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Association of Copper Transporter Expression with Platinum Resistance in Epithelial Ovarian Cancer

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Abstract. *Background: Copper transporters (CTR) also regulate the cellular transport of platinum drugs, but their role in platinum resistance of ovarian cancer has not been elucidated. Materials and Methods: CTR expression in ovarian cancer tissues resected from patients treated by platinum-based chemotherapy was evaluated immunohistochemically. CTR2 expression in ovarian cancer cells was inhibited by bathocuproine disulfonate, and the changes in cisplatin sensitivity were examined. Results: CTR2 expression was increased in chemoresistant patients, but not significantly. However, the CTR2/CTR1 ratio was significantly increased in chemoresistant patients. Cases with positive CTR2 expression or positive CTR2/CTR1 ratio had poor prognoses. When the CTR2 expression in ovarian cancer cells was suppressed, sensitivity to cisplatin was significantly increased. Conclusion: These data suggest that CTR2 contributes to platinum resistance in ovarian cancer. The CTR2/CTR1 ratio is a useful marker for platinum sensitivity and a potential prognostic factor in patients with ovarian cancer.*

Ovarian cancer is known to have the worst prognosis among gynecological malignancies (1). Since there is a lack of characteristic symptoms in the early stage and effective screening methods have not been established, about 70% of patients with ovarian cancer are diagnosed in the advanced stage (1). Following cytoreductive surgery, treatment with platinum and paclitaxel has been recommended for initial chemotherapy (2). First-line chemotherapy with platinum and paclitaxel yields a response rate of >80%. However, despite an initial high response rate, nearly all patients relapse (3). The treatment for recurrent ovarian cancer is often difficult due to resistance to chemotherapeutic agents (4), and the 5-year survival rate for advanced ovarian cancer is less than 30% (1). Therefore, novel strategies for overcoming chemotherapy resistance are needed.

The platinum drugs cisplatin and carboplatin are widely used in ovarian cancer treatment. However, ovarian cancer cells achieve resistance to platinum drugs as well as to other chemotherapeutic agents. The precise mechanisms that account for such drug resistance have not been fully identified. Reduced cellular accumulation of drugs, enhanced detoxification capability, an aberrant apoptosis pathway, and increased DNA repair ability from DNA damage have been proposed (5-12).

The copper transporters (CTRs) are regulators of copper homeostasis, and it has been reported that CTRs also regulate the transport of platinum drugs (13). Thus CTR1, the major copper influx transporter, enhances the influx of platinum drugs into the cell (13). Several reports indicate that enhancing CTR1 expression in the tumor cells increases their sensitivity to platinum drugs (14, 15). On the other hand, a recent study suggested that CTR2 has the opposite role in the transport of platinum drugs by inhibiting the cellular accumulation of platinum drugs (16, 17). CTRs may thus be key regulators of platinum drugs, however, almost all previous reports evaluated CTR function at the cellular level. There are only a few reports available regarding the involvement of CTRs in resistance to platinum drugs in ovarian cancer. Therefore, in this study we investigated the relationship between CTR expression and platinum resistance in patients with ovarian cancer.

Materials and Methods

Patients. Thirty-four cases of ovarian cancer that underwent surgery as their initial treatment at the Osaka City University Medical School Hospital between 2005 and 2011 were reviewed (Table I). Informed consent was obtained from all patients, and the Ethics Committee of the Osaka City University Hospital approved this study (Approval No. 2290). All patients underwent platinum-based chemotherapy after

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Key Words: Ovarian cancer, chemotherapy resistance, copper transporter.

Table I. *Clinical characteristics of patients tested for copper transporter-1 and -2 expression (n=34).*

Characteristic	Number of patients
Age, years	
Median	53 (range 33-81)
Tumour histology	
Serous	11
Mucinous	4
Clear	8
Endometrioid	8
Other	3
Stage	
1	2
2	3
3	23
4	6
Chemotherapy sensitivity	
Sensitive	17
Resistant	17

surgery. In this retrospective study, only patients with measurable lesions that were evaluated before and after chemotherapy were included. Tumor responses to chemotherapy were determined using the Response Evaluation Criteria in Solid Tumors (RECIST) (version 1.0) (18). Patients were staged according to the International Federation of Obstetrics and Gynecology (FIGO) classification (19).

