt of plasma LSCC samples. A $\text{RQ} \geq 2$ was defined as overexpression, $\text{RQ} \leq 0.49$ as underexpression, and RQ between 1.99 and 0.5 as no change in expression.

Statistical analysis

Data analysis was performed with the SPSS software version 23.0 for Windows (IBM SPSS, USA). Wilcoxon, Mann-Whitney, Kruskal-Wallis, Spearman's correlation coefficient and ROC curve analyses were the statistical tests used. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Four oncogenic miRNA isoforms which were previously investigated in laryngeal cancer disease were included in the present study: miR-31-3p, miR-196a-5p, miR-210-3p, and miR-424-5p.¹⁹ Their expression levels were explored for the first time in 22 advanced LSCC plasma samples and 21 healthy volunteers presented as non-oncogenic control group. The LSCC group was composed of 3 females and 19 males (mean age 61 years, age range: 41-76 years), all of them with advanced LSCC (T grade III or IV). Sixteen patients reported tobacco smoking and alcohol consumption daily. Fourteen LSCC patients reported also harmful work exposure. The control group included four females and 18 males with a mean age of 44 years

(age range: 29-68 years). Eighteen controls were positive for tobacco smoking, seventeen were positive for alcohol consumption daily, and two were positive for work exposure. Our criteria for the control group were sex and age matching (over and under 60 years), as well as no detected oncological disease. However, six volunteers from the control group had adjacent diseases (four with inflammation of the swollen lymph nodes, one with diabetes and one with dyskinesia).

Relative expression of studied miRNAs in LSCC tissue samples

We analyzed the relative expression levels of miR-31-3p, miR-196a-5p, miR-210-3p, and miR-424-5p in laryngeal tumour and normal tissues. The results showed that three out of four miRNAs: miR-31-3p ($p < 0.001$), miR-196a-5p ($p = 0.010$), and miR-210-3p ($p = 0.023$) were significantly elevated in laryngeal tumour tissue in comparison to normal tissue (Fig. 1).

Relative expression of studied miRNAs in LSCC plasma samples

The oncogenic miR-31-3p, miR-196a-5p, miR-210-3p, and miR-424-5p explored in our study were found to have elevated levels in most of LSCC plasma samples with mean RQ of 11.10, 3.03, 6.28, and 3.41, respectively. We found that only miR-31-3p and miR-196a-5p were significantly deregulated in LSCC patients in comparison to the non-oncogenic control group ($p < 0.001$ for both miRNAs), as mean RQ values of miR-31-3p and miR-196a-5p in the controls were found to be 1.36 and 1.10. The mean level of miR-210-3p in the control group ($\text{RQ} = 5.62$) was similar to the expression in LSCC group ($\text{RQ} = 6.28$), and the difference did not reach statistical significance ($p = 0.451$). miR-424-5p mean control RQ level was 2.29 and also was not significantly different ($p = 0.109$) in comparison with the LSCC group (Fig. 2).

The expression levels of miR-31-3p were elevated in 20 and normally expressed in 2 LSCC patients. miR-196a-5p expression levels were found elevated in 17 and normally expressed in 5 LSCC patients. No underexpression for both miRNAs was detected in plasma LSCC samples. Levels of miR-210-3p were upregulated in 16 LSCC patients whereas elevated miR-424-5p levels were found in 13 LSCC patients. Three LSCC patients showed downregulated miR-424-5p levels and six LSCC patients had normal miR-210-3p and miR-424-5p levels.

Correlation analysis between studied miRNAs in plasma and tissue LSCC samples

In order to determine which miRNAs are positively correlated between studied plasma and tissue LSCC samples, we conducted Spearman's correlation analysis. In the

