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HUMAN GENETICS

Exosomal miRNA-146a and miRNA-424 as Possible Predictors of Immune Checkpoint Inhibitors Therapy Response in Clear Cell Renal Cell Carcinoma

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Abstract—Clear cell renal cell carcinoma (ccRCC) is a malignant kidney tumour with a poor prognosis and difficult to treat. Despite significant advances in the treatment of ccRCC, immune checkpoint inhibitors (ICI) still have limited therapeutic efficacy. A growing number of investigations has demonstrated that exosomal miRNAs are key modulators of tumour signaling and determinants of the tumour microenvironment. Disruption of miRNA regulation may affect ccRCC immunogenicity and response to ICI therapy, making them attractive for use as prognostic molecular genetic biomarkers. We evaluated exosomal miRNAs (miRNA-424, -146a, -503, -144) expression levels before and after ICI therapy in plasma samples obtained from 42 ccRCC patients. Expression analysis was performed using real-time PCR method. The results showed that the expression levels of miRNA-424 and miRNA-146a were upregulated after ICI therapy treatment (miRNA-424 = Mean ± SEM 1.202 ± 0.15 and miRNA-146a = 12.22 ± 1.45) compared expression levels before therapy (miRNA-424 = Mean ± SEM 0.63 ± 0.17; p-value = 0.03 and miRNA-146a = 7.03 ± 0.90; p-value = 0.006). miRNA-424 and miRNA-146a can be used to create a panel of molecular markers for evaluating the effectiveness of immune checkpoint inhibitors therapy. Even though the results are preliminary and requires further studying on a larger cohorts, it further increases the interest in using miRNAs, as additional ICI therapeutic markers capable of modulating immune tolerance.

Keywords: immune checkpoint inhibitors, resistance, exosomal miRNAs, prognostic markers, renal cell carcinoma

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INTRODUCTION

In recent years, a significant evolution has taken place in cancer therapy, primarily focused on the application of immunotherapeutic approaches, either as replacements for conventional treatment modalities such as chemotherapy, radiation therapy, and surgery, or in combination with them [1]. Immune checkpoint inhibitors (ICIs) have proven to be highly effective agents in the treatment of various cancer types, including clear cell renal cell carcinoma (ccRCC). Despite the progress and unprecedented achievements in the field of cancer immunotherapy, resistance remains a substantial clinical challenge [2, 3].

In recent years, non-coding RNAs have emerged as key players in epigenetic gene regulation, with miRNAs (miRNAs) being extensively investigated for their potential roles in regulating various cellular processes in both normal and pathological conditions. MiRNAs, approximately 22 nucleotides in length, represent post-transcriptional regulators of gene expression [4]. The capacity of miRNAs to inhibit the translation of oncogenes and tumour suppressors suggests their involvement in carcinogenesis [5–7]. Previous studies have demonstrated the stability of miRNAs in plasma or serum, their tissue specificity, and their prognostic clinical value as biomarkers [8, 9].

The objective of this study is to assess the expression profile of exosomal miRNAs (miR-424, miR-146a, miR-503, miR-144) in patients with ccRCC before and after immune checkpoint inhibitor therapy to identify additional biomarkers for the efficacy of immunotherapy.

Table 1. Expression analysis of exosomal miRNAs

miRNA	Before ICI therapy (Mean ± SEM)	After ICI therapy (Mean ± SEM)	*p*-value
146a	7.03 ± 0.90	12.22 ± 1.45	**0.006**
424	0.63 ± 0.17	1.202 ± 0.15	**0.03**
503	0.77 ± 0.05	0.71 ± 0.05	0.18
144	2.42 ± 0.46	2.58 ± 0.65	0.668

Bold font indicates statistically significant results.

MATERIALS AND METHODS

The study included 42 patients with histologically confirmed diagnosis of ccRCC, receiving immune checkpoint inhibitor therapy between 2020 and 2023, residing in the Republic of Bashkortostan. Venous blood samples were collected from patients before and after therapy by personnel from the Republican Clinical Oncology Dispensary, Oncology and Urology Department of the Bashkir State Medical University clinic.