Immunohistochemical staining. Ovarian cancer tissue was fixed with 10% buffered formalin and embedded in paraffin. Five-micrometer sections were de-paraffinized, hydrated, and stained according to the DAKO Envision protocol (Dako, Kyoto, Japan) using a 1:250 dilution of rabbit polyclonal antibody to CTR1 (Novus Biologicals, Littleton, CO, USA) and a 1:25 dilution of rabbit polyclonal antibody to CTR2 (Sigma, St. Louis, MO, USA). To determine the specificity of the reaction, the primary antibody was replaced with commercially available negative control reagent containing rabbit immunoglobulins (Dako). The quantitative analysis of CTR expression was performed as follows. The percentage of positively-stained tumor cells was determined in three separate fields at ×200 magnification and assigned to one of the following categories: 0, no immunostaining; 1, fewer than 25% positive cells; 2, 26-50% positive cells ; 3, 51-75% positive cells; 4, 76-100% positive cells. The intensity of immunostaining was scored as follows: 1, weak; 2, moderate; 3, intense. The percentage of positive tumor cells and the staining intensity were multiplied to produce an expression score for each specimen. These analyses were performed independently by three blinded gynecological oncologists. Furthermore, we defined CTR2 expression score divided by CTR1 expression score as the CTR2/CTR1 ratio. A CTR1 expression score of 12 was defined as being positive expression and scores ≤11 as negative expression. A CTR2 expression score of ≤4 was defined as being negative expression and those ≥5 as positive expression. For the CTR2/CTR1 ratio, values ≥0.5 were defined as positive.

Cell culture. The human serous ovarian cancer cell line Caov3 (American Type Culture Collection, Manassas, VA, USA) was maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS, Gibco BRL). The human clear-cell ovarian cancer cell line RMG1 (Health Science Research Resources Bank, Osaka, Japan) was grown in Ham's F12 (Gibco BRL) with 10% FBS. Both cell lines were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37˚C.

Immunofluorescence staining. Cells were seeded in 4-well chamber slides (Nalge Nunc, Rochester, NY, USA), and after 24 h, the media were removed from each chamber. The chambers were added 300 μl of FBS-free medium or FBS-free medium containing 100 μM of the CTR2 inhibitor bathocuproine disulfonate (BCS; Sigma) for 1 h. After drug exposure, the media were removed and the cells were washed three times in phosphate-buffered saline (PBS; Gibco BRL). Cells were fixed with ice-cold methanol for 15 min at -20˚C and then washed three times in PBS. Cells were permeabilized for 10 min in a 0.2% solution of Triton X-100 and blocked with a 3% bovine serum albumin solution. After a series of washing with PBS, the cells were incubated with 10 μg/ml antibody to CTR1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or 5 μg/ml antibody to CTR2 (Abcam, Cambridge, MA, USA) for 1 h at 37˚C. Following washing with PBS, cells were exposed to 1:20 FITC-conjugated anti-rabbit secondary antibody (DAKO) for 1 h at 37˚C. Immunofluorescent images were observed under a fluorescence microscope (BX50; Olympus Corporation, Tokyo, Japan).

Chemosensitivity assay. The sensitivity of cells to cisplatin (Bristol-Myers Squibb, Tokyo, Japan) was determined using the tetrazolium compound WST-8 (Cell Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan). $5x10^3$ Cells were seeded in each well of a 96-well tissue culture plate. After 24 h, the media were removed from each well. The wells were then added 100 μl of medium or medium containing 100 μM BCS and cells were further incubated for 1 h. The cells were then treated with a series of cisplatin concentrations for 24 h (0-10 μg/ml) to obtain a dose-response curve. Subsequently, 10 μl of WST-8 reagent was added, and incubation was continued for another 2 h. The absorbance at 450 nm was then measured with a microplate reader (Corona Electric, Ibaraki, Japan). Dose-response curves were plotted as the percentage of viable cells compared with the control untreated cells. The assays were performed in triplicate for each cell line.

