Suppression of REV7 enhances cisplatin sensitivity in ovarian clear cell carcinoma cells

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Human REV7 (also known as MAD2L2 and MAD2B) is involved in DNA repair, cell cycle regulation, gene transcription, and carcinogenesis. In this study, we evaluated the expression of REV7 in epithelial ovarian cancer (EOC) and analyzed the association between its expression and chemosensitivity in ovarian clear cell carcinoma (CCC) cells. Expression of REV7 in human EOC tissues was assessed by immunohistochemical staining. Expression was detected in the majority of EOCs (92.0%) with especially high levels of expression frequently observed in CCCs (73.5%) compared with that of non-CCCs (53.4%). Enhanced immunoreactivity to REV7 was associated with poor prognosis represented by reduced progression-free survival in advanced stage (stage II–IV) EOC as assessed using Kaplan–Meier curves and log–rank tests. The effects of REV7 knockdown on cell proliferation and chemosensitivity in CCC cells were also analyzed in vitro and in vivo. Knockdown of REV7 in CCC cells decreased cell proliferation without affecting cell cycle distribution. Additionally, the number of apoptotic cells and DNA damaged cells were increased after cisplatin treatment. In a nude mouse xenograft model, inoculated REV7-knockdown tumors showed significantly reduced tumor volumes after cisplatin treatment compared with those of the control group. These findings indicate that depletion of REV7 enhances sensitivity to cisplatin treatment in CCC, suggesting that REV7 is a candidate molecular target in CCC management.

O varian cancer is the fifth most common malignancy in women and the most common cause of gynecologic cancer-related death.1) The four main histological subtypes of epithelial ovarian cancer (EOC), serous, endometrioid, clear cell, and mucinous adenocarcinomas, are considered to be distinct diseases according to epidemiology, clinical characteristics, responses to treatment, prognosis, and molecular features.2–5) However, EOCs have been largely treated as a single disease. Compared with other EOC subtypes, clear cell carcinoma (CCC) is associated with poor outcome and greater resistance to platinum-based chemotherapy.6–9) Recent studies have shown that CCC carries either ARID1A or PIK3CA mutations as well as HNF-1β overexpression,10–12) but there are no biomarkers for chemotherapy response. Therefore, it is important to identify both the clinical indicators of CCC and the molecular pathways involved in its drug sensitivity.

A number of chemotherapeutic agents induce DNA damage in cells, whereby the DNA repair system is associated with their efficiency in cancer therapy. Deregelation of DNA repair proteins results in failure to repair DNA damage in cells and subsequent genetic instability and cell death. Thus, DNA repair inhibitors are expected to improve the outcome of cancer chemotherapy.13–15) REV7 (also known as MAD2L2 and MAD2B) is involved in translesion DNA synthesis (TLS), one of the damage tolerance processes, which completes DNA synthesis through DNA lesions to prevent DNA damage-induced cell death.16,17) REV7 makes a complex with REV3, forming DNA polymerase ζ, one of the specialized low fidelity polymerases, playing an important role in TLS.18–20) Involvement of REV7 in DNA damage tolerance in human cells has been clearly demonstrated. Inactivation of REV7 by RNAi technology increases sensitivity to DNA-damaging agents in nasopharyngeal carcinoma cells.21) Similarly, after siRNA-mediated REV7 depletion, human fibroblast cells show heightened sensitivity to UV-induced cytotoxicity and low sensitivity to UV-induced mutagenesis compared with control cells.22) It was also reported that REV7-depleted glioma cells show enhanced apoptotic response to ionizing radiation.23) Moreover, it was reported that mRNA levels of REV7 are significantly increased in human breast and colorectal cancers.24,25) and that REV7 interacts with cancer-related proteins PRCC (papillary renal cell carcinoma) and HCCA2 (hepatocellular carcinoma-associated gene 2).26,27) These findings suggest that REV7 expression is associated with cancer development and sensitivity to DNA-damaging agents.

