Original Paper

A Feedback Loop Between miR-30a/c-5p and DNMT1 Mediates Cisplatin Resistance in Ovarian Cancer Cells

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Key Words

Ovarian cancer • Cisplatin resistance • microRNA • Methylation • Epithelial-mesenchymal transition

Abstract

Background: Many microRNAs (miRs) are dysregulated in cancers, and aberrant miR expression patterns have been suggested to correlate with chemo-resistance of cancer cells. We aim to study the role of miR-30 family members in cisplatin-resistance of ovarian cancer cells. **Methods:** qRT-PCR was used to compare differential expression levels of miR-30 family members in ovarian cancer cell line A2780 and its cisplatin-resistant derivative CP70. Changes of cisplatin-sensitivity in miR-30a-5p- and miR-30c-5p-overexpressed-CP70 cells and miR-30a-5p- and miR-30c-5p-inhibited-A2780 cells were examined by CCK8 assay and apoptosis analysis using flow cytometry; targets of miR-30a/c-5p were analyzed by western blotting and luciferase reporter assay; methylation regulation of pre-miR-30a/c-5p was examined by methylation specific PCR. **Results:** miR-30a-5p and miR-30c-5p, in contrast to other miR-30 family members, dramatically decreased in cisplatin-resistant CP70 cells due to overexpressed-DNMT1 induced aberrant methylation. miR-30a/c-5p in turn directly inhibited DNMT1 as well as Snail. Forced expression of miR-30a/c-5p or knocking down of DNMT1 and Snail promoted cisplatin susceptibility and partially reversed epithelial-mesenchymal transition (EMT) in CP70 cells, while inhibition of miR-30a/c-5p or ectopic expression of DNMT1 and Snail induced cisplatin resistance and partial EMT in cisplatin-sensitive A2780 cells. **Conclusions:** A feedback loop between miR-30a/c-5p and DNMT1 is a potent signature for cisplatin-resistance and EMT in ovarian cancer, promising a potential target for improved anti-cancer treatment.

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Introduction

Ovarian cancer is the fifth most common cause of cancer-related deaths among women [1]. Despite the use of modern therapeutic approaches, patients suffering from ovarian cancer still have poor prognosis, with a 5-year survival rate lower than 30% [2]. The high mortality of ovarian cancer is mainly due to the difficulty in early diagnosis, metastasis, and resistance to chemotherapeutics [3]. Although chemotherapy is the preferred option for the treatment of advanced ovarian cancer, the evolution of chemo-resistance remains clinically challenging as it prevents a successful long-term treatment of ovarian cancer [4].

Platinum-containing agents currently are the first-line chemotherapeutics for ovarian cancer [5], yet their efficacy is plagued by the emergence of resistance. Cisplatin is the first platinum-based compound employed for the treatment of ovarian cancer, and refractory reactions to cisplatin are a particular concern for clinical use [6]. Cisplatin binds to double-stranded DNA to form DNA adducts and interferes with DNA replication and RNA transcription, ultimately triggering apoptosis [7]. Most studies focus on the effects of cisplatin to address the molecular mechanisms mediating the rise of cisplatin-resistance, and many have been characterized including defective DNA damage response, increased anti-apoptotic regulators activity, among others. In the past few years, epithelial-mesenchymal transition (EMT) was implicated as a key mechanism mediating chemo-resistance [8, 9]. More recent work, however, has recognized microRNAs (miRs), a new class of post-transcriptional regulators, as central players in chemo-resistance development and EMT induction of cancers [10, 11].

EMT is a process where epithelial cells lose their cell polarity and cell-cell adhesion, and acquire migratory and invasive capabilities [12-14]. EMT can be triggered by a range of environmental stimuli and its molecular signatures include loss of epithelial cell markers such as E-cadherin and gain of mesenchymal cell markers such as vimentin coupled with upregulation of E-cadherin transcription repressors such as Snail [15]. EMT is closely related to cancer metastasis, and has been deemed an important mechanism contributing to chemo-resistance [16, 17].

miRs are 18 to 25-ribonucleotide non-coding RNAs that suppress gene expression via base pairing with the 3'- untranslated region (3'UTR) of mRNA targets to facilitate mRNAs degradation or inhibit protein translation [18, 19]. The abundance of many miRs is altered under various pathophysiological conditions [20-22], and a growing list of miRs are reported as key regulators of ovarian carcinoma-associated EMT [23] as well as cisplatin-resistance [24-26].

