Showa Univ J Med Sci **25**(2), 109～118, June 2013

Original

Overexpression of DNA Polymerase ζ Affects Cisplatin Resistance in Ovarian Cancer : An Immunohistochemical Study

Minoru NAGASHIMA[1)], Tsuyoshi OKUDA[1)], Masaaki NAGATSUKA[1)], Miki KUSHIMA[2)], Takahiko TONOIKE[2)] and Takashi OKAI[1)]

Abstract : DNA polymerase ζ (Pol ζ) participates in translesional bypass replication. Pol ζ has been shown to be an important contributor to *cis*-diamminedichloroplatinum (II) (DDP ; cisplatin) -induced genomic instability and the subsequent emergence of resistance *in vitro*. We immunohistochemically examined the expression of Pol ζ in ovarian cancer tissues to determine whether its expression affects the DDP resistance of human ovarian cancers and also to determine whether Pol ζ expression is a prognostic factor for ovarian cancers. We assessed 76 archival, formalin-fixed, paraffin-embedded tissue samples obtained from patients with epithelial ovarian cancers who underwent their first operation between 2003 and 2011. An ovarian cancer tissue array was also used in this study. Immunohistochemical staining of Pol ζ was performed using an anti-human Pol ζ monoclonal rabbit antibody. The strength of expression of Pol ζ was compared with the DDP resistance and clinical features of the study population. The Pol ζ over-expression in ovarian cancer tissue which compared with epithelial cells in normal ovaries was not affected by the histological types, FIGO stage, or patient age, but Pol ζ was significantly more overexpressed in the DDP-resistant group than in the DDP-sensitive group (P=0.043). Pol ζ over-expression did not significantly affect the survival rate of the ovarian cancer patients ; however, the Pol ζ positive group tended to have a poorer long-term prognosis. In conclusion, ovarian carcinoma patients with Pol ζ over-expression are likely to be resistant to DDP, especially in cases of recurrent disease. These results confirm the previous findings *in vitro*, wherein Pol ζ modulated the cytotoxicity and mutagenicity of DDP.

Key words : DNA polymerase ζ, ovarian cancer, drug resistance

Introduction

Cis-diamminedichloroplatinum (II) (DDP ; cisplatin) is one of the major chemotherapeutic agents widely used for the treatment of a broad range of malignant diseases, including testicular, ovarian, lung, and bladder cancers[1)]. However, the use of DDP is sometimes limited by its severe side effects and the development of resistance. DDP exerts antitumor effects by forming

[1)] *Department of Obstetrics and Gynecology, Showa University School of Medicine,* 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8666, Japan.
[2)] *Department of Pathology Clinico-diagnostic Pathology, Showa University School of Medicine.*

intra-strand and inter-strand cross-links in DNA. The most abundant lesions produced in DNA by DDP are intra-strand cross-links, which are believed to be important to both the cytotoxicity and the mutagenicity of the drug[2]. When the platinum combines with DNA, some cells undergo apoptosis, but some cells are able to repair the DNA and continue proliferating.

DNA damage induced by both exogenous and endogenous insults is usually repaired by various DNA repair mechanisms[3] before DNA replication is initiated. However, when the DNA lesions cannot be completely repaired[4], the major replicative DNA polymerases are unable to carry out translesional synthesis. The mutations generated during translesional synthesis are thought to contribute to malignant transformations[5]. A family of DNA polymerases that can mediate such translesion bypass synthesis has been identified in mammalian cells, and includes Polymerase ζ (Pol ζ), Pol η, Pol κ, Pol μ, and Pol ι[6]. Multiple translesion DNA synthesis (TLS) polymerases are implicated in the lesion bypass of DNA intra-strand cross-links, including those generated by DDP. Replicative bypass of DDP adducts requires the cooperative actions of at least three TLS Pol isoforms: Pol η, REV1, and Pol ζ[7–16]. A reduction in Pol ζ or REV1 function renders cells more sensitive to the cytotoxic effects of DDP, and also markedly decreases its mutagenicity *in vitro*[11, 14]. These results suggest that Pol ζ or REV1 or both are responsible for the ability of cells to replicate their DNA and survive in the presence of a large DDP adduct load and that TLS is important for the mutagenicity of DDP and its ability to generate drug-resistant variants in the surviving population of cells[11]. However, the function of the bypass polymerase involved in TLS in human cancer tissues remains poorly understood.

