Effects of Decitabine on Invasion and Exosomal Expression of miR-200c and miR-141 in Oxaliplatin-Resistant Colorectal Cancer Cells

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The effects of decitabine (DAC), a DNA methyltransferase (DNMT) inhibitor, on metastasis and exosomal expression of microRNAs were examined in SW620/OxR cells, a human colorectal cancer (CRC) cell line (SW620) with acquired resistance to oxaliplatin. This cell line shows an invasive phenotype by epithelial–mesenchymal transition. Two CRC cell lines, SW480, derived from primary CRC, and SW620, derived from lymph node metastasis, which were obtained from the same patient, as well as SW620/OxR, were also used in the present study. Cytarabine (Ara-C), a non-DNMT-inhibiting cytidine analog, was used as negative control of DAC. No significant difference was observed in the invasion abilities of SW480 cells treated with DAC or Ara-C. On the other hand, invasion ability was suppressed by treatment with DAC in SW620 and SW620/OxR cells. Up-regulated expression of E-cadherin, microRNA-200c (miR-200c), and miR-141 following DAC treatment indicated the acquisition of epithelial cell-like characteristics in SW620 and SW620/OxR cells. Exosomal expression levels of miR-200c and miR-141 were also up-regulated by DAC treatment in SW620 and SW620/OxR but not in SW480 cells. This increase in exosomal miRNA expression negatively correlated with invasion ability. These results suggest that DNA demethylation treatment caused acquisition of epithelial cell-like characteristics in SW620 and SW620/OxR cells. Furthermore, the observed increased exosomal expression of miR-200c and miR-141 may be an indicator or biomarker candidate for mesenchymal–epithelial transition of CRC cells.

Key words colorectal cancer (CRC); invasion; decitabine; exosome; microRNA; oxaliplatin resistance

Some epithelial-like cancer cells among colorectal cancer (CRC) cells can acquire mesenchymal cell-like characteristics including enhanced migration and invasion activity. This epithelial–mesenchymal transition (EMT) causes the down-regulation of cadherin1 (CDH1), which encodes E-cadherin, by inducing the overexpression of transcription factors such as Zinc finger E-box-binding homeobox 1 (ZEB1), ZEB2, and Snail.1,2) The down-regulated expression of E-cadherin is characterized by the loss of cell–cell adhesion and acquisition of a migratory phenotype. In contrast, EMT causes the up-regulated expression level of vimentin, which is a mesenchymal cell marker. We previously demonstrated that EMT was caused by the acquisition of resistance to oxaliplatin (l-OHP) by the human CRC cell line SW620.3) Yang et al. also attributed EMT to changes in the cellular localization of EMT-related molecules in two CRC cell lines, KM12L4 and HT29, with acquired resistance to l-OHP.4) DNA methylation leads to the inappropriate expression or silencing of genes and is catalyzed by DNA methyltransferase (DNMT). DNA hypermethylation plays an important role in cancer metastasis by silencing the expression of several metastasis-related genes such as CDH1.5–7) Recent studies have shown that decitabine (DAC), which is a cytidine analog that inhibits DNMT1, affects cancer metastasis.7–10) The effects of DAC on migration and invasion has been suggested to differ among cancer cell types. Shin et al. reported that DAC inhibited migration and invasion by increasing the number of tight junctions in gastric carcinoma cells.10) In contrast, Chik et al. reported that DAC induced an increase in the invasive activity of non-invasive breast cancer cells.9) However, it currently remains unclear whether DAC affects the metastatic activity of l-OHP-resistant CRC cells.

MicroRNAs (miRNAs) are small non-coding RNAs that suppress target gene expression at the post-transcriptional level by binding to the 3′-untranslated region of target mRNA. Members of the miR-200 family (miR-200a, miR-200b, miR-141, miR-200c, and miR-429) are known to be powerful markers for epithelial cells and regulators of EMT in CRC cells, as well as ovarian cancer cells, lung cancer cells, and breast cancer cells.11–13) miR-200 family members have been divided into two clusters: miR-200a/200b/429 and miR-200c/141, which are located in human chromosomes 1 and 12, respectively. The expression levels of the miR-200 family were previously shown to be down-regulated by EMT through the hypermethylation of miR-200a/200b/429 promoters and miR-200c/141 promoters.14,15)

Most extracellular miRNAs in serum, plasma, saliva, urine, and other body fluids are embedded in membrane-bound vesicles such as exosomes. Exosomes are small membrane vesicles that are approximately 100 nm in diameter and can embed various proteins, mRNAs, and miRNAs, depending on the cell type. A previous study reported that the level of ΔNp73 mRNA in exosomes from the plasma of patients with CRC was higher than that of healthy controls.16) Changes in the exosomal expression of miRNAs, such as miR-21 and let-7a, have been suggested to mirror pathological changes in CRC patients and be biomarkers for a non-invasive diagnosis of the disease.17) Furthermore, increases in the expression levels of miR-200 family members have been reported in highly invasive cancer cells and their exosomes.18) Although exosomal proteins, such as human leucocyte antigen-1, were previously found to be increased by DAC,19) changes in the exosomal ex-
pression levels of miRNAs from CRC cells by DAC have not yet been reported.