The miR-30 family, composed of miR-30a, b, c, d, and e (miR-30s), are downregulated in several types of cancer [27, 28]. These miR-30s are recognized as inhibitors of EMT in many kinds of malignancies [28, 29] and, more recently, implicated in chemo-resistance [30-32]. However, their molecular mechanism and regulatory network in cisplatin-resistance remain largely unknown. Here, we report a significant reduction of miR-30a-5p and miR-30c-5p in cisplatin-resistant CP70 ovarian cancer cell line compared with cisplatin-sensitive A2780 ovarian cancer cell line. The introduction of miR-30a/c-5p leads to a decrease at the protein level of DNA methyltransferase DNMT1 and Snail, while DNMT1 also reduces miR-30a/c-5p via methylating the promoter region of miR-30a/c-5p precursor genes. The present study provides a better understanding of the role of miR-30s in the development of cisplatin-resistance in ovarian cancer.

Materials and Methods

Cell cultures

A2780 cell line was from American Type Culture Collection (ATCC) (Manassas, VA, USA). Its cisplatin-resistant cell line CP70 was derived by incubation with stepwise increasing cisplatin concentrations (from 10 to 20 μ mol). Cells were cultured in RPMI 1640 (GIBCO; Grand Island, NY USA) supplemented with 10% newborn bovine serum (GIBCO; Grand Island, NY USA). These two cell lines were maintained in the

presence of 5 % CO₂ atmosphere at 37°C. Cisplatin was purchased from Hansoh Pharmaceutical Co. Ltd. (Lianyungang, Jiangsu, China).

Plasmid transfection

The human DNMT1 and Snail expression vectors were obtained from Addgene. Cells were seeded into 6-well plates until 70 %-80 % confluence and transiently transfected with 2 µg plasmids or empty vector using the X-treme GENE HP DNA Transfection Reagent (Roche, Indianapolis, IN, USA). After 24 h of transfection, the cells were treated with cisplatin for another 24 h.

siRNAs and miRs transfection

Knockdown of DNMT1 and Snail were performed using specific siRNAs purchased from GenePharma Company (Shanghai, China). Sequences of siRNAs are as follows: siDNMT1-a, 5'-UCUGUCCGUUCACAUGUGUdTdT-3'; siDNMT1-b, 5'-GGAUGAGAAGAGACGUAGAdTdT-3'; siSnail-a, 5'-GCGAGCUGCAGGACUCUAAUCdTdT-3'; siSnail-b, 5'-AAUGCUCUACUGGGACUCUGUdTdT-3'. A scrambled siRNA (5'-GCGACGAUCUGCCUAGAAdTdT-3') was used in parallel experiments as negative control. miR-30a/c-5p mimics or inhibitors and corresponding negative control were purchased from Ribo-Bio Co. Ltd. (Guangzhou, China). Cells were seeded into 6-well plates to reach 40%-50% confluence after 24 h and then transiently transfected with 5 µg siRNA, or 80 nM miR-30a/c-5p mimics, or 150 nM inhibitors and corresponding negative control using the X-treme GENE siRNA Transfection Reagent (Roche, Indianapolis, IN, USA). After 24 h of transfection, the cells were treated with cisplatin for another 24 h.

Cell viability assay

Cells were seeded (8×10³ per well for A2780, 1×10⁴ per well for CP70) in triplicates in 96-well plates. Cell viability was monitored using the cell counting kit-8 (CCK-8) assay (7seapharmtech Co., Ltd., Shanghai, China) after 24h-transfection followed by 24h-cisplatin treatment according to the manufacturer's instructions.

Apoptosis analysis

Apoptotic cells were detected using an Annexin V-PE/7-AAD apoptosis Detection Kit (BD Biosciences, Bedford, MA, USA) according to the manufacturer's instructions. Briefly, cells were washed with ice-cold PBS twice and stained with 100 µl of 1× binding buffer containing 5 µl PE Annexin V and 5 µl 7-AAD for 15 min at room temperature in the dark. Then 400µl of 1× binding buffer was added to each tube. Flow cytometry was performed to detect cellular apoptosis within 1 h, and the results were analyzed using the Cell-Quest™ Pro software (BD Biosciences, Bedford, MA, USA).

Quantitative real-time PCR (qRT-PCR)

