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Role of MicroRNA miR-27a and miR-451 in the regulation of *MDR1*/P-glycoprotein expression in human cancer cells

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Abstract

MicroRNAs are short non-coding RNA molecules able to affect stability and/or translation of mRNA, thereby regulating the expression of genes involved in many biological processes. We report here that microRNAs miR-27a and miR-451 are involved in activating the expression of P-glycoprotein, the *MDR1* gene product that confers cancer cell resistance to a broad range of chemotherapeutics. We showed that expressions of miR-27a and miR-451 were up-regulated in multidrug resistant (MDR) cancer cell lines A2780DX5 and KB-V1, as compared with their parental lines A2780 and KB-3-1. Treatment of A2780DX5 cells with the antagomirs of miR-27a or miR-451 decreased the expression of P-glycoprotein and *MDR1* mRNA. In contrast, the mimics of miR-27a and miR-451 increased *MDR1* expression in the parental cells A2780. The sensitivity to and intracellular accumulation of cytotoxic drugs that are transported by P-glycoprotein were enhanced by the treatment with the antagomirs of miR-27a or miR-451. Our results demonstrate for the first time the roles of microRNAs in the regulation of drug resistance mediated by *MDR1*/P-glycoprotein, and suggest the potential for targeting miR-27a and miR-451 as a therapeutic strategy for modulating MDR in cancer cells.

Keywords

MicroRNA; *MDR1*; P-glycoprotein; Gene expression; Drug resistance; Chemotherapy

1. Introduction

Drug resistance constitutes a major obstacle to successful chemotherapy in cancer patients. One of the common forms of resistance to chemotherapy is caused by activation of the *MDR1* (*ABCB1*) gene, resulting in overexpression of P-glyco-protein (P-gp), a 170–190 kDa transmembrane glycoprotein that belongs to the ATP-binding cassette superfamily and acts as a multidrug transporter [1]. Overexpression of P-gp confers cancer cell resistance to a broad range of structurally and functionally diverse chemotherapeutic drugs [2]. Multidrug resistance (MDR) mediated by the overexpression of *MDR1*/P-gp can be intrinsic, or induced by a variety of chemical and physical insults such as cytotoxic agents, arsenite, heat shock, and UV

irradiation [3–8], and the transcriptional activation of the *MDR1* gene is a highly regulated complex event and is associated with several signaling pathways. For example, our laboratory demonstrated that activation of the phospholipase C/Raf/mitogen-activated protein kinase pathway stimulates the transcription of the *MDR1* gene and expression of P-gp, and this pathway can be activated by heat shock, platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) [9]. We also showed that the activation of the *MDR1* gene transcription requires involvement of a number of transcriptional factors such as C-terminal-binding protein 1 (CtBP1) [10]. Nevertheless, the precise mechanism(s) underlying the activation of *MDR1* gene expression is still not fully understood. We previously reported the inhibitory effect of small interfering RNA (siRNA) on *MDR1*/P-gp expression and the feasibility of using RNA interference (RNAi) approach to modulate MDR phenotype [11]. These studies have led to a better understanding of the regulation of the expression of *MDR1* gene, and have helped to develop new strategies to inhibit or prevent the induction of *MDR1*/P-gp expression. To further understand the roles of small RNA molecules in the regulation of drug resistance genes, we investigated the involvement of micro-RNAs (miRNA) in controlling MDR phenotype mediated by *MDR1*/P-gp. MiRNAs are a class of endogenous, 19–25 nucleotides RNA molecules that are able to induce mRNA degradation, translational repression, or both, via pairing with partially complementary sites in the 3' UTR of the targeted genes [12]. It is estimated that there are over 600 miRNAs in mammalian cells, and that 30% of all genes are regulated by miRNAs. Because miRNAs have the ability to target numerous mRNAs, these small RNA molecules can operate highly complex regulatory networks and regulate the expression of genes in many pathways that are associated with tumor initiation, development and progression. Through regulating gene expression, miRNAs can have a profound impact on many patho-physiologic processes, including proliferation, apoptosis, and stress response [13,14]. In the current study, we observed that miRNAs were differentially expressed in MDR cancer cells, and demonstrated that expressions of miRNA 27a and 451 are associated with the activation of *MDR1*/P-gp expression and contribute to drug resistance in cancer cells.

