MiR-136 triggers apoptosis in human gastric cancer cells by targeting AEG-1 and BCL2

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Abstract. – OBJECTIVE: Gastric cancer is the second most prevalent cancer across the globe and accounts for about 10% of new cancer cases. It is one among the leading causes of cancer-related deaths around the world. Recently, microRNAs have been identified as important therapeutic targets for the treatment of several cancers owing to their potential to target multiple genes and hinder several biological processes such as proliferation and apoptosis. In the current study, we investigated the potential of miR-136 as therapeutic target for gastric cancer.

MATERIALS AND METHODS: Total RNA was isolated by RNeasy RNA isolation kit and cDNA was prepared byRevertAid cDNA synthesis kit. The transcript analysis was carried out by quantitative RT-PCR. The transfection of miR-136 mimics or plasmids was carried out by using the Lipofectamine 2000 reagent. Apoptosis was detected by DAPI and acridine orange/ethidium bromide (AO/EB) staining. Protein expression was examined by Western blotting.

RESULTS: The results indicated that the expression of miR-136 is significantly downregulated in gastric cancer cells. Transfection and subsequent overexpression of miR-136 in gastric cancer cells significantly promoted apoptosis as evident from DAPI and OA staining. In silico analysis revealed AEG-1 and BCL2 to be the potential targets of miR-136. Therefore, the expression of AEG-1 and BCL2 was determined in untreated control, cisplatin treated control and miR-136 transfected AGS gastric cancer cells. The results revealed that overexpression of miR-136 expression causes significant downregulation of AEG-1 and BCL2 protein expression.

CONCLUSIONS: Taken together, we conclude that miR-136 promotes apoptosis in gastric cancer cells by targeting AEG-1 and BCL2. Therefore, miR-136 may prove as a potential therapeutic target for the treatment of gastric cancer.

Key Words:
  Gastric cancer, MiR-136, Apoptosis, Cisplatin.

Introduction

Gastric cancer (GC) is one of the most heterogeneous cancers and ranks fifth among most prevalent malignancies across the globe. GC accounts for more than half of the cancers in East Asia annually[1,2]. Although it is believed that there has been significant decline in the incidence of GC and GC related mortality, it is still believed to be the second most prevalent cause of cancer-related deaths world over[3,4]. In UK and USA, gastric cardiac tumors constitute about half of all stomach cancers in men[5,6]. One of the main hurdles in the treatment of GC is that GC is often diagnosed at advanced metastatic stages, making it very difficult to treat[7]. Currently, chemotherapy remains the corner stone for treatment of GC at advanced stages but the outcomes are far from descent. Though a number of oncogenic signaling pathways have been identified as potential targets for GC treatment, there has been no significant improvement in the overall survival rate[8,9]. GC has been pathologically categorized into two different types: intestinal type and the diffuse type. Cell lines have been widely used as experimental models to understand the genetics, to identify the potential targets for pharmacological interventions, and to gain insights about the biochemistry GC[9].

Additionally, the microRNAs (miRNAs), which are a class of small regulatory RNA molecules, have been reported to play vital roles in tumorigenesis and progression of several cancer types[10]. In a recent miRNA expression study, miRNA-136 was found to be highly expressed in cancer cells[11]. Moreover, miR-136 was reported to target the tumor suppressor PTEN, indicative of its potential role in cancer progression. However, the role of miR-136 in GC is still unexplored. Therefore, the present work was designed to investigate the role

of miR-136 in gastric cancer. We report that miR-136 expression is significantly downregulated in GC cells and overexpression of mi-136 expression promotes the sensitivity of GC to the anticancer effects of cisplatin. Moreover, bioinformatic analysis revealed that miR-136 targets the two anti-apoptotic genes. Taken together, we propose that miR-136 may prove a potential target for the treatment of gastric cancers.

Materials and Methods

Chemicals, Reagents and Cell Cultures

Cisplatin, DAPI, RNase A. triton X-100, dimethyl and sulfoxide (DMSO) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Primary and secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Fetal bovine serum (FBS), Roswell Park Memorial Institute-1640 (RPMI-1640) medium, L-glutamine, antibiotics were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Eight human gastric cancer cell lines were purchased from Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin and maintained in a humidified atmosphere containing 5% CO_2. All of the reagents were procured from Hyclone (Logan, UT, USA).

