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Research Article
Upregulation of MicroRNA-146a Increases the Sensitivity of Cisplatin-Resistant Lung Cancer Cells to Chemotherapy Through the Toll-Like Receptor-Signaling Pathway

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Abstract

Background and Objective: Cisplatin, a platinum-based chemical, is a key agent in cancer treatment but is often associated with the development of resistance in several cancers. This resistance has been linked to miRNAs, which play crucial roles in gene regulation. In this study, the involvement of miRNAs in cisplatin resistance in NSCLC cells was explored. **Materials and Methods:** The differentially expressed miRNAs (DEMs) identified using the GSE43249 dataset and various bioinformatics tools. The experimental part involved culturing A549 cells, inducing cisplatin resistance, transfecting cells with miR-146a and assessing gene expression changes. Statistical analysis of the data was performed using R software and GraphPad Prism to elucidate the underlying mechanisms of drug resistance. **Results:** The downregulation of specific miRNAs, especially miR-146a. Functional studies revealed that miR-146a could target the TLR signaling pathway, a crucial pathway in cellular response. Notably, transfection with a miR-146a mimic increased the sensitivity of resistant cells to cisplatin, signifying a potential therapeutic avenue. Further, miR-146a was shown to downregulate the expression of key genes like TNF, MYD88 and IRAK1 in the resistant cells. **Conclusion:** This study provides insights into the molecular mechanisms behind cisplatin resistance in NSCLC cells and suggests potential therapeutic strategies leveraging miRNA modulation.

Key words: Cancer, microRNA, cisplatin, chemoresistance, gene expression, toll-like receptor

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Lung cancer is one of the most common cancers and a primary cause of cancer-related mortality worldwide[1]. Various forms of NSCLC exist, making up approximately 85% of the annual lung cancer diagnoses. While, smoking remains the primary culprit behind this ailment, elements like radon and environmental pollution also contribute to its development. Many people are detected at an advanced stage due to inadequate screening methods and late-emerging symptoms, resulting in a bleak outlook for patients[2]. It is recommended that patients with stage IV NSCLC immediately begin a cytotoxic combination chemotherapy regimen[3]. According to the American Society of Clinical Oncology, the treatment for a patient with a PS of 0 or 1 is a regimen that includes a platinum (cisplatin or carboplatin) in addition to paclitaxel, gemcitabine, docetaxel, vinorelbine, irinotecan or pemetrexed. This treatment is administered sequentially[4].

Cisplatin, cis-diamminedichloroplatinum or DDP, is a platinum-based chemical that produces intra- and inter-strand adducts with DNA and as a result, it is a powerful inducer of cell cycle arrest and death in the majority of different types of cancer cells[5]. Chemoresistance is common among cancer patients. This may occur as a side effect of cisplatin treatment or occur naturally in patients with colorectal, lung, or prostate cancer (as is commonly seen in patients who have ovarian cancer)[6]. The mounting evidence indicates that microRNAs (miRNAs or miR), among other things, are responsible for the modification of cisplatin resistance. The miRNAs are small (18-23 nucleotide) strands of endogenous, noncoding RNA. They can function as either a tumor suppressor or an oncogene depending on their effect on the posttranscriptional regulation of gene expression[7]. Reduced cisplatin accumulation, increased detoxification systems (such as glutathione S-transferase (GST) and glutathione peroxidase (GSTp1), decreased DNA damage and/or increased DNA repair are all potential molecular causes of cisplatin resistance. Tumour cells' acquired resistance to cisplatin allows them to evade the drug's cytotoxic effects and avoid death via apoptosis[8]. It has been demonstrated that microRNAs play a significant part in the progression of chemosensitivity and chemoresistance in lung cancer. These regulatory mechanisms are complementary to one another in cancerous cells and tissues, where they can either increase or impede one another's activity[9]. To better understand the role of miRNAs in the development of cisplatin resistance in NSCLC cells, a bioinformatics strategy based on the analysis of non-coding microarray data and molecular interaction identifiers was used in the present investigation. The bioinformatics data suggested the top hits of down regulated miRNAs. Cell-based experiments, transfection and qPCR were used to investigate the potential players in cancer cells chemo resistance and suggested the possible interventions.