In the present study, we evaluated the localization of Pol ζ in ovarian cancer tissues, and examined the relationship between Pol ζ and the clinical outcome of ovarian cancer patients.

Materials and Methods

Patients and Tissue Samples.

In this prospective study, patients with ovarian cancer were eligible for inclusion if they underwent cytoreductive surgery at Showa University Hospital between 2003 and 2011 (76 cases). This study was approved by the Ethics Committee of Showa University of Medicine. The subjects included 23 cases of serous cystoadenocarcinoma, 9 cases of mucinous cystoadenocarcinoma, 12 cases of endometrioid adenocarcinoma, 23 cases of clear cell adenocarcinoma, and 9 cases of the other kinds of ovarian cancer. Tissue samples were obtained during surgery and processed into paraffin-embedded blocks. In all cases, hematoxylin-and-eosin–stained slides were examined to confirm the original diagnosis and to select paraffin blocks with representative tumor tissue.

The clinical stages of ovarian cancer were determined according to the FIGO staging system. The numbers of patients with each surgical stage of ovarian cancer were as follows: stage Ⅰ, 23 cases; stage Ⅱ, 3 cases; stage Ⅲ, 40 cases; stage Ⅳ, 10 cases. Follow-up information on patient survival was obtained from the beginning of the study until November 30, 2012, via direct contact with the patients or their families. According to the NCCN Clinical Practice Guidelines in Ovarian Cancer, the recurrent cases were classified into two groups depending on the length

of time that had elapsed after platinum-based chemotherapy. The patients who developed recurrent tumors within a year after platinum-based chemotherapy were defined as platinum-resistant, and the patients who did not possess recurrent tumors were defined as platinum-sensitive[17]. Only the cases with complete cytoreductive surgery or optimal cytoreductive surgery were selected in order to provide a more precise understanding of the effect of chemotherapy. After primary cytoreductive surgery, all patients received platinum-based chemotherapy.

Histology

The histopathological diagnosis was based on the World Health Organization (WHO) criteria[21]. The classification was performed using 10 microscopic fields (magnification, ×200) from different parts of the primary tumor.

Immunohistochemical Analysis

The expression of Pol ζ was determined in cases that could be examined by immunohistochemical staining. Formalin-fixed, paraffin-embedded tissue was cut into 3-μm sections. Tissue samples were stained on an autostainer (BenchMark LT, Ventana; Tucson, Arizona, USA) with all steps performed at room temperature. Antigen retrieval was performed in CC1 buffer for 60 min before immunostaining. A monoclonal rabbit anti-human DNA polymerase ζ (Assay Biotechnology; Sunnyvale California, USA) (1 : 100 dilution) was used as the primary antibody and tissue slides were incubated in this antibody for 32 min. These sections were further incubated with an iVIEW DAB universal kit. Positive and negative staining controls were included in each staining run. Negative control sections had not been exposed to the primary antibody. In all positive controls, the Pol ζ staining was localized to the cell cytoplasm and nucleus.

Staining was assessed using a consulting microscope (Model BX50; Olympus, Tokyo, Japan), by three of the authors (MN, TO, MK), none of whom had prior knowledge of the clinical or follow-up data of the patients. The relationships between these results and the clinicopathological variables were analyzed.

Positive staining for Pol ζ was expressed as the percentage of positive cells on the whole surface of the slide, and was semiquantified according to the following grading system: the extent of the area stained in the cancer tissues was scored as 0 (< 25%), 1 (25%～49%), 2 (50%～74%), or 3 (≧75%), where 0 and 1 were deemed negative and 2 and 3 were considered to be positive.