RNA isolation, cDNA Synthesis and Quantitative RT-PCR Analysis

Total RNA was isolated by RNeasy RNA isolation kit (Qiagen, China Shanghai) following the manufacturer's instructions. Thereafter, cDNA was synthesized with the help of RevertAid cDNA synthesis kit (Fermentas, MA, USA) with manufacturer's protocol. To carry out the Real-time PCR, the cDNA was diluted 20 times and qRT-PCR was carried out in triplicates in ABI StepOne Real-time (Applied Biosystems, Foster City, CA, USA) using SYBR Green Master Mix (Fermentas, MA, USA) and gene specific primers. The relative quantification method ($\Delta\Delta^{-CT}$) was used to determine quantitative variation between the replicates examined. The β-actin was used as positive control to normalize the data.

Transfection

The miR-136 mimic and negative controls ordered from RiboBio (Guangzhou, China) as for following nucleotide sequences, 5′-ACUC-CAUUUGUUUUGAUGAUGGA-3′ (miR-136 mimic sense strand), 5′-UCCAUCAUCAAAA-CAAAUGGAGU-3′ (miR-136 mimic anti-sense strand); 5′-UUUGUACUACACAAAAGUA-CUG-3. The transfection of miR-136 mimics or plasmids was carried out by using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol.

Apoptotic Assays

AGS gastric cancer cells were seeded at the density of 2×10^5 cells/well in 6-well plates and administrated with different concentrations of cisplatin and incubated for a period of 24 h. The cells were then stained with a mixture of AO and EB. Stained cells were examined under the fluorescent microscope. Similarly, 4',6-diamidi-no-2-phenylindole (DAPI) staining was carried by incubating the cells with DAPI and then washed with PBS and finally fixed in formaldehyde (10%). The DAPI stained cells were then examined by fluorescence microscope. For annexin V/PI similar procedure as that of DAPI was followed except for the cells were stained with annexin V/PI and investigated by flow cytometry.

Identification of miR-136 Targets

To gain insights about the potential targets of m-R-136, it was subject to scanning in online TargetScan search program.

Western Blotting

Western blotting was carried out as described previously in literature[12]. The antibodies used in this investigation were anti-metadherin (AEG-1) (Invitrogen, Carlsbad, CA, USA) and anti-Bcl-2, (Cell Signaling, Danvers, MA, USA).

Statistical Analysis

All experiments were carried out in three biological replicates and expressed as mean ± SD. Statistical analysis was carried out by GraphPad prism 7 using Student's t-test. The values were considered significant at $*p < 0.01$, $**p < 0.001$, $***p < 0.0001$.

Results

Gastric Cancer Cell Lines Show Downregulation of miR-136 Expression

Since the expression of miR-136 has been reported to be downregulated in several types of

cancer such as lung cancer and glioma, we examined the expression of miR-136 in eight different gastric cancer cell lines (MGC80-3, BGC-823, SGC-7901, AGS, SNU-1, SNU-5, SNU-16 and RF-1) and one normal gastric cell line (GES-1). The results of our study indicated that the expression of miR-136 was significantly downregulated in all the gastric cancer cell lines as compared to the normal cell line AGS-1 (Figure 1).

Ectopic Expression of mi-RNA Increases Sensitivity of Gastric Cancer Cells to Cisplatin

Downregulated expression of miR-136 in all the studied gastric cancer cell lines suggested that miR-136 could prove an important therapeutic target. Therefore, to validate the role of miR-136, we transfected AGS gastric cancer cells with miR-136 mimics. The treated control and transfected AGS cells were subjected to 1 μM of cisplatin. The results indicated that as compared to the untreated control AGS, treated control and miR-136 cells showed significant apoptosis as evident from the white colored nuclei in case DAPI and orange fluorescence in AO/EB staining (Figure 2A-B). More interestingly, apoptosis was higher in case of cells transfected with miR-136 as compared to both treated as well as untreated control cells (Figure 2). Taken together, these re-
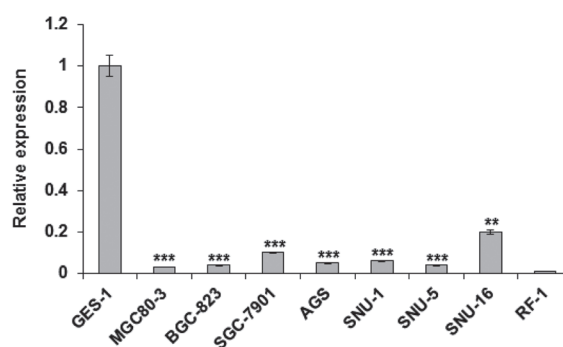