Exosomal miRNAs were extracted from 1 mL of blood plasma, and cDNA synthesis and quantitative real-time PCR were conducted as previously described [10]. The miRCURY LNA kits (Qiagen, Hilden, Germany) were utilized on the Rotor-Gene Q real-time PCR instrument (Qiagen, Hilden, Germany). miRNA-16 and miRNA-1228 served as reference genes (endogenous controls), while UniSp2, UniSp4, UniSp5, UniSp6, and synthetic miRNA-39 were employed as exogenous controls for extraction, reverse transcription, and amplification processes, included in the respective kits (Qiagen, Hilden, Germany). The level of exosomal miRNAs was assessed using the $2^{-\Delta\Delta Ct}$ method.

RESULTS

Expression analysis was conducted using quantitative real-time PCR. A statistically significant dysregulation in the expression of exosomal miRNAs, specifically miRNA-424 and miRNA-146a, was observed. The expression levels of these miRNAs increased following therapy (miRNA-424 = Mean ± SEM 1.202 ± 0.15 and miRNA-146a = 12.22 ± 1.45) compared to before therapy levels (miRNA-424 = Mean ± SEM 0.63 ± 0.17; *p*-value = 0.03 and miRNA-146a = 7.03 ± 0.90; *p*-value = 0.006). Other miRNAs did not demonstrate significant differences in expression levels between the two groups (*p*-value > 0.05) (Figs. 1a–1d, Table 1).

Targeted miRNAs have a wide range of biological functions. It has been identified that many of the studied genes possess functional elements closely associated with ccRCC and immunity. KEGG pathway analysis revealed significant enrichment of miRNA-424 and miRNA-146a in multiple pathways (Table 2, Fig. 2).

To assess the involvement of validated target genes of miRNA-424 and miRNA-146a in relevant signaling pathways that trigger immune-inflammatory responses in cancer, a Gene Ontology (GO) analysis was conducted (Fig. 3).

To evaluate the diagnostic accuracy of exosomal miRNAs as markers of immune checkpoint inhibitors effectiveness, Receiver Operating Characteristic (ROC) curves were constructed. The results indicated that the area under the curve (AUC) for miRNA-424 is 0.804 (95% CI: 0.7082–0.9006), providing 73.8% specificity and 88.1% sensitivity. Diagnostic accuracy of miRNA-424 is higher than that of the miRNA combination (Fig. 4).

DISCUSSION

Despite the high efficacy of immunotherapy in many types of cancer at advanced stages, a subset of patients remains resistant to the treatment or is compelled to discontinue therapy due to severe immune-related side effects. Since the assignment of immunotherapy relies on tumour (tissue) PD-L expression, which is not a specific and precise marker, there is a critical need for more reliable biomarkers to predict the response to immunotherapy. This would enable the selective stratification of patients. The expression of miRNAs in patients receiving immunotherapy and their potential role in patients' response to therapy is currently a relevant topic for investigation. Studies analyzing the expression profile of exosomal miRNAs in patients with ccRCC receiving ICI therapy are limited to individual investigations and are need a more in-depth research of the role of miRNAs in predicting the effectiveness of ICI therapy.

In the studies by Z. Wang et al., it was reported that circulating miRNA-21 functions as a non-invasive prognostic biomarker for response in cancer immunotherapy [11]. L. Chen et al. [12] previously demonstrated that miRNA-200 inhibits PD-L1, preventing epithelial-mesenchymal transition (EMT) and metastasis in lung cancer. It is known that p53 regulates PD-L1 through miRNA-34a [13], which directly binds to the 3'-untranslated region of PD-L1 in non-small cell