Statistical evaluation

The statistical analysis was performed with the SPSS Statistics 20 software program (SPSS IBM; Chicago, IL, USA). Associations were tested by Fisher's exact test, the Chi-square test, or the Wilcoxon signed-rank test. Survival curves were generated according to the Kaplan-Meier method. Statistical significance was established at P values less than 0.05. All P values were calculated by the logrank test.

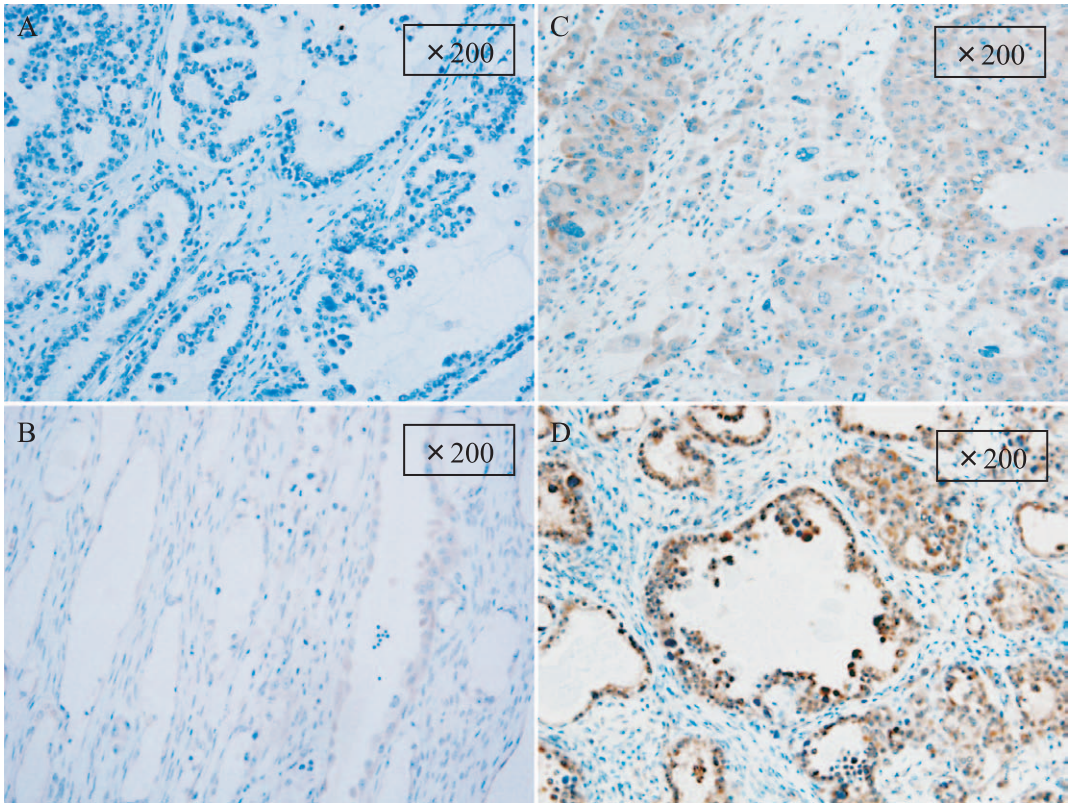

Fig. 1. Examples of immunohistochemical analysis of Pol ζ expression in paraffin-embedded samples of human ovarian clear cell carcinoma. A, score 0; B, score 1; C, score 2; and D, score 3. Magnification, ×200

Table 1. Positive staining rate for Pol ζ in each histological type (N = 67)

Histological type	Positive staining rate	Number
Serous adenocarcinoma	78%	23
Mucinous adenocarcinoma	67%	9
Endometrioid adenocarcinoma	83%	12
Clear cell adenocarcinoma	57%	23

Results

Fig. 1 A～D show representative specimens of ovarian clear cell adenocarcinoma obtained from 45-year-old, 68-year-old, 32-year-old, and 41-year-old patients, respectively. The immunohistochemical staining of Pol ζ was localized in the cytoplasm and was weak in the nucleus of carcinoma cells. The specimens (A to D) were scored 0 to 3, respectively.