2. Materials and methods

2.1. Cell culture and reagents

Human ovarian cancer cell line, A2780, and its multidrug resistant counterpart, A2780DX5, were kindly supplied by Dr. Youcef Rustum (Roswell Park Cancer Institute, Buffalo, NY). Human cervix carcinoma cell line KB-3-1 and its MDR variant KB-V1 were kindly provided by Dr. Michael M. Gottesman (National Cancer Institute, Bethesda, MD). These cell lines were routinely maintained in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Gaithersburg, MD) containing 10% (v/v) fetal bovine serum (Sigma, St. Louis, MO), 100 U/ml of penicillin sodium and 100 µg/ml of streptomycin sulfate (Invitrogen Life Technologies, Gaithersburg, MD), at 37 °C in humidified air containing 5% carbon dioxide air atmosphere. MDR cell lines A2780DX5 and KB-V1 were obtained by step-wise selection with doxorubicin and vinblastine (Sigma, St. Louis, MO), respectively, and were cultured in the continuous presence of doxorubicin (2 µM) or vinblastin (1 µg/ml) to maintain theMDR phenotype. The antagomirs and mimics of miR-27a, miR-451, and control RNA were purchased from Dharmacon Inc. (Lafayette, CO). Rhodamine 123 and verapamil were purchased from EMD Biosciences (La Jolla, CA).

2.2. MiRNA microarray profiling

Total RNA was prepared using the Trizol method (Invitrogen Life Technologies Inc.) according to the manufacturer's instructions. RNA labeling and hybridization on miRNA microarray were performed as described previously [15]. Briefly, 5 µg of total RNA from each sample was biotin-labeled by reverse transcription using 5' biotin end-labeled random octomer oligo primer. Hybridization of biotin-labeled cDNA was carried out on miRNA microarray

chip (OSU version 4.0, The Ohio State University, Columbus, OH), which contains 1600 miRNA oligo probes derived from 474 human and 373 mouse miRNA genes and printed in duplicates. Hybridization signals were detected by biotin binding of a Streptavidin–Alexa 647 conjugate using an Axon Scanner 4000B (Axon Instrument Inc., CA). The images were quantified by GenePix 6.0 software (Axon Instrument Inc., CA).

2.3. MiRNA transfection

Cells in exponential phase of growth were plated in 60mm plates at 1×10^6 cells/plate and cultured for 16 h, and then transfected with the mimics or antagomirs of miR-27a or miR-451, or control RNA (100 nM) using oligofectamine and OPTI-MEM I reduced serum medium (Invitrogen Life Technologies Inc., Carlsbad, CA), according to the manufacturer's protocol. The effects of mimics or antagomirs of miR-27a or miR-451 on MDR were examined 48 h following transfection.

2.4. Real-time RT-PCR

Total RNAs from cells were extracted with TriZol Reagent (Invitrogen Life Technologies, Gaithersburg, MD) following the manufacturer's instruction. First strand cDNA synthesis and amplification were performed using Omniscript RTKit (QIAGEN Valencia, CA). The following human *MDR1* primers were used: forward: 5'-CCC ATC ATT GCA ATA GCA GG-3'; reverse: 5'-TGT TCA AAC TTC TGC TCC TGA-3' [16]. The β -actin primers, designed by our laboratory, were as follows: forward: 5'-GCC AAC ACA GTG CTG TCT GG-3'; reverse: 5'-GCT CAG GAG GAG CAA TGA TCT TG-3'. SYBR Green quantitative PCR amplifications were performed on the Stratagene 3005P Real-Time PCR system. Reactions were carried out in a 25- μ l volume containing 12.5 μ l of 2 $\times$ SYBR Green PCR Master Mix (Bio-Rad). The thermal profile for the real-time PCR was 95 °C for 3min followed by 40 cycles of 95 °C for 20 s, 59 °C for 30 s, and 70 °C for 30 s. The ΔC_t data were collected automatically. The average ΔC_t of each group was calculated by the following formula: ΔC_t = average *MDR1* gene C_t – average of HK (housekeeping) gene' C_t . $\Delta\Delta C_t$ was calculated by $\Delta\Delta C_t$ = ΔC_t of negative control group – ΔC_t of the miRNA transfection group. The fold-change for *MDR1* expression level was calculated using $2^{-\Delta\Delta C_t}$.