MATERIALS AND METHODS

Study area: The study was taken place in Saudi Arabia from October, 2022 to June, 2023.

Dataset selection and differentially expressed miRNA (DEM) analysis: The gene expression omnibus (GEO) database was browsed to retrieve the GSE43249 dataset which is noncoding RNA expression profiling of cisplatin-resistant A549 cells[10]. The micro-array profiling was carried out by GPL14613 ([miRNA-2] Affymetrix Multispecies miRNA-2 Array). Linear Models for Microarray Data (LIMMA) v3.44.3 package of R v4.0.2 was employed to normalize sample values and DEMs[11]. To find DEMs following cut-off criteria were chosen, $|logFC| \geq 0.5$ and $p<0.01$.

Target prediction and functional enrichment analysis: After finding significant DEMs, the targeting ability of selected miRNA was analyzed via the miRTarBase prediction tool[12]. The cut-off criterion for choosing significant targets was the targets that were experimentally validated by qPCR. The significant targets then were functionally analyzed using ClueGO v2.5.9[13] plugin of Cytoscape v3.9.1 (Open source collaborative software)[14]. The involved pathways were retrieved from the Kyoto Encyclopedia of Genes and Genomes (KEGG, Kyoto, Japan) database with the cut-off criterion of $p<0.0001$. Besides, the protein-protein interaction of genes involved in the selected signaling pathway was analyzed by the STRING plugin (confidence score>0.7 as cut-off).

Cell culture: The RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (GIBCO, Carlsbad, California), incubated at 37°C, 5% CO_2 and 90% humidity, was used to develop the A549 cell line, an adenocarcinoma human alveolar basal epithelial cells. Results for every test were gathered when A549 cells were in their logarithmic phase of development.

Transfection of miRNA: The miR-146a Mimic (5'-UGAGAACUG AAUUCCAUGGGUU-3') was developed by and purchased from Bioneer Corporation (Daejeon, South Korea). The transfection reagents and medium used were jetPRIME (Polyplus Co., Illkirch, France) and Opti-MEM (Gibco Life Technologies, Gaithersburg, Maryland). A different number of cells were

Table 1: Primer pairs used in the expression experiments

Gene name	Type	Sequence (5'→3')
MYD88	F	GGCTGCTCTCAACATGCGA
	R	CTGTGTCCGCACGTTCAAGA
IRAK1	F	GCACCCACAACTTCTCGGAG
	R	CACCGTGTTCCTCATCACCG
TNF	F	CCTCTCTCTAATCAGCCCTCTG
	R	GAGGACCTGGGAGTAGATGAG
miR-146a	F	AAGCGACCTGAGAACTGAATTC
	R	GTCGTATCCAGTGCAGGGT
	Stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACCCA
GAPDH	F	CAAGATCATCAGCAATGCCT
	R	GCCATCACGCCACAGTTTCC
U6 snRNA	F	AACAATGTGCTCGCTTCGG
	R	GTCGTATCCAGTGCAGGGT
	Stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAATA

MYD88: Myeloid differentiation primary response 88, IRAK1: Interleukin-1 receptor-associated kinase, TNF: Tumor necrosis factor, miR-146a: MicroRNA 146a, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase and snRNA: Small nuclear RNA

seeded in a cell culture plate with free serum and antibiotics for each test, along with RPMI-1640. The miRNAs Mimic were diluted in jetPRIME dilution buffer and subjected to the jetPRIME reagent for 30 min at room temperature. The Opti-MEM medium's wells were then filled with the miRNA-containing mixtures. After that, the cell culture plates were incubated at 37°C for 5-7 hrs in an incubator. Following that, RPMI-1640 medium with 20% FBS was added to each group of transfected cells.

IC_{50} analysis: The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) technique was carried out to find the IC_{50} of cisplatin in parental cells and whether the chemoresistance was induced. Approximately, 2×104 cells of parental and resistant cells were seeded in a 96-well plate, after which they were treated with a serial dilution of cisplatin. A 24 hrs after treatment, the media of all wells were removed and the cells were exposed to the MTT solution with a concentration of 2 mg mL^{-1}. According to the instruction, the formed formazan crystals were dissolved in Dimethyl Sulfoxide (DMSO) (Merck, Germany) and the absorbance of each well was read by a microplate reader (Tecan, Switzerland).

