MicroRNA-522 reverses drug resistance of doxorubicin-induced HT29 colon cancer cell by targeting ABCB5

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Abstract. MicroRNAs (miRNAs) are small non-coding RNAs, which are important in the development of multidrug resistance in cancer by regulating gene expression at the post-transcriptional level. The present study investigated the functional effects of miR-522 in chemoresistant colon cancer cells. The results demonstrated that miR-522 was significantly downregulated in doxorubicin (DOX) resistant colon cell line, HT29/DOX, compared with the parental HT29 colon cancer cell line. Overexpression of miR-522 in the HT29/DOX cells partially restored DOX sensitivity. miRNA target prediction algorithms suggested that ABCB5 was a target gene for miR-522. A fluorescent reporter assay confirmed that miR-522 was able to specifically bind to the predicted site of the ABCB5 mRNA 3'-untranslated region. When miR-522 was overexpressed in the HT29/DOX cells, the protein expression levels of ABCB5 were downregulated. Furthermore, knockdown of ABCB5 significantly increased the growth inhibition rate of the HT29/DOX cells, compared with the control group. These results suggested that miR-522 may affect the sensitivity of colon cancer cells lines to DOX treatment by targeting ABCB5.

Introduction

Colorectal cancer (CRC) is one of the major types of malignancy worldwide and the incidence of CRC has significantly increased and to become the second leading cause of tumor-associated mortality in Western countries (1). The incidence rates of CRC are also rising rapidly in Asian countries (2), which were formerly considered low-risk areas. Surgical resection is the optimal treatment approach for CRC, and chemotherapy serves as one of the important adjuvant therapies in its treatment, with chemotherapy being the preferred treatment option for malignancies. Due to the complication of cancer cells becoming simultaneously resistant to several structurally and mechanistically unrelated drugs, the efficacy of chemotherapeutic management of cancer often becomes severely limited (2). Several mechanisms, including gene mutation, DNA methylation and histone modification, are important in the resistance of cancer cells to chemotherapeutic agents. A previous study by Climent et al (3) suggested that the increased sensitivity of patients with breast cancer to anthracycline-based chemotherapy may be associated with the deletion of chromosome 11q, a region containing the miR-125b gene. This finding indicated a possible link between miRNA dysregulation and cancer drug resistance.

A class of novel non-coding RNAs, termed microRNAs (miRNAs), have been identified in plants and animals. miRNAs include 18-26 nucleotides, which post-transcriptionally regulate gene expression in multicellular organisms by affecting the stability and translation of mRNAs (4). These miRNAs are transcribed by RNA polymerase II or III in the nucleus (5). The primary transcripts, which are capped and polyadenylated, are cleaved by the Drosha ribonuclease III enzyme to produce a stem-loop precursor miRNA (pre-miRNA) of ~70 nt (6-8), which is further transported to the cytoplasm by Exportin 5 (9). The RNase III enzyme, Dicer, processes the pre-miRNAs into mature miRNAs (~22 nt) (10), and the mature miRNA is incorporated into an RNA-induced silencing complex. The complex, termed miRNP (11,12), recognizes target mRNAs through imperfect base pairing with the 3'-untranslated (3'UTR) regions of the target gene mRNAs and often results in repression of translation or destabilization of the target mRNA (11,13). Considerable evidence has demonstrated the critical functions of miRNAs in diverse biological processes, including proliferation (14), apoptosis (15), angiogenesis (16), cell differentiation (17), adhesion, metastasis (18) and multidrug resistance (MDR) in chemotherapy (19). Therefore, understanding the regulatory role of miRNAs may improve understanding of the molecular events involved in diverse biological processes and suggest that the abnormally expressed miRNAs in human cancer
target transcripts of essential protein coding genes involved in tumorigenesis, including oncogenes and tumor suppressor genes.

