

# Potent Therapeutic Activity Against Peritoneal Dissemination and Malignant Ascites by the Novel Anti-Folate Receptor Alpha Antibody KHK2805<sup>1</sup>



Munetoshi Ando<sup>\*</sup>, Keiko Nagata<sup>\*</sup>, Kaito Nihira<sup>†</sup>, Yui Suzuki<sup>†</sup>, Yutaka Kanda<sup>†</sup>, Maiko Adachi<sup>†</sup>, Tsuguo Kubota<sup>‡</sup>, Naoya Kameyama<sup>§</sup>, Mariko Nakano<sup>¶</sup>, Hiroshi Ando<sup>#</sup>, Kazuya Yamano<sup>\*</sup>, Toshihiko Ishii<sup>\*</sup>, Ryuichiro Nakai<sup>\*</sup> and Kazuyasu Nakamura<sup>\*</sup>

<sup>\*</sup>Oncology R&D Unit, Kyowa Hakko Kirin Co., Ltd., Japan;

<sup>†</sup>Translational Research Unit, Kyowa Hakko Kirin Co., Ltd.,

Japan; <sup>‡</sup>Strategic Product Portfolio Department, Kyowa

Hakko Kirin Co., Ltd., Japan; <sup>§</sup>Immunology & Allergy R&D

Unit, Kyowa Hakko Kirin Co., Ltd., Japan; <sup>¶</sup>Research

Functions Unit, Kyowa Hakko Kirin Co., Ltd., Japan; <sup>#</sup>Tokyo

Research Park, Kyowa Hakko Kirin Co., Ltd., Japan

## Abstract

Many ovarian cancer patients often show peritoneal metastasis with malignant ascites. However, unmet medical needs remain regarding controlling these symptoms after tumors become resistant to chemotherapies. We developed KHK2805, a novel anti-folate receptor  $\alpha$  (FOLR1) humanized antibody with enhanced antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). The primary aim of the present study was to evaluate whether the anti-tumor activity of KHK2805 was sufficient for therapeutic application against peritoneal dissemination and malignant ascites of platinum-resistant ovarian cancer in preclinical models. Here, both the ADCC and CDC of KHK2805 were evaluated in ovarian cancer cell lines and patient-derived samples. The anti-tumor activity of KHK2805 was evaluated in a SCID mouse model of platinum-resistant peritoneal dissemination. As results, KHK2805 showed specific binding to FOLR1 with high affinity at a novel epitope. KHK2805 exerted potent ADCC and CDC against ovarian cancer cell lines. Furthermore, primary platinum-resistant malignant ascites cells were susceptible to autologous ADCC with KHK2805. Patient-derived sera and malignant ascites induced CDC of KHK2805. KHK2805 significantly reduced the total tumor burden and amount of ascites in SCID mice with peritoneal dissemination and significantly prolonged their survival. In addition, the parental rat antibody strongly stained serous and clear cell-type ovarian tumors by immunohistochemistry. Overall, KHK2805 showed cytotoxicity against both ovarian cancer cell lines and patient-derived cells. These translational study findings suggest that KHK2805 may be promising as a novel therapeutic agent for platinum-resistant ovarian cancer with peritoneal dissemination and malignant ascites.

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## Introduction

Many women with ovarian cancer (OC) present with advanced disease (stage III/IV) and often have peritoneal metastasis with ascites, which is associated with a poor prognosis. Epithelial OC, which comprises 90% of all OCs, most frequently disseminates *via* the transcoelomic route, with about 70% of patients having peritoneal metastases at staging laparotomy [1]. A previous report found that more than one-third of women with OC develop malignant ascites

Address all correspondence to: Munetoshi Ando, Oncology R&D Unit, Kyowa Hakko Kirin Co., Ltd., 1188, Shimotogari, Nagaizumi-cho, Sunto-gun, Shizuoka, 411-8731, Japan.

