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ORIGINAL ARTICLE

# Novel insights into laryngeal squamous cell carcinoma from association study of aberrantly expressed miRNAs, lncRNAs and clinical features in Bulgarian patients

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## *Summary*

***Purpose:*** *Laryngeal cancer is one of most common and aggressive head and neck cancers with poor prognosis and great necessity for improvement of treatment modalities. MicroRNAs (miRs) are among the most investigated molecules recently due to their potential as diagnostic and prognostic biomarkers in cancer. The purpose of our study was to explore the association of certain clinicopathological features with the expression levels of some known cancer associated non-coding (nc) RNAs: miR-21 and miR-31 in both of their isoforms, miR-145-5p, miR-55-5p, miR-196a-5p, miR-210-3p, miR-221-3p, miR-222-3p, miR-424-5p, lncRNA MALAT1 and lncRNA HOTAIR.*

***Methods:*** *Expression levels of the chosen markers were investigated in laryngeal squamous cell carcinoma (LSCC) and normal samples in 82 Bulgarian patients via RT-qPCR, and the results were analyzed with SPSS v23.0 statistical software.*

***Results:*** *All of the explored ncRNAs were significantly deregulated in LSCC samples, suggesting their involvement in laryngeal carcinogenesis. New significant association were found between the expression levels of miR-21-5p, miR-222-3p, HOTAIR and family history. Moreover, miR-424-5p showed potential as marker for subglottic LSCC location, and "passenger" miR-31-3p was significantly upregulated in well and moderately differentiated LSCC.*

***Conclusion:*** *Our results enrich the knowledge about ncRNA involvement in LSCC tumorigenesis. Further studies are needed to evaluate the clinical utility of the differently expressed ncRNAs as potential diagnostic and prognostic biomarkers in LSCC.*

***Key words:*** *expression, laryngeal cancer, lncRNAs, miRNAs, novel insights*

## Introduction

Worldwide, cancer of the larynx is the eleventh most common of all malignant neoplasms in men (GLOBOCAN 2012, IACR) [1]. According to the National Cancer Registry of Bulgaria for 2015, 554 (3.1%) patients were diagnosed and 343 (3.3%) were the registered deaths from laryngeal neoplasm [2], which places Bulgaria on the 9th place of laryngeal cancer incidence and mortality in Europe, with 8.9 incidence cases per 100 000 (GLOBOCAN 2018, IACR) [3]. This data shows that Bulgaria has one of the highest morbidity rates in Europe, indicating the social significance of this disease. The clinical features depending on the laryngeal tumour location may vary very early, which is a prerequisite for early diagnosis and good prognosis, but still laryngeal cancer often is diagnosed in advanced stage (III or IV stage) [4], as patients neglect their symptoms. Despite modern medical techniques of cancer surgery and treatment, laryngeal cancer is still a challenging disease with not significantly

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changed 5-year survival for decades, which account about 60% of diagnosed patients [5]. Novel molecular genetic markers are required in order to improve clinical LSCC diagnosis and prognosis.

With the discovery of the oncogenes and tumour supressors involvement in long non-coding RNAs (lncRNAs) [6] and miRs [7] in the early 2000s, it was postulated their potential role as biomarkers due to their deregulation associated with various cellular processes in neoplasms [8]. MiRs are responsible for the expression of two mature miRs: one from the 5' strand and one from the 3' strand of the precursor (miR-5p and miR-3p). However, one of the isoforms, called the "guide" strand, is usually much more prevalent and more biologically active than the other isoform, the "passenger" strand, which is known as miR* [9]. Changes in the expression of these RNA molecules contribute to the formation and progression of cancer and have a key role in tumour microenvironment [10]. Classic example is the article of Medina et al [11] in 2010, which proves that overexpression of only one (miR-21) is enough to give rise to malignant tumours. Additionally, when inhibited, the same tumour lesions regresess - i.e. miR tumour "dependency" was first described [11].

However, in people, the latest release of the miR database (miRBase) has catalogued 1 917 precursors and 2 654 miRNAs (miRBase release 22.1, update October 2018) [12], whereas the number of annotated lncRNAs in NONCODE database (v5) is 172 216 transcripts, coded by 96 308 genes [13]. Despite the great number of research projects, exploring the clinical significance of these ncRNAs, the functional importance of many of those remains to be determined. Understanding the non-coding RNA world, which represents a gold mine for novel biomedical markers and therapeutic strategies, is one of the most important challenges in front of the molecular biology and biomedicine.

The aim of this study was to explore further the role of ncRNAs and their potential as biomarkers in LSCC. We investigated the expression levels of 13 ncRNAs known to be deregulated ncRNAs in cancers, chosen from the literature database searching (PubMed, Scholar, etc.) in laryngeal specimens. Eleven miRs isoforms: miR-21, miR-31, miR-145-5p, miR-155-5p, miR-196a-5p, miR-210-3p, both miR-221-3p/222-3p and miR-424-5p, and two lncRNAs: lncRNA MALAT1 (Metastasis-associated lung adenocarcinoma transcript 1) and lncRNA HOTAIR (Hox antisense intergenic RNA). MiR-21 and miR-31 were investigated in both isoforms (3p and 5p) due to lack of sufficient knowledge about the function of "passegner" isoforms: miR-21-3p and miR-31-3p.

