

Original research article

Overexpression of the *miR-143/145* and reduced expression of the *let-7* and *miR-126* for early lung cancer diagnosis

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Abstract

Introduction: Lung cancer is the leading cause of cancer-related deaths worldwide. For this reason, huge efforts are being invested in discovering suitable blood biomarkers that would allow early diagnosis and treatment. One of the possible promising candidates for this role are microRNA molecules (miRNAs). The aim of the study was to identify individual blood miRNAs that could be used as potential biomarkers for early diagnosis of lung cancer.

Methods: This prospective study analyzed blood samples of 60 patients with early-stage lung cancer, and blood samples of 60 healthy individuals. All study patients with lung cancer had undergone radical pulmonary resection at the University Hospital Ostrava within the study period (2015–2017). Definitive diagnosis of lung cancer was confirmed by histopathology examination of the resected pulmonary specimen. We investigated relative expressions in selected 13 blood miRNAs; the examined miRNAs were *miR-126*, *miR-155*, *miR-221*, *miR-21*, *miR-143*, *miR-145*, *miR-133a*, *let-7a*, *miR-146a*, *miR-31*, *miR-182*, *let-7g* and *miR-19b*.

Results: The outcome of this study showed that the levels of the majority of the tested circulating miRNA in lung cancer patients are significantly altered. The most significant serum miRNA biomarkers for the early detection of lung cancer are as follows: *miR-143*, *let-7g*, *miR-126*, *let-7a*, and *miR-145* (*miR-143* and *miR-145* have oncogene functions, while *miR-126*, *let-7g* and *let-7a* have suppressor functions).

Conclusions: We have demonstrated the excellent diagnostic value of several miRNAs (*miR-126*, *miR-143*, *miR-145*, *let-7a* and *let-7g*). These have an estimated sensitivity and specificity of 75–85% and 0.90–0.93 AUC. However, these individual miRNA biomarkers require further validation in larger prospective cohorts.

Keywords: Blood marker; Screening; Lung cancer; miRNA; *miR-143/145*

Highlights:

- Levels of circulating miRNAs in the serum of lung cancer patients are significantly altered.
- High potential of circulating blood miRNA as screening biomarkers for early lung cancer.
- Most significant serum miRNA biomarker for the early detection of lung cancer are *miR-143*, *let-7g*, *miR-126*, *let-7a*, and *miR-145*.

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide, and the most common cancer in terms of incidence (2.09 million new cases and 1.76 million deaths in 2019) (Bray et al., 2018). The only recommended lung cancer screening test is a low-dose CT. This has a high risk of false positives (Reduced Lung-Cancer Mortality with Low-Dose Computed Tomographic Screening, 2011), which may lead to unnecessary and potentially harmful further diagnostics. Due to this, great efforts have been made to discover suitable blood biomarkers which could potentially facilitate early diagnosis and treatment. Circulating biomarkers may be an integral part of future lung cancer screening programs. One of the suitable candidates for this role are microRNA molecules (miRNAs).

MicroRNAs are a class of small 18–24 nucleotide long, non-coding RNA genes found in all organisms. MiRNAs are involved in gene regulation, apoptosis and maintenance of cell differentiation; they play key roles in the regulation of target genes by suppressing their translation (Bartel, 2004). Overexpression of oncogene miRNAs or loss of tumor suppressor miRNAs are clearly associated with human cancer (Lin and Gregory, 2015). Although miRNA is an intracellular molecule, the cell has several possibilities for its transfer to the extracellular space – miRNAs circulate in body fluids (including blood) and are very stable (out of reach of endogenous RNases). These characteristics predispose miRNAs as very suitable biomarkers for early diagnosis of lung cancer (Gilad et al., 2008).

Until recently, several miRNAs have been used as a diagnostic tool for lung cancer (mostly incorporated in panels of multiple miRNAs). The most sensitive miRNA sets (panels)

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based on the expression ratio of predefined miRNAs in lung cancer exhibit a diagnostic sensitivity and specificity of about 85% (Sozzi et al., 2014). According to literature, individual miRNAs (not incorporated in panels) exhibit significantly lower sensitivity and specificity compared to miRNA panels (Bianchi et al., 2011; Chen et al., 2009; Cho et al., 2009; Harris et al., 2008; Heegaard et al., 2012; Sozzi et al., 2014; Zhu et al., 2016). The aim of the present study was to identify individual blood miRNAs that could be used as potential biomarkers for early diagnosis of lung cancer.