Inducing chemo resistance: The parental cells were exposed to different concentrations of cisplatin, ranging from 10-100% IC_{50}. The procedure involved 3 days of drug exposure followed by 3 days of recovery, repeated approximately three times for each concentration. This process was carried out prior to preparing the parental A549 cells and establishing the IC_{50} of cisplatin in them. After this method, the IC_{50} value should be increased by a factor of at least 10.

qRT-PCR: Upon transfection of resistant cells with the miR-146a, total RNA was harvested from both the transfected and non-transfected groups using the TRIzol reagent (GeneAll, South Korea). Subsequently, complementary DNA (cDNA) was produced using the 2 step 2X RT-PCR Pre-Mix (BioFACT™, South Korea). The expression levels of miR-146a and chosen targets were then assessed using the 2X Real-Time PCR Master Mix (BioFACT™, South Korea) and particular primer pairs (Table 1).

Statistical analysis: The microarray data were analyzed by R software v4.0.2, Biobase[15], GEOquery[16], LIMMA[11] and Affy[17]. The ggplot2 package was also used to illustrate the volcano plot. The expression data was then analyzed by GraphPad Prism v8.0 (GraphPad Software, San Diego, California USA and related statistical tests including t-test, ANOVA one-way and Tukey multiple comparisons. Results were deemed statistically significant at a ($p \leq 0.05$).

RESULTS

Upregulated and downregulated DEMs: The DEMs analysis demonstrated that 211 miRNAs have significantly altered expression after miR-146a mimic transfection, of which 15 miRNAs belong to humans (Fig. 1). In human miRNAs, there were 8 upregulated and 7 downregulated miRNAs in parental cells in comparison with resistant cells (Table 2). The miR-146a which has the most significant p-value was chosen to be transfected to the cisplatin-resistant A549 cells.

miR-146a might target the TLR signaling pathway: After predicting and finding probable targets of miR-146a, the functional enrichment analysis's result demonstrated that targets of miR-146a could significantly participate in the TLR signaling pathway with p = 3.59E-11 (Fig. 2a). The PPI of contributing target genes in TLR signaling pathway including