## Methods

*Patients and tissue samples*

Fresh-frozen tumour and normal tissue was obtained from a total of 82 patients diagnosed with LSCC and enrolled in the current study. Patients were recruited at the Ear, Nose and Throat Department, University Hospital "Queen Joanna - ISUL" Sofia during 2012-2016. Informed consent was obtained from each patient, and the current study was approved by the Ethics Committee of Medical University of Sofia with protocols no. 13/23.04.2015, no./22.04.2016, no.432/2017 and no.435/2017. Samples were stored at -80°C until use at the Molecular Medicine Center biobank at the Department of Medical Chemistry and Biochemistry, Medical University – Sofia. Additional material from formalin-fixed, paraffin-embedded (FFPE) tissue was used to conduct immunohistochemical analysis to determine the clinicopathological characteristics of the samples. None of the patients in this study had received chemotherapy or radiotherapy before surgery.

*Isolation of total RNA from tissue material and reverse transcription*

Total RNA was isolated from fresh frozen tumour and normal tissue using RNA extraction kit from tissue (miRNeasy Micro Kit, Qiagen, Hilden, Germany). The quality and quantity of the total RNA samples were assessed by denaturing electrophoresis on a formaldehyde gel and NanoDrop 2000 (ThermoFisher Scientific, Walmington, DE, USA).

500 ng total RNA of each sample were used to prepare cDNA by using miScript II RT Kit and RT$^2$ First Strand Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Afterward, reverse transcription (RT) samples were processed further or stored at -20°C until use.

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

The expression of mature miRs and lncRNAs was assayed using miScript SYBR Green PCR kit and RT$^2$ SYBR Green Mastermix (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 11 mature miRs: miR-21-3p, miR-21-5p, miR-31-3p, miR-31-5p, miR-145-5p, miR-155-5p, miR-196a-5p, miR-210-3p, miR-221-3p, miR-222-3p and miR-424-5p. The RT$^2$ lncRNA qPCR assays (Qiagen, Hilden, Germany) were used for 2 long non-coding RNAs: lncRNA MALAT1 and lncRNA HOTAIR. Each reaction was performed in triplicate in a total volume of 10μL, according to the manufacturer's protocol. Expression levels of miRs were normalized to the internal control RNU6-2 (Qiagen, Hilden, Germany) and for lncRNAs Actin mRNA (Qiagen, Hilden, Germany) was used as control. The relative quantification (RQ) of miRs and lncRNAs in tumour samples was analyzed by the 2-ΔΔCt method, as previously described [14]. A RQ ≥2 was defined as overexpression, RQ <0.5 as underexpression, and RQ between 1.99 and 0.5 as no change in expression.

*Statistics*

Data analysis was performed with the SPSS software version 23.0 for Windows (IBM SPSS, NY, USA). The expression levels of the studied miRs were evaluated in 82 laryngeal tumour and adjacent normal laryngeal squamous cell tissue specimens, whereas lncRNA MALAT1 and lncRNA HOTAIR were evaluated in 63 tumour and adjacent normal laryngeal samples. Kolmogorov-Smirnov test for normality, Wilcoxon test, Mann-Whitney U test, Kruskal-Wallis or one-way ANOVA test, paired and unpaired T-tests were used as appropriate. A value of $p<0.05$ was considered statistically significant.

## Results

In the current study, 4 females and 78 males, in total 82 LSCC patients, were included. The mean age of the patient group was 61 years (range 41-84). Clinicopathological characteristics of the patients are shown in Tables 1a and 1b together with the expression levels of the explored ncRNAs.

*Relative expression in laryngeal tumour samples*

All of the investigated ncRNAs in LSCC tissue samples were significantly differentially expressed in tumour tissue in comparison to paired normal tissue. However, the relative expression levels of miR-21-3p ($p<0.001$), miR-21-5p ($p<0.001$), miR-31-3p ($p=0.004$), miR-31-5p ($p<0.001$), miR-155-5p ($p=0.003$), miR-210-3p ($p=0.025$), miR-221-3p ($p=0.001$), miR-222-3p ($p=0.001$), miR-196a-5p ($p<0.001$) and lncRNA HOTAIR ($p=0.004$) were significantly upregulated, whereas miR-145-5p ($p<0.001$) and lncRNA MALAT1 ($p<0.001$) were significantly downregulated in laryngeal tumour tissue in comparison to normal tissue. Reversed and normalized dCt values are shown in boxplots (Figure 1). Depending on the normality distribution tests of the samples, Wilcoxon test or paired T-test were used to evaluate the level of significance of the expression differences between tumour and normal laryngeal tissue.

*Association between ncRNA expression levels and clinicopathological characteristics*

Association between RQ data of the studied ncRNAs and the groups based on the clinicopathological characteristics of the LSCC patients is summarized in Tables 1a and 1b. Our data indicates statistically significant association between the expression levels of miR-21-5p, miR-31-3p, miR-31-5p, miR-145-5p, miR-222-3p, miR-424-5p, lncRNA MALAT1, lncRNA HOTAIR and different clinical features. MiR-21-5p showed statistically significant association with tumour differentiation ($p=0.036$), positive family history ($p=0.050$) and work exposures ($p=0.046$). MiR-31-3p was associated with positive nodal metastasis ($p=0.009$) and tumour differentiation ($p=0.034$). The expression

