throughout the world [2]. Therefore, a new strategy for OCCC therapy has been highly anticipated [2].

Heparin-binding epidermal growth factor-like growth factor (HB-EGF), which belongs to the EGF family, is synthesized as an 87 amino acid membrane-anchored glycoprotein [3–5]. Cleavage of membrane-anchored HB-EGF by proteases, called "ectodomain shedding," induces the secretion of soluble mature form of HB-EGF from the cell surface. Through ectodomain shedding, HB-EGF contributes to several biological processes, such as wound healing [6], inflammation [7, 8], embryogenesis [9], and carcinogenesis [10]. In previous reports, we described that the expression of HB-EGF was higher than that of other EGFR ligands in the ascites and tissues of ovarian cancer patients [11, 12]. However, the molecular mechanism(s) underlying the increase of HB-EGF expression in ovarian cancer has remained unclear.

There have been several reports that have described other molecular mechanisms affecting the expression of HB-EGF. For example, inflammatory cytokines, including tumor necrosis factor-α and interleukin-1β, directly or indirectly activated the transcription of HB-EGF [13, 14]. In bladder cancer cells, insulin induces the transcriptional activation of epiregulin, HB-EGF, and amphiregulin (AREG), which may be regulated by transcriptional factors including specific protein 1 (SP1), activator protein-1 (AP-1), and nuclear factor-kappa B (NF-κB) [15]. HB-EGF was rapidly induced following exposure to a variety of chemotherapeutic agents, and the chemotherapy-induced HB-EGF expression was largely dependent on AP-1 and NF-κB [16]. In addition, transcription factors including SP1, AP-1, pancreatic and duodenal homeobox-1 (PDX-1), and myogenic differentiation 1 (MyoD) were detected as direct regulators of HB-EGF expression [15, 17–19]. However, the molecule(s) directly involved in the transcript of HB-EGF has remained unknown in human cancer.

The aim of this study is to assess the association between HB-EGF expression and the cell viability of OCCC cells. Additionally, in order to elucidate transcriptional regulation of HB-EGF in OCCC cells, we also performed a screening assay and identified the transcription factor, SP1, which is responsible for the upregulation of HB-EGF induced by anticancer agent.

Materials and Methods

Cells and cell culture

Eleven OCCC cell lines (RMG-I, RMG-II, OVISE, OVTOKO, KK, TU-OC-1, KOC-7C, HAC-2, OVAS, SMOV-2, ES-2) and 293T cells were used for the studies. The KK cells were a kind gift from Professor Yoshihiro Kikuchi, National Defense Medical College, Japan; the OVAS cells were provided by Professor Hiroshi Minaguchi, Yokohama City University, Japan; KOC-7C by Dr. Toru Sugiyama, Kurume University, Japan; SMOV-2 by Dr. Tomohiro Iida, St. Marianna University, Japan; and HAC-2 by Dr. Masato Nishida, Tsukuba University, Japan. TU-OC-1 cells were derived as described previously [20]. ES-2 cells were purchased from Summit Pharmaceuticals International Corporation (Tokyo, Japan). RMG-I, RMG-II, OVISE, OVTOKO, and 293T cells were purchased from the Health Science Research Resources Bank (HSRRB, Osaka, Japan). All OCCC cell lines were maintained in RPMI-1640 medium (Gibco, Carlsbad, CA), and 293T cells were maintained in DMEM medium (Gibco). The culture media were supplemented with 10% fetal bovine serum (FBS) (ICN Biomedical, Irvine, CA).

Cell viability and apoptosis assay

Paclitaxel (PTX), cisplatin (CDDP), and SN38 (the active metabolite of irinotecan) were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human HB-EGF (rhHB-EGF) was purchased from R&D systems (Minneapolis, MN). Cross-reactive material 197 (CRM197), a specific inhibitor of HB-EGF, was prepared as described previously [21]. In the cell viability assay, 20,000–40,000 cells were incubated in RPMI-1640 medium with 5% FBS and different concentrations of PTX, CDDP, or SN38 on 12-well plates. After a 72-h incubation, the cells were counted using a hemocytometer after the addition of trypan blue exclusion dye to determine the cell viability. In the apoptosis assay, TUNEL-positive cells were quantified as apoptotic cells by a flow cytometric analysis as described previously [22].

DNA extraction, RNA isolation, and cDNA synthesis

ES-2 cells were suspended in 0.1% SDS/Tris-EDTA buffer (pH 8.0). After treatment with proteinase K (Sigma-Aldrich) and RNase A (Invitrogen, Carlsbad, CA), phenol/chloroform extraction and ethanol precipitation were preformed to purify genomic DNA. Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. First-strand cDNA synthesis was performed with 1 μg of total RNA using with Primescript II reverse transcriptase (Takara Bio, Otsu, Shiga, Japan) following the manufacturer's protocol.

Real-time quantitative PCR (qPCR)

qPCR was performed using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and

the samples were analyzed by an Applied Biosystems 7500 Fast Real-time PCR system. TaqMan probes used were as follow: *HB-EGF*: Hs00181813_m1; *AREG*: Hs00950 669_m1; and *GAPDH*: Hs02758991_g1. The methods used for the data analysis were described previously [12].

Western blot analysis

Whole cell lysates (5 μg) were electrophoresed through 10% SDS-PAGE gels and proteins were transferred to a polyvinylidene difluoride membrane using a semi-dry electroblot (Bio-Rad, Hercules, CA). The blot was probed with primary antibodies (anti-human SP1 antibody [#07-645; Millipore, Billerica, MA], anti-human GAPDH antibody [#ABS16; Millipore]) and secondary antibody (anti-rabbit IgG-Peroxidase [A9169; Sigma-Aldrich]), and then the binding to antibody was detected using ECL Western Blotting Detection Regents (General Electric Company, Fairfield, CT) and a Fuji image analyzer (LAS mini-3000; Fujifilm, Tokyo, Japan) according to the manufacturer's protocol.

ELISA

Cells were incubated with culture medium for 24 h. After a 24-h incubation, the concentrations of HB-EGF and AREG in the culture medium were determined using a commercially available sandwich ELISA (DuoSet kit; R&D Systems) according to the manufacturer's instructions.

Vectors

To generate promoter constructs for the *HB-EGF* gene promoter, which were located at −4138 to +205 base pair (bp), −125 to +205 bp, −178 to +205 bp, and −253 to +205 bp from its transcriptional start site (TSS), the sequences were amplified and cloned into pGL4.12 (Promega, Madison, WI). All nucleotide numbering was done with reference to the TSS. The primers used for these PCR assays are listed in Table S1. The pGL4.12 and fragments were digested with *Kpn*I and *Nco*I restriction enzymes and ligated. To create pGL/HB$_{-2585/+205}$ and pGL/HB$_{-454/+205}$, pGL/HB$_{-4138/+205}$ was digested with *Nde*I and *Kpn*I or *Ase*I and *Kpn*I, and self-ligation was performed after blunt-ended treatment with Klenow fragment (Takara Bio). Site-detected mutants of the reporter vector were constructed by inverse PCR with primers described in Table S1. The sequences of all generated vectors were confirmed by DNA sequencing. The expression vectors for SP1 (EX-Y2188-Lv105) and EGFP (EX-EGFP-Lv105) were purchased from GeneCopoeia (Rockville, MD).

Reporter assay

The luciferase assay was performed with ES-2 cells and 293T cells. A total of 25,000 cells were incubated on 24-well plates for 16 h, and then were cotransfected with 1 μg of promoter reporter vector and 25 ng of pRL-TK (Promega), which had a renilla-luciferase gene downstream of the thymidine kinase promoter. Lipofectamine 2000 (Invitrogen) and Opti-MEM (Gibco) were used for transfection. When cells were treated with SN38, the culture medium was changed to RPMI-1640 medium supplemented with 10% FBS and SN38 (10 nmol/L). After a 48-h incubation, Dual-Luciferase Reporter Assay (Promega) was performed according to the manufacturer's protocol.

Small interfering RNA

Stealth RNAi (Invitrogen) against SP1 (siSP1_1: 5′-CAAUGGCAGUGAGUCUUCCAAGAAU-3′, siSP1_2: 5′-GACAGGUCAGUUGGCAGACUCUACA-3′) and control siRNAs (Control_1: Stealth RNAi siRNA Negative Control Lo GC, Control_2: Stealth RNAi siRNA Negative Control Med GC, Invitrogen) were transfected using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol.

Chromatin immunoprecipitation assay

ES-2 cells which were cultured until subconfluent in five 15 cm dishes were fixed in 1% formaldehyde for 10 min at 37°C. Chromatin was sheared using a Digital Sonifier S-250D (Branson, Danbury, CT) to yield DNA fragments of 300–800 bp. Nuclear extraction, immunoprecipitation, reverse cross-linking, and DNA purification were performed using Magna ChIP A kit (Millipore) according to the manufacturer's protocol. An anti-SP1 antibody (#07-645; Millipore), tri-methyl-histone H3 (Lys4) monoclonal antibody (#9751S; Cell Signaling Technology, Boston, MA), and normal rabbit IgG (#2729S; Cell Signaling Technology) were used in the immunoprecipitation reactions. Quantitative real-time PCR was performed using SYBR Premix Ex Taq GC (Takara Bio) to detect the HB-EGF promoter fragments.

Statistical analysis

The two-tailed independent Student's *t*-test was used to calculate all *P*-values. $P < 0.05$ was considered statistically significant.

