Epigenetic silencing of BLU through interfering apoptosis results in chemoresistance and poor prognosis of ovarian serous carcinoma patients

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Abstract
Epithelial ovarian carcinoma is usually present at the advanced stage, during which the patients generally have poor prognosis. Our study aimed to evaluate the correlation of gene methylation and the clinical outcome of patients with advanced-stage, high-grade ovarian serous carcinoma. The methylation status of eight candidate genes was first evaluated by methylation-specific PCR and capillary electrophoresis to select three potential genes including DAPK, CDH1, and BLU (ZMYND10) from the exercise group of 40 patients. The methylation status of these three genes was further investigated in the validation group consisting of 136 patients. Patients with methylated BLU had significantly shorter progression-free survival (PFS; hazard ratio (HR) 1.48, 95% CI 1.01–2.56, \( P \leq 0.013 \)) and overall survival (OS; HR 1.83, 95% CI 1.07–3.11, \( P \leq 0.027 \)) in the multivariate analysis. Methylation of BLU was also an independent risk factor for 58 patients undergoing optimal debulking surgery for PFS (HR 2.37, 95% CI 1.03–5.42, \( P = 0.043 \)) and OS (HR 3.96, 95% CI 1.45–10.81, \( P = 0.007 \)) in the multivariate analysis. A possible mechanism of BLU in chemoresistance was investigated in ovarian cancer cell lines by in vitro apoptotic assays. In vitro studies have shown that BLU could upregulate the expression of BAX and enhance the effect of paclitaxel-induced apoptosis in ovarian cancer cells. Our study suggested that methylation of BLU could be a potential prognostic biomarker for advanced ovarian serous carcinoma.

Key Words
▶ BLU
▶ apoptosis
▶ ovarian carcinoma
▶ paclitaxel
▶ gene methylation

Introduction
Ovarian cancer, especially epithelial ovarian carcinoma, has the highest mortality rate among gynecologic malignancies. It has been regarded as ever important in recent years (Siegel et al. 2012). The majority of the patients are serous type at an advanced stage with overall survival (OS) rates of only 19–30% (Agarwal & Kaye 2003). Current treatments include debulking surgery and adjuvant chemotherapy. Platinum in combination with paclitaxel...
is the most common regimen, which has a response rate of 80% for all patients and 40–60% for advanced-stage patients (Stuart 2003). The patients of advanced stage usually relapse after an initial response and ultimately die of recurrence (Agarwal & Kaye 2003). Elucidating the possible mechanisms and identifying patients who are potentially resistant to chemotherapy may help develop new therapeutic strategies.

Epigenetics define all heritable changes in gene expression that are not coded in the DNA sequence itself. Gene promoter hypermethylation is a major route mediating epigenetic transcriptional silencing in gene expression (Egger et al. 2004). When promoter hypermethylation is present in tumor-suppressor genes, it can inactivate their function and promote the development of malignancy (Herman & Baylin 2003). Aberrant DNA methylation is a common phenomenon in malignancy and methylation profiles are different in various tumors which might be associated with clinical outcomes (Esteller 2007). Although growing numbers of gene hypermethylation in ovarian cancer were investigated, the results of previous literature varied widely (Barton et al. 2008), which could result from different tumor groups, sample processing protocols, and assay platforms (Kagan et al. 2007). The current hypothesis proposed that there could be two possible tumor pathways in the carcinogenesis of ovarian carcinoma including type I and II tumors (Kurman & Shih le 2010). Distinct gene methylation profiles may exist between these two types of tumor pathway (Shih le et al. 2010). Therefore, our study aimed to investigate gene hypermethylation in patients of advanced-stage, high-grade serous ovarian carcinoma, which belongs to type II tumor.

We initially evaluated the methylation status of DAPK (Teodoridis et al. 2005), RASSF1A (Makarla et al. 2005, Teodoridis et al. 2005), RARB (Makarla et al. 2005), CDH1 (Makarla et al. 2005), CDKN2B (Tam et al. 2007), BLU (ZMYND10; Balch et al. 2005), DLEC1 (Kwong et al. 2006), and RUNX3 (Zhang et al. 2009) in the exercise group. These genes were chosen from previous literature, and they were often silenced by gene hypermethylation in several tumor types including ovarian carcinoma. Three potential genes selected form the exercise group, DAPK, CDH1, and BLU, were further investigated in the validation group. DAPK is a tumor-suppressor gene with the ability to sensitize cells to apoptotic signals encountered in tumorigenesis (Bialik & Kimchi 2004). Recovery of DAPK function by demethylating agents has been documented in cancer cell lines that can restore cancer cell sensitivity to chemotherapy (Morita et al. 2006). CDH1 is a calcium-dependent cell–cell adhesion glycoprotein. Loss of its function contributes to carcinogenesis by increasing proliferation, invasion, and metastasis. The effects of dysregulated CDH1 on drug resistance in cancer cell lines were noted in in vitro studies (Nakamura et al. 2003, Green et al. 2004). BLU is located in chromosome 3p21, and its ectopic expression can inhibit the colony formation of cancer cells. This suggests that it could be a candidate of tumor-suppressor gene (Qiu et al. 2004). Our study found that methylation of BLU was associated with chemoresistance, a higher incidence of recurrence, and a poor outcome of advanced serous ovarian carcinoma patients. In vitro assays revealed that the restoration of BLU can improve chemosensitivity by enhancing the paclitaxel-induced apoptosis of ovarian cancer cells.