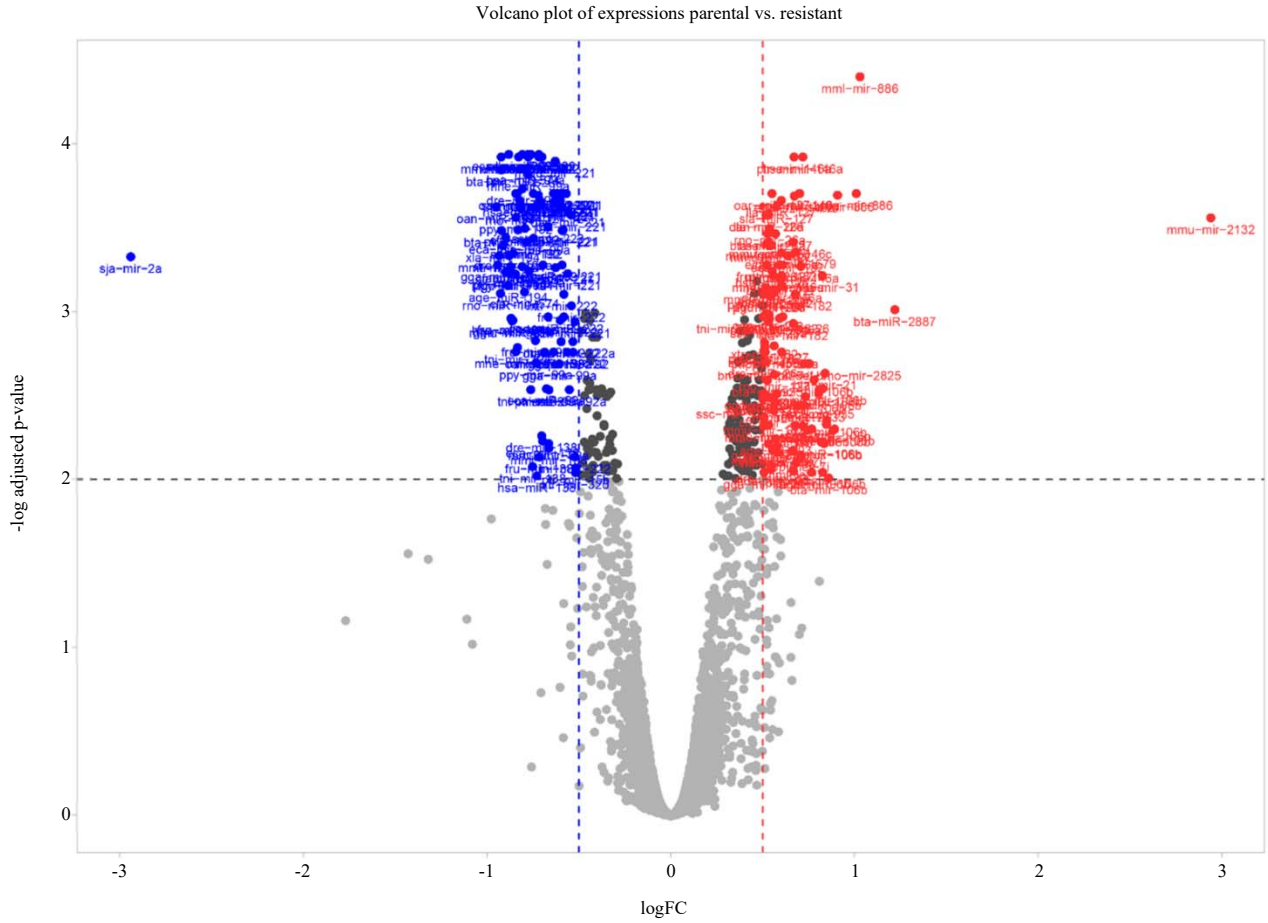

Fig. 1: Volcano plot of DEMs expression, Downregulated DEMs are blue dots on the left side and Upregulated ones are red dots on the other side

Table 2: Differences in expression levels (DEMs) between parental and resistant cells

miRNA	Adjusted p-value	logFC	Status
hsa-mir-146a	0.0001219	0.719	Up
hsa-mir-886	0.0002056	0.908	Up
hsa-mir-127	0.0003487	0.57	Up
hsa-mir-126	0.0005804	0.552	Up
hsa-mir-182	0.0007974	0.678	Up
hsa-mir-106b	0.0044782	0.849	Up
hsa-mir-31	0.0063385	0.58	Up
hsa-let-7i	0.0067244	0.666	Up
hsa-mir-222	0.0001219	-0.718	Down
hsa-mir-574	0.0001427	-0.803	Down
hsa-mir-221	0.000211	-0.623	Down
hsa-mir-192	0.0002208	-0.823	Down
hsa-mir-99a	0.0003705	-0.747	Down
hsa-miR-194	0.0011092	-0.87	Down
hsa-miR-138	0.0095611	-0.729	Down

RAC1, STAT1, IRAK1, NFKB1, TRL2, TRAF6, TNF, IL6, MYD88, IRAK4, TRL4, TAB2 and SPP1 then were evaluated and obtained results predicted that IRAK1, TNF and MYD88 were highly interactive with the number of interaction equal to 11 (Fig. 2b).

miR-146a mimic could increase the sensitivity of resistant cells to cisplatin: The IC_{50} analysis showed that parental cells were sensitive to cisplatin treatment with IC_{50} of 0.1297 μg mL^{-1}. This sensitivity could be changed by the induction of resistance up to 1.049 μg mL^{-1} (approximately

