ORIGINAL ARTICLE

MicroRNA-24 inhibits the proliferation, migration and invasion and enhances chemosensitivity of human gastric cancer by targeting DND1

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Summary

Purpose: *Gastric cancer causes significant human mortality and is the fourth prevalent type of cancer across the globe. The gastric cancer treatment is hurdled by late diagnosis due to unavailability of biomarkers, lack of potent therapeutic targets and adverse effects of chemotherapy. Recent reports have indicated that miR-24 acts a tumor suppressor in different cancers. This study explored the role and therapeutic implications of miR-24 in gastric cancer.*

Methods: *Expression analysis was carried out in gastric cancer tissues and cell lines by qRT-PCR. Proliferation rate was monitored by WST-1 assay. Transwell assay was used to determine cell invasion and wound healing assay was used for cell migration. Protein expression analysis was carried out by western blot analysis.*

Results: *The results showed that miR-24 was significantly suppressed in gastric cancer tissues and cell lines. Overex-pression of miR-24 in SNU-1 gastric cancer cells resulted in decline of proliferation rate in a time-dependent manner. In silico analysis together with the dual luciferase assay revealed RNA binding protein DND1 to be the target of miR-24. Expression analysis of DND1 was found to be significantly overexpressed in gastric cancer tissues and cell lines. Suppression of DND1 suppressed the proliferation of gastric cancer cells. Wound healing and transwell assay revealed that miR-24 overexpression also inhibited the migration and invasion and also enhanced the chemosensitivity of the SNU1 gastric cancer cells.*

Conclusion: *Taken together, miR-24 may prove to be an important therapeutic target for the treatment of gastric cancer and warrants further studies.*

Key words: *gastric cancer, MicroRNA-24, proliferation, migration*

Introduction

Accounting for 7.3 million deaths, gastric cancer is one of the most fatal human malignancies. It is the second major cause of cancer-related mortality across the world [1]. Annually around 1 million new gastric cancer cases are reported worldwide, making gastric cancer the fourth most prevalent cancer around the globe [2]. Except for Japan, the survival rates for gastric cancer are generally poor. Although several factors, such as environmental and genetic, have been implicated in the development of gastric cancer, *Helicobacter pylori* infection, is considered as the main culprit for the initiation of gastric cancer development [3]. Gastric cancer treatment varies depending on the stage of disease. Generally, chemotherapy, surgery or the combination of both is used to treat gastric cancer. However, the treatment is limited by late diagnosis due to unavailability of biomarkers, lack of potent therapeutic targets and adverse effects of chemotherapy [4]. MicroRNAs (miRs) are single stranded 19-25 nucleotides non-coding RNAs produced from endogenous hairpin transcripts. In human miRs are highly conserved and exhibit multiple isoforms [5]. They negatively control the expression of human

genes and it has been reported that around 60% of the human protein-coding genes harbour miR-binding sites [6]. Since, miRs control vital cellular processes in humans, it is not surprising that they control the development of diseases such as cancer [7]. Dysregulation of several miRs may contribute to the development of cancer and this was discovered for the first time in 2002 when deletion of miR-15 and miR-16 were reported to be involved in the development of lymphocytic leukemia [8]. miR-24 is one of the important miRs and regulates cancer growth as indicated by several studies. It has been shown to regulate the growth and metastasis of the bladder cancer cells via targeting FOXM1 [9]. It has also been shown to target the RNA binding protein DND1 to regulate the growth of the tongue squamous cell carcinoma [10]. However, the role of miR-24 in gastric cancer is still largely unknown.

This study was therefore undertaken to investigate the role and therapeutic potential of miR-24 in gastric cancer.

Methods

Tissue samples and cell lines

The snap-frozen gastric cancer and adjacent normal tissues (N1 to N8) were collected from the Department of General Surgery, Tianjin First Central Hospital, Tianjin 300192, China, after obtaining informed consent from the patients. The study was approved by the Research ethics committee of the institute under approval number TFCH02/CA/2018. The SNU-1 human gastric cancer cell line and normal GES-1 cell line used in the present study were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI 1640 medium (Gibco, Carlsbad, CA, USA) containing penicillin (100 U/mL), streptomycin (100 μg/ml) (Sigma-Aldrich, St. Louis, MO, USA), and 10% fetal bovine serum (FBS; Gibco) at 37°C in 5% CO_2.