Previous studies (20,21) have suggested that altered miRNA levels are associated with the response to chemotherapeutic agents and oncogenesis. In several cases, therapy fails due to MDR of cancer cells, either intrinsic or acquired, following an initial round of treatment (22). Xia et al (21) analyzed the possible role of miRNAs in the development of MDR in gastric cancer cells. The profile of miRNA expression was determined, and downregulation of miR-15b and miR-16 were observed in the SGC7901/VCR MDR gastric cancer cell line, compared with its parental cell line, HT29. An in vitro drug sensitivity assay revealed that the overexpression of miR-15b or miR-16 sensitized SGC7901/VCR cells to vincristine, doxorubicin (DOX), etoposide and cisplatin, whereas inhibition of the expression conferred resistance of the SGC7901 cells to the above-mentioned drugs. Zhu et al (23) demonstrated that the expression levels of miR-27a and miR-451 are upregulated in A2780DX5 and KB-V1MDR cancer cell lines, compared with their parental lines, A2780 and KB-3-1, and treatment of A2780DX5 cells with the antagonomers of miR-27a or miR-451 decreases the mRNA expression levels of P-glycoprotein and MDR1. By contrast, mimics of miR-27a and miR-451 increased the expression of MDR1 in the parental A2780 cells. The sensitivity to, and intracellular accumulation of, cytotoxic drugs that are transported by P-glycoprotein are increased following treatment with miR-27a or miR-451 antagonomers. Meng et al (24) revealed that miR-21, miR-141 and miR-200b are markedly overexpressed in malignant cholangiocytes. Inhibition of miR-21 and miR-200b increases sensitivity to gemcitabine, whereas inhibition of miR-141 decreases cell growth.

As noted above, miRNAs constitute a novel regulatory layer of gene expression and may affect the responses of cancer cells to chemotherapy. Therefore, the present study investigated whether miR-522 was differentially expressed in the DOX-resistant human HT29/DOX colon cancer cell line and its parental HT29 cell line. The investigation aimed to determine whether the regulation of miR-522 in colon cancer cells affected sensitivity to DOX, by examining the effects of miR-522 on the DOX-resistance of HT29 colon cancer cells and the identifying its direct target gene. It was hypothesized that miR-522 may assist in the development of novel strategies for preventing chemotherapy resistance to DOX by targeting its target gene in patients with colon carcinoma.

Materials and methods

Cell culture and transfection. The human HT29 colon cancer cell line (American Type Culture Collection, Manassas, VA, USA) was maintained in RPMI-1640 (Gibco Life Technologies, Carlsbad, CA, USA), containing 10% heat-inactivated fetal bovine serum (Gibco Life Technologies), 100 IU/ml penicillin and 0.1 mg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA), in a humidified 5% (v/v) atmosphere of CO2 at 37°C. The DOX-resistant variant, HT29/DOX, was generated in the laboratory from the parent HT29 cell line through continuous exposure of stepwise, increasing concentrations (5, 10, 20 and 40 mg/ml) of DOX (Sigma-Aldrich) over a time-period. To maintain the DOX-resistant phenotype, DOX was added to the culture media, at a final concentration of 40 mg/ml, for the HT29/DOX cells. All transfections were performed using Lipofectamine 2000 reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions.

Fluorescent reporter assay. The cells (2x10⁴) were transfected with miR-522 or a pcDNA3 control vector (Wuhan Cell Marker Biotechnology Co., Ltd, Wuhan, China) in 48-well plates using a pcDNA3/enhanced green fluorescent protein (EGFP)-ABC5 3'UTR or pcDNA3/EGFP-ABC5 3'UTR-mut reporter vector. The pDsRed2-N1 vector (Clontech Laboratories, Inc., Mountain View, CA, USA), expressing red fluorescent protein (RFP) was used for normalization. The intensities of EGFP and RFP fluorescence were detected using an F-4500 Fluorescence Spectrophotometer (Hitachi, Tokyo, Japan).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). To detect the relative expression levels of the transcripts, RT-qPCR was performed. Briefly, a cDNA library was generated by RT using M-MiV Reverse Transcriptase (Promega, Madison, WI, USA) with 2 µg of the large RNA, extracted from the cells using TRIzol reagent (Invitrogen Life Technologies). The resulting cDNA was used for amplification of the ABCB5 gene, and the β-actin gene was used as an endogenous control for the qPCR reaction. The primer sequences were as follows: ABCB5, forward: 5’-TAAATACGACTCACTATAGGGATGTCTGGCTTTTTCCCTTCTTGAC-3’ and reverse: 5’-GATTTAGGTGACACTATAGAAATTCAATGTTGCAGAAATGACCCCCA-3’; actin, forward: 5’-CGTGACATTAAGGAGAAGCTG-3’ and reverse: 5’-CTAGAAGCATTTCGGGTGGAC-3’. The ABCB5 and β-actin primer kits were purchased from Search-LC (Heidelberg, Germany). qPCR was performed under the following conditions: 94°C for 4 min, followed by 40 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min. qPCR was performed and analyzed using the iQ5 Real-Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