Pol ζ positive cells were present in 78% of serous adenocarcinomas, 67% of mucinous adenocarcinomas, 83% of endometrioid adenocarcinomas, and 57% of clear cell adenocarcinomas (Table 1). There were no statistically significant differences in the prevalence of Pol ζ staining

Table 2. Positive staining rate for Pol ζ at each FIGO stage. (N = 96)

Stage	Positive staining rate	Number
I	61%	23
II	67%	3
III	70%	40
IV	80%	10

Table 3. Positive staining rate for Pol ζ at each age. (N = 76)

Age	Positive staining rate	Number
30's	67%	6
40's	61%	18
50's	65%	26
60's	81%	16
70's	70%	10

among the different types of ovarian cancer by Fisher's exact test.

No significant association was observed between the expression of Pol ζ and FIGO stage or patient age by Fisher's exact test (Tables 2 and 3, respectively). In contrast, when cases of recurrence were evaluated, the DDP-resistant group had a higher positive staining rate than the DDP-sensitive group by the Chi-square test ($P = 0.043$; Table 4).

In patients who underwent surgery after recurrence, we examined the positive staining rate of the surgical specimens from both the primary debulking surgery (PDS) and secondary debulking surgery (SDS). There were no significant differences in the prevalence of Pol ζ staining between the PDS (75% Pol ζ positive) and SDS (100% Pol ζ positive) samples by the Wilcoxon signed-rank test (Table 5).

We examined the prognoses of the cases in relation to the differences in the staining for Pol ζ. Only the cases with complete cytoreductive surgery or optimal cytoreductive surgery were evaluated. The study was carried out separately for patients with early stage (stage I/II) and advanced stage (stage III/IV) disease. There were no significant differences in the progression-free survival and overall survival between the Pol ζ positive group and negative group, regardless of the stage (Fig. 2).

Discussion

The mechanism (s) by which cells become resistant to platinum-containing chemotherapeutic agents are still poorly defined. However, the incidence of drug-resistant strains increases when the drug is administered repeatedly to the same cells in experiments using cell lines. The DDP-

Table 4. Clinical background of the DDP-sensitive and -resistant patients with recurrent ovarian cancer

	DDP-sensitive group (N = 11)	DDP-resistant group (N = 19)	*P* value*
Age (years)	52.1 ± 7.1	56.7 ± 10.6	0.22
Height (cm)	152.7 ± 6.7	154.7 ± 5.5	0.38
Weight (kg)	56.5 ± 14.0	50.0 ± 7.9	0.12
BMI	24.3 ± 6.2	20.9 ± 3.0	0.052
CA125 (U/ml)	1464 ± 1720	2429 ± 4083	0.48
(before treatment)			
Stage			
__ I	18.2 % (2)	10.5% (2)	0.61
__ II	0	5.3% (1)	> 0.99
__III	72.7 % (8)	63.2% (12)	0.70
__IV	9.1% (1)	21.1% (4)	0.63
Pathology			
__Serous	36.4% (4)	42.1% (8)	> 0.99
__Mucinous	9.1% (1)	5.3% (1)	> 0.99
__Endometrioid	0	5.3% (1)	> 0.99
__Clear cell	27.3% (3)	26.3% (5)	> 0.99
__Others	27.3% (3)	21.1% (4)	> 0.99
NAC	54.5% (6)	26.3% (5)	0.24
Surgery			
__Complete	9.1% (1)	21.1% (4)	0.63
__Optimal	90.9% (10)	78.9% (15)	0.62
Positive			
staining rate	30.0% (3)	75.0% (10)	0.043

*Chi-square test.

resistant phenotype is the result of genetic changes, and it is known that the TLS pathway plays a role in producing these genetic changes. Specifically Pol ζ or REV1 or both is considered to be important in this pathway in human cells[11, 14, 18]. Disabling REV1 function slowed the emergence of resistance to DDP in a human ovarian carcinoma line[14]. Similar changes in phenotype have been observed in a human fibroblast line expressing antisense RNA to *hREV3*, the catalytic subunit of Pol ζ[11]. However, the role of TLS function in DDP resistance in human carcinoma tissue is still poorly understood. The results of the present study indicate that Pol ζ plays a role in the resistance of human ovarian carcinoma to DDP.