Figure 1. Expression of miR-136 in seven different gastric cancer cell lines (MGC-802, BGC-823, SGC-7901, AGS, SNU-1, SNU-16, and RF-1) and normal gastric cell line (GES-1). The expression was determined by quantitative RT-PCR. The experiment was carried out in triplicates and the values for all gastric cancer cell lines were considered signification in comparison to GES-1 at $*p < 0.01$, $**p < 0.001$, $***p < 0.0001$.

sults indicate that transfection of AGS cells with miR-136 promoted apoptosis.

Ectopic Expression of mi-RNA Promotes Apoptosis

To gain better insights about the role of miR-136 in promotion of apoptosis, we estimated the apoptotic cell populations in untreated control, and cisplatin (1-μM) treated control and miR-136
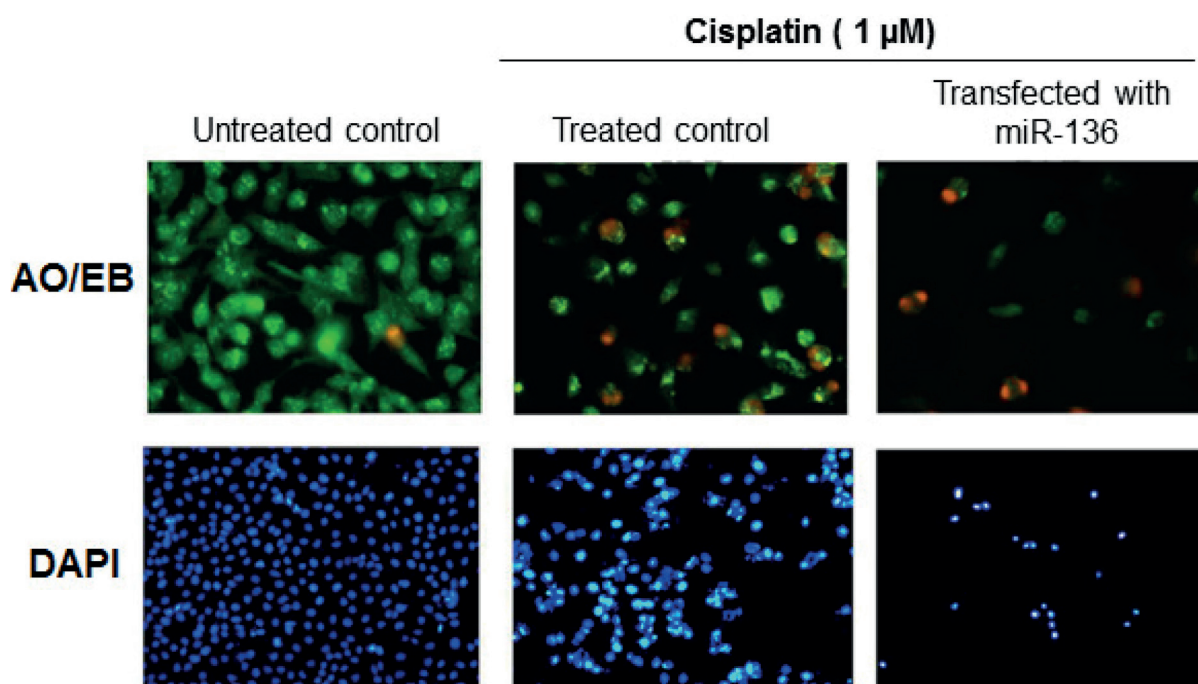

Figure 2. Detection of apoptosis in untreated AGS, cisplatin-treated AGS, and cisplatin-treated AGS cells transfected with miR-136 by AO/EB and DAPI staining. All experiments were carried out in triplicates.

transfected AGS cells by annexin V-FITC/PI staining. The results indicated that the percentage of apoptotic cells was 2.1%, 19.7%, and 37.5% in untreated control and treated control and miR-136 transfected AGS cells, respectively (Figure 3). These results further confirm the role of miR-136 in promotion of apoptosis by increasing the sensitivity of AGS gastric cancer cells to cisplatin.