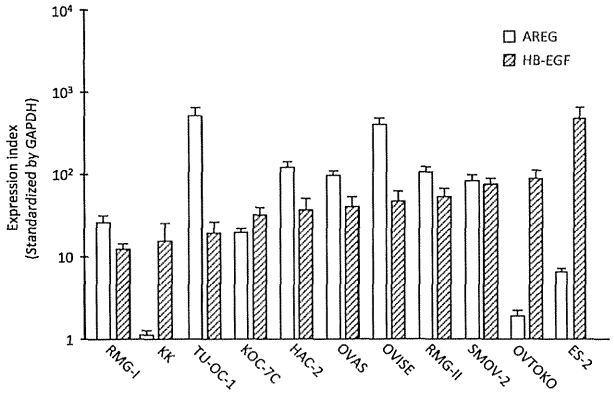


Figure 1. The expression of HB-EGF in 11 ovarian clear cell carcinoma (OCCC) cell lines. The real-time PCR data show the expression of heparin-binding epidermal growth factor-like growth factor (HB-EGF) and amphiregulin (AREG) in OCCC cells. Each value represents the mean (n = 3) and standard deviation (SD) of the mRNA expression index for HB-EGF (diagonal striped bars) and AREG (open bars).

Results

Promotion of HB-EGF expression in response to SN38 treatment

First, we examined the expression of HB-EGF and AREG in 11 cell lines of OCCC. HB-EGF was highly expressed in all of the cell lines, and eight of the 11 cell lines had a high-expression level of AREG (Fig. 1). OVTOKO and ES-2 cells had the highest expression of HB-EGF, while the OVISE and RMG-II cells had higher expression of AREG compared to that of HB-EGF.

To evaluate in vitro anticancer effects of conventional anticancer agents in the OVISE, RMG-II, OVTOKO, and ES-2 cells, cell viability assays were performed using SN38 (Fig. 2A), PTX (Fig. 2B), or CDDP (Fig. 2C). In this analysis, SN38 was a most effective anticancer agent in all four OCCC cell lines. Real-time PCR showed a twofold or higher increase in HB-EGF expression induced by the treatment of the OCCC cells with SN38, and the concentration of HB-EGF also increased more than twofold in the culture medium of RMG-II and ES-2 cells following SN38 treatment (Fig. 3A and B). In contrast, a high concentration of PTX or CDDP did not induce HB-EGF expression in ES-2 cells (Fig. 3C). The addition of the recombinant HB-EGF in cell culture blocked a decrease in cell viability with the treatment of SN38 in OCCC cells (Fig. 3D and E). These results indicated that HB-EGF plays a pivotal role in defense mechanism against the treatment of SN38 in OCCC cells.

To address the potential synergistic anticancer effects of the combination of SN38 and a specific inhibitor of HB-EGF (CRM197), apoptosis assays were performed after treating ES-2 or OVTOKO cells with SN38 and/or CRM197. Treatment with 10 μg/mL of CRM197 and 10 nmol/L of SN38 induced a marked increase in the number of apoptotic ES-2 and OVTOKO cells, compared to

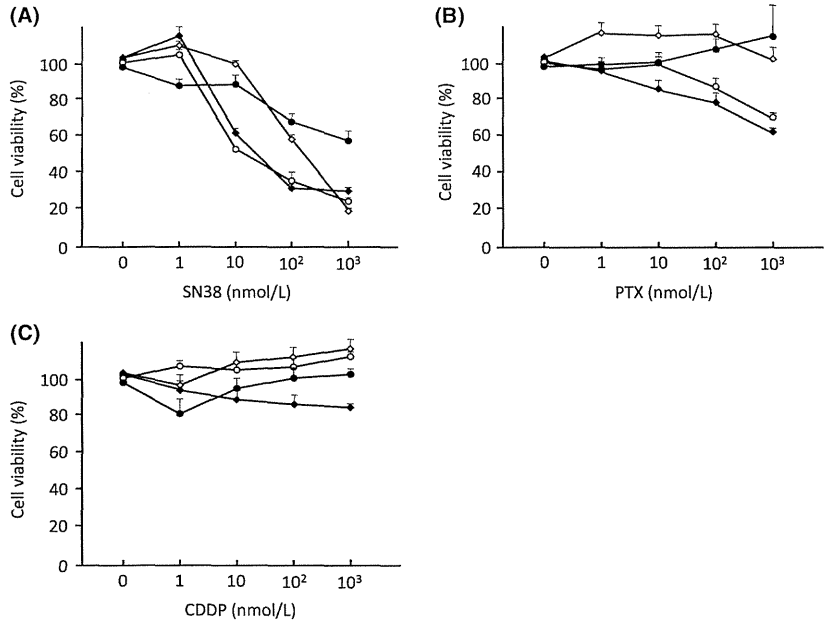


Figure 2. The efficacy of conventional anticancer agents against OCCC cells. Differences in the viability of OVISE (closed squares), RMG-II (closed circles), OVTOKO (open squares), and ES-2 (open circles) OCCC cells after treatment with SN38 (A), paclitaxel (PTX; B), and cisplatin (CDDP; C) for 72 h. Each value represents the mean cell viability rate (n = 3) and the SD. OCCC, ovarian clear cell carcinoma.

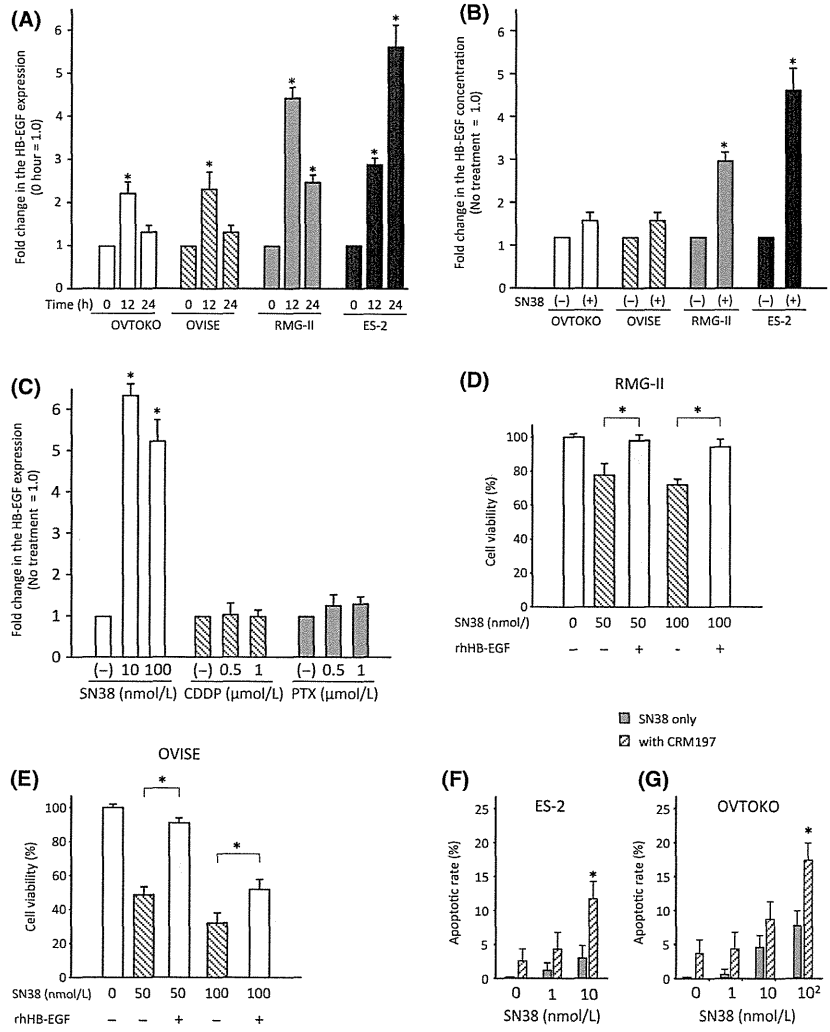
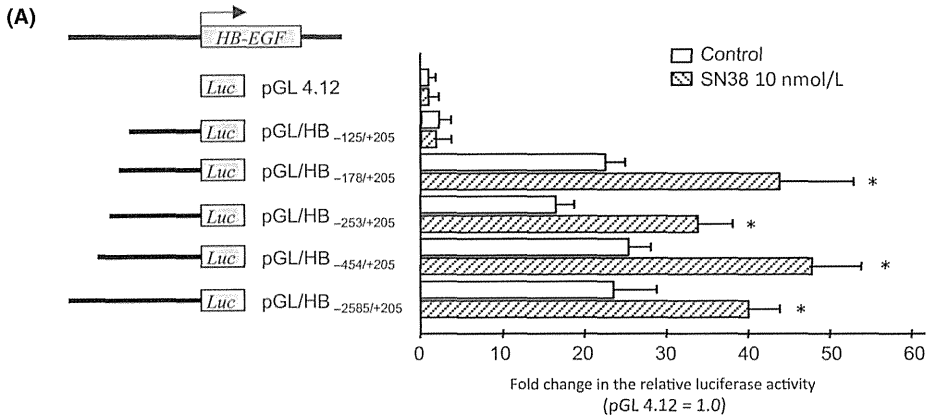


Figure 3. The association between HB-EGF expression and the SN38 treatment of OCCC cells. The induction of HB-EGF mRNA in cells (A) and HB-EGF protein in the culture medium (B) in OVTOKO (open bars), OVISE (diagonal striped bars), RMG-II (gray bars), and ES-2 (closed bars) cells treated with SN38 (10 nmol/L) for 12 or 24 h. Each value represents the mean (n = 3) and SD. (C) The differences in the HB-EGF mRNA expression in ES-2 cells after the treatment with SN38 (open bars), CDDP (diagonal striped bars), or PTX (gray bars). The differences in cell viabilities of RMG-II (D) and OVISE (E), which were cultured with recombinant human HB-EGF (0 and 200 pg/mL) and SN38 (0, 50, and 100 nmol/L). Each value represents the mean fold change (n = 3) and the SD. *P < 0.05. The changes in the apoptosis rate of ES-2 (F) and OVTOKO (G) cells after the treatment with each concentration of SN38 with (closed bars) or without CRM197 (10 μg/mL, diagonal striped bars). Each value represents the mean apoptosis ratio (n = 3) and SD. *P < 0.05, SN38-only versus SN38 with CRM197. HB-EGF, heparin-binding epidermal growth factor-like growth factor; PTX, paclitaxel.

cells with SN38 treatment alone, as determined using TUNEL assay (Fig. 3F and G). These results suggested that the suppression of HB-EGF during the treatment with SN38 leads to a synergistic anticancer effect in OCCC cells.