Expression analysis

TRIzol reagent (Invitrogen, Carlsbad, California, USA) was used for the extract of RNA from the tissues and cell lines. This was followed by purification of the RNA by RNeasy Mini Kit (Qiagen, Hilden, Germany). The complementary (c) DNA was then synthesized with the help of miScript Reverse Transcription Kit (Qiagen). Afterwards, the cDNA was amplified using SYBR Premix Ex *Taq*™ (TaKaRa, Otsu, Shiga, Japan). The expression was estimated by $2^{-\Delta\Delta Ct}$ method and actin was used as an internal control.

Cell transfection

The miR-24 mimics and miR-negative control (NC) were synthesized by RiboBio (Guangzhou, China). Transfection was then carried out using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instruc¬tions. As the SK-BR-3 and CAMA-1 cells reached 80% confluence, the appropriate concentrations of miR-24 mimics or NC were transfected into these cells.

The WST-1 assay

The proliferation rate of SNU-1 cells was monitored by WST-1 assay. In brief, SNU-1 cells were cultured in 96-well plates at a density of 2×10^5 cells/well. The cells were then transfected with miR-NC or miR-24 mimics and again incubated for 24 h at 37°C. This was followed by incubation of the cells with WST-1 at 37°C for another 4 h. The absorbance was then taken at 450 nm using a victor 3 microplate reader to determine the proliferation rate at 0, 12, 24, 48 and 96 h time intervals.

Wound healing assay

The transfected SNU-1 cells were cultured till 80% confluence. This was followed by removal of Dulbecco's modified Eagle's medium (DMEM) and subsequent washing with phosphate buffered saline (PBS). Afterwards, a wound was scratched with a sterile pipette tip and a picture was taken. The plates were then incubated for about 24 h at 37°C and then a picture was taken again with an inverted microscope.

Cell invasion assay

The effects of miR-24 overexpression on the invasion ability of SNU-1 cells was determined by transwell chambers with Matrigel. The SNU-1 cells were transfected with miR-24 mimics and around 200 ml cell culture were placed onto the upper chamber and only DMEM was placed in the bottom chamber. After 24 h of incubation, the cells were removed from the upper chamber and the cells that invaded via the chambers were fixed with methyl alcohol and subsequently stained with crystal violet. Inverted microscope was used to count the number of invaded cells at 200× magnification.

Dual-luciferase reporter assay

The miR-24 target was identified by TargetScan online software (http://www.targetscan.org). The miR-24 mimics or NC were co-transfected with Plasmid pGL3-DND1-5´-UTR-WT or pGL3-DND15´-UTR-MUT into SNU-1 cells. Dual-luciferase reporter assay (Promega, Madison, Wisconsin, USA) was carried out at 48 h after transfection. *Renilla* luciferase was used for normalization.

Western blotting

The normal and gastric cancer tissues and cell lines were lysed in RIPA buffer and then centrifuged at high speed. Then the concentrations of the proteins in the cell extracts were determined and equal concentrations of the proteins were loaded on SDS-PAGE gel (15%). The samples were transferred to polyvinylidene fluoride membranes and blocking was done using 5% skimmed milk powder. This was followed by subjecting of the membrane to incubation with primary antibody at 4°C for 24 h. Next, the membranes were incubated with secondary antibody for 2 h. The membranes were washed with tris-buffered saline and immunoreactive

bands were observed by ECL-PLUS/Kit as per the manufacturer's guidelines.

Statistics

Statistical analysis was performed using a one way analysis of variance (ANOVA) followed by Tukeys's *post-hoc* test using SPSS software package v9.05 (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean±standard deviation, and p<0.05 was considered to indicate a statistically significant difference.