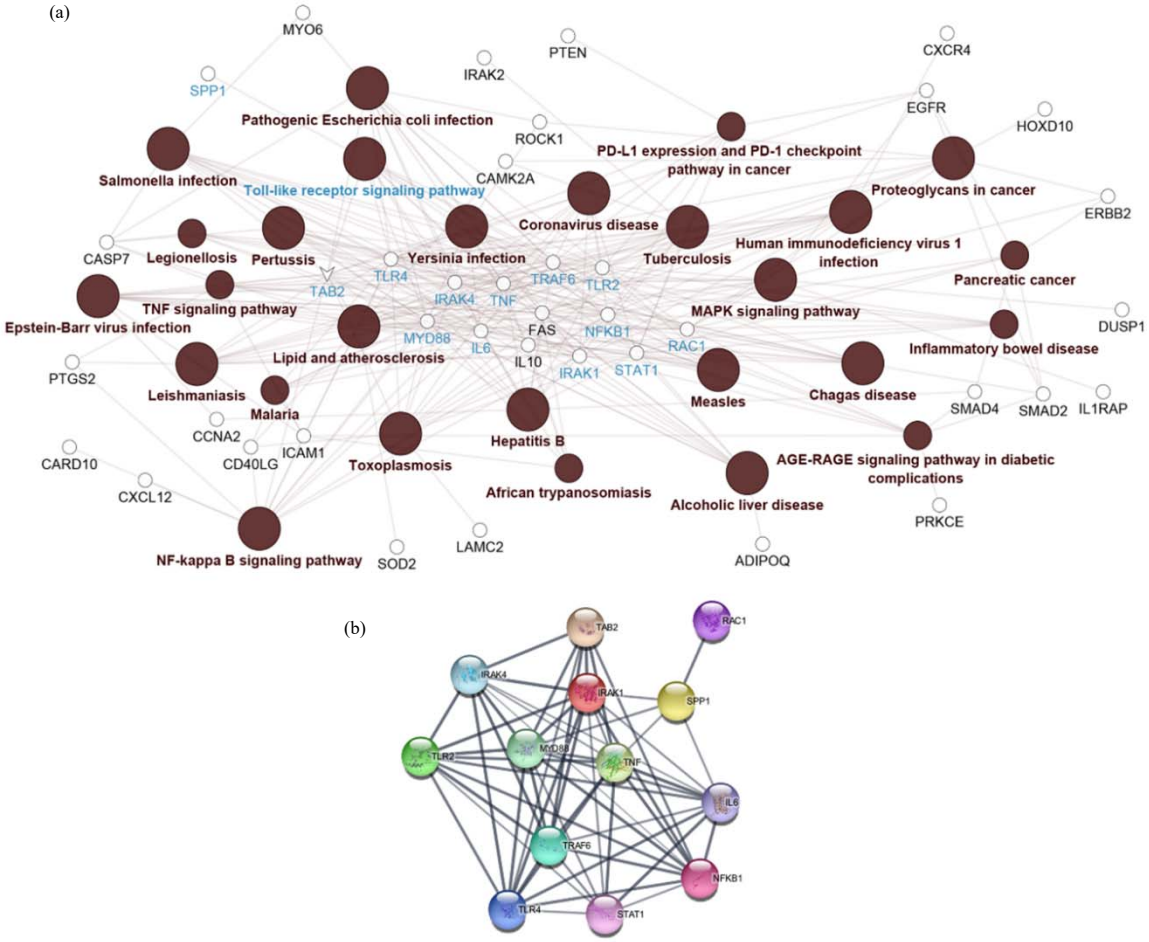

Fig. 2(a-b): Enrichment analysis of targeted genes showed the involvement of (a) Several cellular signaling pathways and diseases and (b) PPI of STRING showed IRAK1, TNF and MYD88 have the highest number of interactions