Materials and methods

Patients and specimens

The study protocol was reviewed and approved by the Institutional Review Board of the hospital. Advanced-staged, high-grade ovarian serous carcinoma patients undergoing debulking surgery and adjuvant chemotherapy in our institute were enrolled and informed consents were obtained. Clinical information was retrieved from medical records in the hospital’s centralized database. Optimal debulking surgery was defined as the maximal diameter of residual tumor <1 cm, or otherwise defined as suboptimal debulking surgery. Histological grading was based on the International Union against Cancer criteria, and staging was based on the criteria of the International Federation of Gynecology and Obstetrics. Abnormal results of imaging studies (including computerized tomography or magnetic resonance image), elevated tumor marker (more than twofold of upper normal limits) of two consecutive tests in 2-week intervals, or tissue proven from biopsy, if possible, were defined as recurrence. Patients with disease progression or recurrence ≤6 months after completing adjuvant chemotherapy were defined as chemoresistant while those without recurrence or recurrence >6 months were defined as chemosensitive. Progression-free survival (PFS) was measured as the period starting from the date of completing chemotherapy to the date of confirmed recurrence, disease progression, or the date of the last follow-up. OS was measured as the period from surgery to the date of death associating with disease or the date of the last follow-up.

The study protocol included two stages. The first stage that consisted of 40 patients, including 20 chemosensitive and 20 chemoresistant patients, was defined as the exercise
The methylation status of DAPK, RASSF1A, RARB, CDH1, CDKN2B, BLU, DLEC1, and RUNX3 were evaluated in this group. Primer sequences are summarized in Table 1. Potential genes were selected and investigated in the validation group including a total of 136 patients of the second stage.

Extraction of DNA in ovarian cancer tissues
Cancerous tissue specimens collected from surgery were frozen and stored at −70 °C until analysis. Genomic DNA of ovarian cancer tissues was isolated using the Qiagen EZ1 DNA Tissue Kit (Qiagen) following the manufacturer’s instructions.

Methylation-specific PCR and capillary electrophoresis
The principle of methylation-specific PCR (MS-PCR) mainly followed the protocol proposed previously (Herman et al. 1996). The processes of MS-PCR in the isolated genomic DNA of ovarian cancer tissues were performed with the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA) following the manufacturer’s instructions. Capillary electrophoresis (CE) was used to analyze MS-PCR products using the HDA system with a GCK-5000 cartridge kit (eGene, Irvine, CA, USA). The gel matrix in the gel cartridge was composed of proprietary linear polymer with ethidium bromide (EtBr) dye. PCR products were diluted 20-fold by deionized water and placed in the sample chamber of the instrument. DNA samples were then injected into the capillary channels and subjected to electrophoresis based on the manufacturer’s protocol. BioCalculator Graphing Software (eGene, Irvine, CA, USA) was used for automatic labeling of peak sizes. In order to confirm the results of CE, the same PCR products for CE were periodically selected to undergo direct DNA sequence analysis using an automated ABI sequencing system.

Extraction of RNA in ovarian cancer tissues
Cancerous tissue specimens were collected, frozen, and stored as described earlier. Total RNA of ovarian cancer tissues was isolated using TRIzol reagent (Invitrogen) following the manufacturer’s instructions.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Sense</th>
<th>Antisense</th>
<th>Tm (°C)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPK</td>
<td>U5-GGAGGATGTTGAGTATTGAGTTAATGTT</td>
<td>5'-CAAATCCTCCCAACACCAAA</td>
<td>56</td>
<td>106</td>
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<tr>
<td></td>
<td>M5-CCCTCCCAACACCAAA</td>
<td>5'-CCCTCCCAACACCAAA</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>RASSF1A</td>
<td>U5-GGGTTTTGTGTTTGGTTT</td>
<td>5'-AACATAACCAATTAACCAATCCA</td>
<td>62</td>
<td>81</td>
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<tr>
<td></td>
<td>M5-CCCTCCCAACACCAAA</td>
<td>5'-CCCTCCCAACACCAAA</td>
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</tr>
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<td>RARB</td>
<td>U5-TGGGATGTTGAGTATTGAGTT</td>
<td>5'-CTTACACAATCCTCAAACAAAACA</td>
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<td>161</td>
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<tr>
<td></td>
<td>M5-CCCTCCCAACACCAAA</td>
<td>5'-CCCTCCCAACACCAAA</td>
<td>149</td>
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<tr>
<td>CDH1</td>
<td>U5-TGGGATGTTGAGTATTGAGTT</td>
<td>5'-ACACCAAATACCATCAATCAACAAA</td>
<td>63</td>
<td>120</td>
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<tr>
<td></td>
<td>M5-CCCTCCCAACACCAAA</td>
<td>5'-CCCTCCCAACACCAAA</td>
<td>112</td>
<td></td>
</tr>
<tr>
<td>CDKN2B</td>
<td>U5-TGGGATGTTGAGTATTGAGTT</td>
<td>5'-ATACAAACCAAAATACCAACAA</td>
<td>60</td>
<td>235</td>
</tr>
<tr>
<td></td>
<td>M5-CCCTCCCAACACCAAA</td>
<td>5'-CCCTCCCAACACCAAA</td>
<td>231</td>
<td></td>
</tr>
<tr>
<td>BLU</td>
<td>U5-TGGGATGTTGAGTATTGAGTT</td>
<td>5'-ATACAAACCAAAATACCAACAA</td>
<td>60</td>
<td>197</td>
</tr>
<tr>
<td></td>
<td>M5-CCCTCCCAACACCAAA</td>
<td>5'-CCCTCCCAACACCAAA</td>
<td>197</td>
<td></td>
</tr>
<tr>
<td>DLEC1</td>
<td>U5-TGGGATGTTGAGTATTGAGTT</td>
<td>5'-CCCTCCCAACACCAAA</td>
<td>60</td>
<td>197</td>
</tr>
<tr>
<td></td>
<td>M5-CCCTCCCAACACCAAA</td>
<td>5'-CCCTCCCAACACCAAA</td>
<td>197</td>
<td></td>
</tr>
<tr>
<td>RUNX3</td>
<td>U5-TGGGATGTTGAGTATTGAGTT</td>
<td>5'-CCCTCCCAACACCAAA</td>
<td>60</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td>M5-CCCTCCCAACACCAAA</td>
<td>5'-CCCTCCCAACACCAAA</td>
<td>115</td>
<td></td>
</tr>
</tbody>
</table>

Tm, melting temperature; bp, base pair; U, primers used for the unmethylated gene promoter; M, primers used for the methylated gene promoter.
performed. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene to compare with BLU. To generate GAPDH, a set of primers, 5'-ACCCAGAAGACTGTGGATGG-3' and 5'-TGCTGTAGC-CAATTCTGTTG-3', for 30 cycles was also performed. PCR products were then analyzed on 1% agarose gel with EtBr staining in TBE solution.

