

**Regular Article***Highlighted Paper selected by Editor-in-Chief***Induction of Epithelial-Mesenchymal Transition and Down-Regulation of miR-200c and miR-141 in Oxaliplatin-Resistant Colorectal Cancer Cells**

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**Epithelial-mesenchymal transition (EMT) and changes in the expression of the microRNA-200 (miR-200) family were examined in the human colorectal cancer (CRC) cell line SW620 with acquired oxaliplatin (L-OHP) resistance. Two CRC cell lines, SW480, derived from primary CRC, and SW620, derived from lymph node metastasis, which were obtained from the same patient, were used in the present study. L-OHP-resistant SW620 cells were obtained by exposure to L-OHP for 155 d. The concentration of L-OHP was increased to 80  $\mu$ M in a stepwise manner. The IC<sub>50</sub> value of L-OHP was increased 16-fold in L-OHP-resistant SW620 cells, which also displayed mesenchymal cell-like characteristics, such as the down-regulation of E-cadherin and up-regulation of vimentin. However, L-OHP-resistant SW480 cells were not obtained when the concentration of L-OHP was increased in a similar stepwise manner. The expression levels of members of the miR-200 family (miR-200a, miR-200b, miR-429, miR-200c, and miR-141) were significantly higher in SW480 cells than in SW620 cells. The expression levels of miR-200c and miR-141 were significantly lower in L-OHP-resistant SW620 cells than in control SW620 cells. L-OHP-resistant SW620 cells did not exhibit cross-resistance to other anti-cancer drugs used to treat CRC, such as 5-fluorouracil, irinotecan, and the active metabolite of irinotecan (SN-38). These results suggest that the down-regulated expression of miR-200c and miR-141 plays a role in selective resistance to L-OHP and EMT in CRC cells during repeated treatments with L-OHP.**

**Key words** colorectal cancer; drug resistance; oxaliplatin; epithelial-mesenchymal transition; microRNA

Oxaliplatin (L-OHP) is an important platinum-based drug that is used to treat colorectal cancer (CRC). FOLFOX, which is a combined therapy with L-OHP, 5-fluorouracil (5-FU), and leucovorin, is a first-line standard chemotherapy for patients with advanced CRC. However, long-term treatments with L-OHP have been shown to lead to the acquisition of resistance to L-OHP in CRC cells.<sup>1,2</sup> Resistance to L-OHP is associated with regulators of the nuclear factor- $\kappa$ B pathway, apoptotic effectors such as caspase 3 and Bax, and DNA repair by excision repair cross complementing group 1 (ERCC1).<sup>3–5</sup> Samimi *et al.* previously reported that L-OHP resistance was caused by overexpression of the copper efflux transporter ATP7A in ovarian cancer cells.<sup>6</sup> Furthermore, using copper influx copper transporter 1 (CTR1) (–)/(–) cells, Larson *et al.* demonstrated that the underexpression of CTR1 was important for resistance to platinum-based drugs.<sup>7</sup>

Epithelial-mesenchymal transition (EMT), through which epithelial cells acquire mesenchymal cell-like characteristics, is important for the migration and invasion processes of cancer cells. EMT is caused by the overexpression of transcription factors including Zinc finger E-box-binding homeobox 1 (ZEB1), ZEB2, and Snail. The expression of E-cadherin was previously shown to be repressed when ZEB1, ZEB2, or Snail bound to the E-box elements of *CDH1*, which encodes E-cadherin.<sup>8,9</sup> The down-regulated expression of E-cadherin is characterized by the loss of cell–cell adhesion and acquisition of a migratory phenotype. Mashita *et al.* reported that the expression levels of *standard CD44 isoform (CD44s) mRNA/variant 9 isoform (CD44v9) mRNA (CD44s/CD44v9) ratio* were higher in the cells of mesenchymal phenotype than

those of epithelial phenotype.<sup>10</sup> CD44 knockdown resulted in significantly reduced migration and invasion of cells.<sup>10</sup> Furthermore, reactive oxygen species (ROS) is associated with the cause of EMT and is regulated by cystine/glutamate transporter (xCT), the cell surface transporter of which might be regulated by CD44 expression.<sup>11</sup> The reversal of EMT *via* the silencing of ZEB1 has been shown to induce an increase not only in the expression of E-cadherin, but also in sensitivity to DNA-damaging drugs.<sup>12</sup> The transcription factors involved in EMT have been reported to lead to multidrug resistance due to the up-regulation of ATP-binding cassette (ABC) transporters, which have an E-box element in their promoters.<sup>13</sup> In addition, the CRC cell lines, KM12L4 and HT29, which acquired L-OHP resistance following long-term treatments with L-OHP, switched from a proliferative phenotype to an invasive phenotype by EMT due to a change in the cellular localization of EMT-related molecules, such as E-cadherin, vimentin, and Snail.<sup>1</sup>