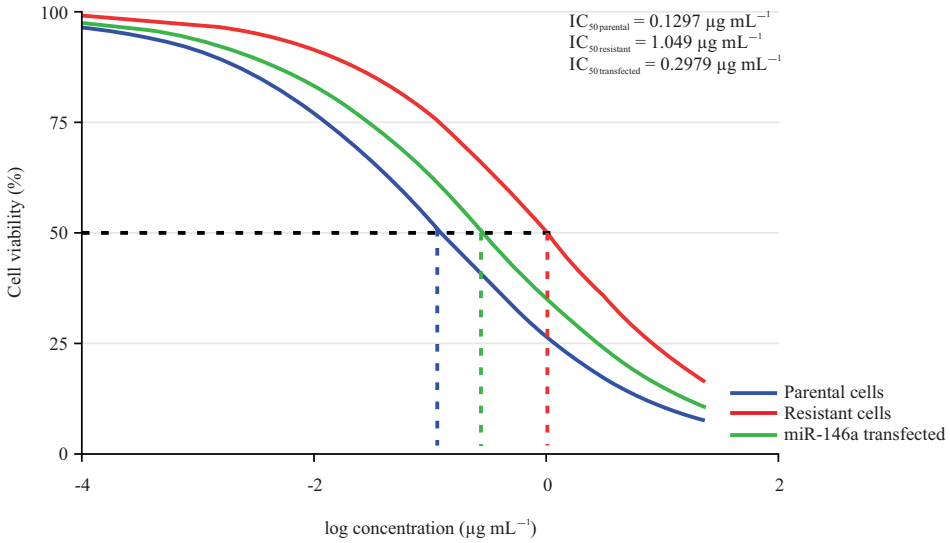

Fig. 3: IC_{50} analysis of cisplatin in parental, resistant and transfected cells

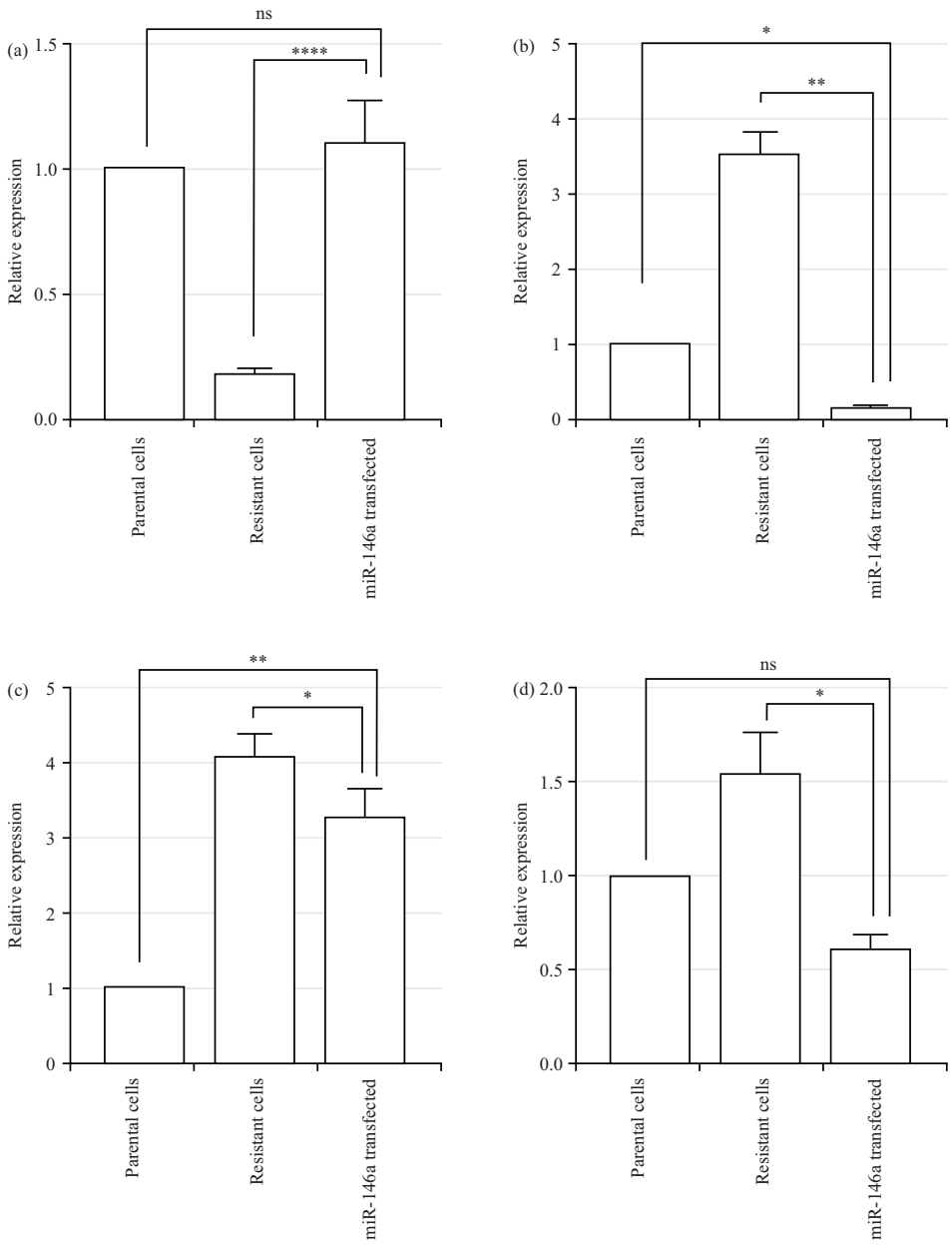

Fig. 4(a-d): Altered expression of (a) miR-146a, (b) TNF, (c) MYD88 and (d) IRAK1 upon the transfection of miR-146a mimics in cisplatin-resistant A549 cells