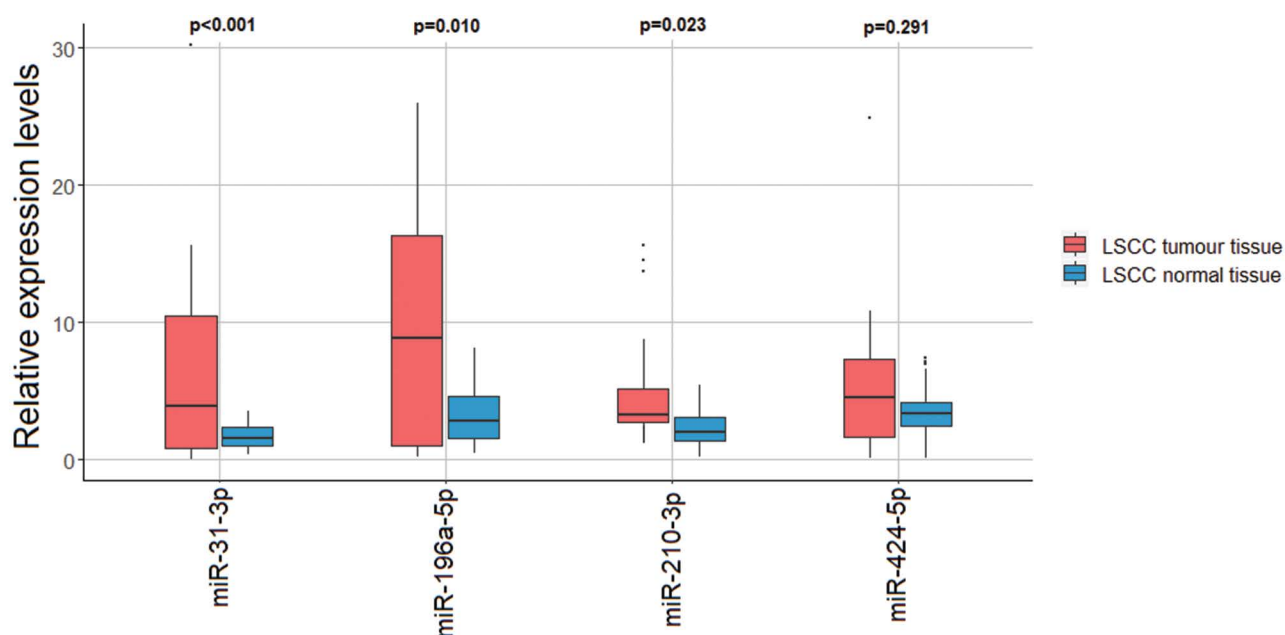


Figure 1. Relative expression levels of miR-31-3p, miR-196a-5p, miR-210-3p, and miR-424-5p in tumour and normal laryngeal tissue. Levels of miR-31-3p ($p<0.001$), miR-196a-5p ($p=0.010$), and miR-210-3p ($p=0.023$) were significantly upregulated in LSCC tumours in comparison to normal laryngeal tissue, whereas miR-424-5p did not reach significance ($p=0.291$) (Wilcoxon test).

