The Akt and ERK Activation by Platinum-based Chemotherapy in Ovarian Cancer is Associated with Favorable Patient Outcome

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Abstract. *Aim: To investigate the phosphatidylinositol 3-kinase (PI3K)-Akt and extracellular signal regulated protein kinase (ERK) activation by chemotherapy, and the relationship between the activation of them and patient outcomes. The effect of chemotherapy on the cell proliferation and apoptosis markers and their role in the biology of ovarian cancer were also investigated. Materials and Methods: This study was carried out in a series of 10 ovarian (or tubal) cancer patients whose specimens were obtained before and after chemotherapy. PI3K-Akt and ERK activation were evaluated by immunohistochemical staining for phosphorylated Akt and ERK. Their correlation with patient outcome was investigated by survival curves using the Kaplan-Meier method. Cell proliferation was evaluated by Ki-67 expression using immunofluorescent staining. Apoptosis was examined by caspase-3 and cleaved Poly ADP ribose polymerase (PARP) using immunofluorescent staining. Results: An increase in Akt and ERK phosphorylation after chemotherapy was observed in 5 and 8 patients, respectively out of 10 patients examined. Akt and ERK activation by chemotherapy were associated with a favorable overall survival. In almost all patients, Ki-67 expression was initially high and largely decreased after chemotherapy. An increase in apoptotic marker expression was observed in almost all patients exposed to chemotherapy. Conclusion: Our findings suggest that Akt and ERK activation by chemotherapy may be associated with favorable prognosis.*

Ovarian cancer is one of the most fatal malignancies in women and is the leading cause of gynecological cancer deaths (1). The current standard therapy in patients with advanced ovarian cancer is primary surgical cytoreduction followed by first-line chemotherapy based on platinum compounds (cisplatin or carboplatin) in combination with taxanes. Although the majority of patients with ovarian cancer respond to the initial chemotherapy, most eventually relapse during the course of treatment, and chemoresistance remains a major hurdle to successful therapy of this disease (2). Sensitivity to platinum is a strong predictor for the prognosis of patients with ovarian cancer (3). Thus, it is important to understand how cancer becomes platinum-refractory in order to develop better molecular targeting strategies.

There still remain unsolved questions concerning the cellular response to cisplatin. The link between adduct formation and triggering of signaling pathways, as well as the network of molecular events that determine whether cells grow or die, have not been fully elucidated. The balance between cellular survival and apoptosis can determine the sensitivity of tumor cells to chemotherapeutic agents. Several studies reported that DNA damage by chemotherapeutic agents results in the activation of several signaling pathways (4-6), including phosphatidylinositol 3-kinase (PI3K)/Akt and extracellular signal-regulated protein kinase 1 and 2 (ERK1/2). The PI3K/Akt pathway is thought to play a major role in the cell cycle (7), glycogen synthesis (8), cell death, and cell survival (9, 10). Overexpression of Akt in ovarian cancer is frequently associated with a poor prognosis and a more aggressive phenotype (11-13). The ERK cascade has been well characterized and is known to be involved in cell survival (14, 15). We have found that both the PI3K-Akt (16) and ERK (17) pathways are activated by cisplatin *in vitro* and *in vivo* (18), and are involved in resistance to cisplatin and paclitaxel (19). Cisplatin induces the phosphorylation of kinases in the Akt and ERK cascades, which may promote cell survival *via* inactivation of the proapoptotic proteins BAD (Bcl-2-associated death protein) and caspase-9 (20, 21).

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In contrast to the results of the *in vitro* studies, recent studies demonstrate that high ERK phosphorylation correlated with favorable prognosis in various types of cancer (22, 23) including ovarian (24). Another study in ovarian cancer has suggested that high Akt phosphorylation is associated with a trend for improved overall survival (25). These reports are results from specimens obtained at the initial surgery, and prior to an administration of chemotherapy. Thus, we examined whether the Akt and ERK pathways are activated by platinum-based chemotherapy, and if changes in Akt and ERK activation by chemotherapy affect the prognosis using clinical samples of ovarian cancer.

To our knowledge, this study is the first report to investigate the change in Akt and ERK phosphorylation as well as Ki-67 and apoptotic markers, such as caspase-3 and Poly ADP ribose polymerase (PARP), expression before and after chemotherapy in patients with ovarian cancers.