MTT assay. The HT29 and HT29/DOX cells were seeded into a 96-well plate at densities of 6,000, 7,000, and 8,000 cells/well 1 day prior to transfection. The cells were transfected with 0.15 µg anti-miR-522 or control vector, or miR-522 or control vector, per well. DOX was subsequently added to produce final concentrations of 1, 2, 4 and 8 µmol/l. An MTT assay was used to determine the cell viability 48 h after transfection. The absorbance (A) at 570 nm was measured using a µQuant Universal Microplate Spectrophotometer (BioTek Instruments, Winooski, VT, USA). The growth inhibition rate was calculated as follows: Growth inhibition rate (%) = (1 - Aexperimental group / Acontrol group) x 100.

Western blotting. The cultured cells were lysed in radioimmunoprecipitation buffer, containing 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 1 mM MgCl2, and 10 mM Tris-HCl (pH 7.4) (Sigma-Aldrich), at 4°C for 30 min. The lysates
were collected and cleared by centrifugation at 1,500 x g for 10 min, and the protein concentration was determined using a Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology, Shanghai, China). The total cell lysates (50 µg) were fractionated using 15% SDS-polyacrylamide gel electrophoresis. The proteins were electroblotted onto nitrocellulose membranes and non-specific binding sites of the membranes were incubated with 5% non-fat milk in Tris-buffered saline with Tween 20 (TBST) solution, containing 100 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.1% Tween-20 (Sigma-Aldrich), followed by incubation for 2 h with rabbit polyclonal anti-ABCB5 (cat. no. sc-104019; 1:1,000) and rabbit polyclonal anti-GAPDH (cat. no. sc-48166; 1:1,000) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) antibodies at room temperature. Following washing with TBST four times, the membranes were incubated with goat anti-rabbit peroxidase-conjugated secondary antibody (1:500; Sigma-Aldrich) in 5% non-fat milk in TBST solution for 1 h at room temperature. The reactions were developed using enhanced chemiluminescence (Perkin-Elmer Life Sciences, Boston, MA, USA).

Small interfering (si)RNA transfection. Transfection of the cells was performed using a SignalSilence ABCB5 siRNA kit (Cell Signaling Technology, Inc., Danvers, MA, USA), according to the manufacturer's instructions. Following incubation for 48 h, the cells were prepared for further analysis. siRNA transfection efficiency was measured using flow cytometry by calculating the percentage of fluorescein-labeled cells. The experiments were repeated three times.

Flow cytometric analysis. At 48 h post-transfection, as described above, the cells were harvested using pancreatin enzyme (Sigma-Aldrich) and washed twice with phosphate-buffered saline (PBS). The washed cells were resuspended in 0.6 ml PBS and fixed with 1.4 ml 100% ethanol at 4°C overnight. The fixed cells were rinsed twice with PBS and resuspended in propidium iodine (PI) solution, containing 50 mg/ml PI and 50 mg/ml RNaseA (Sigma-Aldrich) in PBS without calcium or magnesium, and incubated at 37°C for 30 min in the dark. The stained cells were passed through a nylon mesh sieve to remove cell clumps and were analyzed using a FACScan flow cytometer and Cell Quest analytical software (Becton Dickinson, San Jose, CA, USA). Flow cytometric analysis was performed three times.