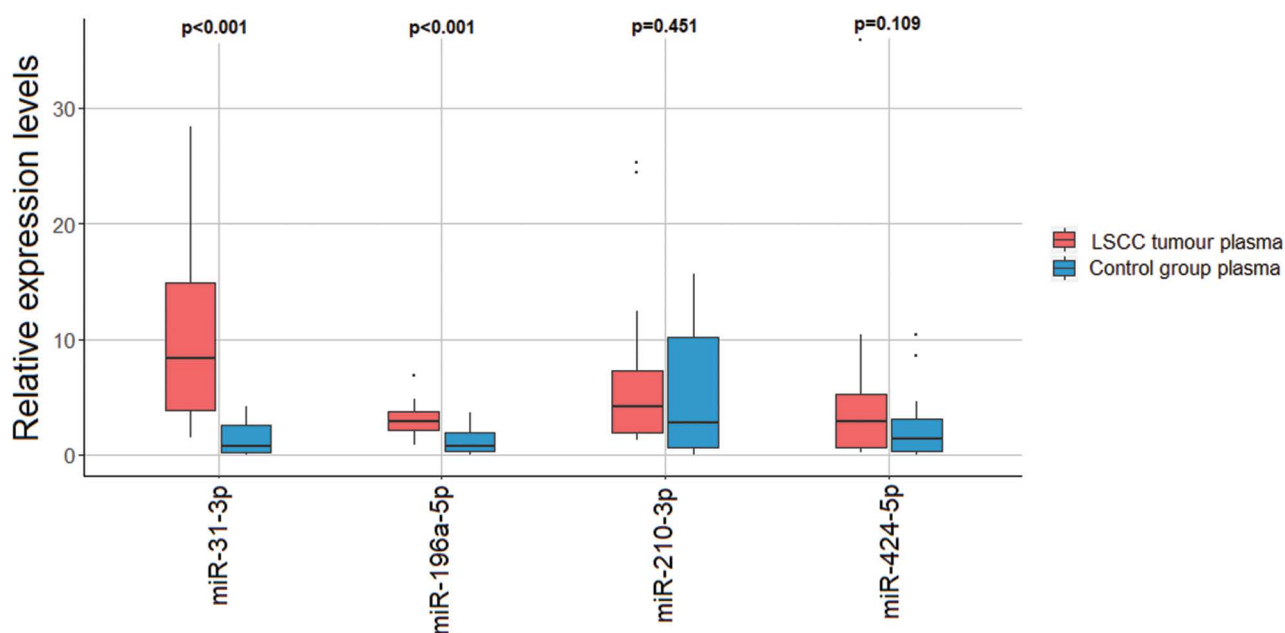


Figure 2. Mean expression levels of miR-31-3p, miR-210-3p, miR-424-5p, and miR-196a-5p in studied plasma LSCC and non-oncological groups. Levels of miR-31-3p and miR-196a-5p were significantly upregulated in LSCC group ($p<0.001$ for both miRNAs) whereas miR-210-3p and miR-424-5p were not significantly upregulated in LSCC group ($p=0.451$ and $p=0.109$) (Mann-Whitney test).

correlation analyses, a significant association was detected for miR-31-3p ($p=0.039$; $r_s=0.443$) and miR-196a-5p ($p=0.008$; $r_s=0.548$) (Fig. 3). Correlation analysis of the rest miR-210-3p and miR-424-5p did not reach significance ($p=0.083$, $r_s=0.384$ and $p=0.477$, $r_s=0.160$).

miR-31-3p and miR-196a-5p as potential non-invasive biomarkers in LSCC disease

In order to explore the power of the most significantly deregulated miR-31-3p and miR-196a-5p in our study as potential plasmatic non-invasive biomarkers in laryngeal cancer, we performed ROC analysis of miRNAs alone

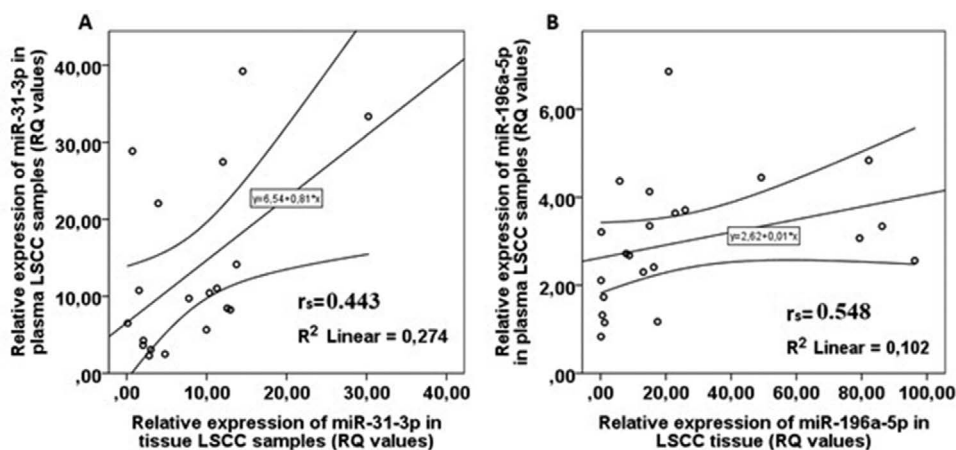


Figure 3. Correlation between investigated LSCC tissue and plasma samples (A) miR-31-3p, $r_s=0.443$, $p=0.039$; (B) miR-196a-5p, $r_s=0.548$, $p=0.008$.

and in combination of both miRNA markers as published previously.²⁰ MiR-31-3p showed better discrimination (AUC=0.934, 95%CI: 0.865–1.000, $p=0.001$) between LSCC cancer patients and control group than miR-196a-5p. At the optimal cut-off value RQ=2.96, sensitivity was 85.7% and specificity was 91.5%. miR-196a-5p plasma levels showed AUC=0.877, 95%CI: 0.775–0.978; $p<0.001$ with 77.3% sensitivity and 81.0% specificity at cut-off RQ value

equal to 2.10 as diagnostic biomarker in our study.