The metastatic activity of SW480 cells, derived from primary CRC, is known to be lower than that of SW620 cells, derived from lymph node metastasis in the same patient. Two CRC cell lines have been used as model cell lines in order to investigate the metastasis of CRC. We herein examined the effects of DAC on the migration and invasion activity and expression levels of EMT-related molecules in 3 human CRC cell lines, SW480, SW620, and L-OHP-resistant SW620 (SW620/OxR). We also investigated whether the exosomal expression levels of the miR-200 family were related to invasion activity in these 3 CRC cell lines.

MATERIALS AND METHODS

Materials Two human CRC cell lines, SW620 and SW480, were purchased from the American Tissue Culture Collection (Manassas, VA, U.S.A.) and DS Pharmabimedical (Osaka, Japan), respectively. SW620/OxR was prepared as previously reported. The IC_{50} values of L-OHP after a 72-h treatment were 47±7 µM, 15±2 µM, and L-OHP-resistant SW620 (SW620/OxR). We also investigated whether the exosomal expression levels of the miR-200 family were related to invasion activity in these 3 CRC cell lines.

Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) for miRNAs Total RNA was extracted from cultured cells using the RNAeasy Mini kit (QIAGEN, Valencia, CA, U.S.A.). Total RNA was reverse-transcribed into cDNA using a ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). Real-time PCR was performed on a MiniOpticon System (BioRad, Tokyo, Japan) using SYBR Green. The PCR conditions used were as follows: Initial denaturation for one cycle of 1 min at 95°C followed by 40 cycles of 10 s at 95°C (denaturation), 10 s at 55°C (annealing), and 20 s at 72°C (extension). After these cycles, a melting curve analysis was performed to confirm the single product. The expression level of each mRNA was normalized by that of ribosomal protein 27 (RP27) as a housekeeping gene. The relative expression level of the target gene was expressed as 2^{-\Delta\Delta Ct}. The sequences of the primers used in this study are listed in Supplementary Table S1.

Migration and Invasion Assays Transwell cell migration and invasion assays were performed using 8.0-µm Transwell inserts, which were uncoated and coated with Matrigel (Corning, New York, NY, U.S.A.), respectively. Cells were suspended in 200 µL of standard growth medium at a density of 2.0×10^5 cells/well and plated in the upper chamber of Transwell inserts. The lower chamber of the wells contained 800 µL of standard growth medium. After 24 h, the medium in the upper Transwell chamber was replaced with serum-free medium, and the medium in the lower chamber of wells was replaced with medium containing 20% FBS. After being incubated for 72 h at 37°C, the membranes were fixed and stained by Diff-Quik reagent (Sysmex, Kobe, Japan) and the cells in each insert were counted at ×200 magnification. Five fields per well were counted manually and averaged.

Western Blot Analysis Proteins were isolated using the M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, Waltham, MA, U.S.A.). Samples were separated by electrophoresis using a 4–12% NuPAGE polyacrylamide gel electrophoresis Bis-Tris gel (Invitrogen) with 4-morpholinepropanesulfonic acid buffer (Invitrogen) and transferred to a polyvinylidene difluoride (PVDF) membrane using iBlot (Invitrogen). The PVDF membrane was blocked with Blocking One (Nacalai Tesque, Kyoto, Japan) at room temperature for 0.5 h and incubated with a primary antibody for 1 h at room temperature or overnight at 4°C. The membrane was washed with Tris-buffered saline–0.1% Tween 20 and incubated with a secondary antibody for 1 h at room temperature. Proteins were visualized using Chemi-Lumi One Super (Nacalai Tesque).

Methylation-Sensitive High Resolution Melting (MS-HRM) Analysis DNA was extracted from cultured cells using DNeasy Blood&Tissue Kit (QIAGEN) and was modified by treatment with sodium sulfite using Epitect Bisulfite Kit.
Exosomal miRNA Isolation from Cell-Cultured Media
Exosome samples were prepared using a stepwise centrifugation method.17) Briefly, culture medium, in which cells were incubated without serum for 48 h at 37°C, was centrifuged at 500 × g for 5 min to remove cell debris, and then centrifuged at 16500 × g for 20 min. The cleared supernatant was passed through a 0.20 µm filter and then ultracentrifuged at 120000 × g for 70 min at 4°C. Exosomal RNA was extracted from the pellet after ultracentrifugation using the Total Exosome RNA & Protein Isolation Kit (Invitrogen).