The cytotoxicity of DDP to cells *in vitro* is proportional to the extent of DDP adduct

Table 5. Differences in the positive staining score for Pol ζ between primary debulking surgery (PDS) and secondary debulking surgery (SDS).

Cases	PDS (score)	SDS (score)	Number of chemotherapy cycles
1	Positive (2)	Positive (2)	4
2	Positive (3)	Positive (3)	6
3	Positive (3)	Positive (2)	6
4	Negative (1)	Positive (2)	6
5	Positive (3)	Positive (2)	6
6	Positive (3)	Positive (2)	6
7	Negative (1)	Positive (2)	4
8	Positive (2)	Positive (2)	6
9	Positive (2)	Positive (3)	12
10	Positive (2)	Positive (2)	11
11	Negative (0)	Positive (2)	9
12	Positive (2)	Positive (2)	8

The extent of the area stained in the cancer tissues was scored as 0 (< 25%), 1 (25%～49%), 2 (50%～74%), or 3 (≧75%), where 0 and 1 were deemed negative and 2 and 3 were considered to be positive.

formation in DNA, and cells with defects in nucleotide excision repair, the major DNA repair mechanism that removes these adducts, are hypersensitive to DDP[19, 20]. Impairment of *hREV1* or Pol ζ function in cells causes an increase in sensitivity to the cytotoxic effect of DDP[14].

Clinically, the effect of platinum-containing drugs in ovarian cancer is dependent on the different histological types. Usually, serous and endometrioid adenocarcinomas are comparatively sensitive to DDP, whereas mucinous and clear cell adenocarcinomas are resistant to DDP. In the current study, the Pol ζ overexpression in ovarian cancer tissue was not related to the histological types. This suggests that the Pol ζ-related error-prone replication system is not involved in the differences in the sensitivity of the different histological types of ovarian cancers to DDP.

In recurrent ovarian cancers, the response rate to chemotherapy is correlated with the disease-free interval. We compared the surgical specimens of PDS and SDS from the same cases, all of whom had relapsed after chemotherapy, in order to investigate the acquisition of drug resistance of the tissues after chemotherapy. We found that the positive staining rate for Pol ζ was 100% (all 12 cases) in the surgical specimens of SDS. Because 9 of these cases were also positive in the PDS specimens, there was no statistically significant difference in the positive staining rate of the two groups; however, the fact that all of the SDS specimens were positive suggested the possibility that the exposure to chemotherapy was involved in the acquisition of drug resistance.