Materials and Methods

Patient selection. This study was carried out in ten histologically confirmed ovarian cancer patients treated at Yamagata University Hospital from 1999 to 2006 whose tumors were sampled both before and after chemotherapy. Tumor samples were collected with written informed consent and approval of the Ethics Committee of Yamagata University. The first sample was obtained at the first operation, and the second obtained at the second operation for recurring tumors (n=4), or from the residual lesion (interval debulking surgery) after the first-line of chemotherapy (n=6). All patients received platinum-based chemotherapy after the first laparotomy, and none had received chemotherapy or radiotherapy prior to the first chemotherapy. Fragments sampled from tumors were fixed in 10% buffered formalin and embedded in paraffin. In each case, hematoxylin and eosin-stained preparations were subjected to histopathological evaluation by a pathologist (H.T). The stage of the tumors was assessed according to FIGO criteria.

Materials. Anti-phospho-Akt and anti-phospho-ERK antibodies, phospho-Akt blocking peptide, and FITC-conjugated anti-Ki-67 antibody were obtained from Cell Signaling (Beverly, MA, USA). Anti-PARP and anti-caspase-3 antibodies were purchased from Promega (Madison, WI, USA).

Immunohistochemical staining. Paraffin-embedded tissue sections were stained with phospho Ser473-specific anti-Akt antibody with or without phospho-Akt (Ser473) blocking peptide, or with phospho-ERK antibody.

Scoring of immunohistochemical staining. The scoring system used in the study was previously described (26, 27). Briefly, the intensity of immunostaining for Akt and ERK phosphorylation was scored as 0 (no signal), +1 (weak), +2 (moderate), and +3 (strong). The +1 pattern was defined as weak, homogeneous cytoplasmic positivity without a granular staining pattern. The +2 and +3 patterns both had strong granular cytoplasmic staining with the +2 having <20% of tumor cells and the +3 >20% of tumor cells. Staining was independently scored by two investigators (O.T. and T.T.) who were blinded to the clinical information. Equivocal or borderline cases were re-examined, and a consensus score was reached between observers. When assessing variables for a given tumor, each observer was blinded to the scoring of the other observer and to the clinical outcome.

Immunofluorescent staining. Paraffin-embedded tissue sections were deparaffinized in xylene and then rehydrated through alcohol. Following deparaffinization, sections were placed in trisodium citrate buffer (0.1 M trisodium citrate and 0.1 M trisodium citrate dehydrate) and heated twice for 10 minutes in a 1 kW microwave oven for antigen retrieval. Endogenous peroxidase activity was blocked by incubation for 20 minutes with H_2O_2 solution in methanol (0.01 M). Non-specific binding was quenched by 20 minutes incubation in 3% skimmed milk powder in phosphate-buffered saline (PBS). After an additional wash with PBS, sections were incubated with the primary antibodies overnight at 4˚C. Anti-PARP and anti-caspase-3 antibodies were used to assess apoptosis. After washing, sections were incubated with Alexa Fluor 488-labeled goat anti-rabbit antibody (1:1,000) for 1 h at room temperature, followed by staining with propidium iodide (1 μg/ml) for 15 minutes at room temperature. Negative control samples exposed to the secondary antibody alone showed no specific staining (data not shown). For Ki-67 immunofluorescent staining, sections were incubated with FITC-conjugated anti-Ki-67 antibody overnight at 4˚C. Fluorescence was visualized using a fluorescence microscope (OLYMPUS). For the apoptosis and Ki-67 assays, three random fields per section were recorded at ×100 magnification, and stained cells were counted using Image J software (National Institutes of Health, USA). The number of PARP-, caspase-3- and Ki-67-positive cells were expressed as the percentage of total cells counted.

Statistics. Statistical analysis was performed using the Mann-Whitney *U*-Wilcoxon Rank Sum W test by suing StatView version 5.0 software (Abacus Concepts Inc, Berkeley, CA, USA). Survival curves were computed using the Kaplan-Meier method, and the log-rank test was used to assess statistical significance using the statistical package Graphpad Prism version 5.0 (GraphPad software Inc, La Jolla, CA, USA) $p<0.05$ was considered significant. Data are expressed as the mean±SE.

Results

Changes in Akt and ERK phosphorylation before and after platinum-based chemotherapy in ovarian cancer patients. Patient characteristics are described in Table I. The mean age of patients was 54 (range, 32 to 68 years). At the time of initial diagnosis, the stages were stage I (one patient), stage II (one patient), stage III (seven patients), and stage IV (one patient). Histology revealed the following findings: eight epithelial ovarian carcinomas and two fallopian adenocarcinomas; serous in six, mucinous in one, endometrioid in two, and clear cell in one patient.