Screening for transcription factors that regulate the HB-EGF expression induced by SN38 treatment

To identify transcription factor(s) that contribute to the HB-EGF induction following treatment with SN38, a promoter analysis was performed using the ES-2 cells. For the reporter gene analysis, a *HB-EGF* promoter fragment (−2585/+205), which is conserved among mammalian species, fused to a luciferase vector, and various truncated constructs were synthesized. The luciferase assay showed that a reporter vector containing promoter fragment of −178/+205 bp from HB-EGF TSS (pGL/HB$_{-178/+205}$) exhibited an about 20-fold increase in luciferase activity compared to that of pGL/HB$_{-125/+205}$ (Fig. 4A). Additionally, treatment with SN38 induced ~twofold increase in the luciferase activity in a reporter vector containing



(B)

MatInspector

```
                    GTGNGCGCBNn ZFP161
                                nSGGGGCGGGGNnnn SP1
                                NVKGGGCGGRGYBNn SP1
                                NNGGGGCGGGGNNnn SP1
                                   GGGCGGGASCn KLF6
                                   nNGSGGGAGGGSGnnnn ZBTB7B
                                     nKGSGAGGGGAGn MAZ
                                                NVKGGGCGGRGYB SP1
                                                NNGGGGCGGGGNN SP1
-125 CAGCCCCCGACCCCGCGGGCGCGGCGGAGGACTGGGCGGGAGGAGAGGGCGGCGG-178
       nnnnnHACCCTVBSBGn KLF1
            nBNBGCGCVTGCGCGNB    NRF1
            nnnnnnNVRGGGGYGGSNYCnnn KLF6
                     GGCGYGG    BRE
```

