Abstract. Background/Aim: We aimed to investigate the prognostic value of biomarkers [Ki-67, adenosine triphosphate-binding cassette sub-family B member 1 (ABCB1), adenosine triphosphate-binding cassette sub-family C member 1 (ABCC1), adenosine triphosphate-binding cassette sub-family C member 2 (ABCC2), p53, cyclin E and v-akt murine thymoma viral oncogene homolog 2 (AKT2)] in patients with advanced epithelial ovarian cancer (EOC). Materials and Methods: The levels of expression of biomarkers in tumor tissues of 47 patients with stage 3 or 4 EOC were estimated via immunohistochemical staining using tissue microarrays. The associations of biomarker expression with progression-free survival (PFS) and overall survival (OS) were evaluated using a log-rank test and Cox regression analysis. Results: Based on multivariate analysis, high expression of Ki-67 (p=0.003) and low expression of ABCC2 (p=0.048) were associated with a prolonged PFS. However, other biomarkers were not associated with PFS. Residual tumor <1 cm (p=0.023) and PFS >6 months (p=0.005) were associated with prolonged OS. However, none of the biomarkers were associated with OS. Conclusion: High expression of Ki-67 and low expression of ABCC2 appear to be useful as markers for prolonged PFS in patients with advanced EOC.

Epithelial ovarian cancer (EOC) is the most fatal cancer among all gynecologic cancers. The prognosis for patients with advanced EOC is poor. Specifically, the 5-year overall survival (OS) of patients with advanced EOC is approximately 50% (1). The standard treatment for advanced EOC is primary debulking surgery followed by platinum-based chemotherapy. Several studies testing the efficacy of chemotherapy in patients with advanced EOC have reported response rates of 70%-80% (2). However, 20%-30% of patients do not respond to chemotherapy and the majority of patients who initially responded to chemotherapy eventually have recurrences (3). Because debulking surgery only removes macroscopic tumors, the tumor response to chemotherapeutic agents is the major determinant of the prognosis of patients with advanced EOC.

In addition to clinical markers, several genes have been suggested as markers for tumor response and prognosis for such patients. Low Ki-67 expression was suggested to be a good prognostic factor based on two studies (4, 5), but a recent study reported that high Ki-67 expression was predictive of complete response in advanced EOC (6). Adenosine triphosphate-binding cassette sub-family B member 1 (ABCB1) is an energy-dependent drug efflux pump and several studies have reported an association between ABCB1 and prognosis in patients with advanced EOC (7, 8). Adenosine triphosphate-binding cassette sub-family C member 1 and 2 (ABCC1 and ABCC2) are associated with anticancer drug resistance (9, 10). p53 is a protein that induces apoptosis in response to DNA damage and may be associated with resistance to chemotherapy (6). Cyclin E is a protein functioning as a regulatory subunit in the G1-to-S transition and several studies have reported that cyclin E overexpression is associated with poor prognosis of patients with advanced EOC (11-13). v-akt murine thymoma viral oncogene homolog 2 (AKT2) is a protein kinase associated with cell survival and apoptosis; previous studies have reported that AKT2 expression is associated with tumor aggressiveness and poor prognosis in patients with EOC (14, 15). However, there are no generally accepted biomarkers predicting the tumor response and prognosis in patients with advanced EOC.
Using immunohistochemical staining, we investigated the prognostic value of these biomarkers in patients with advanced EOC.

**Patients and Methods**

**Patients.** Forty-seven patients with stages 3 or 4 EOC who underwent primary debulking surgery followed by adjuvant paclitaxel plus carboplatin chemotherapy at our institute between June 2003 and March 2008 were included in the study. Age, tumor size, histologic type, stage, and residual tumor after initial surgery were obtained via medical record review. The Institutional Review Board reviewed this protocol in a brief review track and exempted this study from further review (B-1104-125-301).

**Construction of tissue microarrays.** Hematoxylin and eosin (H&E)-stained sections were reviewed by a pathologist (HK) to select representative areas of the tumor. Core tissue biopsies measuring 2 mm in diameter were obtained from matching paraffin blocks (donor blocks) and arranged in new recipient paraffin blocks (tissue array blocks) using a trephine apparatus (Superbiochips Laboratories, Seoul, Korea). Two cores were sampled from each case. Five-micrometer sections were obtained from the microarrays and stained with H&E to confirm the presence of tumor.