Six patients underwent the first operation with a residual tumor of 1 cm in diameter (suboptimal operation), followed by platinum-based chemotherapy and the interval debulking surgery (patients no. 2, 3, 4, 5, 7, and 8) (Table II). The response to first-line chemotherapy was complete in patient

Table I. *Patient characteristics*.

Patient	Age	Histology	Stage	Primary debulking	Numbers of prior platinum-based chemotherapy treatments
1	67	Ovarian cancer, Serous G3	IIIc	Complete	9
2	54	Ovarian cancer, Serous G2	IV	Residual	3
3	32	Ovarian cancer, Endometrioid G2	IIIc	Residual	9
4	56	Fallopian adenocarcinoma, Endometrioid G3	IIIc	Residual	3
5	45	Ovarian cancer, Serous G3	IIb	Residual	3
6	42	Ovarian cancer, Clear cell adenocarcinoma	Ic	Complete	9
7	60	Ovarian cancer, Serous G3	IIIc	Residual	3
8	68	Ovarian cancer, Serous G2	IIIc	Residual	3
9	67	Fallopian adenocarcinoma, Serous G2	IIIc	Complete	6
10	49	Ovarian cancer, Mucinous G2	IIIc	Complete	6

no. 2 and partial in the other patients (no. 3, 4, 5, 7, and 8). Specimens were obtained at the time of initial and interval debulking surgery in these patients.

The other four patients underwent the first operation without residual tumors and subsequently received adjuvant platinum-based chemotherapy (no. 1, 6, 9, and 10). During the follow-up period, these patients had recurring tumors and underwent a second operation. Two patients received secondary platinum-based chemotherapy prior to the second operation (no. 1 and 6). The disease assessment for secondary chemotherapy was stable for patient no. 1 and progressive for no. 6. The other two patients (no. 9 and 10) received a second operation without secondary chemotherapy. Specimens were obtained at the initial and second operations for these four patients.

Figure 1A shows representative pictures of immunohistochemical staining for phospho-Akt and -ERK. Changes in the staining scores from pre- to post chemotherapy are shown in Figure 1B. While immunostaining scores of phospho-Akt after chemotherapy was increased in 5 patients, those of phosho-ERK after chemotherapy was increased in 8 patients. Although Akt phosphorylation was not significantly increased, ERK phosphorylation was significantly ($p<0.01$) increased after chemotherapy.

Association between Akt and ERK activation by platinum-based chemotherapy and patient outcome. We next examined the prognostic effect of the Akt and ERK activation by platinum-based chemotherapy. The median progression-free survival (PFS) was 16 months (range, 8-30 months) and the median overall survival (OS) was 33 months (range, 13-56 months). All patients had a relapse of ovarian cancer at the time of the last follow-up. The median PFS in the patients with Akt activation (patient no. 5, 6, 8, 9, and 10) (18 months) was significantly ($p=0.0342$) longer than that for those without (decrease: no. 3 or no change: no. 1, 2, 4, and 7) Akt activation (11 months) (Figure 2A). The median PFS in patients with ERK activation (patient no. 1, 4, 5, 6, 7, 8, 9, and 10) (17.5 months) tended to be longer ($p=0.0813$) than that for those without (decrease: no. 2 or no change: no. 3) ERK activation (10 months) (Figure 2B). We next analyzed the effect of Akt and ERK activation on OS. Among the ten patients, six had died at the time of the last follow-up. The median OS in patients with Akt activation (49.5 months) was significantly ($p=0.0022$) longer than those without Akt activation (28 months) (Figure 2C). The median OS in patients with ERK activation (43 months) was significantly ($p=0.0062$) longer than those without ERK activation (20.5 months) (Figure 2D). These results suggested that the Akt and ERK activation by platinum-based chemotherapy may be associated with favorable patient outcome.