Statistical analysis. The data are expressed as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference using the Students-Newman-Keuls test. Statistical analyses were conducted using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

miR-522 is downregulated in DOX-resistant HT29/DOX cells. The cell growth inhibition rates of the HT29/DOX cell line and the parental HT29 cell line were assessed (Fig. 1A). The results demonstrated that the HT29/DOX cell line was 1.8-fold resistant to DOX, compared with the HT29 cell line, based on half maximal inhibitory concentration (IC50) values (2.4 vs. 4.1 mg/ml; P<0.05). To further investigate the involvement of miR-522 in the DOX-resistant HT29/DOX colon cancer cell line, RT-qPCR analysis was performed. The results demonstrated that the expression level of miR-522 was higher in the HT29 cells compared with the HT29/DOX cells (Fig. 1B), which suggested that miR-522 may be associated with DOX resistance in colon cancer cells.

Knockdown of miR-522 in the HT29 cells confers resistance to DOX. To determine the association between miR-522 and DOX resistance in HT29 cells, the effect of downregulation of miR-522 on the DOX-sensitive cells was analyzed. The commercially synthesized miR-522 inhibitor, anti-miR-522, was used to alter the levels of miR-522 in the HT29 cells. Following transfection with anti-miR-522 or anti-control into the HT29 cells, the validity of the ectopic expression of miR-522 was confirmed using RT-qPCR (Fig. 2A). The RT-qPCR results revealed that the miR-522 inhibitor reduced the expression of miR-522 in the HT29 cells, compared with the NC group (P<0.05; Fig. 2A). The HT29 cells, which were treated with the miR-522 inhibitor exhibited significantly higher survival rates compared with the anti-control group (Fig. 2B). These results demonstrated that downregulation of the expression of miR-522 conferred DOX-resistance in the HT29 cells.

Overexpression of miR-522 in HT29/DOX cells partially restores DOX sensitivity. To further determine the effects of the overexpression of miR-522 on DOX-induced cell death in the HT29/DOX cells, the miR-522 mimic or miR-control were transfected into the HT29/DOX cells. RT-qPCR revealed that the miR-522 mimics upregulated the expression of miR-522 in the HT29/DOX cells compared with the NC group (P<0.05; Fig. 2C). The HT29/DOX cells transfected with the miR-522 mimic demonstrated significantly lower survival rates compared with the NC group (Fig. 2D). These results suggested that the overexpression of miR-522 partially sensitized the HT29/DOX cells to DOX.

miR-522 directly targets the ABCB5 3'UTR in HT29 colon cancer cells. To determine the target gene mediating the function of miR-522, bioinformatic techniques were used to predict the potential target genes. It was revealed that the 3'UTR of ABCB5 mRNA contained miR-522 complementary binding sites (Fig. 3A). To confirm that ABCB5 was directly targeted by miR-522, an EGFP reporter assay was performed using engineered EGFP reporter vectors, which contained either the wild-type 3'UTR of ABCB5 or the mutant UTR with a five base mutation in the complementary seed sequence (Fig. 3A). pDsRed2-NI was also co-transfected for normalization. The HT29/DOX cells were co-transfected with the pcDNA3/EGFP-ABCB5 3'UTR and miR-522 mimic or miR-control. The overexpression of miR-522 significantly repressed the expression of EGFP, compared with the control (Fig. 3B). By contrast, the expression levels of EGFP in the mutant ABCB5 3'UTR binding sites were not affected by the overexpression of miR-522 (Fig. 3B), indicating that miR-522 bound to the specific sites of the ABCB5 mRNA 3'UTR and negatively regulated the gene expression of ABCB5.
miR-522 exerts a negative regulatory role on ABCB5 at the post-transcriptional level. The predominant functions of miRNAs are to downregulate gene expression in translational repression, cleave mRNA and binding to the 3'UTRs of target genes in various other processes. To determine whether miR-522 depresses the endogenous expression of ABCB5, the HT29 and HT29/DOX cells were transfected with anti-miR-522 and miR-522 mimics, and the mRNA and protein expression levels of ABCB5 were determined using RT-qPCR and western blotting. The results demonstrated that the overexpression of miR-522 reduced the protein expression level of ABCB5 by 90% in the HT29/DOX cells, and that high expression levels of miR-522 in the HT29/DOX cells decreased the endogenous level of ABCB5 mRNA, determined by RT-qPCR (Fig. 3C and D). By contrast, in the HT29 cells transfected with anti-miR-522, the protein expression level of endogenous ABCB5 was significantly increased (Fig. 3D) and the mRNA expression level of ABCB5 was also elevated (Fig. 3C). These results demonstrated a consistent and marked inverse correlation between miR-522 and ABCB5.