E-mail: [munetoshi.ando@kyowa-kirin.co.jp](mailto:munetoshi.ando@kyowa-kirin.co.jp)

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during the course of their disease [2]. Malignant ascites of OC is “blood-like” fluid containing cancer cells, mesothelial cells, fibroblasts, immune cells and red blood cells [3]. It can cause debilitating symptoms, as the substantial volume of the fluid can cause pain, early satiety and respiratory compromise [1]. Although peritoneal dissemination and ascites may be reduced by combination chemotherapies, few options are available for treatment after tumors become resistant to chemotherapies. Indeed, despite an initial response to carboplatin (CBDCA) and paclitaxel (PTX) chemotherapies, over 70% of the patients experience disease recurrence [4]. Therefore, unmet medical needs remain regarding controlling peritoneal metastases and malignant ascites of platinum-resistant OC.

Antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) are known to be key functions of therapeutic antibodies, such as rituximab, trastuzumab, and cetuximab [5,6], and next-generation antibodies have been modified to exhibit enhanced ADCC or CDC. For example, among anti-CD20 antibodies, obinutuzumab is the first approved ADCC-enhanced antibody developed by modifying Fc glycosylation [7,8], and ofatumumab exerts increased CDC and is effective for chronic lymphocytic leukemia patients [9,10]. These antibodies have demonstrated that ADCC or CDC enhancement leads to further clinical benefit, especially for patients with hematological cancer. Natsume et al. found that the engineered constant Fc region as human IgG1/IgG3 chimeric isotypes with nonfucosylated oligosaccharides (113F[-f]) possess ADCC and CDC dual-enhanced cytotoxic functions [11]. This approach is expected to be useful for generating potent therapeutic antibodies, since complement-enhanced ADCC has also been suggested as a synergistic effect of ADCC and CDC [12,13]. However, few studies have explored generating antibodies with such dual enhancement for application to cancer treatment.

Folate receptor  $\alpha$  (FOLR1) is a glycosylphosphatidylinositol anchored cell surface protein known to be overexpressed, especially in epithelial OCs [14–17]. The FOLR1 expression reportedly remains unchanged in epithelial OC after chemotherapy and surgery [18,19]. Furthermore, its expression is normally limited at the luminal surface of nonmalignant epithelial cells and is therefore generally not accessible by molecules in the blood stream, suggesting that targeting FOLR1 by cytotoxic antibodies may be a viable therapeutic approach for epithelial OC [15–17,20]. Indeed, farletuzumab, a clinically developed anti-FOLR1 humanized antibody with ADCC and CDC [21], was suggested to improve the duration of a second response to chemotherapy in a phase II study for patients with platinum-sensitive OC [22]. In addition, farletuzumab had an acceptable safety and pharmacokinetic profile both as a single agent and in combination with chemotherapies [22,23]. However, in a subsequent double-blind randomized phase III study in platinum-sensitive OC, farletuzumab did not meet the criteria for a progression-free survival as the study's primary endpoint, except for in patients with a relatively low CA125 level [23]. These results suggest that while anti-FOLR1 antibodies with conventional ADCC and CDC may have the potential to improve the outcomes for the patient with OC, the effects may be limited.

We therefore hypothesized that a next-generation anti-FOLR1 antibody with enhanced ADCC and CDC would be able to exert more potent cancer depletion activity than conventional antibodies, especially against “blood-like” malignant ascites, thereby improving the prognosis of patients with platinum-resistant OC. In this context, KHK2805 was generated as a novel anti-FOLR1 humanized

antibody with a high binding affinity, novel epitope, and ADCC/CDC dual-enhanced activities by 113F(-f)-engineering. We herein report that KHK2805 showed high cytotoxicity not only against OC cell lines but also in patient-derived samples. KHK2805 had a significant therapeutic effect as monotherapy in a SCID mouse model of platinum-resistant peritoneal dissemination and malignant ascites. These data indicate that KHK2805 may be a novel therapeutic agent for patients with peritoneal dissemination and malignant ascites of platinum-resistant OC.

