Western Blot Analyses

Cells were washed twice with PBS and then lysed in lysis buffer [50 mmol/L Tris-HCl, 150 mmol/L NaCl, 10% glycerol, 1% Nonidet P-40, 2 mmol/L EDTA, 50 mmol/L NaF, 2 mmol/L Na_3VO_4, and protease inhibitors (complete protease inhibitor cocktail tablets [Roche Diagnostics])]. Protein concentrations were measured against a standardized control using a protein assay kit (Bio-Rad Laboratories). A total of 50 μg protein was separated by electrophoresis on a 5% to 20% or 15% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore). All the antibodies that were used came from Cell Signaling Technology, except for mouse antiactin (Sigma)—rabbit anti-PTEN (1:500), rabbit anti-Akt (1:1000), rabbit anti–phosphorylated (p-) Akt (1:500), rabbit anti-mTOR (1:500), rabbit anti–p-mTOR (1:500), rabbit anti–4E-BP1 (1:1000), rabbit anti–p-4E-BP1 (threonine 37/46, 1:1000), rabbit anti–p70 s6 kinase (1:1000), rabbit anti–p-p70 s6 kinase (1:1000), rabbit anticleaved caspase 9 (1:500), rabbit anticleaved PARP (1:1000), and mouse antiactin (1:1000). These were visualized with secondary antimouse or antirabbit immunoglobulin G antibody coupled with horseradish peroxidase using enhanced chemiluminescence according to the manufacturer's recommendation.

Flow Cytometric Analysis

To analyze cell cycle distribution, the cells (2×10^6/L) were trypsinized, collected by centrifugation, fixed in 70% ethanol at 4°C for 1 hour, and resuspended in PBS containing 50 μg/mL propidium iodide and 0.1 mg/mL RNase. After 30 minutes at 37°C, the cells were analyzed with a flow cytometer (EPICS Altra HyperSort; Beckman Coulter, Inc).

Annexin V Staining

The annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (BioVision) was used to assess apoptosis as the externalization of phosphatidylserine residues according to the specifications of the manufacturer. Briefly, cells were suspended in 500 μL of 1 × binding buffer. The cells then were stained with 5 μL annexin V-FITC for 5 minutes in the dark at room temperature. Finally, the cells were analyzed with a flow cytometer (EPICS Altra HyperSort; Beckman Coulter, Inc).

Ovarian Cancer Xenograft Model

This study was carried out at the Laboratory Animal Research Center under the control of the animal research committee in accordance with the Guidelines for Animal Experimentation in the Faculty of Medicine of Tottori University in Yonago, Japan. For these experiments, OMC-1 or RMUG-S cells in log-phase growth were trypsinized, washed twice with PBS, and centrifuged at 250 × g. Viable cells were counted, then 5×10^6 viable cells (in 0.3 mL PBS) were inoculated subcutaneously under aseptic conditions into the left flank of female nude mice. The mice were assigned randomly to 1 of 3 groups (7 mice per group), and treatment was started 7 days later, which are as follows: group 1, oral administration of vehicle (PBS); and groups 2 and 3, oral administration of 25 or 50 mg/kg per day BEZ235 for 3 weeks, respectively (5 days per week). Tumor size was measured with a caliper twice weekly, and tumor volume was calculated according to the following equation: tumor volume (mm^3) = $\pi/6 \times L \times W^2$, where L and W are the long and short dimensions of the tumor, respectively. Two mice of each group were killed on day 25, and tumors were collected and fixed in 10% neutral buffered formalin (Wako Pure Chemical Industries) and embedded in paraffin for immunohistochemical analysis. Paraffin blocks were sliced in 4-μm sections and deparaffinized. The expression of p-Akt, p-mTOR, p-4E-BP1, and p-p70S6K proteins on the tumor tissue sections was detected using the Histofine Simple Stain PO kit (Nichirei Corporation). Slides were counterstained with hematoxylin. The primary antibodies used were anti–p-Akt (dilution, 1:100; Cell Signaling Technology), anti–p-mTOR (dilution, 1:100; Cell Signaling Technology), anti–p-4E-BP1 (dilution, 1:100; Cell Signaling Technology), and anti–p-p70S6K (dilution, 1:100; Cell Signaling Technology).

Statistical Analysis

Statistical analyses were performed using the GraphPad Prism program Version 5 (GraphPad Software, Inc). Data are presented as mean (1 SD). The means for all data were compared by 1-way analysis of variance with post hoc testing. A P value of less than 0.05 was considered statistically significant.

RESULTS

Sensitivity to Targeted Agents and the PI3K/Akt/mTOR Pathway in MAC Cell Lines

The effects of BEZ235 or temsirolimus on the proliferation of 7 MAC cell lines are shown in Figure 1A. NVP-BEZ235 decreased the cell viability rate in a dose-dependent manner in all MAC cell lines tested. Contrarily, reduced cell viability by temsirolimus was significantly less compared with BEZ235 in all lines.

We next determined in each MAC cell line the relationship between sensitivity to BEZ235 or temsirolimus and expression of protein in the PI3K/Akt/mTOR signaling pathway, or the presence of activating mutations of *PIK3CA* and *K-Ras* genes. The IC_{50} of these cell lines to BEZ235 ranged from 18 to 328 nmol/L and more than 10,000 nmol/L for temsirolimus, except for TU-OM-1 cells (530 nmol/L). The protein expression of Akt, mTOR, p70S6K, 4E-BP1, and these phosphorylation forms were confirmed in all cell lines (Fig. 1B). Although the expression levels were not related to the sensitivity to BEZ235 or temsirolimus, 2 cell lines, MCAS and OMC-1, with activating mutations of H1047R and E545K in the *PIK3CA* gene, respectively, exhibited low IC_{50} to BEZ235 (Fig. 1C). Mutations of G12A in the *K-Ras* gene also were observed in MCAS cells.

NVP-BEZ235 Inhibits the PI3K/Akt/mTOR Pathway

We examined the effect of BEZ235 and temsirolimus on the PI3K/Akt/mTOR signaling pathway in MAC cells. Treating OMC-1 and RMUG-S cells with BEZ235 suppressed the protein expression levels of p-Akt, p-p70S6K, and p-4E-BP1 in a dose-dependent manner (Fig. 2A). In contrast, the mTOR inhibitor used alone for temsirolimus treatment slightly down-regulated p-p70S6K and p-4E-BP1, whereas p-Akt

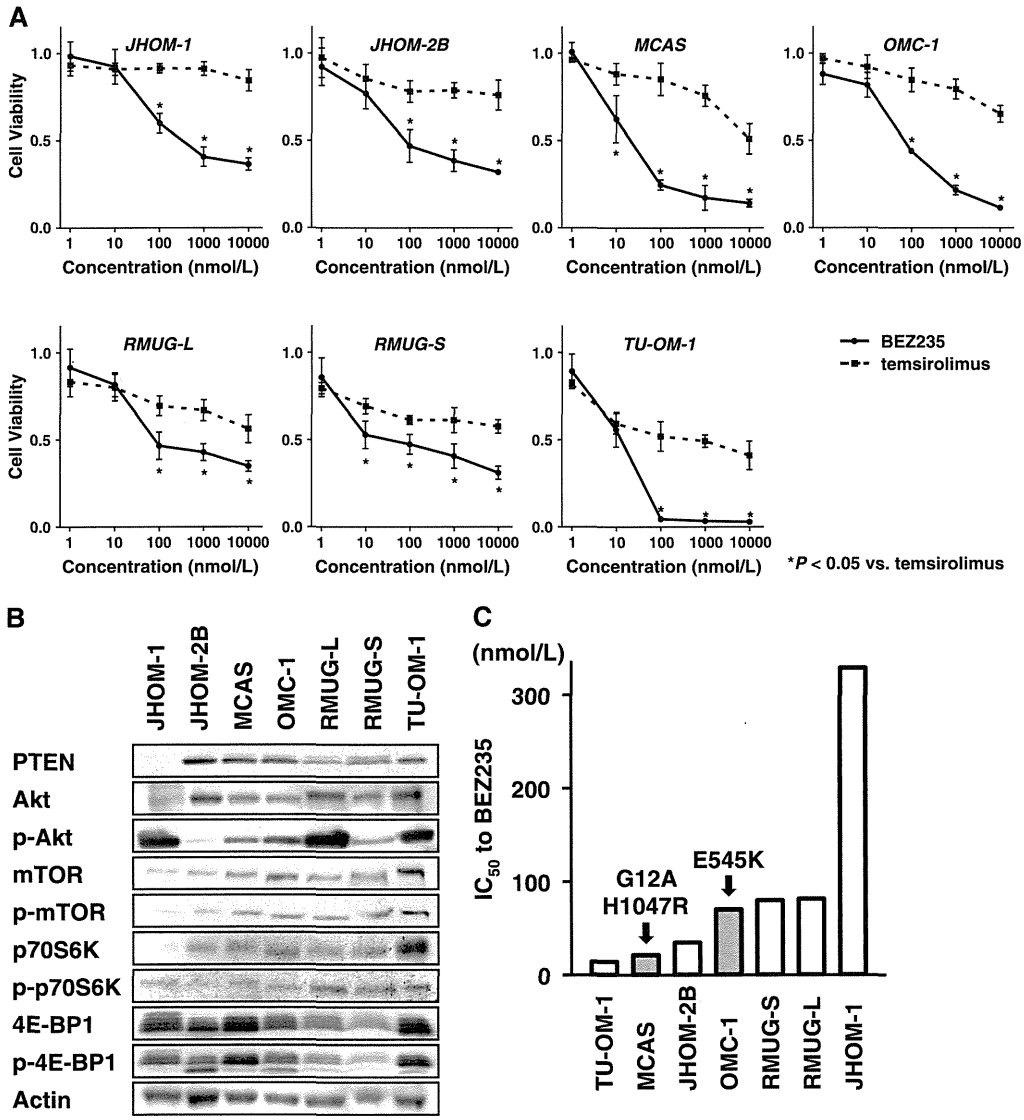

FIGURE 1. The cytotoxicity of BEZ235 and temsirolimus in MAC cell lines and the PI3K, Akt, and mTOR signaling pathways. A, Seven ovarian MAC cell lines were treated with varying concentrations of BEZ235 or temsirolimus, then inhibition of cell growth was determined using the WST-8 assay. The points represent mean (1 SD) from 6 dishes. B, The protein expression of Akt, phosphorylated (p-) Akt, mTOR, p-mTOR, p70S6K, p-p70S6K, 4E-BP1, and p-4E-BP1 in MAC cells was determined by Western blot analysis. The expression of Akt, p-Akt, mTOR, p-mTOR, p70S6K, p-p70S6K, 4E-BP1, and p-4E-BP1 proteins was confirmed in all cell lines. The results shown represent duplicate experiments. C, Half-maximal inhibitory concentrations for the effect of BEZ235 on cell growth were determined from the data in A. Cell lines with mutations activating *PIK3CA* and *K-Ras* are shown as gray columns.

protein expression levels were up-regulated in both cell lines (Fig. 2B). Similar results were obtained in the other 5 cell lines (data not shown).