Results

miR-24 expression is suppressed in gastric cancer

The expression of miR-24 was determined in 15 gastric cancer and 15 normal adjacent tissues and the results showed that the expression of miR-24 was significantly suppressed in gastric cancer tissues. The expression of miR-24 was downregulated in gastric cancer tissues by upto 9-folds
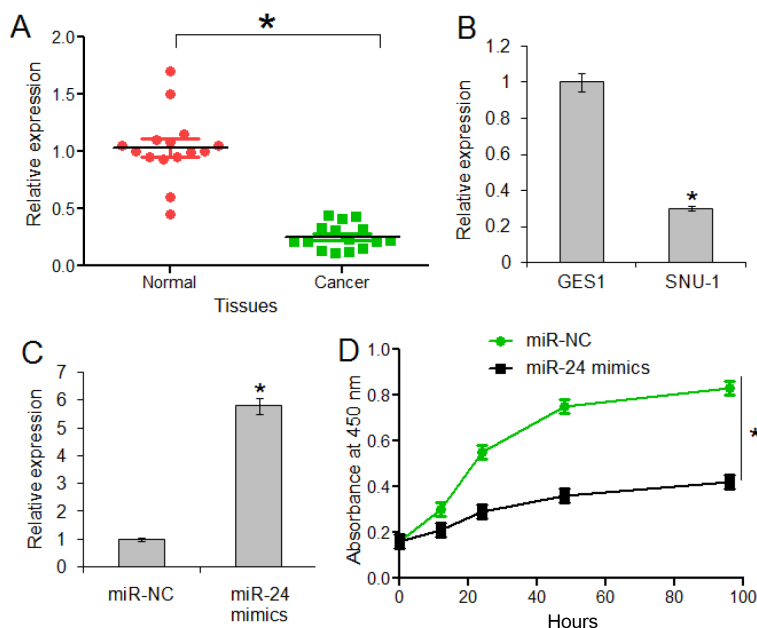

Figure 1. miR-24 inhibits the proliferation of gastric cancer cells. **A:** Expression of miR-24 in normal and gastric cancer tissues as determined by qRT-PCR. **B:** Expression of miR-24 in gastric cancer SNU-1 and normal GES-1 cells. **C:** Expression of miR-24 in miR-NC or miR-24 mimics transfected SNU-1 cells. **D:** Cell viability of the miR-NC or miR-24 mimics transfected SNU-1 cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).
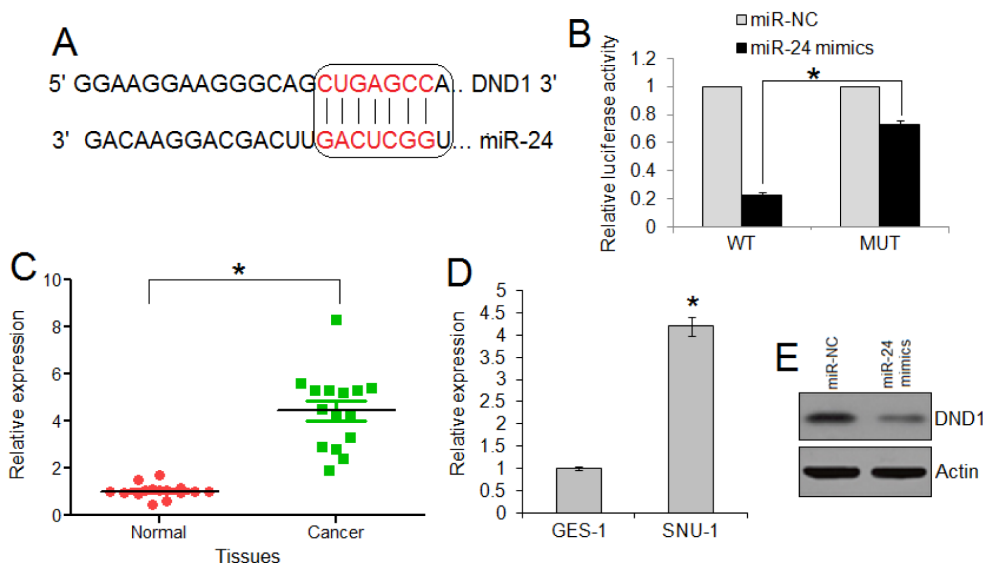