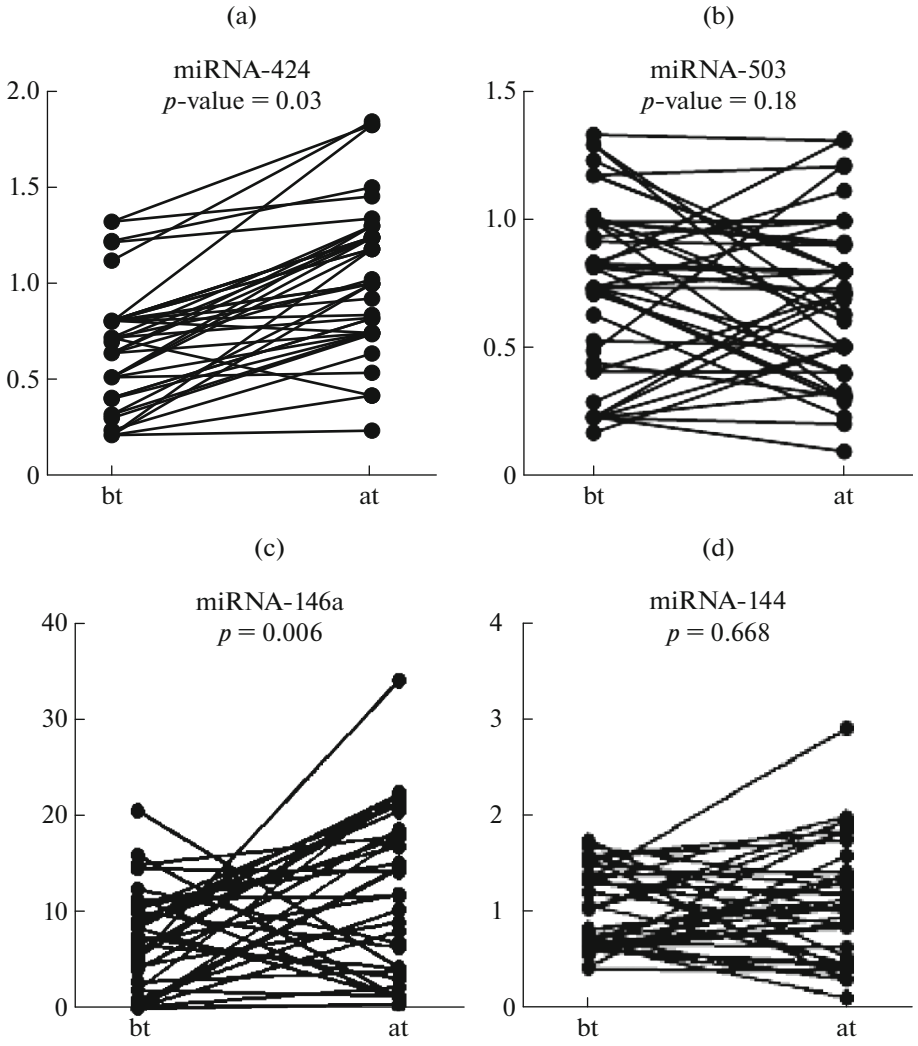

Fig. 1. Expression levels of exosomal miRNAs in patients with ccRCC receiving ICI therapy: (a) miRNA-424; (b) miRNA-503; (c) miRNA-146a; (d) miRNA-144. The significance level of *p*-value was calculated using the Wilcoxon test. bt—before therapy; at—after therapy.