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.<sup>14</sup> Several *in vitro* studies showed that L-OHP resistance is linked to the expression of miRNAs, such as miR-27b, miR-143, miR-181b, miR-203, and miR-1915.<sup>15–18</sup> Furthermore, the responses of patients with CRC to L-OHP-based chemotherapy have been associated with the up-regulated expression of miR-148a, miR-181b, and miR-625-3p.<sup>17,19</sup> However, these miRNAs may not be directly linked to EMT caused by the acquisition of L-OHP resistance in CRC cells because they have no binding sites to the 3'-untranslated

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region of ZEB1. In contrast, members of the miR-200 family (miR-200a, miR-200b, miR-429, miR-200c, and miR-141) and miR-205 play important roles in EMT as regulatory elements, which directly target and inhibit the expression of ZEB1 and ZEB2 in CRC cells as well as ovarian, lung, breast, and kidney cancer cells.<sup>20–24</sup> The role of the miR-200 family and miR-205 in drug resistance has already been reported in various types of cancer cells.<sup>25,26</sup> For example, the down-regulation of miR-200c has been associated with the expression of drug resistance proteins, such as P-glycoprotein (multidrug resistance protein (MDR)1), in human breast cancer cells.<sup>25</sup> A previous study reported that expression of the E-cadherin protein as well as that of miR-200c and miR-205 was lower in docetaxel-resistant prostate cancer cells than in parental prostate cancer cells.<sup>26</sup> However, the roles of the miR-200 family and miR-205 in CRC cells have not yet been examined in terms of EMT after long-term treatments with L-OHP.

Since the potential of SW620 cells, derived from lymph node metastasis, to metastasize is known to be higher than that of SW480 cells, derived from primary CRC in the same patient, these two cell lines were used as model cell lines in a metastasis study of CRC and were caused EMT by transforming growth factor- $\beta$  (TGF- $\beta$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ).<sup>27–30</sup> We herein examined changes in the migration activities and expression levels of EMT-related mRNAs and their proteins as well as the expression levels of the miR-200 family in the human CRC cell line SW620 with acquired L-OHP resistance.

## MATERIALS AND METHODS

**Materials** The human CRC cell lines SW620 and SW480 were purchased from the American Tissue Culture Collection (ATCC) (Manassas, VA, U.S.A.) and DS Pharmabiomedical (Osaka, Japan), respectively. L-OHP was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Leibovitz 15 (L-15) medium was purchased from Life Technologies Corp. (Carlsbad, CA, U.S.A.). Fetal bovine serum (FBS), irinotecan (CPT-11) hydrochloride, and 7-ethyl-10-hydroxycamptothecin (SN-38) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Cisplatin was purchased from Tokyo Chemical Industry Co. (Tokyo Japan). RNeasy Mini kits were purchased from QIAGEN (Valencia, CA, U.S.A.). ReverTra Ace qPCR RT Master Mix with gDNA Remover was purchased from TOYOBO (Osaka, Japan). M-PER Mammalian Protein Extraction Reagent was purchased from Thermo Scientific (Waltham, MA, U.S.A.). Four to twelve percent NuPAGE Bis-Tris gel, 4-morpholinepropanesulfonic acid (MOPS) buffer, and iBlot were purchased from Invitrogen (Carlsbad, CA, U.S.A.). Monoclonal mouse anti- $\beta$ -actin, goat anti-mouse immunoglobulin G-horseradish peroxidase (IgG-HRP), and goat anti-rabbit IgG-HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Monoclonal rabbit anti-E-cadherin, monoclonal rabbit anti-vimentin, and monoclonal rabbit anti-ZEB1 were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). Blocking One, Chemo-Lumi One Super, Sepasol-RNA I Super G, and 5-FU were purchased from Nacalai Tesque (Kyoto, Japan). Mir-X miRNA First Strand Synthesis Kit and DNase I were purchased from TaKaRa Bio Inc. (Otsu, Japan).

