Table I. Characteristics of the OCCC and OSAC cell lines.

Cell line	Original tumor	*KRAS*		*PIK3CA*		IC_{50} of BEZ235 (nM)
		Exon 2	Exon 3	Exon 9	Exon 20	
OVISE	Clear cell carcinoma	wt	wt	wt	wt	44
SMOV-2	Clear cell carcinoma	wt	wt		3141 A>A/T	65
KK	Clear cell carcinoma	wt	wt	1634 A>A/C	wt	74
TU-OC-1	Clear cell carcinoma	wt	wt	1624 G>G/A	wt	131
OVTOKO	Clear cell carcinoma	wt	wt	wt	wt	534
KOC-7c	Clear cell carcinoma	wt	wt	wt	wt	600
OVMANA	Clear cell carcinoma	wt	wt	1634 A>T	wt	641
RMG-I	Clear cell carcinoma	wt	wt	wt	wt	777
KF	Serous adenocarcinoma	wt	wt	wt	wt	779
KOC-2s	Serous adenocarcinoma	wt	wt	wt	wt	989
TU-OS-3	Serous adenocarcinoma	wt	wt	wt	wt	1,004
TU-OS-4	Serous adenocarcinoma	wt	wt	wt	wt	3,951
SHIN-3	Serous adenocarcinoma	34 G>A	wt	wt	wt	25,400

OCCC, ovarian clear cell carcinoma; OSAC, ovarian serous adenocarcinoma; wt, wild-type.

Statistical analysis. Statistical analyses were performed with Prism version 5 (GraphPad Software Inc., San Diego, CA, USA). Data are presented as means ± 1 standard error. Means for all data were compared by one-way analysis of variance with *post hoc* testing or by unpaired t-test. A P-value of <0.05 was considered to indicate a statistically significant result.

Results

Identification of PIK3CA and KRAS mutations in OCCC and OSAC cell lines. We first screened the mutation status of *PIK3CA* (exons 9 and 20) and *KRAS* (exons 2 and 3) in the 8 OCCC and 5 OSAC cell lines. Four out of the 8 OCCC cell lines showed a *PIK3CA* mutation while none of the 5 OSAC cell lines showed the mutation (Table I). One of the 5 OSAC cell lines showed a *KRAS* mutation (34G>A) while none of the 8 OCCC cell lines showed this mutation.

Sensitivity to NVP-BEZ235 or temsirolimus. The IC_{50} values of NVP-BEZ235 in the OCCC cell lines were lower than these values in the OSAC cell lines (Table I). In the OCCC cell lines, the IC_{50} of temsirolimus was higher than that of BEZ235 (Table II). Although the *PIK3CA* mutation was more frequently noted in OCCC than OSAC, the sensitivity of these cell lines to NVP-BEZ235 or temsirolimus was not related to the mutation status.

Expression levels of PI3K-Akt-mTOR pathway molecules in the OCCC and OSAC cell lines. Comparison of the OCCC and OSAC cell lines showed that pHER3 and pAkt expression was more frequent in OCCC than OSAC (Fig. 1A). That is, 7 of the 8 OCCC cell lines expressed pHER3 whereas 2 of the 5 OSAC cells lines exhibited expression. Similarly, 6 of the 8 OCCC cell lines expressed pAkt while 2 of the 5 OSAC cell lines did. The protein expression levels were distributed widely, and did not relate to the sensitivity to NVP-BEZ235 or temsirolimus.

Table II. IC_{50} of temsirolimus in the OCCC cell lines.

Cell line	IC_{50} (nM)	
	BEZ235	Temsirolimus
OVISE	44	9,122
SMOV-2	64	8,924
KK	74	5,929
TU-OC-1	131	7,224
OVTOKO	534	12,776
KOC-7c	600	9,779
OVMANA	641	17,650
RMG-I	777	4,045

When OVISE cells were treated with NVP-BEZ235, expression levels of p-p70S6K and p4E-BP1 were suppressed in a dose-dependent manner (Fig. 1B). Treatment with temsirolimus incompletely suppressed p-p70S6K and p4E-BP1 expression in the OVISE cells. Moreover, treatment with NVP-BEZ235 suppressed pAKT expression, while treatment with temsirolimus did not. Similar results were observed in the KK cells (Fig. 1C).

NVP-BEZ235 induces G_1 phase arrest and apoptosis in OCCC cells. OVISE cells were arrested at the G_1 phase, but did not exhibit apoptosis (denoted by an increased proportion of cells in sub-G_1), after 72 h of treatment with 10 and 100 nM NVP-BEZ235 (Fig. 2A). We observed similar results of G_1 arrest in the KK cells (Fig. 2A). Although the same conditions as those in the cell cycle analysis did not induce apoptosis,

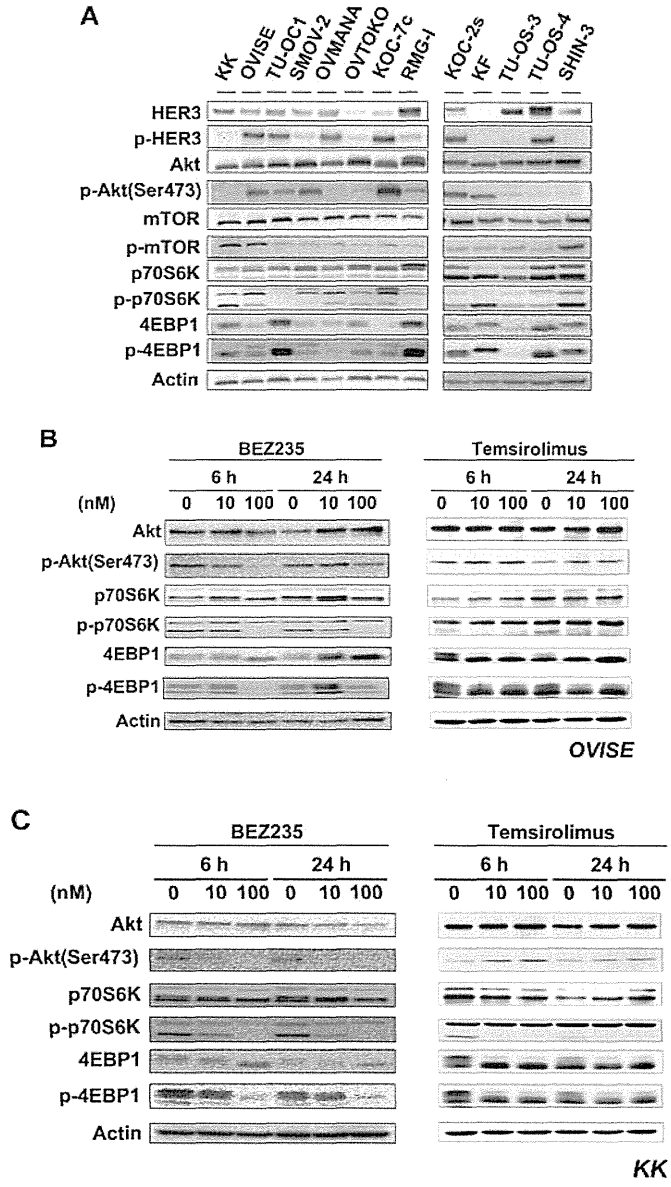

Figure 1. (A) Baseline expression of PI3K-Akt-mTOR pathway molecules in the OCCC and OSAC cell lines. Eight OCCC cell lines (KK, OVISE, TU-OC-1, SMOV-2, OVMANA, OVTOKO, KOC-7c and RMG-I) and 5 OSAC cell lines (KOC-2s, KF, TU-OS-3, TU-OS-4 and SHIN-3) were cultured in DMEM/F12 medium with 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2 at 37°C. Western blot analysis was performed to detect the expression levels of HER3, p-HER3, Akt, p-Akt, mTOR, p-mTOR, p70S6K, p-p70S6K, 4E-BP1 and p-4E-BP1. β-actin was used as a loading control. Each experiment was repeated 3 times independently. (B and C) NVP-BEZ235 suppressed pAkt expression in OCCC cells. Two OCCC cell lines (OVISE and KK) were plated in 6-well plates. The protein samples were collected after treatment with 10 and 100 nM NVP-BEZ235 or temsirolimus for 6 or 24 h. Western blot analysis was performed to detect Akt, p-Akt, p70S6K, p-p70S6K, 4E-BP1 and p-4E-BP1 expression. β-actin was used as a loading control.

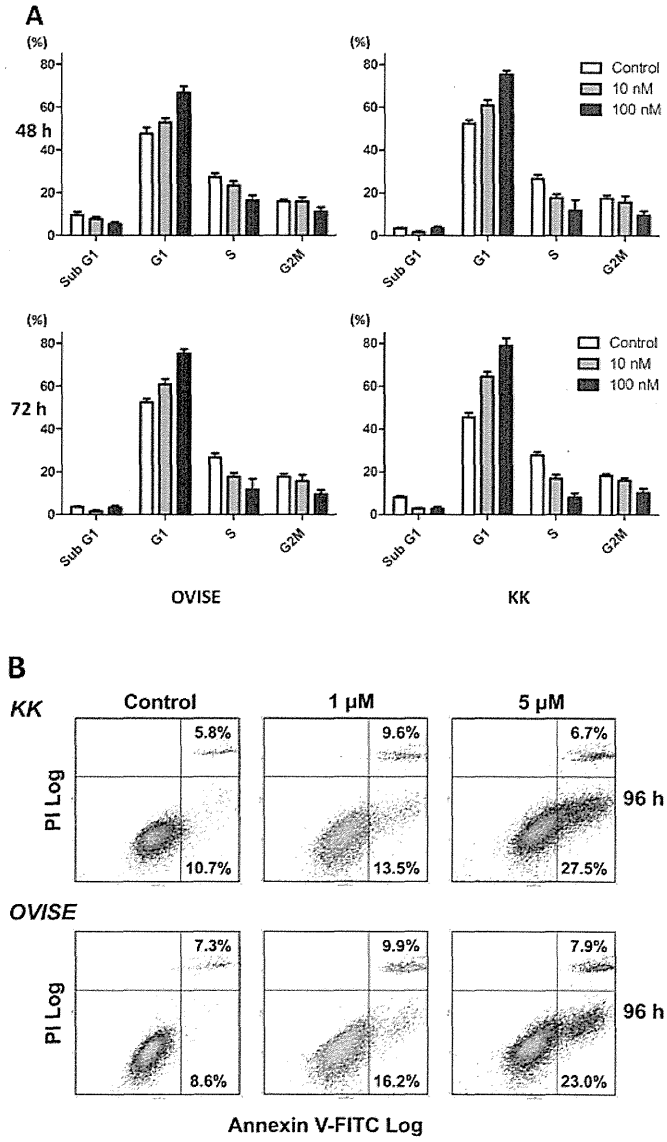

Figure 2. (A) NVP-BEZ235 induced G_1 phase arrest in the OCCC cells. OVISE or KK cells were treated with 10 or 100 nM NVP-BEZ235 for 48 or 72 h. FACScan analysis was performed to detect the cell cycle distribution. (B) NVP-BEZ235 induced apoptosis in the OCCC cells. OVISE and KK cells were treated with 1 or 5 μM NVP-BEZ235 for 96 h. Apoptosis was determined by the Annexin V-FITC Apoptosis Detection kit. Early apoptotic cells were scored as Annexin V-FITC-positive and propidium iodide (PI)-negative to exclude necrotic cells.

treatment of OVISE cells with 1 or 5 μM of NVP-BEZ235 for 96 h increased the number of Annexin V-positive and PI-negative cells (Fig. 2B). Similar results were observed in the KK cells (Fig. 2B).