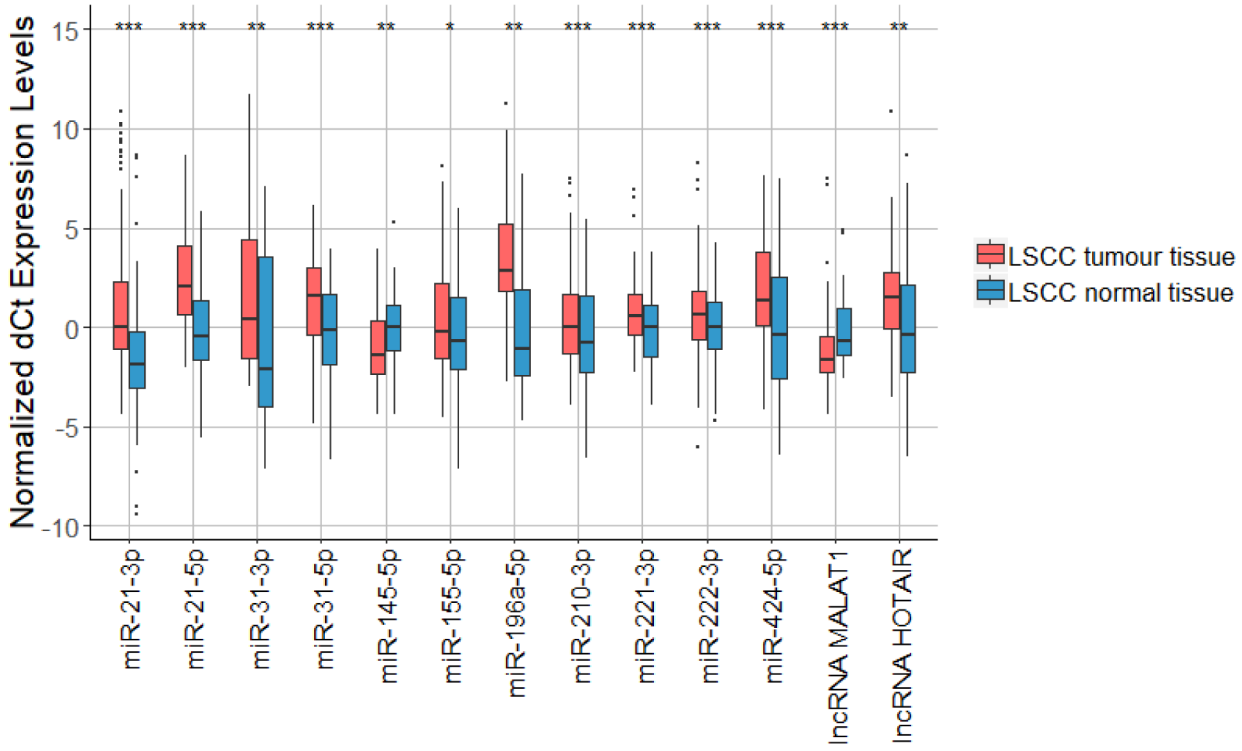


**Figure 1.** Normalized dCt expression levels of investigated ncRNAs in laryngeal tumour and normal tissue with levels of significance: *p<0.05; **p<0.01; ***p<0.001.

**Table 1a.** Association between mean RQ levels of miR-21 and miR-31 isoforms, miR-145-5p, miR-155-5p, miR-196a-5p and clinicopathological characteristics in patients with LSCC