In this study, we established the association between REV7 expression and the chemosensitivity of CCC using clinical materials and in vitro and in vivo experiments. Our findings suggest that REV7 is a potential candidate for molecular target in CCC therapy.
Materials and Methods

Patients and tissue samples. One hundred and thirty-seven ovarian carcinoma tissue samples (47 serous adenocarcinomas, 19 mucinous adenocarcinomas, 22 endometrioid adenocarcinomas, and 49 CCCCs) were obtained from patients who underwent surgical treatment at Nagoya University Hospital (Nagoya, Japan) between 1998 and 2003 following informed consent. The patients’ ages ranged from 23 to 82 years, with a median age of 54 years. The histological types were assigned according to the World Health Organization classification criteria. Clinical stage was assigned on the basis of the International Federation of Gynecology and Obstetrics staging system.

Immunohistochemical staining. Formalin-fixed and paraffin-embedded tissues were sliced at a thickness of 4 μm. For antigen retrieval, they were heated in Target Retrieval Solution pH 9.0 (Dako, Copenhagen, Denmark) for 40 min at 98°C. Endogenous peroxidase was inhibited using 3% H2O2 in methanol for 15 min. After blocking with 10% normal goat serum for 10 min at room temperature (RT), sections were incubated with primary antibodies for 90 min at RT and then incubated with the secondary antibody conjugated to HRP-labeled polymer (EnVision + anti-rabbit; Dako) for 15 min at RT. Reaction products were visualized using diaminobenzidine (Dako), and nuclei were counterstained with hematoxylin. The staining intensity of REV7 was scored as 0 (negative), 1 (weak), 2 (medium), or 3 (strong) and then further classified into two categories: low, expression scores 0 and 1; or high, expression scores 2 and 3 (Fig. 1a, see Data S1 for antibody information). The REV7 expression levels were evaluated by two independent blinded observers.

Cell proliferation and viability assay. Cells were seeded in 96-well plates at a density of 2 × 10² cells in 100 μL medium. Twenty-four hours after seeding, the cell proliferation assay was carried out using WST-1 Reagent (Roche, Basel, Switzerland) according to the manufacturer’s instructions. For the cell viability assay, 5 × 10⁵ cells per well were seeded in 96-well plates and treated with the indicated concentrations of cisplatin (cis-diaminedichloroplatinum, CDDP) for 48 h. Cell viability was determined by the WST-1 assay. Absorbance was measured at 450 nm using a microplate reader (Tecan, Palm Springs, CA, USA).

Fluorescence immunocytochemistry. Cells were seeded in 96-well plates for cleaved caspase-3 staining and transferase-mediated biotin-16-dUTP nick-end labeling (TUNEL) assay or on coverslips for phospho-H2AX foci analysis. After CDDP treatment at the indicated concentrations for 24 h, the cells were fixed in 4% paraformaldehyde for 20 min at 4°C, washed in PBS, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. After blocking with 1% BSA, the cells were incubated with anti-cleaved caspase-3 or anti-phospho-H2AX antibody for 90 min at RT. The cells were then incubated with secondary antibody conjugated to Alexa Fluor 488 for 30 min at RT. Cellular DNA was counterstained with DAPI. The TUNEL assay was carried out following the manufacturer’s instruction (In Situ Cell Death Detection Kit, Fluorescein; Roche). To assess the immunoreactivity of cleaved caspase-3 or TUNEL, the cells were counted using a Cellomics Array Scan VTI (Cellomics-Thermo-Fisher, Walling, MA, USA). To assess the positivity for phospho-H2AX, the cells with more than 10 foci were counted using a fluorescence microscope (Olympus, Tokyo, Japan).

Mouse tumor xenografts. TOV-21G cells (1 × 10⁷) with short-hairpin RNA (shRNA)-mediated REV7 depletion (shREV7) and control cells (shCont) in 200 μL PBS were injected s.c. into the right flank of eight 6-week-old female nude mice (Crlj:CD1-Foxn1nu; Charles River Laboratories, Yokohama, Japan). When tumor volumes reached approximately 100 mm³, the mice were given PBS or CDDP (2 mg/kg body weight) i.p. four times every 2 days (n = 4/group). We then measured the diameters of each tumor every 4 days after beginning treatment and calculated their estimated volumes using the following formula: volume = length × width × height/206.
width \times 1/2. The mice were maintained in accordance with the institutional guidelines of Nagoya University Graduate School of Medicine, and experiments were carried out according to approved experimental protocols.