BLU RNA expression and methylation status in ovarian cancer cell lines

SKOV-3 and OVCAR-3 cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The transcription and the methylation status of BLU in SKOV-3 and OVCAR-3 were examined using RT-PCR and MS-PCR as described earlier.

Demethylation by 5-aza-2'-deoxycytidine in SKOV-3 ovarian cancer cells

For the demethylation experiments, SKOV-3 cells were treated with 5-aza-2'-deoxycytidine (5-aza-dC) for 72 h and SKOV-3 cells treated with PBS were used as the control. The transcription levels of BLU in SKOV-3 treated with 5-aza-dC were also determined using RT-PCR.

Transfection of BLU in SKOV-3 cells

To generate pcDNA3-BLU, BLU was first amplified by PCR using SKOV-3 cell cDNA as the template with 5'-GCATGAGAACCTGGAGAAGC-3' and 5'-GCTAAGCATAAGCTCAAGG-3' as primers. The amplified product was then cloned into the XbaI/BamH1 sites of the pcDNA3 vector (Invitrogen Life Technologies). The transfection of BLU was performed using Lipofectamine 2000 Transfection Reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. SKOV-3 cell transfection with pcDNA3 (mock) was used as the control. RNA transcription levels of BLU in various SKOV-3 transfectants were examined by RT-PCR. The stable clones of various SKOV-3 transfectants were used in the following experiments.

Knockdown of BLU by RNA interference in OVCAR-3 cells

OVCAR-3 cells were transfected with siRNA for BLU (Silencer siRNA, ID: s28018 s28019, and s28020; Life Technologies Corporation) according to the manufacturer's instructions. Briefly, OVCAR-3 cells were grown and transfected with 50 pM siRNA using 12 μg Lipofectamine 2000 Transfection Reagent (4:1) in a total volume of 2 ml of serum-free RPMI-1640 medium. After incubation at 37 °C, 5% CO2 for 6 h, 2 ml of RPMI-1640 medium containing 20% normal growth medium were added. Samples were collected 48 h later to assess the inhibition of BLU expression by RT-PCR, and then the cells were used in the following experiments.

Apoptotic assays of the ovarian cancer cells

Apoptotic assays of the ovarian cancer cells treated with cytotoxic agents were performed by detecting caspase activity and annexin V in these cells. Briefly, ovarian cancer cells were harvested and treated with 2.5 nM paclitaxel or 12.5 μM cisplatin. Paclitaxel and cisplatin were purchased from Sigma–Aldrich. The preparation and the dosage of these drugs were according to the manufacturer's instructions and previous literature (Kelland & Abel 1992, Mistry et al. 1992, Smith et al. 2005). The ovarian cancer cells treated with PBS were used as the negative control. The treated cells were incubated with Vybrant FAM Caspase-3 and -7 Assay Kit V-35118 (Molecular Probes, Invitrogen, Carlsbad, CA, USA) and FITC-conjugated Annexin V (PharMingen; BD Biosciences, San Jose, CA, USA) respectively according to the manufacturer's instructions, and were then analyzed by flow cytometry (FACScan; Becton Dickinson, Franklyn Lakes, NJ, USA).

Immunoprecipitation and immunoblotting

Immunoblotting assays of the original and various SKOV-3 transfectants treated with paclitaxel were performed. Briefly, the original and various SKOV-3 transfectants were seeded overnight and treated with 2.5 nM paclitaxel for 24 h. The cells were then lysed in the immunoprecipitation assay buffer, and protein extracts were quantified using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Then, 50 μg of each cell lysate were resolved by SDS/PAGE (12% gel), transferred onto a PVDF/nylon membrane (Millipore, Billerica, MA, USA), and probed with antibodies specific to BCL-2, MCL-1, BAX, BAK, and β-ACTIN (Upstate Biotechnology, Lake Placid, NY, USA). The membrane was then probed with either a HRP-conjugated goat anti-mouse or goat anti-rabbit antibody. Specific bands were visualized using an ECL western blotting system (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Protein levels were measured by densitometric analysis and normalized to the levels of β-actin (control) by ImageQuant 5.0 Software (Molecular Dynamics, Inc., Sunnyvale, CA, USA). The expression level of each molecule was presented as folds in comparison.
with the density of β-ACTIN, and expression levels in the original SKOV-3 cells was used as a reference.

**Statistical analysis**

Data were analyzed by Statistical Package of Social Studies Software (SPSS 15.0; SPSS, Inc.). Student’s *t*-test was used for continuous variables and Fisher’s exact test for categorical variables. Survival curves were generated using the Kaplan–Meier method and differences in survival curves were calculated using the log-rank test. Cox regression analyses were used to evaluate prognostic factors for recurrence and death. In the *in vitro* experiments, one-way ANOVA was used to analyze the percentages of apoptosis in cells treated with cytotoxic agents. All data are expressed as mean ± S.E.M. from at least three different experiments. A *P* value < 0.05 was considered to be statistically significant.