**Immunohistochemical staining.** Deparaffinization and antigen retrieval were performed via a heat-induced epitope retrieval method. Specifically, sections were treated with Dako Envision FLEX target retrieval solution high pH (50x, K8004; Dako, Glostrup, Denmark) using Dako PT Link at 97˚C for 20 min. After rinsing with a washing buffer [Dako EnVision FLEX wash buffer (20x)], sections were treated with 3% hydrogen peroxide for 5 min to remove the intrinsic peroxidase. The sections were then incubated with primary antibodies against Ki-67, ABCB1, ABCC1, ABCC2, p53, cyclin E and AKT2 for 30 min. Details of the primary antibodies are summarized in Table I. After rinsing with a washing buffer, a secondary antibody [Dako EnVision+ System-HRP (DAB) anti-mouse (K4006) for Ki-67, ABC1, ABCC1, ABCC2, p53, cyclin E and AKT2; Dako EnVision+ System-HRP (DAB) anti-rabbit (K4100) for cyclin E; Dako polyclonal rabbit anti-goat immunoglobulins/biotinylated (E0466), dilution 1:200 for ABCB1] was applied for 30 min. In addition, for ABCB1, sections were treated with Dako LSAB2 peroxidase-conjugated streptavidin (K1016). Sections were stained for 10 min with 3',3-diaminobenzidine tetrahydrochloride, then counterstained with hematoxylin. Sections were washed, dehydrated, and mounted. Appropriate positive and negative controls were used for all markers.

**Grading of expression level.** Each core of the microarray was scored individually, and the results are presented as the mean of the two replicate core samples. For Ki-67 and cyclin E, the percentage of stained nuclei was estimated. For ABCB1, ABC1, ABCC2 and AKT2, the intensities of cytoplasmic or membranous staining were scored (0, negative; 1, weakly positive; 2, moderately positive; and 3, strongly positive), and the proportion of stained cells was scored in a semi-quantitative manner (0.0%; 1, 1%-33%; 2, 34%-66%; and 3, >66%). The immunoscore was calculated for each core as the product of the intensity score and the proportion score, and ranged from 0-9. For p53, the level of expression was determined by the percentage of cells with stained nuclei (0, <10%; 1, 10%-25%; 2, 25%-50%; 3, 50%-75%; 4, >75%). All grading was performed by a pathologist (HK) who was blinded to the clinical data.

**Table I. Details of the primary antibodies* used for immunohistochemical staining.**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Dilution</th>
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<tbody>
<tr>
<td>Ki-67</td>
<td>Mouse monoclonal Ab (sc-23900)</td>
<td>1:100</td>
</tr>
<tr>
<td>ABCB1</td>
<td>Goat polyclonal Ab (sc-1517)</td>
<td>1:100</td>
</tr>
<tr>
<td>ABCC1</td>
<td>Mouse monoclonal Ab (sc-18836)</td>
<td>1:100</td>
</tr>
<tr>
<td>ABCC2</td>
<td>Mouse monoclonal Ab (sc-59608)</td>
<td>1:50</td>
</tr>
<tr>
<td>p53</td>
<td>Mouse monoclonal Ab (sc-236)</td>
<td>1:100</td>
</tr>
<tr>
<td>Cyclin E</td>
<td>Rabbit polyclonal Ab (sc-198)</td>
<td>1:100</td>
</tr>
<tr>
<td>AKT2</td>
<td>Mouse monoclonal Ab (sc-5270)</td>
<td>1:100</td>
</tr>
</tbody>
</table>

*All antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

**Analysis.** All variables were dichotomized using median values. Progression-free survival (PFS) was defined as the interval from the last cycle of adjuvant chemotherapy to the first documented progression. OS was defined as the interval from the last cycle of adjuvant chemotherapy to any cause of death. The PFS and OS curves were depicted using the Kaplan-Meier method. A p-value <0.05 was regarded as being statistically significant and all statistical analyses were performed using SPSS (version 18.0) (SPSS Inc., Chicago, IL, USA).

**Results**

**Clinicopathologic characteristics.** The median age was 53 years and the median tumor size was 9 cm. Thirty-four out of 47 patients (72%) had serous-type tumors and 39 patients (83%) had stage 3 disease. After primary debulking surgery, residual tumors <1 cm in size were achieved in 21 patients (45%) (Table II). After surgery, all patients received adjuvant paclitaxel plus carboplatin chemotherapy, but 7 patients received <6 cycles of chemotherapy due to toxicities.

**Expression of biomarkers.** Biomarkers showed diverse levels of expression (Table III and Figure 1).

**Association of variables with PFS and OS.** Based on univariate analysis, Ki-67 expression was associated with PFS (p=0.002). Based on multivariate analysis, high expression of Ki-67 [p=0.003; odds ratio (OR)=0.300; 95% confidence interval (CI)=0.136-0.662] and low expression of ABCC2 (p=0.048; OR=2.451; 95% CI=1.009-5.954) were associated with prolonged PFS.