When using non-oncological control samples as calibrators, the combination of both miRNAs – miR-31-3p and miR-196a-5p – outperformed previous results of discriminative potential of miR-31-3p and miR-196a-5p alone (AUC=0.978, 95% CI: 0.945–1.000, $p<0.001$ with 100% sensitivity and 81% specificity at cut-off: RQ=2.99). The results are shown in Fig. 4.

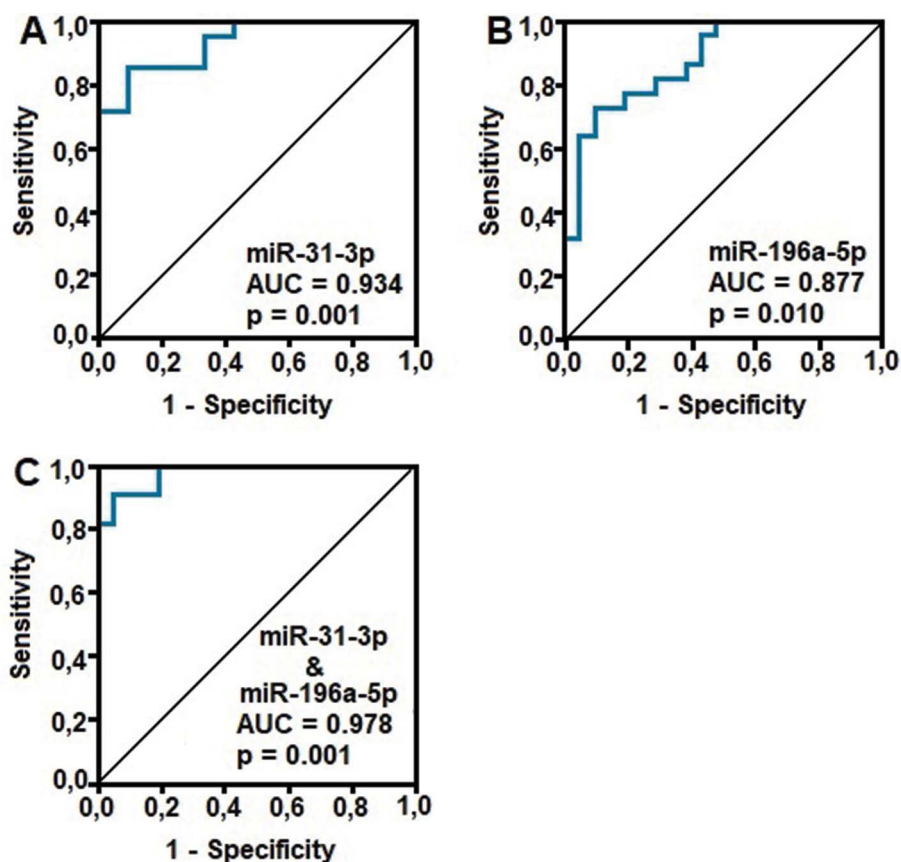


Figure 4. ROC analysis of (A) miR-31-3p (AUC=0.943, 95% CI: 0.865–1.000, $p=0.001$); (B) miR-196a-5p (AUC=0.877, 95% CI: 0.775–0.978, $p<0.001$); (C) Combination of miR-31-3p and miR-196a-5p (AUC=0.978, 95% CI: 0.945–1.000, $p<0.001$).

Association between studied miRNAs and clinicopathological characteristics of studied LSCC plasma samples

We performed non-parametric Mann-Whitney U test and Kruskal-Wallis test to determine whether the levels of the two plasma miR-31-3p and miR-196a-5p alone and

in combination (miR-31-3p and miR-196a-5p) can predict any of the included clinical and pathological characteristics of the LSCC patients. Results are presented in Table 1. But unfortunately, none of the miRNAs showed significant association, which suggest that they could not be used as markers in clinicopathological diagnosis of LSCC patients.