Materials and methods

Study population

This study included blood samples of 60 patients with early-stage lung cancer (study group), and blood samples of 60 healthy individuals (control group). According to the ESMO Consensus Guidelines, stage I–II was defined as early-stage lung cancer (Eberhardt et al., 2015). Signed, written informed consent was obtained from all included study participants, while ensuring anonymity. The study was approved by the Ethical board of the University Hospital Ostrava (ref. number 373/2014), and the Jessenius Faculty of Medicine in Martin of the Comenius University in Bratislava (ref. number IRB00005636).

All study patients with lung cancer had undergone radical pulmonary resection at the University Hospital Ostrava within the study period (2015–2017). A definitive diagnosis of lung cancer was confirmed by histopathology examination of the resected pulmonary specimen. The blood samples were peripheral venous blood collected from all study patients before their respective treatments (e.g., surgery, adjuvant chemotherapy). PAXgene® Blood RNA Tubes were used for blood collection. We investigated the relative expressions of 13 selected blood miRNAs; the examined miRNAs included *miR-126*, *miR-155*, *miR-221*, *miR-21*, *miR-143*, *miR-145*, *miR-133a*, *let-7a*, *miR-146a*, *miR-31*, *miR-182*, *let-7g* and *miR-19b*.

The patient study group consisted of 43 men and 17 women; the mean age was 65.8 ± 7.8 years. The histopathology findings of resected lung tumors in our study group corresponded to the normal proportion of lung cancer in the population – the most common tumor type was adenocarcinoma (46.7%), followed by squamous cell carcinoma (36.7%). The demographic and clinical characteristics of the patients in the study group are shown in Table 1.

Preparation and RNA isolation

After the blood samples had been collected, they were incubated for 24 h at room temperature and frozen in a vertical position at -20°C . At this temperature, samples were stored and transported to the Martin Biomedical Center for final processing and evaluation.

Table 1. Demographics and clinical data of study patients

Age (years, mean \pm SD)	65.8 \pm 7.8
Sex, n (%)	
Male	43 (71.7)
Female	17 (28.3)
Histopathology findings, n (%)	
Adenocarcinoma	28 (46.7)
Squamous cell carcinoma	22 (36.7)
Adenosquamous carcinoma	2 (3.3)
Small cell carcinoma	6 (10.0)
Neuroendocrine carcinomas	2 (3.3)

The PAXgene Blood RNA kit was used to isolate RNA from blood samples. The isolated RNA were quantified and assayed for purity using a NanoDrop spectrophotometer (Thermo Fisher Scientific, MA, U.S.A.) and then stored at -80°C . The concentration and absorption spectrum of RNA was measured spectrophotometrically (NanoDrop® ND-2000) at 260 nm in ng/ μL . The purity of RNA was verified based on A260/A280 and A260/A230 absorbance ratios. Samples with a ratio of A260/A280 in the range of 1.8–2.2 and a ratio of A260/A230 in the range of 1.7–2.9 were used to determine selected miRNAs.

Reverse transcription and statistical analysis

Reverse transcription products were prepared using a C 1000™ Thermal Cycler and TaqMan MicroRNA RT kit and TaqMan MicroRNA Assays. The kits contained a specific miRNA RT primer for a particular miRNA. The RNA was diluted with DEPC-Treated water to a concentration of 2 ng/ μL . The expression levels of the miRNAs were determined by using IQ5 Multicolor Real-Time PCR Detection System and TaqMan MicroRNA Assays, which contained a fluorescently labeled TM probe (20× TagMan MicroRNA Assays mix).

Relative expression of a targeted miRNA was computed using the equation $2^{[-(C_t - C_{t_{REF}})]}$. C_t values were defined as the fractional cycle number needed for the fluorescence to cross the fixed threshold. Since relative expressions are non-gaussian, the non-parametric Mann–Whitney test was used to test the H_0 : the mean relative expressions of study cases are the same as the mean relative expressions of controls for each miRNA; against the two-sided alternative.

To estimate the test validity for biomarkers, we used the area under the ROC curve analysis (AUC). The miRNA importance plot was determined by the out-of-bag estimate in RandomForest. Computations and graphs were completed using R (R-project.org., 2019) and R libraries MASS (Venables and Ripley, 2002), beeswarm (Eklund, 2016), pROC (Robin et al., 2011), and randomForestSRC (Ishwaran and Kogalur, 2016).