Table II. *Summary of objective clinical response of tumors to chemotherapy and the change in Ki-67 and apoptotic markers expression in patients with residual tumors at initial surgery.*

Patient	Objective response	Ki-67	Cleaved caspase-3	Cleaved PARP
2	Complete	Large decrease	Large increase	Large increase
3	Partial	Large decrease	Increase	Small increase
4	Partial	Large decrease	Small increase	Increase
5	Partial	Large decrease	Small increase	Increase
7	Partial	Small decrease	Large increase	Increase
8	Partial	Large decrease	Increase	Increase

Ki-67 expression before and after platinum-based chemotherapy in ovarian cancer patients. We further examined the effect of platinum-based chemotherapy on cell proliferation by analyzing the expression of Ki-67, a cell proliferation-specific marker (28). Representative photographs of Ki-67 immunofluorescent staining are shown in Figure 3A. Before chemotherapy, nine patients showed high percentages (12 to 53%) of Ki-67-positive cells (Figure 3B). After chemotherapy,

the percentage of Ki-67-positive cells was significantly ($p<0.05$) reduced. In samples obtained from recurring lesions not exposed to chemotherapy (patients no. 9 and 10), the percentage of Ki-67-positive cells was higher than that at the primary surgery. Patient no.6, whose histology showed clear cell adenocarcinoma, had a notably low percentage of Ki-67 positive cells both pre- and post-chemotherapy.

Caspase-3 and PARP expression (apoptotic markers) before and after platinum-based chemotherapy in ovarian cancer patients. PARP is a caspase substrate; caspase-3 activation results in PARP cleavage. Cleaved PARP (molecular weight 85 kDa) has been established as a hallmark of apoptosis (29, 30). We investigated the effects of platinum-based chemotherapy on apoptosis *in vivo* by analyzing the expression of both caspase-3 and PARP. Representative photographs of immunofluorescent staining for cleaved caspase-3 are shown in Figure 4A. The percentages of cells positive for caspase-3 cleavage before and after chemotherapy are shown in Figure 4B. Before chemotherapy, the percentage of positive cells was low in all ten patients. After chemotherapy, expression of cleaved caspase-3 significantly ($p<0.05$) increased: expression was greatly increased in two patients (no. 2 and 7), mildly in three (no. 1, 3, and 8), and slightly in two (no. 4 and 5). In samples obtained from recurring lesions not exposed to secondary platinum-based chemotherapy (no. 9 and 10), the percentage of caspase-3-positive cells did not change. The percentage of caspase-3-positive cells was stably low at pre- and post-chemotherapy in patient no. 6, whose histology showed clear cell adenocarcinoma.

The expression of cleaved PARP was assessed by immunofluorescent staining, and representative photographs are shown in Figure 5A. Changes in staining intensity before and after chemotherapy are shown in Figure 5B. Before chemotherapy, the percentage of cells positive for cleaved PARP was low in all ten patients. After chemotherapy, the percentage of cleaved PARP-positive cells was significantly ($p<0.05$) increased: it increased greatly in patient no. 2, mildly in five patients (no. 1, 4, 5, 7, and 8), and slightly in patient No. 3. However, the percentage of cleaved PARP-positive cells did not change in three patients (no. 6, 9, and 10).

Objective clinical response to chemotherapy and changes in Ki-67 and apoptotic marker expression in patients with residual tumors. It is possible to accurately examine the relationship between the clinical response of tumors to chemotherapy and changes in Ki-67 and apoptotic marker expression in patients with a large (>1 cm) residual tumor at initial surgery. We investigated whether the changes in Ki-67 and apoptotic marker expression by chemotherapy are related to the clinical response of tumors to chemotherapy in these patients (Table II). All these patients showed that a decrease in the expression of Ki-67 and an increase in apoptotic markers, those were associated with complete or partial responses to the first platinum-based chemotherapy. These results suggested that changes in Ki-67 expression and apoptotic markers might be a predictive factor of sensitivity to chemotherapy.

Discussion

This is the first report to investigate the changes in Akt and ERK activation as well as cell proliferation and apoptosis using clinical samples of ovarian cancer before and after platinum-based chemotherapy. We found that Akt and ERK activation by chemotherapy may be associated with favorable patient outcome. In the present study, Akt was activated by the applied chemotherapy in five patients, and ERK was activated in eight patients. These findings were consistent with previous reports that cisplatin induces Akt and ERK phosphorylation *in vitro* (16, 17) and *in vivo* (18).

Another interesting finding of the present study is that the Akt and ERK activation by platinum-based chemotherapy may be associated with favorable prognosis in ovarian cancer. Several studies indicate that patients with high Akt phosphorylation showed a trend for improved OS in ovarian cancer (25) and ERK activation in effusions correlated with better OS (24).