Statistical analysis. We used \( \chi^2 \)-tests to analyze the associations between the REV7 status and clinicopathological parameters. The overall survival (OS) was defined as the time between the date of surgery and the last date of follow-up or date of death due to cancer. The progression-free survival (PFS) was defined as the time interval between the date of surgery and the last date of follow-up or recurrence or death of last follow-up. Survival analysis was carried out according to the life tables and Kaplan–Meier methods using SPSS software (version 20; SPSS Inc., Armonk, NY, USA) and the log–rank test. A multivariate analysis was carried out using a Cox proportional hazards model. For data from in vivo and in vitro experiments, statistical comparisons among groups were carried out using non-paired Student’s \( t \)-test or ANOVA with Bonferroni corrections. Differences were considered significant at \( P < 0.05 \). Data are indicated as the mean \( \pm \) SD.

Results

REV7 is frequently expressed in EOC, especially CCC. Ovarian cancer specimens from 137 patients were immunohistochemically stained for REV7. REV7 was mainly expressed in the nuclei of cancer cells, but not in the surrounding non-tumor cells (Fig. 1a). The specific reaction of rabbit polyclonal anti-REV7 antibody was shown by comparison with negative control staining using the preimmune serum or normal rabbit IgG (Fig. S1). The immunohistochemical analyses revealed that 11 of 137 specimens (8.0%) were REV7-negative and 126 (92.0%) were REV7-positive. Fifty-four cases (39.4%) were classified into the high REV7 expression group (REV7\textsuperscript{high}), and 83 cases (60.6%) were classified into the high REV7 expression group (REV7\textsuperscript{low}) (Table 1). The association between REV7 expression and clinicopathological parameters were statistically analyzed. All 49 CCC cases (100%) were REV7-positive, with 13 (26.5%) in the REV7\textsuperscript{low} group and 36 (73.5%) in the REV7\textsuperscript{high} group. In contrast, 77 of 88 non-CCC cases (87.5%) were REV7-positive, with 41 (46.6%) in the REV7\textsuperscript{low} group and 47 (53.4%) in the REV7\textsuperscript{high} group. The association between CCC histopathological type and high REV7 expression was statistically significant (\( P = 0.021 \)), indicating that REV7 is highly expressed in CCC (Table 1). However, REV7 expression was not associated with other clinicopathological parameters.

Next, we examined whether or not REV7 expression was associated with prognosis of EOC. When the association between REV7 expression and OS or PFS was assessed by Kaplan–Meier methods and the log–rank test, no significant association was found (OS, \( P = 0.711 \); PFS, \( P = 0.237 \)). However, analysis of the association between REV7 expression and PFS in 87 cases with advanced stage EOC (stage II–IV) revealed that PFS in the REV7\textsuperscript{high} group (\( n = 48 \)) was significantly shorter than that in the REV7\textsuperscript{low} group (\( n = 39 \)) (\( P = 0.017 \), Fig. 1b). Then, we analyzed the factors that contributed to the prognosis using a multivariate Cox proportional hazards model (Table S1). The results showed that REV7 expression was independently associated with PFS (\( P = 0.012 \)). In addition, 1-year PFS was significantly associated with REV7 expression in cases with advanced stage EOC treated with platinum-based chemotherapy (\( P = 0.022 \), Table S2). These results indicate that REV7 expression is implicated in the prognosis of EOC at advanced stage.

Depletion of REV7 suppresses cell proliferation but does not affect the cell cycle in CCC cells. As it was revealed that REV7 is strongly expressed in CCC, we investigated the significance of REV7 expression in CCC biology. REV7-knockdown cells were generated using three CCC cell lines, ES-2, KOC-7C, and 547.