### Cell Culture and Establishment of L-OHP-Resistant

**CRC Cell Lines** SW620 cells or SW480 cells were grown in L-15 medium plus 10% FBS, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin at 37°C in 100% air. SW620 cells or SW480 cells were exposed to L-OHP in L-15 medium plus 10% FBS, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin. SW620 cells or SW480 cells were initially seeded at a density of  $1.0 \times 10^3$  cells/60-mm dish and cultured with 5  $\mu$ M L-OHP for 2 weeks. After 2 weeks,  $4.0 \times 10^5$  cells were then subcultured on a 60-mm dish.<sup>31</sup> The surviving cells were subcultured at a density of  $4.0 \times 10^5$  cells/60-mm dish or  $1.2 \times 10^6$  cells/100-mm dish with L-OHP. The concentration of L-OHP to which SW620 cells were exposed was increased in a stepwise manner to 5  $\mu$ M (for 38 d), 20  $\mu$ M (for 36 d), 40  $\mu$ M (for 42 d), and finally 80  $\mu$ M (for 39 d).<sup>1</sup> The concentration of L-OHP to which SW480 cells were exposed was increased in a stepwise manner to 5  $\mu$ M (for 41 d), 10  $\mu$ M (for 40 d), 20  $\mu$ M (for 36 d), and finally 40  $\mu$ M (for 39 d).<sup>1</sup> We defined the acquisition of L-OHP resistance as a more than 10-fold increase in the IC<sub>50</sub> value of L-OHP.

**Cytotoxicity Assay** The cytotoxicity of anti-cancer drugs in cultured cells was evaluated using the WST-8 assay. Cells ( $3.0 \times 10^3$  cells/well) were seeded on a 96-well plate in 100  $\mu$ L culture medium. Twenty-four hours later, anti-cancer drugs diluted with culture medium were added to each well. The WST-8 colorimetric assay was performed following a 72-h incubation at 37°C. The culture medium in each well was replaced with 100  $\mu$ L culture medium without anti-cancer drugs and 10  $\mu$ L WST-8 reagent solution was added to each well. Absorbance was determined 1 to 4 h later at 450 nm with a reference wavelength of 655 nm using a Model 550 Microplate Reader (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The IC<sub>50</sub> values of anti-cancer drugs in cells were calculated according to the sigmoid inhibitory effect model;  $E = E_{\max} \times C^\gamma / (C^\gamma + IC_{50}^\gamma)$ , by means of a nonlinear least-squares fitting method (Solver, Microsoft Excel 2010).<sup>32</sup>  $E$  and  $E_{\max}$  represented the surviving fraction (% of control) and its maximum, respectively.  $C$  and  $\gamma$  represented the drug concentration in the medium and sigmoid factor, respectively.

**Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) for mRNAs** Total RNA was extracted from cultured cells using RNeasy Mini kits. Total RNA was reverse-transcribed into cDNA using a ReverTra Ace qPCR RT Master Mix with gDNA Remover. Real-time PCR was performed on a MiniOpticon System (BioRad, Tokyo, Japan) using SYBR Green. The PCR conditions 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, the melting curve was examined to confirm a single product. The expression level of each mRNA was normalized to that of ribosomal protein 27 (RPL27) as a housekeeping gene.<sup>33</sup> The relative expression level of the target gene was expressed as  $2^{-\Delta\Delta Ct}$ . The sequences of the primers for mRNA expression used in this study are listed in supplemental Table 1.

**Proliferation Assay** Cells ( $5.0 \times 10^3$  cells/well) were seeded on a 96-well plate in 100  $\mu$ L culture medium and incubated for 24, 48, or 72 h. At the end of the incubation, the culture medium in each well was replaced with 100  $\mu$ L culture medium and 10  $\mu$ L WST-8 reagent solution was added to each well. Absorbance was determined 1 h later at 450 nm with a reference wavelength of 655 nm using a Model 550 Microplate

Reader.