NVP-BEZ235 Increased Gap $(G)_0/G_1$-Phase Fraction and Up-regulates the Apoptotic Pathway

We then assessed the cell cycle distribution by flow cytometry to confirm whether treatment with BEZ235 influenced cell cycle progression. After treatment with BEZ235, the proportion of the cells in G_0/G_1 phase increased markedly and in the S phase was decreased in OMC-1 and RMUG-S cells (Fig. 3A–C). Moreover, 72 hours after treatment with BEZ235, the $subG_1$ population was significantly increased compared with untreated controls.

To confirm whether the apoptotic pathway was activated by BEZ235, we assessed the protein expression levels of cleaved PARP and caspase 9 and the proportion of annexin V-positive cells among MAC cells. Forty-eight hours after

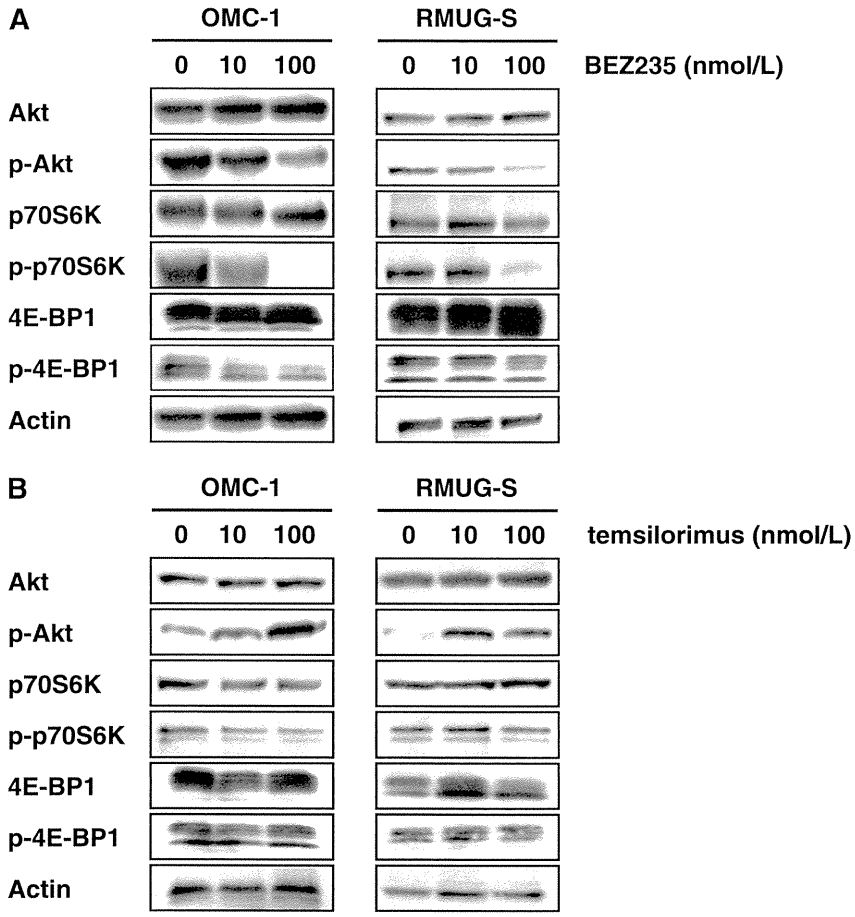

FIGURE 2. BEZ235 inhibited the PI3K, Akt, and mTOR signaling pathways. A, Phosphorylated (p-) Akt, p-p70S6K, and p-4E-BP1 in OMC-1 and RMUG-S cells were down-regulated in a dose-dependent manner 24 hours after being treated with BEZ235. B, Although p-p70S6K and p-4E-BP1 were suppressed 24 hours after treatment with temsirolimus, p-Akt increased in OMC-1 and RMUG-S cells. The results shown represent duplicate experiments.

treatment with BEZ235 at higher doses, the expression of cleaved PARP and caspase 9 were up-regulated in OMC-1 and RMUG-S cells, and the proportion of annexin V-positive cells increased in a dose-dependent manner (Fig. 4A and B). Similar results were obtained in the other 5 cell lines (data not shown).

NVP-BEZ235 Suppressed Tumor Growth in MAC Xenograft Models

After confirming that BEZ235 reduced cell viability and enhanced apoptosis in vitro, we examined the effect of treatment of BEZ235 in xenograft models of MAC. Female nude mice were given subcutaneous injections of OMC-1 or RMUG-S cells and then treated with daily oral PBS or BEZ235 (25 or 50 mg/kg per day). There were no signs of overt toxicity (weight loss or gross clinical signs) in any group (Fig. 5A).

To confirm the inhibition of PI3K/Akt/mTOR pathway by BEZ235 in vivo, we performed immunohistochemical analysis of tumor tissues (Fig. 5B). As expected, p-Akt, p-mTOR, p-4E-BP1, and p-p70S6K proteins were down-regulated in tumors from mice treated with BEZ235.

In nude mice bearing OMC-1 or RMUG-S, the mean volume of subcutaneous tumors in the group treated with BEZ235 25 or 50 mg/kg per day doses was significantly smaller than what was in the group treated with PBS ($P < 0.05$; Fig. 5C). These findings indicated that BEZ235 suppressed growth of subcutaneous tumors in nude mice bearing OMC-1 or RMUG-S cells.

Combination Effects of BEZ235 and Anticancer Agents

We analyzed the synergistic activities of combining BEZ235 with each anticancer agent by calculating CI values by using the method of Chou and Talalay.[26] Data representative of BEZ235 combined with paclitaxel or cisplatin in OMC-1 cells are shown in Figure 6A. The CI value at an effective dose of 50 (effective dose being the percentage inhibition of cell growth using the drug combinations in the

actual experiment) was less than 0.9 (synergism) for 6 cell lines treated with paclitaxel, 5 cell lines with cisplatin and SN-38, representing an active metabolite of irinotecan, 7 cell lines with etoposide, and 3 lines with gemcitabine (Fig. 6B).

DISCUSSION

In this exploration of the effects of BEZ235, we found that dual inhibition of PI3K and mTOR significantly inhibited proliferation activity in a panel of MAC cells. We also showed that BEZ235 induced cell cycle arrest at the G_0/G_1 phase and activated apoptotic pathways. The effectiveness of this agent was confirmed in xenograft models of MAC. NVP-BEZ235 markedly suppressed tumor growth in these mice compared with control animals. To our knowledge, this is the first study to show that the novel dual PI3K/mTOR inhibitor BEZ235 is effective against MAC, both in vitro and in vivo.

Numerous molecular-targeted agents have been developed and have already entered clinical practice.[29,30] The PI3K/Akt/mTOR pathway has been a focus for attractive treatment options for cancers.[20,21] The activation of PI3K caused phosphorylation and increased the activity of Akt, and

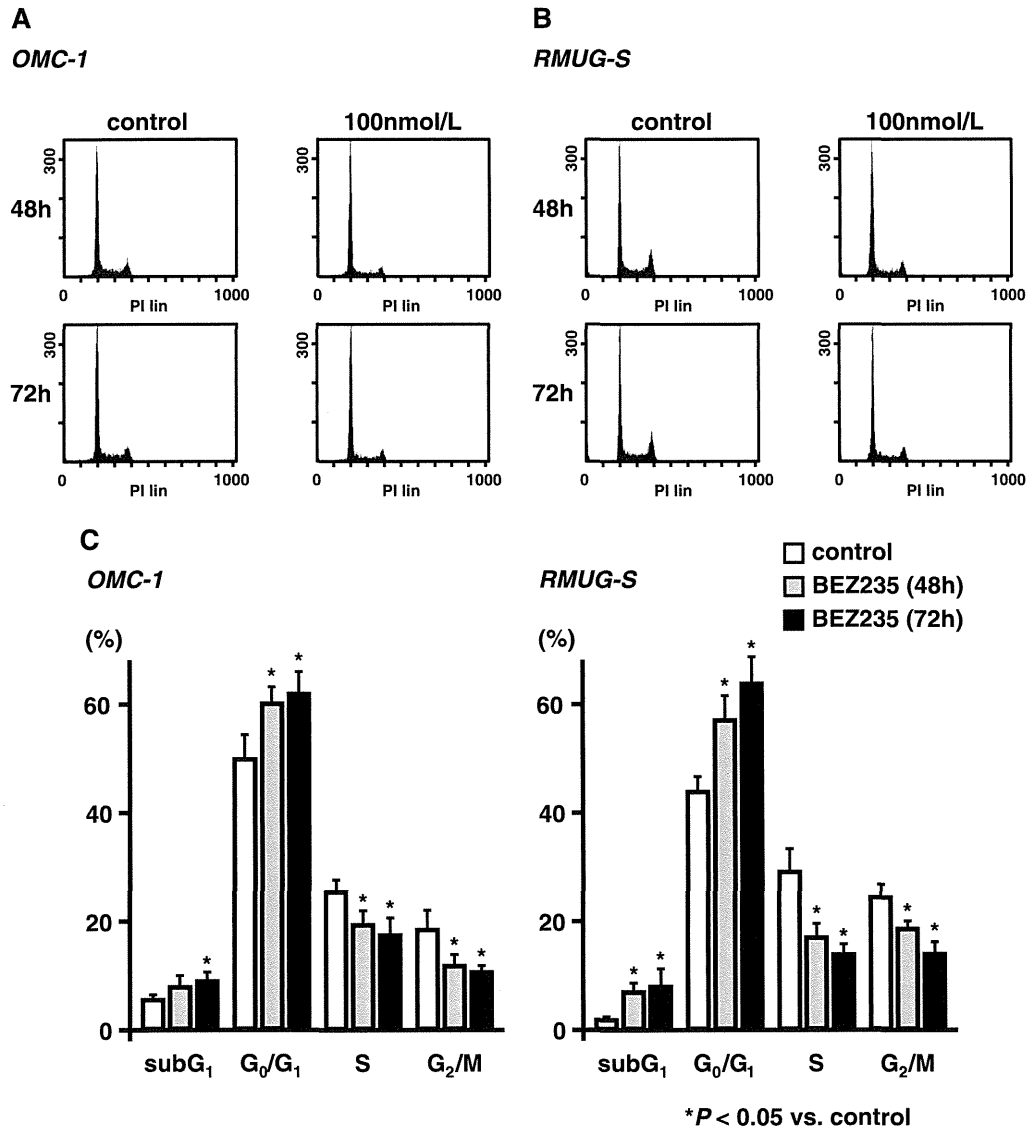

FIGURE 3. The effects of BEZ235 on the cell cycle distribution. The MAC cells OMC-1 and RMUG-S were treated with PBS (control) or 100 nmol/L BEZ235. The cell cycle distributions of drug-treated cells were then determined by flow cytometry. Representative cytometry data are shown for OMC-1 (A) and in RMUG-S cells (B). C, BEZ235 decreased the G_0/G_1 phase fraction for 48 and 72 hours, and then, the $subG_1$ phase was increased for 72 hours in OMC-1 and RMUG-S cells. Points represent mean (1 SD) from 3 independent experiments.