Table 1. Association between plasmatic miR-31-3p and miR-196a-5p with clinicopathological characteristics of the studied LSCC patients

Clinicopathological features	LSCC patients (n; %)	miR-31-3p expression	miR-196a-5p expression	miR-31-3p&miR-196a-5p expression
Gender				
Female	3 (13.64)	9.26±7.17	3.03±1.52	11.352±8.607
Male	19 (86.36)	11.41±9.45	3.42±0.91	14.256±12.861
		<i>p</i> =0.989	<i>p</i> =0.784	<i>p</i> =0.712
Age				
≤60	11 (50.00)	8.91±6.03	3.09±1.02	9.769±4.689
>60	11 (50.00)	13.46±11.37	2.95±1.89	17.950±15.998
		<i>p</i> =0.372	<i>p</i> =0.461	<i>p</i> =0.119
Tumour stage				
T3	11 (50.00)	11.76±8.66	2.94±1.29	11.784±7.745
T4	11 (50.00)	10.37±9.85	3.11±1.67	15.936±15.662
		<i>p</i> =0.933	<i>p</i> =0.534	<i>p</i> =0.440
Nodal stage				
N0	10 (45.45)	12.22±9.05	3.09±1.16	12.407±7.830
N1-3	12 (54.55)	10.08±9.34	2.96±1.73	15.071±15.246
		<i>p</i> =0.902	<i>p</i> =0.628	<i>p</i> =0.605
Tumour differentiation				
G1	7 (31.82)	13.27±8.28	2.67±1.54	12.632±7.452
G2	11 (50.00)	9.26±9.07	3.44±1.43	11.456±8.999
G3	4 (18.18)	12.29±12.66	2.30±1.21	22.618±23.264
		<i>p</i> =0.551	<i>p</i> =0.326	<i>p</i> =0.294
Tumour localization				
Glottis	10 (45.45)	13.38±10.42	2.82±1.21	12.201±7.551
Supraglottis	12 (55.55)	8.98±7.87	3.12±1.76	15.794±15.953
		<i>p</i> =0.425	<i>p</i> =0.823	<i>p</i> =0.514
Family history				
Deny	16 (72.73)	13.20±9.75	2.88±1.24	15.335±12.870
Report	6 (27.27)	5.85±3.98	3.38±1.97	9.926±10.344
		<i>p</i> =0.127	<i>p</i> =0.693	<i>p</i> =0.330
Tobacco smoking				
Deny	6 (27.27)	16.80±10.11	2.73±1.48	15.210±7.411
≤20 cigarettes per day	7 (31.82)	9.333±8.98	3.06±1.33	9.578±7.052
21 to 40 cigarettes per day	9 (41.00)	8.87±8.14	3.21±1.67	16.290±17.255
		<i>p</i> =0.333	<i>p</i> =0.895	<i>p</i> =0.549
Alcohol consumption				
Deny	6 (27.27)	16.80±10.11	2.73±1.48	15.210±7.411
≤100 mL per day	6 (27.27)	13.48±10.20	3.30±0.92	12.881±8.639
>100 mL per day	10 (45.45)	5.71±3.74	3.03±1.80	13.637±16.608
		<i>p</i> =0.620	<i>p</i> =0.676	<i>p</i> =0.949
Work exposures				
Deny	8 (36.37)	12.60±10.49	2.71±1.17	11.257±7.067
Report	14 (63.63)	10.18±8.33	3.21±1.61	15.347±14.444
		<i>p</i> =0.692	<i>p</i> =0.714	<i>p</i> =0.384

DISCUSSION

In this study, we evaluated the expression levels of four miRNAs: miR-31-3p, miR-196a-5p, miR-210-3p, and miR-424-5p plasma and tissue (tumour and normal) samples obtained from a group of patients (n=22), diagnosed with LSCC as well as in non-oncological control group (n=21). After literature search in archive databases (PubMed, Scholar, etc.), we chose miRNAs previously demonstrated as oncogenic miRNAs in tissue laryngeal cancer disease and malignant laryngeal cell lines²¹⁻²⁴, but was not previously studied as non-invasive biomarkers in patients with laryngeal neoplasms. In the current study for the first time, we presented the potential of some of the studied miRNAs as diagnostic biomarkers, alone and in combination.

In summary, we demonstrated that the plasma miRNA levels are in strong correlation with their expression in laryngeal tumour tissue in the same patient laryngeal group. Our results showed that all four miRNAs are upregulated in the majority of plasma samples obtained from LSCC patients but only two out of four (miR-31-3p and miR-196a-5p) are significantly upregulated in LSCC plasma in comparison to the non-oncological control group. Moreover, we show for the first time their potential as non-invasive biomarkers, as in combination they showed the best accuracy rate for discrimination of advanced LSCC with 100% sensitivity and 81% specificity.