Table 1. Association between REV7 expression and clinicopathological factors in patients with ovarian cancer (\( n = 137 \))

| Variables                  | Patients (n) | REV7 expression score | REV7 expression level | \( P \)-value |
|----------------------------|--------------|-----------------------|-----------------------|--------------|
| Age, years                 |              |                       |                       |              |
| <60                        | 96           | 7                     | 35                    | 42 (43.8)   |
| \( \geq 60 \)              | 41           | 4                     | 8                     | 12 (29.3)   |
| FIGO stage                 |              |                       |                       |              |
| I                          | 50           | 5                     | 10                    | 15 (27.8)   |
| II–IV                      | 87           | 6                     | 33                    | 39 (44.8)   |
| Histological type          |              |                       |                       |              |
| Clear cell                 | 49           | 0                     | 13                    | 13 (26.5)   |
| Non-clear cell             | 88           | 11                    | 30                    | 41 (46.6)   |
| Serous                     | 47           | 4                     | 15                    | 19 (40.4)   |
| Mucinous                   | 19           | 2                     | 4                     | 6 (31.3)    |
| Endometrioid               | 22           | 5                     | 11                    | 16 (72.7)   |
| Residual tumor             |              |                       |                       |              |
| Absent                     | 96           | 8                     | 30                    | 38 (39.6)   |
| Present                    | 41           | 3                     | 13                    | 16 (39.0)   |
| CA125, U/mL                |              |                       |                       |              |
| <50                        | 27           | 1                     | 6                     | 7 (25.9)    |
| \( \geq 50 \)              | 110          | 10                    | 37                    | 47 (42.7)   |
| Chemotherapy (platinum-based) |          |                       |                       |              |
| Absent                     | 18           | 2                     | 5                     | 7 (38.9)    |
| Present                    | 119          | 9                     | 38                    | 47 (39.5)   |

Table S1. Association between REV7 expression and clinicopathological parameters in patients with ovarian cancer (\( n = 137 \))

Table S2. Association between REV7 expression and clinicopathological parameters in patients with ovarian cancer (\( n = 137 \))

FIGO, International Federation of Gynecology and Obstetrics.
and TOV-21G, by RNAi technology (Data S1). Expression levels of REV7 in the three CCC cell lines were similar and were upregulated compared with other cell lines derived from yolk sac tumor and serous adenocarcinoma (Fig. S2). Expression of REV7 was significantly suppressed in all CCC cell lines with shRNA targeting REV7 (Fig. 2a). Then, we assessed the effects of REV7 knockdown on cell proliferation, in which shREV7 cells grew more slowly than shCont cells, indicating that REV7 affects cell proliferation (Fig. 2b). Cell cycle distribution was also analyzed in shREV7 and shCont cells by flow cytometry, but it was not affected by REV7 depletion (Fig. 2c, Data S1).

Knockdown of REV7 enhances chemosensitivity and increases CDDP-induced apoptosis in CCC cells. Next, we investigated the effect of REV7 expression on chemosensitivity to DNA damaging agents in CCC cells. REV7-knockdown and control cells were treated with CDDP at various concentrations for 48 h, and cell viability was assessed. REV7 knockdown rendered cells more sensitive to CDDP, and the IC50 values were decreased by REV7 depletion compared with those in the shCont cells (1.61-fold, 1.98-fold and 1.93-fold decrease in IC50 in ES-2, KOC-7C, and TOV-21G cells, respectively) (Fig. 3a). The chemosensitivity of REV7-knockdown cells was confirmed by colony formation assay, in which the number of colonies formed after CDDP treatment was decreased by REV7 depletion (Fig. S3, Data S1). In addition, chemosensitivity was also examined in shCont and shREV7 ES-2 cells with ectopic REV7 expression. Ectopic REV7 expression rescued enhanced chemosensitivity in shREV7 cells, however, its expression did not significantly affect chemosensitivity in shCont cells, although the IC50 of REV7-expressing shCont cells was elevated a little (Fig. S4), suggesting that endogenous REV7 expression is high enough for chemoresistance in ES-2 cells.

We then assessed CDDP-induced apoptosis in REV7-knockdown and control cells. After the cells were treated with CDDP at concentrations of 0, 20, 40, and 80 μM for 48 h, the cleaved poly(ADP-ribose) polymerase (PARP) fragment could be detected in REV7-knockdown cells at lower concentrations of CDDP than in control cells (Fig. 3b). Similar results were observed in cells treated with UV irradiation at doses of 0, 10, and 20 J/m² (Fig. 3b). To assess the enhancement of apoptosis in REV7-knockdown cells quantitatively, shREV7 and shCont cells were immunofluorescently stained for TUNEL and cleaved caspase-3 before and after CDDP treatment. The number of TUNEL-positive cells significantly increased in REV7-depleted cells after CDDP treatment (ES-2, 20 μM; KOC-7C and TOV-21G, 50 μM) when compared with that of control cells (Fig. 3c). The percentages of cleaved caspase-3-positive cells after CDDP treatment dose-dependently increased in control cells and were significantly elevated by suppression of REV7 in all three cell lines (Fig. 3c). These results indicate
that CDDP-induced apoptosis is enhanced by REV7 depletion in CCC cells.