| *Clinical features* | *Number of patients (%)* | *Relative expression of miR-21-3p (mean±SD)* | *Relative expression of miR-21-5p (mean±SD)* | *Relative expression of miR-31-3p (mean±SD)* | *Relative expression of miR-31-5p (mean±SD)* | *Relative expression of miR-145-5p (mean±SD)* | *Relative expression of miR-155-5p (mean±SD)* | *Relative expression of miR-196a-5p (mean±SD)* |
|---|---|---|---|---|---|---|---|---|
| Gender | | p=0.666 | p=0.613 | p=0.754 | p=0.797 | p=0.178 | p= 0.393 | p= 0.314 |
| Female | 4 (7.88) | 5.000 ± 0.169 | 6.055 ± 2.863 | 6.750±8.131 | 6.825±8.167 | 0.248 ± 0.061 | 0.946 ± 0.964 | 48.606±67.352 |
| Male | 78 (95.12) | 6.468 ± 6.345 | 8.166 ± 9.434 | 7.390±13.956 | 6.939±10.384 | 1.020 ± 1.331 | 4.478 ± 6.353 | 32.979±39.498 |
| Age, years | | p=0.446 | p=0.902 | p=0.380 | p=0.305 | p=0.738 | p=0.313 | p=0.314 |
| ≤60 | 49 (59.76) | 6.926 ± 5.161 | 8.575 ± 9.131 | 7.950±10.949 | 8.771±11.134 | 0.815 ± 0.879 | 5.925 ± 7.382 | 42.274±47.878 |
| >60 | 33 (40.24) | 6.104 ± 6.908 | 8.372±10.610 | 6.432±13.117 | 6.202±10.153 | 0.563 ± 0.521 | 3.097 ± 4.264 | 27.731±33.167 |
| Tumour stage | | p=0.301 | p=0.832 | p=0.985 | p=0.048 | p=0.035 | p=0.642 | p=0.746 |
| T1/2 | 11 (13.41) | 4.065 ± 2.597 | 7.284 ± 4.976 | 9.518±19.366 | 2.007±1.870 | 1.253 ± 1.408 | 3.957 ± 4.092 | 23.635±38.040 |
| T3 | 28 (34.15) | 7.902 ± 6.481 | 8.681±11.655 | 6.303±8.884 | 6.643±9.745 | 0.741 ± 0.684 | 6.390 ± 8.151 | 40.030±46.452 |
| T4 | 43 (52.45) | 5.976 ± 6.579 | 7.482 ± 9.518 | 7.589±15.376 | 9.443±8.121 | 0.459 ± 0.319 | 2.752 ± 3.410 | 29.967±39.456 |
| Nodal stage | | p=0.957 | p=0.834 | p=0.009 | p=0.805 | p=0.007 | p=0.948 | p=0.597 |
| N0 negative | 56 (68.29) | 6.294 ± 5.514 | 8.238 ± 7.618 | 8.749±15.585 | 6.858±10.090 | 1.102 ± 1.315 | 4.645 ± 6.233 | 31.126±39.661 |
| N1-3 positive | 26 (30.71) | 6.838 ± 8.209 | 6.441 ± 4.603 | 3.319±3.568 | 8.318±12.071 | 0.485 ± 0.502 | 3.006 ± 4.385 | 40.639±41.387 |
| Tumour differentiation | | p=0.429 | p=0.036 | p=0.034 | p=0.797 | p=0.722 | p=0.374 | p=0.219 |
| G1 well differentiation | 31 (37.80) | 4.410 ± 4.326 | 7.421 ± 8.011 | 8.553±19.122 | 7.163±10.175 | 0.790 ± 0.789 | 4.066 ± 5.130 | 26.399±35.983 |
| G2 moderate differentiation | 40 (48.78) | 8.233 ± 7.365 | 7.352 ± 6.491 | 7.314±9.642 | 7.602±11.497 | 0.725 ± 0.781 | 4.465 ± 6.668 | 40.863±43.937 |
| G3 poor differentiation | 11 (13.41) | 4.332 ± 3.471 | 15.480±8.724 | 1.535±2.209 | 4.791±4.346 | 0.232 ± 0.158 | 3.300 ± 1.863 | 16.216±10.771 |
| Tumour localization | | p=0.259 | p=0.376 | p=0.288 | p=0.516 | p=0.288 | p=0.821 | p=0.641 |
| Supraglottis | 39 (47.56) | 6.361 ± 7.414 | 7.375 ± 8.064 | 4.205±4.257 | 5.327±7.014 | 0.423 ± 0.418 | 2.986 ± 4.554 | 33.632±39.336 |
| Glottis | 45 (54.87) | 6.437 ± 6.284 | 7.686 ± 6.717 | 9.061±16.736 | 8.250±11.889 | 0.819 ± 0.781 | 5.495 ± 6.750 | 35.248±44.407 |
| Subglottis | 8 (9.75) | 6.590 ± 2.278 | 9.181 ± 6.050 | 7.544±14.361 | 7.334±12.024 | 0.957 ± 1.153 | 1.636 ± 1.299 | 25.816±16.863 |
| Family history | | p=0.370 | p=0.050 | p=0.653 | p=0.721 | p=0.141 | p=0.705 | p=0.987 |
| Deny | 63 (60.97) | 6.399 ± 6.681 | 5.354 ± 3.852 | 7.804±14.941 | 6.765±10.574 | 0.715 ± 0.696 | 3.675 ± 4.450 | 34.281±42.603 |
| Report | 19 (23.18) | 6.532 ± 4.880 | 9.167 ± 8.046 | 6.085 ± 9.721 | 8.591±10.675 | 0.711 ± 0.957 | 5.849 ± 8.637 | 31.400±32.158 |
| Missing data | 13 (15.85) | | | | | | | |
| Tobacco smoking | | p=0.744 | p=0.205 | p=0.516 | p=0.687 | p=0.048 | p=0.678 | p=0.920 |
| Deny | 4 (4.87) | 5.700 ± 2.880 | 3.910 ± 3.631 | 3.166 ± 3.085 | 4.673 ± 6.161 | 1.450 ± 1.753 | 2.431 ± 1.979 | 12.344±13.935 |
| ≤20 cigarettes per day | 26 (31.70) | 7.316 ± 6.702 | 10.148±7.889 | 9.599±17.740 | 6.543±5.778 | 0.950 ± 0.913 | 4.846 ± 5.412 | 40.942±49.079 |
| 21< ≥40 cigarettes per day | 30 (36.58) | 5.866 ± 5.075 | 6.522 ± 4.110 | 5.356±10.160 | 9.361±15.564 | 0.653 ± 0.435 | 2.851 ± 3.694 | 38.790±43.680 |
| ≥41 cigarettes per day | 13 (15.85) | 7.007 ± 8.722 | 7.604 ± 9.332 | 4.294 ± 3.197 | 7.144±11.791 | 0.318 ± 0.291 | 5.408 ± 8.979 | 20.553±22.720 |
| Missing data | 9 (10.97) | | | | | | | |
| Alcohol consumption | | p=0.600 | p=0.768 | p=0.714 | p=0.754 | p=0.295 | p=0.278 | p=0.670 |
| Deny | 13 (15.85) | 5.062 ± 3.224 | 6.916 ± 4.696 | 6.332±12.692 | 6.922±10.650 | 0.758 ± 1.054 | 4.775 ± 9.738 | 16.235±16.313 |
| <100 mL per day | 20 (24.39) | 7.173 ± 5.025 | 10.280±9.674 | 7.622±10.690 | 6.052 ± 5.113 | 0.840 ± 0.962 | 3.678 ± 5.049 | 41.079±46.826 |
| 100 - 200 mL per day | 19 (23.17) | 5.697 ± 4.826 | 8.944 ± 7.814 | 9.117±19.506 | 8.686±14.673 | 0.627 ± 0.518 | 3.543 ± 4.194 | 34.454±52.613 |
| >200 mL per day | 17 (20.73) | 6.727 ± 7.886 | 6.548 ± 5.371 | 4.190 ± 4.886 | 9.866±12.675 | 0.811 ± 0.793 | 5.571 ± 5.112 | 38.359±33.988 |
| Missing data | 13 (15.85) | | | | | | | |
| Work exposures | | p=0.258 | p=0.046 | p=0.081 | p=0.794 | p=0.666 | p=0.863 | p=0.481 |
| Deny | 46 (40.25) | 7.283 ± 7.225 | 5.594 ± 3.711 | 5.818 ± 3.911 | 7.827±11.751 | 0.827 ± 1.154 | 3.468 ± 4.022 | 27.671±32.926 |
| Report | 36 (43.90) | 5.334 ± 4.558 | 10.473±7.386 | 8.404±13.510 | 6.458 ± 8.897 | 1.110 ± 1.307 | 5.210 ± 7.534 | 41.138±47.161 |
| Missing data | 13 (15.85) | | | | | | | |