**Results**

**Chemoresistant serous ovarian carcinoma patients had a higher percentage of DAPK, CDH1, or BLU gene methylation in the exercise group**

We first selected the potential genes correlated with chemoresponse from the exercise group. The methylation status of the respective genes and the clinical characteristics of the 40 patients are shown in Table 2. The percentages of gene methylation in chemoresistant patients were 35% for DAPK, 30% for RASSF1A, 15% for RARB, 55% for CDH1, 35% for CDKN2B, 65% for BLU, 80% for DLEC1, and 35% for RUNX3. The percentages of gene methylation in chemoresistant patients were significantly higher for DAPK (35 vs 5%, *P* = 0.044, χ² test), CDH1 (55 vs 20%, *P* = 0.048), and BLU (65 vs 15%, *P* = 0.003) when compared with chemosensitive patients.

Our results revealed that chemoresistant patients had higher percentages of gene methylation for DAPK, CDH1, or BLU in the exercise group. These three genes were further investigated in the validation group consisting of 136 patients with advanced-stage, high-grade serous ovarian carcinoma.

**Decreased RNA transcription levels of BLU correlated with methylation of BLU in serous ovarian cancerous tissues**

Representative images of MS-PCR by CE analysis are shown in Fig. 1A. After the reaction of the C–T conversion agent, the cytosine bases (C) of CpG dinucleotides in the unmethylated gene promoter were converted to thymidine (T), which was detected by the primers used for the unmethylated gene promoter. In contrast, cytosine (C) in the methylated gene promoter did not change after the C–T conversion agent and these sites were detected by the primers used for the methylated gene promoter. We

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Chemosensitive</th>
<th>Chemoresistant</th>
<th><em>P</em> value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cases</td>
<td>20</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Age (y/o)</td>
<td>52.8 ± 13.8</td>
<td>59.2 ± 11.1</td>
<td>0.12²</td>
</tr>
<tr>
<td>Preoperative CA-125</td>
<td>1551 (87–9368.5)</td>
<td>2609 (128.5–11 646)</td>
<td>0.64²</td>
</tr>
<tr>
<td>Debulking surgery</td>
<td></td>
<td></td>
<td>0.33³</td>
</tr>
<tr>
<td>Optimal</td>
<td>10 (50%)</td>
<td>6 (30%)</td>
<td></td>
</tr>
<tr>
<td>Suboptimal</td>
<td>10 (50%)</td>
<td>14 (70%)</td>
<td></td>
</tr>
<tr>
<td>Platinum-based chemotherapy</td>
<td></td>
<td></td>
<td>0.41³</td>
</tr>
<tr>
<td>Without paclitaxel</td>
<td>5 (25%)</td>
<td>2 (10%)</td>
<td></td>
</tr>
<tr>
<td>With paclitaxel</td>
<td>15 (75%)</td>
<td>18 (90%)</td>
<td></td>
</tr>
<tr>
<td>Gene promoter hypermethylation</td>
<td></td>
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</tr>
<tr>
<td>DAPK</td>
<td>1 (5%)</td>
<td>7 (35%)</td>
<td>0.044⁴</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>11 (55%)</td>
<td>6 (30%)</td>
<td>0.20⁴</td>
</tr>
<tr>
<td>RARB</td>
<td>0 (0%)</td>
<td>3 (15%)</td>
<td>0.23⁵</td>
</tr>
<tr>
<td>CDH1</td>
<td>4 (20%)</td>
<td>11 (55%)</td>
<td>0.048⁵</td>
</tr>
<tr>
<td>CDKN2B</td>
<td>3 (15%)</td>
<td>7 (35%)</td>
<td>0.27⁵</td>
</tr>
<tr>
<td>BLU</td>
<td>3 (15%)</td>
<td>13 (65%)</td>
<td>0.003⁶</td>
</tr>
<tr>
<td>DLEC1</td>
<td>11 (55%)</td>
<td>16 (80%)</td>
<td>0.18⁷</td>
</tr>
<tr>
<td>RUNX3</td>
<td>10 (50%)</td>
<td>7 (35%)</td>
<td>0.52⁷</td>
</tr>
</tbody>
</table>

y/o, years old; age value is presented as mean ± s.d.; preoperative CA-125 is presented as median (minimum–maximum). Other values are presented as number (percentage).

²Student’s *t*-test.
³Mann–Whitney *U* test.
⁴χ² test.
⁵²×² test.
⁶Fisher’s exact test.
defined gene methylation when the sample showed a positive result in the primers used for the methylated gene promoter and negative in the primers used for the unmethylated gene promoter at the same time.

In order to confirm the results of MS-PCR, the transcriptional levels of BLU in cancer tissues were checked by RT-PCR. Also, the same MS-PCR products for CE analysis were sent for direct DNA sequence analysis. Representative images of RT-PCR for BLU in ovarian cancerous tissues are shown in Fig. 1B. The transcription levels of BLU in patients with the methylated BLU promoter (patients 1–3) were lower than that in patients without it (patients 4 and 5). (C) Representative images of the DNA sequence in the BLU promoter. After the reaction of the C–T conversion agent used in this investigation, the cytosine base of unmethylated CpG dinucleotides was converted to thymidine, but the cytosine base of methylated CpG dinucleotides did not. The arrows indicate these cytosine bases of CpG dinucleotides.

Methylation of BLU and suboptimal debulking surgery were two independent factors for the outcome of advanced-stage, high-grade serous ovarian carcinoma patients

Patient characteristics and gene methylation in the validation group are shown in Table 3. The mean age of these 136 patients at diagnosis was 55.9 years old. Twenty-five patients were FIGO stage IV while others were stage III (1, 8, and 102 for stages IIIa, IIIb, and IIIc). The median OS was 34 months, and median PFS was 10 months. Ninety-two (67.6%) patients were chemosensitive and 44 (32.4%) of them were chemoresistant. The mean age, serum level of preoperative CA-125, or chemotherapeutic regimens (with or without paclitaxel) showed no difference between the chemosensitive and chemoresistant patients. Chemosensitive patients had higher percentages of optimal
We then evaluated whether gene methylation was associated with the prognosis of the patients. Patients with the methylation of BLU had significantly shorter PFS (P=0.012; Fig. 2A) and OS (P=0.024; Fig. 2B) than those without the methylation of BLU by Kaplan–Meier survival analysis. Within the 116 patients who underwent platinum–paclitaxel chemotherapy, those with methylation of BLU also had significantly shorter PFS (P=0.048; Fig. 2C) and OS (P=0.042; Fig. 2D) by Kaplan–Meier survival analysis.

