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MiR-375 is epigenetically downregulated due to promoter methylation and modulates multi-drug resistance in breast cancer cells via targeting YBX1

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Abstract. – OBJECTIVE: In this study, we firstly verified how miR-375 is downregulated in breast cancer cells with multi-drug resistance (MDR) and further investigated the regulative effect of miR-375 on Ybx1 expression.

MATERIALS AND METHODS: MiR-375 expression and promoter methylation status were studied by retrieving data in NCBI GEO Datasets, qRT-PCR and Methylation-Specific PCR (MSP) assay. Drug sensitivity of the cancer cells was assessed using MTT assay. The binding between miR-375 and YBX1 gene was predicted using Targetscan 7.1 and verified using western blot and dual luciferase assay.

RESULTS: MiR-375 is significantly downregulated in both MCF-7/ADM and MCF-7/PTX cells than in MCF-7 cells. MCF-7/ADM and MCF-7/PTX cells had significantly higher level of promoter methylation than MCF-7 cells. 5-AZA-dC treatment significantly reduced the methylation in MCF-7/ADM and MCF-7/PTX cells and increased miR-375 expression. MiR-375 can directly target 3'UTR of YBX1 and thereby decrease its expression in MCF-7/ADM and MCF-7/PTX cells. Both miR-375 overexpression and YBX1 knockdown significantly decreased P-gp expression and increased chemosensitivity of the cancer cells.

CONCLUSIONS: MiR-375 is downregulated in MCF-7/ADM and MCF-7/PTX cells, and its downregulation is a result of promoter methylation. MiR-375 can directly target 3'UTR of YBX1 and thereby decrease its expression, which might be an important mechanism of MDR in breast cancer cells.

Key Words:
MiR-375, Methylation, MDR, YBX1, Breast cancer.

Introduction

Dysregulated epigenetic modulations, such as methylation, histone modification and non-coding RNAs at transcriptional and post-transcriptional levels are associated with carcinogenesis and multi-drug resistance (MDR) of the cancer cells[1,2]. In breast cancer, DNA methylation and microRNA (miRNA) silencing are among the important mechanisms directly contribute to chemoresistance or indirectly modulate the pathological processes related to chemoresistance, such as epithelial mesenchymal transition (EMT)[3,4]. For example, miR-149 is downregulated in breast cancer cells due to hypermethylation of its 5'-UTR and it can decrease chemoresistance through targeting GlcNAc N-deacetylase/N-sulfotransferase-1 (NDST1)[5]. The miR-200c/141 cluster is downregulated in breast cancer cells due to hypermethylation in the promoter region, which is associated with ectopic expression of the EMT transcription factor Twist and subsequently enhanced EMT[6].

To investigate the network of methylation and miRNA regulation in breast cancer cells during the acquisition of chemoresistance, Suzuki et al[7] performed high-throughput reduced representation bisulfite sequencing (RRBS), and RNA sequencing (RNA-Seq) to get the transcriptome profiles of both mRNAs and non-coding small RNAs in chemoresistant and chemosensitive breast cancer cell lines. According to their data, miR-375 is significantly downregulated in both MCF-7 derived adriamycin (ADM) resistant MCF-7/ADM and paclitaxel (PTX) resistant MCF-7/PTX cells than in the parent MCF-7 cells[7]. Actually, miR-375 has previously been identified as an important miRNA modulating trastuzumab resistance, tamoxifen resistance and EMT property of breast cancer cells[8-10]. Due to the complex regulative networks of miRNAs, its downstream regulation in breast cancer has not been fully understood.

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Y-box binding protein 1 (Ybx1) is encoded by the YBX1 gene. Previous researches[11,12] found that the expression of Ybx1 is corrected drug resistance and patient outcome in breast cancer. Zhu et al[13] found that its expression is regulated by miR-137 in breast cancer cells and miR-137 restoration can sensitize MCF-7/ADM cells to anti-cancer agents via downregulating Ybx1 level. In this study, we firstly verified how miR-375 is downregulated in MDR breast cancer cells and further investigated the regulative effect of miR-375 on Ybx1 expression.

Materials and Methods

Cell Culture and Treatment

The human breast cancer cell line MCF-7 was obtained from American Type Culture Collection (Manassas, VA, USA). The MCF-7 derived adriamycin resistant MCF-7/ADM and paclitaxel resistant MCF-7/PTX cells were generated by using a conventional stepwise method as described in a previous study[14], which induces drug resistance by increasing concentrations of ADM and PTX over 8 months. The cells were cultured in RPMI supplemented with 10% FBS, 100 μg/mL penicillin, and 100 U/mL streptomycin.