NVP-BEZ235 suppresses tumor growth in an OCCC xenograft model. To assess short-term systemic toxicity of the agent, we recorded body weight changes of mice in addition to visual observation. After treatment, no mice had detectable changes in body weight, implying that there was no severe toxicity (Fig. 3A). At doses of 25 or 50 mg/kg/day, NVP-BEZ235 significantly inhibited subcutaneous tumor growth in mice bearing OVISE cells ($P<0.05$ for 25 mg/kg/day, $P<0.01$ for 50 mg/kg/day) (Fig. 3B). TU-OC-1 tumor volume in the 50 mg/kg/day group was significantly lower than that of the vehicle control although that in the 25 mg/kg/day group was not ($P<0.01$ for 50 mg/kg/day) (Fig. 3C).

Discussion

Many authors have reported poorer prognoses for patients with advanced stage OCCC (4,20,21). Low survival rates in OCCC

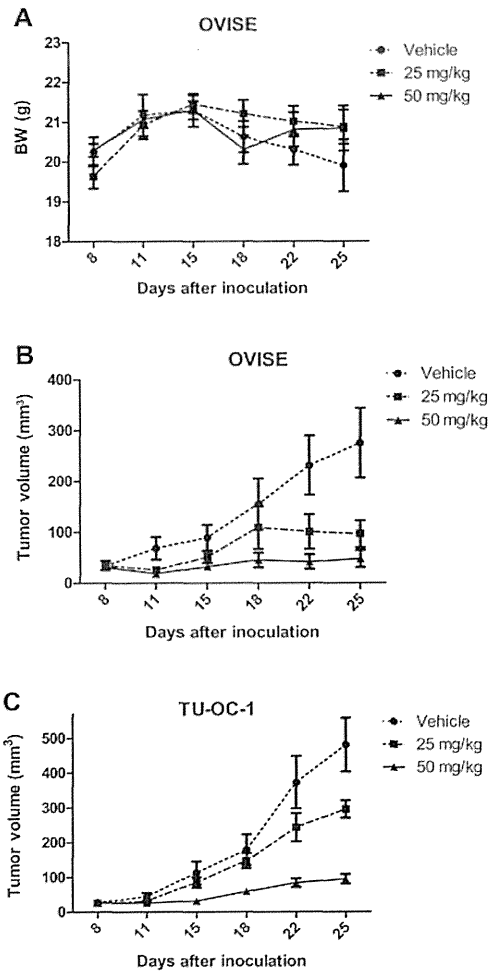

Figure 3. NVP-BEZ235 inhibits tumorigenicity in an OCCC xenograft model without producing toxic effects. Female athymic nude mice were injected subcutaneously with $4x10^6$ OVISE or TU-OC-1 cells. The experimental groups were treated with vehicle control (10% 1-methyl-2-pyrrolidone-90% polyethylene glycol 300), or 25 or 50 mg/kg/day NVP-BEZ235 for 3 weeks beginning 7 days after cell injection. (A) Mean body weight in each treatment group. (B and C) The tumor volume in OVISE or TU-OC-1 xenografts was measured with a caliper twice weekly. Error bars represent the standard error.

may, in part, reflect its lack of sensitivity to platinum-based chemotherapy. There are no antineoplastic agents definitely active and effective to treat OCCC. Therefore, novel therapeutic strategies, including targeted therapy, are needed to improve the prognosis of patients with OCCC.

It is known that mutations of *PIK3CA* are common molecular genetic alterations identified in OCCC (11). Expression of phospho-mTOR was found to be more prominent in OCCC than in OSAC (22). mTOR exists in two distinct complexes, mTOR complex 1 (mTORC1) and mTORC2. The downstream targets of mTORC1 are p70 ribosomal S6 kinase 1 (p70S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), both of which are crucial to the regulation of protein synthesis.

In practice, inhibitors of mTORC1, such as temsirolimus and everolimus, have been used for renal cell carcinoma. A phase II study (GOG268) combining temsirolimus with carboplatin and paclitaxel following temsirolimus consolidation as first-line therapy is underway in patients with OCCC (23). However, the efficacy of mTORC1 blockade may be attenuated due to the loss of an mTORC1-dependent negative feedback loop on PI3K signaling and the mTORC2-mediated activation of Akt (24). p70S6K inhibits insulin receptor substrate 1 (IRS-1) by phosphorylating it, by inducing it to degrade and by altering its localization (25,26). The inhibition of IRS by p70S6K attenuates PI3K-AKT activation. Rapamycin (and its analogs temsirolimus and everolimus) stops this negative feedback loop from the p70S6K to the PI3K signaling pathway, resulting in activation of proliferative and prosurvival effectors such as AKT.

NVP-BEZ235 is a dual pan-class I PI3K and an mTOR kinase inhibitor that has the possible advantage of inhibiting PI3K, mTORC1 and mTORC2. Therefore, it should turn off this pathway completely and overcome feedback inhibition that is normally observed with mTORC1 inhibitors (e.g. rapamycin analogs). It is known that NVP-BEZ235 displays significant antitumor activities in glioblastoma, lung, breast, renal cell and uterine endometrial carcinomas (12,14,13,27).

In the present study, IC_{50} of temsirolimus was markedly higher than NVP-BEZ235 in all OCCC cell lines. In contrast, NVP-BEZ235 effectively suppressed proliferation of OCCC cells. Additionally, treatment with temsirolimus increased expression of pAKT while p-p70S6K and p4E-BP1 were suppressed. Treatment with NVP-BEZ235 suppressed pAkt, p-p70S6K and p4E-BP1. Accordingly, NVP-BEZ235 may be the more effective agent.

We found that NVP-BEZ235 suppressed tumor growth in an OCCC xenograft model. A few authors have reported on the antitumor activity of this compound in ovarian carcinoma. Montero *et al* (28) showed that NVP-BEZ235 effectively suppressed proliferation of 4 ovarian carcinoma cell lines which were not derived from OCCC. Santiskulvong *et al* (29), investigated the *in vivo* effects of NVP-BEZ235 on an immunocompetent transgenic murine ovarian endometrioid adenocarcinoma model. They also examined *in vitro* activity of the compound in 17 human ovarian carcinoma cell lines including 2 OCCC cell lines (ES-2 and OV207). Unfortunately, these studies did not focus on OCCC. Recently, Rahman *et al* (30) investigated the frequency of *PIK3CA* mutations in patients with OCCC and the relationship between the mutations and clinicopathological or biological variables. They also examined the *in vitro* sensitivity of 9 OCCC cell lines to LY294002, temsirolimus and NVP-BEZ235. No relationship was observed between the mutation status and sensitivity to these inhibitors. We also examined the mutation status of *PIK3CA* and *KRAS* genes and baseline protein expression levels of the PI3K/Akt/mTOR pathway molecules. Although the *PIK3CA* mutation was more common in OCCC than in OSAC in our series, there were no relationships between the mutation status or protein expression levels and sensitivity to NVP-BEZ235. These findings supported those of a previous report (30).

Our results revealed that NVP-BEZ235 effectively suppressed not only p-p70S6K and p4E-BP1, but also pAKT expression in OCCC cell lines and suppressed tumor growth in an OCCC xenograft model. This is the first report to demonstrate the efficacy of NVP-BEZ235 in OCCC.

We conclude that the PI3K-AKT-mTOR pathway is a potential therapeutic target for OCCC and that NVP-BEZ235 warrants investigation as a therapeutic agent.

Acknowledgements

We thank Dr Kazuyuki Kitatani of the Medical Megabank Organization at Tohoku University for providing technical advice and Dr Yuji Nakayama and Ms. Hiromi Miyauchi of the Division of Functional Genomics, Research Center for Bioscience and Technology at Tottori University for assisting with the cell cycle analysis. The present study was supported by the Project for Development of Innovative Research on Cancer Therapeutics, from the Ministry of Education, Culture, Sports, Science and Technology of Japan and Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (25462594 to T.O.).

References

1. Scully RE: World Health Organization classification and nomenclature of ovarian cancer. J Natl Cancer Inst Monogr 42: 5-7, 1975.
2. McGuire V, Jesser CA and Whittemore AS: Survival among U.S. women with invasive epithelial ovarian cancer. Gynecol Oncol 84: 399-403, 2002.
3. Kennedy AW, Biscotti CV, Hart WR and Webster KD: Ovarian clear cell adenocarcinoma. Gynecol Oncol 32: 342-349, 1989.
4. Sugiyama T, Kamura T, Kigawa J, *et al*: Clinical characteristics of clear cell carcinoma of the ovary: a distinct histologic type with poor prognosis and resistance to platinum-based chemotherapy. Cancer 88: 2584-2589, 2000.
5. Courtney KD, Corcoran RB and Engelman JA: The PI3K pathway as drug target in human cancer. J Clin Oncol 28: 1075-1083, 2010.
6. Nakayama K, Nakayama N, Kurman RJ, *et al*: Sequence mutations and amplification of PIK3CA and AKT2 genes in purified ovarian serous neoplasms. Cancer Biol Ther 5: 779-785, 2006.
7. Zhang L, Huang J, Yang N, *et al*: Integrative genomic analysis of phosphatidylinositol 3'-kinase family identifies *PIK3R3* as a potential therapeutic target in epithelial ovarian cancer. Clin Cancer Res 13: 5314-5321, 2007.
8. Campbell IG, Russell SE, Choong DYH, *et al*: Mutation of the *PIK3CA* gene in ovarian and breast cancer. Clin Cancer Res 64: 7678-7681, 2004.
9. Altomare DA, Wang HQ, Skele KL, *et al*: AKT and mTOR phosphorylation is frequently detected in ovarian cancer and can be targeted to disrupt ovarian tumor cell growth. Oncogene 23: 5853-5857, 2004.
10. Mabuchi S, Kawase C, Altomare DA, *et al*: mTOR is a promising therapeutic target both in cisplatin-sensitive and cisplatin-resistant clear cell carcinoma of the ovary. Clin Cancer Res 15: 5404-5413, 2009.
11. Kuo KT, Mao TL, Jones S, *et al*: Frequent activating mutations of *PIK3CA* in ovarian clear cell carcinoma. Am J Pathol 174: 1597-1601, 2009.
12. Maira SM, Stauffer F, Brueggen J, *et al*: Identification and characterization of NVP-BEZ235, a new orally available dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent *in vivo* antitumor activity. Mol Cancer Ther 7: 1851-1863, 2008.
13. Brachmann S, Hofmann I, Schnell C, *et al*: Specific apoptosis induction by the dual PI3K/mTOR inhibitor NVP-BEZ235 in HER2 amplified and PIK3CA mutant breast cancer cells. Proc Natl Acad Sci 106: 22299-22304, 2009.
14. Cho DC, Cohen MB, Panka DJ, *et al*: The efficacy of the novel dual PI3-kinase/mTOR inhibitor NVP-BEZ235 compared with rapamycin in renal cell carcinoma. Clin Cancer Res 16: 3628-3638, 2010.
15. Itamochi H, Kato M, Nishimura M, *et al*: Establishment and characterization of a novel ovarian serous adenocarcinoma cell line, TU-OS-4, that overexpresses EGFR and HER2. Hum Cell 25: 111-115, 2012.
16. Itamochi H, Kato M, Nishimura M, *et al*: Establishment and characterization of a novel ovarian clear cell carcinoma cell line, TU-OC-1, with a mutation in the *PIK3CA* gene. Hum Cell 26: 121-127, 2013.
17. Itamochi H, Oishi T, Shimada M, *et al*: Inhibiting the mTOR pathway synergistically enhances cytotoxicity in ovarian cancer cells induced by etoposide through upregulation of c-Jun. Clin Cancer Res 17: 4742-4750, 2011.
18. McIntyre AJ, Summersgill BM, Spendlove HE, *et al*: Activating mutations and/or expression levels of tyrosine kinase receptors *GRB7*, *RAS*, and *BRAF* in testicular germ cell tumors. Neoplasia 7: 1047-1052, 2005.
19. Li VSW, Wong CW, Chan TL, *et al*: Mutations of *PIK3CA* in gastric adenocarcinoma. BMC Cancer 5: 29, 2005.
20. Rauh-Hein AJ, Winograd D, Growdon WB, *et al*: Prognostic determinants in patients with uterine and ovarian clear carcinoma. Gynecol Oncol 125: 376-380, 2012.
21. Pectasides D, Fountzilas G, Aravantinos G, *et al*: Advanced stage clear-cell epithelial ovarian cancer: the Hellenic Cooperative Oncology Group experience. Gynecol Oncol 102: 285-291, 2006.
22. Miyazawa M, Yasuda M, Fujita M, *et al*: Therapeutic strategy targeting the mTOR-HIF-1α-VEGF pathway in ovarian clear cell adenocarcinoma. Pathol Int 59: 19-27, 2009.
23. Itamochi H and Kigawa J: Clinical trials and future potential of targeted therapy for ovarian cancer. Int J Clin Oncol 17: 430-440, 2012.
24. Efeyan A and Sabatini DM: mTOR and cancer: many loops in one pathway. Curr Opin Cell Biol 22: 169-176, 2010.
25. Harrington LS, Findlay GM, Gray A, *et al*: The TSC1-2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins. J Cell Biol 166: 213-223, 2004.
26. Hartley D and Cooper GM: Role of mTOR in the degradation of IRS-1: regulation of PP2A activity. J Cell Biochem 85: 304-314, 2002.
27. Shoji K, Oda K, Kashiyama T, *et al*: Genotype-dependent efficacy of a dual PI3K/mTOR inhibitor, NVP-BEZ235, and an mTOR inhibitor, RAD001, in endometrial carcinomas. PLoS One 7: e37431, 2012.
28. Montero JC, Chen X, Ocaria A, *et al*: Predominance of mTORC1 over mTORC2 in the regulation of proliferation of ovarian cancer cells: therapeutic implications. Mol Cancer Ther 11: 1342-1352, 2012.
29. Santiskulvong C, Konecny GE, Fekete M, *et al*: Dual targeting of phosphoinositide 3-kinase and mammalian target of rapamycin using NVP-BEZ235 as a novel therapeutic approach in human ovarian carcinoma. Clin Cancer Res 17: 2373-2384, 2011.
30. Rahman M, Nakayama K, Rahman MT, *et al*: Clinicopathologic and biological analysis of *PIK3CA* mutation in ovarian clear cell carcinoma. Hum Pathol 43: 2197-2206, 2012.