**Table 1b.** Association between mean RQ levels of miR-210-3p, miR-221-3p, miR-222-3p, miR-424-5p, lncRNA MALAT1, lncRNA HOTAIR and clinicopathological characteristics in patients with LSCC

| *Clinical features* | *Number of patients* (%) | *Relative expression of miR-210-3p (mean±SD)* | *Relative expression of miR-221-3p (mean±SD)* | *Relative expression of miR-222-3p (mean±SD)* | *Relative expression of miR-424-5p (mean±SD)* | *Relative expression of lncRNA MALAT1 (mean±SD)* | *Relative expression of lncRNA HOTAIR (mean±SD)* |
|---|---|---|---|---|---|---|---|
| Gender | | p=0.386 | p=0.829 | p=0.023 | p=0.795 | p= 0.077 | p=0.060 |
| Female | 4 (7.88) | 2.379 ± 0.585 | 1.926 ± 0.807 | 1.566 ± 0.828 | 6.731 ± 1.595 | 0.209 ± 0.113 | 2.529 ± 3.507 |
| Male | 78 (95.12) | 3.167 ± 3.975 | 2.691 ± 3.036 | 3.502 ± 5.577 | 9.906 ± 12.037 | 0.739 ± 0.986 | 6.472 ± 8.638 |
| Age, years | | p=0.574 | p=0.817 | p=0.052 | p=0.817 | p=0.024 | p=0.130 |
| ≤60 | 49 (59.76) | 2.369 ± 2.959 | 2.541 ± 2.629 | 3.189 ± 3.970 | 10.071 ± 10.851 | 1.076 ± 1.008 | 7.674 ± 9.580 |
| >60 | 33 (40.24) | 3.651 ± 4.394 | 2.878 ± 3.208 | 5.035 ± 4.118 | 9.604 ± 12.601 | 0.634 ± 1.123 | 5.432 ± 7.762 |
| Tumour stage | | p=0.383 | p=0.144 | p=0.857 | p=0.873 | p=0.072 | p=0.918 |
| T1/2 | 11 (13.41) | 1.586 ± 1.650 | 2.002 ± 1.058 | 1.701 ± 1.711 | 6.626 ± 4.683 | 0.431 ± 0.648 | 3.576 ± 4.429 |
| T3 | 28 (34.15) | 3.031 ± 3.872 | 1.477 ± 0.877 | 4.798 ± 5.265 | 11.382 ± 11.620 | 0.975 ± 1.381 | 6.437 ± 8.644 |
| T4 | 43 (52.45) | 3.603 ± 4.299 | 5.002 ± 7.985 | 2.890 ± 3.763 | 9.446 ± 13.249 | 0.514 ± 0.442 | 6.940 ± 9.271 |
| Nodal stage | | p=0.562 | p=0.743 | p=0.597 | p=0.012 | p=0.915 | p=0.392 |
| N0 negative | 56 (68.29) | 3.128 ± 3.865 | 3.281 ± 5.744 | 2.456 ± 6.062 | 9.288 ± 13.175 | 0.696 ± 1.043 | 5.582 ± 7.271 |
| N1-3 positive | 26 (30.71) | 3.166 ± 4.169 | 4.026 ± 8.231 | 3.361 ± 4.757 | 4.733 ± 3.663 | 0.598 ± 0.507 | 8.515 ± 11.493 |
| Tumour differentiation | | p=0.795 | p=0.126 | p=0.139 | p=0.689 | p=0.481 | p=0.243 |
| G1 well differentiation | 31 (37.80) | 2.989 ± 3.131 | 5.251 ± 9.919 | 4.618 ± 5.429 | 7.333 ± 10.337 | 0.558 ± 0.514 | 4.028 ± 5.220 |
| G2 moderate differentiation | 40 (48.78) | 3.355 ± 4.601 | 2.303 ± 2.078 | 2.672 ± 3.158 | 7.787 ± 8.934 | 0.790 ± 1.179 | 7.801 ± 10.093 |
| G3 poor differentiation | 11 (13.41) | 3.076 ± 1.836 | 2.879 ± 1.811 | 2.905 ± 1.447 | 9.901 ± 16.039 | 0.371 ± 0.288 | 7.366 ± 8.989 |
| Tumour localization | | p=0.886 | p=0.702 | p=0.293 | p=0.015 | p=0.530 | p=0.417 |
| Supraglottis | 39 (47.56) | 3.141 ± 3.956 | 1.961 ± 1.734 | 1.796 ± 1.273 | 4.216 ± 4.125 | 0.648 ± 0.457 | 9.265 ± 10.490 |
| Glottis | 45 (54.87) | 2.873 ± 3.385 | 4.577 ± 8.278 | 4.729 ± 5.327 | 9.661 ± 13.161 | 0.746 ± 1.180 | 3.978 ± 5.034 |
| Subglottis | 8 (9.75) | 4.403 ± 6.023 | 2.240 ± 1.578 | 1.659 ± 1.182 | 12.023 ± 6.780 | 0.393 ± 0.434 | 9.610 ± 12.960 |
| Family history | | p=0.952 | p=0.917 | p=0.015 | p=0.987 | p=0.868 | p=0.031 |
| Deny | 63 (60.97) | 3.117 ± 3.850 | 3.181 ± 5.769 | 2.567 ± 4.022 | 9.541 ± 11.922 | 0.703 ± 1.027 | 4.748 ± 5.678 |
| Report | 19 (23.18) | 3.201 ± 4.209 | 4.319 ± 8.139 | 4.754 ± 5.408 | 10.522 ± 11.963 | 0.577 ± 0.593 | 10.145 ±11.211 |
| Missing data | 13 (15.85) | | | | | | |
| Tobacco smoking | | p=0.453 | p=0.279 | p=0.499 | p=0.174 | p=0.086 | p=0.275 |
| Deny | 4 (4.87) | 6.894 ± 8.849 | 3.295 ± 1.822 | 2.282 ± 1.605 | 10.673 ± 9.034 | 0.123 ± 0.087 | 4.606 ± 6.775 |
| ≤20 cigarettes per day | 26 (31.70) | 3.567 ± 4.494 | 4.302 ± 7.917 | 4.297 ± 5.803 | 14.022 ± 16.123 | 0.939 ± 1.419 | 5.515 ± 9.937 |
| 21< ≥40 cigarettes per day | 30 (36.58) | 2.502 ± 2.600 | 2.329 ± 2.162 | 2.947 ± 4.104 | 8.976 ± 9.986 | 0.653 ± 0.532 | 7.411 ± 7.146 |
| ≥41 cigarettes per day | 13 (15.85) | 3.151 ± 3.214 | 4.525 ± 9.297 | 3.725 ± 5.382 | 4.888 ± 5.974 | 0.395 ± 0.255 | 4.005 ± 4.067 |
| Missing data | 9 (10.97) | | | | | | |
| Alcohol comsumption | | p=0.146 | p=0.154 | p=0.506 | p=0.892 | p=0.108 | p=0.391 |
| Deny | 13 (15.85) | 2.990 ± 5.342 | 2.046 ± 1.383 | 2.257 ± 1.679 | 9.990 ± 6.572 | 0.758 ± 1.054 | 5.317 ± 6.982 |
| <100 mL per day | 20 (24.39) | 2.590 ± 1.912 | 5.295 ± 10.256 | 4.674 ± 4.949 | 8.335 ± 8.433 | 0.946 ± 0.997 | 9.981 ± 12.399 |
| from 100 to 200 mL per day | 19 (23.17) | 3.484 ± 3.992 | 2.634 ± 2.473 | 3.532 ± 4.810 | 11.953 ± 14.090 | 1.067 ± 1.356 | 3.469 ± 4.847 |
| >200 mL per day | 17 (20.73) | 4.272 ± 4.749 | 5.222 ± 9.002 | 4.286 ± 5.121 | 12.632 ± 18.264 | 1.223 ± 1.832 | 5.464 ± 4.533 |
| Missing data | 13 (15.85) | | | | | | |
| Work exposures | | p=0.501 | p=0.620 | p=0.020 | p=0.455 | p=0.302 | p=0.218 |
| Deny | 46 (40.25) | 3.001 ± 3.456 | 2.370 ± 1.858 | 2.159 ± 3.033 | 9.090 ± 10.143 | 0.727 ± 1.154 | 5.614 ± 8.037 |
| Report | 36 (43.90) | 3.315 ± 4.440 | 4.893 ± 9.363 | 5.076 ± 7.727 | 10.696 ± 13.886 | 0.599 ± 0.542 | 7.251 ± 9.202 |