MiR-375 mimics, miR-375 inhibitors (anti-miR-375), YBX1 siRNA and the corresponding scramble negative controls were purchased from Ribobio (Guangzhou, China). MCF-7 cells were transfected with 50 nM miR-375 inhibitors, while MCF-7/ADM and MCF-7/PTX were transfected with 100 nM miR-375 mimics or 100 nM YBX1 siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). For 5-AZA-dC treatment, the cells were cultured 2.5 μM 5-AZA-dC (Sigma-Aldrich, St. Louis, MO, USA) for 48h, and then were harvested for the MSP assay and qRT-PCR analysis of miR-375 expression.

Bioinformatics Analysis

The data of RNA sequencing (RNA-Seq) and high-throughput reduced representation bisulfite sequencing (RRBS) of methylation patterns of miRNAs in chemoresistant and chemosensitive MCF-7 cell lines were retrieved in NCBI GEO Datasets (http://www.ncbi.nlm.nih.gov/gds, GSE68815). The possible targets of miR-375 were predicted using TargetScan 7.1 (http://www.targetscan.org/).

QRT-PCR Analysis of miR-375 Expression

Total RNA from cells samples was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The cDNA was reversely transcribed with miRNAs specific stem-loop primers and the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The mature miR-375 level was determined using qRT-PCR analysis with TaqMan MicroRNA Assay Kit (Applied Biosystems). The $2^{-\Delta\Delta Ct}$ method was used to calculate relative miRNA expression.

Methylation-Specific PCR (MSP)

Genomic DNA was isolated from cell samples using a DNeasy tissue kit (Qiagen, Hilden, Germany). Bisulfite reaction was carried out on 5 μg of genomic DNA using EpiTect Bisulfite Kit (Qiagen) according to manufacturer's instruction. The MSP was performed following the methods introduced in one previous study[15] with methylated-specific primers (M): forward, 5'-AGCGGCGTATAGTTTTTTTTATTC-3' and reverse, 5'-CGAACCTAAACGTTT-TATTCGTT-3'; and unmethylated-specific primers (U): forward, 5'-TGGAGTGGTG-TATAGTTTTTTTTATTT-3' and reverse, 5'-AC-CAAACCTAAACATTTTATTCATT-3'. PCR products were verified by 2.5% agarose gel electrophoresis.

Drug Sensitivity Assay

48 h after indicating transfection, the cells were seeded in a 96-well plate. After 24 hours incubation, the cells with treated with varying concentrations of ADM (0, 0.1, 0.25, 0.5, 1, 5, 10 and 20 μM) or PTX (0, 0.05, 0.1, 0.5, 1, 2, 5 and 10 μM) for 48 hours. Then, cell viability was measured using a conventional MTT (Sigma-Aldrich, St. Louis, MO, USA) assay. Absorbance was recorded at 490 nm using a microplate reader. The IC50 value was determined by creating dose-response curves.

Dual Luciferase Assay

According to the bioinformatics prediction, two short sequences of YBX1 3'UTR containing the wild-type or mutant predicted targeting sites of miR-375 were chemically synthesized. Then, the sequences were cloned into the downstream of the luciferase gene of pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA). The recombinant vectors were named as pGLO-YBX1-WT and pGLO-YBX1-MT respectively. MCF-7/ADM and MCF-7/PTX cells were co-transfected with

200 ng luciferase reporter vector and 100 nM miR-375 mimics or the negative controls. Luciferase activity was examined 24 hours after the transfection using the Dual-Luciferase Assay Kit (Promega, Madison, WI, USA) according to manufacturer's instruction.

Western Blot Analysis

The cells were lysed for protein extraction using a lysis buffer (Beyotime, Shanghai, China). Then, the protein samples (20 μg of total protein loaded per lane) were separated by 10% SDS-PAGE and then electrophoretically transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat milk powder and incubated overnight with primary antibodies against Ybx1 (#4202, Cell Signaling, Danvers, MA, USA), P-gp (#13342, Cell Signaling) and β-actin (ab8227, Abcam, Cambridge, UK). The second HRP conjugated secondary antibodies were purchased from Abcam. Protein bands were visualized by super ECL detection reagent (Applygen, Beijing, China). The relative gray scale of the bands was analyzed using ImageJ software.