Figure 2. miR-24 exerts its effects by targeting DND1. **A:** TargetScan analysis showing that miR-24 targets DND1. **B:** Dual luciferase assay. **C:** Expression of DND1 in normal and gastric cancer tissues. **D:** expression of DND1 in normal GES-1 and SNU-1 gastric cancer cells. **E:** Expression of DND1 in miR-NC or miR-24 mimics transfected SNU-1 cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).
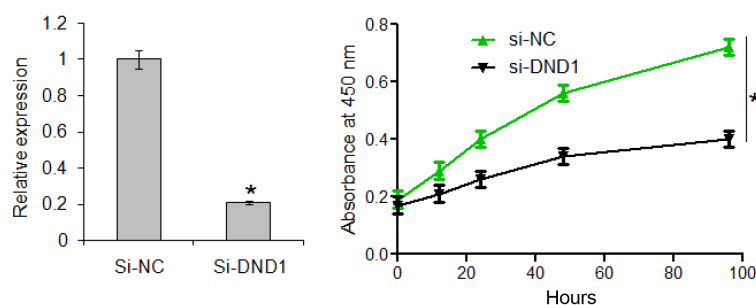
Figure 3. A: Expression of DND1 in si-NC or si-DND1 transfected SNU-1 gastric cancer cells. **B:** cell viability of the si-NC or si-DND1 transfected SNU-1 cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).
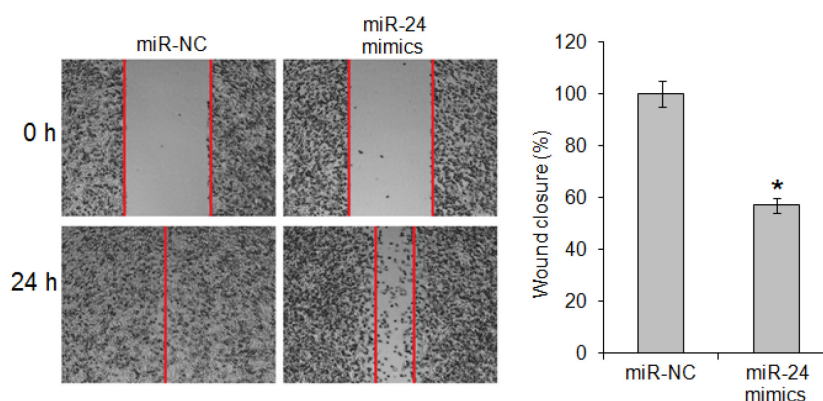

Figure 4. Wound healing assay showing cell migration in miR-NC and miR-24 mimics transfected SNU-1 gastric cancer cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

(Figure 1A). The expression of miR-24 was also investigated in the normal GES-1 and gastric cancer SNU-1 cell lines and found more than 3-folds in gastric cancer cells (Figure 1B).

miR-24 inhibits the proliferation of the SNU-1 gastric cancer cells

To assess the effects of miR-24 on the proliferation of the SNU-1 gastric cancer cells, miR-24 was overexpressed in SNU-1 gastric cancer cells by transfection with miR-24 mimics. The overexpression of miR-24 was validated by qRT-PCR (Figure 2A). Next, the effects the miR-24 overexpressing SNU-1 cells were subjected to WST-1 assay. The results showed that miR-24 overexpression caused time-dependent inhibition of the proliferation of the SNU-1 cells (Figure 2B).

miR-24 targets RNA binding protein DND1 in gastric cancer

Target identification by TargetScan online software revealed that miR-24 targets the RNA binding protein in SNU-1 gastric cancer (Figure 3A). Dual luciferase assay also confirmed the interaction between miR-24 and DND1 (Figure 3B). Next, the expression of DND1 was examined in the gastric cancer tissues and cell lines and it was found that the

expression of DND1 was upregulated in both the gastric cancer tissues and cell lines (Figure 3C and Figure 3D). Nonetheless, overexpression of miR-24 caused remarkable downregulation of RNA binding protein DND1 (Figure 3E).

miR-24 inhibits the migration and invasion of SNU-1 cells

The effects of miR-24 on the migration of the SK-BR-3 cells were determined by wound healing assay. The results showed that miR-24 caused significant decrease in the migration of the SNU-1 cells as evidenced from the wound width (Figure 4). Transwell assay was used to investigate the cell invasion of the SNU-1 cells and the results showed that the migration of the SNU-1 was significantly inhibited. The migration in miR-24 overexpressing SNU-1 cells was found to be 27% relative to control (Figure 5).