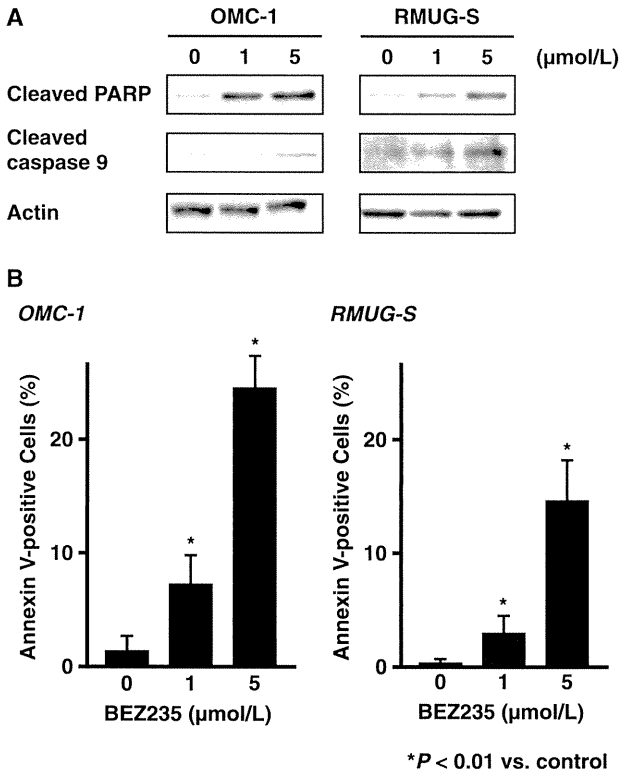

FIGURE 4. BEZ235 activated apoptotic pathways. A, The MAC cells OMC-1 and RMUG-S were treated with PBS (control) or 1 or 5 μmol/L BEZ235 for 48 hours. BEZ235 up-regulated the expression of cleaved PARP and caspase 9 proteins. B, Cells were treated with PBS (control) or 1 or 5 μmol/L BEZ235 for 48 hours and then stained with annexin V- FITC. The number of apoptotic cells increased after treatment with BEZ235 in a dose-dependent manner. Points represent mean (1 SD) from 3 independent experiments.

thus activated mTORC1.[10] Rapamycin and its derivatives, such as temsirolimus and everolimus, bind to FK506-binding protein 12, a member of the immunophilin protein family, and this complex inhibits kinase activity of mTORC1 allosterically by binding directly to mTOR. When the downstream signaling of mTOR such as 4E-BP1 and p70S6K1 is blocked, it leads to arrest in the G_1-phase cell cycle and cell death.[21] However, several studies have reported that mTOR inhibition alone up-regulated the expression of p-Akt in some cells because a feedback loop that depends on mTORC1 is induced that limits activation of PI3K and/or continued activation of Akt mediated by mTORC2. Activated Akt may attenuate the antitumor effect of rapalogues.[24,31–33] In addition, rapalogues may cause feedback activation of the PI3K/Akt pathway mediated by insulinlike growth factor 1 receptor signaling.[34] Indeed, we found that p-Akt increased in 6 of 7 cell lines after being treated with temsirolimus and that those 6 cells resisted cell cycle arrest induced by temsirolimus. These results suggested that the effect of the mTOR inhibitor alone might be limited to treat patients with MAC.

In contrast to the mTOR inhibitor alone, the dual PI3K/mTOR inhibitor, BEZ235, down-regulated p-Akt expression and downstream signaling of mTOR (p-4E-BP1 and p-p70S6K) and suppressed proliferation activity of MAC cells. Moreover, BEZ235 induced cell death by up-regulating the apoptotic pathway in MAC cells. Consistent with our findings, other studies have reported that BEZ235 had more pronounced effects compared with an mTOR inhibitor alone on cell growth in breast cancer and EOC.[22,24] This convergence of results suggested that dual inhibition of PI3K and mTOR may be required for therapeutic efficacy in patients with MAC.

Several studies have reported that activating mutations of the *PIK3CA* gene may predict enhanced sensitivity to treatment with inhibitors of the PI3K/Akt/mTOR pathways in patients with advanced cancers, including EOC.[24,35–37] *PIK3CA* is located on chromosome 3q26.32 and encodes the p110α catalytic subunit of the class IA PI3K. Most *PIK3CA* mutations are at E542K and E545K in the helical domain (exon 9) and H1047R in the kinase domain (exon 20), resulting in kinase activation of p110α, which then activates Akt.[38] Indeed, 2 cell lines with *PIK3CA* mutations at an E545K or H1047R showed low IC_{50} to BEZ235. However, we observed that BEZ235 was effective (IC_{50} < 100 nmol/L) in 4 of the other 5 MAC cell lines with wild-type *PIK3CA*. Similar effectiveness of BEZ235 on some cancer cells without mutations in this gene has been reported.[24,39] Our findings suggested that BEZ235 may have clinical benefit not only in

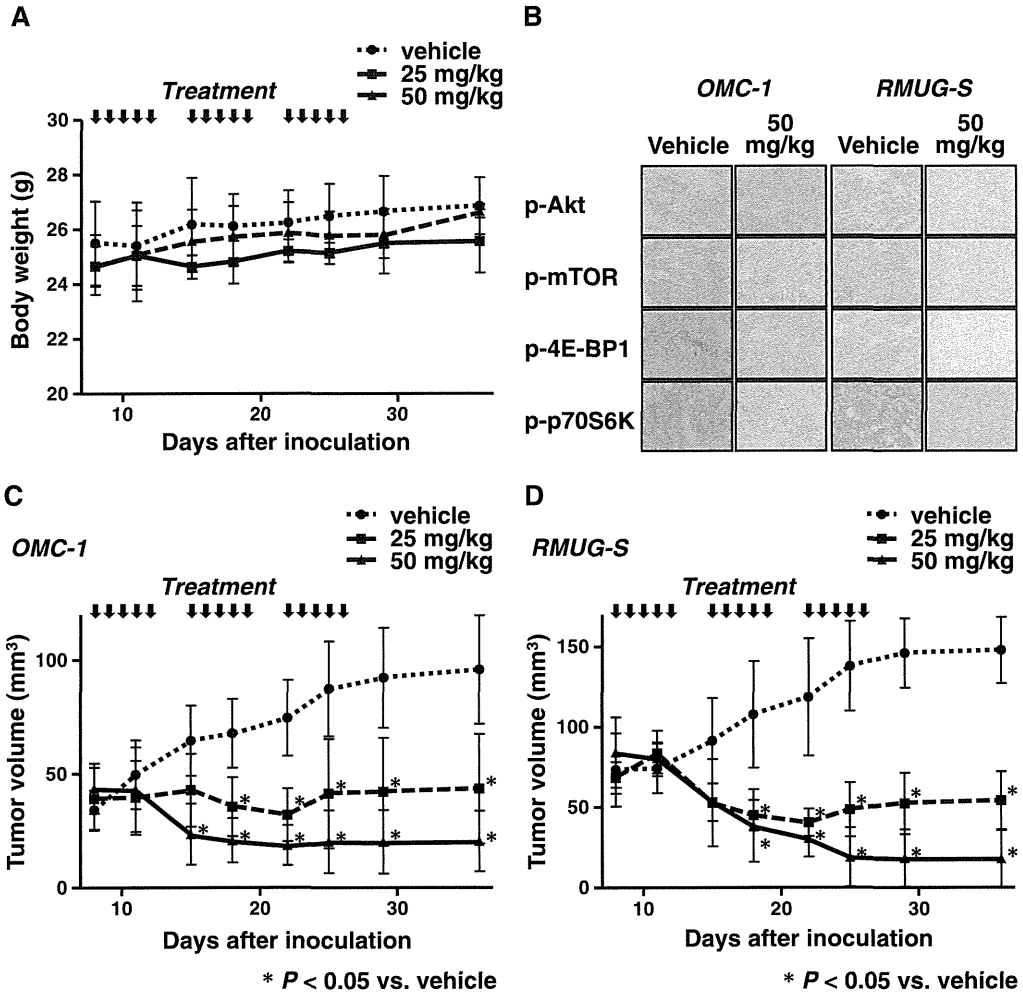

FIGURE 5. Treatment with BEZ235 suppressed the growth of subcutaneous tumors in mice with implanted OMC-1 or RMUG-S cells. Female nude mice (7 per group) were given a subcutaneous inoculation of 5×10^6 OMC-1 or RMUG-S followed by daily oral administration of PBS or 25 or 50 mg/kg per day BEZ235 for 3 weeks. A, The mean body weight of each treatment group. Error bars represent 1 SD. B, Immunohistochemical stains of representative tumor tissue samples from mice implanted with OMC-1 or RMUG-S cells and treated with PBS or 50 mg/kg per day BEZ235. The brown staining indicates phosphorylated (p-) Akt, p-mTOR, p-4E-BP1, and p-p70S6K. C and D, Tumor size was measured twice weekly. In mice inoculated with OMC-1 (C) and RMUG-S cells (D), tumor volume of the subcutaneous tumors was significantly lower in the mice treated with BEZ235 than with PBS (control; $P < 0.05$).

patients with MAC with activating mutation in *PIK3CA* but also for those with wild-type *PIK3CA*.

