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HIGHER EXPRESSION LEVELS OF MIR-1246 IN ADVANCED LARYNGEAL CARCINOMA ARE ASSOCIATED WITH ELEVATED RISK OF LOCOREGIONAL METASTASIS

Gergana Stancheva, Silva Kyurkchiyan, Veronika Petkova, Tzvetomir Marinov*, Maya Belitova*,#, Sylvia Skelina, Radka Kaneva, Diana Popova***, Todor M. Popov*****

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Abstract

Worldwide, laryngeal squamous cell carcinoma (LSCC) is the second most common head and neck cancer (HNC). Advanced forms of LSCC, especially HPV-negative types, frequently involve locoregional lymph node metastasis, which are associated with treatment failure. Despite recent development and improvement in treatment protocols [1], still advanced LSCC is a global oncological burden, associated with challenges and complications. The study group included 60 cases of predominantly HPV-negative tumours with all patients having history of long-term smoking. We found that miR-1246 was significantly dysregulated in advanced LSCC – almost 72% of the patients in the study group showed overexpression of miR-1246 in tumour tissue above relative quantity of 2 (RQ>2). Additionally, miR-1246 showed significant heterogeneity in expression levels between tumour depth and surface and moreover higher expression levels of the molecule correlated with higher chance of locoregional metastasis. In conclusion, we explored the expression levels of miR-1246 in advanced LSCC and adjacent normal tissue, revealing new tumour heterogeneity characteristic. We demonstrated potential association between miR-1246 expression levels and presence of locoregional lymph nodal metastasis.

#Corresponding author.

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Key words: laryngeal carcinoma, head and neck cancer, field cancerization, locoregional metastasis survival, miR-1246

Introduction. Worldwide, laryngeal squamous cell carcinoma (LSCC) is the second most common head and neck cancer (HNC). Advanced forms of LSCC, especially HPV-negative types, frequently involve locoregional lymph node metastasis, which are associated with treatment failure. Despite recent development and improvement in treatment protocols [1], still advanced LSCC is a global oncological burden, associated with challenges and complications.

MicroRNAs (miRNAs) are investigated and studied as regulatory small molecules with strong therapeutic potential in cancer disease. Despite some limitations of the use of miRNAs as treatment option, like delivery efficiency, multitargeting effects, toxicity and level of clearance in blood system [2], miRNAs could be a step forward in cancer disease treatment [2]. For instance, miRNA target solid cancer therapies were developed and involved in clinical trials, like MRX34 (developed by Mirnarx Therapeutics, Inc., entered the Phase I clinical trials in 2013; ClinicalTrials.gov, NCT number: 01829971) [3] and TargomiRs (developed by EnGeneIC, phase I clinical trial, ClinicalTrials.gov, NCT number: 02369198) [4].

One of the miRNAs that our team highlight is miR-1246, which was found for the first time in 2008 in human embryonic stem cells [5]. A number of studies described miR-1246 oncogenic characteristics, and its essential role in various cancer diseases. Potential cell mechanisms and targets through which miR-1246 could regulate key signalling in cancerogenesis are explored and described [6]. Studies revealed the potential of miR-1246 as a marker for overall survival (OS) and existence of locoregional metastasis, including in early and advanced LSCC tissues and cell lines (TU212 and AMC-HN-8) [7]. Moreover, miR-1246 was analyzed as potential biomarker for cellular heterogeneity in aggressive breast cancer [8], and miR-1246 heterogeneity was associated with radio resistance [9].

The aim of this study is to investigate the expression levels of miR-1246 in larger independent advanced LSCC group and explore its gene expression levels with clinicopathological characteristics of the patients, and level of heterogeneity in patients tissue samples. Our team has already published data of a full microRNA profiling in advanced LSCC. The result analysis revealed a set of significantly deregulated miRNAs [10], and part of it was miR-1246.

Materials and methods. ***Sample collection.*** Sixty patients (mean age was 64.6 ± 8.7 years) with pathologically verified primary advanced laryngeal carcinoma were enrolled in the current study. All patients underwent primary laryngectomy in 2018–2019 at the Department of ENT, Head & Neck Surgery, Medical University-Sofia. During surgery four samples from each patient were obtained: two from the tumour site – surface and depth; the third sample was taken from histologically healthy peritumour mucosa within 1 cm from the border of the tumour, and the fourth sample was paired normal laryngeal mucosa distant