*p<0.05, **p<0.01, ****p<0.0001 and ns: Not significant

10 times). Besides, miR-146a mimic transfection was able to reduce the IC_{50} to about 0.2979 μg mL^{-1} (Fig. 3).

miR-146a mimics decrease the expression of TNF, MYD88 and IRAK1: The qRT-PCR revealed that the transfection of miR-146a mimic to cisplatin-resistant A549 cells could significantly decrease the expression of TNF, MYD88 and IRAK1 in these cells in comparison with untransfected resistant cells. Besides induction of cisplatin resistance affected the expression of miR-146a, TNF and MYD88; however, no significant change was seen in the expression of IRAK1 after inducing resistance (Fig. 4).

DISCUSSION

The current study revealed that cisplatin resistance could affect the expression of several miRNAs and among them, miR-146a was highly affected. The miR-146A gene that is

found in humans may be found on chromosome 5. The miR-146a, in its mature form, is highly conserved across all vertebrate species. Since the primary transcript (pri-miR-146a) is generated as a standalone unit rather than from a protein-coding gene's intron (miRtron), it is regulated by a unique promoter region[18]. In NSCLC cell lines, it was shown that miR-146a promoted programmed cell death while discouraging cell proliferation and cell migration[19]. These three behaviors are considered to be essential characteristics of cancer. Downregulation of miR-146a was observed in several NSCLC cell lines and correlated strongly with poor prognosis in patients with advanced lung cancer and decreased progression-free survival[20]. This was demonstrated in several NSCLC cell lines. It has been hypothesized that miR-146a may play a significant part in a number of the characteristics that are associated with lung cancer, including the ability to avoid growth barriers, cell proliferation, resistance to cell death, promotion of angiogenesis, inflammation, cancer immune tolerance, imbalance in energy metabolism (the Warburg effect), metastasis and genome fragility[21]. The results of the present study showed that the upregulation of this miRNA could significantly affect genes, that belong to the TLR, signaling pathway. These genes include TNF, MYD88 and IRAK1.

The TNF-α can be generated in the microenvironment of a tumor by cancerous cells, immune cells that have infiltrated the tumor and stroma cells. Patients who have more than one form of advanced cancer have a higher than normal level of adaptor protein known as MYD88[22]. The TLRs can initiate a signaling cascade by bringing the cytoplasmic toll-interleukin 1 receptor (TIR) domains together after ligand binding by inducing the dimerization of their ectodomains, which then leads to the activation of signal adaptor molecules. The MYD88, TIR production of miR-146 is induced in the THP1 cell line by the same TLRs that are listed above: TLR2, TLR4, TLR5, TLR7-TLR8 and TLR9. These proteins are essential components of the MYD88-dependent pathway for NF-κB activation that occurs downstream of these TLRs. Following bacterial infection, it has been hypothesized that miR-146 can operate as a negative regulator of the MYD88-NF-κB signaling pathway[23]. The TIR-domain-containing adaptor protein (TIRAP; also known as MAL), TIRAP-inducing IFN-β (TRIF) and TRIF-related adaptor molecule are the four primary TLR adaptor molecules. The MYD88 is an adapter protein that contains the TIR domain (TRAM). These signaling pathways may be roughly categorized as either being dependent on MYD88 or being independent of MYD88[24]. The TNF-α expression in both their biopsies and their plasma[25] even though different TLRs make use of these adaptors in a variety of different combinations. The expression of MYD88, which is involved in the immune response to cancer, was found to be modulated by miR-146a in several different investigations[26,27]. The IL-1R-associated kinase 1 (IRAK1) and TNFR-associated factor 6 (TRAF6) are the cellular components that are targeted by miR-146[23]. It is common knowledge that TNF-α has a role in all stages of the formation of tumors, including carcinogenesis, proliferation, angiogenesis, metastasis and the suppression of immune responses[25]. In addition, TNF-α causes melanoma cells to become resistant to BRAF inhibitors and causes malignant pleural mesothelioma cells to become resistant to the chemotherapeutic drug cisplatin[28]. Even though the bulk of studies have focused on the role of TNF-α in carcinogenesis and the formation of tumors, far less is known about the actions of TNF-α in cancer, especially in chemoresistance. The miR-146a has been shown to inhibit TNF expression as well[29].