## Materials and Methods

### OC Cell Lines

IGROV1 and OVMANA cells were obtained from the National Cancer Institute (NCI) and Japanese Collection of Research Bioresources, respectively. SKOV3 (HTB-77) and OVCAR3 (HTB-161) cells were purchased from American Type Culture Collection.

### Antibodies

KM8188 was produced by CHO/DG44 cells and purified by a protein-A column from the culture supernatant. The amino acid sequences of heavy- and light-chain KM8188 were quoted from International Publication Number WO2012/054654A2, which specifies the MORAb-003/farletuzumab sequence, and ligated into an expression vector. CHO/DG44 cells were obtained from Dr. Lawrence Chasin and Gail Urlaub Chasin (Columbia University, New York City, NY, USA) [24]. ADCC/CDC dual-enhanced KM8188 (KM8188/113F[-f], Supplemental Figure S1) was produced through 113F(-f)-engineering by FUT8-knockout CHO/DG44 cells [11,25]. KHK2805, a humanized and CDR-modified antibody derived from anti-FOLR1 rat monoclonal antibody KM4193 with 113F(-f)-engineering, and a defucosylated anti-dinitrophenyl (DNP) human IgG1 were produced as described above.

### Recombinant Proteins

cDNAs of FOLR1 (NM\_016725.2), FOLR2 (NM\_000803.2) and FOLR3 (BC148785.1) were purchased from Origene (SC122853, SC119666) and Open Biosystems (100015838). Polyhistidine-tag (six-histidine residues) or human IgG1 Fc-tag was added by polymerase chain reaction (PCR) at the C-terminus of FOLR1 (1 to 233 amino acids) (FOLR1-His or FOLR1-Fc, respectively). FOLR1-His or FOLR1-Fc was produced by CHO/DG44 cells and purified from the culture supernatant using Ni-NTA Agarose (Thermo Fisher Scientific, Waltham, MA, USA) or ProSep-vA High Capacity (MILLIPORE, Darmstadt, Germany), respectively. A similar method was applied to obtain FOLR2-Fc (1 to 227 amino acids of FOLR2 with the Fc-tag) or FOLR3-Fc (1 to 243 amino acids of FOLR3 with the Fc-tag) (Supplemental Figure S2).

### Enzyme-Linked Immunosorbent Assay (ELISA)

FOLR1-Fc, FOLR2-Fc or FOLR3-Fc recombinant protein (2  $\mu$ g/mL each) was coated onto an ELISA plate. After blocking in 1% BSA-PBS, KHK2805 was reacted at room temperature for 1 h. Goat Anti-Human Kappa-Horseradish Peroxidase (HRP; SouthernBiotech, Birmingham, AL, USA) and the following SureBlue Reserve TMB Microwell Peroxidase Substrate (KPL, Gaithersburg, MD, USA) was used for the visualization. After adding a stop solution, the absorbance at 450 nm (A450 nm) was measured.

### Flow Cytometry

The FOLR1 expression was evaluated by flow cytometry (FCM, FACSVerser; BD Biosciences, San Jose, CA, USA). OC cells were prepared for  $2 \times 10^5$  cells in FCM buffer (PBS [Thermo Fisher Scientific] supplemented with 5% [vol] heat-inactivated FCS [Thermo Fisher Scientific], 1 mmol/L EDTA [Thermo Fisher Scientific]). KHK2805, KM8188 or KM8188/113F(-f) (10  $\mu$ g/mL final concentration) was used for staining at 4 °C for 1 h. After staining with PE-labeled anti-human IgG (BD Biosciences), the PE intensity in the live-cell-population was measured by FCM.