AliBaba 2.1

```
-125 CAGCCCCCGACCCCGCGGGCGCGGCGGAGGACTGGGCGGGAGGAGAGGGCGGCGG -178
      ======SP1=====
                ====WT1===
                ======SP1====
                 ===EGR2===
                      =====SP1===
                         ====GAL4==
                              ======SP1=====
                                   ===TEAD2==
                                     ======SP1=====
                                                =====SP1==
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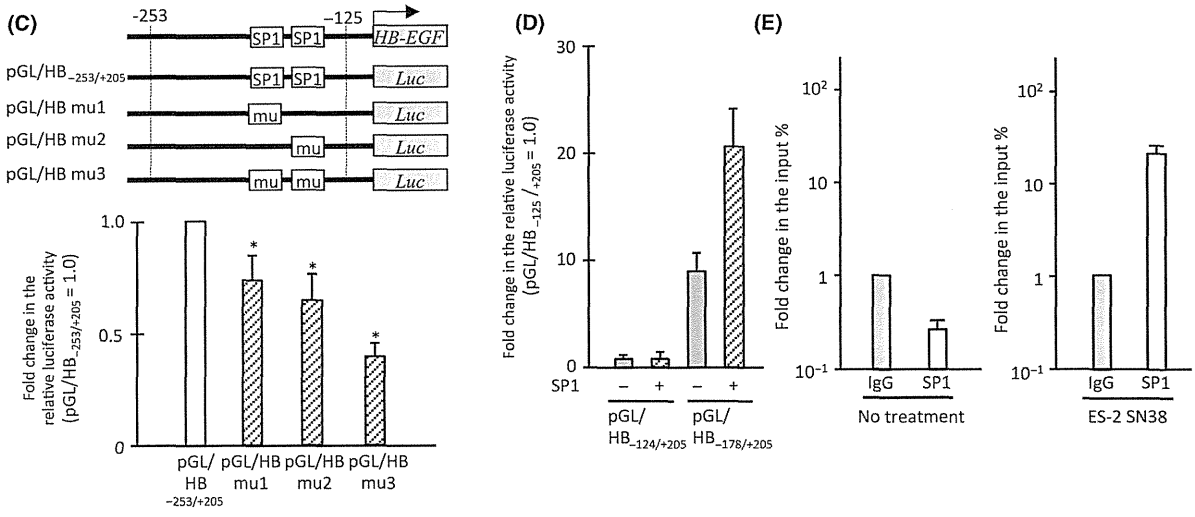

pGL/HB$_{-178/+205}$ compared to the control (Fig. 4A). Accordingly, the sequence located −178 to −125 bp from the TSS of *HB-EGF* was recognized as a promoter sequence bound by transcription factors.

Using the MatInspector (http://www.genomatix.de) and AliBaba 2.1 (http://www.gene-regulation.com) in silico analysis programs, we found that SP1-binding sites are commonly found in this promoter sequence (Fig. 4B). Since the HB-EGF promoter has a TATA-less and GC-rich promoter, which are characteristics of SP1-binding promoters, we focused on SP1 as the candidate transcription factor inducing HB-EGF in SN38-treated ES-2 cells [17, 23, 24].

Reporter vectors with site mutations of each SP1-binding site showed significantly reduced luciferase activity compared to the control (Fig. 4C). Moreover, the luciferase activity significantly decreased, compared to the control, when the two putative binding sites of SP1 were replaced with mutant sites (Fig. 4C). Forced expression of SP1 activated the luciferase activity in 298T cells transfected with pGL/HB$_{-178/+205}$, although no difference was found between the forced expression of SP1 and control in 293T cells transfected by pGL/HB$_{-125/+205}$ (Fig. 4D).

To detect direct interaction between the SP1 protein and HB-EGF promoter in ES-2 cells, a chromatin immunoprecipitation assay was performed in cells with or without treatment with SN38. Quantitative real-time PCR showed that, during treatment with SN38, the level of HB-EGF promoter bound to SP1 significantly increased compared to that of control cells (Fig. 4E). These results indicated that the HB-EGF expression promoted by the treatment with SN38 was regulated by SP1 in OCCC cells.

Contribution of SP1 to the HB-EGF expression and SN38 sensitivity of OCCC cells

To confirm whether SP1 affects the sensitivity of cells to SN38 via the induction of HB-EGF expression, the expression and the cell viability assays were performed after the transfection of small interfering RNA (siRNA) targeting SP1. A Western blot analysis showed that the ES-2 cells transfected with siSP1_1 and siSP1_2 had reduced expression of the SP1 protein compared to the cells transfected with control siRNAs (Fig. 5A). In the ES-2 cells transfected with siSP1_2, the HB-EGF expression was reduced to approximately half the control level, although the HB-EGF expression was not altered in the ES-2 cells transfected with siSP1_1 (Fig. 5B). No induction of HB-EGF expression was found in the presence of SN38 in the ES-2 cells transfected with either siSP1_1 or siSP1_2 (Fig. 5C). Additionally, we examined alterations in the cell viabilities using RMG-II cells, which had the least antitumor effect with the treatment of SN38. Following the treatment with SN38, the number of RMG-II cells transfected with siSP1_1 and siSP1_2 significantly decreased compared to that in the RMG-II cells transfected with control siRNAs (Fig. 6). These results indicate that SP1 regulates the drug sensitivity of SN38 by regulating the HB-EGF expression in OCCC cells.

Discussion

In this study, HB-EGF was attributable for the escape from cell death, as SN38 damaged OCCC cells. SP1 activated HB-EGF expression through its binding to multiple transcription sites within the promoter of HB-EGF in OCCC cells. In addition, the suppression of HB-EGF as well as SP1 enhanced the sensitivity of SN38 in OCCC cells.

SP1, which belongs to the family of SP1/Kruppel-like factor (KFL) transcriptional factors, regulates the expression of numerous genes involved in cell proliferation, apoptosis, and differentiation [25]. Phorbol 12-myristate 13 acetate (PMA) induced an increase in the transcriptional activity of SP1 through the deacetylation of SP1, and also provoked the ectodomain shedding of HB-EGF; it also increased the expression of HB-EGF [26, 27].

Figure 4. The interaction of SP1 with the *HB-EGF* promoter region following SN38 treatment. (A) The alterations in the luminescence of ES-2 cells for each reporter vector in cells with (open bars) and without (diagonal striped bars) SN38 (10 nmol/L) treatment. A schematic diagram showing the reporter vectors on the left side. (B) A schematic diagram obtained from the in silico analysis including the MatInspector and AliBaba 2.1 programs. (C) The luciferase reporter analysis of the site mutants in ES-2 cells. The schematic diagram shows the wild-type SP1-binding sites as "SP1" and the mutated binding sites as "mu" in each vector. The alterations in the luminescence of ES-2 cells among the wild-type reporter vector (open bar) and the mutated reporter vectors (diagonal striped bars) in the presence of SN38 (10 nmol/L). Each value represents the mean (n = 3) and SD. (D) The differences in the relative luciferase activity of 293T cells transfected with pGL/HB$_{-178/+205}$ and the SP1 expression vector (diagonal striped bars). An EGFP expression vector was used as a control (gray bars). Each value represents the mean (n = 3) and SD. All relative luciferase activities were normalized to the renilla luminescence. (E) The results of the ChIP analysis of ES-2 cells with or without SN38 (10 nmol/L) treatment. The fold change in the immunoprecipitated DNA for SP1 (open bars) against templates due to the nonspecific binding with IgG (gray bars). Each value represents the mean (n = 3) and SD. *P < 0.05, versus control. HB-EGF, heparin-binding epidermal growth factor-like growth factor; SP1, specific protein 1.

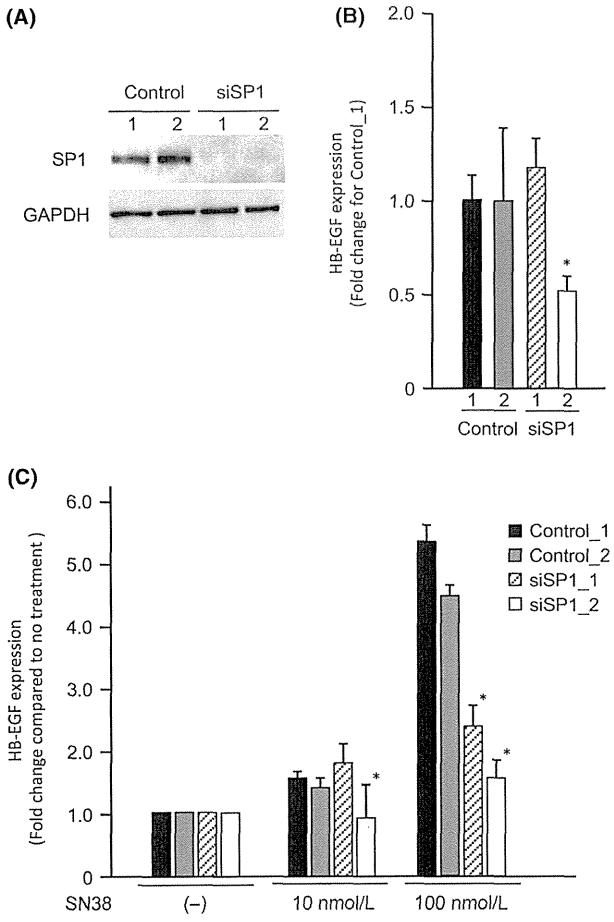

Figure 5. The contribution of SP1 to the HB-EGF induction following the treatment with SN38. (A) A Western blot analysis was performed to confirm the SP1 reduction in the transfected ES-2 cells induced by treatment with siRNAs against SP1 or a control siRNA. (B) The alterations in the HB-EGF mRNA expression in the transfected ES-2 cells induced by treatment with the siRNAs against SP1 or the control siRNA. (C) The induction of HB-EGF mRNA in the RMG-II cells transfected with siRNAs against SP1 or the control siRNA following the treatment of SN38. Each value represents the mean (n = 3) and SD. Closed bars, control_1 siRNA; gray bars, control_2 siRNA; diagonal striped bars, siSP1_1 si RNA; open bars, siSP1_2 siRNA. *P < 0.05, control_1 versus each siRNA. SP1, specific protein 1.

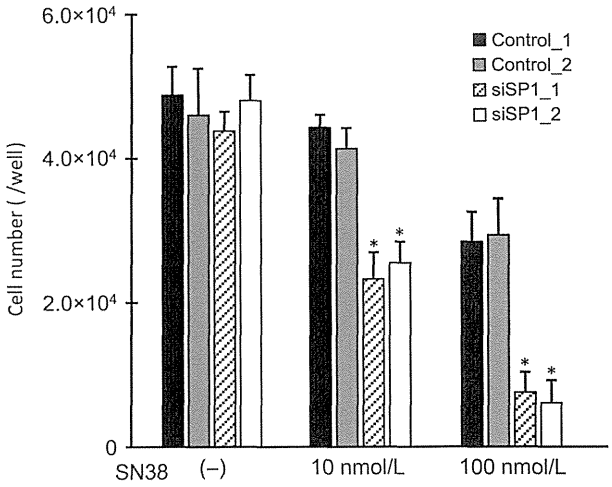

Figure 6. The contribution of SP1 to the cell viabilities following the treatment with SN38. The alterations in the numbers of RMG-II cells transfected with siRNAs against SP1 or a control siRNA after treatment with SN38. Each value represents the mean (n = 3) and SD. Closed bars, control_1 siRNA; gray bars, control_2 siRNA; diagonal striped bars, siSP1_1 si RNA; open bars, siSP1_2 siRNA. *P < 0.05, control_1 versus each siRNA. SP1, specific protein 1.

Additionally, NF-κB induced the expression of HB-EGF, and the NF-κB and SP1 binding sequence was shown to be the same GC-rich element in colon cancer cells [16, 28]. This evidence suggests that SP1 augments the expression of HB-EGF through the deacetylation of SP1 or via an interaction with NF-κB. In mice, SP1 was reported to be directly bound to the promoter regions of HB-EGF [24]. In humans, SP1 also functions as a direct regulator for the expression of HB-EGF, possibly through various posttranslational modifications of SP1 and by interactions with other transcriptional factors.

Dysregulation of SP1 is found in many types of cancer, including ovarian, breast, and gastric cancer, for which HB-EGF is a rational therapeutic target [22, 29, 30]. The hypoxia-inducible factor 2α (HIF2α)–SP1 complex activated coagulation factor VII promoter in OCCC and estrogen receptor α also form complexes with SP1 in other types of ovarian cancer [31, 32]. Additionally, previous reports showed that activation of SP1 promoted breast cancer development and the metastasis of gastric cancer [33, 34]. On the other hand, the SP1 level was highly upregulated in patients with early stage and minimally invasive lung cancer cells and in patients with stage I lung cancer compared to that in lung cancers with high invasiveness and in patients with stage IV lung cancer [35]. These previous findings show that the significance of SP1 involvement in cancer progression have been controversial. In this study, the suppression of SP1 enhanced the sensitivity of OCCC cells to SN38. Therefore, it seems to be important that the suppression of SP1 itself, as well as the inhibition of the posttranslational modifications of SP1, are critical for cancer therapy.