The hazard ratios (HRs) of various risk factors by the multivariate Cox regression model for these 136 patients are shown in Table 4. Methylation of BLU (HR 1.48, 95% CI 1.01–2.56, P=0.013) and suboptimal debulking surgery (HR 1.66, 95% CI 1.06–2.60, P=0.007) were two independent risk factors for disease recurrence. Methylation of BLU (HR 1.83, 95% CI 1.07–3.11, P=0.027) and suboptimal debulking surgery (HR 2.99, 95% CI 1.68–5.32, P<0.001) were also the risk factors for the death of the patients. Methylation of neither DAPK nor CDH1 was a risk factor for the recurrence or death of these patients.

Our results revealed that BLU methylation and suboptimal debulking surgery were two factors correlated with the prognosis of advanced-stage, high-grade ovarian serous carcinoma patients.

**Methylation of BLU was the only independent risk factor on the outcome of patients undergoing optimal debulking surgery**

We further focused on the risk factors for the outcome of 58 patients undergoing optimal debulking surgery. By Kaplan–Meier survival analysis, methylation of BLU in these patients also had shorter PFS (P=0.038; Fig. 2E) and shorter OS (P=0.003; Fig. 2F). Methylation of BLU was the only independent risk factor for disease recurrence (HR 2.37, 95% CI 1.03–5.42, P=0.043) and the death of the patients (HR 3.96, 95% CI 1.45–10.81, P=0.007) by the multivariate analysis (Table 5).
RNA transcription levels and gene methylation of **BLU** in ovarian cancer cell lines

RNA transcription levels and the status of **BLU** gene methylation were further studied in ovarian cancer cell lines (Fig. 3). Promoter methylation and decreased RNA expression of **BLU** were noted in SKOV-3 cells. In contrast, the transcription of **BLU** was not downregulated in OVCAR-3 cells in which the **BLU** gene was unmethylated.

The demethylating agent, 5-aza-dC was added to SKOV-3 cells to confirm whether the downregulated RNA expression of **BLU** was due to methylation of the gene. Patients with **BLU** methylation had shorter PFS than those without it ($P = 0.012$). (B) OS in a total of 136 patients. Patients with **BLU** methylation had shorter OS than those without it ($P = 0.024$). (C) PFS in 116 patients undergoing adjuvant platinum–paclitaxel chemotherapy. Patients with **BLU** methylation had shorter PFS than those without it ($P = 0.048$). (D) OS in 116 patients undergoing adjuvant platinum–paclitaxel chemotherapy. Patients with **BLU** methylation had OS than those without it ($P = 0.042$). The difference was calculated by the log-rank test.

**Table 4** Multivariate Cox regression model for the risk factors on recurrence and death in the 136 patients with advanced serous ovarian carcinoma.

<table>
<thead>
<tr>
<th></th>
<th>Recurrence</th>
<th>Death</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Hazard ratio (95% CI)</td>
</tr>
<tr>
<td><strong>Debulking surgery</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optimal</td>
<td>58</td>
<td>1.00</td>
</tr>
<tr>
<td>Suboptimal</td>
<td>78</td>
<td>1.66 (1.06–2.60)</td>
</tr>
<tr>
<td><strong>Platinum-based chemotherapy</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without paclitaxel</td>
<td>20</td>
<td>1.00</td>
</tr>
<tr>
<td>With paclitaxel</td>
<td>116</td>
<td>1.45 (0.75–2.78)</td>
</tr>
<tr>
<td><strong>Gene promoter hypermethylation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DAPK</strong></td>
<td></td>
<td></td>
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<tr>
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<td>114</td>
<td>1.00</td>
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<tr>
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<td>1.29 (0.71–2.36)</td>
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<tr>
<td><strong>CDH1</strong></td>
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<tr>
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<tr>
<td><strong>BLU</strong></td>
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<tr>
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<td>1.00</td>
</tr>
<tr>
<td>Methylated</td>
<td>38</td>
<td>1.48 (1.01–2.56)</td>
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transcription of BLU could be reversed. As shown in Fig. 3B, the RNA transcription level of BLU could be restored by 5-aza-dC, and it could not be observed in SKOV-3 cells treated with PBS only.

Our results indicated that the downregulated RNA transcription level of BLU by gene methylation in SKOV-3 cells could be restored with the demethylating agent.
**BLU enhanced the apoptosis of human ovarian cancer cells treated with cytotoxic drugs**

To investigate the possible mechanism and function of BLU in the chemoresistance of ovarian cancer, BLU-transfected SKOV-3 cells were generated for in vitro apoptotic assays. As shown in Fig. 3B, the RNA transcription level of BLU in SKOV-3/BLU transfectants was higher than those of the mock-transfected and original SKOV-3 cells.

Representative images of flow cytometric analysis for detecting caspase 3- and 7-positive cells in SKOV-3 cells are shown in Fig. 4A. The incremental percentages of caspase 3- and 7-positive cells were significantly higher in SKOV-3 treated with 5-aza than those in the original SKOV-3 cells, when treating the cells with paclitaxel for 24 h (original SKOV-3: 3.33 ± 0.27%, SKOV-3 (5-aza): 5.61 ± 1.00%, *P* = 0.022). The incremental percentages in the SKOV-3/BLU transfectants were significantly higher than those in the mock-transfected SKOV-3 cells (SKOV-3 (mock): 4.24 ± 0.67%, SKOV-3/BLU transfectant 1: 6.85 ± 0.97%, SKOV-3/BLU transfectant 2: 7.28 ± 1.32%, *P* = 0.041; Fig. 4B). Besides, the trend was also noted in cells treated with paclitaxel for 12 h (original SKOV-3: 1.57 ± 0.11%, SKOV-3 (5-aza): 2.75 ± 1.67%, *P* = 0.239; SKOV-3 (mock): 2.07 ± 0.34%, SKOV-3/BLU transfectant 1: 2.78 ± 0.73%, SKOV-3/BLU transfectant 2: 3.10 ± 0.41%, *P* = 0.124), even though the difference was not statistically significant.