Dual Inhibition of Phosphatidylinositol 3′-Kinase and Mammalian Target of Rapamycin Using NVP-BEZ235 as a Novel Therapeutic Approach for Mucinous Adenocarcinoma of the Ovary

Akiko Kudoh, MD, Tetsuro Oishi, MD, PhD,* Hiroaki Itamochi, MD, PhD,* Seiya Sato, MD, PhD,* Jun Naniwa, MD, PhD,* Shinya Sato, MD, PhD,* Muneaki Shimada, MD, PhD,* Junzo Kigawa, MD, PhD,† and Tasuku Harada, MD, PhD**

Abstract: Ovarian mucinous adenocarcinoma (MAC) resists standard chemotherapy and is associated with poor prognosis. A more effective treatment is needed urgently. The present study assessed the possibility of molecular-targeted therapy with a novel dual inhibitor of phosphatidylinositol 3′-kinase (PI3K) and mammalian target of rapamycin (mTOR), NVP-BEZ235 (BEZ235) to treat of MAC. Seven human MAC cell lines were used in this study. The sensitivity of the cells to BEZ235, temsirolimus, and anticancer agents was determined with the WST-8 assay. Cell cycle distribution was assessed by flow cytometry, and the expression of proteins in apoptotic pathways and molecules of the PI3K/Akt/mTOR signaling pathways was determined by Western blot analysis. We also examined the effects of BEZ235 on tumor growth in nude mice xenograft models. The cell lines showed half-maximal inhibitory concentration values of BEZ235 from 13 to 328 nmol/L. Low half-maximal inhibitory concentration values to BEZ235 were observed in MCAS and OMC-1 cells; these 2 lines have an activating mutation in the *PIK3CA* gene. NVP-BEZ235 down-regulated the protein expression of phosphorylated (p-) Akt, p-p70S6K, and p-4E-BP1, suppressed cell cycle progression, up-regulated the expression of cleaved PARP and cleaved caspase 9, and increased apoptotic cells. Synergistic effects were observed on more than 5 cell lines when BEZ235 was combined with paclitaxel or cisplatin. The treatment of mice bearing OMC-1 or RMUG-S with BEZ235 significantly suppressed tumor growth in MAC xenograft models without severe weight loss. We conclude that the PI3K/Akt/mTOR pathway is a potential therapeutic target and that BEZ235 should be explored as a therapeutic agent for MAC.

Key Words: PI3K, mTOR, NVP-BEZ235, Ovarian mucinous adenocarcinoma, Molecular-targeted therapy

Received September 11, 2013, and in revised form December 19, 2013.
Accepted for publication December 19, 2013.

(*Int J Gynecol Cancer* 2014;24: 444–453)

*Department of Obstetrics and Gynecology, Tottori University School of Medicine; and †Tottori University Hospital Cancer Center, Yonago, Japan.
Address correspondence and reprint requests to Hiroaki Itamochi, MD, PhD, Department of Obstetrics and Gynecology, Tottori University School of Medicine, 36-1 Nishicho, Yonago 683-8504 Japan.
E-mail: itamochi@med.tottori-u.ac.jp.

ISSN: 1048-891X
DOI: 10.1097/IGC.0000000000000091
This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (24592517 to H. Itamochi).
The authors declare no conflicts of interest.

Mucinous adenocarcinoma of the ovary (MAC) is the third most common type of epithelial ovarian cancer (EOC), comprising 10% to 12% of EOC.[1–3] Mucinous adenocarcinoma of the ovary seems to have a clinical behavior distinct from other EOC. Several studies have shown that MAC often is diagnosed at an early stage, and therefore, confers a relatively good prognosis. However, advanced MAC has a poorer prognosis than other histopathologic subgroups.[4–6] Poor response (13% to 42%) to conventional platinum- or taxane-based chemotherapy is associated with poor prognosis because chemosensitivity is one of the main prognostic factors for patients with advanced EOC.[4–9] Thus, novel treatment strategies (eg, incorporation of molecular-targeted agents) for advanced MAC are needed.

The phosphatidylinositol 3′-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathway plays a key role in cellular metabolism, proliferation, survival, and motility.[10] This pathway is hyperactivated by several mechanisms, such as deletion or decreased function of phosphatase and tensin homolog (PTEN), activating mutations of PI3K or Akt, and activating receptor tyrosine kinases (eg, epidermal growth factor receptor, human epidermal growth factor receptor 2).[10,11] Activating this cascade is indicated to relate drug resistance and poor prognosis in many cancers including EOC.[10,12] The frequent mutation of *PIK3CA* has been reported in clear cell carcinoma (33%) and endometrioid adenocarcinoma (20%) of the ovary, and this cascade is thought to be a potential therapeutic target.[13,14] Several studies have shown that inhibiting the Akt/mTOR signaling pathway is one of these promising therapeutic targets in clear cell carcinoma of the ovary.[15,16] Therefore, we focused on this cascade to treat MAC. In MAC, overexpressed proteins of the epidermal growth factor receptor family and activated downstream signaling of Akt/mTOR have been found in 48% of tumors and were associated with poor patient outcomes.[17,18] This pathway could be an attractive target for therapy for MAC.

Recently, a novel imidazoquinoline derivative, NVP-BEZ235 (BEZ235), was developed and has entered clinical trials.[19–21] NVP-BEZ235 is an orally bioavailable agent that targets the ATP-binding clefts of the class 1 PI3K and mTOR kinases, thereby inhibiting the activity of PI3K, mTOR complex (mTORC) 1, and mTORC2. Several studies have shown potential antitumor activities by BEZ235 in vitro and in vivo in a variety of cancers, including EOC.[19,22–25] However, the effects of a dual inhibitor of PI3K and mTOR have not been evaluated systematically in MAC. We therefore conducted the present study to assess the possibility of molecular-targeted therapy with BEZ235 to treat MAC.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The 7 human MAC cell lines used in this study were obtained as follows: JHOM-1 and JHOM-2B from Riken BioResource Center cell bank (Tsukuba City, Ibaraki, Japan); MCAS from Health Science Research Resources Bank (Chiyoda-ku, Tokyo, Japan); OMC-1 from Dr Tsuyoshi Saito (Sapporo Medical University, Sapporo, Japan); and RMUG-L and RMUG-S from Dr Daisuke Aoki (Keio University, Tokyo, Japan). TU-OM-1 was established by our department. These cell lines were maintained in DMEM/Ham's F-12 medium (Wako Pure Chemical Industries, Ltd) with 10% fetal bovine serum, 100 IU/mL penicillin, and 50 μg/mL streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C.

Dose-Response Studies

The sensitivity of the cell lines to BEZ235 (LC Laboratories) and temsirolimus (LC Laboratories) was determined by a cytotoxicity assay using Cell Counting Kit-8 (Dojindo Laboratories). Briefly, cells were diluted with culture medium to a seeding density of 3 to 5 × 10^4/mL, plated on 96-well tissue culture plates at 180 μL per well, and incubated at 37°C for 24 hours. The next day, the cells were treated continuously with 20 μL of various concentrations of the agents to obtain a dose-response curve for each agent. The concentration for each agent was 1 to 10,000 nmol/L. After being incubated for 72 hours, 20 μL of Cell Counting Kit-8 solution was added to each well, and the plates were incubated for another 1 to 2 hours. Absorbance was measured at 450 nm with a microplate reader (iMark Microplate Absorbance Reader; Bio-Rad). Inhibition of cell growth was calculated as the percentage of viable cells compared with the percentage in untreated cultures.

Dose-Effect Analysis

NVP-BEZ235 was combined with each of the different anticancer agents at a fixed ratio that spanned the individual half-maximal inhibitory concentrations (IC_{50}) of each drug. The IC_{50} was determined from dose-effect curves created by a cytotoxicity assay. Median effect plot analyses and calculated combination indices (CI) were analyzed by the method of Chou and Talalay.[26] CalcuSyn software (Biosoft) was used to analyze data from the cytotoxicity assays in which cells were exposed to agents alone or combined with the anticancer drugs and BEZ235. CalcuSyn provided a measure of the combined agents in an additive or synergistic manner. We used the Chou and Talalay[26] definition of CI as synergistic (CI < 0.9), additive (0.9 < CI < 1.1), or antagonistic (CI > 1.1).