Several compounds that inhibit the transcriptional activity of SP1 have been developed for cancer therapy [25], for example, arsenic trioxide downregulates the expression of SP1 [36]. A phase III trial of arsenic trioxide was performed for patients with acute promyelocytic leukemia classified as having low-to-intermediate risk, and the results suggested that all-*trans*-retinoic acid plus arsenic trioxide may be superior to all-*trans*-retinoic acid

plus other chemotherapy [37]. Bortezomib has been already used for the treatment of patients with multiple myeloma [38, 39]. Bortezomib has been shown to decrease the expression of SP1 and disrupt the interaction of SP1 with NF-κB [40]. Other inhibitory compounds for SP1 will be clinically applied for various diseases, in addition to cancer [41, 42].

We have performed a clinical phase II trial using CRM197 in patients with recurrent and advanced ovarian cancer. To explore the importance of the posttranslational modifications of SP1 in the induction of HB-EGF expression and to search for compounds that can inhibit such modifications, a preclinical study should be performed using combination therapy with CRM197, irinotecan, and a compound that inhibits SP1, such as arsenic trioxide or bortezomib. Such a combination would likely improve the prognosis of patients with OCCC.

Acknowledgments

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Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primer sequences.

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ORIGINAL ARTICLE

Efficacy and safety of triple therapy with aprepitant, palonosetron, and dexamethasone for preventing nausea and vomiting induced by cisplatin-based chemotherapy for gynecological cancer: KCOG-G1003 phase II trial

Nobuhiro Takeshima · Maki Matoda · Masakazu Abe · Yasuyuki Hirashima · Kentaro Kai · Kaei Nasu · Masashi Takano · Kenichi Furuya · Seiya Sato · Hiroaki Itamochi · Hiroshi Tsubamoto · Kosei Hasegawa · Kiminari Terao · Takeo Otsuki · Keiko Kuritani · Kimihiko Ito

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Abstract
Purpose Prevention of chemotherapy-induced nausea and vomiting (CINV) is crucial for maintaining the quality of life of cancer patients. Female patients have been underrepresented in previous clinical studies of aprepitant or palonosetron. We performed a prospective multicenter study to investigate the efficacy and safety of triple therapy comprising these two agents and dexamethasone in female cancer patients receiving chemotherapy that included cisplatin (≥50 mg/m^2).
Methods Aprepitant was administered at a dose of 125 mg before chemotherapy on day 1 and at 80 mg on days 2 and 3. Palonosetron (0.75 mg) was given before chemotherapy on day 1. Dexamethasone was administered at a dose of 9.9 mg before chemotherapy on day 1 and at 6.6 mg on days 2–4. The

N. Takeshima · M. Matoda (✉)
Department of Gynecology, Cancer Institute Hospital, 3-8-31 Ariake, Koto-ku, Tokyo 135-8550, Japan
e-mail: maki.matsumura@jfcr.or.jp

N. Takeshima
e-mail: nobuhiro.takeshima@jfcr.or.jp

M. Abe · Y. Hirashima
Division of Gynecology, Shizuoka Cancer Center Hospital, 1007 Shimonagakubo, Nagaizumi-cho, Sunto-gun, Shizuoka 411-8777, Japan

M. Abe
e-mail: ma.abe@scchr.jp

Y. Hirashima
e-mail: y.hirashima@scchr.jp

K. Kai · K. Nasu
Department of Obstetrics and Gynecology, Faculty of Medicine, Oita University, Idaigaoka 1-1, Hasama-machi, Yufu-shi, Oita 879-5593, Japan

K. Kai
e-mail: kenta9sp@oita-u.ac.jp

K. Nasu
e-mail: nasu@oita-u.ac.jp

M. Takano · K. Furuya
Department of Obstetrics and Gynecology, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359-8513, Japan

M. Takano
e-mail: mastkn@ndmc.ac.jp

K. Furuya
e-mail: furuyac@nifty.com

S. Sato · H. Itamochi
Department of Obstetrics and Gynecology, Tottori University, School of Medicine, 36-1 Nishicho, Yonago, Tottori 683-8504, Japan

S. Sato
e-mail: sseiya@med.tottori-u.ac.jp

H. Itamochi
e-mail: itamochi@hotmail.com

H. Tsubamoto
Department of Obstetrics and Gynecology, Hyogo College of Medicine, Mukogawa 1-1, Nishinomiya, Hyogo 663-8501, Japan
e-mail: tsuba@hyo-med.ac.jp

primary endpoint was the the proportion of patients with a complete response (CR no vomiting and no use of rescue medication) throughout the overall period (0–120 h post-chemotherapy).
Results Ninety-six women (median age 55 years) were enrolled. The overall CR rate was 54.2 %. CR was obtained during the acute phase (0–24 h post-chemotherapy) and the delayed phase (24–120 h post-chemotherapy) in 87.5 and 56.3 % of the patients, respectively. The most common adverse reactions were constipation and fatigue (reported by three patients each).
Conclusions Exhibition of a favorable overall CR rate over existing two-drug combinations suggests that the triple therapy regimen used in the present study is effective and tolerable in patients with gynecological malignancies receiving cisplatin-based chemotherapy. Female patients may have a higher risk of developing CINV.

Keywords Aprepitant · Palonosetron · Nausea · Vomiting · CINV · Cisplatin

Introduction

Chemotherapy-induced nausea and vomiting (CINV) occurs with a high frequency following chemotherapy for cancer and is one of the adverse reactions that causes hardship for patients receiving chemotherapy. Failure to prevent CINV may result in worsening of the physical and mental state of the patient and may even become an obstacle to the continuation of chemotherapy. Thus, prevention or alleviation of CINV is extremely important for maintenance of the quality of life of patients and for continuation of their treatment [1, 2].

Antineoplastic agents cause vomiting via two pathways. In one pathway, enterochromaffin cells of the gastrointestinal mucosa are stimulated by a chemotherapy agent and release 5-hydroxytryptamine (serotonin, 5-HT), a neurotransmitter that activates gastrointestinal 5-hydroxytryptamine type 3 (5-HT_3) receptors and transmits signals to the vomiting center in the lateral reticular formation of the medulla via vagal afferents or via the chemoreceptor trigger zone (CTZ). In the other pathway, a drug directly stimulates the CTZ, and then, signals are transmitted to the vomiting center via dopamine receptors and 5-HT_3 receptors. Furthermore, antineoplastic agents can promote the secretion of substance P in the area postrema and the nucleus solitarius of the medulla oblongata, after which substance P binds to neurokinin-1 (NK-1) receptors and induces vomiting. Attention has recently been paid to this mechanism as a new target for antiemetic therapy [3].

Aprepitant is a selective NK-1 receptor antagonist. Clinical trials of this agent with a new mechanism of action for the prophylaxis for CINV have been undertaken outside Japan, and it has been shown to be effective for both acute CINV and also delayed CINV, which responds poorly to existing medications [4–7]. In Japan, the efficacy of aprepitant was demonstrated in Japanese patients by a phase II trial [8], and authorization for manufacturing/marketing was gained in October 2009.

Palonosetron is a new second-generation 5-HT_3 receptor antagonist that differs from other 5-HT_3 receptor antagonists by showing higher receptor-binding affinity, as well as having an extended half-life of about 40 h (four to five times longer than dolasetron, granisetron, or ondansetron) and an excellent safety profile [9, 10]. In Japan, the efficacy of palonosetron was demonstrated by a randomized, parallel-group, comparative, multicenter study using granisetron hydrochloride as the comparator [11], and manufacturing/marketing authorization was obtained in January 2010.

Combined administration of NK-1 receptor antagonists, 5-HT_3 receptor antagonists, and steroids is recommended for the prevention of CINV associated with the administration of highly or moderately emetogenic antineoplastic agents in the international guidelines for antiemetic therapy issued by the American Society of Clinical Oncology (ASCO), Multinational Association of Supportive Care in Cancer (MASCC), and National Comprehensive Cancer Network (NCCN) [12–14]. In clinical studies of aprepitant or palonosetron, thus far reported in or outside Japan, there have been relatively few female patients, and the efficacy of these drugs for CINV in patients with gynecological cancer has not yet been established. In addition, no information is available in or outside Japan concerning the clinical efficacy of triple therapy with the

K. Hasegawa
Department of Gynecologic Oncology, Saitama Medical University International Medical Center, 1397-1 Yamane, Hidaka-shi, Saitama 350-1298, Japan
e-mail: koseih@gmail.com

K. Terao
Miyazaki Prefectural NOBEOKA Hospital, Shinkouji 2-1-10, Nobeoka-shi, Miyazaki 882-0835, Japan
e-mail: k-terao@pref-hp.nobeoka.miyazaki.jp

T. Otsuki
Department of Obstetrics and Gynecology, Tohoku University, School of Medicine, 1-1, Seiryo-machi, Sendai 980-8574, Japan
e-mail: takeootsuki@med.tohoku.ac.jp

K. Kuritani · K. Ito
Kansai Rosai Hospital, 3-1-69 Inabaso, Amagasaki, Hyogo 660-8511, Japan