Total cellular RNA was extracted using the Trizol reagent (Invitrogen; Carlsbad, CA, USA) according to manufacturer's instruction. Total RNA concentration was quantified by absorbance at 260 nm using spectrophotometer. 1 ng of total RNA was subjected to reverse transcription using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara; Dalian, China) according to the manufacturer's instructions. Each cDNA generated was amplified by quantitative PCR using SYBR Premix Ex Taq™ II kit (Takara; Dalian, China) on a CFX96 real-time PCR system (Bio-Rad; Hercules, CA, USA). The coding genes were normalized to β-actin, while miR-30s were normalized to small nuclear U6. The expression levels were automatically quantified using 2^{-ΔΔCt}. The primers for the coding genes were synthesized by Beijing Genomics Institute (Beijing, China), and the primer sequences were listed below: DNMT1 forward: 5'-CCTGAGGCCTTCACGTTCAA-3'; DNMT1 reverse: 5'-ACTTGTGGGTGTTCTCAGGC-3'; Snail forward: 5'-TCCAGAGTTTACCTTCCAGCA-3'; Snail reverse: 5'-CTTTCCCACTGTCTCATCTG-3'; β-actin forward: 5'-TCCCTGGAGAAGAGCTACGA-3'; β-actin reverse: 5'-AGCACTGTGTTGGCGTACAG-3'. The primers for miR-30a-, 30b-, 30c-, 30d- and 30e-5p, and U6 reverse transcription and amplification were designed and synthesized by Ribo-Bio Co. Ltd. (Guangzhou, China).

Western blotting

Cell pellets were lysed in RIPA buffer containing 1 mM PMSF and 1% complete protease inhibitor cocktail (Roche Applied Science; Indianapolis, IN, USA). After quantification, equal amounts of lysate were separated by SDS-PAGE and then transferred onto NC membranes (Pall Life Science, Port Washington,

NY, USA). Membranes were blocked in 5% nonfat milk and incubated with anti-DNMT1(1:1000, Active Motif; Carlsbad, CA, USA), anti-E-cadherin(1:1000, Cell Signaling Technology; Beverly, MA, USA), anti-vimentin(1:1000, Cell Signaling Technology; Beverly, MA, USA), anti-Snail(1:400, Cell Signaling Technology; Beverly, MA, USA), and anti- β actin (1:1000, Cell Signaling Technology; Beverly, MA, USA) primary antibodies, followed by incubation with goat-anti-rabbit or goat-anti-mouse secondary antibody conjugated with horseradish peroxidase (HRP) (1:2000, Pierce; Rockford, IL, USA). The protein signals were visualized using enhanced chemiluminescence reagents (Millipore, Billerica, MA, USA) by a chemiluminescence imaging system (Bio-Rad, Richmond, CA, USA).

Methylation specific PCR (MSP)

DNA bisulfite modification and purification were performed using an EZ DNA methylation-Direct kit (Zymo Research Corporation, Irvine, California, USA) according to the instruction. Concentration of DNA was evaluated by absorbance at 260 nm on a UV spectrophotometer (BioRad Inc., Hercules, CA, USA). The sets of primers for miR-30a/c-5p gene were flanking the 3kb regions of the 5' upstream regions from the start of pre-miR-30a/c-5p sequences. The primers for methylation-specific PCR were designed by MethPrimer and the sequences were as follows: miR-30a methylated (M)-forward (F): 5'-TAAATATTTTAAGGATAGAGAGCGA-3', miR-30a methylated (M)-reverse (R): 5'-AAAATTTAAAAACAACCCGAA-3', miR-30a unmethylated (U)- forward (F): 5'-AAATATTTTAAGGATAGAGAGTGA-3', miR-30a unmethylated (U)- reverse (R): 5'-ATAAAAAATTTAAAAACAACCCAAA-3'; miR-30c methylated (M)-forward (F): 5'-AGGCGTTTGTAGTTTATGTTATTCG-3', miR-30c methylated (M)-reverse (R): 5'-CGAAATAAAATCTCGCTCTATCG-3', miR-30c unmethylated (U)- forward (F): 5'-GTGTTTGTAGTTTATGTTATTTGG-3', miR-30c unmethylated (U)- reverse (R): 5'-TATACTCCAACACTACTCACACCACG-3. DNA amplification was performed with Epi Taq HS (Takara Biotechnology Co. Ltd., Dalian, China) under the following condition: 94 °C for 5 min; 40 cycles of 94 °C for 45 s, 44.3 °C (miR-30a U)/ 48 °C (miR-30a M)/ 36 °C (miR-30c U)/ 50 °C (miR-30c M)/ for 45 s, 72 °C for 1 min; and 72 °C for 10 min. The PCR products were separated by 2.0 % agarose gel electrophoresis and visualized by a chemiluminescence imaging system (Bio-Rad, Richmond, CA, USA).