This study highlights the potential caveats in the investigation of NSCLC cell responses to cisplatin and miRNA modulation. However, the study could impose some limitations, specifically, the research used the A549 cell line, which might not fully capture NSCLC's cellular heterogeneity. Moreover, the study's findings are grounded in *in vitro* data, which might not translate directly to *in vivo* conditions, especially considering the complexities of the tumor microenvironment.

Apart from TLR3, all TLRs relay their signals through an adaptor protein known as MYD88[22]. The TLRs can initiate a signaling cascade by bringing the cytoplasmic TIR domains together after ligand binding by inducing the dimerization of their ectodomains, which then leads to the activation of signal adaptor molecules. The MYD88, TIRAP, TRIF and TRIF-related adaptor molecule are the four primary TLR adaptor molecules. The MYD88 is an adapter protein that contains TRAM. Cell signaling routes can typically be divided into two groups: Those that require MYD88 to function and those that function without relying on MYD88[24], although various TLRs utilize these adapters in a diverse array of assortments. The expression of MYD88, which is involved in the immune response to cancer, was found to be modulated by miR-146a in several different investigations[26,27]. The IRAK1 and TRAF6 are the cellular components that are targeted by miR-146[23]. The production of miR-146 is induced in the THP1 cell line by the same TLRs that are listed above: TLR2, TLR4, TLR5, TLR7-TLR8 and TLR9. These proteins are essential components of the MYD88-dependent pathway for NF-κB activation that occurs downstream of these TLRs. Following bacterial infection, it has been hypothesized that miR-146 can operate as a negative regulator of the MYD88-NF-κB signaling pathway[23].

The study imposes several future recommendations. Despite the *in vitro* evidence presented, the study acknowledges the need for *in vivo* validation through animal models or patient-derived xenografts to confirm the findings in a more complex biological context. Clinical studies are recommended to establish miR-146a's clinical relevance as a biomarker or therapeutic target, involving the correlation of miR-146a expression levels with patient prognosis and response to cisplatin.

CONCLUSION

When it comes to the treatment of solid malignancies like NSCLC, cisplatin is widely regarded as one of the most efficient and widely utilized chemotherapeutic medications available. Microarray data (GSE43249) that was collected from cisplatin-resistant A549 cells were used in this study. We selected the most significantly downregulated member of the DEMs to test its efficacy in reversing chemoresistance in cancer cells. The results of this study depicted that miR-146a could act as a key element in the regulation of cisplatin resistance in NSCLC by modulating the TLR signaling pathway. Finding this molecular axis as miR-146a/TNF/MYD88/IRAK1 sheds a light on the investigation of new therapeutic approaches in patients suffering from NSCLC, particularly using a combination of miRNAs and chemotherapy to enhance the effectiveness of treatment and dealing with the probable induction of chemoresistance.

SIGNIFICANCE STATEMENT

This study unveils a significant knowledge in our understanding of cisplatin resistance in Non-Small Cell Lung Cancer (NSCLC) by shedding light on the pivotal role of microRNAs (miRNAs), specifically miR-146a. The research demonstrates that the downregulation of miR-146a contributes to cisplatin resistance in NSCLC cells, potentially through its modulation of the Toll-Like Receptor (TLR) signaling pathway. Moreover, the study shows that introducing a miR-146a mimic can sensitize resistant cells to cisplatin, suggesting a promising therapeutic approach. Furthermore, the findings reveal miR-146a's ability to downregulate key genes involved in resistance, including TNF, MYD88 and IRAK1. Overall, this research offers valuable insights into the molecular mechanisms underlying cisplatin resistance and presents an exciting avenue for future therapeutic interventions centered around miRNA manipulation.

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