MiR-136 Targets AEG-1 and Bcl-2

The targets of miR-136 were predicted by TargetScan search program. The results indicated that the potential targets of miR-136 are the two anti-apoptotic genes AEG-1 and BCL2 (Figure 4). To further confirm that miR-136 promotes apoptosis by targeting AEG-1 and BCL2, we determined the expression of AEG-1 and BCL2 in untreated control, treated control and miR-136 transfected cells. The results indicated that the expression of both AEG-1 and BCL2 were comparatively higher in untreated control as compared to treated control and miR-136 transfected cells subjected to 1 µM cisplatin (Figure 5). Therefore, these results clearly indicate that miR-136 triggers apoptosis via downregulation of the expression of AEG-1 and BCL2.

Discussion

Gastric cancer is one of the lethal cancers and is the second most common cause of cancer-related deaths across the globe[13]. The treatment of GC is often limited by it diagnosis at advanced metastatic stage, upsurge of drug resistance, limited availability of drugs, side effects associated with the currently used drugs and frequent relapses after treatment[14]. Therefore, there is need to develop more efficient drug options and to explore novel therapeutic targets for the treatment of GC. MiR-136 has been reported to be downregulated in several types of cancers such as glioma and lung cancer cells[15]. The present study was, therefore, designed to investigate the expression of miR-136 in gastric cancer cells and explore the potential of miR-136 as a therapeutic target. In this context, the expression of miR-136 was determined in 8 different gastric cancer cell lines and one normal GES-1 cell line. The results of the study indicated that the expression of miR-136 was significantly downregulated in all the gastric cancer cell lines, indicating the potential of miR-136 as therapeutic target. Our results are in confirmation with previous studies wherein miR-136 expression was reported to be suppressed in cancer cells[16]. To find out the role of miR-136 in progression and tumorigenesis, one gastric cancer cell line AGS was selected and transfected with miR-136 constructs. The miR-136 transfected and non-transfected control cells were subjected to treatment with cisplatin (1 µM). It was observed that although cisplatin induced apoptosis in both control and miR-136 transfected cells, the apoptosis was more pronounced in miR-136 transfected cells, indicating that miR-136 promotes apoptosis in gastric cancer AGS cells. Similar results were
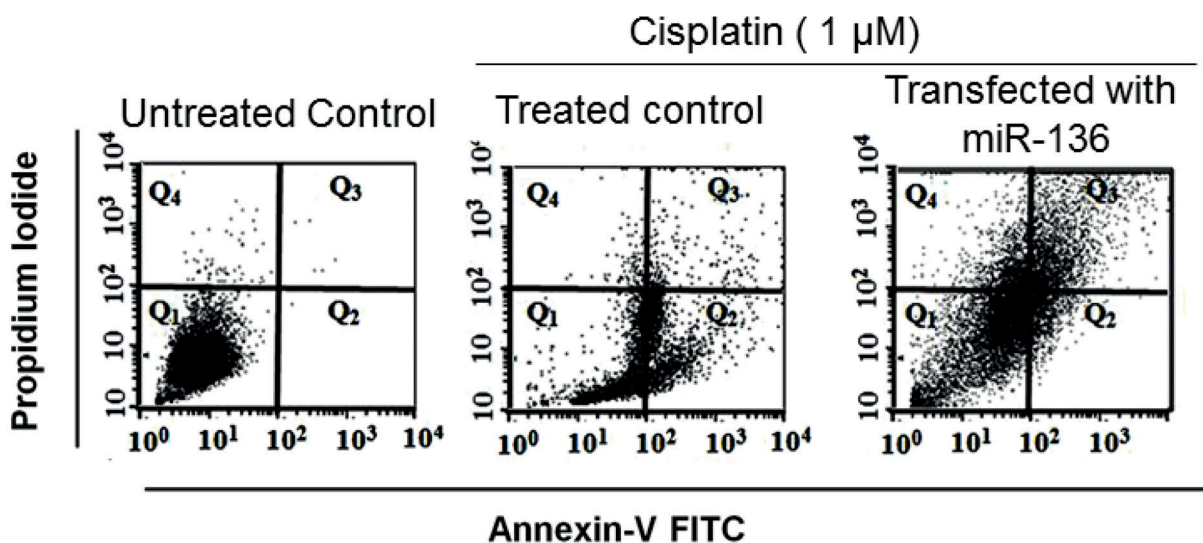