to the tumour (contralateral, at least 3 cm distance). All samples were stored in RNAlater Solutions (Thermo Fisher Scientific, Massachusetts, USA) and frozen at −20 °C for a short period. Collected samples were transported to the Molecular Medicine Center, Department of Medical Chemistry and Biochemistry, Medical University-Sofia, and maintained at −80 °C until use. The study was approved by the Ethical Committee of Medical University-Sofia, and written informed consent was signed by every patient. The enrolled cohort was a single-surgeon consecutive series and the inclusion criteria were advanced-stage laryngeal squamous cell carcinoma (T3 or T4 stage). Surgical treatment included laryngectomy with free resection margins and neck dissection ipsilaterally (2–5 levels). In cases of tumours crossing the median line, contralateral neck dissection was also performed (2–5 levels). Additionally, if the tumour extended into the subglottic or retrocricoid region, a full paratracheal lymph node dissection (levels 6–7) was carried out. All patients underwent postoperative radiotherapy or combined chemoradiotherapy according to the protocol.

The follow-up period was an average of 24 months with a standard deviation of 13 months. Patients were followed-up every month during the first six months after surgery and every three months after this period. Every six months, PET-CT was scheduled for radiological evaluation.

RNA extraction and RT-qPCR. Total RNA (including miRNAs) was isolated from 60 fresh-frozen tumour materials and adjacent normal tissue using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. The quantity and quality of the RNA was assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and Qubit™RNA HS Assay Kit, Qubit™2.0 Fluorometer (Thermo Fisher Scientific, USA).

Copy DNA (cDNA) was synthesized from 400 ng of total RNA using the miScript II RT Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. miScript Primer assays (Qiagen, Hilden, Germany) and miScript SYBR Green PCR kit (Qiagen, Hilden, Germany) were used for real time-qPCR on a 7900HT Fast Real-Time PCR System (Applied Biosystems, USA). Each sample was performed in triplicate in a total volume of 10 µL. RNU6 (Qiagen, Hilden, Germany) was used as endogenous control. Those samples, which were analyzed with extreme RQ values, were tested two more times. Negative controls were also evaluated. The relative quantification (RQ) of miRNAs in samples was analyzed by the 2-ΔΔCt method, as previously described [11]. RQ over 2.00 was defined as overexpression, and RQ less than 0.5 was defined as underexpression.

Statistical analysis. Data analysis was performed with SPSS software v23.0 for Windows (IBM SPSS, USA) and GraphPad Prism software. A two-tailed p-value ≤ 0.05 was considered significant.

Results. The study group included 60 cases of predominantly HPV-negative tumours with all patients having history of long-term smoking. In terms of tumour staging, the majority of the cases (87.1%) were classified as pT4a, one case was

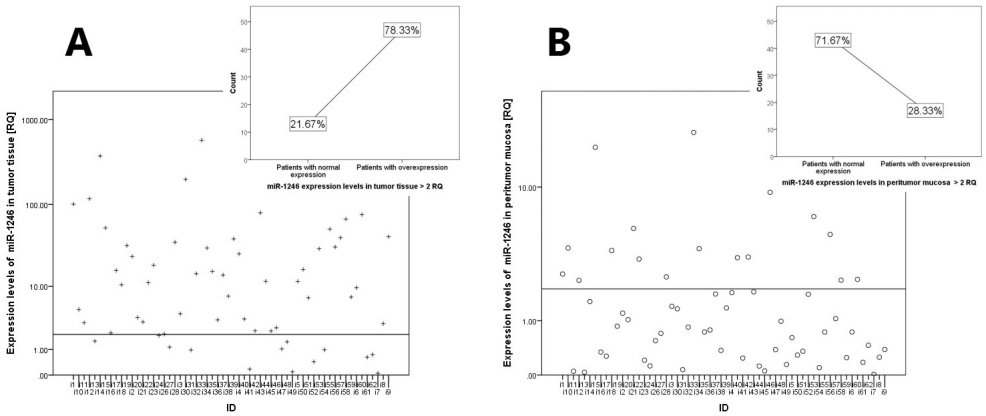

Fig. 1. Expression levels of miR-1246 in tumour tissue (A) and in peritumour normal laryngeal mucosa (B)

pT4b, and the rest were staged as pT3. Almost half of the group, 48.3% of the cases, had pathologically verified metastatic processes, and the distribution of N status was as follows: N1 (28.6%); N2a/N2b/N2c (57.1%), N3 (14.3%).

MiR-1246 is significantly dysregulated in advanced LSCC. Almost 72% of the patients in the study group show overexpression of miR-1246 in tumour tissue above relative quantity of 2 (RQ>2) (Fig. 1B). Mean RQ value for the whole group is 37.2 (std. dev. 90.2), median is 10.06 and minimum/maximum is 0.05/571.3. Interestingly, 28.3% of the patients also showed significant overexpression of miR-1246 in normal peritumour mucosa when compared to paired distant control laryngeal mucosa (Fig. 1A).