Almost all patients had an initial high percentage of Ki-67-positive cells that remarkably deceased after the chemotherapy (Figure 3). This result is consistent with a previous finding that rapidly proliferating cells are sensitive to chemotherapy (31). Patients with large residual tumors (no. 2, 3, 4, 5, 7 and 8) had an initial high percentage of Ki-67 expression that decreased after the chemotherapy, and these patients also responded well to chemotherapy. Ki-67 expression did not decrease in recurring tumors not exposed to chemotherapy (no. 9 and 10). Patient no. 6 (clear cell adenocarcinoma) showed stably low Ki-67 expression but maintained disease progression after chemotherapy. This finding is consistent with previous reports of low proliferative activity for clear cell adenocarcinoma in spite of its more aggressive clinical course and poorer prognosis (31). These results suggest that initial high Ki-67 expression with reduction after chemotherapy may be indicative of a favorable outcome in ovarian cancer patients.

All patients had an initially low percentage of apoptotic cells that increased after chemotherapy (Figure 4 and 5). This finding is consistent with several reports that chemotherapeutic agents induce apoptosis in cancer cells (32, 33). Patients with large residual tumors (no. 2, 3, 4, 5, 7, and 8) had an initially low percentage of apoptotic cells that subsequently increased; these patients also responded well to chemotherapy. Patient no. 6, who showed disease progression after chemotherapy, also showed no change in