Mutational Analysis

Mutation screening for *PIK3CA* was performed at exons 9 and 20 and *K-Ras* at exons 2 and 3, covering the mutational hot spots in human cancers. These exons of *PIK3CA* and *K-Ras* were amplified using polymerase chain reaction (PCR) for genomic DNA. The primers for PCR and sequencing were prepared according to previous reports.[27,28]

The PCR conditions were as follows: 1 cycle at 94°C for 5 minutes, 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 90 seconds, followed by 1 cycle at 72°C for 7 minutes. The PCR products subsequently were subjected to direct sequencing PCR with BigDye terminator v3.1 cycle sequencing reagents (Applied Biosystems). The samples were finally analyzed on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems) with Sequence Scanner Software v1.0 (Applied Biosystems).

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

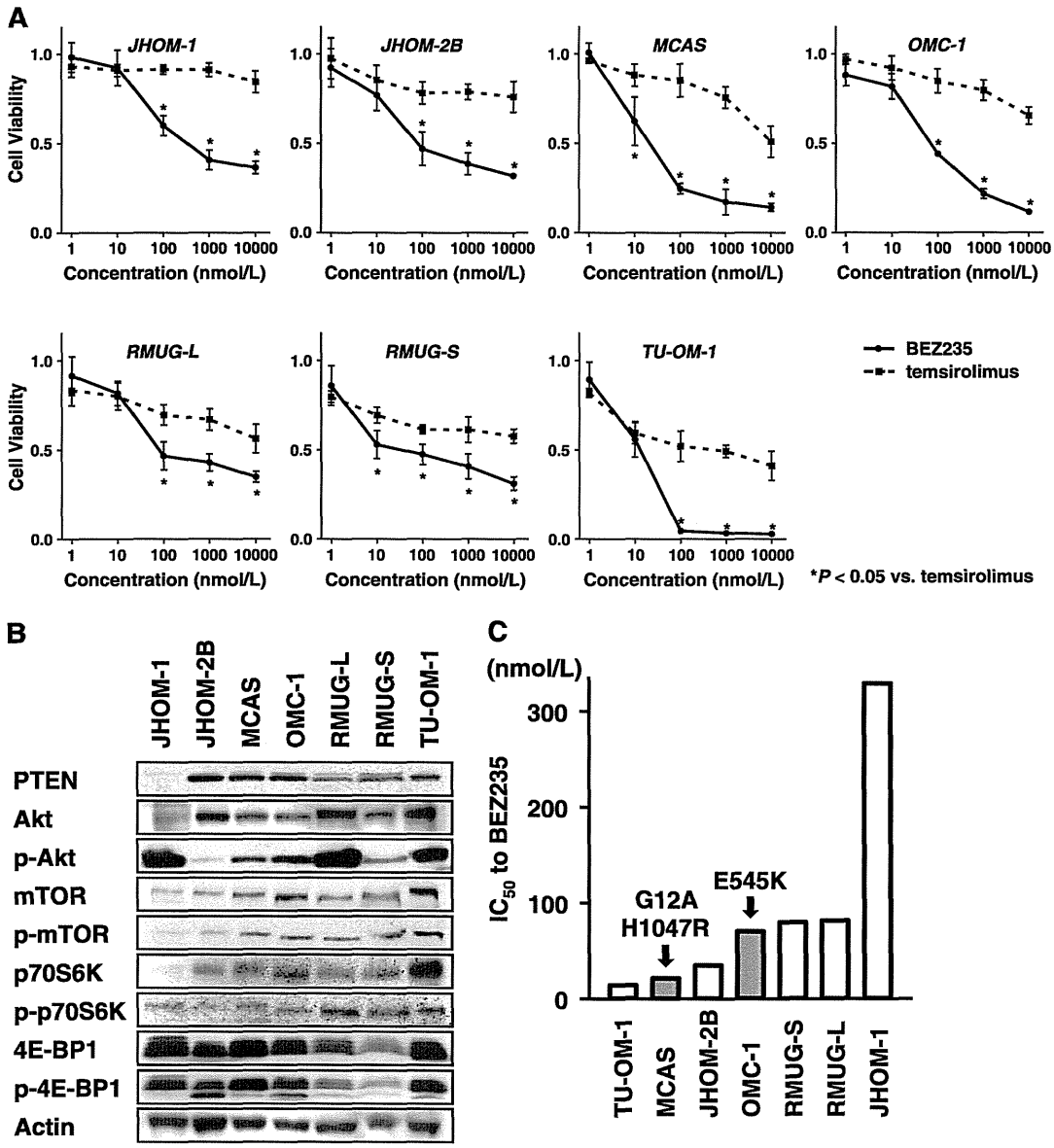

FIGURE 1. The cytotoxicity of BEZ235 and temsirolimus in MAC cell lines and the PI3K, Akt, and mTOR signaling pathways. A, Seven ovarian MAC cell lines were treated with varying concentrations of BEZ235 or temsirolimus, then inhibition of cell growth was determined using the WST-8 assay. The points represent 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

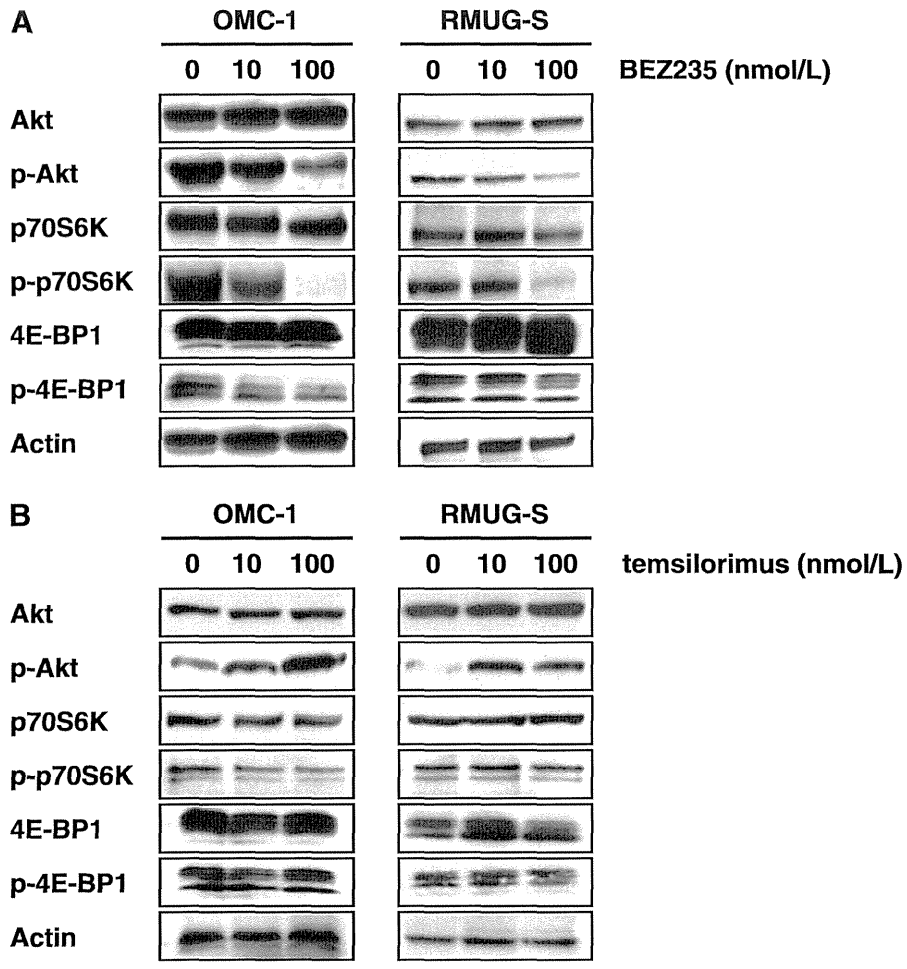

FIGURE 2. BEZ235 inhibited the PI3K, Akt, and mTOR signaling pathways. A, Phosphorylated (p-) Akt, p-p70S6K, and p-4E-BP1 in OMC-1 and RMUG-S cells were down-regulated in a dose-dependent manner 24 hours after being treated with BEZ235. B, Although p-p70S6K and p-4E-BP1 were suppressed 24 hours after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

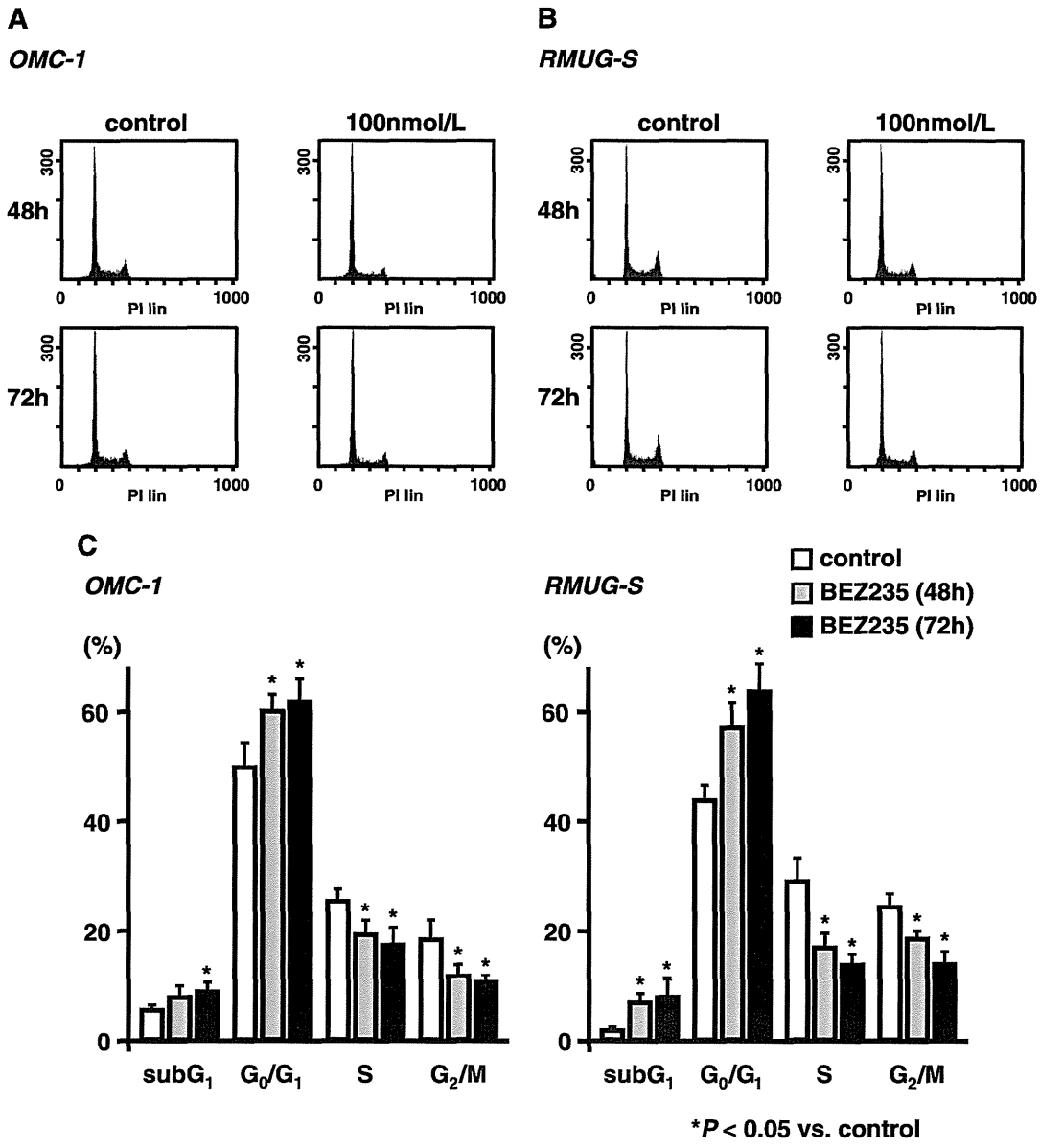

FIGURE 3. The effects of BEZ235 on the cell cycle distribution. The MAC cells OMC-1 and RMUG-S were treated with PBS (control) or 100 nmol/L BEZ235. The cell cycle distributions of drug-treated cells were then determined by flow cytometry. Representative cytometry data are shown for OMC-1 (A) and in 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.