Statistical analysis. The significance of differences in CTR expression and CTR2/CTR1 ratio was calculated by the Mann–Whitney *U*-test. Survival curves were generated using the Kaplan-Meier method, and the significance of differences in survival curves were calculated using the generalized Wilcoxon test. The statistical significance of differences in the chemosensitivity assay was calculated by Student's *t*-test. *p*-Values less than 0.05 were considered statistically significant.

Results

CTR expression in ovarian cancer cells correlates with chemotherapy response rate and histological subtype. Immunoreactivity for CTR1 and CTR2 was observed in the cytoplasm and cell membrane of the ovarian cancer cells (Figures 1A and B, and 2A and B). The effect of platinum-based chemotherapy was evaluated according to the RECIST

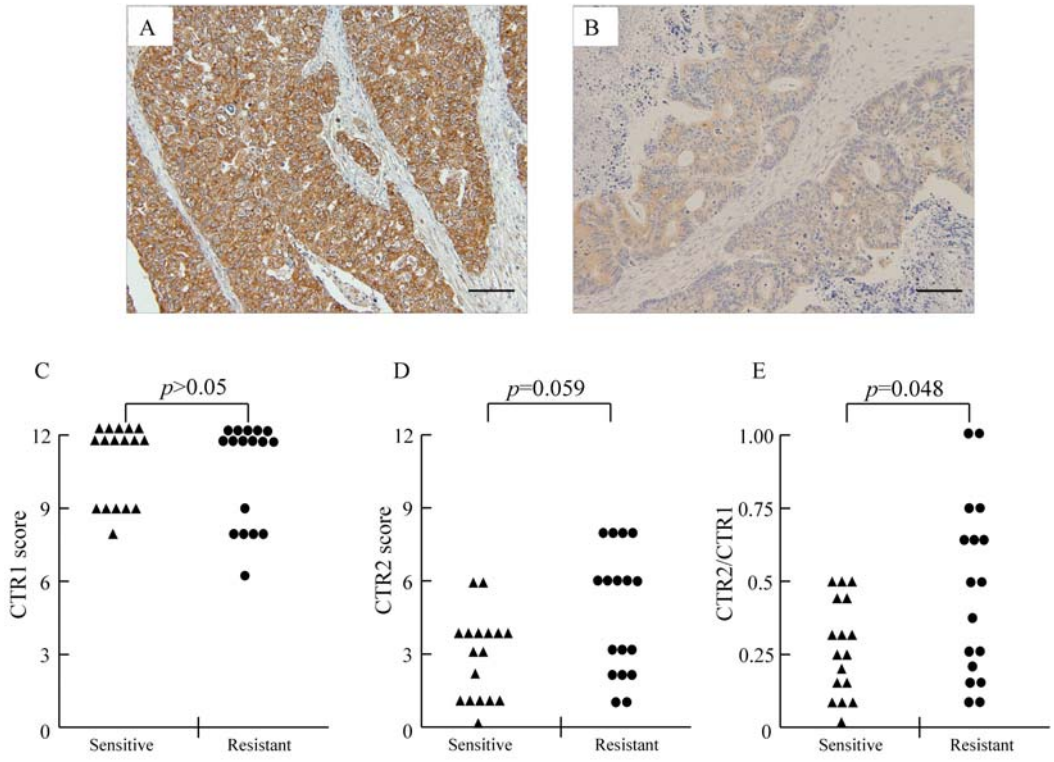

Figure 1. *A: Stage IIIc endometrioid adenocarcinoma. Strong expression of copper transporter (CTR)-1 was observed in ovarian cancer tissues. B: Stage IIIc endometrioid adenocarcinoma. Weak CTR2 expression can be seen in tumor cells (original magnification, ×200, scale bar=100 μm). C: There was no difference in CTR1 expression between the chemoresistant group and the chemosensitive group. D: CTR2 expression was increased in the chemoresistant group compared to the chemosensitive group, but there was no significant difference between them. E: The CTR2/CTR1 ratio was significantly increased in the chemoresistant group compared to the chemosensitive group.*

guidelines. The patients who presented with complete response or partial response were grouped as the chemosensitive group. The patients with stable disease or progressive disease were grouped as the chemoresistant group. As shown in Figure 1C, there was no difference in CTR1 expression between the two groups. On the other hand, CTR2 expression was increased in the chemoresistant group compared to the chemosensitive group, but there was no significant difference (Figure 1D). However, as shown in Figure 1E, the CTR2/CTR1 ratio was significantly increased in the chemoresistant group compared to the chemosensitive group. Next, we compared the CTR expression among histological subtypes. CTR2 expression and the CTR2/CTR1 ratio were significantly increased for the clear cell subtype compared to other histological subtypes (Figure 2D and E), but there was no significant difference in CTR1 expression among histological subtypes (Figure 2C).