Previously, miR-31 was investigated in patients with oral squamous cell carcinoma (OSCC) and control group as non-invasive biomarker in plasma and saliva for early detection of OSCC. Researchers found that miR-31 was highly abundant, its levels were reduced in body fluids after removal of the oral tumour, which makes it a marker of potential interest.²⁵ Elevated miR-31 was shown also in other squamous cell carcinoma as oesophageal squamous cell carcinoma. In the study it was demonstrated that miR-31 could be found in OSCC patients with AUC of 0.902 (95% CI: 0.857–0.936)²⁶, similar to the data from our ROC analysis results for miR-31-3p alone. In addition, we found that miR-31-3p in investigated plasma LSCC samples was found in almost all patients with relatively high expressions in comparison to the rest of the explored miRNAs, which is in line with the previous findings. Most of the authors do not specify which miRNAs they investigate, which could influence the precise knowledge about the miRNAs and their way of functioning. However, the authors from the study of Chang et al.²⁷, explored both miR-31 isoforms (-5p and -3p), and showed that miR-31-3p was less abundant than miR-31-5p, but still miR-31-3p had an oncogenic role in OSCC. Its target is 3'UTR of RhoA and this miRNA is able to downregulate RhoA expression which decreases both proliferation and migration of OSCC SAS and Fadu cell lines²⁷, which suggest its key role in tumorigenesis.

miR-31 has been also associated with therapy response in colorectal cancer (CRC) and laryngeal cell lines. Recently, the study of Daneberga et al.²⁸ showed that plasma miR-31 overexpression was associated with therapy response in

CRC, due to cancer cell autophagy, which process lead to high concentration of miR-31, whereas its overexpression could be a suitable non-invasive marker for cancer progression and therapy resistance. Also, circulating miR-31 was suggested as marker for metastasis and survival, but this finding is controversial due to other research studies and meta-analysis which do not validate this data.^{29,30} Previously miR-31-3p was investigated as one of the most significantly upregulated miRNA after paclitaxel treatment in laryngeal cancer cell line Hep-2 and was suggested as potential marker of resistance to drug treatment.³¹ Our results add to the knowledge about the potential role of circulating miR-31-3p in laryngeal carcinomas and show that preferentially miR-31-3p could be part of non-invasive panel set for LSCC screening.

The study of Saito et al.³² demonstrated that both miR-196a isoforms are the most useful markers for diagnostics and treatment of laryngeal cancer. Downregulation of miR-196a inhibited cancer cell proliferation in laryngeal cancer cell lines and xenograft model.³² Therefore, miR-196a could strongly promote laryngeal cancer growth and the inhibition of miR-196a effective to reduce in vivo growth of LSCC. Higher plasma miR-196a expression in oral cancer was recorded, in comparison to oral-cancer lesions and in combination with miR-196b was hypothesized as potential non-invasive marker for determination and prediction of potential malignancy with 91% sensitivity and 85% specificity.³³ The obtained discrimination accuracy of miR-196a, alone in this study was AUC=0.864³³, which is similar to our results for circulating miR-196a-5p in laryngeal plasma AUC=0.877.

The same combination of miR-196a and miR-196b was investigated also in gastric cancer (GC), where miR-196a alone exhibited higher discriminative potential than combined miR-196a/b, nevertheless the combination of both markers was found to be strongly associated with advanced GC stages, metastasis potential and poorer survival.³⁴ The data from both studies suggest that miR-196a and miR-196b levels alone or in combination may vary in different cancer diseases and need to be validated in larger samples.

The rest of the investigated miRNAs in our study, miR-210-3p and miR-424-5p, did not reach significantly different expression in the plasma LSCC samples in comparison to the non-oncological group. miR-210 is one of the most investigated “hypoxic” miRNA and its hypoxic association is shown in laryngeal disease.²¹ miR-210 under hypoxia decreases proliferation through inducing cell cycle arrest and apoptosis through targeting FGFRL1 (fibroblast growth factor receptor-like 1).²¹ However, in glioma cancers, circulating serum miR-210 was associated with high grade and poor patients outcome³⁵, but its non-invasive potential was not determined in LSCC.

Ectopic miR-424 expression was published previously in LSCC, which was related to the influence of p53 signalling pathway¹⁵, and the multidrug resistance was associated with upregulation of miR-424-3p²². Circulating miR-424 could be potential marker for early breast cancer³⁶, but

similarly to miR-210, miR-424-5p potential in LSCC has not been investigated in details yet.