2.5. Western blot analysis

Cells were washed twice with PBS containing 1 mM phenyl-methylsulphonyl fluoride, and then lysed in CelLytic™ MT Cell Lysis Reagent (Sigma–Aldrich, St. Louis, MO). The lysates were transferred to Eppendorf tubes and clarified by centrifugation at $12,000 \times g$ for 30 min at 4 °C. Identical amounts (25 μ g protein) of cell lysates were resolved by 8% SDS-PAGE, and then transferred to nitrocellulose. The membranes were incubated in blocking solution consisting of 5% powered milk in PBST (PBS plus 0.1% Tween 20) at room temperature for 1 h, then immunoblotted with monoclonal anti-P-gp antibody C219 (Calbiochem, San Diego, CA) or anti- β -actin antibody (Sigma–Aldrich, St. Louis, MO). Detection by enzyme-linked chemiluminescence was performed according to the manufacturer's protocol (ECL; Pierce Biotechnology Inc., Rockford, IL). Quantifications of protein bands were performed using the ImageJ software (<http://rsb.info.nih.gov/ij>).

2.6. Drug sensitivity assay

A2780DX5 cells were transfected with the antagomir of miR-27a or a control RNA. At the end of transfection, the cells were plated in 96-well plates in growth medium and incubated at 37 °C in a humidified 5% CO₂ atmosphere for 60 h in the presence of varying concentrations of vinblastine or hydroxyurea (Sigma, St. Louis, MO). The viability of cells was measured using the MTT assay.

2.7. Doxorubicin accumulation

Doxorubicin accumulation was assayed by a method described previously by our laboratory [11]. Briefly, cells transfected with the mimics or antagomirs of miR-27a or miR-451, or control RNA were incubated with 25 μ M of doxorubicin for 2 h. At the end of incubation, cells were washed thrice with PBS and observed under a fluorescence microscope with 400 $\times$ lens (Nikon ECLIPSE TE200 microscope; Nikon Inc., Melville, NY). Quantifications of doxorubicin fluorescence intensity were performed using the ImageJ software.

2.8. Rhodamine 123 (Rh-123) efflux

The transport activity of P-gp was determined by assaying the efflux of rhodamine 123 (Rh-123), as described previously [17]. Briefly, cells were incubated with 2 μ M of Rh-123 for 30 min at 37 $^{\circ}$ C. At the end of incubation, cells were washed thrice with PBS to remove free Rh-123. Cells were kept in dye-free medium at 37 $^{\circ}$ C. The fluorescence of remaining Rh-123 in the cells was measured by FACS using a flow cytometer (Coulter Cytomics FC500, Beckman Coulter, Miami, FL).

3. Results and discussion

To explore the roles of miRNAs in MDR phenotype, we first compared the profiling of miRNA expression between the human MDR cancer cells and their parental drug sensitive cells. Using a miRNA microarray (OSUCCC-microRNA version 4.0), we observed a differential expression pattern between the MDR cells and parental cells. As shown in Table 1, the expressions of miR-27a, miR-99a, miR-100, miR-125b1 and miR-451 were increased (2.0–7.8-fold) in human MDR ovarian cancer cell line A2780DX5 and cervix carcinoma cell line KB-V1, as compared to their respective parental cell lines A2780 and KB-3-1. The up-regulations of miR-27a, miR-99a, miR-100, miR-125b1 and miR-451 in the MDR cancer cells suggest a role for these small RNA molecules in the activation of expressions of *MDR1* gene and/or other genes associated with the development of drug resistance in these cancer cells.