CTR expression in ovarian cancer cells correlates with survival period. CTR expression in ovarian cancer tissue and the patients' prognoses were examined. As shown in Figure 3A, there was no significant difference in the patients' prognoses between CTR1-positive and -negative groups. On the other hand, compared to the CTR2-negative group, the CTR2-positive group had a significantly shorter overall survival (Figure 3B). As shown in Figure 3C, the group with positive CTR2/CTR1 ratio had significantly shorter overall survival compared to the negative group.

Inhibition of CTR2 enhances the cisplatin sensitivity of ovarian cancer cells. The expression of CTR2 protein in two types of ovarian cancer cell lines, Caov3 and RMG1, was examined by immunofluorescence analysis. The CTR2 protein was expressed in both types of cells (Figure 4A and C). A 1-h incubation with 100 μM BCS resulted in almost total disappearance of CTR2 (Figure 4B and D). These effects caused by BCS on CTR2 expression have already been confirmed in other reports (18, 20). We studied whether the sensitivity of ovarian cancer cells to cisplatin would change due to CTR2 inhibition. When CTR2 expression in Caov3 cells was inhibited by BCS, their sensitivity to cisplatin was significantly enhanced (Figure 4E). Similarly, the sensitivity of RMG1 cells to cisplatin was significantly enhanced by exposure to BCS (Figure 4F).