Plasmids construction and Luciferase reporter assay

The 3' UTR of human DNMT1 gene containing predicted miR-30a/c-binding site was amplified by PCR from cDNA of CP70 cells using the following primers: forward (wt-F): 5'-CCCAAGCTTGGGGTCACCCCTGTTTCTGGCA-3'; and reverse (wt-R): 5'-CGAGCTCGCAAGGCCACAAACACCATGT-3'. The PCR products were digested with Hind III and Sac I enzymes and inserted into pMIR-REPORTM Luciferase vectors (Ambion; Austin, TX, USA) to construct a DNMT1 WT-3' UTR vector. The transversion mutant in the miR-30a/c-5p seed region was generated by overlap extension method to construct DNMT1 MUT-3' UTR vector, and the primers were listed as follows: mu1 forward (mu1-F, containing the mutant first binding site): 5'-CCCAAGCTTGGGGTCACCCGTGGGCTGGCACC-3'; mu2 forward (mu2-F, containing the second binding site): 5'-ACTGATGTTGGTGGGTTAACATGTC-3'; mu2 reverse (mu2-R, containing the second binding site): 5'-GACATGTTAACCCACCAACATCAGT-3'; mu3 reverse (mu3-R, containing the third binding site): 5'-CGAGCTCG CAAGGCCACCCACCCATGTACC-3'. All the construct sequences were confirmed by sequencing. Snail WT-3' UTR and MUT-3' UTR vectors were constructed as previously reported[33].

To perform luciferase reporter assay, cells grown in a 24-well plate were cotransfected with 180 ng of recombinant wild-type (WT-3' UTR) or mutation (MUT-3' UTR) reporter vectors, 20 ng of pRL-TK vector and 20 nM miR mimics using X-treme GENE siRNA Transfection Reagent (Roche, Indianapolis, IN, USA). 24 h after transfection, cells were harvested, and reporter assays were performed using a dual-luciferase reporter gene assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Luciferase activity was measured on the GloMax® 20/20 Luminometer (Promega, Madison, WI, USA).

Statistical analysis

The graphical presentations were prepared using GraphPad Prism 5.0. Data were presented as the means $\pm$ SD and were analyzed using SPSS 22.0 software (Chicago, IL, USA). Statistical differences were analyzed by Chi-square test, or two-tailed t-test using SPSS software (Chicago, IL, USA). Differences were considered significant at $P < 0.05$ (*) and highly significant at $P < 0.01$ (**) for all comparison.

Results

miR-30a/c-5p reduction enhanced cisplatin resistance of ovarian cancer cells

We first compared A2780 and CP70 cells with respect to their resistance to cisplatin. The IC₅₀ values for A2780 and CP70 cells treated by cisplatin were 21.92 and 206.0 μ M, respectively (Fig. 1A). 100 μ M of cisplatin rendered a 2-fold increase in apoptosis of A2780 cells compared with CP70 cells (Fig. 1B). The level of miR-30s in cisplatin-sensitive A2780 cells and cisplatin-resistant CP70 cells was then examined. Of the five members of the miR-30 family, only miR-30a/c-5p displayed a drastic reduction of more than 60% in CP70 cells relative to A2780 cells, while a moderate 30% decrease in the level of miR-30d and no significant change of miR-30b and -30e were observed under the same conditions (Fig. 1C).

Fig. 1. miR-30a/c-5p impair resistance of ovarian cancer cells to cisplatin. (A) Cisplatin-sensitive A2780 cells and cisplatin-resistant CP70 cells were treated with cisplatin of indicated concentrations for 24 h. Compared with A2780 cells, a significant increase in cell survival was persistently observed with CP70 cells at all cisplatin concentrations. (B) Flow cytometry analysis showed that treatment of ovarian cancer cells with 100 μ M cisplatin for 24 h caused more apoptotic death to A2780 than to CP70 cells. The lower right quadrant in the image represented early apoptosis, while the upper right quadrant signified late apoptosis. The percentage of apoptotic cells was calculated by including cells in both early and late apoptosis. (C) qRT-PCR results showed that miR-30a/c-5p were significantly reduced in CP70 cells relative to A2780 cells. (D) qRT-PCR displayed 2000- and 300-fold increase in miR-30a-5p and miR-30c-5p, respectively, in miR-30a/c-5p mimics transfected-CP70 cells. (E) CCK8 assays showed that overexpression of miR-30a/c-5p suppressed CP70 cell survival under cisplatin treatment and that mimics of miR-30a-5p and miR-30c-5p could synergistically sensitize CP70 cells to cisplatin. The IC₅₀ values for negative control cells, miR-30a-5p mimic, miR-30c-5p mimic and miR-30a+c mimic-transfected CP70 cells were 237.6, 112.1, 104.3 and 78.36 μ M, respectively. (F) Flow cytometry analysis showed that transfection by miR-30a-5p mimic and/or miR-30c-5p mimic significantly increased apoptotic cell counts in CP70 cells, with the combination of miR-30a+c mimic being most effective. (G) qRT-PCR results showed the downregulation of miR-30a/c-5p via transfection of their inhibitors into A2780 cells. (H) CCK8 assays showed that downregulation of miR-30a/c-5p increased cisplatin resistance of A2780 cells. The IC₅₀s for negative control cells, miR-30a-5p inhibitor, miR-30c inhibitor and miR-30a+c inhibitors-transfected A2780 cells were 36.38, 58.99, 51.98 and 58.09 μ M, respectively. (I) Flow cytometry analysis showed that inhibition of miR-30a/c-5p significantly suppressed cell apoptosis in A2780 cells where miR-30a/c-5p functioned synergistically. All experiments were carried out in triplicate and the results were presented as means $\pm$ SD. Calculation of statistical significance was performed using a two-tailed t-test.