To determine the functions of these differentially expressed miRNAs in regulating MDR phenotype, we tested the effects of antagomirs and mimics of miR-27a, miR-99a, miR-100, miR-125b1 and miR-451 on *MDR1* gene and P-gp expression in sensitive and MDR A2780 cells. Among the miRNAs tested, we only found that miR-27a and miR-451 affected *MDR1*/P-gp expression; miR-99a, miR-100 and miR-125b1 did not show any detectable effect in the same tests (data not shown). We demonstrated that transfection of drug resistant A2780DX5 cells with the antagomirs of miR-27a or miR-451 decreased the expression of the *MDR1* gene product, P-gp, as determined by Western blot (Fig. 1), suggesting that expressions of miR-27a and miR-451 positively regulate the expression of P-gp. To examine the effects of miR-27a and miR-451 on *MDR1* mRNA expression, we performed qRT-PCR analysis following treatment of the MDR cells with either the antagomirs or mimics of miR-27a and miR-451. Fig. 2 shows that treatment with the antagomirs of miR-27a (Fig. 2A and B) or miR-451 (Fig. 2C and D) for 48 h reduced the level of *MDR1* mRNA in both A2780DX5 (Fig. 2B and D) and A2780 (Fig. 2A and C) cells. By contrast, the mimic of miR-27a increased the *MDR1* mRNA level in the parental, drug sensitive cell line A2780 (Fig. 2A), but did not further increase the expression of P-gp (Fig. 1A) and *MDR1* mRNA (Fig. 2B) in the MDR cell line A2780DX5, probably due to the already high basal level of *MDR1*/P-gp expression in these cells. Similarly, the mimic of miR-451 did not have any detectable effect on P-gp level in A2780DX5 cells (Fig. 1B); however, the miR-451 mimic caused an increase in *MDR1* mRNA in A2780 (Fig. 2C) and A2780DX5 cells (Fig. 2D). The effects of these antagomirs or mimics on *MDR1* mRNA expression were most evident 48 h following transfection, but were almost undetectable 72 h after the treatment (data not shown), probably due to the fast degradation of these RNA molecules. MiRNAs are known to have the ability to reduce protein expression by

causing translational inhibition or degradation of the target mRNA, and inhibition of a miRNA typically leads to an increase in target protein expression. Interestingly, here we found that expressions of miR-27a and miR-451 were increased in MDR cancer cells (Table 1), and inhibition of these two miRNAs with their antagomirs resulted in reductions of both P-gp and *MDR1* mRNA expression (Fig. 1 and Fig. 2). Therefore, the effects of miR-27a and miR-451 on *MDR1* expression appear to be indirect, and are likely mediated through inhibiting expression of some transcriptional factors involved in suppressing *MDR1* gene activation. This may also explain the incomplete inhibition of P-gp expression by the antagomirs of these two miRNAs. The precise mechanisms and pathways by which miR-27a and miR-451 participate in the activation of the *MDR1* gene expression require further investigation. Although the expressions of miR-99a, miR-100 and miR-125b1 were also found to be increased in MDR cancer cells, we did not detect their effects on *MDR1*/P-gp expression. The cause for the lack of detectable effects of these miRNAs on *MDR1*/P-gp expression is unclear at present. It is likely that the up-regulations of miR-99a, miR-100 and miR-125b1 in MDR cells represent a response of cancer cells to drug toxicity, and may play regulatory roles in other events associated drug resistance, since these MDR cell lines were established through step-wise selection with cytotoxic agents and harbor multiple mechanisms of drug resistance [18,19].