Results

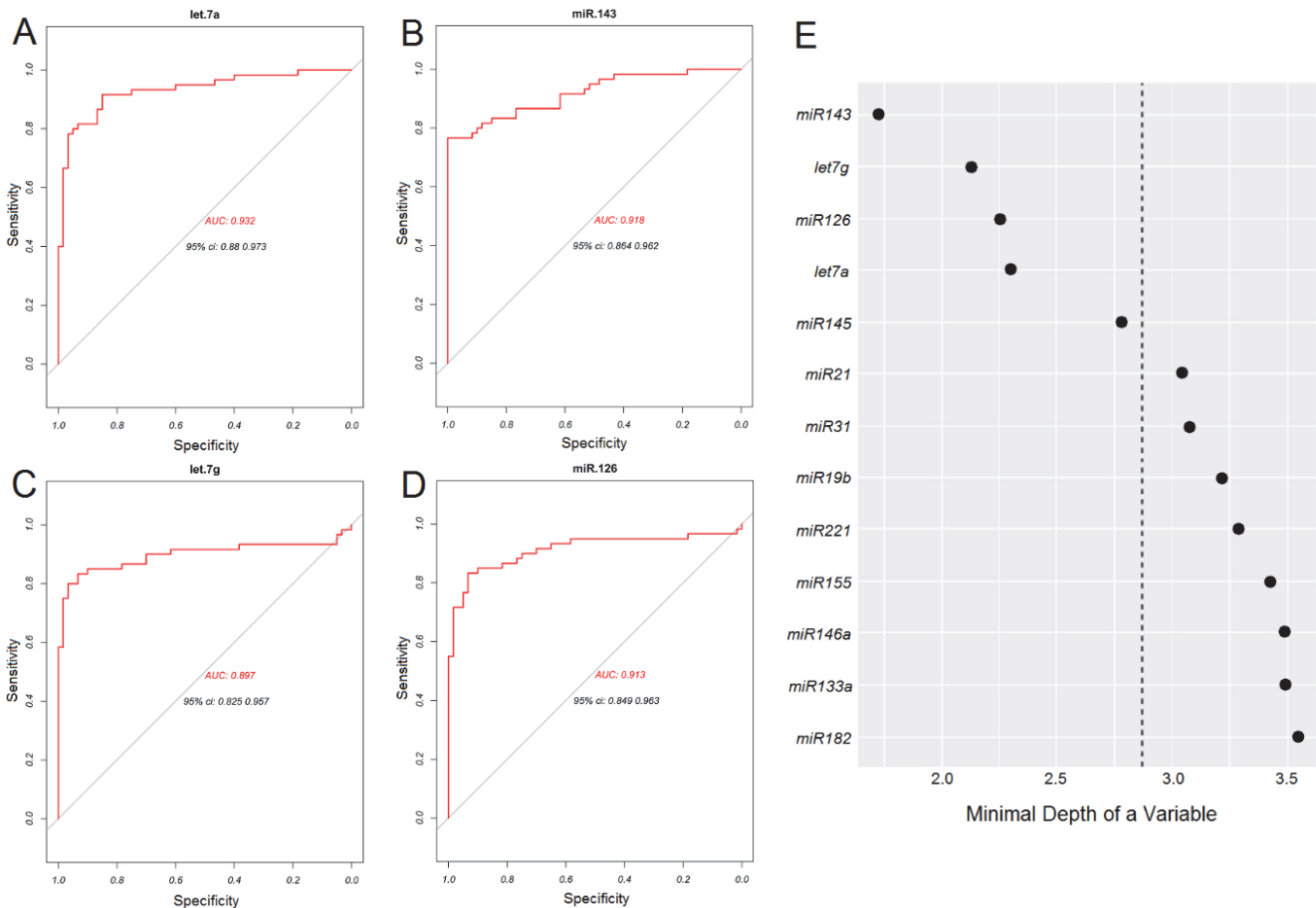
The relative expression of individual miRNAs in our study, along with the statistical evaluation of the differences (patients' group vs. control group), are presented in Table 2.