Statistical Analysis

Data were presented in the form of means ± standard deviation (SD) Group difference was assessed using the unpaired *t*-test. A two-sided *p*-value of <0.05 was considered statistically significant.

Results

MiR-375 is Downregulated in MCF-7/ADM and MCF-7/PTX Cells

One previous study[7] analyzed miRNAs expression profiles and methylation status by using high-throughput sequencing in MCF-7/ADM and MCF-7/PTX cells. To further analyze the miRNAs profiles, we retrieved the data in NCBI GEO Datasets (http://www.ncbi.nlm.nih.gov/gds, GSE68815). We firstly screened the most dysregulated miRNAs (with at least 3 fold changes) in MCF-7/ADM and MCF-7/PTX (Figure 1A-B). MiR-375 had over 5 folds downregulation in both MCF-7/ADM and MCF-7/PTX compared with the parent MCF-7 cells (Figure 1A and B). Previous studies[8,9] observed that miR-375 is an important miRNA modulating EMT[10] and drug resistance of breast cancer. Therefore, we decided to further explore the role of miR-375 in breast cancer. Then, we performed qRT-PCR to confirm the expression of miR-375. The results confirmed that miR-375 is significantly downregulated in both MCF-7/ADM and MCF-7/PTX than in MCF-7 cells (Figure 1C).

MiR-375 Downregulation is a Result of Promoter Methylation in MCF-7/ADM and MCF-7/PTX Cells

By analyzing the high-throughput RRBS data, we found that the level of methylation in the promoter region of miR-375 in MCF-7/ADM (Figure 2A) and MCF-7/PTX (Figure 2B) cells were significantly higher than in MCF-7 cells. To further verify the effect of methylation, we performed MSP assay. The results showed that MCF-7/ADM and MCF-7/PTX cells had significantly higher levels of promoter methylation than MCF-7 cells (Figure 2C). The 5-AZA-dC treatment reduced significantly the methylation in MCF-7/ADM and MCF-7/PTX cells (Figure 2C). In addition, 5-AZA-dC treatment also sig-

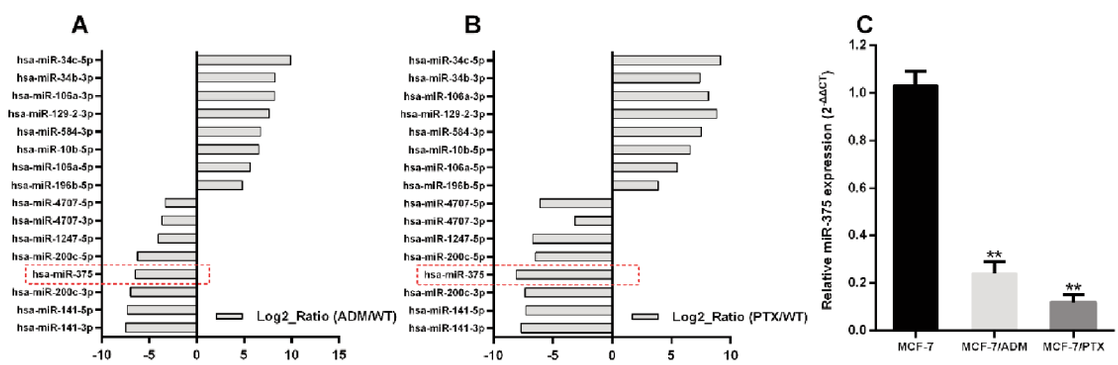

Figure 1. MiR-375 is downregulated in MCF-7/ADM and MCF-7/PTX cells. ***A-B,*** The most upregulated (8) and the most dowmregulated (8) miRNAs in MCF-7/ADM (A) and MCF-7/PTX cells (B) compared with wild type (WT) MCF-7 cells. (Data retrieved from NCBI GEO Dataset, with GEO accession number: GSE68815). ***C,*** QRT-PCR analysis of miR-375 expression in parent MCF-7 cells, MCF-7/ADM and MCF-7/PTX cells. $^{**}p<0.01$.