Quantitative RT-PCR for Exosomal miRNAs
Reverse transcription was performed using the miScript Reverse Transcription Kit (QIAGEN). cDNA was synthesized from the maximum volume of exosomal RNA. Real-time PCR was performed with the miScript SYBR Green Kit (QIAGEN). The expression level of each miRNA was normalized to that of miR-451a.17) The sequences of the primers used in this study are listed in Supplementary Table S2.

Statistical Analysis
All data are expressed as the mean±standard error of the mean (S.E.M.). A one-way ANOVA followed by the Student–Newman–Keuls test was used to compare more than two groups. Correlations were determined using Pearson’s correlation test.

RESULTS

Expression of the DNA Genome-Wide Demethylation Marker by the DAC Treatment
Melanoma-associated antigen I (MAGEA1) mRNA expression has been correlated with genome-wide demethylation in many cancer cell lines.26,27) MAGEA1 mRNA expression was not detected in the control or Ara-C-treated cells from the three cell lines studied (Fig. 1). On the other hand, MAGEA1 mRNA expression was detected in DAC-treated cells from the three cell lines (Fig. 1). This result suggested that MAGEA1 DNA was demethylated by the DAC treatment in SW480 cells, SW620 cells, and SW620/OxR cells.

Effects of DAC on Epithelial Cell-Like Characteristics
In SW480 cells, no significant differences were observed in the protein expression levels of E-cadherin or vimentin between Ara-C-treated cells and DAC-treated cells (Fig. 2A). The protein expression level of E-cadherin was up-regulated by the DAC treatment in SW620 cells and SW620/OxR cells (Fig. 2A). Since high intracellular levels of miR-200c and miR-141 indicate epithelial cell-like characteristics,11) we examined the intracellular expression levels of miR-200c and miR-141. The DAC treatment up-regulated the intracellular expression of miR-200c and miR-141 in SW620 cells and SW620/OxR cells, but not in SW480 cells (Fig. 2B). These results suggested that DAC treatment led to the acquisition of epithelial cell-like characteristics in SW620 cells and SW620/OxR cells. Furthermore, the intracellular expression levels of miR-200c and miR-141 negatively correlated with invasion ability (Supplementary Fig. S2).

Effects of DAC on CDH1 DNA Methylation
In three cell lines, the percentages of CDH1 DNA methylation were about 30%, and no significant differences were observed in the CDH1 DNA methylation among control cells, Ara-C-treated cells and DAC-treated cells (Fig. 3B). These results suggested that up-regulation of E-cadherin protein expression level was not caused by DNA demethylation of CDH1 in SW620 cells and SW620/OxR cells.

Exosomal Expression Levels of miR-200c and miR-141

![Fig. 1. Effects of the DAC Treatment on the mRNA Expression Level of MAGEA1 in CRC Cells](image-url)

Samples of total RNA from cultured cells after a 72-h treatment with 2.0 µM DAC or 0.3 µM Ara-C were prepared as described in Materials and Methods. MAGEA1 mRNA expression levels were detected by a real-time PCR analysis and normalized by RPL27 mRNA expression levels. Each column represents the mean±S.E.M. of three independent experiments. ND: Not detected.
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**Fig. 2. Effects of the DAC Treatment on EMT-Related Molecules in CRC Cells**

(A) The protein expression levels of E-cadherin and vimentin were detected by Western blotting. β-Actin was used as a loading control. (B) The intracellular expression levels of miR-200c and miR-141 were detected by real-time RT-PCR and were normalized by U6 snRNA expression levels. Samples of proteins from cultured cells after the 72-h treatment with 2.0 µM DAC or 0.3 µM Ara-C were prepared as described in Materials and Methods. Each column represents the mean ± S.E.M. of three independent experiments (Student–Newman–Keuls test, *p < 0.05, **p < 0.01).