**Migration and Invasion Assay** Transwell cell migration and invasion assays were done using 8.0- $\mu$ m transwell inserts, which were uncoated and coated with matrigel, respectively. Cells were suspended in 200  $\mu$ L of standard growth medium at density of  $2.0 \times 10^5$  cells/well and plated in upper chamber of transwell inserts. The lower chamber of wells contained 800  $\mu$ L of standard growth medium. After 24 h, the medium in 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.<sup>34</sup> After incubation for 72 h at 37°C, the membranes were fixed and stained by Diff Quik reagent and the cells on each insert were counted at 200 $\times$ magnification. Five fields per well were counted manually and averaged.

**Western Blot Analysis** Protein was isolated using M-PER Mammalian Protein Extraction Reagent. Samples were separated by electrophoresis using a 4–12% NuPAGE Bis-Tris gel with MOPS buffer and transferred to a polyvinylidene difluoride (PVDF) membrane using iBlot. The PVDF membrane was blocked with Blocking One 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.

**Quantitative RT-PCR for miRNAs** Total RNA containing RNA molecules smaller than 200 nucleotides was extracted from cultured cells using Sepasol-RNA I Super G. Total RNA was first digested with DNase I. To amplify miRNA, DNase I-digested total RNA was reverse-transcribed using Mir-X miRNA First Strand Synthesis Kit. The expression level of each miRNA was normalized to that of U6 small nuclear RNA (snRNA). The sequences of the primers for miRNA expression used in this study are listed in supplemental Table 2. The primer in the Mir-X miRNA First Strand Synthesis Kit was used for U6 snRNA expression.

**Statistical Analysis** All data are expressed as the mean $\pm$ S.E.M., and differences between two groups were evaluated by the unpaired Student's *t*-test.

## RESULTS

**SW620 Cells with Acquired L-OHP Resistance** SW620 cells or SW480 cells were treated with L-OHP for more than 4 months. The sensitivity of SW620 cells or SW480 cells to L-OHP during L-OHP treatment was measured using the WST-8 assay (Table 1, supplemental Fig. 1, and supplemental Table 3). As shown in Table 1, SW620 cells treated with

L-OHP for 155 d became resistant to L-OHP and the IC<sub>50</sub> value of L-OHP was approximately 16-fold higher than that of control SW620 cells. No significant differences were observed in  $\gamma$  values (data not shown). The IC<sub>50</sub> value of L-OHP in SW480 cells was not markedly increased by the long-term treatment with L-OHP for more than 4 months (supplemental Table 3). L-OHP-resistant SW620 cells exposed to L-OHP for more than 4 months were used in subsequent experiments. The mRNA expression levels of *CTRI*, *CTR2*, and *ATP7A* were lower in L-OHP-resistant SW620 cells than in control SW620 cells (supplemental Fig. 2). No significant differences were observed in sensitivity to cisplatin between L-OHP-resistant SW620 cells (IC<sub>50</sub> value:  $32.3 \pm 2.2 \mu$ M) and control SW620 cells (IC<sub>50</sub> value:  $30.6 \pm 3.5 \mu$ M). The proliferation profiles of L-OHP-resistant SW620 cells were compared with those of control SW620 cells at 24, 48, and 72 h (supplemental Fig. 3). The doubling time of L-OHP-resistant SW620 cells was approximately 28 h while that of control SW620 cells was approximately 20 h. These results showed that the growth rate of L-OHP-resistant SW620 cells was slower than that of control SW620 cells.