For OS, residual tumor <1 cm in size [p=0.023; OR=6.366; 95% CI=1.293-31.353 (multivariate analysis)] and PFS >6
months \[ p=0.005; \text{OR}=0.142; \text{95\% CI}=0.037-0.548 \] (multivariate analysis) were associated with prolonged OS in univariate and multivariate analyses. However, none of the biomarkers were associated with OS (Table IV).

**Discussion**

**ABCC2.** ABCC2 mediates active efflux of anticancer drugs. Studies on the prognostic value of ABCC2 in patients with advanced EOC have reported conflicting results. An in vitro study using an ovarian cancer cell line showed that the inhibition of ABCC2 expression with short hairpin RNA significantly enhanced cellular cisplatin accumulation and induced the apoptosis of cells (9). In addition, a study using 58 ovarian cancer specimens showed that ABCC2 expression was associated with poor prognosis (16). However, the association of ABCC2 expression with prognosis in patients with EOC was negative in two studies (8, 17).

The strengths of the current study are the standardized treatment which was a primary debulking surgery followed by paclitaxel plus carboplatin chemotherapy and the use of tissue microarray to minimize the variability of staining techniques. The current study suggested ABCC2 expression predicts a shorter PFS. However, a larger study is necessary to clarify the clinical implication of ABCC2 expression in patients with advanced EOC.

**Ki-67.** Ki-67 is an antigen expressed during G\textsubscript{1}-to-M phases and reflects the rate of cell proliferation. Several studies have investigated the association of Ki-67 expression with prognosis in patients with advanced EOC, but reported conflicting results. For example, two studies investigating Ki-67 expression revealed that the level of Ki-67 expression was lower in long-term than short-term survivors (4, 5). In addition, a study including 105 patients with advanced EOC revealed that low expression of Ki-67 was associated with prolonged survival (18). However, a recent study reported that high expression of Ki-67 is predictive of a complete response in advanced EOC (6). The current study suggested that high expression of Ki-67 was associated with prolonged PFS. The basis for the conflicting results between studies is unclear.

**Studies on other biomarkers.** ABCB1 is one of the major mechanisms for drug resistance in cancer. Many studies have examined the association of ABCB1 expression with prognosis in patients with EOC and reported conflicting results (7, 8). In the current study, ABCB1 expression was not associated with PFS or OS.

ABCC1 is also an ABC transporter and is associated with anticancer drug resistance. There are few studies on the association of ABCC1 expression with prognosis in patients with EOC. Some studies demonstrated an inverse correlation between ABCC1 expression and survival in patients with EOC (8, 19). In the current study, ABCC1 expression was not associated with PFS or OS.

p53 is a protein that induces apoptosis in response to DNA damage and is associated with resistance to chemotherapy. The clinical significance of p53 expression in patients with EOC has been assessed in many studies, but remains controversial (4-6). No correlation between p53 expression and prognosis was found in the current study.

Cyclin E is a protein functioning as a regulatory subunit in the G\textsubscript{1}-to-S transition. Alteration of cyclin E expression has been observed in many tumors, and the protein is thought to be related in oncogenesis (20). Several studies have reported that high expression of cyclin E is associated with poor prognosis in patients with advanced EOC (11-13). In the current study, cyclin E expression was not associated with PFS and OS, due to small sample size.

AKT2 is a protein kinase associated with cell survival and apoptosis. Few studies have reported AKT2 expression in EOC and suggested that AKT2 expression is associated with tumor aggressiveness and poor prognosis (14, 15). The
The current study showed that AKT2 was expressed in tumors of most patients with advanced EOC, but did not demonstrate a correlation between AKT2 expression and prognosis.

**Summary.** High expression of Ki-67 and low expression of ABCC2 were not associated with OS but prolonged PFS. Other biomarkers (ABCB1, ABCC1, p53, cyclin E and AKT2) were associated with neither PFS nor OS. Residual tumor <1 cm in size and PFS >6 months were reaffirmed to be predictors of prolonged OS. Ki-67 and ABCC2 appear to be useful as markers for prolonged PFS in patients with advanced EOC.

**Conflict of Interest**
None.

**Acknowledgements**
The current study was supported by grant no. 04-2007-005 from the SNUBH Research Fund.
Figure 1. High expression of biomarkers in selected cases. A: Ki-67 (×400), B: ABCB1 (×400), C: ABCC1 (×400), D: ABCC2 (×400), E: p53 (×400), F: Cyclin E (×400) and G: AKT2 (×400).
References


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