Mutational activation of *K-Ras* has been reported to predict poor response to PI3K/Akt inhibitors,[37] and *K-Ras* is thought to mutate frequently (average, 43%) in MAC.[40] In this study, however, only 1 cell line had alterations in *K-Ras* and *PIK3CA* genes, and it was sensitive to BEZ235. Consistent with our results, Santiskulvong et al[24] showed that BEZ235 was effective to established ovarian tumor disease using transgenic and immunocompetent LSL-*K-Ras*$^{G12D/+}$ *Pten*$^{loxP/loxP}$ mice. However, further studies are needed to elucidate the effect of BEZ235 in MAC with *K-Ras* mutation.

Further, we confirmed the effect of BEZ235 in vivo on MAC in murine xenograft models. NVP-BEZ235 decreased the expression of p-Akt and p-mTOR in the tumors of these mice compared with those treated with PBS alone. The present study provided clear evidence that BEZ235 down-regulated PI3K/Akt/mTOR signaling and suppressed proliferation of MAC cells both in vitro and in vivo.

Although MAC is known to resist platinum- and taxane-based chemotherapy, patients with MAC are usually treated with the standard chemotherapy regimen as used for EOC. Interestingly, a synergistic effect was observed from combining BEZ235 at even lower doses with paclitaxel (6 cell

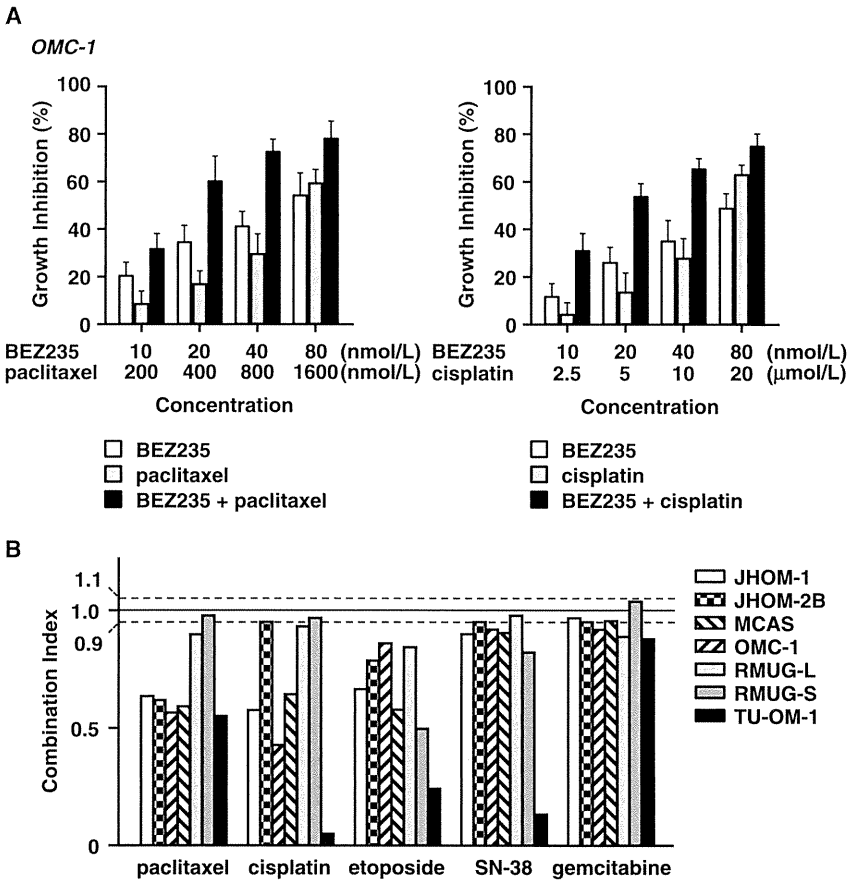

FIGURE 6. The effects of BEZ235 are synergistic or additive with those of each anticancer agent. Cells were incubated with increasing concentrations of BEZ235 with paclitaxel or cisplatin at a fixed ratio for 72 hours. The inhibition of growth was analyzed by using Cell Counting Kit-8. A, Representative data from BEZ235 combined with paclitaxel or cisplatin in OMC-1 cells. Results are mean (1 SD) of 3 independent experiments. B, Data analyzed by using CalcuSyn software to determine the CI. Chou and Talalay[26] defined CI as less than 0.9, 0.9 less than CI less than 1.1, and CI greater than 1.1 as synergism, additivity, and antagonism of the 2 agents, respectively.

lines) or cisplatin (5 cell lines) on cell proliferation. Several researchers also have reported that BEZ235 could sensitize the cytotoxic effects of paclitaxel or cisplatin in EOC and breast cancer cells, suggesting that dually inhibiting PI3K/mTOR might overcome resistance to anticancer agents.[24,25,41] In contrast, we previously found antagonistic effects when the mTOR inhibitor rapamycin was combined with paclitaxel or cisplatin on more than 4 of 6 ovarian serous adenocarcinoma cell lines.[42] However, combining the PI3K inhibitor LY294002 with paclitaxel or cisplatin had an additive inhibitory effect on cell growth in serous adenocarcinoma cells.[43,44] These observations suggested that BEZ235 could be incorporated into first-line chemotherapy for MAC.

In summary, our study showed that the dual PI3K/mTOR inhibitor BEZ235 has growth inhibition and antitumor effects, and it enhances the cytotoxic effect of chemotherapeutic agents in MAC cells. We also found that BEZ235 is therapeutically effective in both *PIK3CA* mutant and *PIK3CA* wild-type MAC cells. Therefore, we concluded that BEZ235 is worth exploring in a therapeutic strategy for MAC. We hope that such therapies will improve the survival of patients with advanced MAC.

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For a full list of references, please contact itamochi@med.tottori-u.ac.jp.

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ORIGINAL ARTICLE

Pathology-oriented treatment strategy of malignant ovarian tumor in pregnant women: analysis of 41 cases in Japan

Asuka Morikawa · Kazu Ueda · Kazuaki Takahashi · Masaharu Fukunaga · Mitsutoshi Iwashita · Yoichi Kobayashi · Kimihiro Takechi · Satoshi Umezawa · Fumitoshi Terauchi · Kazushige Kiguchi · Daisuke Aoki · Hiroyuki Nomura · Hiroyuki Yoshikawa · Toyomi Satoh · Toshiko Jobo · Hiroyuki Fujiwara · Yuji Takei · Seiryu Kamoi · Yasuhisa Terao · Seiji Isonishi

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Abstract
Background The aim of this study was to investigate the impact of the histological findings on the treatment of malignant ovarian tumors in pregnant women.
Methods This is a retrospective study of 41 patients diagnosed and treated for ovarian malignancy during pregnancy between 1985 and 2010.
Results The median age of the study group was 30 years old, ranging from 20 to 41. Thirty-eight (92 %) patients were diagnosed with stage I, and one (2 %) with each of stages II, III, and IV. Twenty-five (61 %) patients had borderline malignancy, 8 (20 %) were diagnosed with epithelial ovarian cancer, 7 (17 %) with germ cell tumor, and one with sex cord stromal tumor. All patients received primary surgery; 7 (17 %) patients had cystectomy, 32 (78 %) had unilateral salpingo-oophorectomy, and 3 (7 %) underwent hysterectomy with bilateral salpingo-oophorectomy. Thirty-one (76 %) patients delivered live newborns; 21 had borderline tumor (84 %), 2 had ovarian cancers (25 %), and 8 had non-epithelial tumor (100 %). Six cases were terminated in order to perform the standard treatment for ovarian malignancy and 2 cases aborted spontaneously.
Conclusion In pregnant women, ovarian cancer is exceptionally less frequent compared with non-pregnant

A. Morikawa · K. Ueda · K. Takahashi · S. Isonishi (✉)
Department of Obstetrics/Gynecology, Jikei Daisan Hospital, 4-11-1 Izumi-Honchou, Komae, Tokyo 201-8601, Japan
e-mail: isonishi@jikei.ac.jp

M. Fukunaga
Department of Pathology, Jikei Daisan Hospital, 4-11-1 Izumi-Honchou, Komae, Tokyo 201-8601, Japan

M. Iwashita · Y. Kobayashi
Department of Obstetrics and Gynecology, Kyorin University Hospital, 6-20-2 Shinkawa, Mitaka, Tokyo 181-0004, Japan

K. Takechi
Department of Obstetrics and Gynecology, Showa General Hospital, 2-450 Tenjincho, Kodaora, Tokyo 187-8510, Japan

S. Umezawa
Department of Obstetrics and Gynecology, Japanese Red Cross Musashino Hospital, 1-26-1 Kyonancho, Musashino, Tokyo 180-8610, Japan

F. Terauchi
Department of Obstetrics and Gynecology, Tokyo Medical University Hospital, 6-7-1 nishi-shinjyuku, shinjyuku-ku, Tokyo 160-0023, Japan

K. Kiguchi
Department of Obstetrics and Gynecology, St Marianna University School of Medicine Hospital, 2-16-1 Sugao, Miyamae-ku, Kawasaki, Kanagawa 216-8511, Japan

D. Aoki · H. Nomura
Department of Obstetrics and Gynecology, Keio University Hospital, 35 Sinanomachi, Shinjyuku-ku, Tokyo 160-8582, Japan

H. Yoshikawa · T. Satoh
Department of Obstetrics and Gynecology, Tsukuba University Hospital, 2-1-1 Amakubo, Tsukuba, Ibaraghi 305-8576, Japan

T. Jobo
Department of Obstetrics and Gynecology, Social Insurance Sagamino Hospital, 1-2-30 Fuchinobe, Chuou-ku, Sagamihara, Kanagawa 252-0206, Japan

H. Fujiwara · Y. Takei
Department of Obstetrics and Gynecology, Jichi Medical University Hospital, 3311-1 Yakushiji, Shimono, Tochigi 329-0498, Japan

women, i.e. age-matched, statistically-corrected controls based on the Japanese annual report [8/33 (24 %) vs. control (60 %); ovarian cancer/(ovarian cancer + borderline tumor), $P = 0.001$]. The pregnant women with ovarian cancer chose to prioritize treatment of ovarian cancer at the sacrifice of their babies while those with borderline tumor or non-epithelial tumor were able to successfully deliver live newborns.