miR-24 enhances the chemosensitivity of the SNU-1 cells

The chemosensitivity of miR-24 overexpressing cells was also assessed by WST-1 assay. The miR-24 mimics-transfected, cisplatin (2 μM) treated or miR-24-transfected plus cisplatin-treated SNU-1 cells were subjected to WST-1 assay. The
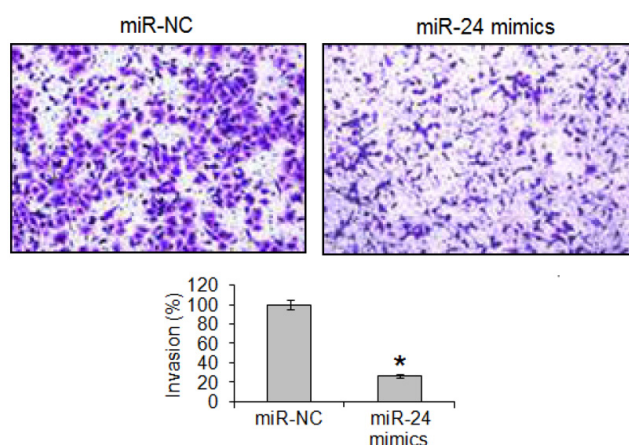
Figure 5. Transwell assay showing cell invasion in miR-NC and miR-24 mimics transfected SNU-1 gastric cancer cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).
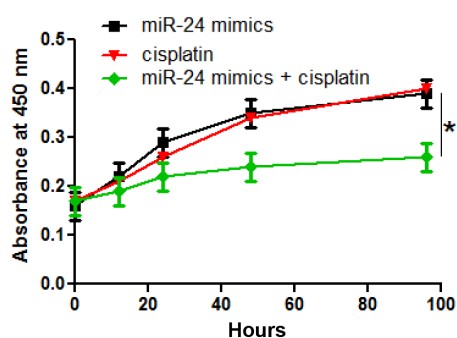

Figure 6. miR-24 enhances the cisplatin resistance in SNU-1 gastric cancer cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

results showed that proliferation rate of miR-24 mimics-transfected plus cisplatin-treated cells was decreased more profoundly than miR-24 mimics transfected or cisplatin-treated cells (Figure 6). These results indicate that miR-24 overexpression enhances the chemosensitivity of the SNU-1 gastric cancer cells to cisplatin.

Discussion

Being the fourth most prevalent type of cancer, gastric cancer causes 0.73 million deaths annually world over [2]. The treatment of gastric cancer is obstructed by its diagnosis at advanced stages, lack of therapeutic targets and side effects of the chemotherapeutic targets [11]. Over the years miRs have shown great promise to be developed as therapeutic targets for the treatment of fatal diseases such as cancer [12]. Herein, the role of miR-24 was explored in gastric cancer. The expression of miR-24 was found to be significantly upregulated in the gastric cancer tissues and cell lines. These results are

in agreement with previous investigations carried out on the miR-24. For instance, miR-24 has also been shown to act as biomarker in colorectal cancer [13]. In addition, miR-24 has been shown to be significantly downregulated in bladder cancer [14]. Next, to gain insights about the role of miR-24 in gastric cancer, miR-24 was found overexpressed in the SNU-1 gastric cancer cells. The results showed that upon overexpression of miR-24 in gastric cancer SNU-1 cells, their proliferation was decreased time-dependently. Previously, miR-24 was reported to suppress the proliferation of lung cancer cells by targeting PKC-alpha [15]. Nonetheless, there are also some studies which have shown that miR-24 promotes the proliferation of lung cancer cells by targeting NAIF1 [16]. Bioinformatic analysis revealed the RNA binding protein to be the target of miR-24 which was also confirmed by dual luciferase assay. The expression of DND1 was aberrantly upregulated in gastric cancer tissues and cell lines and overexpression of miR-24 could suppress the expression of DND1. Silencing of DND1 in SNU-1 gastric cancer cells suppressed their proliferation, migration and invasion. DND1 has been reported to regulate the activity of miRs by binding to the sequences on the 3´UTR of the targeted mRNAs [17]. Studies have shown that DND1 is imperative for the survival of germ cells in zebrafish [18], and regulates the development of the testicular germ cell tumors in mice [19].

Finally, the effects of miR-24 overexpression were also investigated on the migration, invasion and chemosensitivity of the human gastric cancer cells. The results showed that overexpression of miR-24 enhanced the chemosensitivity and inhibited the migration and invasion of the SNU-1 gastric cancer cells to cisplatin. This is also supported by an investigation carried out earlier wherein miR-24 has been shown to regulate the cisplatin resistance of the tongue squamous cell carcinoma [20].

Conclusion

The findings of the present study suggest that miR-21 is suppressed in the gastric cancer tissues and cell lines and acts a tumor suppressor. Furthermore, it suppresses the migration and invasion of gastric cancer cells by targeting DND1. Taken together these results suggest that miR-21 may prove to be an important therapeutic target for the gastric cancer management.

Conflict of interests

The authors declare no conflict of interests.

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