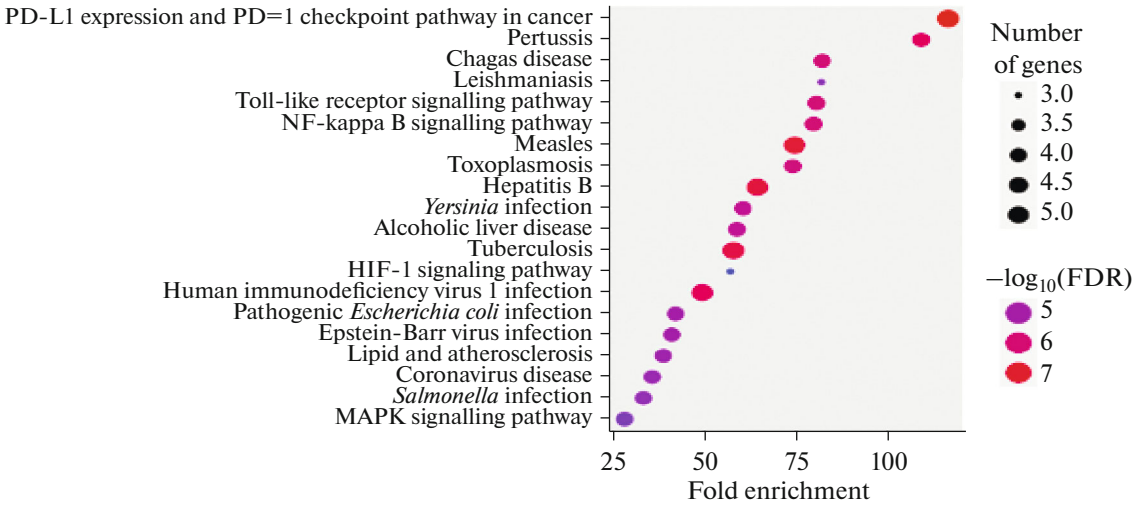

Fig. 2. GO signaling pathway enrichment analysis and a diagram showing the results of KEGG pathway enrichment analysis performed for miRNA-146a and miRNA-424 and their validated targets.

Table 2. KEGG enrichment pathway analysis for exosomal miRNA-146a and miRNA-424

FDR	Number of target genes	Number of signaling pathway genes	Name of the signaling pathway
2.9×10^{-8}	5	89	PD-L1 expression and checkpoint signaling pathway in oncology
4.8×10^{-7}	4	76	Whooping cough
1.1×10^{-6}	4	101	Chagas disease
2.5×10^{-5}	3	76	Leishmaniasis
1.1×10^{-6}	4	103	Toll-like receptor signaling pathway
1.1×10^{-6}	4	104	NF-kappa B signaling pathway
1.4×10^{-7}	5	139	Measles
1.4×10^{-6}	4	112	Toxoplasmosis
1.9×10^{-7}	5	161	Hepatitis B
2.8×10^{-6}	4	137	Yersiniosis infection
2.9×10^{-6}	4	141	Alcoholic liver disease
2.4×10^{-7}	5	179	Tuberculosis
6.7×10^{-5}	3	109	HIF-1 signaling pathway
4.4×10^{-7}	5	210	Human immunodeficiency virus 1 infection
1.0×10^{-5}	4	197	Pathogenic *Escherichia coli* infection
1.0×10^{-5}	4	202	Epstein-Barr virus infection
1.2×10^{-5}	4	214	Lipids and atherosclerosis
1.6×10^{-5}	4	232	Coronavirus infection
2.0×10^{-5}	4	249	*Salmonella* infection
3.4×10^{-5}	4	294	MAPK signaling pathway

lung cancer (NSCLC), suppressing the expression of PD-L1.

Drug resistance is one of the most significant challenges in clinical practice. It has been demonstrated that the loss of miRNA-424(322)/503 contributes to the development of chemoresistance by regulating two of its targets—the apoptosis regulator BCL-2 and the insulin-like growth factor-1 receptor IGF1R. Targeted therapy that inhibits the aberrant activity of these targets restores sensitivity to chemotherapy [14].

There is evidence suggesting that miRNA-424 is involved in the development of drug resistance in gastric cancer patients receiving platinum-based chemotherapy. One of the miRNA-424 target genes is *SMURF1*, which participates in ubiquitin ligase activity. In patients resistant to cisplatin, reduced expression of miRNA-424 increased the expression of the E3 ubiquitin-protein ligase gene *SMURF1* and stimulated the expression of the *RHOA* gene, a member of the Ras homolog family, which plays a role in drug resistance [15]. In another study, it was shown that decreased expression of miRNA-424-3p prevents the upregulation of the *ABCC2* gene, encoding a protein associated with multidrug resistance, leading to drug resistance and tumour progression. However, contrasting results were obtained by Y. Li et al., who demonstrated that both in vivo and in vitro overexpression of miRNA-424-3p plays a crucial role in gastric cancer cell resistance to cisplatin [16].