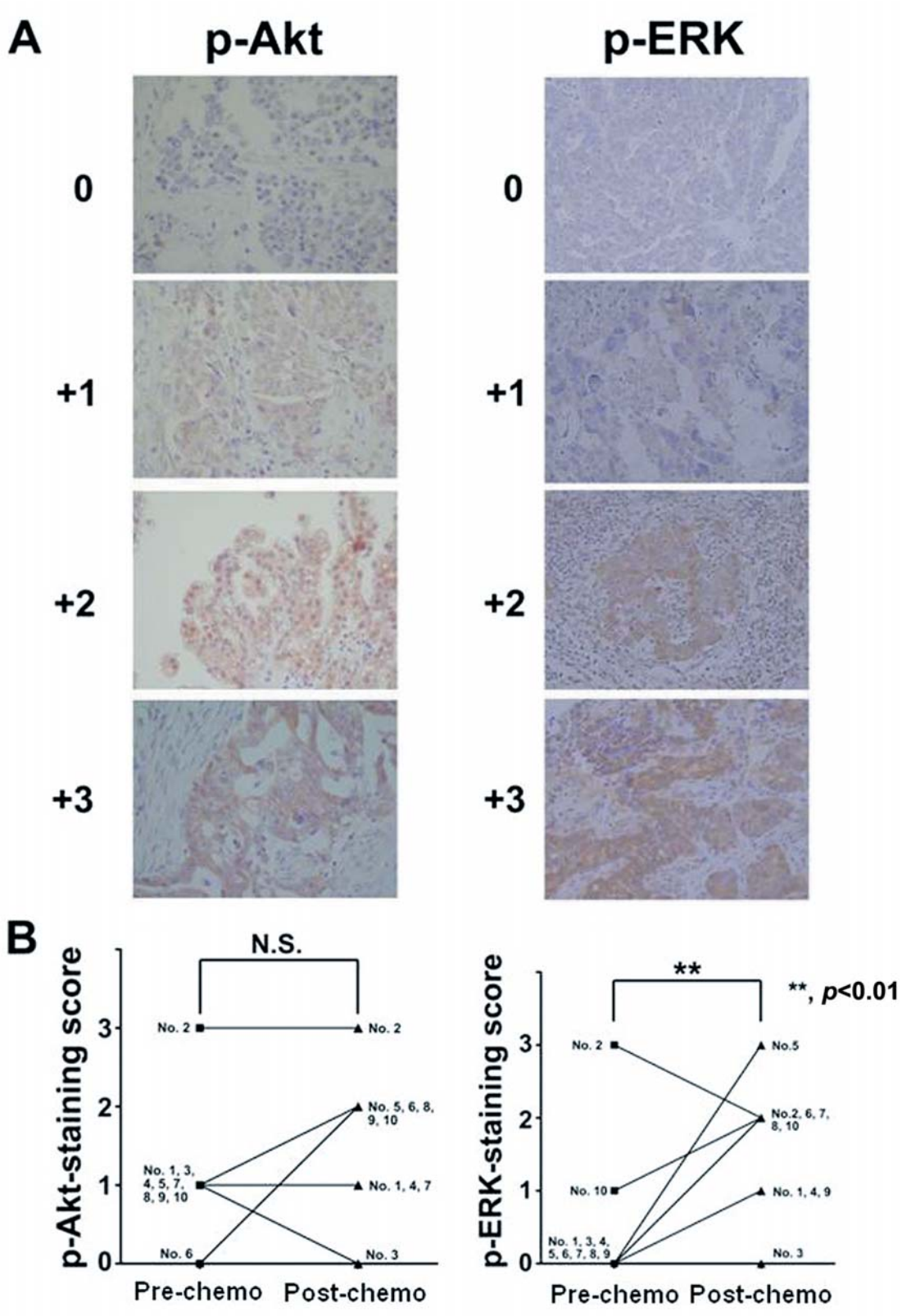

Figure 1. *Scoring of immunostaining intensity for phospho-Akt (p-Akt) and phospho-ERK (p-ERK) and changes in p-Akt and p-ERK staining intensity before and after chemotherapy. A, Representative images of staining intensity score (0, +1, +2, +3) for p-Akt and p-ERK are shown. Magnification, ×200. B, p-Akt and p-ERK staining scores before and after chemotherapy in each patient.*