levels of miR-31-3p were higher in patients positive for work exposures but statistical significance was not reached (p=0.081). We found significant association between miR-31-5p (p=0.048) and miR-145-5p (p=0.035) and tumor stage. MiR-145-5p was significantly decreased with progression of T stage (p=0.035), positive nodal metastasis (p=0.007), and heavy tobacco smoking (p=0.050). MiR-222-3p was significantly associated with gender (p=0.023), positive family history (p=0.015), work exposures (p=0.020), and borderline associated with age (p=0.052). Decreased expression of miR-424-5p was associated with positive nodal metastasis (p=0.012), and a trend was shown for association with tumor localization (p=0.015), as higher expression was reached in subglottic tumors. lncRNA MALAT1 was positively associated with age (p=0.024), whereas lncRNA HOTAIR with positive family history (p=0.031). However, miR-21-3p, miR-155-5p, miR-196a-5p, miR-210-3p and miR-221-3p did not show any significant association with the explored clinical features.

## Discussion

The aim of the current study was to explore and analyze the association between the expression levels of certain isoforms of commonly deregulated miRNAs and lncRNAs and the clinicopathological features of Bulgarian patients with laryngeal squamous cell carcinoma.

Development of LSCC involves various genetic and epigenetic changes, including aberrant expression of ncRNAs, including miRs and lncRNAs. NcRNAs have an important, but still widely uncovered regulatory role in adjusting the canonical cascades of laryngeal carcinogenesis. The obtained results showed statistically different expression of all studied ncRNAs in LSCC tumour tissue in comparison to adjacent normal samples, which suggests their involvement in laryngeal carcinogenesis. Still most promising miRs associated with patient features were: miR-21-5p, miR-31-3p, miR-31-5p, miR-145-5p, miR-222-3p, miR-424-5p, and lncRNA HOTAIR.