At the significance level 0.05, the mean of the relative expression of *miR-221* and *miR-133a* was comparable in the patient and control group (the expression difference was not significant). As shown in Table 2, the differences in the expression levels of all remaining miRNAs reached a high level of significance. Thus, we have confirmed that the levels of the majority of the tested circulating miRNA in lung cancer patients were significantly altered. We discovered that *miR-21*, *miR-143* and *miR-145* have oncogene functions (expression levels were significantly elevated in lung cancer patients). In contrast, *miR-126*, *miR-155*, *let-7a*, *miR-146*, *miR-31*, *miR-182*, *let-7g*, and *miR-19b* have suppressor functions (expression levels were significantly reduced in lung cancer patients).

The individual tested miRNAs exhibited AUC values from 0.539 to 0.932 AUC (in distinguishing lung cancer patients from healthy individuals), revealing 48–85% sensitivity and 42–92% specificity. However, the top five miRNAs (*miR-126*, *miR-143*, *miR-145*, *let-7a* and *let7g*) achieved sensitivity and specificity of 75–85% and 0.90–0.93 AUC (Fig. 1). Thus, the diagnostic value of these top miRNAs can be considered excellent.

Table 2. The expression difference of blood miRNAs between patient group and control group, the AUC values, sensitivity and specificity

miRNAs	Median of miRNA level		<i>p</i>	AUC (95% CI)	Sensitivity	Specificity
	Patient group	Control group				
<i>miR-126</i>	0.001	0.093	0.000	0.913 (0.851–0.965)	0.8500000	0.8666667
<i>miR-155</i>	0.003	0.012	0.000	0.729 (0.630–0.826)	0.6666667	0.7500000
<i>miR-221</i>	0.012	0.017	0.461	0.539 (0.432–0.642)	0.4833333	0.5666667
<i>miR-21</i>	0.175	0.066	0.000	0.761 (0.668–0.844)	0.7000000	0.6833333
<i>miR-143</i>	0.003	0.000	0.000	0.918 (0.861–0.959)	0.7833333	0.9000000
<i>miR-145</i>	0.056	0.020	0.000	0.846 (0.767–0.909)	0.7500000	0.7833333
<i>miR-133a</i>	0.001	0.002	0.222	0.565 (0.461–0.667)	0.6333333	0.5000000
<i>let-7a</i>	0.026	0.714	0.000	0.932 (0.883–0.971)	0.8500000	0.8666667
<i>miR-146a</i>	0.011	0.027	0.017	0.626 (0.519–0.729)	0.6500000	0.6000000
<i>miR-31</i>	0.001	0.002	0.017	0.626 (0.511–0.732)	0.7000000	0.4166667
<i>miR-182</i>	0.126	0.357	0.000	0.719 (0.622–0.811)	0.7333333	0.7166667
<i>let-7g</i>	0.013	0.487	0.000	0.897 (0.827–0.955)	0.8333333	0.9166667
<i>miR-19b</i>	0.076	0.440	0.000	0.741 (0.638–0.835)	0.7666667	0.6500000

**Fig. 1.** ROC curve analysis of four miRNAs (**A** – *let-7a*, **B** – *miR-143*, **C** – *let-7g*, **D** – *miR-126*) in case group. The area under the ROC curve (AUC) for each miRNA conveys its accuracy for discriminating lung cancer; **E** – miRNA importance plot based on OOB (out-of-bag estimate) showing most significant serum miRNA biomarkers.

The potential panel of the top five miRNAs produces a sensitivity of nearly 100%. Using the OOB error (out-of-bag estimate), a variable elimination of the least important miRNAs was performed, and a miRNA importance plot was determined. As the most significant serum miRNA biomarker for the early detection of lung cancer, we confirmed the following miRNAs: *miR-143*, *let-7g*, *miR-126*, *let-7a*, and *miR-145* (*miR-143* and *miR-145* have oncogene functions, while *miR-126*, *let-7g* and *let-7a* have suppressor functions). Our findings confirmed the high potential of circulating blood miRNA as screening biomarkers for early lung cancer.

Discussion

Our study data confirmed 11 differentially expressed miRNAs in patients with early lung cancer. Five of the investigated miRNAs seem to have excellent diagnostic value, evident by their individual AUC value of above 0.9.