K. Kuritani
e-mail: keiko0630@bcb.bai.ne.jp

K. Ito
e-mail: kimihiko-ito@kanrou.net

combination of aprepitant, palonosetron, and dexamethasone in patients with gynecological cancer.

Therefore, we planned the present study to evaluate the efficacy and safety of combined therapy with these three agents for prevention of CINV in patients with gynecological malignancies receiving chemotherapy containing cisplatin, a highly emetogenic antineoplastic agent.

Methods

Patients

Patients aged 20 years or older were enrolled if they were scheduled to receive more than one cycle of highly emetogenic chemotherapy at any of ten facilities related to Kansai Clinical Oncology Group (KCOG) between July 1, 2010 and June 30, 2012. All patients had gynecological cancer and were scheduled to receive cisplatin at a dose of 50 mg/m^2 or more. Patients who fulfilled any of the following criteria were excluded from the study: previous cisplatin use, severe hepatic insufficiency (Child-Pugh score >9), pre-enrollment alanine aminotransferase (glutamic-pyruvic transaminase) or aspartate aminotransferase (glutamic-oxaloacetic transaminase) level >3 times the upper limit of normal, pre-enrollment total bilirubin >2 times the upper limit of normal, and pre-enrollment serum creatinine level >1.5 times the upper limit of normal.

Study treatment

Aprepitant was administered orally, with a dose of 125 mg being given at 60–90 min before chemotherapy on day 1 and 80 mg being administered once daily on days 2 and 3. Palonosetron was administered intravenously with a dose of 0.75 mg at 30–60 min before chemotherapy on day 1. Dexamethasone was administered orally or intravenously, with a dose of 9.9 mg being given at 30–60 min before chemotherapy on day 1 followed by 6.6 mg once daily on days 2–4.

Parameters assessed

The primary endpoint of the study was the proportion of patients with a complete response (CR), which was defined as no episodes of vomiting and no rescue therapy for nausea, throughout the study period from 0 to 120 h after cisplatin administration (overall CR rate). The secondary endpoints were the proportion of patients with CR in the acute phase (0–24 h after cisplatin administration) and in the delayed phase (24–120 h after cisplatin administration) of the study, as well as the proportion of patients with complete protection (CP no vomiting, no rescue therapy, and no significant nausea (visual analog scale score <25 mm)) throughout the study and in the acute and delayed phases. The proportion of patients who gave the response "Little or no effect on activities of daily living" when completing the Functional Living Index-Emesis (FLIE) questionnaire on day 6 was also determined to assess the influence on the quality of life (QOL).

Evaluation of safety

Adverse events and laboratory data were compiled according to the Common Terminology Criteria for Adverse Events (version 4).

Statistical analysis

It was calculated that 81 patients were needed to detect a difference of $P \leq 0.05$ (two-sided) with a 90 % power if the CR rate for antiemetic therapy was assumed to be 70 %. By estimating the rate of exclusion from analysis as about 20 %, the target number of subjects for enrollment was set at 100. In the main analysis, the study therapy would be judged to be effective if the proportion of patients with a CR throughout the study period (0–120 h) exceeded the proportion of patients with a CR with standard therapy (dual therapy with a first-generation 5-HT$_3$ receptor antagonists and dexamethasone) in previous reports.

Ethical considerations

The present study was conducted in accordance with ethical principles based on the Declaration of Helsinki and the "Ethical Guidelines for Clinical Studies." It was approved by an appropriate institutional review board and ethics committee at each participating center after assessment of the protocol and written information provided for the patients. All patients gave written informed consent prior to inclusion in the study. This study was registered with the University Hospital Medical Information Network (UMIN) clinical trial registry (no. UMIN000003820).

Results

Patient characteristics

A total of 96 patients were enrolled, and their characteristics are summarized in Table 1. All patients were female and their median age was 55 years (range 32–75 years). They were treated for the following gynecological malignancies: endometrial cancer in 61 patients (63.5 %), cervical cancer in 14 patients (14.6 %), and ovarian cancer in 19 patients (19.8 %). Among them, 49 patients (51.0 %) had a history of morning

Table 1 Patient characteristics

		Number	Percent
Total		96	100.0
Age (years)			
	Median	55	
	Range	32–75	
Performance status			
	0	91	94.8
	1	5	5.2
Gynecological malignancy			
	Endometrial cancer	61	63.5
	Ovarian cancer	19	19.8
	Cervical cancer	14	14.6
	Others	2	2.1
Cisplatin dose			
	≥50 and <60	57	59.4
	≥60 and <70	31	32.3
	≥70	8	8.3
	Mean	56.0	
Chemotherapy regimen			
	Cisplatin/Adriamycin	46	47.9
	Cisplatin/Irinotecan	26	27.1
	Cisplatin/Docetaxel	12	12.5
	Cisplatin/Taxol	7	7.3
	Cisplatin alone	2	2.1
	Cisplatin/5-fluorouracil	2	2.1
	Cisplatin/Doxorubicin	1	1.0
Prior chemotherapy			
	Vomiting—Yes	19	19.8
	Vomiting—No	15	15.6
No prior chemotherapy		62	64.6
Drinking alcohol			
	Yes	17	17.7
	No	79	82.3
Motion sickness			
	Yes	30	31.3
	No	66	68.8
morning sickness			
	Yes	49	51.0
	No	47	49.0

sickness during pregnancy, 30 (31.3 %) had a history of motion sickness, and 17 (17.7 %) drank alcohol. Furthermore, 34 patients (35 %) had received prior anticancer chemotherapy, and 19 patients (19.8 %) had experienced nausea. The mean dosage of cisplatin was 56 mg/cm^2, and the other drug used in combination with cisplatin was adriamycin in 46 patients (47.9 %), irinotecan in 26 patients (27.1 %), and docetaxel in 12 patients (12.5 %).

Table 2 Efficacy data

	Study phase	Percent	95 % confidence interval
CR	Acute	87.5	(79.2–93.4)
	Delayed	56.3	(45.7–66.4)
	Overall	54.2	(43.7–64.4)
CP	Acute	82.3	(73.2–89.3)
	Delayed	45.8	(35.6–56.3)
	Overall	44.8	(34.6–55.3)
No emesis	Acute	90.6	(82.9–95.6)
	Delayed	71.9	(61.8–80.6)
	Overall	71.9	(61.8–80.6)
No rescue therapy	Acute	95.8	(89.7–98.9)
	Delayed	65.6	(55.2–75.0)
	Overall	62.5	(52.0–72.2)
No nausea	Acute	74.0	(64.0–82.4)
	Delayed	33.3	(24.3–44.1)
	Overall	30.2	(21.5–40.8)
No significant nausea	Acute	89.6	(81.7–94.9)
	Delayed	62.5	(52.6–72.8)
	Overall	62.5	(52.6–72.8)

The results for patients with no nausea and with no significant nausea are based on data for 95 patients because there was one omission

CR complete response, *CP* complete protection

Antiemetic effect

The antiemetic effect of study therapy is summarized in Table 2. The overall CR rate, which was the primary endpoint, was 54.2 %, while the acute CR rate and delayed CR rate were 87.5 and 56.3 %, respectively. The proportion of patients with no emesis was 90.6, 71.9, and 71.9 % in the acute phase, delayed phase, and overall, respectively, while the corresponding CP rates were 82.3, 45.8, and 44.8 %. The proportion of patients with no nausea was 74.0, 33.3, and 30.2 % in the acute phase, delayed phase, and overall, respectively. The proportion of patients with no nausea on a daily basis for 5 days after administration of chemotherapy is shown in Fig. 1. Control of CINV was poorest at 4 days after the administration of chemotherapy. The proportion of patients who did not need rescue therapy was 95.8, 65.6, and 62.5 % in the acute phase, delayed phase, and overall, respectively. The proportion of patients who gave the response "Little or no effect on activities of daily living" when completing the FLIE questionnaire on day 6 was determined to assess QOL. It was 82.3 % for the vomiting domain, 43.8 % for the nausea domain, and 59.4 % for the combined nausea/vomiting domain. With regard to the nine items in the nausea domain of the FLIE questionnaire, the impact of nausea on daily activities was greatest for "Ability to enjoy a meal" (Fig. 2).

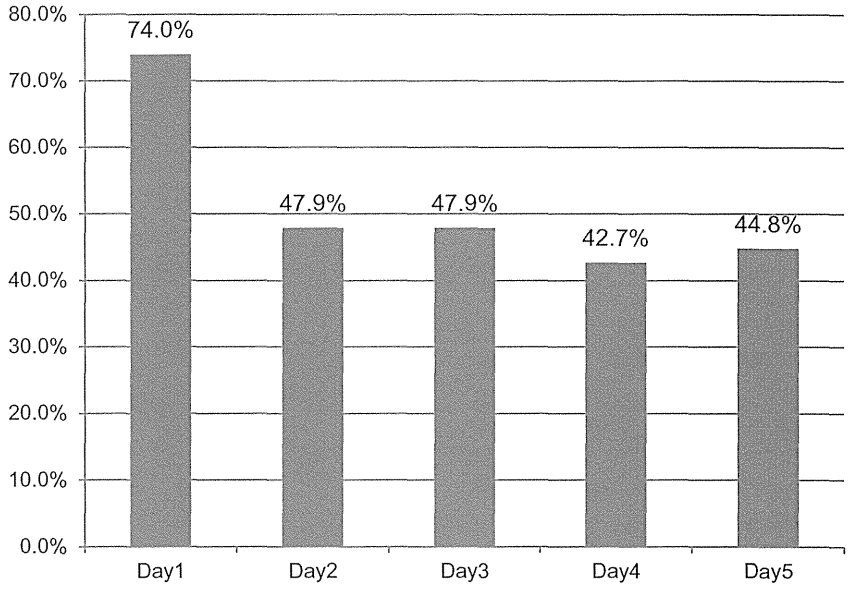

Fig. 1 Proportion of patients with no nausea

Safety

Triple therapy with aprepitant, palonosetron, and dexamethasone showed good tolerability throughout the study period. The most frequently reported adverse reactions were constipation and fatigue, each of which was noted by three patients (3 %).

Discussion

The present multicenter clinical study aimed to evaluate the effect of triple therapy with aprepitant (an NK-1 receptor antagonist), palonosetron (a second-generation 5-HT_3 receptor antagonist), and dexamethasone on CINV in patients with female malignancies who received cisplatin-based chemotherapy. Female sex is known as a risk factor for CINV. In addition, cisplatin is an antineoplastic agent that frequently causes CINV. Accordingly, the subjects of the present study may represent a population of patients in whom CINV would be difficult to control. Cisplatin-containing regimens are classified as highly emetogenic chemotherapy in the guidelines for antiemetic therapy issued by the ASCO [12], MASCC [13], NCCN [14], and Japan Society of Clinical Oncology [15].