**Table 1. Effect of DAC Treatment on Migration Ability of CRC Cells**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Migration cells/High-power field (×200)</th>
<th>Control</th>
<th>Ara-C</th>
<th>DAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW480</td>
<td>8.8 ± 0.9</td>
<td>7.4 ± 0.5</td>
<td>5.5 ± 0.8*</td>
<td></td>
</tr>
<tr>
<td>SW620</td>
<td>17.5 ± 1.9</td>
<td>15.7 ± 1.8</td>
<td>12.1 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>SW620/OxR</td>
<td>20.8 ± 1.2</td>
<td>20.2 ± 0.7</td>
<td>16.1 ± 0.7*†</td>
<td></td>
</tr>
</tbody>
</table>

Cells were treated as described in Materials and Methods, and migration assay was performed. Each value represents the mean ± S.E.M. of three independent experiments (Student–Newman–Keuls test, *p < 0.05 significantly different from control cells, †p < 0.05 significantly different from Ara-C treatment cells).

**Table 2. Effect of DAC Treatment on Invasion Ability of CRC Cells**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Invasion cells/High-power field (×200)</th>
<th>Control</th>
<th>Ara-C</th>
<th>DAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW480</td>
<td>4.7 ± 0.4</td>
<td>2.4 ± 0.2**</td>
<td>1.9 ± 0.3**</td>
<td></td>
</tr>
<tr>
<td>SW620</td>
<td>36.1 ± 2.9</td>
<td>30.6 ± 1.7</td>
<td>20.9 ± 0.6**†</td>
<td></td>
</tr>
<tr>
<td>SW620/OxR</td>
<td>65.5 ± 3.3</td>
<td>45.9 ± 2.5**</td>
<td>31.4 ± 2.4**††</td>
<td></td>
</tr>
</tbody>
</table>

Cells were treated as described in Materials and Methods, and invasion assay was performed. Each value represents the mean ± S.E.M. of three independent experiments (Student–Newman–Keuls test, **p < 0.01 significantly different from control cells, †p < 0.05, ††p < 0.01 significantly different from Ara-C treatment cells).

Fig. 2. Effects of the DAC Treatment on EMT-Related Molecules in CRC Cells

(A) The protein expression levels of E-cadherin and vimentin were detected by Western blotting. β-Actin was used as a loading control. (B) The intracellular expression levels of miR-200c and miR-141 were detected by real-time RT-PCR and were normalized by U6 snRNA expression levels. Samples of proteins from cultured cells after the 72-h treatment with 2.0 µM DAC or 0.3 µM Ara-C were prepared as described in Materials and Methods. Each column represents the mean ± S.E.M. of three independent experiments (Student–Newman–Keuls test, *p < 0.05, **p < 0.01).
No significant differences were observed in the exosomal expression levels of miR-200c and miR-141 between SW480 cells treated with DAC and control SW480 cells or SW480 cells treated with Ara-C (Fig. 4A). On the other hand, the exosomal expression levels of miR-200c and miR-141 in SW620 cells and SW620/OxR cells were increased by the DAC treatment (Fig. 4A). Furthermore, these exosomal expression levels of miR-200c and miR-141 negatively correlated with invasion ability (Fig. 4B). The intracellular expression levels of miR-200c positively correlated with the exosomal expression levels of miR-200c \((r=0.957, p<0.001)\), as well as those of miR-141 \((r=0.854, p=0.003)\).

**DISCUSSION**

We herein demonstrated that the DAC, a DNA methylation inhibitor, suppressed cell invasion activities through the acquisition of epithelial cell-like characteristics in not only SW620 cells, but also SW620/OxR cells (Fig. 2, Tables 1, 2). Furthermore, the exosomal expression levels of miR-200c and miR-141 were increased by the DAC treatment in SW620 cells and SW620/OxR cells (Fig. 4A). These expression levels negatively correlated with invasion ability (Fig. 4B). To the best of our knowledge, this is the first study to show that DNA demethylation by DAC suppressed the invasion ability of 1-OHP-resistant CRC cells and up-regulated the exosomal expression levels of miR-200c and miR-141.

DAC did not affect E-cadherin, miR-200c, and miR-141 expression levels in SW480 cells, while DAC treatment inhibited cell migration and invasion activities of SW480 cells (Fig. 2, Tables 1, 2). Since SW480 cells display more epithelial cell-like characteristics than SW620 cells and SW620/OxR cells,\(^4,21\) SW480 cells did not acquire additional epithelial cell-like characteristics after DAC treatment. In addition, no significant differences were observed in the cell migration or invasion ability of DAC-treated SW480 cells and Ara-C-treated SW480 cells. These results suggested that DNA demethylation caused by DAC treatment was not associated with cell migration or invasion ability in SW480 cells.

Previous studies reported that some miRNAs were selectively secreted into exosomes.\(^{18,28,29}\) However, the intracellular expression levels of miR-200c and miR-141 positively correlated with their exosomal expression levels in this study, suggesting that miR-200c and miR-141 were not selectively incorporated into exosomes.