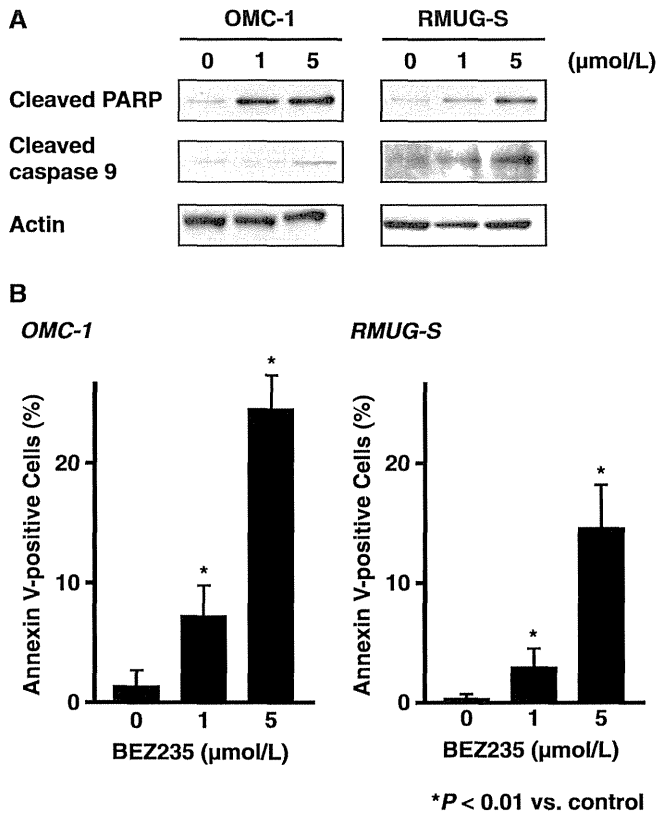

FIGURE 4. BEZ235 activated apoptotic pathways. A, The MAC cells OMC-1 and RMUG-S were treated with PBS (control) or 1 or 5 μmol/L BEZ235 for 48 hours. BEZ235 up-regulated the expression of cleaved PARP and caspase 9 proteins. B, Cells were treated with PBS (control) or 1 or 5 μmol/L BEZ235 for 48 hours 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

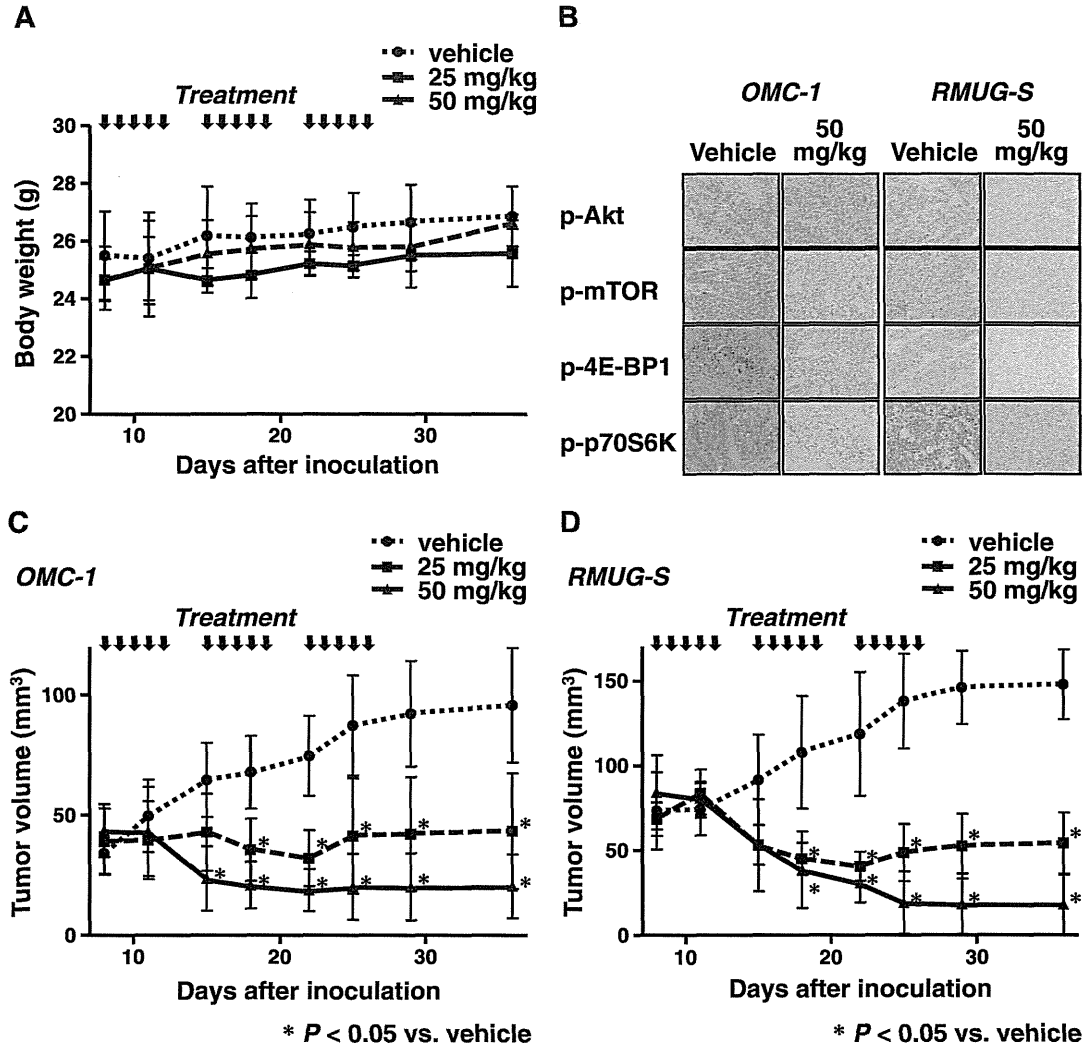

FIGURE 5. Treatment with BEZ235 suppressed the growth of subcutaneous tumors in mice with implanted OMC-1 or RMUG-S cells. Female nude mice (7 per group) were given a subcutaneous inoculation of 5×10^6 OMC-1 or RMUG-S followed by daily oral administration of PBS or 25 or 50 mg/kg per day BEZ235 for 3 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

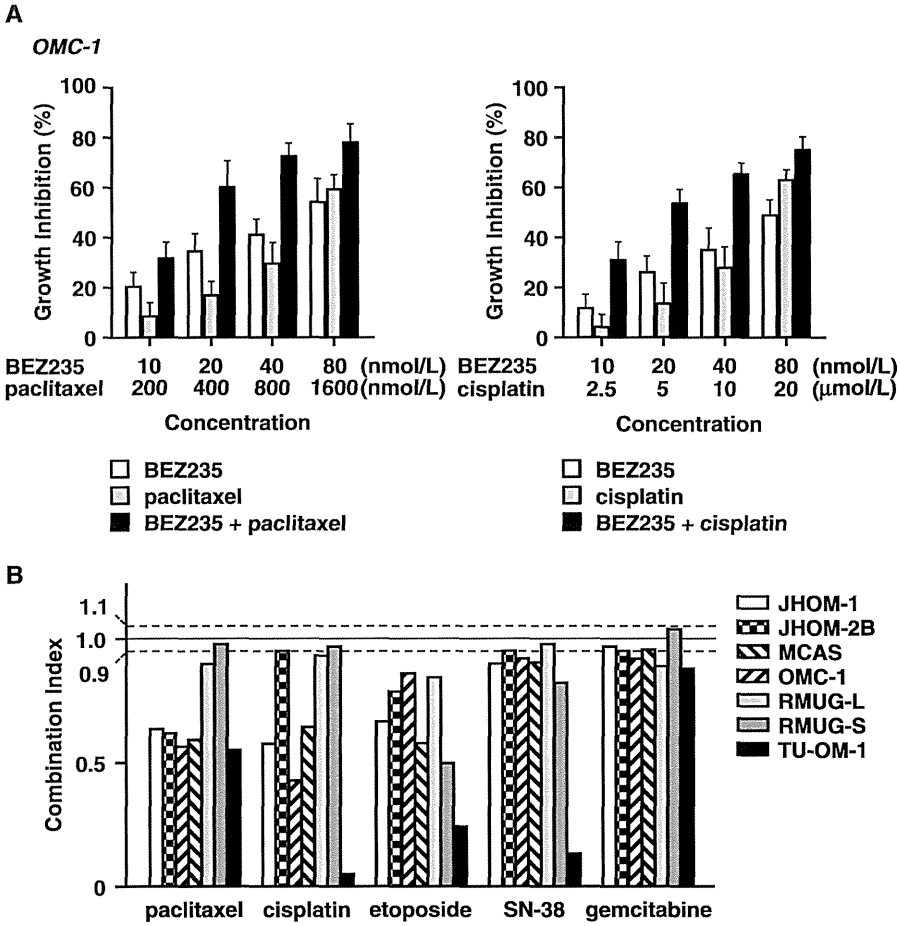

FIGURE 6. The effects of BEZ235 are synergistic or additive with those of each anticancer agent. Cells were incubated with increasing concentrations of BEZ235 with paclitaxel or cisplatin at a fixed ratio for 72 hours. The inhibition of growth was analyzed by using Cell Counting Kit-8. A, Representative 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.

REFERENCES

1. McGuire V, Jesser CA, Whittemore AS. Survival among U.S. women with invasive epithelial ovarian cancer. *Gynecol Oncol.* 2002;84:399–403.
2. Seidman JD, Kurman RJ, Ronnett BM. Primary and metastatic mucinous adenocarcinomas in the ovaries: incidence in routine practice with a new approach to improve intraoperative diagnosis. *Am J Surg Pathol.* 2003;27:985–993.
3. Chan JK, Teoh D, Hu JM, et al. Do clear cell ovarian carcinomas have poorer prognosis compared to other epithelial cell types? A study of 1411 clear cell ovarian cancers. *Gynecol Oncol.* 2008;109:370–376.
4. Hess V, A'Hern R, Nasiri N, et al. Mucinous epithelial ovarian cancer: a separate entity requiring specific treatment. *J Clin Oncol.* 2004;22:1040–1044.