In the present study, a CR rate of 54.2 % was achieved by triple therapy with aprepitant, palonosetron, and dexamethasone. This study demonstrated that the triple-agent strategy is

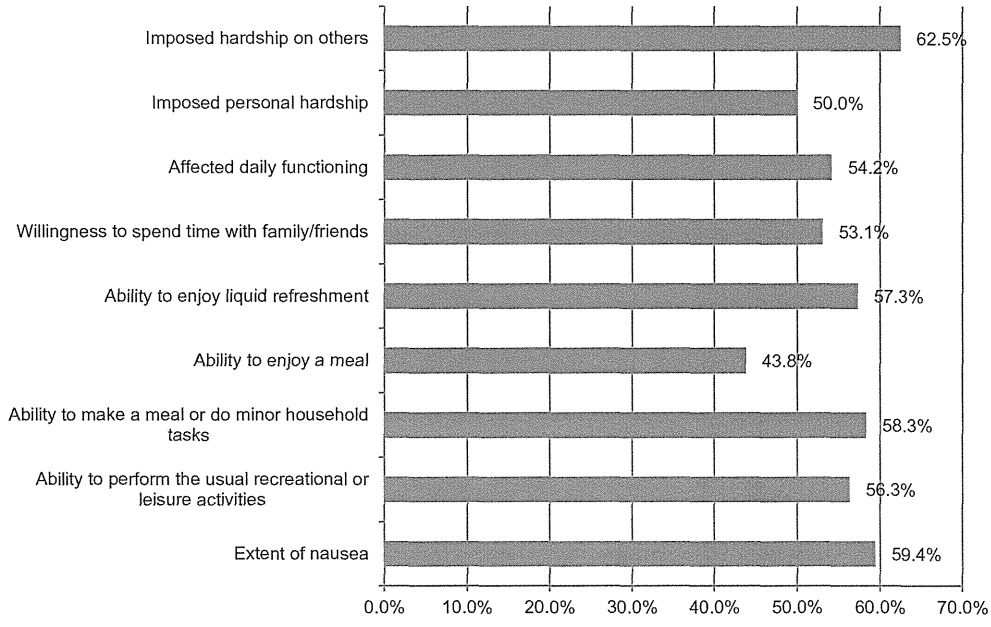

Fig. 2 Nine items in the nausea domain of the Functional Living Index-Emesis questionnaire

Table 3 Comparison of triple therapy regimens

	No. of patients	Percentage of female patients	Cisplatin ≥70 mg/m^2	CR	No emesis	No nausea	No significant nausea	5-HT$_3$ receptor antagonist
Hesketh et al. (2003)	260	48	70 %	73 %	78 %	48 %	73 %	Ondansetron
Poli-Bigelli et al. (2003)	261	37	82 %	63 %	66 %	49 %	71 %	Ondansetron
Schmoll et al. (2006)	243	39	75 %	72 %	77 %	–	73 %	Ondansetron
Takahashi et al. (2011)	149	24	100 %	71 %	77 %	34 %	69 %	Granisetron
Present study	96	100	9 %	54 %	72 %	30 %	63 %	Palonosetron

CR complete response

useful, because it achieved similar CR rates to standard therapy (with a first-generation 5-HT$_3$ receptor antagonist plus dexamethasone) that was used as the comparator in previous clinical trials of aprepitant or palonosetron: Hesketh et al. [4] and Poli-Bigelli et al. [5], respectively, reported a CR rate of 52.3 and 43.3 % with the standard ondansetron-dexamethasone combination. In addition, a CR rate of 40.3 % was reported with the combination of palonosetron and dexamethasone [11]. However, the triple therapy used in the present study achieved a lower CR rate than other triplet regimens, since the CR rate was 72.7, 62.7, and 72.0 % with triple therapy using ondansetron (a first-generation 5-HT$_3$ receptor antagonist) as reported by Hesketh et al. in 2003 [4], Poli-Bigelli et al. in 2003 [5], and Schmoll et al. in 2006 [16], respectively. In addition, a CR rate of 70.5 % for a triplet regimen using granisetron (another first-generation 5-HT$_3$ receptor antagonist) was reported by Takahashi et al. in 2011 (Table 3) [8]. One reason for this difference from other studies may be that our subjects were all female patients. Women are known to be susceptible to CINV, and stratified analysis has shown that dual therapy with a first-generation 5-HT$_3$ receptor antagonist and dexamethasone achieves a lower CR rate in women than in men [17]. In other clinical studies with a lower proportion of women than the present study, the CR rate for triple therapy that included palonosetron as the first-generation 5-HT$_3$ receptor antagonist was reported to be 70.3 % (percentage of women in the study population 23.4 %) by Longo et al. [18] and 81.0 % (percentage of women 23 %) by Miura et al. [19]. Since patients with previous chemotherapy were also enrolled in the present study, an analysis was conducted to compare the subgroups with and without prior chemotherapy, but similar results were obtained (Table 4). Accordingly, although the fact that the subjects of the present study were all women should have an influence, the possibility of other factors cannot be ruled out because it has been reported that the addition of aprepitant to dual therapy with a first-generation 5-HT$_3$ receptor antagonist and dexamethasone can overcome the increased risk of CINV associated with the female gender [17].

In the present study, only 30 % of patients had no nausea throughout the observation period, and adequate control of nausea was not achieved. Rescue medication was administered at the discretion of the attending physician at each participating center because the present study was a multicenter investigation. Accordingly, one reason for the low CR rate may be that rescue therapy was provided without careful consideration of patient's complaints about nausea at some centers. Therefore, different measures should be taken for the control of nausea in future studies.

In another study of patients undergoing highly emetogenic chemotherapy (TRIPLE), comparison was performed between granisetron (a first-generation 5-HT$_3$ receptor antagonist) and palonosetron (a second-generation 5-HT$_3$ receptor antagonist) with basal antiemetic therapy using aprepitant and dexamethasone [20]. The overall CR rate was the primary endpoint, and this showed no statistically significant difference between the two groups. Palonosetron group was significantly superior to the granisetron group with regard to the nausea domain rates for complete control and total control. In view of our present finding that the control of nausea was even poor with

Table 4 Results for patients with and without prior chemotherapy

	No. of patients	Percentage of female patients	Cisplatin ≥70 mg/m^2	CR	No emesis	No nausea	No significant nausea	Prior chemotherapy
Longo et al. (2010)	222	23	98 %	70 %	93 %	60 %	91 %	Chemo-naïve
Miura et al. (2013)	64	23	95 %	81 %	–	54 %	67 %	Chemo-naïve
Present study	96	100	9 %	54 %	72 %	30 %	63 %	Cisplatin-naïve
	62*	100	8 %	55 %	68 %	31 %	63 %	Chemo-naïve

CR complete response

palonosetron, the influence of female gender is considered to be strong.

Recently, the efficacy of olanzapine for controlling nausea has been reported. This agent is one of the multi-acting receptor antipsychotics (MARTA) used for the treatment of schizophrenia, which can block dopamine receptors, serotonin receptors, histamine receptors, adrenergic receptors, and other receptors associated with CINV. Olanzapine was recently reported to be effective for preventing CINV based on its mechanism of action [21]. Therefore, concomitant use of olanzapine is another option that is available.

In order to achieve further improvement of the control of CINV, several additional treatments are likely to be introduced. Since nausea often reaches a peak at 4 days after the administration of anticancer agents (Fig. 2), treatment with aprepitant for 5 days is also likely to be a useful option. Furthermore, administration of dexamethasone for a period of up to 5 days is recommended by guidelines established in Japan, and this regimen is also available.

The present study was the first to investigate the usefulness of triple therapy with aprepitant, palonosetron, and dexamethasone for prevention of CINV in Japanese patients with gynecological cancer receiving cisplatin-containing chemotherapy. This triple therapy was ascertained to be effective compared with the current standard therapy using first-generation 5-HT_3 receptor antagonists and dexamethasone. Nevertheless, the preventive effect of triple therapy tended to be weaker in the present study than that reported previously. All of the subjects were female and therefore may have been predisposed to develop CINV, but the reason is unclear. Control of delayed nausea is an important measure against CINV in the gynecology field. Thus, it may be necessary for medical and co-medical staff, including pharmacists and nurses, to conduct further follow-up of patients.

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Conflict of interest The authors have no conflicts of interest to declare.

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The PI3K/mTOR dual inhibitor NVP-BEZ235 reduces the growth of ovarian clear cell carcinoma

TETSURO OISHI[1], HIROAKI ITAMOCHI[1], AKIKO KUDOH[1], MICHIKO NONAKA[1], MISAKI KATO[2], MAYUMI NISHIMURA[2], NAO OUMI[2], SEIYA SATO[1], JUN NANIWA[1], SHINYA SATO[1], MUNEAKI SHIMADA[1], JUNZO KIGAWA[2] and TASUKU HARADA[1]

[1]Department of Obstetrics and Gynecology, Tottori University School of Medicine;
[2]Tottori University Hospital Cancer Center, Yonago, Tottori 683-8504, Japan

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Abstract. Patients with clear cell carcinoma of the ovary (OCCC) have poor survival due to resistance to standard chemotherapy. OCCC has frequent activating mutations of the *PIK3CA* gene. The present study was conducted to clarify the efficacy of the inhibition of the PI3K-AKT-mTOR pathway in OCCC. We used 8 OCCC cell lines and 5 ovarian serous adenocarcinoma (OSAC) cell lines. The mutation status of the *PIK3CA* and *KRAS* genes was examined by direct sequencing. The IC_{50} values of NVP-BEZ235 (BEZ235) and temsirolimus were determined by WST-8 assay. Protein expression levels of PI3K-AKT-mTOR pathway molecules were examined by western blotting. Cell cycle distribution was analyzed by flow cytometry. Annexin V staining was used for detecting apoptosis. We also investigated the effects of BEZ235 on OCCC tumor growth in a nude mouse xenograft model. Four of the 8 OCCC cell lines showed a *PIK3CA* mutation while none of the 5 OSAC cell lines showed a mutation. The IC_{50} values of BEZ235 for the OCCC cell lines were lower than these values for the OSAC cell lines. The IC_{50} value of temsirolimus was higher than BEZ235 in the OCCC cell lines. The *PIK3CA* mutation was more frequently noted in OCCC than OSAC cells, but the sensitivity of these cell lines to BEZ235 or temsirolimus was not related to the mutation status. pHER3 and pAkt proteins were expressed more frequently in OCCC compared with OSAC. However, protein expression levels were distributed widely, and were not related to the sensitivity. Treatment with BEZ235 suppressed expression of pAkt, although treatment with temsirolimus did not. OCCC cells exhibited G_1 phase arrest after treatment with BEZ235 and apoptosis with a higher concentration of the agent. BEZ235 significantly inhibited tumor growth in mice bearing OVISE and TU-OC-1 cell tumors. The present study indicated that the PI3K-AKT-mTOR pathway is a potential target for OCCC, and that BEZ235 warrants investigation as a therapeutic agent.