**Mesenchymal Cell-Like Characteristics of L-OHP-Resistant SW620 Cells** The shape of L-OHP-resistant SW620 cells was more elongated than that of control SW620 cells (Fig. 1A). *CDH1* mRNA expression levels were lower in L-OHP-resistant SW620 cells than in control SW620 cells (Fig. 1B). On the other hand, the mRNA expression levels of *vimentin* and *ZEB1* were up-regulated in L-OHP-resistant SW620 cells (Fig. 1B). No significant changes were observed in the mRNA expression levels of *Snail* in L-OHP-resistant SW620 cells (Fig. 1B). The mRNA expression levels of *CDH2*, which encodes N-cadherin, and *ZEB2* were not detected in L-OHP-resistant SW620 cells or control SW620 cells (data not shown). Similarly, the protein expression level of E-cadherin was down-regulated in L-OHP-resistant SW620 cells (Fig. 1C). The protein expression levels of vimentin and *ZEB1* were also up-regulated after the acquisition of L-OHP resistance (Fig. 1C). Migration or invasion activity of L-OHP-resistant SW620 cells was higher than that of control SW620 cells (Fig. 1D). The CD44s/CD44v9 ratio was markedly higher in L-OHP-resistant SW620 cells than in control SW620 cells (supplemental Fig. 4). These results suggested that L-OHP-resistant SW620 cells showed mesenchymal cell-like characteristics. Insignificant changes in intracellular ROS level and mRNA expression levels of *xCT* were observed in L-OHP-resistant SW620 cells (supplemental Fig. 5). On the other hand, *CDH1* mRNA expression level was higher in SW480 cells treated with L-OHP than in control SW480 (supplemental Fig. 6A). The migration ability of SW480 cells treated with L-OHP did not change significantly from that of control SW480 cells (supplemental Fig. 6C). These results suggested that SW480 cells after the treatment with L-OHP did not show mesenchymal cell-like characteristics.

**Expression Level of miR-200 Family Members and miR-205 in L-OHP-Resistant SW620 Cells** Since the expression levels of *ZEB1* mRNA and its protein were up-regulated in L-OHP-resistant SW620 cells, we next examined the expression levels of miR-200 family members and miR-205, which down-regulate the expression of *ZEB1* mRNA and its protein. The expression levels of miR-200 family members were higher in SW480 cells than in SW620 cells (supplemen-

Table 1. IC<sub>50</sub> Values of L-OHP in SW620 Cells Treated with L-OHP

L-OHP treatment	IC <sub>50</sub> ( $\mu$ M)	Relative resistance
Control	13.4	1.0
Day 33	30.2	2.2
Day 68	40.2	3.0
Day 110	110.0	8.2
Day 155	215.6	16.1

The IC<sub>50</sub> values of L-OHP were calculated as described in Materials and Methods. Relative resistance was calculated by dividing each IC<sub>50</sub> value in SW620 cells treated with L-OHP by that in control SW620 cells.

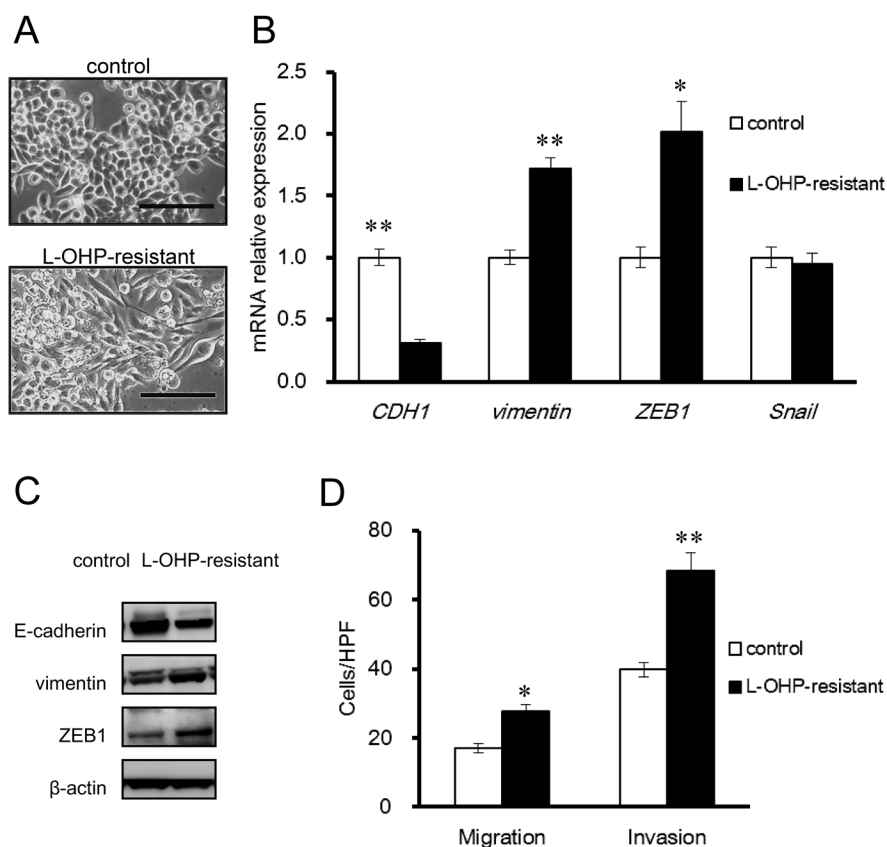


Fig. 1. Changes in EMT-Related Molecules and Migration Activity in L-OHP-Resistant SW620 Cells