Keywords Ovarian cancer · Preganancy · Pathology · Progesterone

Introduction

Malignant ovarian tumors diagnosed during pregnancy are extremely rare [1, 2] which is reflected in the paucity of reports in the literature. The estimated occurrence of ovarian carcinoma in pregnancy is reported to be between 1:10,000 and 1:50,000 [3, 4] pregnancies with a reported incidence of 0.0179 [5] to 0.11 [6] per 1000 pregnancies. Germ cell tumors are reported as more prevalent than other histological types of ovarian malignancy diagnosed during pregnancy [7, 8], which is consistent with the age-matched, non-pregnant women [9, 10]. Epithelial ovarian malignancy is reported to occur in 1:12,000 to 1:50,000 pregnancies [1, 11, 12]. The low incidence of epithelial ovarian malignancy detected during pregnancy appears to reflect the low prevalence of ovarian cancer in young women [6], although childbearing among older women is increasing these days. On the other hand, ovarian epithelial tumors diagnosed during pregnancy are reported in the majority to be borderline malignant tumors (BLM). BLM during the reproductive age (20–40 years old) is more frequent than in the age bracket (40–60 years old), which is more susceptible to epithelial ovarian cancer (EOC). However, in Japan, the real number of ovarian invasive cancers is still much higher than BLM even in the reproductive age group. Since the degree of malignancy is a particularly important factor in deciding management methods for ovarian cancer during pregnancy, we have reviewed the clinical courses of 41 cases, focusing on the treatment options based on pathological findings in order to strike a balance between maternal and fetal outcome.

S. Kamoi
Division of Reproductive Medicine, Perinatology and Gynecologic Oncology, Graduate School of Medicine, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan

Y. Terao
Department of Obstetrics and Gynecology, Juntendo University School of Medicine, 2-1-1 Hongou, Bunkyo-ku, Tokyo 113-8412, Japan

Materials and methods

Clinical and pathological profiles of the patients

This is a retrospective review of the clinical history of patients with ovarian cancer during pregnancy who were diagnosed and treated in the Gynecologic Oncology Departments of 12 institutions over a 25-year period between 1985 and 2010. This study was conducted in compliance with the principles outlined in the Declaration of Helsinki and was approved by each of the universities and hospitals involved. The following clinical and pathological information was collected from the patients' records: patient's age, gestational age at diagnosis, histological type of tumor, FIGO stage, type of surgery and chemotherapy, and perinatal outcome.

All pathological diagnoses were performed by specialists from the Pathology Department at each hospital. For EOC, the available specimens were reviewed again by a surgical pathologist to obtain the pathological diagnosis along with the type I/II classification. Follow-up information at each hospital was recorded up to the date of last contact or death.

Differences in proportions were evaluated by the chi-squared test as deemed appropriate.

Results

Patient demographics

The median age of the patients was 30 years, ranging from 20 to 41 years. Table 1 shows the patients' distribution by stage and histological type. Thirty-eight patients (92 %) were in stage I, and one patient (2 %) was in each of stages II, III, and IV. Twenty-five (61 %) were diagnosed with BLM and 8 (20 %) had EOC, one of whom was diagnosed with type II high-grade serous carcinoma. Seven (17 %) had germ cell tumors and one had sex cord stromal tumor. It should be noted that only three cases had advanced ovarian cancer (stage ≥II); one case in each of the BLM, EOC and non-epithelial categories. When the ratios of EOC/(EOC + BLM) are compared with those in non-pregnant, age-matched statistically corrected controls, based on the Japanese Annual Report of Gynecologic Malignancies 2010 [13], EOC was significantly less frequent in pregnant women [8/33 (24 %) vs. 440/740 (60 %)] ($P = 0.001$).

Clinical features

Table 2 summarizes the clinical features in relation to the histological type. No significant differences were noted

Table 1 Patients' demography by stage and histological type

FIGO stage	Borderline malignancy (25)			Epithelial cancer (8)				Non-epithelial cancer (8)				Total (%)
	Serous	Mucinous	Mix	SA	MA	EA	CA	IT	MT	YS	SL	
Ia	9	9		1	1	1		2	1		1	25 (61)
Ib	1											1 (2)
Ic	1	3	1			1	3		1	2		12 (29)
IIc								1				1 (2)
IIIc	1											1 (2)
IV				1								1 (2)
Total	12	12	1	2	1	2	3	3	2	2	1	41

SA serous adenocarcinoma, *MA* mucinous adenocarcinoma, *EA* endometrioid adenocarcinoma, *CA* clear cell adenocarcinoma, *IT* immature teratoma, *MT* mature cystic teratoma with malignant transformation, *YS* yolk sac tumor, *SL* Sertoli-Leydig cell tumor

among obstetric data of gestation and delivery, and 26 (63 %) patients were pregnant for the first time. In 31 patients (76 %), ovarian tumor was detected in first trimester and was removed immediately after the diagnosis when the patient did not want the baby (8 cases). However, when patients desired to keep their pregnancy, laparotomy was performed at around 14 weeks of gestation when hormonal function begins in the placenta. In 6 patients, ovarian tumor was detected in the 2nd trimester and, in 4 of them, the tumor was removed immediately after diagnosis. One of them had abdominal pain at the late phase of the 2nd trimester and she underwent unilateral salpingo-oophorectomy at 28 weeks of gestation, revealing the EOC. Another patient with a yolk sac tumor had an extremely high level of AFP in the late phase of the 2nd trimester and a germ cell tumor was strongly suspected at 28 weeks of gestation. She received a Cesarean section with concurrent salpingo-oophorectomy and omentectomy at 28 weeks of gestation. She received chemotherapy after delivery. In 3 patients, ovarian tumor was detected at 38 weeks of gestation when Cesarean section was performed. All of these cases showed no apparent clinical symptoms during pregnancy.

Although the tumor markers usually rise during pregnancy, the average serum CA125 in pregnant women with EOC was higher than in those with BLM (176 u/ml vs. 56 u/ml), suggesting that a substantially higher level of CA125 might be an indicator of EOC, although there is a lack of statistical evidence due to the small sample size. Similarly, there were two cases of yolk sac tumor showing extremely high levels of AFP $>$ 6000 ng/ml, indicating that an extraordinarily increased AFP could be an important warning for the presence of yolk sac tumor. Clinical symptoms of abdominal pain were seen in one case of EOC and genital bleeding in one case of BLM; the latter might be attributable to an obstetric cause. Among 31 patients who gave birth to a healthy baby, 20 were selected for Cesarean section. Eight patients had obstetric indications, while 5 patients terminated pregnancy at the earliest opportunity in order to treat the ovarian malignancy after termination, and 4 patients selected Cesarean section for concurrent second surgery. There was no patient with a history of infertility or a family history of hereditary cancer syndrome.

One exceptional case with stage IV, high-grade serous adenocarcinoma underwent immediate salpingo-oophorectomy and omentectomy immediately after diagnosis in the 12th week of gestation. She terminated the pregnancy at 16 weeks of gestation, when multiple metastases were detected in lung and liver. She received platinum-based chemotherapy; however, the tumor grew further, she developed brain metastasis, and died of the disease 6 months after diagnosis.

Surgery

Surgery was the primary treatment in all cases and the type of surgery is listed in Table 3. The basic surgery was composed of either or both of total abdominal hysterectomy and/or salpingo-oophorectomy. Seven patients (17 %) had cystectomy, 31 patients (76 %) had salpingo-oophorectomy, and 3 patients (7 %) had total abdominal hysterectomy. Of the 7 cystectomies, 6 were performed in initial laparotomy as a core surgery under the preoperative diagnosis of benign ovarian tumor. However, after pathological diagnosis of ovarian malignancy, these patients received additional surgery composed of not less than salpingo-oophorectomy. One case with bilateral BLM, underwent cystectomy and salpingo-oophorectomy to preserve hormonal function at the initial surgery without any further treatment. Additionally, 13 patients (31 %) underwent omentectomy and 1 patient (2 %) had lymphadenectomy. Thirty-nine patients (95 %) underwent conservative surgery as initial treatment. Surgery was performed during the prenatal period in 27 cases, at the time of delivery in 6, and postpartum in 3.

Table 2 Clinical characteristics

	BLM tumor ($n = 25$)	Epithelial cancer ($n = 8$)	Non-epithelial cancer ($n = 8$)	Total
Parity				
0	16	5	5	26
1	6	2	1	9
2	3	1	2	6
≥3	0	0	0	0
Gravidity				
0	13	4	4	21
1	7	2	1	10
2	3	1	3	7
≥3	2	1	0	3
Tumor detection				
1st trimester	20	6	5	31
2nd trimester	2	2	2	6
3rd trimester	2	0	1	3
Unknown	1	0	0	1
Tumor marker				
CA125				
≥35 u/ml	8	4	3	15
<35 u/ml	7	1	0	8
AFP				
≥20 ng/ml	0		2	2
<20 ng/ml	5		0	5
Symptoms				
Abdominal pain	0	1	0	1
Bleeding	1	0	0	1
Delivery				
Transvaginal	9	1	1	11
CS	12	1	7	20
Obstetric indication	7	0	1	8
Early termination	2	0	3	5
Concurrent second operation	2	1	1	4
Chemotherapy[a]	0	0	2	2
Unclear	1	0	0	1

CS Cesarean section

[a] CS performed in order to minimize the toxicity of chemotherapy to the fetus in utero

Table 3 Type of surgery

Surgery	BLM tumor	Epithelial cancer	Non-epithelial cancer	Total (%)
Core surgery				
Cystectomy	4[a]	0	3	7 (17)
USO	17	8	5	30 (73)
BSO	1	–	–	1 (2)
TAH + BSO	3	–	–	3 (7)
Additional surgery				
Lymphadnectomy	–	1	–	1 (2)
Omentectomy	6	4	3	13 (31)

TAH total abdominal hysterectomy, *BSO* bilateral salpingo-oophorectomy, *USO* unilateral salpingo-oophorectomy, *BLM* borderline malignancy

[a] Oophorectomy and cystectomy

Table 4 Clinical profiles of 12 cases treated with chemotherapy

Histological type	Stage	Regimen	Fetal outcome	Maternal outcome
BLM tumor				
Serous	Ia	CAP	CS	NED
Serous	IIIc	Unknown	CS	NED
Mucinous	Ia	5-FU	T	NED
Epithelial ovarian cancer				
Serous	Ia	TC	T	NED
Serous	IV	TC	T	DOD
Endometrioid	Ic	TC	VD	NED
Clear cell	Ic	CAP	T	NED
Clear cell	Ic	TC	SA	NED
Non-epithelial ovarian cancer				
Immature	IIc	BEP[a]	CS	NED
Immature	Ia	VIP	CS	NED
Yolk sac	Ic	PVB[a]	CS	NED
Yolk sac	Ic	BEP	CS	NED

BLM borderline malignancy, *T* termination, *SA* spontaneous abortion, *VD* vaginal delivery, *CS* cesarean section, *TC* paclitaxel + carboplatin, *CAP* cyclophosphamide + doxorubicin + cisplatin, *BEP* bleomycin + etoposide + cisplatin, *VIP* ifosfamide + etoposide + cisplatin, *PVB* cisplatin + vinblastine + bleomycin, *NED* no evidence of disease, *DOD* died of disease