Introduction

Clear cell carcinoma of the ovary (OCCC) is recognized in the World Health Organization classification of ovarian tumors as a distinct histological entity. Its clinical behavior is distinctly different from other epithelial ovarian cancers (1). OCCC accounts for 3.7-12.1% of epithelial ovarian cancers (2,3). We found that response rates for platinum-based chemotherapy were 11.1% for OCCC and 72.5% for serous adenocarcinoma (SAC), suggesting that OCCC resists conventional platinum-based chemotherapy (4). A novel therapeutic strategy is needed to improve the prognosis of patients with OCCC.

PIK3CA is located at the 3q26.3 locus and encodes the catalytic subunit of the phosphatidylinositol 3-kinase (PI3K), p110α (5). In response to an extracellular signal, the activated p110α phosphorylates PIP2 to generate PIP3. The PIP3 recruits AKT to the plasma membrane, where it is phosphorylated and activated by phosphatidylinositol-dependent kinase 1 (PDK1) and PDK2. Activated AKT can directly activate the mammalian target of rapamycin (mTOR) by phosphorylation at Ser2448. mTOR is a serine/threonine kinase that acts as an effector in the PI3K/Akt pathway. Aberrations of the PI3K pathway are frequently present in many different types of cancer. A number of studies have shown amplification or mutations of the *PIK3CA* gene in ovarian cancers (6-8). AKT and mTOR are also hyperactivated in ovarian cancer (9,10). Additionally, a high frequency of activating mutations of *PIK3CA* has been observed in OCCC (11).

NVP-BEZ235 is an imidazoquinoline derivative that potently and reversibly inhibits class 1 PI3K and mTOR catalytic activity by competing at its ATP-binding site (12). It has been demonstrated to reduce tumor growth in several xenograft models and is currently in clinical trials (12-14). The present study was conducted to clarify the efficacy of NVP-BEZ235 treatment on OCCC.

Correspondence to: Dr Tetsuro Oishi, Department of Obstetrics and Gynecology, Tottori University School of Medicine, 36-1 Nishicho, Yonago, Tottori 683-8504, Japan
E-mail: tetsuro@med.tottori-u.ac.jp

Abbreviations: PI3K, phosphatidylinositol 3-kinase; PDK1, phosphatidylinositol-dependent kinase 1; mTOR, mammalian target of rapamycin; OCCC, ovarian clear cell carcinoma; OSAC, ovarian serous adenocarcinoma

Key words: clear cell carcinoma, PI3K, mTOR, ovarian carcinoma

Materials and methods

Cell lines and cell cultures. Eight human OCCC cell lines (OVISE, SMOV-2, KK, TU-OC-1, OVTOKO, KOC-7c, RMG-I and OVMANA) and five OSAC cell lines (KF, KOC-2s, TU-OS-3, TU-OS-4 and SHIN-3) were used. Cells were obtained as follows: OVISE and OVTOKO from Dr Hiroshi Minaguchi (Yokohama City University, Yokohama, Japan); SMOV-2 from Dr Tomohiro Iida (St. Marianna University, Kawasaki, Japan); KK and KF from Dr Yoshihiro Kikuchi (National Defense Medical College, Tokorozawa, Japan); KOC-7c and KOC-2s from Dr Toru Sugiyama (Kurume University, Kurume, Japan); RMG-I from Dr Shiro Nozawa (Keio University, Tokyo, Japan); and SHIN-3 from Dr Yasuhiko Kiyozuka (Nara Medical University, Kashihara, Japan). TU-OC-1, TU-OS-3, and TU-OS-4 cells were established by our department (15,16). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO_2 at 37˚C.

Mutation screening. Screening for mutations was performed as previously described (17). Genomic DNA was purified from all cell lines using a DNeasy Tissue kit (Qiagen, Valencia, CA, USA). PCR primers used to amplify the sequence of interest (exons 9 and 20 of the *PIK3CA* gene, exons 2 and 3 of the *KRAS* gene) were the same as reported in the literature (18,19). DNA was amplified in reactions of 30 sec at 94˚C; 30 sec at 55˚C; followed by 90 sec at 72˚C for 30 cycles. Then, PCR products were subjected to sequencing using BigDye Terminator v3.1 Cycle Sequencing kit and an Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems Foster City, CA, USA).

Reagents. NVP-BEZ235 and temsirolimus were purchased from LC Laboratories (Woburn, MA, USA). Stock solutions were prepared in dimethyl sulfoxide (DMSO) and stored at -20˚C for the *in vitro* experiments. The drugs were diluted in fresh medium immediately before each experiment. In all the experiments, the final DMSO concentration was <0.1%.

Dose-response studies. The cytotoxicities of NVP-BEZ235 and temsirolimus were assessed by the WST-8 assay using Cell Counting Kit-8 (Dojindo Laboratories, Tabaru, Japan) as previously described (17). Cells ($2\text{-}4x10^3$ cells/80 μl) were seeded into each well of a 96-well tissue culture plate, cultured overnight, and then treated with 20 μl of NVP-BEZ235 or temsirolimus solution at a final concentration of 0.001, 0.01, 0.1, 1 or 10 μM for 72 h. After that, 20 μl of Cell Counting Kit-8 solution was added to each well, and the plates were incubated for another 1-2 h. Absorbance was measured at 450 nm with a microplate reader (iMark Microplate Absorbance Reader). Cell viability was calculated as the percentage of cells killed by the treatment. All experiments were conducted in triplicate. Median inhibitory concentrations were determined from these calculations.

Western blot analysis. Cells were washed twice with ice-cold PBS. Cell pellets were then lysed in a buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA, 50 mM NaF, 2 mM Na_3VO_4 and protease inhibitors (Complete Protease Inhibitor Cocktail Tablets; Roche Diagnostics)] as previously described (17). Protein concentrations were measured against a standardized control using a protein assay kit (Bio-Rad Laboratories). A total of 50 mg protein was separated by electrophoresis on a 5-20% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore). The antibodies were as follows: rabbit anti-erbB3 antibody (C17) (diluted 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-β-actin (AC-40) antibody (1:1,000; Sigma-Aldrich, St. Louis, MO, USA); and anti-phospho-erbB3 (Tyr1289) (21D3) antibody (1:1,000), rabbit anti-AKT antibody (1:1,000), rabbit anti-phospho-AKT (Ser473) antibody (1:500), rabbit anti-mTOR antibody (1:500), rabbit anti-phospho-mTOR (Ser2448) antibody (1:500), rabbit anti-p70S6K antibody (1:500), rabbit anti-phospho-p70S6K (Thr389) antibody (1:500), rabbit anti-4E-BP1 antibody (1:1,000) and rabbit anti-phospho-4E-BP1 (Thr37/49) antibody (1:1,000) (all from Cell Signaling Technology (Danvers, MA, USA). Signals were detected with secondary anti-mouse or anti-rabbit immunoglobulin G antibody coupled with horseradish peroxidase, using an Ez-Capture II chemiluminescent imaging system (ATTO, Tokyo, Japan).

Cell cycle distribution analysis. Cell cycle distribution was analyzed by flow cytometry. Briefly, cells were plated in a 6-well plate, cultured overnight, and then treated with NVP-BEZ235 or left untreated for 48 or 72 h (final concentration of 10 or 100 nM). Floating and adherent cells were fixed overnight in ice-cold 70% ethanol. The cells were then resuspended in PBS containing propidium iodide (PI, 25 μg/ml) supplemented with 0.1% RNase A and incubated at 37˚C for 30 min. DNA content was measured with a FACSCalibur flow cytometer with CellQuest software (Becton-Dickinson, Franklin Lakes, NJ, USA). Cell fit analysis determined the percentage of the cell count in a specific phase of the cell cycle.

Annexin V staining. The Annexin V-FITC Apoptosis Detection kit (BioVision, Mountain View, CA, USA) was used to assess apoptosis as the externalization of phosphatidylserine residues, according to the specifications of the manufacturer. Briefly, cells were suspended in 500 ml of 1X binding buffer. The cells then were stained with 5 ml Annexin V-FITC (fluorescein isothiocyanate) and 5 ml PI (50 mg/ml) for 5 min in the dark at room temperature. Finally, the cells were analyzed with a flow cytometer (FACSCalibur; Becton-Dickinson).

Ovarian cancer xenograft model. OVISE or TU-OC-1 cells in log-phase growth were trypsinized, washed twice with PBS and centrifuged at 250 x g. For subcutaneous tumor development, $4x10^6$ viable cells (in 0.1 ml of PBS) were injected subcutaneously under aseptic conditions into female athymic mice. Seven days after the injection, we confirmed the development of measurable tumors, and then treatment was initiated with NVP-BEZ235 at doses of 25 or 50 mg/kg/day, and continued for 3 weeks. Mice treated with vehicle (10% 1-methyl-2-pyrrolidone-90% polyethylene glycol 300) were used as the control group. All agents were administered by oral gavage. Ten mice were used in each experimental group. The tumor volume was measured with a caliper twice weekly. The body weight of mice was also measured twice weekly.