ABCB5 is a key signaling molecule in DOX-resistance of HT29/DOX cells. To determine whether ABCB5 is important in
Figure 3. ABCB5 is a direct target of miR-522. (A) Predicted binding sites of miR-522 on ABCB5 mRNA. The mutant UTR contains five bases in the complementary seed sequences. (B) Effects of miR-522 on ABCB5 were assessed using a luciferase reporter system. The miR-522 mimics, together with the luciferase reporter vector or the mutant vector, were co-transfected into the HT29/DOX cells (*P<0.05, vs. miR-control). (C) Reverse transcription-quantitative polymerase chain reaction measured the mRNA expression levels of ABCB5 in the HT29 and HT29/DOX cells, following transfection with the miR-522 mimics or control (*P<0.05, vs. miR-522). (D) Protein expression levels of ABCB5 were assessed in the HT29 and HT29/DOX cells. HT29 cells were transfected with the miR-522 inhibitor or control. HT29/DOX cells transfected with the miR-522 mimics or control. Data are expressed as the mean ± standard deviation. DOX, doxorubicin; UTR, untranslated region; miR, microRNA; CMV, cytomegalovirus; WT, wild-type; mut, mutant.

Figure 4. ABCB5 is important in DOX-resistance in HT29/DOX cells. (A) Protein expression levels of ABCB5 in the HT29/DOX cells following treatment with ABCB5 siRNA or a scrambled siRNA (*P<0.05). (B) HT29/DOX cells were treated with various concentrations of DOX (1, 2, 4 or 8 µmol/l) 48 h after transfection. The cell growth inhibition rate was determined using an MTT assay (*P<0.05). (C) Apoptosis was detected using flow cytometry. HT29/DOX cells were transfected with the control mimic, miR-522 mimic or siR ABCB5 for 48 h and apoptotic cells were detected using flow cytometry. The lower right quadrant indicates the pro-apoptotic cell rate. Data are expressed as the mean ± standard deviation. DOX, doxorubicin; miR, microRNA; siR, small interfering RNA; PI, propidium iodide.
miR-522-regulated DOX-resistance, the HT29/DOX cells were transfected with either ABCB5 siRNA or an siRNA-control, and the cell growth inhibition rate was measured in various concentrations of DOX. Western blotting revealed that ABCB5 siRNA effectively reduced the protein level of ABCB5 (Fig. 4A), and ABCB5 knockdown significantly increased the growth inhibition rate of the HT29/DOX cells, compared with the control group (Fig. 4B). This suggested that miR-522 may modulate DOX-resistance in HT29/DOX cells by down-regulating ABCB5. The HT29/DOX cells transfected with the miR-522 mimic and siRNA-ABCBI exhibited significantly higher apoptotic rated, compared with the NC group (P<0.05; Fig. 4C).

Discussion

Drug resistance is a multifactorial process, which is responsible for the absence of chemoresponse in primary and secondary types of tumor. The usage of DOX, a DNA intercalating anthracycline antibiotic, in combination with other anticancer agents has been demonstrated to exert a good therapeutic effect for the treatment of advanced CRC (25,26). However, the drug resistance to treatment with DOX is one of the predominant causes of chemotherapy failure in the treatment of advanced colon cancer (27,28). Therefore, it is critical to identify novel strategies to increase the effectiveness of DOX for therapeutic purposes. During the process of tumor formation, abnormal genetic expression and weakening of the dynamic balance between oncogenes and tumor suppressor genes usually leads to the development of cancer (29). As for many other genes, miRNAs are involved in the regulation of oncogenes and tumor suppressor genes. Increasing numbers of studies have indicated that aberrant miRNAs are also associated with the chemotherapeutic response. The present study attempted to to identify a novel miRNA, which regulates the expression of ABCB5 and evaluate its effects on treatment with DOX using HT29 cells. Additionally, the suppression of ABCB5 and upregulation of miR-522 acted in concert to trigger the chemotherapeutic sensitivity to DOX. As a result, miR-522 sensitized HT29 cells to treatment with DOX.