We further determined whether inhibition of miR-27a or miR-451 could modulate the sensitivity of the MDR cancer cells to chemotherapeutic drugs. Following transfection of the drug resistant A2780DX5 cells with the antagomir of miR-27a or miR-451, we treated the cells with a series of concentrations of cytotoxic drugs vinblastine or hydroxyurea. Fig. 3 shows that treatment with the antagomir of miR-27a increased the sensitivity of A2780DX5 cells to vinblastine, a drug that is transported by P-gp (IC_{50} : 0.7nM vs. 20 nM). By contrast, the sensitivity to hydroxyurea, a non-P-gp substrate chemotherapeutic agent, was not affected by inhibition of miR-27a with its antagomir (Fig. 3; IC_{50} : 1030 μ M vs. 1010 μ M). The effect of the miR-451 antagomir on sensitivity to vinblastine was not significant (data not shown). Because the drug sensitivity assays lasted for 72 h, the lack of significant effect of the miR-451 antagomir on drug sensitivity was probably due to the faster turnover of this antagomir molecule and the indirect mode of action of miR-451 on *MDR1* expression. The stability of RNA molecules is known to vary, in particular in response to cellular stress [20]. To further confirm the roles of miR-27a and miR-451 in regulating P-gp expression, we assessed the transport activity of P-gp by measuring intracellular accumulation of doxorubicin, a chemotherapeutic drug that is transported by P-gp, in the MDR cells following treatment with the antagomirs and mimics of miR-27a or miR-451. Fig. 4A and B shows that the intracellular accumulation of doxorubicin was increased in A2780DX5 cells treated with the antagomirs of miR-27a or miR-451 in comparison with the cells treated with the control RNA or the mimics. The accumulation of Rhodamine 123, a P-gp substrate fluorescent dye, was also increased in A2780DX5 cells treated with the antagomirs as compared with the cells treated with the control RNA or the mimics, as analyzed by FACS (Fig. 4C). P-gp antagonist, verapamil, was used as positive control in these experiments. The results of these functional studies further support a role for miR-27a and miR-451 in regulating the MDR phenotype.

The evidence of the roles for miRNAs in determining drug sensitivity/resistance has been emerging recently. For example, Mo and colleagues reported that in addition to promoting tumor cell proliferation and growth, expression of miR-21 renders tumor cells insensitivity to topotecan, a chemotherapy agent that acts as a topoisomerase I inhibitor, and anti-miR-21 oligonucleotide could enhance the inhibitory effect of topotecan on growth of tumor cells [21]. Mishra et al. demonstrated that alteration of the miR-24 miRNA binding site in dihydrofolate reductase gene results in resistance to methotrexate, a commonly used anti-cancer agent [22]. More recently, it was reported that the up-regulation of miR-214 promotes survival of ovarian cancer cells and induces resistance to cisplatin [23]. The role of miRNAs in the regulation of ATP-binding cassette genes has also attracted attention recently. For

instance, Narvaiza et al. showed the involvement of miRNAs in the adenovirus-mediated RNA interference on mouse multidrug resistance protein 2 gene (*Abcc2*) and their effect on liver bilirubin transport [24]. We now demonstrate that the increased expressions of miR-27a and miR-451 contribute to the MDR phenotype in cancer cells. To our knowledge, this is the first study reporting the effects of miRNAs on *MDR1*/P-gp expression and the MDR phenotype. The exact roles and mechanisms underlying the effects of miR-27a and miR-451 remain to be studied.

In summary, our observation that miR-27a and miR-451 function as activators of *MDR1*/P-gp expression implies that approach to targeting these two miRNAs may offer novel therapeutic opportunities for the treatment of MDR cancers.

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Fig. 1.