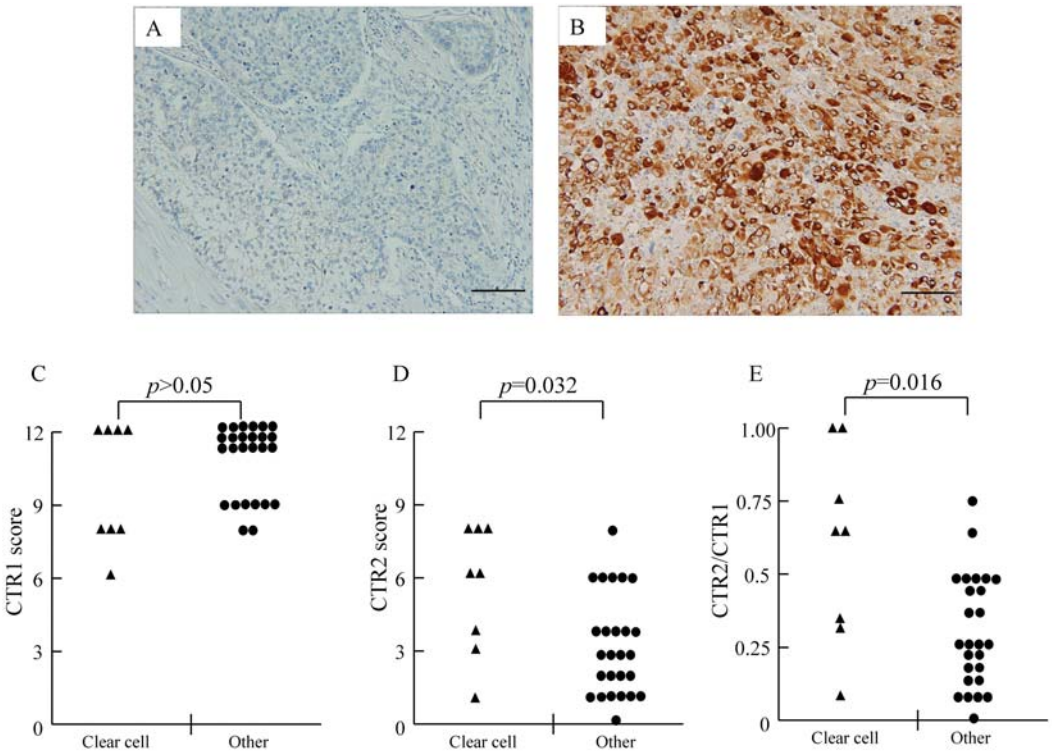

Figure 2. *A: Stage IIIc endometrioid cystadenocarcinoma. Very weak expression of copper transporter (CTR)-2 was observed in ovarian cancer tissues. B: Stage IIc clear cell adenocarcinoma. Strong CTR2 expression can be seen in tumor cells (original magnification, ×200, scale bar=100 μm). C: There was no difference in CTR1 expression between the clear cell sub-type and other histological sub-types. D: CTR2 expression was significantly increased in the clear-cell sub-type compared to other histological sub-types. E: The CTR2/CTR1 ratio was significantly increased in the clear-cell sub-type compared to other histological sub-types.*

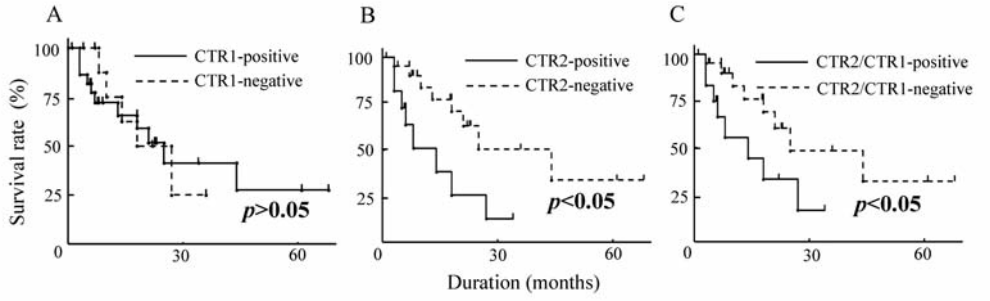

Figure 3. *A: Kaplan-Meier survival analysis showing that there was no significant difference in the overall survival between copper transporter (CTR)-1-positive and -negative groups of patients with ovarian cancer. B: The CTR2-positive group had a significantly shorter overall survival compared to the CTR2-negative group. C: The group with positive CTR2/CTR1 ratio had a significantly shorter overall survival compared to the negative group.*

Discussion

In this study, we have shown that CTR2 expression in ovarian cancer tissues was increased in patients who showed resistance to platinum-based chemotherapy, but there was no significant difference. We suggest that the CTR2 result is not significant because of the small number of patients. On the other hand, there was no difference in CTR1 expression between the chemotherapy-resistant and -sensitive groups. Therefore, in this study, CTR1 is not a good indicator of chemosensitivity in patients with ovarian cancer. In contrast, CTR2 is a useful, but somewhat weak indicator of chemosensitivity. However, the CTR2/CTR1 ratio was significantly increased in the chemoresistant group