Intriguing for the first time, we found that the expression levels of miR-21-5p, miR-222-3p and lncRNA HOTAIR were statistically significantly associated with positive family cancer history of the LSCC patients. Family history is an important risk factor in cancer, however knowledge about ncRNA expression related to family history is still scarce. In our study, LSCC patients who were positive to family cancer history, reported other solid tumours, including head and neck cancers (thyroid, lip, throat), lung, breast, bladder, kidney, uterus, stomach, prostate, leukemia, duodenum and colorectal cancer in their relatives. We suggest that the expression levels of miR-21-5p, miR-222-3p and lncRNA HOTAIR is potentially associated with aberrantly expressed target mRNA. One of the common targets of miR-21 and miR-222 is PTEN, which often is deregulated in various tumours, including head and neck neoplasms, due to somatic mutation, deletions or epigenetic silencing [15], and is associated with family cancer [16]. In addition, two lncRNA HOTAIR polymorphisms are published, rs7958904C>G and rs4759314G>A, that lead to higher expression associated with other family solid tumours [17]. Previously, miRs expression in patients with and without family breast cancer was explored, but statistically different expression was not found, including miR-21 [18], whereas in another study miR-155 was found significantly deregulated in patients with family lung cancer [19]. Our data could promote additional investigation on aberrant expression of promising ncRNAs in cases with family cancer history.

In addition to previous findings, miR-222-3p expression levels were marginally positively associated with aging, whereas lncRNA MALAT1 was significantly negatively associated with age. MiR-222 is reported as "aging miRNA" [20], whereas downregulation of lncRNA MALAT1 in relation to age was not published previously and deserves further investigation. The factor "age" should also be taken into account when comparing results for these ncRNAs between studies, in addition to ethnicity and clinical characteristics. MiR-222-3p, X-chromosome-related miR, has been previously reported to be associated with gender. Elevated expression was observed in male compared to female patients in a study of Khalifa et al in 2016. Significant relationship between FOXP3 promoter polymorphism rs3761548A/C, the expression of miR-222 and gender effect was detected [21]. lncRNA MALAT1 expression levels were not associated previously with gender, but still both markers should be validated additionally in larger female LSCC group.

MiR-424-5p was upregulated in LSCC patients with positive and negative node status, but the results showed significant negative association with positive nodal metastasis group. Our flinging is in line with previously described decreased expression levels of miR-424-5p in nodal metastatic esophageal squamous cell carcinoma (ESCC) and epithelial ovarian cancer [22,23]. However, we identified for the first time strong association between the expression levels of miR-424-5p and laryngeal tumour location. We showed gradual increase in the expression level from supraglottis, through glottis and subglottis. Subglottic nodal metastases are not common in LSCC and they are less metastatic

tumours in comparison to supraglottic, which are more lymph-enriched [24]. We could assume that marked overexpression of miR-424-5p could be potential protective marker for nodal metastasis and invasion. The other isoform, miR-424-3p, is reported as one of the most positively correlated supraglottic miRs [25], whereas we investigated miR-424-5p in all LSCC locations, and found its expression was significantly negatively associated with supraglottic laryngeal location. We believe that aberrant miR-424 isoforms expression could potentially be associated with LSCC specific location, and could be used as surrogate localization marker in clinical practice.

In the current study, miR-31-3p and miR-145-5p levels were negatively associated with nodal metastasis. Moreover, miR-145-5p was downregulated with elevated T-stage, and heavy smoking, while the "active" isoform miR-31-5p was overexpressed in advanced T-stage tumors. Paclitaxel sensitivity and drug resistance are related to aberrantly expressed miR-31 in investigated LSCC patients [26], and colorectal cancer (CRC) patients [27], whereas downregulation of miR-145 is found to be related with development and progression of CRC and increases the sensitivity to cetuximab in CRC independently of KRAS status through downregulating BCL2 expression [28,29]. These two miRs could be suggested as potential markers of treatment and prognosis.

miR-21 and miR-31 are reported as aberrantly expressed miRs due to environment exposure, tobacco smoking and the development of cancer [30]. Our results confirm more specifically miR-21-5p isoform association with harmful work exposures, whereas the expression levels of miR-31-3p isoform were higher in patients positive for harmful work exposures, but could not reach statistical significance. In addition, the positively associated miR-222 in LSCC patients who report work exposures (p=0.020) is in line with a previous report by Bollati et al in 2010. They investigated the changes in the expression levels of miR-222 as candidate biomarker in peripheral blood leukocytes obtained from 63 postexposure workers [31].

Our data indicated elevated expression of miR-21-5p associated with LSCC poor tumour differentiation, whereas on the contrary, upregulated levels of "passenger" miR-31-3p were more typical for well and moderately differentiated LSCC, suggesting its strong oncogenic activity during early stages of carcinogenesis. Our findings are in agreement with previously published data about miR-31, but not miR-21 association with tumour differentiation in verrucous head and neck cancer [32]. Both, miR-21 and miR-31, are among the most investigated miRs, widely expressed in most SCCs, including laryngeal cancer [33], but still the knowledge about their isoform association with different clinicopathological features in LSCC is elusive.

The rest of the investigated miR isoforms (miR-21-3p, miR-155-5p, miR-196a-5p, miR-210-3p and miR-221-3p) were aberrantly expressed in LSCC tumor tissue, but were not associated with any of the clinicopathological features.