Fig. 2. miR-30a/c-5p target Snail to attenuate cisplatin resistance. (A) Western blotting results showed that CP70 cells expressed a substantially higher level of Snail protein than did A2780 cells. (B) Ectopic expression of miR-30a/c-5p inhibited Snail protein expression in CP70 cells. (C) Transfection of miR-30a/c-5p inhibitors rescued Snail protein level in A2780 cells. (D) Schematic representation of Snail WT-3'UTR and MU-3'UTR sequences used for the construction of luciferase report vectors. Luciferase reporter assay showed that miR-30a/c-5p mimics significantly diminished luciferase activity of wild type Snail 3' UTR, with no effect on that of the mutated Snail 3' UTR. (E) Western blotting results showed that Snail protein level in CP70 cells was significantly reduced by siSnails transfection. (F) CCK8 results showed that knocking down of Snail increased cisplatin activity against CP70 cells. The IC₅₀s for negative control cells, siSnail-a, siSnail-b-transfected CP70 cells were 318.1, 273.2, and 230.0 μ M, respectively. (G) Flow cytometry analysis showed that inhibition of Snail in CP70 cells resulted in a 1.5-fold increase in cell apoptosis. (H) Western blotting results showed that Snail was overexpressed when Snail expression plasmid was transfected into A2780 cells. (I) CCK8 results showed that Snail overexpression significantly increased resistance of A2780 cells to cisplatin at concentrations of 40 and 80.0 μ M. The IC₅₀ values for negative control cells and Snail-overexpressed A2780 cells were 45.66 and 74.84 μ M, respectively. (J) Flow cytometry analysis showed that Snail overexpression reduced apoptotic cell counts in cisplatin-treated A2780 cells. (K) CCK8 results showed that Snail overexpression partly reversed the inhibitory effect of miR-30a-5p on cisplatin resistance of CP70 cells. (L) CCK8 results showed that Snail overexpression partly reversed the inhibitory effect of miR-30c-5p on cisplatin resistance of CP70 cells. The statistical differences were calculated using a two-tailed t-test.

The effects of miR-30a/c-5p on cisplatin resistance were further investigated. Forced expression of miR-30a/c-5p (Fig. 1D) sensitized CP70 cells to cisplatin, as shown by a significant reduction in IC₅₀ values (Fig. 1E) and an increase in apoptotic cell counts (Fig. 1F) in miR-30a/c-5p mimics-transfected CP70 cells compared with negative control cells. Of note, synergistic effects of miR-30a/c-5p mimics on cisplatin sensitivity were observed in CP70 cells (Fig. 1E and 1F). By contrast, suppression of miR-30a/c-5p (Fig. 1G) substantially enhanced cell survival (Fig. 1H) and reduced cell apoptosis (Fig. 1I) in cisplatin-treated A2780 cells. Similarly, miR-30a and miR-30c inhibitors were also synergistic on apoptosis as observed by flow cytometry.

Fig. 3. miR-30a/c-5p inhibit EMT. (A) Western blotting results showed that CP70 cells expressed higher levels of vimentin than did A2780 cells, indicating occurrence of partial EMT in the former. (B) In vitro migration assay showed that cell motility was enhanced in CP70 cells relative to A2780 cells (original magnification $\times 400$). (C) Western blotting results showed that vimentin was decreased in miR-30a/c-overexpressed CP70 cells. (D) In vitro migration assay showed that cell motility was impaired in miR-30a/c-overexpressed CP70 cells (original magnification $\times 400$). (E) Western blotting results showed that vimentin was upregulated in miR-30a/c-underexpressed A2780 cells. (F) In vitro migration assay showed that cell motility was enhanced in miR-30a/c-underexpressed A2780 cells (original magnification $\times 400$). The statistical differences were calculated using a two-tailed t-test.