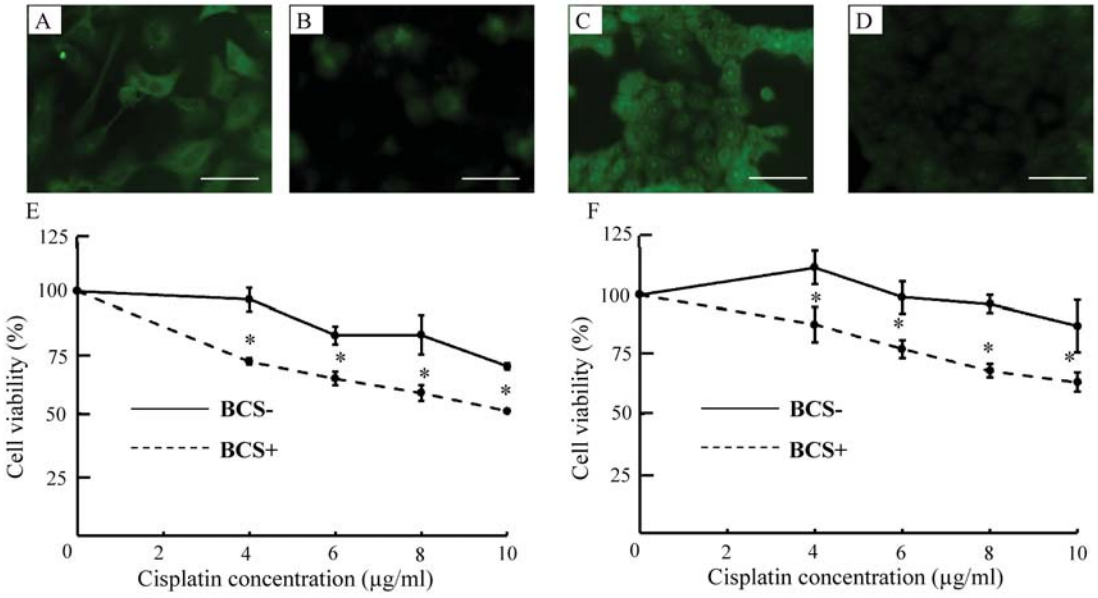

Figure 4. *The expression of copper transporter (CTR)-2 protein in Caov3 (A) and RMG1 (C) cell was confirmed by immunofluorescence analysis. One-hour exposure to 100 μM bathocuproine disulfonate (BCS) resulted in almost total disappearance of CTR2 in Caov3 (B) and RMG1 (D) cells (original magnification, ×400, scale bar=50 μm). When CTR2 expression was inhibited by BCS, the sensitivity to cisplatin of Caov3 (E) and RMG1 (F) cells was significantly enhanced. Bars=SD. *p<0.05 compared with cells not exposed to BCS (Student's t-test).*

compared to the chemosensitive group and may, thus, be a sensitive and useful indicator of chemosensitivity in patients with ovarian cancer. In addition, we have also shown that inhibition of CTR2 in ovarian cancer cell lines leads to increased sensitivity to cisplatin, which supports the results obtained in patients with ovarian cancer. The precise role of CTR2 in platinum resistance is still unclear, but as suggested in previous reports (16, 17), CTR2 may contribute to cisplatin resistance of ovarian cancer by reducing the cellular accumulation of cisplatin. Further studies are now on-going in our laboratory. Moreover, the patients who were positive for CTR2 expression and had a positive CTR2/CTR1 ratio had significantly poorer prognoses than did the patients who were negative for CTR2 and had a negative CTR2/CTR1 ratio. Chemotherapy resistance caused by CTR2 expression may contribute to a worse prognosis for patients with ovarian cancer. These findings indicate that CTR2 has an important role in platinum resistance in ovarian cancer and that the CTR2/CTR1 ratio is a potential prognostic factor in ovarian cancer.

In this study, CTR1 was expressed in almost all ovarian cancer tissues, and there was no correlation between the chemotherapy response and CTR1 expression. Several reports have suggested that CTR1 is associated with sensitivity to platinum-based chemotherapy, however, almost all reports were based on *in vitro* or animal studies (14, 21). Only a few reports have been published on the relation between chemotherapy resistance in patients with ovarian cancer and CTRs. Lee *et al*. demonstrated that expression of CTR1, not CTR2, was associated with chemosensitivity and good prognosis of patients with ovarian cancer (22). The discrepancy from our results may be caused by differences in the experimental procedure and the criteria for patient inclusion. Lee *et al*. evaluated only mRNA expression and the serous type of ovarian cancer. On the other hand, we evaluated CTR expression at the protein level and in all histological types of ovarian cancer.

Clear-cell adenocarcinoma is known to be resistant to platinum-based chemotherapy (23). Several mechanisms have been suggested for this drug resistance (24), such as enhanced drug detoxification and increased DNA repair activity (25, 26), however, the exact mechanisms are still unclear. In this study, clear cell adenocarcinoma showed increased expression of CTR2 and a higher CTR2/CTR1 ratio compared with other histological subtypes. Although further studies are needed to elucidate the details of the mechanism involved in this event, to our knowledge, this is the first report indicating an association between CTRs and chemoresistance of clear-cell adenocarcinoma. In this study we have confirmed that CTR2 appears to contribute to platinum resistance in patients with ovarian cancer.

The prognosis for patients with ovarian cancer has shown a tendency towards improvement with the development of novel treatments. However, the long-term prognosis is still poor. With further research on CTRs, it is expected that new strategies for diagnosis and treatment of ovarian cancer will emerge.

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