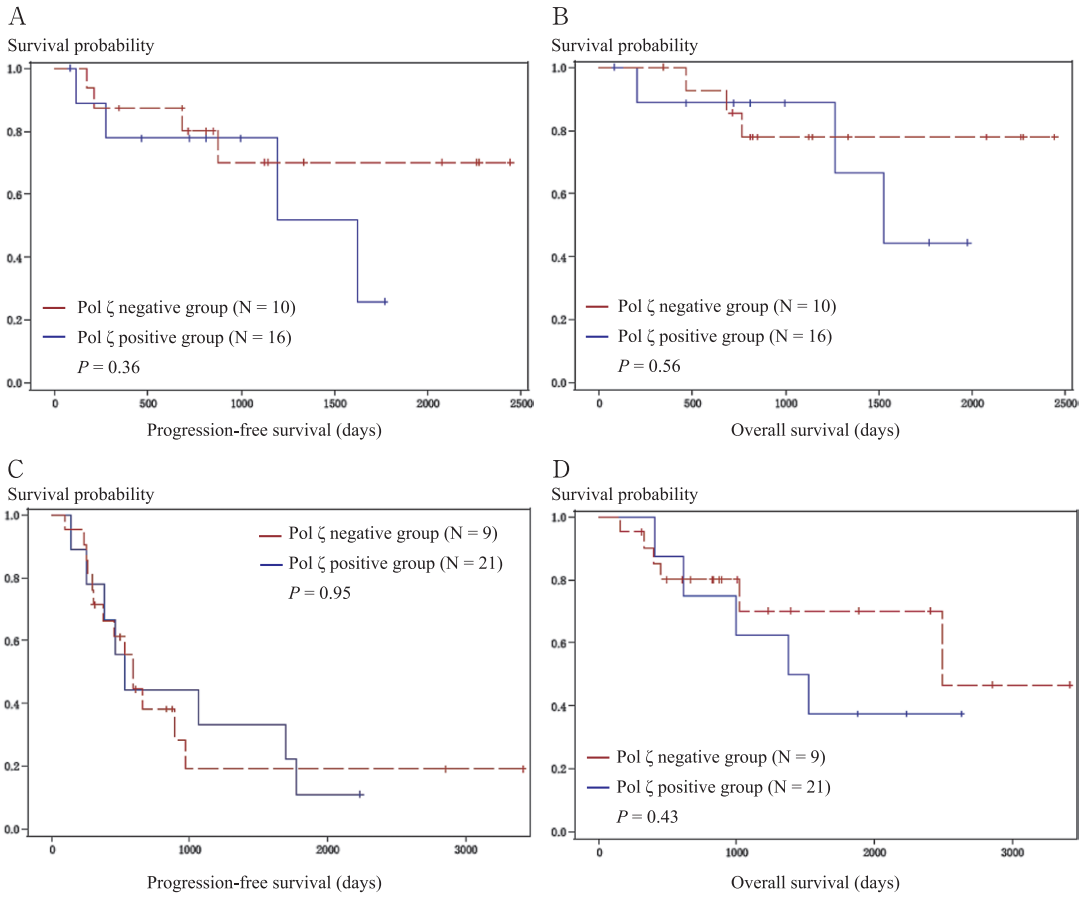

Fig. 2. Kaplan Meier Curves for progression-free survival and overall survival comparing Pol ζ positive group versus Pol ζ negative group.
A, B. Patients with early stage cancer (stages Ⅰ and Ⅱ) analyzed for progression-free survival (A) and overall survival (B). C, D. Patients with advanced stage cancer (stages Ⅲ and Ⅳ) analyzed for progression-free survival (C) and overall survival (D).

In the current study, the Pol ζ over-expression in the DDP-resistant group was significantly higher than that in the DDP-sensitive group. However, Pol ζ over-expression was not related to FIGO staging. The present results support those of *in vitro* studies, in which Pol ζ modulated the cytotoxicity and mutagenicity of DDP[11].

We examined the association between the clinical variables and Pol ζ expression to assess its potential prognostic significance. There were no significant differences between the clinical variables with regard to the Pol ζ expression in the ovarian cancer patients. Moreover, Pol ζ over-expression did not significantly affect the survival of the ovarian cancer patients. However, the Pol ζ-positive group tended to have a poorer long-term prognosis. This is the only study to analyze Pol ζ protein in human tissue. For the detailed understanding of the TLS pathway in the DDP resistance in human, further studies including REV1 or related TLS pathway proteins are required.

In conclusion, ovarian carcinoma patients with Pol ζ over-expression are considered likely

to have resistance to DDP, especially in the recurrent state. This is the first report of the Pol ζ-related error-prone replication system in human tissues of cancer patients. Based on our present findings and those of previous studies, Pol ζ is an attractive target for therapeutic investigation to simultaneously enhance DDP sensitivity and reduce the risk of developing drug resistance. Studies are now needed to examine how the expression of Pol ζ varies among different types of tumors and whether its expression is linked to the clinical response.

Conflict of interest

The authors have declared no conflict of interest.