miR-30a/c-5p directly inhibited Snail to attenuate cisplatin resistance

Snail was predicted by TargetScan (<http://www.targetscan.org/>) and previously verified as a direct target of miR-30s. We therefore examined the association of miR-30a/c-5p with Snail as well as the role of Snail in cisplatin resistance. As shown in Figure 2A, the protein level of Snail was higher in CP70 cells than in A2780 cells. However, Snail protein decreased in miR-30a/c-overexpressed CP70 cells (Fig. 2B) and increased in miR-30a/c-underexpressed A2780 cells (Fig. 2C). A direct suppression of miR-30a/c-5p on Snail expression was further verified using luciferase reporter assay. Compared with the negative control, miR-30a/c-5p significantly diminished the luciferase activity of wild-type Snail 3' UTR, whereas it had no effect on that of a mutated Snail 3' UTR (Fig. 2D). As expected, knocking down of Snail (Fig. 2E) sensitized CP70 to cisplatin-induced cell death (Fig. 2F and 2G), while ectopic expression of Snail (Fig. 2H) promoted cisplatin-resistance in A2780 cells (Fig. 2I and 2J). Furthermore, the activity of miR-30a/c-5p mimics on cisplatin sensitization was antagonized by concomitant overexpression of Snail in CP70 cells (Fig. 2K and 2L). These data verified that miR-30a/c-5p directly suppressed Snail expression and consequently enhanced cisplatin sensitivity.

miR-30a/c-5p negatively regulated EMT

Since Snail, the direct target of miR-30a/c-5p, is a key pro-EMT factor, we examined the effect of miR-30a/c-5p on the expression pattern of EMT markers. CP70 cells displayed a comparable level of E-cadherin and a higher level of vimentin relative to A2780 cells (Fig. 3A) as well as an increased capability of migration (Fig. 3B), indicating occurrence of partial EMT in CP70 cells. Overexpression of miR-30a/c-5p in CP70 cells decreased vimentin (Fig. 3C) and inhibited cell migration (Fig. 3D), while under-expression of miR-30a/c-5p in A2780 cells increased vimentin (Fig. 3E) and promoted cell migration (Fig. 3F). These data suggested that miR-30a/c-5p inhibited Snail to not only weaken cisplatin resistance but also reverse EMT.

Fig. 4. Methylation regulation mediated by DNMT1 is responsible for miR-30a/c-5p under-expression in CP70 cells. (A) qRT-PCR showed that miR-30a/c-5p increased more than 3 folds after 48 h treatment with 2μM 5-Aza-dC. (B) MSP assay showed that compared with A2780 cells, methylation levels in the promoter regions of miR-30a/c-5p precursor genes were significantly increased in CP70 cells. U= unmethylated. M= methylated. (C) Western blotting results indicated CP70 expressed higher levels of DNMT1 than did A2780 cells. (D) Western blotting results showed that DNMT1 protein level in CP70 cells was significantly reduced by siDNMT1 transfection. (E) qRT-PCR results showed the miR-30a/c-5p significantly increased in DNMT1-suppressed CP70 cells. (F) MSP assay showed that methylation of miR-30a/c-5p precursor genes significantly decreased in DNMT1-suppressed CP70 cells as opposed to negative control cells. U=unmethylated. M= methylated. (G) Western blotting results showed that DNMT1 was overexpressed when DNMT1 expression plasmid was transfected into A2780 cells. (H) qRT-PCR results showed that miR-30a/c-5p were reduced by more than 50% in DNMT1-overexpressed A2780 cells. (I) MSP results showed that compared with negative controls, methylation of miR-30a/c-5p precursor genes increased in DNMT1-overexpressed A2780 cells. U=unmethylated. M= methylated. The statistical differences were calculated using a two-tailed t-test or Chi-square test.

miR-30a/c-5p was downregulated by DNMT1-maintained methylation

Next, we investigated the mechanism responsible for miR-30a/c-5p reduction in cisplatin-resistant CP70 cells. Since potential methylation sites in the promoter region of miR-30a/c-5p precursor genes were found using online software (<http://www.urogenet.org/methprimer/>), we studied the contribution of methylation regulation to the under-expression of miR-30a/c-5p in CP70 cells. When CP70 cells were treated with the DNA methylation inhibitor 5-Aza-dC, both miR-30a-5p and miR-30c-5p were elevated by more than 3-fold (Fig. 4A). Methylation specific PCR (MSP) assay showed that methylation levels in the promoter regions of miR-30a/c-5p precursor genes were increased in CP70 cells (Fig. 4B). A much higher level of the maintenance methyltransferase DNMT1 was detected in CP70 cells than in A2780 cells (Fig. 4C).