### Surface Plasmon Resonance (SPR)

Biacore T100 (GE Healthcare, Buckinghamshire, England) was used to evaluate the binding affinity to FOLR1-His. A Human Antibody Capture Kit (GE Healthcare) was immobilized onto a Series S CM5 chip (GE Healthcare) by amine coupling. Subsequently, KHK2805 or KM8188 was captured on the chip. FOLR1-His prepared for 5-step dilution (12.3, 37, 111, 333 and 1000 ng/mL) by HBS-EP+ buffer (GE Healthcare) was then applied as an analyte. Kinetic constants ( $k_a$ ,  $k_d$ ,  $KD$ ) were calculated by a 1:1 binding model in single-cycle kinetics using the Biacore T100 Evaluation software program (GE Healthcare).

### Epitope Evaluation

Rat/human-chimeric FOLR1-His proteins were produced and purified. Rat2-FOLR1-His was prepared by substituting the amino acid sequence at 55 to 62 (EQCRPWVK) of human FOLR1 to the sequence at 53 to 60 (DQCSPWKT) in rat FOLR1 (NM\_133527), and a His-tag was added to obtain FOLR1-His. Similarly, Rat4-FOLR1-His was prepared by substituting the sequence at 91 to 95 (EMAPA) of human FOLR1 to the sequence at 89 to 93 (TMTPE) in rat FOLR1.

For the binding analysis, FOLR1-His, Rat2-FOLR1-His and Rat4-FOLR1-His were prepared at 10  $\mu$ g/mL and coated onto an ELISA plate. After blocking in 1% BSA-PBS, the anti-FOLR1 antibodies were reacted at room temperature for 1 h. Goat anti-human IgG(H + L)-Horseradish Peroxidase (HRP) (American Qualex, San Clemente, CA, USA) or Rabbit anti-mouse immunoglobulin-HRP (Agilent Technologies, Santa Clara, CA, USA) was used as the secondary antibody for anti-FOLR1 humanized or mouse antibody, respectively. ABTS (2,2-azinobis [3-ethylbenzothiazole-6-sulfonic acid] ammonium; Wako, Tokyo, Japan) substrate solution (1 mmol/L ABTS, 0.1 mol/L citrate buffer [pH 4.2], 0.1%  $H_2O_2$ ) was used for the visualization. After adding a stop solution, A415 nm was measured. The mouse anti-FOLR1 monoclonal antibody MOv18 (ALEXIS Biochemicals, San Diego, CA, USA) was used as a control.

### In Vitro ADCC Assay (Healthy Donor PBMCs)

Frozen human peripheral blood mononuclear cells (PBMCs) were purchased from Precision Bioservices (Frederick, MD, USA). The PBMCs were thawed and washed with medium containing DNase (Roche Diagnostics, Mannheim, Germany). After overnight incubation, the PBMCs were used as effector cells. OC cells, as target cells, were labeled with approximately 3.7 MBq of  $Na_2^{51}CrO_4$  (PerkinElmer, Waltham, MA, USA) for 1 h at 37 °C. These target cells ( $1.0 \times 10^4$  cells) were incubated with the anti-FOLR1 antibodies and PBMCs at an E:T ratio of 25:1 for 4 h at 37 °C. After incubation, each supernatant was transferred onto a LumaPlate (PerkinElmer) and dried sufficiently. The  $^{51}Cr$  radioactivity was measured using a microplate scintillation counter (TopCount NXT, PerkinElmer).

After subtracting the radioactivity of the medium background from the radioactivity of each sample, the cytotoxicity was calculated using the following formula: % cytotoxicity =  $100 \times (S - T_{spo}) / (Total - T_{spo})$ , where S is the radioactivity of the experimental sample well,  $T_{spo}$  is the mean spontaneously released radioactivity of the target cells in the absence of antibody and effector cells (target cells incubated with medium alone), and Total is the mean radioactivity of the target cell lysis (target cells incubated with detergent).