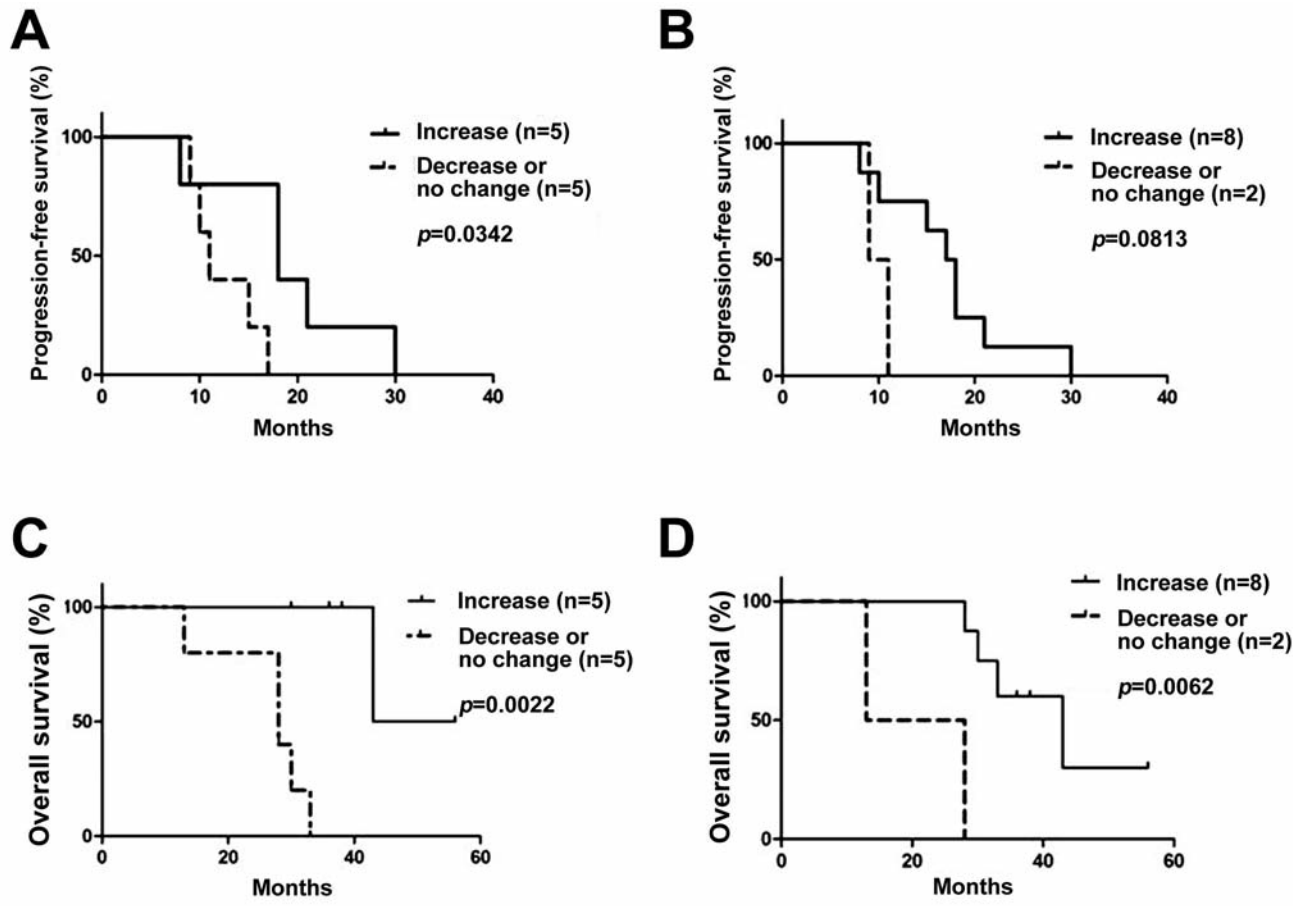

Figure 2. *The prognostic effects of the change in Akt and ERK activation by platinum-based chemotherapy on ovarian cancer patients. Kaplan-Meier plots of progression free survival of groups defined by change in Akt (A) and ERK (B) activation. Kaplan-Meier plots of overall survival of groups defined by change in Akt (C) and ERK (D) expression. The tumors were divided into two groups: with (increase) or without (decrease or no change) in Akt and ERK activation.*

the expression of apoptotic markers. These findings suggested a relationship between the changes in the expression of apoptotic markers by chemotherapy and the clinical response to chemotherapy.

Our novel finding is that Akt and ERK activation by chemotherapy induced apoptosis that was associated with favorable patient outcome using clinical samples. This is in contrast to the *in vitro* results of ours and other researchers (34, 35), which suggest that the Akt and ERK activation is involved in cell survival, a more aggressive phenotype and resistance to chemotherapy (11-18). However, recent studies demonstrated that Akt and ERK activation improved patient outcomes in breast (23, 36), endometrial (22, 37), lung (38) and ovarian (24, 25) cancer. Akt and ERK cascades are involved in both cell survival and apoptosis, and the role of these types of signaling may be different *in vitro* and *in vivo*. Recently, even *in vitro* study suggests that insulin-like growth factor-I inhibits cell growth in a lung cancer cell line with sustained Akt activation (39).

Several recent studies have shown that ERK activation induces the expression of the cell cycle inhibitor $p21^{WAF1/CIP}$ in various cellular models (40, 41) including ovarian cancer cells (42). Overexpression of $p21^{WAF1/CIP}$ enhances cisplatin-induced apoptosis through the induction of TNFRSF9 gene and activation of caspase-7 in human laryngeal squamous carcinoma cells (43). We also examined the effect of platinum-based chemotherapy on $p21^{WAF1/CIP}$ expression by immuno-histochemical staining. Expression of $p21^{WAF1/CIP}$ after chemotherapy was observed in three out of ten patients. An association among $p21^{WAF1/CIP}$ expression, ERK activation and an increase in apoptosis markers was confirmed in these patients (no. 1, 5, and 7, data not shown). The result suggests that ERK activation by chemotherapy may induce apoptosis *via* $p21^{WAF1/CIP}$ in human samples of ovarian cancer.

Although our findings have suggested several important factors for determining sensitivity to platinum-based chemotherapy in ovarian cancer patients, this study was

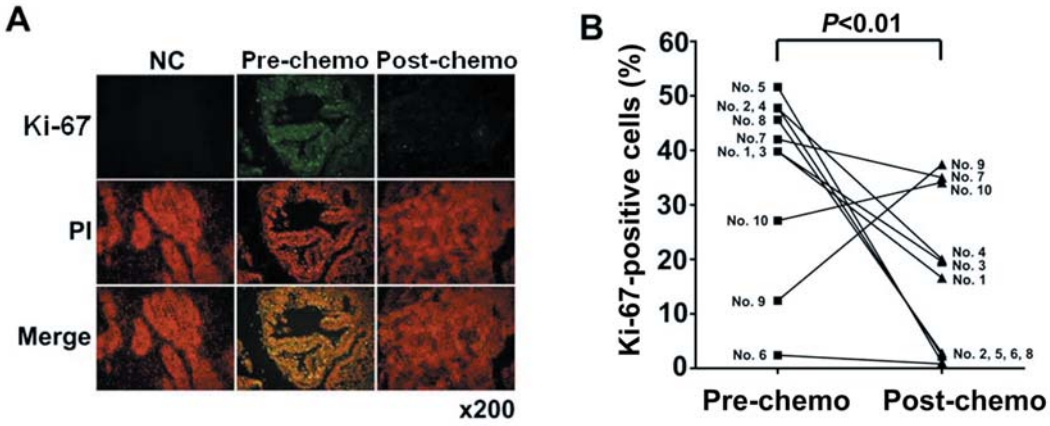

Figure 3. *The immunofluorescent staining and changes in the percentages of Ki67-positive cells before and after chemotherapy. A, Paraffin-embedded tissue sections were deparaffinized in xylene and double-stained with FITC-conjugated anti-Ki-67 antibody (green) and propidium iodide (PI) (red). Magnification, ×200. B, The number of Ki-67-positive cells is expressed as the percentage of total cells and shown for each patient pre- and post-chemotherapy.*