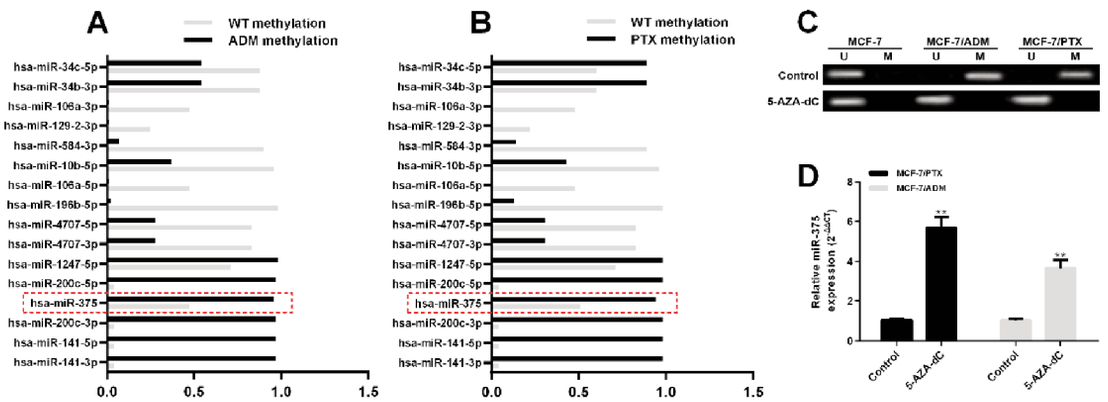

Figure 2. **MiR-375 downregulation is a result of promoter methylation in MCF-7/ADR and MCF-7/PTX cells.** ***A-B,*** The promoter methylation status of the most upregulated (8) and the most dowmregulated (8) miRNAs in MCF-7/ADM (A) and MCF-7/PTX cells ***(B)*** compared with wild type (WT) MCF-7 cells. (Data retrieved from NCBI GEO Dataset, with GEO accession number: GSE68815). ***C,*** The methylation status of the miR-375 promoter was detected in MCF-7, MCF-7/ADM and MCF-7/PTX cells with or without the treatment of 5-AZA-dC by MSP. M, methylation-specific primer amplification; U, unmethylation-specific primer amplification. ***D,*** QRT-PCR analysis of miR-375 expression in MCF-7/ADM and MCF-7/PTX cells with or without the treatment of 5-AZA-dC. $^{**}p<0.01$.

nificantly increased miR-375 expression in both MCF-7/ADM and MCF-7/PTX cells (Figure 2D).

MiR-375 Modulates MDR in Breast Cancer Cell Lines

Considering the dysregulated miR-375 in MCF-7/ADM and MCF-7/PTX cells, we then investigated the effect of miR-375 on drug sensitivity of the cells. MTT assay showed that MCF-7 cells with knockdown of miR-375 had decreased sensitivity to ADM (Figure 3A) and PTX (Figure 3B). MCF-7/ADM and MCF-7/PTX cells with enforced miR-375 expression had increased drug sensitivity (Figure 3C-D). These results suggest that miR-375 can modulate MDR in breast cancer cell lines.

YBX1 is a Functional Target of miR-375 Modulating MDR

Then, we further explored the downstream regulation of miR-375 in breast cancer. Online bioinformatics analysis showed that the 3'UTR of YBX1 has a highly conserved targeting site of miR-375 (Figure 4A). In both MCF-7/ADM and MCF-7/PTX cells, miR-375 overexpression significantly decreased Ybx1 protein expression (Figure 4B-C). To further verify the regulative effect of miR-375 on YBX1, we constructed dual luciferase reporters carrying either wild-type or mutant YBX1 3'UTR sequences. The following assay showed that miR-375 significantly suppressed relative luciferase activity of the reporter with wild type sequences, but had no suppressive effect on the reporter with mutant sequences in