Depletion of REV7 causes accumulation of DNA double-strand breaks after CDDP treatment in CCC cells. As CDDP induces DNA damage of intrastrand and interstrand cross-links, which are repaired by nucleotide excision repair and homologous recombination repair machineries, it is possible that REV7 depletion causes dysfunction of DNA repair machinery and accumulation of DNA damage in cells, causing enhancement of apoptosis. DNA damage of double-strand breaks was assessed in shREV7 and shCont cells by immunofluorescence staining using anti-phospho-H2AX antibody. TOV-21G-derived shREV7 and shCont cells were treated with CDDP for 48 h and used for Western blotting (upper panels). The shREV7 and shCont CCC cells were also treated with UV at the indicated doses and used for Western blotting 12 h after UV irradiation (lower panels). The blots of β-actin are indicated as internal controls. (c) Assessment of apoptosis in the shREV7 and shCont CCC cells after CDDP treatment. Immunoreactivity for TUNEL and cleaved caspase-3 in cells treated with CDDP was fluorescently analyzed. The means ± SDs of the percentage of TUNEL-positive (upper panels) or cleaved caspase-3-positive (lower panels) cells are shown. *P < 0.05; **P < 0.01.

Depletion of REV7 confers CDDP sensitization in vivo. To test whether REV7-depleted tumors are sensitive to systemic therapy of DNA damaging agents, a mouse-tumor model was established using shCont and shREV7 TOV-21G cells, and the effect of systemic CDDP treatment was assessed. We found that REV7-depleted tumors grew more slowly than control tumors, and CDDP treatment further drastically suppressed growth of REV7-depleted tumors (Fig. 5a,b). Tumor volumes of control and REV7-depleted tumors at day 32 were reduced to 39.7 ± 8.9% and 15.3 ± 10.2% by CDDP treatment, respectively (P < 0.05) (Fig. 5c). Immunohistochemical analyses of the tumor graft tissues at day 32 revealed that REV7-depleted tumors contained
In this study, we showed that most EOC types expressed REV7 protein and that high expression levels were frequently observed in mucinous adenocarcinoma (Table 1). Both CCC and mucinous adenocarcinoma tend to be resistant to antineoplastic agents. Moreover, we found that the PFS in the REV7high group was significantly shorter than that in the REV7low group in advanced EOC cases. In current treatment, platinum/paclitaxel-based chemotherapy is required for advanced cases, and the existence or development of chemoresistance is an important factor for poor prognosis. Our results indicate that high-level expression of REV7 contributes to poor prognosis of EOC at advanced stages, suggesting that REV7 expression may be associated with chemoresistance in EOC. However, no significant difference was observed between the PFS of REV7high and REV7low groups in only CCC cases at advanced stages (data not shown). A possible reason for this result is that a large portion of CCC cases were in early stage (stage I) and only 23 cases (46.9%) were in advanced stage (stage II–IV). To clarify the data, larger prospective studies for outcome analysis in CCC should be carried out in the future.

Development of chemoresistance is evoked by enhanced drug efflux, reduced drug uptake, aberrant apoptotic pathways, induction of drug-detoxifying mechanisms, and increased repair of DNA damage. Although it has been reported that p53 mutation, low expression of ARID1A, or upregulation of FGF1, annexin A3, bcl-2, or Ret finger protein can be responsible for platinum-based chemoresistance of EOC, the mechanisms of CDDP resistance in human ovarian cancer still remain unclear. Moreover, there are no promising biomarkers for predicting chemoresistance. It has been reported that REV7 affects the anticancer effect of DNA-damaging agents including CDDP, mitomycin C, and doxorubicin in nasopharyngeal carcinoma cells and of ionizing radiation in glioma cells. Therefore, we investigated the effect of REV7 expression on CDDP-induced anticancer effects in CCC cells. We showed that REV7 depletion suppressed proliferation of CCC cells without obvious alterations to the cell cycle, suggesting that REV7 depletion reduces cell proliferation by spontaneous stresses in the absence of DNA-damaging agents. In contrast to control cells, REV7-depleted cells subjected to CDDP treatment contained a large number of cells positive for cleaved caspase-3 and TUNEL and showed increased cleaved PARP by Western blotting. They also showed enhanced immunoreactivity for phospho-H2AX-positive cells than control tumors (P < 0.01) (Fig. 5d-f). These results indicate that REV7 depletion confers enhanced sensitivity to CDDP treatment in CCC cells in vivo.