### Autologous ADCC Assay (Patient-Derived Ascites)

OC ascites-derived frozen cells, which were indicated as platinum-resistant (Supplemental Table S1), were purchased from Molecular Response (San Diego, CA, USA). These cells were thawed, and  $2 \times 10^6$  cells/0.5 mL was seeded into a 24-well plate with the antibody. After 24-h incubation, FOLR1-positive (FOLR1[+]) cancer, NK or the T cell population was detected by FCM. The FOLR1(+) cancer cells were identified as 7-AAD-negative (7-AAD[-]) FS(high)CD45(-)CD14(-)FOLR1(+) cells. Both NK and T cells were defined as CD3(-)CD16(+) and CD3(+)CD16(-) cells in a CD45(+)CD14(-) population, respectively. PE-conjugated anti-FOLR1 antibody (Clone #548908, Cat. No. FAB5646P), which does not compete with either KHK2805 or KM8188 (data not shown), was purchased from R&D Systems (Minneapolis, MN, USA). The other FCM reagents, including mouse control IgGs, were purchased from BD Biosciences. The number of FOLR1(+) cancer cells was normalized by the number of ascites lymphocytes (gated in FS/SS as 7-AAD[-]CD45[+]CD14[-] cell population) in each sample.

### In Vitro CDC Assay (Healthy Donor Sera)

OC cells ( $1 \times 10^4$ ) labeled with  $Na_2^{51}CrO_4$ , as described in the *in vitro* ADCC assay method, were incubated with the anti-FOLR1 antibodies and complement sera human (Sigma-Aldrich, Saint Louis, MO, USA) at a final dilution of 1/6. After 2 h incubation, the  $^{51}Cr$  radioactivity was measured as described in the *in vitro* ADCC assay method. After subtracting the radioactivity of the serum background from the radioactivity of each sample, the cytotoxicity was calculated using the following formula: % cytotoxicity =  $100 \times (S - T_{spo}) / (Total - T_{spo})$ , where S is the radioactivity of the experimental sample well,  $T_{spo}$  is the mean spontaneously released radioactivity of the target cells in the absence of antibody and effector cells (target cells incubated with medium alone), and Total is the mean radioactivity of the target cell lysis (target cells incubated with detergent).

### In Vitro CDC Assay (Patient-Derived Serum or Ascites)

Ten serum samples and two supernatants of malignant ascites from patients with OC were purchased from ProteoGenex (Culver City, CA, USA) and Tissue Solutions (Glasgow, UK), respectively. IGROV1 cells ( $1 \times 10^4$ ) were incubated with the anti-FOLR1 antibodies and serum or malignant ascites at a final dilution of 1/6 or 1/3. After 2-h incubation, CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) was used to quantify the cell viability by measuring the A490 nm. After subtracted the average absorbance background, the % cytotoxicity was calculated as  $100 \times (T - S) / T$ , where T is the mean A490 nm of serum or ascites and target cells without antibody and S is the A490 nm of the experimental sample well.

### Peritoneal Dissemination Model

SCID mice (C.B-17/Icr-scld/scld Jcl, female, 6 weeks old) were purchased from CLEA Japan. IGROV1 cells (5 or  $10 \times 10^6$  cells/



0.1 mL/mouse) were intraperitoneally inoculated into the mice. Five days after the cell inoculation, the intravenous administration of KHK2805 was started (defined as day 0). The intraperitoneal tumor burden and amount of ascites were measured on Day 24 or 21 (Figure 5, A or B). In the survival model, mice in a moribund state (hypoactivity and/or abdominal distention) were euthanized for ethical reasons. The day when the mice were euthanized was designated as the date of death. The administration of KHK2805 was continued until the mice died.

All of the animal studies were performed in accordance with Standards for Proper Conduct of Animal Experiments at Kyowa Hakko Kirin Co., Ltd., which is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International, under the approval of the company's Institutional Animal Care and Use Committee.