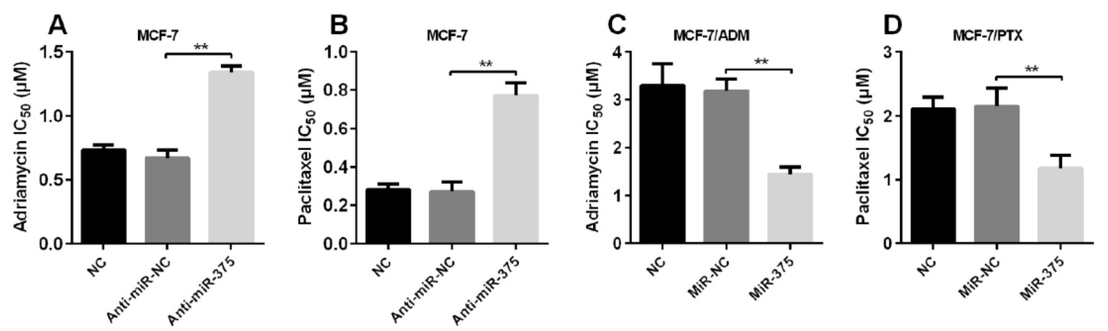

Figure 3. **MiR-375 modulates MDR in breast cancer cell lines.** ***A-D.*** Adriamycin (ADM) IC50 (A and C) and paclitaxel (PTX) IC50 (B and D) in MCF-7 cells transfected with miR-375 inhibitors (A-B) and in MCF-7/ADM (C) and MCF-7/PTX cells (D) transfected with miR-375 mimics. $^{**}p<0.01$.

both MCF-7/ADM and MCF-7/PTX cells (Figure 4D-E). In fact, YBX1 has been demonstrated as an important gene modulating chemosensitivity of multiple types of cancer[12,16,17]. Then, we investigated further the functional role of YBX-1 in MCF-7/ADM and MCF-7/PTX cells. MTT assay showed knockdown of endogenous YBX1 significantly decreased drug resistance (Figure 4 F-G). Following western blot analysis also confirmed that both miR-375 overexpression and YBX1 knockdown significantly decreased P-gp expression in MCF-7/ADM and MCF-7/PTX cells (Figure 4H).

Discussion

Although the use of chemotherapeutic agents and development of combined chemotherapy regimens have significantly improved survival outcome of patients with breast cancer, the development of MDR is still one of the major reasons of therapy failure[18]. The dysregulated epigenetic modulations play critical roles in the development of MDR[19,20]. MiRNAs are a group of small and highly conserved non-coding RNAs that suppress gene expression via inhibiting transcription or inducing degradation of targeting mRNAs[21]. Some recent investigations found that several miRNAs are epigenetically silenced in breast cancer and are involved in the development of MDR[22], such as miR-149 and miR-200c/141 cluster[5,6]. MiR-149 is downregulated due to hypermethylation of its 5'-UTR, which reduces MDR via targeting NDST1[1], while the miR-200c/141 cluster is decreased as a result of promoter hypermethylation and their downregulation directly leads to ectopic expression of the EMT transcription factor Twist and subsequently enhanced EMT[2]. In fact, miR-149 can also suppress EMT via downregulating FOXM1[23]. The regulative network of miRNAs are quite complex and their biological roles in chemoresistance have not been fully elucidated.

Suzuki et al[7] suggests that miR-375 is significantly downregulated in both MCF-7/ADM and MCF-7/PTX cells than in the parent MCF-7 cells, and its downregulation is associated with promoter hypermethylation. In this study, besides retrieving the RNA-Seq and high-throughput