Discussion

The DNA repair system is required by cells to maintain genetic stability. Impairment of the DNA repair system causes increased susceptibility to DNA damaging agents, resulting in genetic instability and cell death. The REV7 protein is involved in TLS and homologous recombination repair, and REV7-deficient mice show numerous apoptotic cells and accumulation of DNA damage in both germ cells and somatic cells suggesting that REV7 dysfunction enhances cellular sensitivity to DNA damage.

In this study, we showed that most EOC types expressed REV7 protein and that high expression levels were frequently detected in CCC. Our results also showed that high REV7 expression was frequently observed in mucinous adenocarcinoma.
immunoreactivity for cleaved caspase-3 and phospho-H2AX. These in vivo results were compatible with those obtained from in vitro experiments, suggesting that REV7 expression in CCC tumors may affect chemosensitivity to systemic CDDP treatment. Because REV7 expression in human cancer tissues can be detected easily by immunohistochemistry in comparison with REV1 and REV3, we propose that REV7 can be a good marker for chemosensitivity and a novel molecular target for CCC therapy. Further analyses of chemosensitivity in REV7-depleted tumors are necessary to confirm the association between REV7 expression and the outcome of chemotherapy in CCC.

Fig. 5. Suppression of REV7 enhances cisplatin (CDDP) sensitivity in vivo. (a) Growth of inoculated tumors in nude mice with and without systemic CDDP treatment. REV7-knockdown (shREV7) or control (shCont) ovarian clear cell carcinoma TOV-21G cells (1 × 10⁷) were used in this analysis. The means ± SDs of tumor volume are shown. *P < 0.05; **P < 0.01. (b) Macroscopic images of transplanted tumors excised at day 32. (c) Reduction of tumor volume by CDDP treatment. Tumor volumes of the CDDP-treated group relative to the mean tumor volume of the PBS-treated group were calculated, and the means ± SDs of relative tumor volumes are shown. *P < 0.05. (d) Immunohistochemical analysis of excised tumors for REV7, cleaved caspase-3, and phospho-H2AX. Excised tumor tissues at day 32 were immuno-histochemically stained with anti-REV7 (upper panels), anti-cleaved caspase-3 (middle panels), and anti-phospho-H2AX (lower panels) antibodies. Scale bar = 50 μm. (e, f) Quantitative assessment of cleaved caspase-3-positive cells (e) and phospho-H2AX-positive cells (f) in shREV7 and shCont tumors. Cells were counted under high power fields (HPF). The data obtained from five separate fields are shown as means ± SDs. **P < 0.01.

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Disclosure Statement

The authors have no conflict of interest.
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Supporting Information

Additional supporting information may be found in the online version of this article:
Data. S1. Supplementary materials and methods.
Fig. S1. Specific immunohistochemical reaction of rabbit polyclonal anti-REV7 antibody.
Fig. S2. Expression of REV7 in ovarian cancer cell lines.
Fig. S3. Depletion of REV7 enhances chemosensitivity in colony formation assay.
Fig. S4. Expression of REV7 conferred resistance to cisplatin (CDDP).
Fig. S5. Immunohistochemical and immunofluorescence analyses of control (shCont) and REV7-knockdown (shREV7) tumors for REV7 expression.
Table S1. Multivariante analysis of several clinicopathologic parameters in relation to survival of patients with advanced stage epithelial ovarian cancer.
Table S2. Association between REV7 expression and 1-year progression-free survival in patients with advanced stage epithelial ovarian cancer who received platinum-based chemotherapy.