### Immunohistochemistry

Mouse xenograft tissue of SKOV3 and SNU16 cells (human gastric cancer) was used as positive and negative control tissue for FOLR1 staining, respectively. A formalin-fixed and paraffin-embedded (FFPE) human OC tissue array was purchased from US Biomax (Rockville, MD, USA). For immunohistochemistry, KM4193 (a monoclonal rat anti-human FOLR1 antibody) was utilized as the primary antibody. Specimens were deparaffinized, rehydrated, heated for target retrieval and then processed for immunohistochemical (IHC) staining. The IHC staining protocol included the sequential application of a peroxidase-blocking reagent, the primary antibody or negative control antibody, a rabbit anti-rat IgG linker (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and an HRP-conjugated anti-rabbit polymer reagent (Nichirei Biosciences, Tokyo, Japan). Immunoreactivity was visualized with a 3,3'-diaminobenzidine colorimetric reaction. All of the specimens were counterstained with hematoxylin and examined microscopically. IHC specificity of the protocol was confirmed using FOLR1-His (Supplemental Figure S3).

### Statistical Analyses

The tumor burden and amount of ascites were assessed using the Kruskal-Wallis test + the Steel-Dwass test or Steel test. The mouse survival was analyzed by the Kaplan-Meier method, and the survival curves were compared using the log-rank test with Bonferroni correction. The SAS software program (Release 9.4, SAS Institute, Cary, NC, USA) was used for the statistical analyses. In our study,  $P < .05$  was considered significant. Graphs were described using the Graph-Pad Prism software program (ver. 4.03; La Jolla, CA, USA).

## Results

### Binding Characterization of KHK2805

To evaluate the binding specificity of KHK2805, recombinant FOLR1 and its family receptors, FOLR2 and FOLR3 (also known as FR $\beta$  and FR $\gamma$ , respectively), were prepared (FOLR1-, FOLR2- and FOLR3-Fc, respectively). As shown in Figure 1A, KHK2805 bound to FOLR1-Fc but did not bind to either FOLR2- or FOLR3-Fc. This result demonstrates that KHK2805 bound specifically to FOLR1 without cross reactivity to FOLR2 or FOLR3.

We then confirmed whether KHK2805 binds to cancer cells. Four OC cell lines (IGROV1, OVMANA, SKOV3 and OVCAR3) were used for the FCM analysis. KHK2805 bound to these OC cells as strongly as KM8188 (Figure 1B), which has an equivalent amino acid

sequence to farletuzumab as the positive control for existing anti-FOLR1 humanized antibody. In addition, KM8188 with the 113F(-f)-engineering (KM8188/113F[-f]) showed equivalent binding activity to both KHK2805 and KM8188, indicating that the 113F(-f)-engineering does not affect the binding activity.

KHK2805 was then subjected to SPR to clarify the binding affinity against recombinant FOLR1 (FOLR1-His). As shown in Figure 1C, the binding responses of both KHK2805 and KM8188 were observed. The kinetics calculation revealed that the mean  $K_D$  of 5 lots of KHK2805 was 0.647 nmol/L in a 1:1 binding model, whereas the  $K_D$  of KM8188 was 31.4 nmol/L (Figure 1C). This result indicates that KHK2805 has greater affinity for FOLR1 than the existing anti-FOLR1 humanized antibody.

### Epitope of KHK2805

KHK2805 binding disappeared on an ELISA when a chimeric recombinant protein, which substituted the N-terminal of FOLR1 for a FOLR2 sequence, was used (data not shown). This result indicates that the epitope of KHK2805 is located at the N-terminal of FOLR1. Since the parental monoclonal antibody KM4193 was established in a rat, we hypothesized that KHK2805 recognizes amino acid differences between human and rat FOLR1 in the N-terminal. In this context, the differences were classified into three regions, and three rat/human chimeric FOLR1 recombinant proteins (Rat2-FOLR1, Rat3-FOLR1 and Rat4-FOLR1) were constructed (Figure 2A). Although Rat3-FOLR1 could not be prepared in our system (reason unknown, data not shown), Rat2-FOLR1 and Rat4-FOLR1 were prepared and used for ELISA.