Recently published studies offer outcomes of miRNAs incorporated in panels (sets) for detection of lung cancer. Authors of these panels exhibit a diagnostic sensitivity and specificity of about 85% (Bianchi et al., 2011; Chen et al., 2009; Cho et al., 2009; Harris et al., 2008; Heegaard et al., 2012; Sozzi et al., 2014; Yu et al., 2018; Zhu et al., 2016). In general, the biomarker panel has a significantly higher sensitivity compared to individual miRNAs. However, authors of each of the above studies recommend different miRNAs panels (Yu et al., 2018), which causes very difficult interpretation and comparison of different study outcomes. Due to this, the main aim of our study was to discover individual miRNAs with the highest diagnostic accuracy. These individual miRNAs should be definitively validated in a prospective-sufficiently powered multicenter clinical trial.

So far, only a few individual miRNAs with sensitivity and specificity of over 80% have been confirmed (Leidinger et al., 2016; Yu et al., 2018). We offer at least three more. Our results support the findings of Shen et al. (2011) and Zhu et al. (2016) who recommended *miR-126* as a suitable biomarker. In our study, *miR-126* showed sensitivity and specificity of above 85%. The atypical expression of *miR-126* is involved in the pathogenesis of NSCLC through the regulation of vascular cell adhesion molecule 1 (Harris et al., 2008). Likewise, we must agree with other authors (Bianchi et al., 2011; Heegaard et al., 2012) who recommend the *let-7* family as a significant biomarker. In tumorigenesis, both of these miRNAs act as tumor suppressors.

Generally, *miR-143* and *miR-145* are known as tumor suppressors. They are mostly down-regulated in tumor tissues, as has been reported by authors of most published studies. Reduced expression of these miRNAs limits the tumor-forming capacity of diverse tumor cell types (Chen et al., 2009; Cho et al., 2009). In our study, the relative expression of *miR-143* and *miR-145* increased significantly in the patient population, which is indicative of their oncogenic potential. Dimitrova et al. (2016) had already described similar findings in 2015. Dimitrova identified a novel role for *miR-145* and *miR-143* as key modulators of endothelial function in cancer biology. *In vivo* removal of *miR-143/145* resulted in decreased neoangiogenesis, increased apoptosis, and limited tumor size expansion. These findings showed that *miR-143/145* promotes tumorigenesis, and even cautioned against using these miRNAs as agents in cancer therapeutics. Lawson et al. (2017) also confirmed these results. Lawson showed that the transfer of *miR-143/145* in extracellular vesicles from pulmonary adeno-

carcinoma cells to endothelial cells decreases *CAMK1D* levels, and thus increases endothelial cell proliferation. Published reports on *miR-143/145* are contradictory, and miRNAs can seemingly act both as a tumor suppressor and have an oncogenic role in tumorigenesis. The explanation for this discrepancy remains unclear.

Another possible oncogenic miRNAs in the development of lung cancer is *miR-21*. In mammals, this is one of the first identified miRNAs. It promotes carcinogenesis by suppressing apoptosis and inhibiting the negative regulators *RAS/MEK/ERK*. Thus, it acts mainly antiapoptically by activating epidermal growth factor receptors (*EGFRs*) (Seike et al., 2009) and by inhibiting the suppressors *PTEN*, *PDCD4* and *TPM1* (Zhang et al., 2010; Zhu et al., 2008). According to the aforementioned studies, the *miR-21* oncogene may be essential in suppressing apoptosis, and thus plays an important role in the natural defense against tumor growth. Unfortunately, these characteristic of *miR-21* regulation were not confirmed in our study.

Another interesting group of pulmonary miRNA oncogenes is the duet *miR-221/222*. It is increased mainly in non-small cell carcinoma. They modify suppressors for *PTEN* and *TIMP3*. Increased transcription of the *TIMP3* gene induces an increased response to mitogenic stimulation, thereby multiplying the tumor's metastatic potential (Garofalo et al., 2009). In our study, the relative expression of the *miR-221* molecule did not differ from the control cohort. Therefore, we cannot confirm any of its oncogenic potential.

The remaining tested miRNA molecules do not have a confirmed oncogenic potential, although there are some studies available which have shown their potential oncogenicity. For example, high *miR-155* expression may predict recurrence and low survival in non-small cell lung cancer (Yang et al., 2013). It can also inhibit the sensitivity of lung cancer cells to cisplatin *miR-133a* and *miR-31*. In turn, it can suppress cell proliferation, migration, and invasion in human lung cancer (Xu and Wang, 2013; Yu et al., 2016).