Overexpression of miR-1246 is significantly higher in T4 stage compared to T3 stage and correlates with tumour size. The relation between tumour size and miR-1246 expression levels was evaluated using the bivariate correlation coefficient Spearman's rank test. There was a moderately strong association between both variables ($p < 0.005$, $r_s = .350$). Additionally, there was a significant difference in expression RQ values when compared patients with stage T3 vs. T4a/T4b (Fig. 2).

MiR-1246 shows significant heterogeneity in expression levels between tumour depth and surface. Subsequently, we analyzed the heterogeneity of the tumour tissue in our group of patients by performing a pairwise comparison (Wilcoxon signed rank test) between samples taken from the surface of the tumour and its depth. The expression levels of miR-1246 were significantly higher at the tumour depth than at the tumour surface ($z = 2.393$, $p = 0.017$) (Fig. 3). We could conclude from our data that tumour depth exhibits to some degree a more pronounced dysregulation pattern in the expression levels.

MiR-1246 expression levels are positively associated with higher risk of locoregional metastasis but are not a prognostic marker for sur-

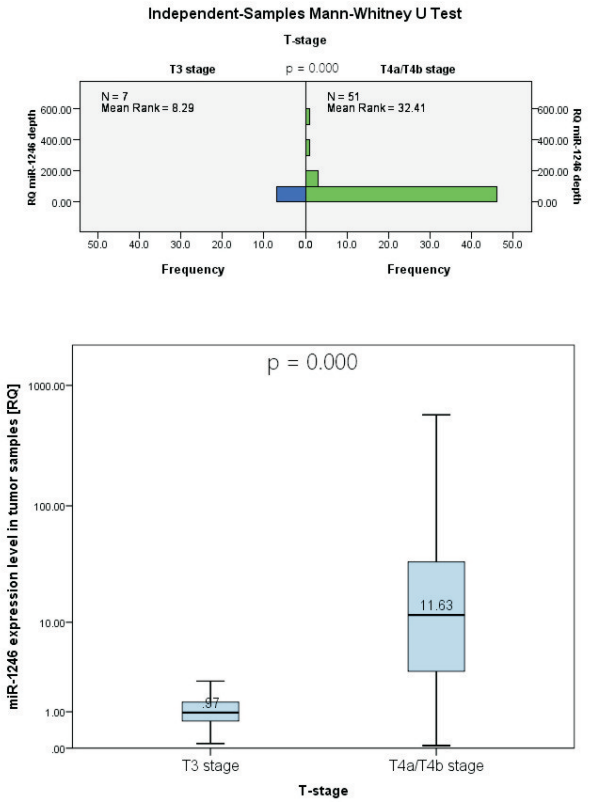

Fig. 2. Difference in expression levels of miR-1246 depending on the T-stage

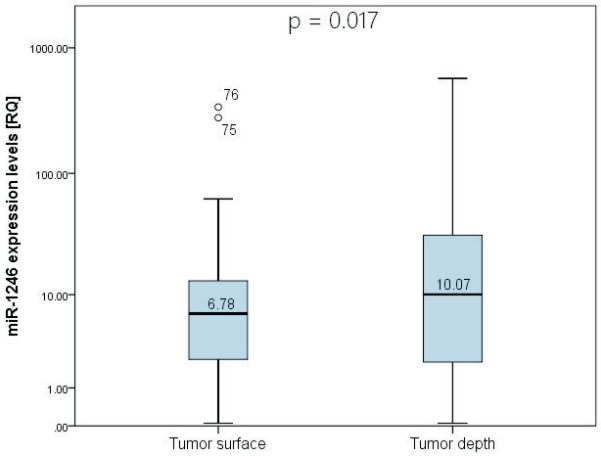

Fig. 3. Heterogeneity of miR-1246 expression levels in tumour tissue – tumour depth versus tumour surface

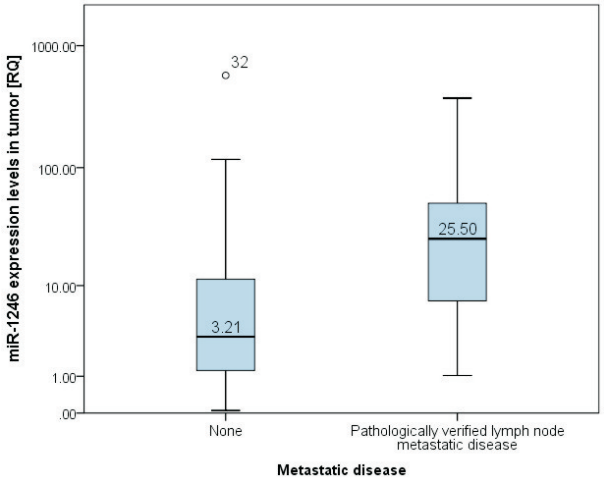

Fig. 4. Significantly higher expression levels of miR-1246 in patients with lymph node metastasis