Representative images of flow cytometric analysis for detecting annexin V-staining cells in SKOV-3 cells, which were defined as apoptotic cells, are shown in Fig. 4C. The incremental percentages of annexin V-positive cells were significantly higher in SKOV-3 treated with 5-aza than those in the original SKOV-3 cells, when treating the cells with paclitaxel for 24 h (original SKOV-3: 4.42 ± 0.43%, SKOV-3 (5-aza): 10.26 ± 2.10%, *P* = 0.042). The incremental percentages in the SKOV-3/BLU transfectants were significantly higher than those in the mock-transfected SKOV-3 cells (SKOV-3 (mock): 7.08 ± 2.18%, SKOV-3/BLU transfectant 1: 19.57 ± 7.08%, SKOV-3/BLU transfectant 2: 20.73 ± 2.17%, *P* = 0.011). The trend was still significant in cells treated with paclitaxel for 48 h (original SKOV-3: 8.42 ± 2.40%, SKOV-3 (5-aza): 14.81 ± 2.11%, *P* = 0.048; SKOV-3 (mock): 8.65 ± 2.84%, SKOV-3/BLU transfectant 1: 21.33 ± 1.38%, SKOV-3/BLU transfectant 2: 26.70 ± 4.37%, *P* = 0.025; Fig. 4D).

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**Figure 4**

*In vitro* apoptosis assays of SKOV-3 cells treated with cytotoxic drugs. (A) Representative images of flow cytometric analysis for caspase 3- and 7-positive cells when treated with 2.5 nM paclitaxel. (B) The bar graph of the incremental percentages of caspase 3- and 7-positive cells when treated with 2.5 nM paclitaxel for 12 and 24 h. The 5-aza-treated and BLU-transfected SKOV-3 cells had higher caspase 3- and 7-positive cells when treated with paclitaxel for 24 h. (C) Representative images of flow cytometric analysis for annexin V-positive cells when treated with 2.5 nM paclitaxel. (D) The bar graph of the incremental percentages of annexin V-positive cells when treated with 2.5 nM paclitaxel for 24 and 48 h. The 5-aza-treated and BLU-transfected SKOV-3 cells had higher annexin V-positive cells when treated with paclitaxel for 24 and 48 h. (E) The bar graph of the incremental percentages of caspase 3- and 7-positive cells when treated with 12.5 μM cisplatin for 12 and 24 h. There were no differences among the original, 5-aza-treated, mock-transfected, and BLU-transfected SKOV-3 cells. (F) The bar graph of the incremental percentages of annexin V-positive cells treated with 12.5 μM cisplatin for 24 and 48 h. There were no differences among the original, 5-aza-treated, mock-transfected, and BLU-transfected SKOV-3 cells.
The incremental percentages of caspase 3- and 7-positive cells were not different between the original SKOV-3 cells, SKOV-3 treated with 5-aza, and SKOV-3/BLU transfectants when treating the cells with cisplatin for 12 h (original SKOV-3: 0.31 ± 0.04%, SKOV-3 (5-aza): 0.88 ± 0.09%, *P* = 0.501; SKOV-3 (mock): 0.55 ± 0.06%, SKOV-3/BLU transfectant 1: 0.68 ± 0.19%, SKOV-3/BLU transfectant 2: 0.91 ± 0.17%, *P* = 0.840) and 24 h (original SKOV-3: 0.32 ± 0.16%, SKOV-3 (5-aza): 0.51 ± 0.17%, *P* = 0.473; SKOV-3 (mock): 0.64 ± 0.15%, SKOV-3/BLU transfectant 1: 0.74 ± 0.21%, SKOV-3/BLU transfectant 2: 0.93 ± 0.15%, *P* = 0.275; Fig. 4E). The incremental percentages of annexin V-positive cells were not different between the original SKOV-3 cells, SKOV-3 treated with 5-aza, and SKOV-3/BLU transfectants when treating the cells with cisplatin for 24 h (original SKOV-3: 1.95 ± 0.94%, SKOV-3 (5-aza): 2.75 ± 1.95%, *P* = 0.164; SKOV-3 (mock): 3.21 ± 0.40%, SKOV-3/BLU transfectant 1: 6.61 ± 1.28%, SKOV-3/BLU transfectant 2: 10.43 ± 5.39%, *P* = 0.283) and 48 h (original SKOV-3: 5.14 ± 4.35%, SKOV-3 (5-aza): 6.91 ± 1.67%, *P* = 0.704; SKOV-3 (mock): 3.88 ± 0.82%, SKOV-3/BLU transfectant 1: 14.72 ± 8.44%, SKOV-3/BLU transfectant 2: 17.34 ± 9.34%, *P* = 0.084; Fig. 4F).

Our results indicated that the paclitaxel-induced apoptosis of human ovarian cancer cells could be enhanced in the BLU-expressed SKOV-3 cells through activating the caspase 3 and 7 pathway.

Knockdown of BLU expression decreased the apoptosis of human ovarian cancer cells treated with cytotoxic drugs

siRNA BLU-transfected OVCAR-3 cells were generated for in vitro apoptotic assays. The RNA transcription level of BLU in OVCAR-3/siRNA BLU transfectants was lower than those of OVCAR-3 treated with PBS and the original OVCAR-3 cells (Fig. 3B).