Currently, the only screening method recommended for lung cancer is low-dose computed tomography – declared by the European Lung Cancer Screening Situation Statement (Oudkerk et al., 2017). The authors of this statement show a decent capture of lung nodularities, and describe the diagnostic boundaries with a lesion volume above 200 mm³. However, there are oppositions to this screening method in available literature, which point out various shortcomings (Ruano-Ravina et al., 2018). Firstly, no study has shown a significant survival improvement. As Ruano-Ravina demonstrates, approximately 30–35% of the lung cancers found in these screening programs were in advanced stages (stages III or IV) – for which the benefits of screening are negligible. Harris (2015) reports only a 5% drop in lung cancer mortality, even with full implementation of this method in the American population.

Secondly, the number of false positives is still relatively high. Doubts about the relevance of low dose CT persist, and will continue to do so in the future. Therefore, efforts to identify suitable miRNA as a biomarker for lung cancer screening seem to be paramount.

A possibility for early diagnosis of lung cancer is the detection of free-circulating tumor cells that are highly specific for lung cancer. However, this cannot be used as a screening method since the capture rate of circulating cells in lung cancer patients is only approximately 50% (Gorges et al., 2016). Research of free-circulating tumor DNA detection seems to be more promising. The efficiency of this analysis is currently similar to that of miRNA at the experimental level, although meta-analyses show that circulating tumor DNA may be con-

sidered as an effective biomarker for detecting mutations in lung cancer (Qiu et al., 2015). Detection of early stages of the tumor remains a problem, because the percentage of successful detection, even under laboratory conditions, is less than 50% (Haber and Vasulescu, 2014). The last major method for detecting lung cancer from peripheral blood is to evaluate a set of specific antibodies. However, the accuracy and sensitivity of this method is even lower than in the previously mentioned methods.

By confirming miRNA as a suitable biomarker, it can be used not only in secondary prevention (screening), but also in tertiary prevention as a prognostic marker and indicator of disease recurrence. Our study did not deal with late parameters and prognostic validity of results. However, many previous studies have provided these conclusions. It has been shown that miRNA can be considered as a prognostic marker (Liu et al., 2017), and even an indicator of response to treatment or recurrence of the disease (Dejima et al., 2017). Another possible use of miRNA is its therapeutic application. Suppressor miRNAs have a proven protective function in oncogenesis. An artificial increase in the level of suppressor miRNAs could lead to attenuation of tumor growth and metastasis (Rupaimoole and Slack, 2017).

Although our data are very promising, our study group contained a relatively small number of samples, so various other factors may have skewed the results. Individual miRNA biomarkers therefore require further evaluation in larger prospective cohorts. To confirm our conclusions, it would be desirable to perform a control prospective study specifically focused on the given miRNAs (*miR-126*, *miR-143*, *miR-145*, *let-7a* and *let-7g*). In addition to determining the relative expression of miRNA molecules at the time of the disease, it would be appropriate to take peripheral blood samples and determine the relative expression, even after the tumor has been removed and the patient has been cured (remission phase of the disease). Decreased levels of these markers after therapy would suggest certain significance in tumor oncogenesis. It would also be appropriate to divide the group of patients according to the types of lung cancer, as individual miRNAs can only affect some of its subtypes. The authors do not rule out such a study in the future.

Discussions of current possibilities of early diagnosis of lung cancer show the urgent need for a better and more specific marker. Our knowledge of miRNA interactions and roles is severely limited due to the specificity of miRNA regulation. According to our outcomes, miRNAs seem very promising in terms of being a suitable biomarker.

Conclusions

We have shown that levels of circulating miRNAs in the serum of lung cancer patients are significantly altered. We have demonstrated the excellent diagnostic value of several miRNAs (*miR-126*, *miR-143*, *miR-145*, *let-7a* and *let-7g*), which have reached the sensitivity and specificity of 75–85% and 0.90–0.93 AUC.

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Ethical aspects and conflict of interests

The authors have no conflict of interests to declare.

Author contributions

LT wrote this paper. PI made contributions to the conception of the paper and critically revised the manuscript. AD contributed to data preparation and critically revised the manuscript. TM critically revised the manuscript. All authors read and approved the final version of the manuscript.

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