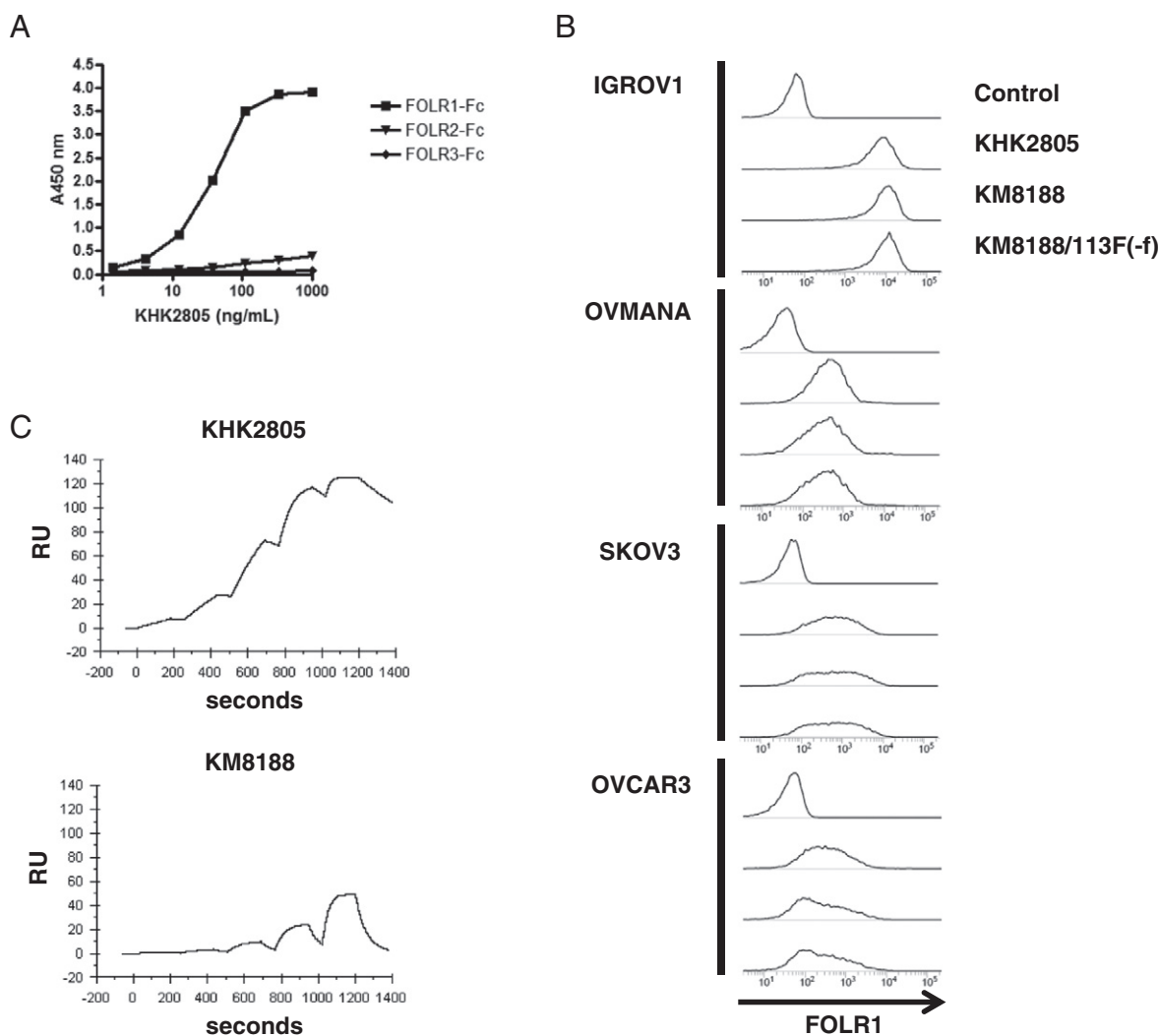
The binding of KHK2805 was completely abolished when Rat2-FOLR1 was used. In contrast, KM8188 bound to Rat2-FOLR1, although the binding was abolished when Rat4-FOLR1 was the target (Figure 2B). In addition, MOv18 bound to all of the chimeric recombinant proteins. These results demonstrate that the critical epitope of KHK2805 is located at positions 55 to 62 (EQCRPWRK) of FOLR1 and differ from the binding site of both KM8188 and MOv18. Regarding the FOLR1 structure, the epitope of KHK2805 is located at the opposite side of the folic acid binding-pocket on FOLR1 [26] (Figure 2C).