Recent studies reported that miR-21-3p is an oncogene which is highly associated with microsatellite instability and promotes cellular mobility through epithelial-mesenchymal transition [34], and is shown to be significantly associated with the depth of tumor invasion, nodal metastasis and clinical stage in gastric cancer [35]. Previously, miR-155 was investigated in 63 laryngeal tumor specimens and the results showed significant positive correlation to T-stage and poor tumor differentiation, and it was suggested as oncogene promoting laryngeal invasiveness through targeting SOCS1 and STAT3 [36]. One possible explanation of the discrepant results could be that in this study the expression levels in tumors were compared to only 21 laryngeal normal tissue [36], while in the current study we compared equal numbers of 63 tumor and adjacent normal tissues. In our study samples, RQ levels of miR-196 were extremely elevated with no significant association with patients' clinical features. In 2013 in the study of Saito et al, miR-196a was presented as the most promising laryngeal cancer biomarker with high expression levels in early T-stages [35]. We also found higher miR-196a-5p levels in T1 and T2 stages, which could support its active role in very early stages of LSCC disease progression. In renal cancer, miR-210 is associated with cancer metastasis, whereas miR-221 is suggested as suitable biomarker for lower cancer-specific treatment [38], but we did not find any significant association with the investigated features. The obtained results could be due to limitations of the sample size.

In summary, based on the existing studies and the current observations, we could not clarify the role of miR-21-3p, miR-155-5p, miR-196a-5p, miR-210-3p and miR-221-3p in laryngeal carcinogenesis, which could be proven by further more extensive investigations. However, at this stage they could not be chosen as suitable markers for LSCC diagnosis and prognosis.

We found that MALAT1 was underexpressed in the investigated LSCC tumour tissues. In many articles MALAT1 was previously described as a cancer-promoting and metastasis-promoting lncRNA, while other recent reports suggested a tumor-suppressing role of MALAT1 [39-41].

The findings in the literature are controversial about the role of MALAT1 in cancer. In the study of Xu et al in 2015 in breast cancer the levels of MALAT1 were downregulated, associated with short relapse-free survival and suggested for target therapy biomarker [42]. In addition, downregulation of MALAT1 was predicted as stemness and proliferative marker associated with activation of LTBP3 gene and suppressed expression of Sox2 and Nestin in glioma mesenchymal stem SHG139S cell line. Moreover, decreased levels of MALAT1 led to activation of ERK/MAPK signaling pathway and promoted proliferation [43]. On the other hand, in multiple myeloma, knockdown of MALAT1 induced apoptosis, but not proliferation, through the activation of mitochondrial-controlled apoptosis by upregulation of Bax, Caspase-3/-9 expression and downregulation of Bcl-2 [44]. In 2016 Fang et al showed that knockdown of MALAT1 in tongue squamous cell carcinoma cell lines (CAL27 and SCC-25) is associated with upregulation of SPRR2A protein, which influenced distant metastasis of TSCC [45]. Controversially, previous investigations in 2015 of Pang et al found elevated RQ levels of MALAT1 in pancreatic cancer, and its oncogenic potential was discussed for targeted strategy [46].

There are only limited studies on MALAT1 expression in laryngeal cancer. Intriguingly Feng et al published in 2012 aberrantly upregulated expression of MALAT1 in 72 LSCC specimens, associated with poor tumour differentiation and active proliferation [47].

We have carefully examined the experimental settings and approaches that have been used to study MALAT1 and could speculate that the discrepancies of the observations between studies could be due to various reasons, some related to experimental and statistical issues. In the study of Feng et al 18S was used as endogenous control [47], while in our study we compared the levels of MALAT1 to ACTB control, which was suggested recently as suitable endogenous control [48]. Zhou et al published in 2015 upregulation of MALAT1 expression in 54 randomly collected OSCC samples with only 12 normal mucosa samples, but they used GADPH endogenous control. The tumour and normal samples were not from the same patients and the numbers of normal and tumour samples were not equal [49]. The statistical power of such sample to detect true differences in expression is very limited.

In addition, several molecular mechanisms by which MALAT1 regulates tumor progression and metastasis have been suggested, including its action as competitive endogenous (ceRNA) or decoy miRs; its interaction with the Polycomb repressive complex 2 (PRC2), catalyzing histone methylation and playing important role in transcriptional repression and cancer; binding and inactivating TEAD transcription factor; regulating multiple signaling pathways in cancer such as Hippo-YAP, PI3K-AKT, MAPK, WNT, and NF-κB pathways [39].

It is still unclear if the conserved and highly abundant lncRNA in normal tissues MALAT1 acts as tumour suppressor or tumour inducer. Comparing the obtained and published results, we could conclude that the expression of MALAT1 may differ in different tissues, cancer types and genetic backgrounds. Unraveling its precise mechanism of action and contribution to laryngeal cancer needs more comprehensive studies.

In conclusion, we explored the expression levels of 11 miRs and two lncRNAs (MALAT1 and HOTAIR) in LSCC and adjasent normal tissue in Bulgarian patients, revealing new associations between the studied ncRNAs and clinicopathological features. We demonstrated new potential association between family history and the expression levels of miR-21-5p, miR-222-3p and lncRNA HOTAIR, downregulation of MALAT1 with age and potential gender effect, upregulation of "passenger" miR-31-3p in well and moderately differentiated LSCC tumours, and potential role of miR-424-5p isoform in LSCC location screening. Our research confirms some previous findings and shows controversial results to other investigations. MiR-145-5p was negatively related to T-stage and tobacco smoking, while miR-31-3p association with nodal metastasis could serve as potential marker for cancer treatment. We validated the potential role of miR-21-5p and miR-222-3p with harmful work exposures.

These results contribute to better understand the mechanisms of carcinogenesis in laryngeal cancer as well as to elucidate the potential role of ncRNA as biomarkers in the clinical practice.

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## Conflict of interests

The authors declare no conflict of interests.

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