[a] Chemotherapy was performed with fetus in utero

Chemotherapy

Table 4 lists 11 cases who received chemotherapy. Two patients received chemotherapy with the fetus in utero. One patient with yolk sac tumor, stage Ic, received 4 courses of cisplatin, vincristine and bleomycin (PVB) from 20 to 28 weeks of gestation after confirmation from the histological diagnosis obtained on unilateral salpingo-oophorectomy at 17 weeks of gestation. She underwent Cesarean section at 31 weeks of gestation in order to obtain the intact survival of the baby while minimizing the toxic effects of PVB to the fetus. No residual tumor was detected at the time of Cesarean section. Another patient with grade 3 immature teratoma, stage IIc, received 4 courses of bleomycin, etoposide and cisplatin (BEP) starting at 27 weeks of gestation after confirmation of histological diagnosis on cystectomy at 23 weeks of gestation. She underwent Cesarean section at 33 weeks of gestation for the same reasons as the previous

Table 5 Fetal outcome

Pathology	Fetus			Newborns			
	Abortion	Termination	Total (%)	>37 weeks	≤37 weeks	Unclear	Total (%)
BLM tumor (25)	0	2	2 (8)	7	8	6	21 (84)
Epithelial ovarian cancer (8)	2	4	6 (75)	1	0	1	2 (25)
Non-epithelial ovarian cancer (8)	0	0	0 (0)	3	4	1	8 (100)
Total (41)	2	6	8	11	12	8	31

BLM borderline malignancy

case. Synchronously, she received unilateral salpingo-oophorectomy and the resection of peritoneal disseminated tumor. She continued BEP therapy after delivery to complete the course. Out of 13 cases with stage Ic disease, 5 patients received chemotherapy, and all 13 cases are alive with no evidence of the disease.

Fetal outcome

Table 5 summarizes fetal outcomes in all patients. Thirty-one patients gave birth to a healthy baby after the removal of their tumors in order to confirm the stage and the histological findings, while 8 patients failed to deliver their babies. Out of 8 cases, 2 patients aborted spontaneously and 6 patients terminated their pregnancy in the first or second trimester to prioritize the treatment of ovarian cancer. Among 8 EOC cases, 6 did not continue pregnancy (75 %), whereas, only 2 patients with BLM (8 %) and no patients with non-epithelial ovarian cancer failed to continue pregnancy. For both of the cases with BLM, it was strongly suspected that they had invasive ovarian cancer on first diagnosis, which discouraged the patients from continuing the pregnancy.

Discussion

The data presented here demonstrate that, in pregnant women, the BLM tumor is the most frequent ovarian malignancy (25 cases), followed by EOC (8 cases) and germ cell tumor (8 cases). This trend is consistent with other reports [6, 14], although it is of particular interest that our data shows that the ratio of BLM:EOC (25:8) is more than three times that found in the non-pregnant controls (300:440). Although the average age of childbearing was older than previously during the period of our investigation (1985–2010), this high incidence rate of BLM had not been expected. For ovarian cancer in pregnancy, primary surgery is indispensable to determine whether the patients are able to continue their pregnancies. When surgery reveals that the patient has BLM or a non-epithelial tumor, this is indicative that a safe normal delivery is possible. By contrast, in cases with EOC, 75 % of our patients discontinued their pregnancies. The core point in treating ovarian malignancy in pregnant women is to confirm the pathological diagnosis, particularly whether the tumor is EOC. Here we focused on two important points: (a) the clinical features and pathology-oriented treatment modalities, and (b) the scarcity of EOC during gestation.

Firstly, several lines of enquiry are suggested by our clinical surveillance of 41 gravid cases, despite some paucity of data and the lack of imaging data.

All but 2 cases followed an asymptomatic course for an average of 8 weeks of gestation after the patients had been preoperatively diagnosed as possibly having ovarian malignancy. Two cases experienced bleeding or abdominal pain in BLM and EOC, respectively, but these symptoms were not specific for their ovarian malignancies. Physicians in charge of gravid patients would not expect an extremely rare case of ovarian malignancy during pregnancy and it is quite normal that they did not check tumor markers routinely. However, our collected data indicate that extremely high levels of CA125 are found in EOC compared with BLM, and much higher than the natural elevation of CA125 in pregnant women. The fact that most of these cases were detected during early pregnancy (1st or early 2nd trimester) indicates that there may have been a pre-operative warning of EOC. This is more apparent with AFP level in the case of germ cell tumor. An extreme increase in AFP strongly indicates that the patient has germ cell tumor. When ovarian malignancy is suspected, immediate removal of the tumor is recommended in order to achieve a better outcome. It is essential to secure a pathological diagnosis as well as the stage of the disease as soon as possible. Initial surgery could avoid rapid progression of the disease even when it is cystectomy. However, this is undoubtedly insufficient surgery for the treatment of malignant ovarian tumor and patients must receive follow-up surgery. Cesarean section was occasionally performed as concurrent second surgery to complete the surgical treatment and to obtain more detailed staging information in order to determine whether the patient needed adjuvant chemotherapy. This is an acceptable strategy to treat malignant ovarian tumor during pregnancy without delay.

Secondly, the possible protective effect of pregnancy on tumorigenecity and progression of EOC is an important issue these days [15]. During the gestational period,

maternal gonadotropin decreases strongly, while massive placental production of progesterone begins. With regard to gonadotropin stimulation, evidence has shown that animals subjected to excessive gonadotropin have lower or no tumor occurrence when treated with depot gonadotropin-releasing hormone agonists [16, 17].

The tumors seen in these animals are tubular adenomas, which grow within and replace the ovarian stroma, but which do not invade in the uncontrolled manner characteristic of malignant cancers and do not metastasize. The findings are quite similar to those in ovarian BLM. On the other hand, progesterone is implicated as a protective factor against ovarian cancer, which is not mediated through suppression of ovulation. Progesterone or the response of ovarian surface epithelial cells to steroids affords protection against ovarian cancer development or progression, and increased parity is associated with a reduction in ovarian cancer risk [18, 19]. Although our data did not include EOC in a twin pregnancy, women with a history of twin pregnancies exhibit a lower risk for developing ovarian cancer, possibly due to the higher levels of progesterone in maternal circulation during twin pregnancies than during singleton pregnancies [18, 20]. Furthermore, women with natural dizygotic twins appear to have higher gonadotropin levels during their reproductive years [21, 22] and may be more likely to have double ovulation [23] compared with women experiencing singleton pregnancies. However, in a record-linkage study in women with dizygotic twins, no excess of ovarian cancer cases appeared [24], and a case–control study that examined the history of twin pregnancies found a slightly decreased risk with this factor [25].

In our retrospective study, along with the literature review, we have investigated the pathological features of malignant ovarian tumors during pregnancy to determine whether the treatment modality could be managed on the basis of histological findings and how to cope with the ovarian malignancy while preserving the baby.

Our data has provided several lines of thought-provoking issues that might be beneficial for future studies on ovarian cancer in pregnant women.

Conflict of interest The authors declare that they have no conflict of interest.

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Administration of standard-dose BEP regimen (bleomycin + etoposide + cisplatin) is essential for treatment of ovarian yolk sac tumour

Toyomi Satoh [a,*], Yoichi Aoki [b], Takahiro Kasamatsu [c], Kazunori Ochiai [d], Masashi Takano [e], Yoh Watanabe [f], Fumitaka Kikkawa [g], Nobuhiro Takeshima [h], Masayuki Hatae [i], Harushige Yokota [j], Toshiaki Saito [k], Nobuo Yaegashi [l], Hiroaki Kobayashi [m], Tsukasa Baba [n], Shoji Kodama [o], Tsuyoshi Saito [p], Noriaki Sakuragi [q], Toshiyuki Sumi [r], Toshiharu Kamura [s], Hiroyuki Yoshikawa [a]

[a] *Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tsukuba, Tsukuba, Japan*
[b] *Department of Obstetrics and Gynecology, Graduate School of Medical Science, University of the Ryukyus, Naha, Japan*
[c] *Department of Gynecologic, National Cancer Center Hospital, Tokyo, Japan*
[d] *Department of Obstetrics and Gynecology, The Jikei University School of Medicine, Tokyo, Japan*
[e] *Department of Obstetrics and Gynecology, National Defense Medical College, Saitama, Japan*
[f] *Department of Obstetrics and Gynecology, Kinki University Faculty of Medicine, Osaka, Japan*
[g] *Department of Obstetrics and Gynecology, Nagoya University Graduate School of Medicine, Nagoya, Japan*
[h] *Department of Gynecology, Cancer Institute Hospital, Tokyo, Japan*
[i] *Department of Obstetrics and Gynecology, Kagoshima City Hospital, Kagoshima, Japan*
[j] *Department of Gynecology, Saitama Cancer Center, Ina, Japan*
[k] *Gynecology Service, National Kyushu Cancer Center, Fukuoka, Japan*
[l] *Department of Obstetrics and Gynecology, Tohoku University, Sendai, Japan*
[m] *Department of Obstetrics and Gynecology, Graduate School of Medical Sciences Kyushu University, Fukuoka, Japan*
[n] *Department of Gynecology and Obstetrics, Kyoto University Graduate School of Medicine, Kyoto, Japan*
[o] *Department of Gynecology, Niigata Cancer Center Hospital, Niigata, Japan*
[p] *Department of Obstetrics and Gynecology, Sapporo Medical University School of Medicine, Sapporo, Japan*
[q] *Department of Gynecology and Obstetrics, Hokkaido University, Sapporo, Japan*
[r] *Department of Obstetrics and Gynecology, Osaka City University Graduate School of Medicine, Osaka, Japan*
[s] *Department of Obstetrics and Gynecology, Kurume University School of Medicine, Kurume, Japan*

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* *Corresponding author at:* Department of Obstetrics and Gynecology, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8575, Japan. Tel.: +81 29 853 3073; fax: +81 29 853 3072.
E-mail address: toyomi-s@md.tsukuba.ac.jp (T. Satoh).

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KEYWORDS
Ovarian yolk sac tumour
BEP regimen
Prognosis
Fertility

Abstract ***Aim:*** The aim of this study was to investigate prognostic factors, including postoperative chemotherapy regimen, for the treatment of ovarian yolk sac tumour (YST), and resulting fertility outcome.