5. Cannistra SA. Cancer of the ovary. *N Engl J Med.* 2004;351:2519–2529.
6. Winter WE 3rd, Maxwell GL, Tian C, et al. Prognostic factors for stage III epithelial ovarian cancer: a Gynecologic Oncology Group study. *J Clin Oncol.* 2007;25:3621–3627.
7. Pectasides D, Fountzilas G, Aravantinos G, et al. Advanced stage mucinous epithelial ovarian cancer: the Hellenic Cooperative Oncology Group experience. *Gynecol Oncol.* 2005;97:436–441.
8. Pisano C, Greggi S, Tambaro R, et al. Activity of chemotherapy in mucinous epithelial ovarian cancer: a retrospective study. *Anticancer Res.* 2005;25:3501–3505.
9. Shimada M, Kigawa J, Ohishi Y, et al. Clinicopathological characteristics of mucinous adenocarcinoma of the ovary. *Gynecol Oncol.* 2009;113:331–334.
10. Vivanco I, Sawyers CL. The phosphatidylinositol 3-kinase AKT pathway in human cancer. *Nat Rev Cancer.* 2002;2:489–501.
11. Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev Cancer.* 2005;5:341–354.
12. Chandarlapaty S, Sawai A, Scaltriti M, et al. AKT inhibition relieves feedback suppression of receptor tyrosine kinase expression and activity. *Cancer Cell.* 2011;19:58–71.
13. Kuo KT, Mao TL, Jones S, et al. Frequent activating mutations of PIK3CA in ovarian clear cell carcinoma. *Am J Pathol.* 2009;174:1597–1601.
14. Campbell IG, Russell SE, Choong DY, et al. Mutation of the *PIK3CA* gene in ovarian and breast cancer. *Cancer Res.* 2004;64:7678–7681.
15. Mabuchi S, Kawase C, Altomare DA, et al. mTOR is a promising therapeutic target both in cisplatin-sensitive and cisplatin-resistant clear cell carcinoma of the ovary. *Clin Cancer Res.* 2009;15:5404–5413.
16. Nagaraja AK, Creighton CJ, Yu Z, et al. A link between mir-100 and FRAP1/mTOR in clear cell ovarian cancer. *Mol Endocrinol.* 2010;24:447–463.
17. Alshenawy HA. Immunohistochemical expression of epidermal growth factor receptor, E-cadherin, and matrix metalloproteinase-9 in ovarian epithelial cancer and relation to patient deaths. *Ann Diagn Pathol.* 2010;14:387–395.
18. Lee ES, Lee Y, Suh D, et al. Detection of *HER-2* and *EGFR* gene amplification using chromogenic in-situ hybridization technique in ovarian tumors. *Appl Immunohistochem Mol Morphol.* 2010;18:69–74.
19. Maira SM, Stauffer F, Brueggen J, et al. Identification and characterization of NVP-BEZ235, a new orally available dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent in vivo antitumor activity. *Mol Cancer Ther.* 2008;7:1851–1863.
20. Courtney KD, Corcoran RB, Engelman JA. The PI3K pathway as drug target in human cancer. *J Clin Oncol.* 2010;28:1075–1083.
21. Benjamin D, Colombi M, Moroni C, et al. Rapamycin passes the torch: a new generation of mTOR inhibitors. *Nat Rev Drug Discov.* 2011;10:868–880.
22. Serra V, Markman B, Scaltriti M, et al. NVP-BEZ235, a dual PI3K/mTOR inhibitor, prevents PI3K signaling and inhibits the growth of cancer cells with activating PI3K mutations. *Cancer Res.* 2008;68:8022–8030.
23. Faber AC, Li D, Song Y, et al. Differential induction of apoptosis in HER2 and EGFR addicted cancers following PI3K inhibition. *Proc Natl Acad Sci U S A.* 2009;106:19503–19508.
24. Santiskulvong C, Konecny GE, Fekete M, et al. Dual targeting of phosphoinositide 3-kinase and mammalian target of rapamycin using NVP-BEZ235 as a novel therapeutic approach in human ovarian carcinoma. *Clin Cancer Res.* 2011;17:2373–2384.
25. Montero JC, Chen X, Ocaña A, et al. Predominance of mTORC1 over mTORC2 in the regulation of proliferation of ovarian cancer cells: therapeutic implications. *Mol Cancer Ther.* 2012;11:1342–1352.
26. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul.* 1984;22:27–55.
27. Li VS, Wong CW, Chan TL, et al. Mutations of PIK3CA in gastric adenocarcinoma. *BMC Cancer.* 2005;5:29.
28. McIntyre A, Summersgill B, Spendlove HE, et al. Activating mutations and/or expression levels of tyrosine kinase receptors GRB7, RAS, and BRAF in testicular germ cell tumors. *Neoplasia.* 2005;7:1047–1052.
29. Ma WW, Adjei AA. Novel agents on the horizon for cancer therapy. *CA Cancer J Clin.* 2009;59:111–137.
30. Itamochi H. Targeted therapies in epithelial ovarian cancer: Molecular mechanisms of action. *World J Biol Chem.* 2010;1:209–220.
31. Sun SY, Rosenberg LM, Wang X, et al. Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition. *Cancer Res.* 2005;65:7052–7058.
32. O'Reilly KE, Rojo F, She QB, et al. mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. *Cancer Res.* 2006;66:1500–1508.
33. Guertin DA, Sabatini DM. Defining the role of mTOR in cancer. *Cancer Cell.* 2007;12:9–22.
34. Wan X, Harkavy B, Shen N, et al. Rapamycin induces feedback activation of Akt signaling through an IGF-1R-dependent mechanism. *Oncogene.* 2007;26:1932–1940.
35. Di Nicolantonio F, Arena S, Tabernero J, et al. Deregulation of the P13K and KRAS signaling pathways in human cancer cells determines their response to everolimus. *J Clin Invest.* 2010;120:2858–2866.

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

Pathol. Oncol. Res.
DOI 10.1007/s12253-014-9854-y

RESEARCH

Immunophenotype and Human Papillomavirus Status of Serous Adenocarcinoma of the Uterine Cervix

Shinichi Togami · Yuko Sasajima · Takahiro Kasamatsu · Rie Oda-Otomo · Satoshi Okada · Mitsuya Ishikawa · Shun-ichi Ikeda · Tomoyasu Kato · Hitoshi Tsuda

Received: 22 November 2013 / Accepted: 14 October 2014

Abstract Serous adenocarcinoma of the cervix (SACC) is a very rare tumor. Our study aimed to characterize the immune profile and human papillomavirus (HPV) status of SACC, in comparison with other serous adenocarcinomas arising in the female genital tract. The pathological specimens obtained from 81 patients with serous carcinoma of the uterine cervix (n=12), 29 endometrium, 20 ovary and 20 patients with mucinous carcinoma of the uterine cervix were reviewed. We assessed the expression of WT-1, p53, p16, HER2, CEA, and CA125 by immunohistochemistry and HPV DNA by PCR in 12 SACC samples. Their immune profile was compared with that of uterine papillary serous carcinoma (UPSC), ovarian serous adenocarcinoma (OSA), and mucinous endocervical adenocarcinoma (MEA). WT-1 and HER2 were expressed in very few SACC samples (0 and 0 %, respectively), but p16, CA125, CEA and p53 were present in 100, 92, 58 and 50 %, respectively. The difference in WT-1 expression between SACC and UPSC, MEA is not significant, but SACC differ significantly from OSA (p<0.01). HPV DNA (type 16 or 18) was detected in 4 of the 12 SACC. The immunophenotype of SACC was similar to UPSC, whereas the frequency of expression of WT-1 was significantly lower in SACC than OSA. It appeared that p53 expression was associated with worse clinical outcome in patients with SACC, and that HPV infection was related to its occurrence.

Keywords Serous adenocarcinoma · Uterine cervix · Immunohistochemical features · Human papillomavirus

S. Togami (✉) · T. Kasamatsu · S. Okada · M. Ishikawa · S.-i. Ikeda · T. Kato
Department of Gynecology, National Cancer Center Hospital, 5-1–1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan
e-mail: togami@m3.kufm.kagoshima-u.ac.jp

S. Togami
Department of Obstetrics and Gynecology Department of Molecular and Cellular Pathology, Field of Oncology, Faculty of Medicine, Kagoshima University, Kagoshima, Japan

Y. Sasajima · R. Oda-Otomo · H. Tsuda
Department of Pathology and Clinical Laboratories, National Cancer Center Hospital, Chuo-ku, Tokyo, Japan

Y. Sasajima
Department of Pathology, Teikyo University School of Medicine, Itabashi-ku, Tokyo, Japan

Published online: 05 November 2014

Introduction

Serous adenocarcinomas are one of the most aggressive gynecological cancer types, very rare in the uterine cervix, but common in the ovary, fallopian tube, and peritoneum. It represents <10 % of endometrial carcinomas [1]. Zhou et al. [2] reported the first detailed clinicopathological features of 17 cases of serous adenocarcinoma of the uterine cervix (SACC). Our previous retrospective study assessed the clinicopathological features and prognosis of 12 patients with SACC who underwent hysterectomy [3]. However, little is known about the immunoprofile and human papillomavirus (HPV) involvement in SACC.

In the present study, we characterized the immunohistochemical features of 12 SACCs, using seven antibodies against WT-1, p53, p16, human epidermal growth factor receptor 2 (HER2), carcinoembryonic antigen (CEA), and CA125 and compared the immunoprofile with uterine papillary serous carcinoma (UPSC), ovarian serous adenocarcinoma (OSA) and mucinous endocervical adenocarcinoma (MEA). In this way, we aimed to characterize the immunoprofile and HPV status of SACC, in comparison with other serous adenocarcinomas arising in the female genital tract and MEA.

Materials and Methods

Patients and Tissue Samples

We reviewed the medical records and pathological specimens obtained from 81 patients with serous carcinoma of the uterine cervix (n=12), 29 endometrium, 20 ovary and 20 patients with mucinous carcinoma of the uterine cervix. All 81 patients were treated in the Department of Gynecology and diagnosed in the Department of Pathology and Clinical Laboratories, National Cancer Center Hospital, Tokyo, Japan, between 1985 and 2005 and underwent surgical staging according to the International Federation of Gynecology and Obstetrics (FIGO) system. All patients with serous carcinomas of the cervix had radical hysterectomy and bilateral salpingo-oophorectomy in order to exclude a primary neoplasm in the corpus, ovary or tube. All hematoxylin-eosin-stained slides were reviewed in all cases, and final diagnoses were confirmed by two observers (S.T. and Y.S.).

The diagnosis of SACC was made only when an invasive endocervical adenocarcinoma exhibited a prominent papillary structure and/or slit-like glandular spaces, and usually moderate to marked cytologic atypia (Fig. 1a) without either intra- or extra-cytoplasmic mucin. Absence of concurrent or previous primary endometrial, ovarian, fallopian tubal or peritoneum serous carcinoma was a prerequisite for the diagnosis of SACC. At least 10 % of the tumor area had to be of papillary serous type for inclusion as a SACC in this study. Seven of 12 SACC cases were classified as pure serous adenocarcinomas, and the other 5 were mixed serous adenocarcinoma. Uterine and ovarian serous adenocarcinoma and mucinous endocervical adenocarcinoma were diagnosed according to the World Health Organization International Histological Classification of Tumors [4]. Eleven cases of mixed types were included among the 29 UPSCs, but mixed serous adenocarcinoma and mucinous adenocarcinomas were excluded from this study among the cases of OSA. All cases were included only when destructive or frank stromal invasion was observed.