The incremental percentages of caspase 3- and 7-positive cells were significantly lower in the OVCAR-3/siRNA BLU transfectants than those in the original OVCAR-3 cells and mock-transfected OVCAR-3 cells, when treating the cells with paclitaxel for 24 h (original OVCAR-3: 7.84 ± 1.34%, OVCAR-3 (mock): 7.25 ± 2.28%, OVCAR-3/siRNA BLU-1: 2.79 ± 0.15%, OVCAR-3/siRNA BLU-2: 2.44 ± 0.25%, *P* = 0.022). There was no difference in cells treated for 12 h (original OVCAR-3: 2.83 ± 0.13%, OVCAR-3 (mock): 2.09 ± 0.11%, OVCAR-3/siRNA BLU-1: 2.44 ± 0.09%, OVCAR-3/siRNA BLU-2: 1.18 ± 0.72%, *P* = 0.337; Fig. 5A).

The incremental percentages of annexin V-positive cells were significantly lower in the OVCAR-3/siRNA BLU transfectants than those in the original OVCAR-3 cells and mock-transfected OVCAR-3 cells after treating with paclitaxel for 24 h (original OVCAR-3: 11.06 ± 2.16%, OVCAR-3 (mock): 9.36 ± 1.93%, OVCAR-3/siRNA BLU-1: 4.08 ± 0.81%, OVCAR-3/siRNA BLU-2: 2.85 ± 0.98%, *P* = 0.039) and 48 h (original OVCAR-3: 14.89 ± 1.33%, OVCAR-3 (mock): 13.02 ± 1.09%, OVCAR-3/siRNA BLU-1: 4.69 ± 0.74%, OVCAR-3/siRNA BLU-2: 2.03 ± 0.61%, *P* = 0.008; Fig. 5B).

The incremental percentages of caspase 3- and 7-positive cells were not different in these cells when treated with cisplatin for 12 h (original OVCAR-3: 0.32 ± 0.09%, OVCAR-3 (mock): 0.36 ± 0.08%, OVCAR-3/siRNA BLU-1: 0.36 ± 0.26%, OVCAR-3/siRNA BLU-2: 0.40 ± 0.10%, *P* = 0.443) and 24 h (original OVCAR-3: 0.64 ± 0.48%, OVCAR-3 (mock): 0.45 ± 0.02%, OVCAR-3/siRNA BLU-1: 0.76 ± 0.14%, OVCAR-3/siRNA BLU-2: 0.62 ± 0.06%, *P* = 0.178; Fig. 5C). The incremental percentages of annexin V-positive cells were not different in these cells when treated with cisplatin for 24 h (original OVCAR-3: 4.87 ± 1.25%, OVCAR-3 (mock): 4.51 ± 1.09%, OVCAR-3/siRNA BLU-1: 3.66 ± 1.39%, OVCAR-3/siRNA BLU-2: 3.99 ± 1.29%, *P* = 0.401) and 48 h (original OVCAR-3: 6.85 ± 2.07%, OVCAR-3 (mock): 5.02 ± 1.31%, OVCAR-3/siRNA BLU-1: 4.08 ± 1.29%, OVCAR-3/siRNA BLU-2: 4.29 ± 1.24%, *P* = 0.263; Fig. 5D).

Our results indicated that the paclitaxel-induced apoptosis of human ovarian cancer cells decreased in the siRNA BLU-knockdown OVCAR-3 cells through inhibiting the caspase 3 and 7 pathway.

**BLU prompts paclitaxel-induced apoptosis of ovarian cancer cells by upregulating the BAX pro-apoptotic molecule**

We then evaluated whether BLU could regulate apoptosis-related molecules in the apoptotic pathway. Representative images of the expressions of apoptosis-related molecules by immunoblotting, including BCL-2, MCL-1, BAX, and BAK, in the original SKOV-3 cells and various SKOV-3 transfectants treated with paclitaxel are shown in Fig. 6A. When ovarian cancer cells were treated with PBS, the protein levels of Bax in SKOV-3 treated with 5-aza were significantly higher than those in the original SKOV-3 cells (original SKOV-3: 1, SKOV-3 (5-aza): 1.84 ± 0.10, *P* = 0.003). Also, the protein levels of the SKOV-3/BLU transfectants were significantly higher than those in the mock-transfected SKOV-3 cells (SKOV-3 (mock): 1.13 ± 0.21, SKOV-3/BLU transfectant 1: 2.04 ± 0.16, SKOV-3/BLU transfectant 2: 2.09 ± 0.19, *P* = 0.006;
When ovarian cancer cells were treated with paclitaxel, the protein levels of BAX in SKOV-3 treated with 5-aza were significantly higher than those in the original SKOV-3 cells (original SKOV-3: 1, SKOV-3 (5-aza): 2.69 ± 0.23, P < 0.001). Also, the protein levels of the SKOV-3/BLU transfectants were significantly higher than those in the mock-transfected SKOV-3 cells (SKOV-3 (mock): 1.52 ± 0.09, SKOV-3/BLU transfectant 1: 5.35 ± 0.39, SKOV-3/BLU transfectant 2: 4.65 ± 0.36, P < 0.001; Fig. 6C). However, the protein levels of BCL-2, MCL-1, or BAK were not different in ovarian cancer cells treated with PBS or paclitaxel.

Our results indicated that BLU could enhance the expression of BAX in ovarian cancer cells when encountered with paclitaxel.

Discussion
The current proposed mechanism of pathogenesis in epithelial ovarian carcinoma includes type I and II tumor pathways (Kurman & Shih Ie 2010) containing different histological types respectively. Type I tumor is composed of low-grade serous, low-grade endometrioid, clear cell, mucinous, and Brenner carcinomas that generally have an indolent behavior and the majority are confined to the ovary at initial presentation. However, type II tumor consists of high-grade serous carcinoma, undifferentiated carcinoma, and carcinosarcoma that have highly aggressive behaviors and are almost at the advanced stage. Previous literature about epigenetics in ovarian cancer showed the pattern of gene methylation varied in different histological types of ovarian cancer. For example, aberrant gene methylation of BRCA1, CDKN2A, and SFN is frequent in ovarian serous carcinoma, but SFN,
TMS1, and WTI are more frequent in ovarian clear cell carcinoma. (Barton et al. 2008, Asadollahi et al. 2010, Houshdaran et al. 2010, Seeber & van Diest 2012). Therefore, our study recruited only patients of advanced-stage, high-grade ovarian serous carcinoma, and the association of gene methylation and clinical outcomes can be interpreted without the influence of other histological types of ovarian carcinoma.