Intriguingly, our results showed that miR-210-3p was significantly upregulated in tumour LSCC tissue in comparison to normal tissue, which may suggest its involvement in laryngeal cancerogenesis but was not significantly elevated in plasma LSCC samples in comparison to non-oncological control group. We did not find significant upregulation in plasma and tissue samples for miR-424-5p. Four volunteers from the control group with adjacent non-oncologic disease were found with elevated levels of miR-210-3p and miR-424-5p, which may contribute to the observed lack of significant differences between the expression in target plasma LSCC group and the control group. Aside from miR-210 and miR-424 cancer association, these miRNAs also influence inflammatory processes in autoimmune diseases and inflammatory mechanisms following ischemic stroke.³⁷⁻³⁹ Perhaps miR-424-5p and miR-210-3p, as validated oncogenic miRNAs in cancer disease, may have a notable role also in inflammatory pathways. Hence, we could suggest that expression of both miRNAs could not be considered specific for laryngeal cancer only. We should note that three volunteer were found with higher than 2.0 RQ level for miR-31-3p and two showed elevated levels for miR-196a-5p, but still the expression levels of plasma miR-31-3p and miR-196a-5p were much more elevated in the LSCC group, and we hypothesize their stronger cancer association. Unfortunately, the levels of miR-31-3p and miR-196a-5p could not be associated with any clinicopathological features, perhaps due to the small investigated group. Therefore, these pilot data could serve as a starting point for validation in larger cohorts.

CONCLUSIONS

In conclusion, the expressions of miR-31-3p and miR-196a-5p were significantly upregulated in laryngeal tumour samples as well as plasma samples of patients with LSCC, which suggests their involvement in laryngeal tumorigenesis. The combination of both circulating markers showed the highest accuracy rate for detection of advanced LSCC in our group. The levels of expression of the other investigated miR-210-3p and miR-424-5p did not differ between plasma samples of LSCC patients and controls and could not be used as non-invasive biomarkers in LSCC. Our pilot data could serve as a starting point for validation in larger cohorts.

Acknowledgments

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Пилотное исследование показывает потенциал miR-31-3p и miR-196a-5p в качестве неинвазивных биомаркеров при распространённом раке гортани

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Резюме

Введение: В последнее время микроРНК стали популярными молекулами, которые используются в качестве неинвазивных биомаркеров рака.

Цель: Целью исследования было изучить экспрессию четырёх изоформ микроРНК: miR-31-3p, miR-196a-5p, miR-210-3p и miR-424-5p в образцах ткани плазмы от пациентов с распространённой плоскоклеточной карциномой гортани (РПКГ) и здоровых людей.

Материалы и методы: Свежезамороженная опухоль и нормальная ткань гортани, а также образцы плазмы, взятые у 22 пациентов с диагнозом распространённого РПКГ. В контрольную группу вошли образцы плазмы от 21 добровольца, не поражённого раком. Выделение общей РНК (включая микроРНК), обратная транскрипция и qPCR в реальном времени были лабораторными методами, использованными в исследовании. Полученные результаты были проанализированы с помощью SPSS v. 23.

Результаты: Мы обнаружили, что уровни miR-31-3p, miR-196a-5p и miR-210-3p были значительно повышены в опухолевой ткани гортани, но только уровни miR-31-3p и miR-196a-5p были значительно выше в плазме целевой группы с РПКГ. Положительная корреляция была получена для miR-31-3p ($r_s=0.443$, $p=0.039$) и miR-196a-5p ($r_s=0.548$; $p=0.008$) между образцами плазмы и прилегающей опухолевой тканью из РПКГ. Анализы ROC использовали для оценки различительной способности обеих микроРНК в отдельности и в комбинации. Комбинация miR-31-3p и miR-196a-5p показала наилучшие результаты при AUC=0.978 (95% CI: 0.945–1.000, $p<0.001$) со 100% чувствительностью и предельной специфичностью (cut-off): RQ=2.99.

Заключение: На основании этого miR-31-3p и miR-196a-5p предлагаются в качестве потенциальных биомаркеров для валидации среди более крупных групп с РПКГ и могут быть включены в набор неинвазивных микроРНК для обнаружения РПКГ.

Ключевые слова

плоскоклеточный рак гортани, LSCC, миРНК, неинвазивный биомаркер, плазма