Immunohistochemistry

All tumor tissue specimens having been fixed in formalin and embedded in paraffin were cut into 4-μm-thick serial sections for immunohistochemical staining, in addition to the usual hematoxylin and eosin staining. This study was performed with the approval of the Internal Review Board on ethical issues. Antibodies used for immunohistochemistry were WT-1 (clone 6 F-H2, 1:50, Dako, Glostrup, Denmark), p53 (clone DO-7, 1:100, Dako), p16 (clone 16P07, 1:100, Neomarkers Inc., Fremont, CA), HercepTest (Dako), CEA (polyclonal, 1:5,000, Dako), and CA125(clone M11, 1:20, Dako). Immunohistochemical staining for WT-1, p53, p16, HER2, CEA, and CA125 products was performed with an autoimmunostainer (Autostainer Link 48, Dako) according to the manufacturers' instructions.

Scoring of the Results

The results of the immunohistochemical staining were evaluated as the percentage of positively stained neoplastic cells. In mixed type tumors, only the serous component was evaluated

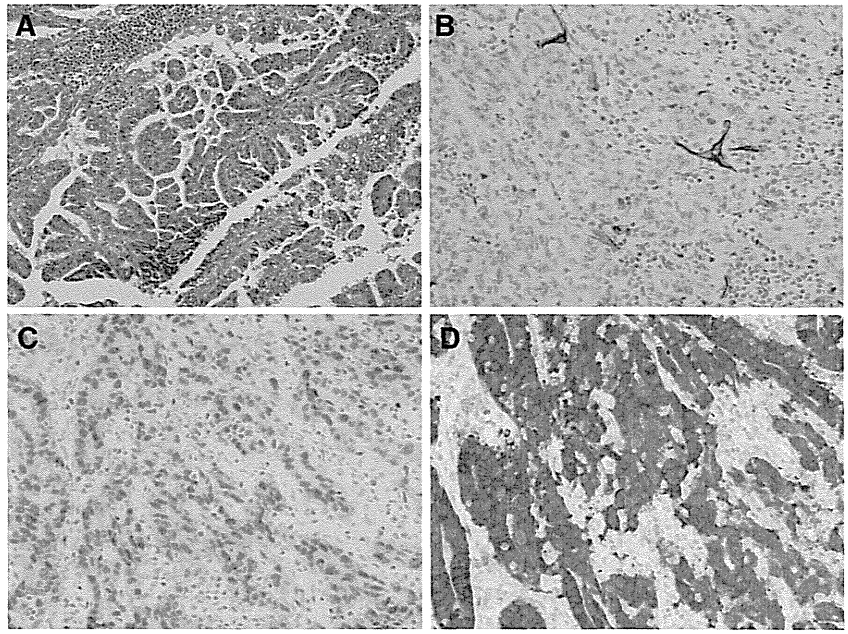

Fig. 1 Histopathological presentation of SACC. **a** H&E staining. (x100). **b** Immunohistochemical staining showing lack of WT-1 expression (x200). **c** Immunohistochemical staining showing expression of p53 (x200). **d** Immunohistochemical staining showing expression of p16 (x200)

in this study. For all antibodies except HER2, the level of expression was graded according to the percentage of immunoreactive neoplastic cells of the serous carcinoma component as follows: 0, <10 %; 1+, 10–25 %; 2+, 26–50 %; 3+, >50 %. Tumors with >10 % stained cells were considered positive for expression of that antigen. Immunoreactivity for HER2 was scored semiquantitatively as follows: 0, no immunostaining, or membrane staining in <10 % of cells; 1+, weak or barely perceptible staining in ≥10 % of cells, the cells stained in only part of the membrane; 2+, weak or moderate staining in the whole membrane in ≥10 % of tumor cells; 3+, strong staining in the whole membrane in ≥10 % of tumor cells [5]. We defined cases scoring 2+ and 3+ as HER2–positive. The immunohistochemical evaluation was performed by two observers (S.T. and Y.S.) separately, and the median value was used.

Polymerase Chain Reaction and Sequencing Analysis

DNA samples were extracted from paraffin embedded sections using the QIAamp DNA FFPE tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. PCR was performed using HPV consensus primers GP5+/6+ as previously described [6]. DNA samples obtained from cervical squamous cell carcinoma with HPV 16 infection and from the HeLa cell line, which is positive for HPV 18 DNA, were used as positive controls. *GAPDH* was amplified to ensure proper DNA extraction using the primer pair 5′-GCAG TGGGGACACGGAAGGC-3′ and 5′-ACTGTGGATG GCCCCTCGG-3′. The PCR products were electrophoresed in a 2 % (w/v) agarose gel and visualized under ultraviolet light with ethidium bromide staining.

The PCR products were purified using QIAquick Spin (Qiagen) and bidirectionally sequenced with the same primers as used for amplification. Sequence data were analyzed by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Statistics

Inter-group comparisons were made by Fisher's exact test. $P<0.05$ was considered statistically significant.

Results

Expression of WT-1, p53, p16, HER2, CEA, and CA125 in Cancer

Table 1 shows a comparison of staining with each antibody in SACC, UPSC, OSA and MEA. WT-1 and p53 staining was nuclear, p16 staining was both cytoplasmic and nuclear, HER2, CEA and CA125 staining was only at the membrane.

Table 1 Immunohistochemical findings

Molecule	Number of cases (%)			
	Immunohistochemistry score			
	0 (0–9 %)	1 (10–25 %)	2 (26–50 %)	3 (50 % <)
SACC (n=12)				
WT-1	12 (100)	0 (0)	0 (0)	0 (0)
p53	6 (50)	2 (17)	0 (0)	4 (33)
p16	0 (0)	0 (0)	2 (17)	10 (83)
HER2	11 (92)	1 (8)	0 (0)	0 (0)
CEA	5 (42)	3 (25)	1 (8)	3 (25)
CA125	0 (0)	1 (8)	0 (0)	11 (92)
UPSC (n=29)				
WT-1	23 (80)	2 (7)	1 (3)	3 (10)
p53	6 (21)	0 (0)	3 (10)	20 (69)
p16	1 (3)	1 (3)	2 (7)	25 (87)
HER2	23 (80)	1 (3)	1 (3)	4 (14)
CEA	20 (69)	4 (14)	2 (7)	3 (10)
CA125	1 (3)	0 (0)	2 (7)	26 (90)
OSA (n=20)				
WT-1	0 (0)	0 (0)	0 (0)	20 (100)
p53	4 (20)	0 (0)	0 (0)	16 (80)
p16	0 (0)	5 (25)	1 (5)	14 (70)
HER2	18 (90)	0 (0)	1 (5)	1 (5)
CEA	18 (90)	2 (10)	0 (0)	0 (0)
CA125	0 (0)	0 (0)	0 (0)	20 (100)
MEA (n=20)				
WT-1	20 (100)	0 (0)	0 (0)	0 (0)
p53	18 (90)	1 (5)	0 (0)	1 (5)
p16	1 (5)	1 (5)	0 (0)	18 (90)
HER2	19 (95)	1 (5)	0 (0)	0 (0)
CEA	1 (5)	4 (20)	1 (5)	14 (70)
CA125	1 (5)	0 (0)	1 (5)	18 (90)

SACC Serous adenocarcinoma of the cervix, *HER2* human epidermal growth factor receptor 2, *CEA* carcinoembryonic antigen, *UPSC* uterine papillary serous carcinoma, *OSA* ovarian serous adenocarcinoma, *MEA* endocervical adenocarcinoma

Serous Adenocarcinoma of the Uterine Cervix (SACC)

Representative immunohistochemical stainings for each antibody are shown in Figs. 1 and 2. WT-1 and HER2 were negative in all SACC (Figs. 1b and 2a). Six SACC cases (50 %) were positive for p53 (Fig. 1c), with 4 of them showing strong (3+) expression. In contrast, p16 had intermediate (2+) or strong expression in all 12 SACC (Fig. 1d), and 11/12 were strongly positive for CA125 (Fig. 2d).

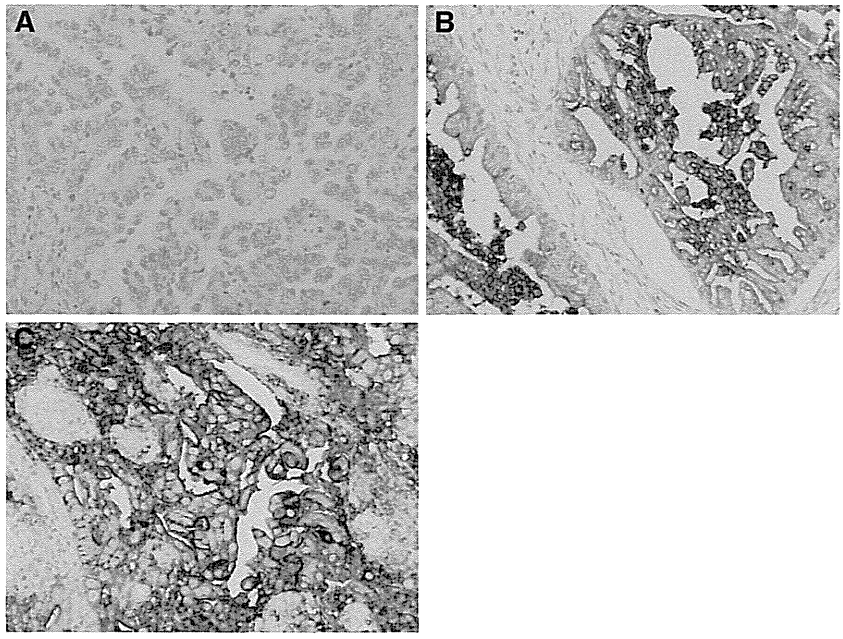

Fig. 2 Histopathological presentation of SACC. (**a**) Immunohistochemical staining showing negative expression of HER2. (x200). Immunohistochemical staining showing expression of (**b**) CEA (x200), (**c**) CA125 (x200)

Uterine Papillary Serous Carcinoma (UPSC)

Representative immunohistochemical stainings for each antibody are shown in Fig. 3. Only 21 % (6/29) of UPSC cases were positive for WT-1 (Fig. 3b) and only one case of UPSC was CK5/6-positive (Fig. 3b). Similarly, 17 % (5/29) of UPSC s were positive for HER2 (Fig. 3d), with 4 cases having strong expression. In contrast, p16 and CA125 showed intermediate or strong positive expression in the majority of cases (93 % and 97 %, respectively). These findings in UPSC are thus similar to SACC. p53 was positive in 23 cases of UPSC (79 %)(Fig. 3c), and strongly positive in most of these (69 %).