References

1) Kelland L. The resurgence of platinum-based cancer chemotherapy. Nat Rev Cancer. 2007;**7**:573-584.
2) Lin X, Trang J, Okuda T, *et al*. DNA polymerase zeta accounts for the reduced cytotoxicity and enhanced mutagenicity of cisplatin in human colon carcinoma cells that have lost DNA mismatch repair. Clin Cancer Res. 2006;**12**:563-568.
3) Lindahl T, Wood RD. Quality control by DNA repair. Science. 1999;**286**:1897-1905.
4) Hicks JK, Chute CL, Paulsen MT, *et al*. Differential roles for DNA polymerases eta, zeta, and REV1 in lesion bypass of intrastrand versus interstrand DNA cross-links. Mol Cell Biol. 2010;**30**:1217-1230.
5) Nelson JR, Lawrence CW, Hinkle DC. Thymine-thymine dimer bypass by yeast DNA polymerase zeta. Science. 1996;**272**:1646-1649.
6) Friedberg EC, Feaver WJ, Gerlach VL. The many faces of DNA polymerases: strategies for mutagenesis and for mutational avoidance. Proc Natl Acad Sci U S A. 2000;**97**:5681-5683.
7) Simpson LJ, Sale JE. Rev1 is essential for DNA damage tolerance and non-templated immunoglobulin gene mutation in a vertebrate cell line. EMBO J. 2003;**22**:1654-1664.
8) Sonoda E, Okada T, Zhao GY, *et al*. Multiple roles of Rev3, the catalytic subunit of polzeta in maintaining genome stability in vertebrates. EMBO J. 2003;**22**:3188-3197.
9) Bassett E, King NM, Bryant MF, *et al*. The role of DNA polymerase eta in translesion synthesis past platinum-DNA adducts in human fibroblasts. Cancer Res. 2004;**64**:6469-6475.
10) Niedzwiedz W, Mosedale G, Johnson M, *et al*. The Fanconi anaemia gene FANCC promotes homologous recombination and error-prone DNA repair. Mol Cell. 2004;**15**:607-620.
11) Wu F, Lin X, Okuda T, *et al*. DNA polymerase zeta regulates cisplatin cytotoxicity, mutagenicity, and the rate of development of cisplatin resistance. Cancer Res. 2004;**64**:8029-8035.
12) Albertella MR, Green CM, Lehmann AR, *et al*. A role for polymerase eta in the cellular tolerance to cisplatin-induced damage. Cancer Res. 2005;**65**:9799-9806.
13) Nojima K, Hochegger H, Saberi A, *et al*. Multiple repair pathways mediate tolerance to chemotherapeutic cross-linking agents in vertebrate cells. Cancer Res. 2005;**65**:11704-11711.
14) Okuda T, Lin X, Trang J, *et al*. Suppression of hREV1 expression reduces the rate at which human ovarian carcinoma cells acquire resistance to cisplatin. Mol Pharmacol. 2005;**67**:1852-1860.
15) Chen YW, Cleaver JE, Hanaoka F, *et al.* A novel role of DNA polymerase eta in modulating cellular sensitivity to chemotherapeutic agents. Mol Cancer Res. 2006;**4**:257-265.
16) Doles J, Oliver TG, Cameron ER, *et al*. Suppression of Rev3, the catalytic subunit of Pol{zeta}, sensitizes drug-resistant lung tumors to chemotherapy. Proc Natl Acad Sci U S A. 2010;**107**:20786-20791.
17) Chuang YT, Chang CL. Extending platinum-free interval in partially platinum-sensitive recurrent ovarian cancer by

a non-platinum regimen: its possible clinical significance. Taiwan J Obstet Gynecol. 2012;**3**:336–341.
18) Lin X, Okuda, Trang J, *et al*. Human REV1 modulates the cytotoxicity and mutagenicity of cisplatin in human ovarian carcinoma cells. Mol Pharmacol. 2006;**69**:1748–1754.
19) Damia G, Guidi G, D'Incalci M. Expression of genes involved in nucleotide excision repair and sensitivity to cisplatin and melphalan in human cancer cell lines. Eur J Cancer. 1998;**34**:1783–1788.
20) Furuta T, Ueda T, Aune G, *et al*. Transcription-coupled nucleotide excision repair as a determinant of cisplatin sensitivity of human cells. Cancer Res. 2002;**62**:4899–4902.
21) Scully RE. Surface epithelial-stromal tumours. In Histological typing of ovarian tumours. 2nd ed. Berlin: Springer; 1999. pp11–19.

[Received January 16, 2013 : Accepted January 25, 2013]