The relationship of DNMT1 with miR-30a/c-5p in the occurrence of cisplatin resistance was therefore further studied. Knocking down of DNMT1 (Fig. 4D) increased mRNA level of miR-30a/c-5p (Fig. 4E) and decreased the methylation levels of promoter regions in the miR-30a/c-5p precursor gene (Fig. 4F). Ectopic expression of DNMT1 (Fig. 4G) repressed miR-30a/c-5p (Fig. 4H) and elevated the methylation levels in the promoters of miR-30a/c-5p precursor genes (Fig. 4I) in A2780 cells. These data demonstrated that DNMT1 downregulated miR-30a/c-5p via maintaining high levels of methylation in the promoter regions of miR-30a/c-5p precursor genes.

Fig. 5. DNMT1 promotes cisplatin resistance and EMT in ovarian cancer. (A) CCK8 results showed that DNMT1 inhibition increased cisplatin activity against CP70 cells. The IC₅₀ values for negative control cells, siDNMT1-a and siDNMT1-b-transfected CP70 cells were 268.5, 248.0 and 164.4 μM , respectively. (B) Flow cytometry analysis showed that DNMT1 inhibition promoted apoptosis in cisplatin-treated CP70 cells. (C) CCK8 results showed that DNMT1 overexpression increased resistance to cisplatin of A2780 cells. The IC₅₀ values for negative control cells and DNMT1-overexpressed A2780 cells were 48.03 and 73.25 μM , respectively. (D) Flow cytometry analysis showed that DNMT1 overexpression reduced apoptotic cell counts in cisplatin-treated A2780 cells. (E) Knocking down of DNMT1 in CP70 cells decreased vimentin expression. (F) Knocking down of DNMT1 weakened CP70 cell motility (original magnification $\times 400$). (G) Western blotting results showed that vimentin was upregulated in DNMT1-overexpressed A2780 cells. (H) In vitro migration assay showed that cell motility was enhanced in DNMT1-overexpressed A2780 cells (original magnification $\times 400$). The statistical differences were calculated using a two-tailed t-test.

DNMT1 contributed to cisplatin resistance and partial EMT

Since DNMT1 reversely regulated miR-30a/c-5p, the influence of DNMT1 on cisplatin resistance and EMT was then examined. Inhibition of DNMT1 sensitized CP70 cells to cisplatin as shown by decreased cell survival (Fig. 5A) and increased cell apoptosis (Fig. 5B) under cisplatin pressure. Overexpression of DNMT1 introduced resistance to cisplatin in A2780 cells as illustrated by improved cell survival (Fig. 5C) and reduced cell apoptosis (Fig. 5D). Additionally, knocking down of DNMT1 in CP70 cells depressed vimentin expression (Fig. 5E) and cell motility (Fig. 5F), while ectopic expression of DNMT1 in A2780 cells enhanced vimentin expression (Fig. 5G), and augmented cell migration (Fig. 5H). These data indicated that DNMT1 promoted cisplatin resistance and partial EMT in ovarian cancer cells.

miR-30a/c-5p directly inhibited DNMT1

We investigated the possible effect of miR-30a/c-5p on DNMT1 expression as well, and found loss of DNMT1 in miR-30a/c-overexpressed CP70 cells (Fig. 6A) and gain of DNMT1 in miR-30a/c-underexpressed A2780 cells (Fig. 6B). Although DNMT1 was not predicted as a miR-30s target by online miR target prediction tools, we manually located three potential

Fig. 6. DNMT1 is a direct target of miR-30a/c-5p. (A) Western blotting results showed that a decrease of DNMT1 in miR-30a/c-overexpressed CP70 cells. (B) Western blotting results showed that an increase of DNMT1 in miR-30a/c-downregulated A2780 cells. (C) Schematic representation of DNMT1 WT-3'UTR and MU-3'UTR sequences used for the construction of luciferase report vectors. (D) Luciferase reporter assay showed that miR-30a/c-5p significantly diminished luciferase activity of wild type DNMT1 3' UTR. The statistical differences were calculated using a two-tailed t-test. (E) Schematic representation of interactions among DNMT1, miR-30a/c-5p, and Snail in cisplatin resistance and EMT of ovarian cancer.

binding sites of miR-30a/c-5p in the 3'UTR of DNMT1 (Fig. 6C). Luciferase reporter assay demonstrated a direct suppression of miR-30a/c-5p on DNMT1 expression. Compared with the negative control, miR-30a/c-5p mimics significantly diminished the luciferase activity of wild-type DNMT1 3' UTR, whereas it had no effect on that of the mutated DNMT1 3' UTR (Fig. 6D).