Ovarian Serous Adenocarcinoma (OSA)

Representative immunohistochemical stainings for each antibody are shown in Fig. 4. WT-1 (Fig. 4b) and CA125 were positive in all OSA. Thus, the frequency of WT-1 expression was significantly higher in OSA than SACC ($p<0.01$). p53 and p16 were strongly positive in 80 % (16/20) and 70 % (14/20) of OSA cases, respectively (Fig. 4c and d). In contrast,

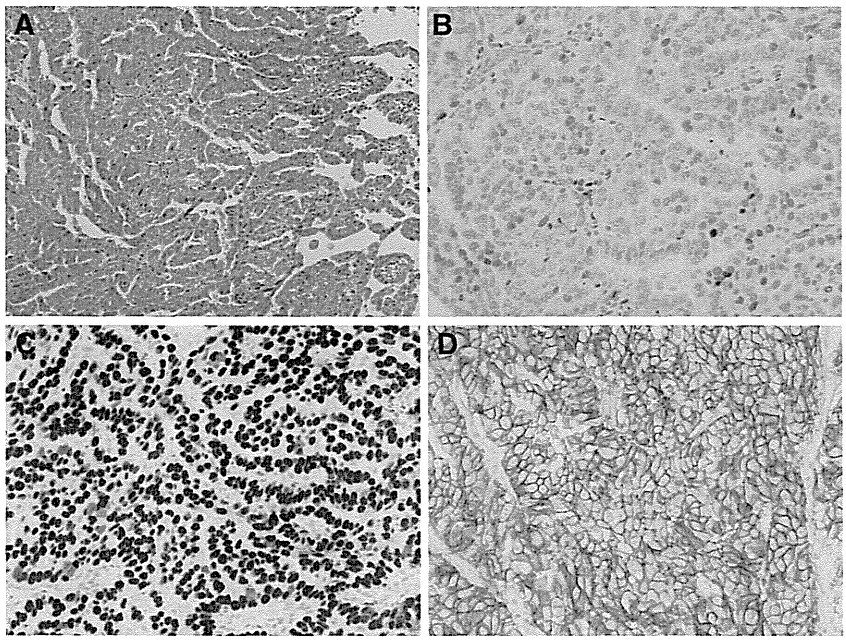

Fig. 3 Histopathological presentation of UPSC. (**a**) H&E staining. (x100). Immunohistochemical staining showing expression of (**b**) WT-1 (x200), (**c**) p53 (x200), (**d**) HER2 (x200)

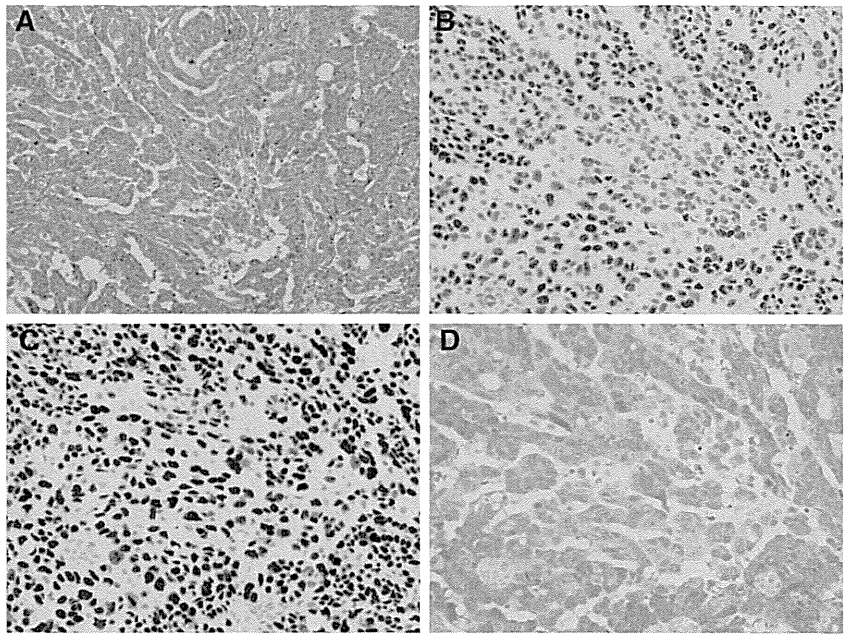

Fig. 4 Histopathological presentation of OSA. (**a**) H&E staining. (x100). Immunohistochemical staining showing expression of (**b**) WT-1 (x200), (**c**) p53 (x200), (**d**) p16 (x200)

HER2 and CEA expression was rare, at only 10 % (2/20) each.

Mucinous Endocervical Adenocarcinoma (MEA)

Representative immunohistochemical stainings for each antibody are shown in Fig. 5. WT-1 and HER2 were negative in all MEA, p53 expression was rare, at 10 % (2/20). In contrast, p16 and CA125 both showed strong positive expression in 90 % (18/20) of cases (Fig. 5b and d).

We suggested binarizing the immunostaining results as positive vs negative, and comparing using the Fisher's exact test. WT-1 and p53 appear to show differences in percent of cases expressing these proteins between SACC, UPSC, OSA and MEA. The difference in WT-1 expression between SACC and UPSC, MEA is not significant, but SACC differ significantly from OSA ($p<0.01$). In the case of p53, overexpression (3+ staining) is seen in 4/12 SACC which differs significantly compared to either endometrial (20/29) or ovarian (16/20) serous carcinomas. There is a tendency between recurrence and p53

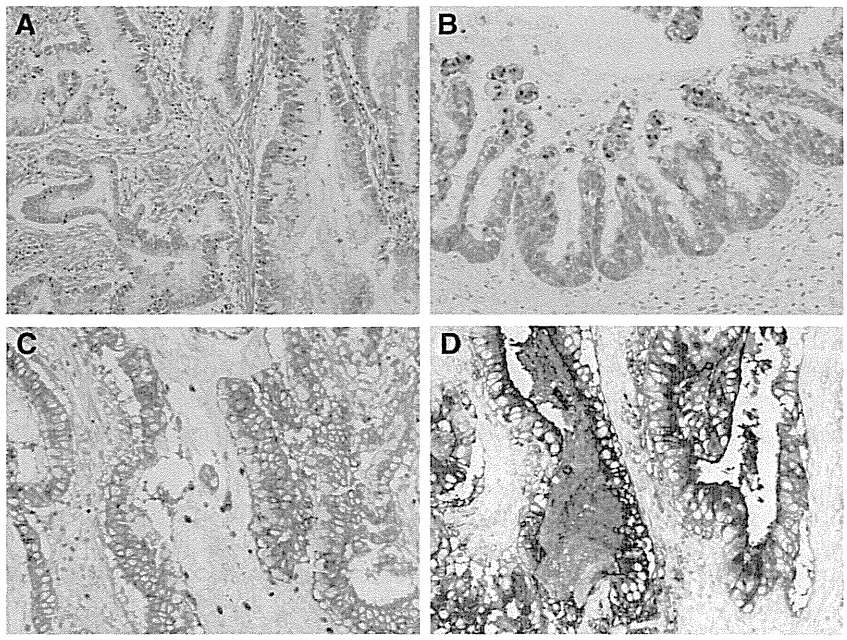

Fig. 5 Histopathological presentation of MEA. (**a**) H&E staining. (x100). Immunohistochemical staining showing expression of (**b**) p16 (x200), (**c**) CEA (x200), (**d**) CA125 (x200)

overexpression (p=0.06). The differences in frequency of HER2 expression are not significant.

HPV Infection

GAPDH was negative in the PCR for 2 of 12 SACC (cases 2 and 6), suggesting poor DNA preservation. These were excluded from further analysis. Among the remaining ten cases, four (cases 1, 3, 7 and 9) were positive when using the HPV consensus primers GP5+/6+ (Fig. 6). Sequencing of the PCR products showed that they had been derived from HPV16 in two samples (cases 1 and 9) and from HPV18 in the other two (cases 3 and 7).

Discussion

The p53 tumor suppressor gene plays a major role in cell cycle control and growth arrest following DNA damage. Mutations of this gene are the most common genetic alterations in human cancers [7]. Overexpression of p53, as detected by immunohistochemistry, has been proposed to indicate a worsened prognosis in some malignancies [8]. In our study, 50 % (6/12) cases of SACC were positive for p53, among them 4 with strong expression. In contrast, 90 % of MEA were negative for p53. Hunt et.al. [8] reported that p53 was not expressed in 86 % (30/35) of their uterine cervical adenocarcinomas, implying a difference in the pathogenetic mechanisms between SACC and MEA in the context of p53 inactivation. However, there is a report that the rates of p53 gene mutation and p53 nuclear immunoreactions in adenocarcinomas of the uterine cervix are relatively high, at 46 % and 32 %, respectively [9]. Therefore, further studies are needed to clarify differences in the pathogenetic mechanisms in SACC and MEA.

Interestingly, 3 of 4 SACC cases which showed strong p53 expression had died, and there is a tendency between recurrence and p53 expression (p=0.06). Zhou et al. [2] reported that nuclear immunoreactivity for p53 was present in 5 of 12 SACC cases, and, of the five patients with p53 positive tumors, 4 developed metastases. Batistatou et al. [10] reported a deceased case of SACC with p53 expression. Recently,

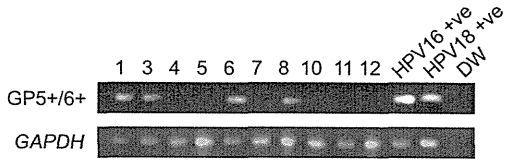

Fig. 6 Detection of HPV DNA by polymerase chain reaction, Four cases (cases 1, 3, 7 and 9) were positive using the HPV consensus primers GP5+/6+. DW, distilled water (no DNA template); +ve, positive control. *GAPDH* served as a positive control

Nofech-Mozes et al. [11] reported p53 immunostaining in 9 of 10 SACC cases, of which 3 had strong expression (>50 % of cells positive). That study [11] included 3 deceased cases of SACC, of whom two had strong expression (>50 % of cells). Thus, the strong p53 expression in SACC seemed to be associated with worse clinical outcome. Establishing p53 status may therefore contribute to prognostic indicators in this disease.

In addition, overexpression of p16 induced by HPV has been found to be associated with cervical squamous carcinoma [12–15]. It was also reported to be expressed in cervical adenocarcinoma [16–18]. In our study, 90 % (18/20) of MEA and 83 % (10/12) of SACC were strongly positive for p16, with no significant difference between SACC and MEA. Chiesa-Vottero et al. [19] reported that p16 overexpression was present in uterine and ovarian high grade serous adenocarcinomas. In our study, both OSA and UPSC showed p16 expression, with no significant differences among serous adenocarcinomas arising from different female genital tract organs and MEA.

Some studies have shown that persistent infection with high risk HPV is an important etiological factor for the occurrence of cervical adenocarcinoma [20, 21]. In the present study, 2 cases had HPV 16 DNA and 2 had HPV 18 DNA (both high risk HPV types). Nofech-Mozes et al. [22] reported that high-risk HPV DNA was found in 3 of 4 SACCs. Another study reported that HPV was detected in 1 of 3 SACCs [23]. We suggest that high risk HPV infection affects the occurrence of SACC.

Despite different origins, serous adenocarcinomas of the female genital tract show similar morphologic features, characterized by the presence of a prominent papillary structure and/or slit-like glandular spaces, and usually moderate to marked cytologic atypia. It has been shown that among the various histological types of ovaian carcinoma, the incidence of WT-1 positive tumors is highest in ovarian serous adenocarcinoma [24–27]. Nofech-Mozes et al. [28] concluded that strong WT-1 expression was associated with OSA rather than UPSC. In our study, WT-1 was positive in all OSA, whereas only 3 of 29 (10 %) UPSC showed strong expression of this antigen. In addition, WT-1 was negative in all SACC in this study. Nofech-Mozes et al. [11] also reported that only two cases of 10 SACC showed immunoreactivity to WT-1 where staining was seen in <50 % of all neoplastic cells. This is similar to the findings in our study. We suggest that SACC has biological features similar to UPSC. These may be associated with its embryologic developmental origin, in that both the uterine cervix and the uterine corpus are derived from the mullerian duct. On the other hand, the ovary is derived from indifferent gonad.

Generally, a majority of endocervical adenocarcinomas is CEA positive [29]. Alkushi et al. [30] reported that all endocervical type cervical adenocarcinomas expressed CEA