vival. Independent Mann–Whitney U Test was conducted to compare expression level between patients with pathologically verified lymph node metastasis at the moment of surgery vs. patients with none after the neck dissection. Expression levels of miR-1246 were significantly higher among patients with metastatic disease compared to patients with free of metastatic neck lymph nodes (mean RQ 25.5 vs. 3.21, $p = 0.001$) (Fig. 4). Recurrence-free survival was 63.9% for the whole study group. Neither Kaplan–Maier method, nor Cox regression, showed significant association of the expression levels of this microRNA with survival outcomes.

Discussion. Development of LSCC includes a broad range of genetic and epigenetic changes, including deregulation of miRNAs. Still the regulatory role in signalling mechanisms as potential targets in laryngeal cancerogenesis is not revealed. In our study, we focused on advanced- LSCC to achieve maximum homogeneity of the group. Moreover, in order to investigate potential tumour heterogeneity we analyzed per two samples from each tumour (surface and depth) and laryngeal normal tissue, distant from the tumour bulk.

The obtained results showed statistically different expression of miR-1246 in advanced LSCC tissues in comparison to adjacent normal samples, which suggest its involvement in laryngeal cancerogenesis. In majority of patients (72%) expression levels of miR-1246 was overexpressed, which is consistent with other cancer research studies [6]. Moreover, with the current study our team investigates for the first time significant upregulation of miR-1246 in part of the normal laryngeal samples (28.3%) in comparison to paired distant control mucosa. Significant degree of heterogeneity was evaluated between tumour depth and tumour surface samples. Additional tests and studies could be used for the assesement of

miR-1246 in biomedical and clinical practice.

Promising data was found, when we compared miR-1246 levels between patients with and without presence of pathologically verified locoregional metastasis. LSCC patients with lymph nodal metastasis expressed much higher miR-1246 levels (Fig. 3), and our results are in line with recently published results from oral cancer and colorectal cancer [12,13].

MiR-1246 is found to be a strong marker of stemness and invasiveness in other solid cancers [14,15]. High miR-1246 expression was related to shorter survival rates and poor prognosis in OSCC and lung cancer [9,12]. Recently Wu et al. [7], investigated that miR-1246 regulated cell proliferation, apoptosis and migration in laryngeal cancer tissues and cell lines. Additionally, miR-1246 overexpression was associated with short overall survival (OS) [6].

Additionally, miR-1246/CCNG2 axis was presented as regulator of cell proliferation and cell cycle progression, but also is involved in EMT and chemoresistance. In details, miR-1246 could influence RAF/MEK/ERK, GSK3β, Wnt/β-catenin, JAK/STAT, PI3K/AKT, THBS2/MMP, and NOTCH2 signalling pathways [6].

Conclusion. In conclusion, we explored the expression levels of miR-1246 in advanced LSCC and adjacent normal tissue, revealing new tumour heterogeneity characteristic. We demonstrated potential association between miR-1246 expression levels and presence of locoregional lymph nodal metastasis. Significant association between miR-1246 upregulation and poor OS was not reached.

These results contribute to better understand the mechanisms of carcinogenesis in advanced LSCC as well as to elucidate the potential role of miR-1246 as a biomarker in the clinical practice.

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Molecular Medicine Center
Medical University-Sofia
2 Zdrave St
1431 Sofia, Bulgaria
e-mail: g.stancheva@medfac.mu-sofia.bg
s.kyurkchiyan@medfac.mu-sofia.bg
v.petkova@medfac.mu-sofia.bg
r.kaneva@medfac.mu-sofia.bg

**Department of Anesthesiology*
and Intensive Care
Medical University-Sofia
University Hospital
"Tsaritsa Yoanna – ISUL"
8 Byalo More St
1527 Sofia, Bulgaria
e-mail: ts.marinov@medfac.mu-sofia.bg
mbelitova@medfac.mu-sofia.bg
belitova@mail.bg

**Department of Neurology
Medical University-Sofia
1 St. G. Sofiiski Blvd
1431 Sofia, Bulgaria
e-mail: s.skelina@medfac.mu-sofia.bg

***Department of ENT
Medical University-Sofia
10 Adriana Budevska St
1463 Sofia, Bulgaria
e-mail: d.popova@medfac.mu-sofia.bg
t.popov@medfac.mu-sofia.bg