It is known that hypoxia induces the miRNA-424 expression, and overexpression of this miRNA suppresses the tumour suppressor *PDCD4*, involved in apoptosis. In the study by D. Zhang et al., the downregulation of miRNA-424 regulation increased cell death under doxorubicin treatment, attributed to enhanced apoptosis [17].

In another study, the stimulation of miRNA-424-3p expression contributed to the sensitization of ovarian cancer cells to cisplatin by reducing the expression of galectin-3, encoded by the *LGALS3* gene [18]. Additionally, a group of miRNAs (miRNA-34a, -34b, -187, -199a, -199b, -146a, -15b, -130a, -214, -424) was identified to be differentially expressed during doxorubicin treatment. Investigating the biological significance of these identified miRNAs revealed their correlation with the function of cardiomyocytes and drug-induced cardiotoxicity. This suggests that specific miRNA signatures could be used as potential biomarkers for drug-induced cardiotoxicity [19].

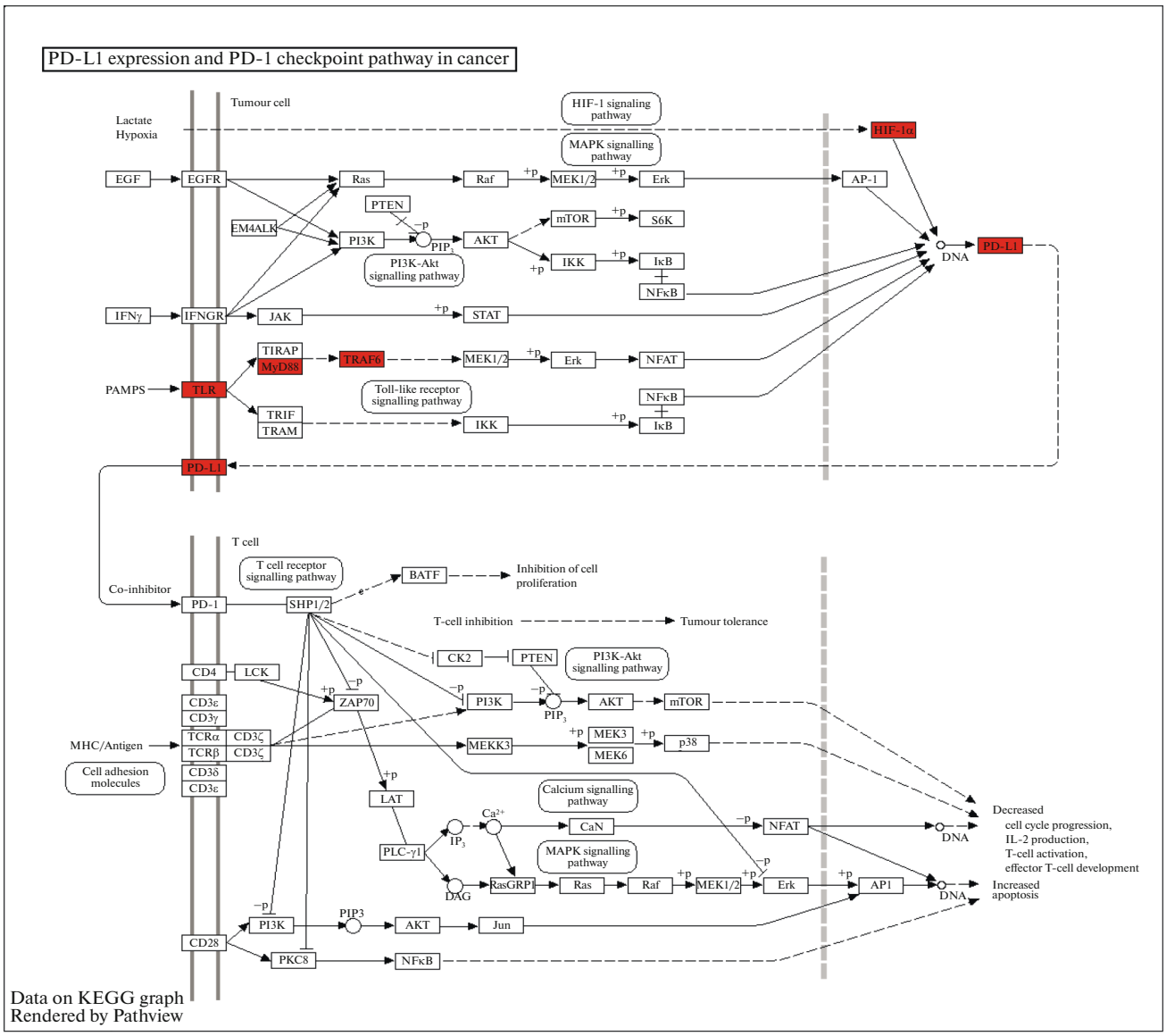

Fig. 3. Signaling pathway of PD-L1 expression and PD-1 checkpoints including validated miRNA-146a and miRNA-424 target genes (highlighted in red), according to the KEGG database.

It is known that the long non-coding RNA (lncRNA) LINC01116 plays a significant role in the development of drug resistance in osteosarcoma. LINC01116 inhibits the expression of miRNA-424-5p by binding to the *EZH2* gene, encoding histone-lysine N-methyltransferase, thereby increasing the resistance of osteosarcoma cells to doxorubicin [20]. B. Ralla et al. investigated the role of miRNA-9-5p in resistance