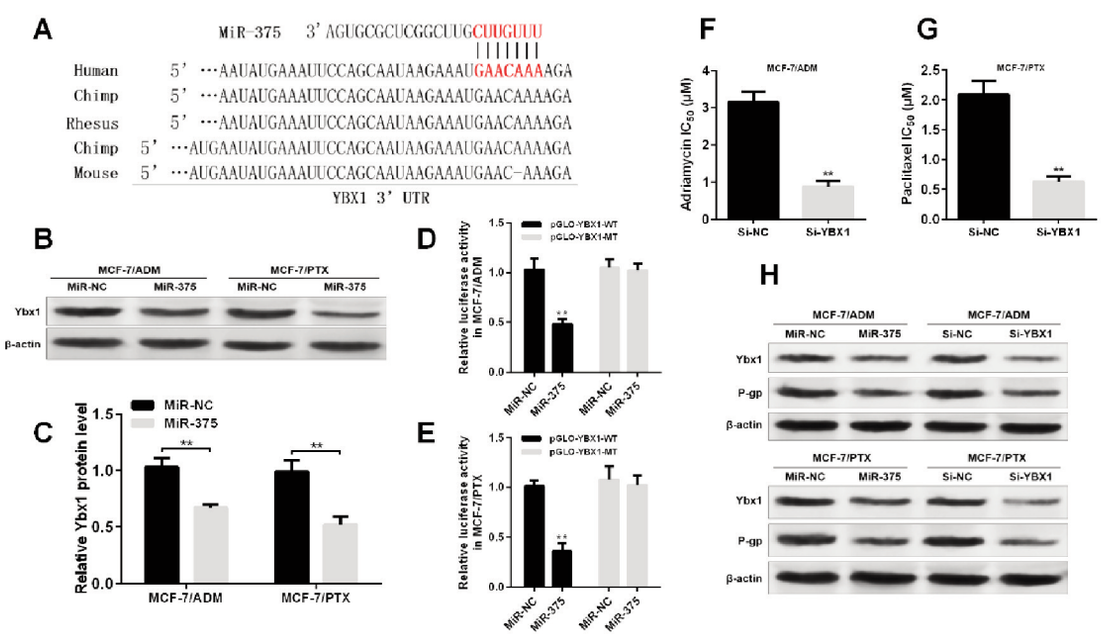

Figure 4. YBX1 is a functional target of miR-375 modulating MDR. ***A,*** Bioinformatics analysis of the possible binding sites between miR-375 and 3'UTR of YBX1. ***B* and *C,*** The representative images (B) and quantification (C) of western blot analysis of Ybx1 protein level in MCF-7/ADM and MCF-7/PTX cells transfected with miR-375 mimics. ***D-E,*** Dual luciferase assay of relative luciferase activity in MCF-7/ADM (D) and MCF-7/PTX cells (E) transfected with miR-375 mimics (100 nM) in combination with pGLO-YBX1-WT and pGLO- YBX1-MT. ***F-G,*** ADM IC50 (F) and PTX IC50 (G) in MCF-7/ADM and MCF-7/PTX cells transfected with YBX1 siRNA. ***H,*** Western blot analysis of Ybx1 and P-gp expression in MCF-7/ADM and MCF-7/PTX cells transfected with miR-375 mimics or YBX1 siRNA. $^{**}p<0.01$.

RRBS data, we further performed qRT-PCR and MSP assay to examine the expression of miR-375 and its promoter methylation status. Our data confirmed that miR-375 is downregulated in MCF-7/ADM and MCF-7/PTX cells and its downregulation is a result of promoter methylation. Previous reports[24,25] found that miR-375 has paradoxical roles in the regulation of chemoresistance in different types of cancer. For example, in cervical cancer, paclitaxel treatment can induce miR-375 upregulation, which facilitates EMT process via directly targeting E-cadherin consequently results in enhanced chemoresistance. However, in colorectal cancer, ovarian cancer and breast cancer, miR-375 overexpression can inhibit EMT and reduce chemoresistance[9,26,27]. Typically, in breast cancer, miR-375 can directly target SHOX2, an EMT inducer, which promotes EMT via upregulating transforming growth factor beta receptor I (TbetaR-I) expression[10]. Enforced miR-375 expression can reverse both tamoxifen resistance and accompanying EMT-like properties in breast cancer cells via downregulating metadherin (MTDH)[9]. In addition, Overexpression of miR-375 can restore the sensitivity of breast cells to trastuzumab via downregulating insulin-like growth factor 1 receptor (IGF1R), a common feature of trastuzumab-refractory cells[8]. Since miR-375 exerts complex regulative roles in breast cancer cells, we decided to further investigate its downstream regulation.

By performing bioinformatics analysis, we found that YBX1 is a possible target of miR-375. Ybx1 upregulation is corrected with drug resistance and patient outcome in breast cancer[11,12]. Overexpression of YBX1 in MCF-7 and MDA-MB-231 cells bestowed cisplatin resistance in such cells[17], while enforced YBX1 expression in human mammary epithelial cells (HMECs) resulted in triple negative breast cancer (TNBC) tumor formation, which also demonstrated a MDR phenotype[28]. These findings suggest that YBX1 is an important gene modulating MDR in breast cancer cells. Our Western blot analysis and dual luciferase assay confirmed that miR-375 can directly target 3'UTR of YBX1 mRNA and thereby decrease its expression in MCF-7/ADM and MCF-7/PTX cells. In addition, we also observed that both miR-375 overexpression and YBX1 knockdown significantly decreased P-gp expression, a MDR marker[29], and reduced MDR. Based on these findings, we infer that YBX1 is a functional target gene of miR-375 modulating MDR in breast cancer cells.

Conclusions

MiR-375 is downregulated in MCF-7/ADM and MCF-7/PTX cells, and its downregulation is a result of promoter methylation. MiR-375 can directly target 3'UTR of YBX1 and, thereby, decrease its expression, which might be an important mechanism of MDR in breast cancer cells.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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