(A) Morphological characteristics of L-OHP-resistant SW620 cells and control SW620 cells were observed by phase contrast microscopy (scale bar: 100  $\mu$ m). (B) The mRNA expression of *CDH1*, *vimentin*, *ZEB1*, and *Snail* in L-OHP-resistant SW620 cells and control SW620 cells was detected by real-time PCR analysis and normalized by *RPL27* mRNA expression. (C) The protein expression of E-cadherin, vimentin, and ZEB1 was detected in L-OHP-resistant SW620 cells and control SW620 cells by Western blotting.  $\beta$ -Actin was used as a loading control. (D) Migration assay and invasion assay were performed in L-OHP-resistant SW620 cells and control SW620 cells. Each column represents the mean  $\pm$  S.E.M. of three independent experiments (unpaired Student's *t*-test, \* $p$ <0.05, \*\* $p$ <0.01 significantly different from control SW620 cells).

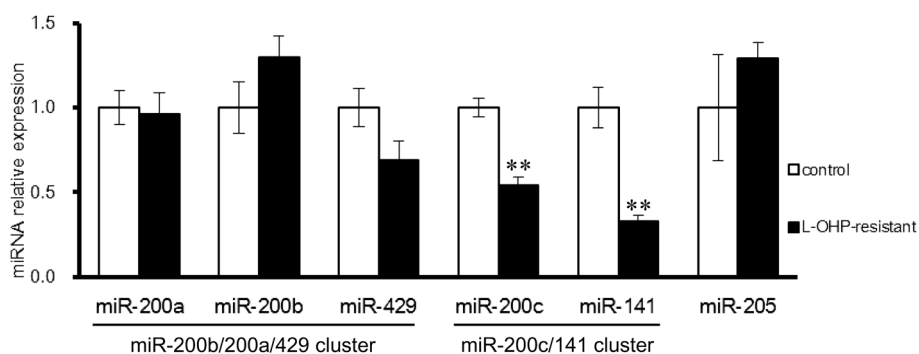


Fig. 2. Decreased Expression Levels of miR-200 Family Members in L-OHP-Resistant CRC Cells

The expression of miR-200 family members and miR-205 was detected in L-OHP-resistant SW620 cells (closed column) and control SW620 cells (open column) by real-time RT-PCR and normalized by U6 snRNA expression. Each column represents the mean  $\pm$  S.E.M. of three to five independent experiments (unpaired Student's *t*-test, \*\* $p$ <0.01 significantly different from control SW620 cells).

tal Table 4). The expression levels of miR-200c and miR-141 were markedly lower in L-OHP-resistant SW620 cells than in control SW620 cells (Fig. 2). No significant differences were observed in the expression levels of miR-200a, miR-200b, miR-429, and miR-205 between L-OHP-resistant SW620 cells and control SW620 cells (Fig. 2).

**Sensitivity of L-OHP-Resistant SW620 Cells to Anti-cancer Drugs** We examined the sensitivity of L-OHP-resistant SW620 cells to anti-cancer drugs, such as 5-FU, CPT-11, and SN-38 which is an active metabolite of CPT-11, as poten-

tial treatments for CRC. The  $IC_{50}$  value of 5-FU was slightly higher in L-OHP-resistant SW620 cells than in control SW620 cells (Table 2). The  $IC_{50}$  values of CPT-11 and SN-38 were slightly lower in L-OHP-resistant SW620 cells than in control SW620 cells (Table 2). These results suggested that L-OHP-resistant SW620 cells exhibited selective resistance to L-OHP.