Initially, RT-qPCR was performed to demonstrate that miR-522 was significantly downregulated in human colon cancer DOX resistant HT29/DOX cells compared with the parental HT29 cell line. The results suggested that miR-522 may be important in the development of colon cancer chemotherapy resistance. Therefore, it was hypothesized that miR-522 is an inhibitory factor of DOX resistance in HT29 cells as a result of the low expression levels of miR-522 in HT29/DOX. The cell growth inhibition rate was determined by an MTT assay to detect the association between miR-522 and the growth inhibition capacity of colon cancer cell lines with or without DOX resistance. The cell growth inhibition rate of HT29 cells transfected with the anti-miR-522 was significantly decreased when compared with the control group (Fig. 2B). In addition, overexpression of miR-522 affected cell growth inhibition positively when compared with the control group (Fig. 2D). miR-522 is closely associated with DOX resistance. Secondly, bioinformatic analyses predicted an miR-522 binding site on the ABCB5 transcript (Fig. 3A). Experimental evidence indicated that ABCB5 was indeed a target of miR-522. The ability of miR-522 to regulate the expression of ABCB5 was direct, since it bound the 3'UTR of ABCB5 mRNA complementarily to the miR-522 seed region. The EGFP fluorescence intensity of EGFP-ABCBI-3'UTR was specifically responsive to the overexpression of miR-522 (Fig. 3B). Furthermore, mutation of the miR-522 binding site abolished the effect of miR-522 on the regulation of EGFP fluorescence intensity (Fig. 3B). The endogenous protein expression levels of ABCB5 were decreased in the HT29/DOX cells transfected with miR-522, whereas it was increased in HT29 cells transfected with anti-miR-522 (Fig. 3D). In addition, changes in the expression levels of miR-522 affected the mRNA expression level of ABCB5. These results suggested that miR-522 regulated the protein expression level of ABCB5 at the post-transcriptional level.

The mechanism of MDR is highly complex and is closely associated with genes, cytokines and transporters. ATP-binding cassette (ABC) transporters are known to be crucial in the development of MDR. In MDR, patients, which are on medication eventually develop resistance not only to the drug they are being administered, but also to several different types of drug. This is caused by several factors, one of which is increased excretion of the drug from the cell by ABC transporters. For example, the ABCBI protein, P-glycoprotein, functions in pumping tumor suppressor drugs out of the cell. ABCBI is an ABC transporter and P-glycoprotein family member predominantly expressed in physiological skin and human malignant melanoma (30-32). A previous study demonstrated that ABCB5 is functionally relevant to carcinogenesis, as demonstrated in colorectal cancer, where it was revealed to act as a mediator of 5-FU patient chemoresistance. This protein exerted a further direct role in tumorogenesis, as demonstrated by shRNA-mediated CRC cell-line ABCB5 knockdowns, which impeded tumorogenesis in human-to-mouse xenografts (33). These data revealed multiple roles for ABCB5 in cancer progression and chemoresistance, making it an attractive target for combined therapy. Analysis of ABC transporters (34), which may confer to chemical drug resistance in colon cancer may be used to predict the chemosensitivity prior to cancer chemotherapy.

The present study uncovered the association between miRNAs and ABCB5 in colon cancer cells. Knock down of ABCB5 improved the DOX sensitivity of HT29/DOX cells, which is consistent with the results of miR-522 overexpression.

In conclusion, the present study demonstrated that miR-522 reduced cell survival and DOX resistance in human colon cancer by directly targeting ABCB5. It was also revealed that the miR-522 mimics may be used as a therapeutic approach for colon cancer. The exact roles and mechanisms underlying the effects of miR-522 and other miRNAs requires elucidation in an increased number of colon cancer cell lines and clinical colon cancer biopsies.

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References