Methylation of BLU was associated with recurrence and the death of the patients with advanced ovarian serous carcinoma. The correlation of poor clinical outcomes and BLU methylation was also observed in patients who underwent chemotherapy with paclitaxel. This indicated that transcriptional silence of BLU by gene methylation may play an important role in ovarian cancer cells resistant to chemotherapeutic agents. Currently, the function of BLU is still under investigation. This gene is located in chromosome 3p21 containing eight candidate tumor-suppressor genes, including HYAL2, FUS1, RASSF1, BLU/2MYND10, NPRL2, 101F6 (CYB561D2), PL6 (TMEM115), and CACNA2D2. The MYND domain of BLU is a potential zinc-binding motif composed of cysteine and histidine residues, and it appears to act as transcriptional regulators by interacting with chromatin remodeling and transcription factors (Hesson et al. 2004, 2007). Ectopic expression of BLU can inhibit the colony formation of cancer cells and tumor formation in mice xenograft (Qiu et al. 2004, Hesson et al. 2007). The cytotoxicity of paclitaxel acts through the stabilization of microtubules, cell-cycle arrest in G2/M-phase, and the activation of pro-apoptotic signaling (Wang et al. 2000). Drug resistance is the flaw of paclitaxel in clinical managements of ovarian cancer patients, and several mechanisms have been proposed including overexpression of the multidrug-resistance gene (Yamamoto et al. 2000), increased DNA repair (Vikhanskaya et al. 2001), and suppression of apoptotic pathways (O’Gorman & Cotter 2001). Paclitaxel can upregulate the levels of BAX and BAK, which made the situation favorable for apoptosis (Jones et al. 1998). Stimulation of BAX expression led to enhanced sensitivity to paclitaxel in cancer cells (Strobel et al. 1996, De Feudis et al. 2000). Our results indicated that BLU can enhance the expression of BAX in ovarian cancer cells especially when treating with paclitaxel. It meant that BLU could upregulate the expression of BAX in favor of the pro-apoptotic pathway to promote paclitaxel-induced apoptosis in ovarian cancer cells. Transcriptional silencing of BLU by gene methylation would suppress the pro-apoptotic function, resulting in the resistance of ovarian cancer cells to paclitaxel. Restoration of transcription by demethylating agents would resume the pro-apoptotic ability of BLU to potentially enhance the therapeutic effects of paclitaxel on ovarian cancer. Our study also showed a trend for BLU in promoting the apoptosis of ovarian cancer cell treated with cisplatin, although the difference was not significant. SKOV-3 and OVCAR-3 cells are both originally resistant to cisplatin according to the ATCC (OVCAR-3, www.atcc.org/ATCCAdvancedCatalogSearch/ProductDetails/tabid/452/Default.aspx?ATCCNum=HTB-161&Template=cellBiology; SKOV-3, www.atcc.org/ATCCAdvancedCatalogSearch/ProductDetails/tabid/452/Default.aspx?ATCCNum=HTB-77&Template=cellBiology),
and the basic characteristic of the two cell lines might have a detrimental effect on evaluating the ability of BLU in the regulation of cisplatin-induced apoptosis. Paclitaxel enhanced apoptosis probably involving the BAX gene through a PS3-independent pathway, but cisplatin-induced apoptosis depended more on the status of PS3 in ovarian cancer cells (Jones et al. 1998, Takahashi et al. 2000, Gadducci et al. 2002, Kupryjaczyk et al. 2003). The conclusive clinical association between BAX and cisplatin sensitivity is currently lacking (Galluzzi et al. 2012). Further studies are needed to figure out the complex mechanism of cisplatin-induced apoptosis involving BLU, PS3, and apoptosis-related molecules.

The current managements for ovarian cancer are cytoreduction surgery and adjuvant chemotherapy. It is well known that residual tumor volume has a negative impact on the survival of patients with advanced ovarian cancer. The meta-analyses demonstrated that with each 10% increase in maximal cytoreduction, a 5.5–6.0% increase in median survival time was observed (Bristow et al. 2002). The goal of primary surgery is to remove as much tumor masses as possible and perfectly obtain the absence of residual disease, but the ability to successfully perform optimal cytoreduction ranges from 20 to 90% of the patients depending on the experience of the surgeon (Wakabayashi et al. 2008). In addition to optimal cytoreduction, chemoresistance is another obstacle in the treatments of ovarian cancer where several novel agents are now under investigation to overcome it (Agarwal & Kaye 2003). Epigenetically silenced genes can be re-expressed by demethylating agents in vitro and in vivo studies, and several clinical trials of epigenetic therapy are undergoing (Matei & Nephew 2010). Our study indicated that BLU methylation was an independent prognostic factor in advanced serous ovarian carcinoma, even in patients undergoing optimal debulking. The in vitro experiments also proved that BLU could help the ovarian cancer cells sensitize to paclitaxel through upregulating BAX. The novel findings could be incorporated into the design trials of epigenetic therapy in patients with advanced ovarian carcinoma. Based on the BLU methylation of ovarian cancer tissue, appropriate candidates, especially in patients undergoing optimal debulking, can be selected to receive a combination of paclitaxel-based chemotherapy and demethylating agents. Further clinical trials are needed to prove the utility of BLU methylation in the practice of managing ovarian cancer patients.

Taken together, methylation of BLU can be an epigenetic biomarker to predict prognosis and has the potential to develop a new strategy by combing demethylating agents in managing advanced serous ovarian cancer.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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