## DISCUSSION

SW620 cells were categorized as an epithelial-like CRC



Table 2. IC<sub>50</sub> Values of Anti-cancer Drugs for CRC Treatments in L-OHP-Resistant SW620 Cells

Cell	IC <sub>50</sub> value			
	L-OHP ( $\mu$ M)	5-FU ( $\mu$ M)	CPT-11 ( $\mu$ M)	SN-38 (nM)
Control	13.8 $\pm$ 2.2	8.4 $\pm$ 1.6	4.3 $\pm$ 0.2	10.0 $\pm$ 0.7
L-OHP-resistant	260.8 $\pm$ 13.7	16.8 $\pm$ 1.9	3.2 $\pm$ 0.3	4.9 $\pm$ 0.1
<i>p</i> Value	0.0001	0.026	0.032	0.002

The IC<sub>50</sub> values of anti-cancer drugs were calculated as described in Materials and Methods. Each value represents the mean $\pm$ S.E.M. of three independent experiments.

cell and were caused EMT by TGF- $\beta$  or TNF- $\alpha$ .<sup>29,30)</sup> The results of the present study indicated that SW620 cells with acquired L-OHP resistance displayed mesenchymal cell-like characteristics and expressed high levels of *ZEB1* mRNA and its protein. Furthermore, L-OHP-resistant SW620 cells expressed lower levels of miR-200c and miR-141 than control SW620 cells. To the best of our knowledge, this is the first study to demonstrate that miR-200 family members were down-regulated in L-OHP-resistant CRC cells, which suggests that the down-regulation of these two miRNAs plays a role in the resistance to L-OHP and EMT observed in this study. L-OHP-resistant SW620 cells also exhibited selective resistance to L-OHP.

Copper transporters have been shown to mediate the initial influx or efflux of platinum-based drugs, such as L-OHP and cisplatin.<sup>6,7)</sup> The mRNA expression levels of *CTR1*, *CTR2*, and *ATP7A* were also down-regulated in L-OHP-resistant SW620 cells. However, the sensitivity of L-OHP-resistant SW620 cells to cisplatin was not significantly different from that of control SW620 cells. These results suggested that sensitivity to platinum-based drugs such as cisplatin was not affected by changes in expression levels of copper transporters in SW620 cells.

In the present study, L-OHP-resistant SW480 cells were not obtained by long-term treatments with L-OHP. In addition, expression levels of miR-200 family members were higher in SW480 cells than in SW620 cells. A recent study suggested that mesenchymal-like breast cancers were more likely to develop drug resistance.<sup>35)</sup> These findings indicate that SW620 cells display more mesenchymal characteristics than SW480 cells and are more likely to develop L-OHP resistance. Therefore, EMT may be easily induced in SW620 cells treated with L-OHP. These results demonstrated that the up-regulated expression of miR-200 family members inhibited the acquisition of L-OHP resistance by blocking EMT.

Zhou *et al.* compared the miRNA expression profiles, including miR-200c, of three CRC cell lines (HT29, HCT116, and RKO) with those of their L-OHP-resistant derivative cells.<sup>18)</sup> They reported that the expression of miR-200c in these L-OHP-resistant CRC cells was not down-regulated.<sup>18)</sup> Regulators of the nuclear factor- $\kappa$ B pathway, caspase 3, Bax, and ERCC1 have been linked to the development of insensitivity to L-OHP.<sup>3-5)</sup> We did not determine whether these were involved in the development of L-OHP resistance in L-OHP-resistant SW620 cells in the present study because caspase 3, Bax, and ERCC1 are not considered to play an important role in EMT caused by the acquisition of L-OHP resistance. The low expression levels of caspase 3 and Bax were reported to be involved in L-OHP resistance and cisplatin resistance.<sup>3)</sup> However, no significant differences were observed in sensitivity to cisplatin between L-OHP-resistant SW620 cells and con-

trol SW620 cells in this study. The expression level of ERCC1 was corrected with that of Snail, not with that of ZEB1.<sup>36)</sup> We will intend to investigate the relationship between expression levels of nuclear factor- $\kappa$ B and EMT using L-OHP-resistant SW620 cells.

The results of the present study suggest that changes in the expression levels of miR-200c and miR-141 represent novel targets of chemotherapy, and that these two miRNAs could be candidates for biomarkers of acquired selective L-OHP resistance and EMT caused by acquired L-OHP resistance in patients with CRC. However, additional studies are required to develop an effective chemotherapy for patients with L-OHP-resistant CRC and prevent acquired L-OHP resistance and EMT.

**Conflict of Interest** The authors declare no conflict of interest.

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

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