to tyrosine kinase inhibitors (TKIs) in comparison with other clinical features (age, gender, tumour stage, metastatic status, etc.). The overexpression of miRNA-9-5p and the reduced expression of miRNA-489-3p were associated with a lack of response to TKI therapy in patients with metastatic ccRCC [21]. In another study conducted by A. Gámez-Pozo et al., the prognostic ability of models incorporating miRNA expression regarding resistance to sunitinib therapy was examined. When comparing these models with the MSKCC risk classification for predicting overall survival, the model including miRNA-192, -193-3p, and -501-3p demonstrated good ability to identify patients with a poor response to therapy and accurate overall survival prediction [22]. According to the study by J. Kovakova et al., miRNA-376b-3p can be used to predict the response to sunitinib therapy in metastatic tumours [23].

J. He et al. [24] hypothesized that miRNA-31-5p, transported by extracellular vesicles, is involved in the development of resistance to the multitarget receptor tyrosine kinase sorafenib. Y. Liu et al. investigated a

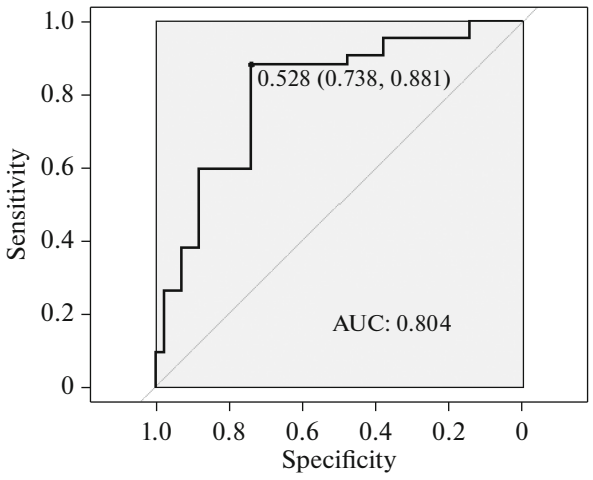

Fig. 4. ROC analysis to predict response to ICI therapy in ccRCC based on analysis of exosomal miRNA-424 expression.

drug delivery system in the form of nanovesicles loaded with a combination of anti-PD-L1 and miRNA-424. These nanovesicles activated T cells, which released a large amount of cytokines such as IFN-γ and IL-2, to activate macrophages and NK cells, thereby inhibiting the growth of subcutaneously transplanted hepatocellular carcinoma in mice [25].