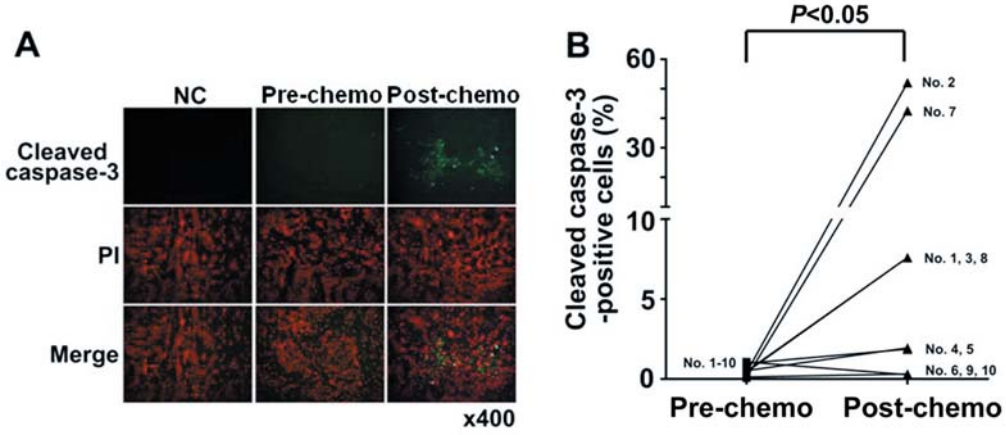

Figure 4. *Immunofluorescent staining and changes in the percentages of caspase-3-positive cells before and after chemotherapy. A, Paraffin-embedded tissue sections were deparaffinized in xylene and double-stained with anti-caspase-3 antibody followed by fluorescein (FITC)-conjugated goat anti-mouse IgG (green) and propidium iodide (PI) (red). Magnification, ×400. B, The number of caspase-3-positive cells is expressed as the percentage of total cells and shown for each patient pre- and post-chemotherapy.*

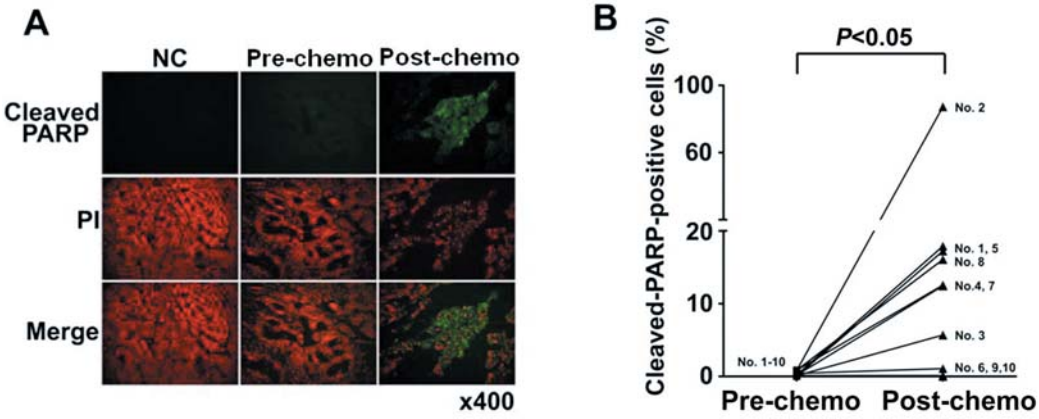

Figure 5. *Immunofluorescent staining and changes in the percentages of PARP-positive cells before and after chemotherapy. A, Paraffin-embedded tissue sections were deparaffinized in xylene and double-stained with anti-PARP antibody followed by fluorescein (FITC)-conjugated goat anti-mouse IgG (green) and propidium iodide (PI) (red). Magnification, ×400. B, The number of PARP-positive cells was expressed as the percentage of total cells and shown for each patient pre- and post-chemotherapy.*

performed in a small number of patients, thus additional work is required to identify the subset of patients who will benefit most from such treatment.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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