Discussion

Cisplatin is a mainstay of treatment for many solid tumors including ovarian cancer [34]. However, its clinical efficiency is frequently influenced by the onset of chemoresistance of tumor cells to cisplatin [35]. Overcoming cisplatin resistance necessitates the elucidation of the molecular determinants refractory to cisplatin. Recently, miR-30s have been found involved in the regulation of cisplatin-sensitivity of some cancers, where individual members of the miR-30 family assist tumor cells in both cisplatin re-sensitization [36, 37] and cisplatin insensitivity [38, 39]. Indeed, the inconsistency on the role of miR-30a/c-5p in tumor cell sensitivity to other chemo-therapeutics also exists in various cancers. For example, miR-30a-5p was inversely correlated with gemcitabine [40] and paclitaxel [41] sensitivity, while high expression of miR-30a/b/c-5p was resistant to gemcitabine in CD133+ pancreatic cancer stem-like cells [42]. Here, the downregulation of miR-30a/c-5p was found in cisplatin-resistant ovarian cancer cells, and miR-30a/c-5p overexpression re-sensitized cells to cisplatin. Of note, upregulation of miR-30a-5p by transfection of its mimic not only increased itself by more than 1500-fold but also increased miR-30e-5p to 100-fold (data not

shown). Since miR-30a mimics predominantly enhanced miR-30e, and A2780 and CP70 cells expressed comparable level of miR-30e-5p, the effect of miR-30a-5p mimic on miR-30e-5p was considered to be insignificant in cisplatin resistance.

The regulatory mechanisms responsible for miR-30s downregulation in cisplatin-resistant cancers remain largely unresolved. Reported studies have revealed the involvement of miRs and their epigenetic regulation in the development of chemoresistance [43]. Methylation of DNA at cytosine residues is an epigenetic modification critical for the regulation of gene expression [44, 45]. Mammalian DNA methyltransferase DNMT1 functions as a maintenance methyltransferase that is overexpressed [46] and hyperactivated [47] in many cancers, resulting in hypermethylation of promoter CpG islands and gene silence. Since DNMT1 promotes cisplatin-resistance [48, 49], we examined the connection between miR-30a/c-5p and DNMT1 in the onset of cisplatin-resistance of ovarian cancer cells, and established a feedback loop between DNMT1 and miR-30a/c-5p as shown in Fig. 6E. Upregulated DNMT1 in cisplatin-refractory CP70 cells epigenetically attenuated miR-30a/c-5p via methylating CpG-rich regions at the promoter regions of miR-30a/c-5p precursor genes, which blocked the inhibitory effect of miR-30a/c-5p on DNMT1 expression. Although DNMT1 was previously found to induce miR-30b promoter methylation and downregulate miR-30b [50], DNMT1 was directly targeted by some miRs involved in ovarian cancer cisplatin resistance [51]. Our data revealed the existence of the feedback loop between miR-30a/c-5p and DNMT1 which was first related to the cisplatin-resistance of ovarian cancer cells. Of note, miR-30s can be repressed via various mechanisms, as illustrated by the findings that miR-30s downregulation could be induced by hypoxia in a hypoxia-inducible factors dependent manner [52], oncogenic signals such as epidermal growth factor and hepatocyte growth factor, or competitive endogenous RNA [53]. Our observation provided an additional regulatory pathway controlling miR-30s expression.

The mechanisms promoting cisplatin-sensitization of miR-30s include inhibition of EMT [36], autophagy [31], and other pro-chemoresistance molecules [54]. EMT is a dynamic process necessary for embryonic development and certain pathological events such as fibrosis and tumorigenesis [55]. Accumulating evidence indicates that EMT plays a pivotal role in metastasis and chemoresistance [56]. Snail is a well-known E-cadherin-transcriptional repressor and EMT inducer that is significantly upregulated during EMT process [57]. We found Snail was targeted by miR-30a/c-5p acting as antagonists of EMT in the ovarian cancer cells. However, the role of miR-30s in EMT is controversial in that miR-30a/c-5p can function either as antagonist [54, 58-61] or agonist [62] of EMT, suggesting that miR-30s might be involved in different pathways via targeting different genes.

A better understanding of the molecular mechanisms underlying cisplatin-sensitivity is necessary for developing successful strategies to treat ovarian cancer. Our cell-based observations implicated miR-30a/c-5p as part of the regulatory signaling events involved in cellular response of ovarian cancer cells to cisplatin, thus underscoring a possible alternative therapeutic strategy for the treatment of cisplatin-resistant ovarian cancer patients.

Conclusions

MiR-30a/c-5p are aberrantly methylated and thus silenced by overexpressed DNMT1, which relieves the inhibitory effect of miR-30a/c-5p on DNMT1 and Snail, leading to cisplatin resistance and partial EMT in ovarian cancer. Our study implicates miR-30a/c-5p and DNMT1 as promising therapeutic targets for the treatment of cisplatin-refractory ovarian cancer and control of metastasis.

Disclosure Statement

No conflict of interest.

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