Methods: A multi-institutional retrospective investigation was undertaken to identify patients with ovarian pure or mixed YST who were treated between 1980 and 2007. Postoperative chemotherapy regimen and other variables were assessed in univariate and multivariate analyses. Additionally, the reproductive safety of the BEP (bleomycin, etoposide and cisplatin) regimen was evaluated.

Results: There were 211 patients enrolled from 43 institutions. The BEP regimen and a non-BEP regimen were administered to 112 and 99 patients as postoperative chemotherapy, respectively. In univariate and multivariate analyses, age $\geqslant$ 22, alpha-fetoprotein $\geqslant$ 33,000 ng/ml, residual tumours after surgery and non-BEP regimen were independently and significantly associated with poor overall survival (OS). BEP was significantly superior to non-BEP in 5-year OS (93.6% versus 74.6%, $P = 0.0004$). Reduced-dose BEP (<75% standard-dose bleomycin and < 50% etoposide dose) was significantly associated with poorer 5-year OS compared with standard-dose BEP (89.4% versus 100%, $P = 0.02$ and 62.5% versus 96.9%, $P = 0.0002$). All patients who underwent fertility-sparing surgery recovered their menstrual cycles. Sixteen of 23 patients receiving BEP (70.0%) and 13 of 17 patients receiving non-BEP (76.5%) who were nulliparous at fertility-sparing surgery and married at the time of investigation gave birth to 21 and 19 healthy children, respectively.

Conclusions: The results of the present study suggest that standard-dose BEP should be administered for ovarian YST. BEP is as safe as non-BEP for preserving reproductive function.

1. Introduction

Until the early 1970s, patients with ovarian yolk sac tumour (YST) had miserable prognosis [1–4]. However, after a triple combination regimen, such as vincristine + actinomycin D + cyclophosphamide (VAC) or cisplatin + vinblastine + bleomycin (PVB), was introduced as postoperative chemotherapy during the 1970s, the survival of patients with YST drastically improved [5–7]. Furthermore, the bleomycin + etoposide + cisplatin (BEP) regimen was developed in the 1980s, and the survival of patients with YST revolutionarily improved [8,9]. However, recent reports have suggested that patients with YST have poorer prognosis than patients with other malignant ovarian germ cell tumours. Peccatori showed that the mortality rates of YST and dysgerminoma were 13.0% (3/23) and 5.3% (3/57), respectively [10]. Mangili reported that the 5-year overall survival (OS) rates were 69.6% and 94.2% for patients with YST and other germ cell tumours, respectively ($P < 0.001$) [11].

The standard strategy for treating YST is administration of BEP following primary surgery in all stages. BEP for patients with YST has been recommended by various guidelines. The recommended dose and administration schedule in the National Comprehensive Cancer Network (NCCN) guideline is bleomycin 30 units per week; etoposide 100 mg/m^2/day daily for days 1–5; and cisplatin 20 mg/m^2/day daily for days 1–5 for 3–4 cycles [12]. However, the BEP regimen is sometimes administered with a reduced dose of bleomycin and/or etoposide because of the potential for serious adverse reactions such as severe bone marrow suppression, pulmonary fibrosis or secondary leukaemia [13–17]. Therefore, the present study investigated whether the use of BEP, especially standard-dose BEP, is associated with OS in patients with YST, in addition to other prognostic factors.

Fertility-sparing surgery (FSS) is considered for patients with YST, even in advanced disease. Therefore, an additional purpose of the present study was to investigate the reproductive safety of postoperative chemotherapy for YST.

2. Methods

2.1. Patient population

Between 1980 and 2007, 211 patients with YST who underwent treatment in 43 institutions belonging to the Japan Clinical Oncology Group or who were referred to these institutions immediately after primary surgery performed elsewhere were enrolled into this study. Patients who received preoperative chemotherapy and/or no postoperative chemotherapy were not included in this study.

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Before the study subjects were enrolled into the present study, reassessment of histological type was performed in each institution according to the World Health Organisation criteria. Staging was determined according to the FIGO classification (1987).

Institutional review board approval was obtained from each institution before initiating the present investigation.

2.2. Definition of standard BEP and non-standard BEP

In the present study, standard BEP was defined as 3 or 4 cycles of chemotherapy consisting of bleomycin (20 mg/m^2/day or 30 mg/day) given on days 2, 9 and 16, etoposide (100 mg/m^2/day) given on days 1–5 and cisplatin (20 mg/m^2/day) given on days 1–5. The cycles were repeated every 3 weeks. Regarding the standard number of cycles of BEP, we administered three cycles for patients without residual tumour and four cycles for patients with residual tumour at primary surgery, allowing one or two additional courses until achieving normal AFP level. The serum AFP was measured every course of BEP in almost all patients.

Patients who received BEP were divided into the standard BEP group ($n = 37$) and the non-standard BEP group. The non-standard BEP group ($n = 70$) received less than the standard dose and/or less than the standard number of cycles. The following five patients who received BEP were excluded from both the standard BEP and non-standard BEP group: two patients were given an excessive dose of bleomycin, and three patients received BEP with an uncertain dose.

2.3. Matters for analysis

We investigated postoperative chemotherapy regimen and other variables as prognostic factors in all 211 patients. Regarding chemotherapy regimen, we compared 5-year OS between BEP and non-BEP, and between standard BEP and non-standard BEP. Other variables were age, stage, tumour size, serum AFP level before treatment, FSS and residual tumour at primary surgery. ROC curve was used for searching cut-off levels for age and AFP and we found the level of age was 22 years and the level of AFP was 33,000 ng/ml. We also investigated whether the doses of bleomycin, etoposide and cisplatin were associated with OS in patients who received BEP.

The reproductive safety of BEP and other regimens was retrospectively reviewed from the medical records of the patients who provided information on menstruation and reproductive outcomes.

2.4. Statistical analysis

Statistical analysis of data was performed using the JMP statistics package (SAS Institute, Cary, NC, USA). Two-sided probability values were calculated throughout and considered to be significant at the level of $P < 0.05$. Survival estimates were generated using Kaplan–Meier methods. To test differences between groups, we used log-rank testing for the univariate analysis and the Cox proportional hazard regression method for the multivariate analysis.

3. Results

3.1. Patient characteristics

A total of 211 patients with YST were entered into the study (Fig. 1). Table 1 summarises the main characteristics of patients and tumours. The median duration of follow-up after excluding patients who died was 93 (4–333) months from primary surgery.

The serum AFP level before treatment was measured in 174 of the 211 study patients. The AFP level of the patients with pure YST was similar to that of the patients with mixed YST having $\geqslant 50\%$ of the YST component, however the level was significantly higher than in the patients with mixed YST having <50% of YST component ($P < 0.01$).

Complete surgical staging was not done like a epithelial ovarian cancer in most patients with YST, therefore staging was determined by limited information from surgical and pathological findings. No residual tumour, residual tumour within 2 cm and residual tumour over 2 cm after initial surgery was 77.7%, 12.5% and 9.8% in BEP group, 68.7%, 10.1% and 21.2 in non-BEP group, 89.1%, 5.4% and 5.4% in standard BEP group and 87.1%, 0% and 12.9% in non-standard BEP group.

Table 2 shows that comparative demographics for the BEP group versus non-BEP group and standard BEP group versus non-standard BEP group were similar.

3.2. Clinical outcomes

The estimated 5-year OS of the patients in each stage was 92.5% in stage I, 87.8% in stage II, 74.7% in stage III and 44.5% in stage IV. Overall, 33 deaths (15.6%) occurred from the following causes: disease progression of YST ($n = 31$, 14.2%), pulmonary fibrosis during BEP ($n = 1$, 0.5%) that developed after the patient was given 330 mg of bleomycin in total, and breast cancer ($n = 1$, 0.5%) that occurred 7 years after complete remission following BEP.

Of 99 patients who received non-BEP, 12 patients after remission (normalisation of AFP) and 15 patients without remission progressed their disease. Six patients after remission and 2 patients without remission progressed their disease among 70 patients who received non-standard BEP. We experienced no recurrent patients in the standard BEP group. Only two of 27 (7.4%) relapsed patients in non-BEP group and two of

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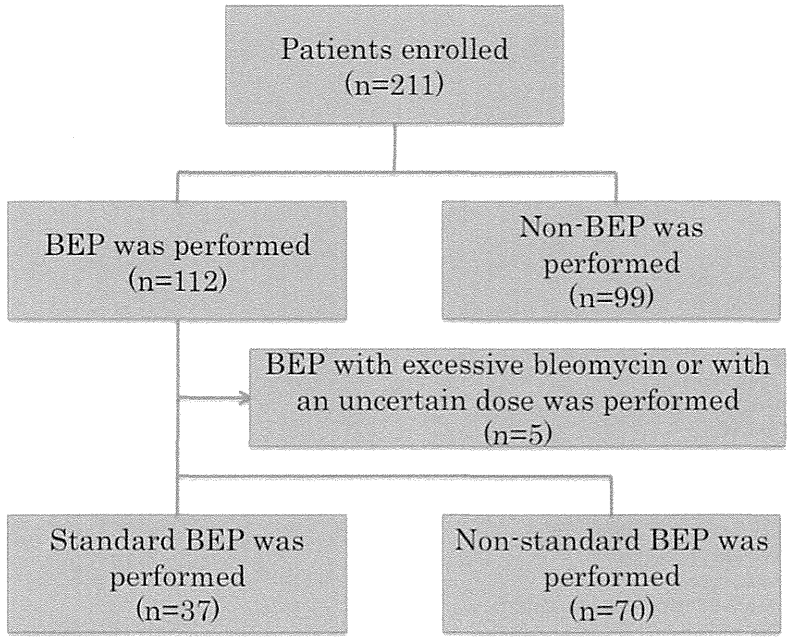

Fig. 1. CONSORT diagram.

eight (25%) relapsed patients in non-standard BEP group had long-term progression-free survival after receiving salvage therapy and the remaining 31 patients died of disease within 44 months.

As for the two rescued patients in the non-BEP group, one patient with stage Ic who had three courses of platinum-based non-BEP after surgery progressed the disease and the patient received six courses of PVB and had complete remission. Another patient with stage 1c who received five courses of platinum-based non-BEP after surgery, recurrent tumour developed in the contralateral ovary 128 months after surgery. She underwent the tumour resection from the ovary, fertility-sparing surgery, followed by three courses of VAC.