Figure 3. Estimation of apoptotic cell populations in untreated AGS, cisplatin-treated AGS, and cisplatin-treated AGS cells transfected with miR-136 by annexin V/PI staining. All experiments were carried out in triplicates.
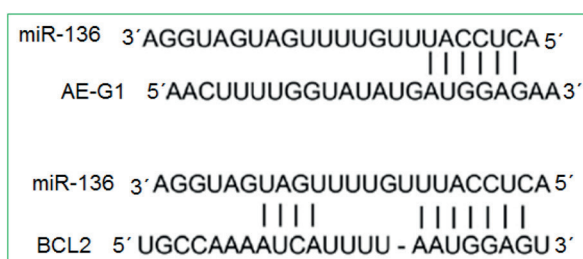
Figure 4. MiR-136 targets AEG1 and BCL2 as confirmed by TargetScan online software.

obtained in a previous study wherein miR-136 has been reported to promote apoptosis in cancer cells[16]. Next, to identify the potential targets of miR-136, we used TargetScan software. AEG-1 and BCL2 were found to be the potential targeted of miR-136. To experimentally validate that
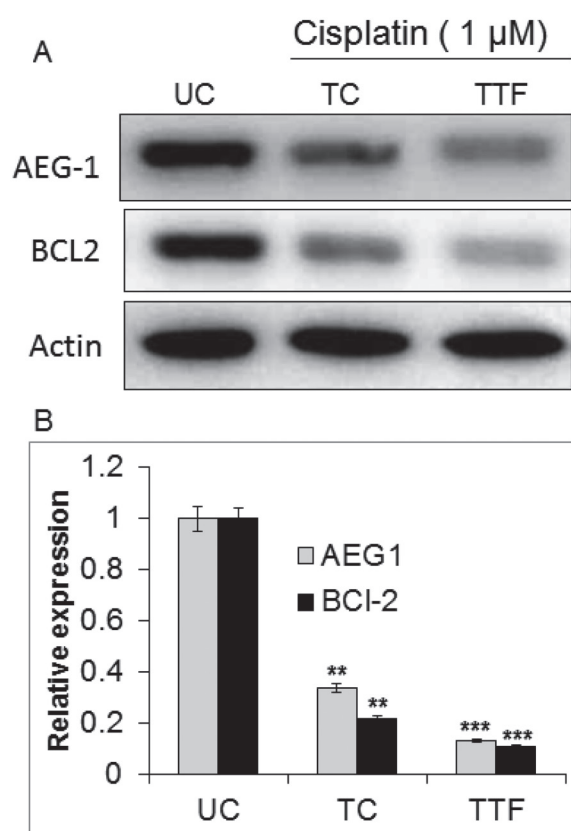

Figure 5. Expression of AEG1 and BCL2 in untreated AGS (UC), cisplatin-treated AGS (TC), and cisplatin-treated AGS cells transfected with miR-136 (TTF) by annexin V/ PI staining *(A)* Western blots *(B)* Quantification of Western blots by densitometer. All experiments were carried out in triplicates. The values were considered significant at $*p < 0.01$, $**p < 0.001$, $***p < 0.0001$.

AEG-1 and BCL2 are the targets of miR-136 we determined the expression of AEG-1 and BCL2 in AGS gastric cancer cells. The results indicated that the expression of both AEG-1 and BCL2 was significantly downregulated in miR-136 cells treated with cisplatin as compared to both untreated and treated control AGS cells. In previous studies the higher expression of AEG-1 and BCL2 in cancer cells has anti-apoptotic effects have been reported[16]. Therefore, our results that ectopic expression of miR-136 downregulates the expression of AEG-1 and BCL2, suggest that miR-136 promotes apoptosis by downregulation of these anti-apoptotic genes.

Conclusions

We demostrated that miR-136 was significantly downregulated in gastric cancer cells. Moreover, restoring of miR-136 expression promotes apoptosis of gastric cancer cells by downregulating the expression of anti-apoptotic proteins AEG-1 and BCL2. Taken together, these results indicate that miR-136 may prove a potential therapeutic target for treatment of gastric cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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