Effects of antagomirs and mimics of miR-27a (A) and miR-451 (B) on P-gp expression in MDR cancer cells. A2780DX5 cells were transfected with 100 nM of mimics or antagomirs of miR-27a or miR-451, or a control RNA. Forty-eight hours later, cell lysates were prepared from the transfected cells. Equal amounts (25 μ g proteins) of cell lysates were separated by 8% SDS-polyacrylamide gel electrophoresis, and then transferred onto nitrocellulose membrane. The membranes were immunoblotted with a monoclonal anti-P-gp antibody, C219. Detection of P-gp was performed using enzyme-linked chemiluminescence. β -Actin was used as a loading control. Protein expression was quantified using the ImageJ software. P-gp/actin ratios of the samples treated with a control RNA was arbitrarily set at 1, and the P-gp/actin ratios of the mimic or antagomir-treated samples were normalized to the control. Results represent the mean $\pm$ S.D. of triplicate determinations from one of three identical experiments.

Fig. 2.

Effects of antagomirs and mimics of miR-27a and miR-451 on *MDR1* mRNA expression in MDR cancer cells. A2780 or A2780DX5 cells were transfected with 100 nM of mimics or antagomirs of miR-27a or miR-451, or a control RNA. Forty-eight hours later, total RNAs were extracted from the treated cells and quantitative real-time RT-PCR analysis of *MDR1* mRNA was performed as described in Section 2. *MDR1* mRNA level of the samples treated with a control RNA was arbitrarily set at 1, and the *MDR1* mRNA levels of the mimic or antagomir-treated samples were normalized to the control. Results are the mean $\pm$ S.D. of triplicate determinations from one of three identical experiments.

Fig. 3.

Effects of antagomir of miR-27a on sensitivity of MDR cancer cells to vinblastine and hydroxyurea. miR-27a antagomir or a control RNA-treated A2780DX5 cells were plated in 96-well plates in growth medium and incubated at 37 °C in a humidified 5% CO₂ atmosphere for 60 h in the presence of vehicle or a series of concentrations of vinblastine or hydroxyurea. At the end of incubation, the viability of cells was measured using the MTT assay. Each point represents the mean $\pm$ S.D. of quadruplicate determinations. Results are the representative of two similar experiments.

Fig. 4.

Effects of antagomirs and mimics of miR-27a and miR-451 on accumulation of doxorubicin and rhodamine 123 (Rh-123) in MDR cancer cells. (A and B) Drug resistant A2780DX5 cells treated with the antagomirs or mimics of miR-27a or miR-451, and the parental, drug sensitive A2780 cells, were incubated with 25 μ M of doxorubicin for 2 h. At the end of incubation, the cells were washed thrice with PBS, and then, (A) observation under a fluorescence microscope with 400 $\times$ lens; (B) quantification of doxorubicin fluorescence intensity. Bars, mean $\pm$ S.E.M. $**p < 0.05$. (C) Cells were incubated with 2 μ M of Rh-123 for 30 min at 37 $^{\circ}$ C. At the end of incubation, the cells were washed thrice with PBS to remove the free Rh-123. The fluorescence

of remaining Rh-123 in the cells was measured by FACS. Results are the representative of two similar experiments.

Table 1

Differential expressions of miRNAs in multidrug resistant cancer cells

Name/sequence	Fold change	
	KB-V1/KB-3-1	A2780DX5/A2780
hsa-miR-27a, UUCACAGUGGCUAAGUCCGC	3.8	5.1
hsa-miR-99a, AACCCGUAGAUCCGAUCUUGUG	2.8	6.2
hsa-miR-100, AACCCGUAGAUCCGAACUUGUG	4.1	7.8
hsa-miR-125b1, UCCCUGAGACCCUAAUUGUGA	6.9	2.9
hsa-miR-451, AAACCGUUACCAUACUGAGUU	2.2	2

Total RNAs was prepared were prepared from sensitive and MDR cancer cells and biotin-labeled by reverse transcription using 5' biotin end-labeled random octomer oligo primer. Hybridization of biotin-labeled cDNA was carried out on OSU version 4.0 miRNA microarray chips. Hybridization signals were detected by biotin binding of a Streptavidin–Alexa 647 conjugate and the images were quantified using GenePix 6.0 software.