In many studies, miRNA-146a is considered as a negative regulator of immune activation, comparable to immune checkpoint molecules. miRNA-146a is known to play a central role in the STAT1/IFNγ axis in the melanoma microenvironment, influencing migration, proliferation, mitochondrial function, and the level of PD-L1 [26]. It has been established that a decrease in the expression of miRNA-146a leads to significantly more severe immune-related adverse events (irAEs). The polymorphic variant (SNP) rs2910164, causing a decrease in the expression of miRNA-146a, is associated with an increased risk of developing severe irAEs [27]. In our study, we also confirmed that the reduced expression of miRNA-146 in patients with ccRCC with severe irAEs and the SNP rs2910164 correlate with an increased risk of developing severe complications [10]. It has been previously described that the regulation of miRNA-146a plays a crucial role in enhancing immune suppression by increasing the population of regulatory T cells and may modulate the drug resistance of tumour cells [28].

In another study, a decrease in miRNA-150, -146a, and -424 was identified in peripheral blood mononuclear cells in patients with type 1 diabetes. This reduction is likely associated with the positivity of autoantibodies and the impairment of endogenous residual beta-cell function, suggesting the involvement of these miRNAs in the regulation of the immune response [29].

X.-X. Peng et al. evaluated the potential use of blood plasma exosomal miRNAs as biomarkers in patients with NSCLC receiving immunotherapy [30]. In this study, three miRNAs from the hsa-miRNA-320 family (miRNA-320d, -320c, and -320b) were identified as potential predictors of response, as their levels were significantly elevated in the group of patients with progressive disease compared to the group with partial response at baseline before treatment initiation. Additionally, it was observed that the level of miRNA-125b-5p was decreased in patients responding to ICI treatment. A.R. Halvorsen et al. conducted next-generation sequencing (NGS) with miRNA profiling in serum samples collected from NSCLC patients before the initiation of nivolumab immunotherapy [31]. They identified a signature consisting of seven miRNAs (miRNA-215-5p, -411-3p, -493-5p, -494-3p, -495-3p, -548-5p, and -93-3p) that was statistically significantly associated with overall survival.

M. Boeri et al. prospectively assessed the expression of plasma miRNAs in patients with NSCLC before starting ICI therapy. The analysis of the miRNA profile allowed the assessment of the relationship with parameters such as overall response rate, progression-free survival, and overall survival. The expression level of miRNAs during therapy decreased and remained low until tumour progression in patients responding to the treatment [32].

T. Rajakumar et al. also evaluated the expression profile of various miRNAs in NSCLC and identified five miRNAs (miRNA-2115-3p, -218-5p, -224-5p, -4676-3p, 6503-5p) for assessing the risk, enabling the prediction of overall survival in stage IV NSCLC patients receiving monotherapy with a PD-1 inhibitor [33]. Other Italian researchers recognized exosomal miRNA-625-5p as a novel independent biomarker for response and survival in patients receiving ICI therapy in the first, second, or third line of treatment for NSCLC [34].

The response to immunotherapy is likely determined by the complex interaction between the tumour and immune-dependent factors, thereby limiting the prediction of the response based on individual biomarkers and unilateral tumour parameters. Additionally, sample limitations, tumour heterogeneity, and interpopulation differences also necessitate further in-depth investigation to fill knowledge gaps. Clearly, the identification and validation of additional prognostic biomarkers for assessing the effectiveness and safety of immunotherapy will be an active research direction in the years to come. In the era of personalized medicine, further research and confirmation of results in a larger independent cohort of patients, as well as in vitro and in vivo modeling, are required. This will enable the integration of miRNAs for monitoring and predicting responses to treatment and therapy resistance in clinical settings.

FUNDING

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Ethics Committee of the Institute of Biochemistry and Genetics—Subdivision of the Ufa Federal Research Centre of the Russian Academy of Sciences, protocol No. 11 dated October 27, 2014. Informed consent was obtained from all individual participants involved in the study. All participants were adults.

Written informed consent form for the collection of biological samples and molecular-genetic analysis was obtained from each patient.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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