As for the two rescued patients in the non-standard BEP group, one patient with stage IIIa who received three courses after surgery had recurrent tumour in the pelvis 17 months after surgery. She received three courses of non-standard BEP after the recurrent tumour was removed surgically. Another patient with stage IIIc who received five courses after surgery had recurrent tumour in a paraaortic lymph node 42 months after surgery. The tumour was completely removed by surgery. She did not receive postoperative chemotherapy because the pathological diagnosis of the removed tumour was mature cystic teratoma with a very small part of YST.

These four patients were alive without disease 85, 68, 60 and 52 months after salvage therapy, respectively.

3.2.1. Analysis of prognostic factors

Table 3 shows the results of univariate and multivariate analyses for OS. In the univariate analysis, five variables—age $\geqslant$ 22 years, FIGO stage III/IV, AFP $\geqslant$ 33,000 ng/ml, residual tumour at primary surgery, and chemotherapy regimens other than BEP were significantly associated with poor OS. Subsequently, we performed multivariate analysis using the above significant five variables in the univariate analysis. In the multivariate analysis, age $\geqslant$ 22, AFP $\geqslant$ 33,000 ng/ml, residual tumour at primary surgery, and regimens other than BEP were independently and significantly associated with poor OS of patients with YST.

3.2.2. BEP and non-BEP

There were 112 patients who received BEP and 99 patients who received non-BEP. Non-BEP chemotherapy regimens were PVB ($n = 33$), peplomycin + etoposide + cisplatin ($n = 20$), paclitaxel + carboplatin ($n = 8$), vinblastine + actinomycin D + bleomycin ($n = 7$), VAC ($n = 4$), peplomycin + vinblastine + cisplatin ($n = 4$), etoposide + cisplatin ($n = 4$), other regimens with platinum ($n = 16$) and other regimens without platinum ($n = 3$). Of 99 patients who received non-BEP, 72 patients who gave substantial information received additional 0–7 cycles (median: 2) of chemotherapy after AFP normalisation. As shown in Table 3, BEP was significantly superior to non-BEP with respect to 5-year OS (93.6% versus 74.6%, $P = 0.0004$). In 71 patients with stage III/IV, 5-year OS was 94.0% with BEP ($n = 35$), 66.7% with PVB ($n = 9$), 50.0% with PEP ($n = 6$) and 43.5% in other regimens ($n = 21$) (Fig. 2A). The 5-year OS of 56 patients with residual tumour at primary surgery was 91.8% with BEP ($n = 25$), 50% with PVB ($n = 8$), 40.0% with PEP ($n = 5$) and 33.3% with the other regimens ($n = 18$) (Fig. 2B). In

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Table 1
Patient characteristics ($n = 211$).

Median age (range)	23 (11 months-68 years)
FIGO stage	
I	123 (58.3%)
II	17 (8.1%)
III	60 (28.4%)
IV	11 (5.2%)
Ascites	
Present	163 (77.3%)
⩾500 ml	50 (23.7%)
<500 ml	89 (42.2%)
Unknown	24 (11.4%)
Absent	44 (20.9%)
Unknown	4 (1.9%)
Histological features	
Pure YST	144 (68.2%)
Mixed YST	67 (31.8%)
Proportion of YST in mixed YST	
YST component ⩾50%	21 (31.3%)
YST component <50%, ⩾5%	29 (43.3%)
YST component <5%	5 (7.5%)
Unknown	12 (17.9%)
Median AFP level before treatment	
Pure YST ($n = 117$)	22,829 (403–540,000)
Mixed YST	
YST component ⩾50% ($n = 18$)	22,318 (1,399–146,665)
YST component <50%, ⩾5% ($n = 25$)	7,350 (136–80,300)
YST component <5% ($n = 5$)	228 (36–1,488)
YST component: unknown ($n = 9$)	5,145 (308–55,700)
Postoperative chemotherapy regimen in primary treatment	
BEP (Bleomycin + Etoposide + Cisplatin)	112 (53.1%)
PVB (Cisplatin + Vinblastine + Bleomycin)	33 (15.6%)
PEP (Peplomycin + Etoposide + Cisplatin)	20 (9.5%)
TC (Paclitaxel + Carboplatin)	8 (3.8%)
VAB (Vinblastine + Actinomycin D + Bleomycin)	7 (3.3%)
PVP (Peplomycin + Vinblastine + Cisplatin)	4 (1.9%)
VAC (Vinblastine + Actinomycin D + Cyclophosphamide)	4 (1.9%)
EP (Etoposide + Cisplatin)	4 (1.9%)
Other	19 (9.0%)
Fertility-sparing therapy at initial treatment (n = 196)	
Yes	157 (80.1%)
No	39 (19.9%)

Abbreviations: FIGO, International Federation of Gynecology and Obstetrics; YST, yolk sac tumor; NAC, neoadjuvant chemotherapy followed by surgery.

addition, BEP was significantly superior to platinum-based non-BEP in 5-year OS (93.6% versus 75.9%, $P = 0.0009$, Table 3).

3.2.3. Standard BEP and non-standard BEP

In this comparison, we excluded five patients as described earlier. Of the remaining 107 patients who received BEP, six patients died of YST at 5–44 months after primary surgery, and one patient died of breast cancer (the same patient described in 'Clinical Outcomes'). The median duration of follow-up after excluding the seven patients who died was 80.5 (4–178) months from the day of primary surgery. Median number of cycles is four (3–6) for standard BEP and four (1–6) for non-standard BEP.

Median (range) total doses of bleomycin, etoposide and cisplatin at the first course of non-standard BEP group were 35 (3–60) mg/course or 21 (15–60) mg/m^2/course, 500 (80–775) mg/m^2/course and 80 (15–150) mg/m^2/course, respectively, and median (range) cycles of non-standard BEP is 4 (1–6).

Table 4 shows a comparison of the 5-year OS between the standard BEP group and the non-standard BEP group; 100% of the standard BEP group survived to 5 years, and 91.0% of the non-standard BEP group survived to 5 years ($P = 0.049$) (Fig. 3A). Considering the dose of each drug, <75% of the standard dose of bleomycin and <50% of the dose of etoposide were significantly associated with poor 5-year OS (100% versus 89.4%, $P = 0.02$, and 96.9% versus 62.5%, $P = 0.0002$) (Fig. 3B, C). Regarding the administration schedule of BEP, the non-standard administration schedule of bleomycin was associated with poor 5-year OS (97.2% versus 88.0%, $P = 0.02$) and the non-standard administration

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Table 2
Proportion of patient characteristics in each regimen.

Patient characteristics	BEP group ($n = 112$)	Non-BEP group ($n = 99$)	*P*-Value	Standaard BEP group ($n = 37$)	Non-standard BEP group ($n = 70$)	*P*-Value
Median age (range)	23 (1 months - 57 years)	22 (7 years - 68 years)	0.73	22 (12 years - 39 years)	23 (11 months - 57 years)	0.73
FIGO stage			0.55			0.49
IA	28	17 (58.3%)		10	17	
IC	41	35		16	22	
I unknown substage	0	2		0	0	
II	8	9 (8.1%)		2	6	
III	31	29 (28.4%)		7	23	
IV	4	7 (5.2%)		2	2	
Histological features			0.64			0.72
Pure YST	78	66		24	41	
Mixed YST	34	33		13	19	
Median AFP (range, [n]), ng/ml	18,273 (36.3–367,464, [98])	21,490 (101–540,000, [76])	0.76	19,549 (198.8–344,880, [32])	18,048 (36.3–367,464, [63])	0.74

Abbreviations: FIGO, International Federation of Gynecology and Obstetrics; YST, yolk sac tumor; BEP, Combination chemotherapy with Bleomycin, Etoposide and Cisplatin.

schedule of etoposide tended to be associated with poor 5-year OS (96.3% versus 87.5%, $P = 0.054$).

All patients who received standard BEP became normalised in AFP levels, whereas two of 70 (2.9%) patients who received non-standard BEP failed to be normalised in AFP levels because of residual tumour.

Three patients suffered from pulmonary fibrosis caused by bleomycin. Two of the three patients were diagnosed at 3 or 4 months after the last cycle of BEP, and the other patient developed pulmonary fibrosis after the first cycle of BEP. In all three patients, the pulmonary fibrosis was successfully treated by steroid hormone therapy after the completion of chemotherapy. The patient who developed the pulmonary fibrosis after the first cycle was treated by chemotherapy only with etoposide and cisplatin without bleomycin. All three patients had no evidence of recurrence.

As for the five patients excluded from the present study, one of the two patients who received an excessive dose of bleomycin died of pulmonary fibrosis after the 4th cycle of BEP. The other patient developed pulmonary fibrosis, however she recovered, and is alive without disease. The other three of the five patients who received uncertain doses of drugs are alive without disease.

3.3. Reproductive outcomes of the patients with BEP and non-BEP

We excluded 38 patients from the 112 patients who received the BEP regimen and 64 patients from the 99 patients who received the non-BEP regimen for the following reasons: primary amenorrhea, age > 40 years at diagnosis, non-FSS, death during the study period and loss of information. Therefore, we assessed the reproductive safety and outcomes in 74 patients who received the BEP and 35 patients who received the non-BEP. As for the menstruation, 106 of 109 patients recovered almost the same cycles as before treatment within 6 months ($n = 85$, 78.0%), 7–12 months ($n = 19$, 17.4%) and over 12 months ($n = 2$, 1.8%) after treatment, two patients (1.8%) had menarche, and a patient (0.9%) who received irradiation for metastatic pelvic and para-aortic lymph nodes after chemotherapy did not recover menstruation.

Sixteen of 23 patients (70.0%) receiving BEP who were nulliparous at FSS and married at the end of the study period achieved 26 pregnancies and gave birth to 21 healthy children during follow-up. Thirteen of 17 patients (76.5%) receiving the non-BEP who were nulliparous at FSS and married at the end of the study period achieved 20 pregnancies; 12 gave birth to 19 healthy children during follow-up.

4. Discussion

In univariate and multivariate analyses, we revealed that age ⩾ 22, AFP ⩾ 33,000 ng/ml, residual tumours at primary surgery, and non-BEP were independently and significantly associated with poor OS of patients with YST.

Regarding malignant ovarian germ cell tumours, Chan [18] reported that older age (age > 40) predicted poorer survival. In the present study, we also confirmed that the elder age was one of prognostic factors. However, the cut-off level (age ⩾ 22) was younger compared with Chan's results. These results might be due to that this study was focused on YST histology alone.

The prognostic value of the high level of pretreatment AFP in patients with YST has not been well evaluated. In two studies [19,20] including non-YST germ cell tumours in most of the study patients, a high AFP level was a significantly poor prognostic factor, using 1000 ng/ml as the cut-off level. Three other studies reported that preoperative AFP levels had no significant

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