Ara-C, used as the negative control of DAC, caused up-regulation of E-cadherin and vimentin protein expression levels in SW620/OxR cells (Fig. 2A). Although Ara-C treatment slightly affected these proteins expression levels, the effect of Ara-C treatment on E-cadherin protein expression level was lower than that of DAC in this study. This result suggested that DNA demethylation caused up-regulation of E-cadherin protein expression level in SW620/OxR cells.

We did not examine the expression of miR-200a, miR-200b,
or miR-429, which are miR-200 family members, in the present study. Hur et al. previously reported that these miRNAs were not considered to play an important role in metastasis caused by DNA methylation in vivo. We previously showed that the expression levels of miR-200c and miR-141 were markedly lower in SW620/OxR cells than in SW620 cells, and no significant differences were observed in the expression levels of miR-200a, miR-200b, and miR-429 between SW620/OxR cells and SW620 cells. Therefore, miR-200a, miR-200b, and miR-429 may not play a significant role in EMT caused by the acquisition of L-OHP resistance.

In this study, the CDH1 DNA methylation was not regulated by DAC treatments in SW480 cells, SW620 cells as well as SW620/OxR cells (Fig. 3B). The reason could be low methylation of CDH1 DNA in each control cell. In addition, these results suggested that the acquisition of epithelial cell-like characteristics up-regulated the protein expression level of E-cadherin in SW620 cells and SW620/OxR cells. Therefore, miR-200a, miR-200b, and miR-429 may not play a significant role in EMT caused by the acquisition of L-OHP resistance.

Fig. 4. Effects of the DAC Treatment on Exosomal Expression Levels of miR-200c and miR-141 in CRC Cells

Samples of exosomes from cultured cells after the 72-h treatment with 2.0 μM DAC or 0.3 μM Ara-C were prepared as described in Materials and Methods, and exosomal RNA was extracted. (A) The exosomal expression levels of miR-200c and miR-141 were detected by real-time RT-PCR and normalized by miR-451a expression levels. Each column represents the mean±S.E.M. of three independent experiments (Student–Newman–Keuls test, *p<0.05, **p<0.01). (B) Logarithmic correlations were performed using the data shown in (A) and Table 2. Each point represents the mean±S.E.M. of three independent experiments. The expression level of miRNA was transformed into log10 before performing Pearson’s correlation test. r: Pearson’s correlation coefficients.

or miR-429, which are miR-200 family members, in the present study. Hur et al. previously reported that these miRNAs were not considered to play an important role in metastasis caused by DNA methylation in vivo. We previously showed that the expression levels of miR-200c and miR-141 were markedly lower in SW620/OxR cells than in SW620 cells, and no significant differences were observed in the expression levels of miR-200a, miR-200b, and miR-429 between SW620/OxR cells and SW620 cells. Therefore, miR-200a, miR-200b, and miR-429 may not play a significant role in EMT caused by the acquisition of L-OHP resistance.

In this study, the CDH1 DNA methylation was not regulated by DAC treatments in SW480 cells, SW620 cells as well as SW620/OxR cells (Fig. 3B). The reason could be low methylation of CDH1 DNA in each control cell. In addition, these results suggested that the acquisition of epithelial cell-like characteristics up-regulated the protein expression level of E-cadherin in SW620 cells and SW620/OxR cells. We did not determine the miR-200c/141 promoters DNA methylation of three cell lines in the present study, although DNA methylation of miR-200c/141 promoters may be demethylated by DAC treatment. SW620 cells and SW620/OxR cells acquired epithelial cell-like characteristics by DAC treatment. SW620 cells and SW620/OxR cells acquired epithelial cell-like characteristics by DAC treatment. In addition, previous studies reported DNA methylation of miR-200c/141 promoters was regulated by acquisition of epithelial cell-like characteristics. Thus, DNA of miR-200c/141 promoters was directly demethylated by DAC treatment and/or was indirectly demethylated through acquired epithelial cell-like characteristics. Therefore, DNA methylation of miR-200c/141 promoters makes no difference in the conclusion of this study that the increased exosomal expression of miR-200c and miR-141 may be an indicator or biomarker candidate for the mesenchymal-epithelial transition (MET) of CRC cells.

FLIS et al. reported that a combination of DNMT inhibitors including DAC with L-OHP had synergistic effects on
co-treatment with DAC in 4 CRC cell lines (SW480, HT29, SW48, and HCT116). We intend to investigate the effects of DAC on the cytotoxicity of 1-OHP in SW620/OxR cells in future studies.

The results presented here suggest that the increased exosomal expression of miR-200c and miR-141 may be an indicator or biomarker candidate for the MET of CRC cells.

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

REFERENCES