### KHK2805-Mediated ADCC Against OC Cells In Vitro

The ADCC of KHK2805, KM8188 and KM8188/113F(-f) against OC cells was evaluated using healthy volunteers' PBMCs as the effector cells. As shown in Figure 1B, IGROV1, OVMANA, SKOV3 and OVCAR3 cells had a sequentially decreasing expression of FOLR1 and were used as the target cells to clarify whether the FOLR1 expression affects the ADCC. KHK2805 showed the highest ADCC against all tested OC cells among the three anti-FOLR1 humanized antibodies. In addition, both KHK2805 and KM8188/113F(-f) showed comparable or better ADCC than KM8188 against IGROV1 cells (Figure 3A). These data suggest that the ADCC of KHK2805 is higher than that of KM8188, regardless of the FOLR1 expression on target OC cells. In addition, KHK2805 was able to induce higher ADCC against OC cells with middle to low expression of FOLR1 than KM8188/113F(-f), indicating that potent ADCC of KHK2805 is not wholly reliant on 113F(-f)-engineering but also its unique F(ab')<sub>2</sub> activity.

### Ex Vivo Autologous ADCC of KHK2805 in Malignant Ascites Cells

Platinum-resistant cells (Supplemental Table S1) isolated from malignant ascites of six patients with OC were used for the autologous



**Figure 1.** Binding characterization of KHK2805. (A) Recombinant proteins (2  $\mu$ g/mL, FOLR1-Fc, FOLR2-Fc or FOLR3-Fc) were coated onto an ELISA plate. Various concentrations of KHK2805 were reacted for 1 h. Anti-Human Kappa-HRP was used as the secondary antibody, followed by TMB substrate reaction. After stopping the reaction, the A450 nm was measured. Each point represents the mean  $\pm$  SD of triplicate experiments. (B) The binding activity of anti-FOLR1 antibodies (KHK2805, KM8188 and KM8188/113F[-f]) against four OC cell lines was evaluated by FCM. The cells were treated with anti-FOLR1 antibodies (10  $\mu$ g/mL) at 4  $^{\circ}$ C for 1 h. PE-labeled anti-human IgG was used as the secondary antibody. Anti-DNP human IgG1 was used as the control. (C) SPR was used to measure the binding affinity of anti-FOLR1 antibodies against FOLR1-His. Anti-human IgG antibody was immobilized onto a sensor chip, and then KHK2805 or KM8188 was captured. Five concentrations of FOLR1-His (12.3, 37, 111, 333 and 1000 ng/mL in order) were applied as analytes. The affinity was calculated by single-cycle kinetics in a 1:1 binding model. Representative data of five lots of KHK2805 are shown. RU: response units.

depletion assay. These cells were incubated with KHK2805 or KM8188 for 24 h. In sample FZ32, the assay was conducted only at an antibody concentration of 100 ng/mL, because of the limited number of sample cells. After the incubation, the numbers of FOLR1(+) cancer, NK and T cells (Supplemental Table S2) were measured by FCM. As shown in Figure 3B, both KHK2805 and KM8188 showed autologous depletion activity against FOLR1(+) cancer cells. Furthermore, KHK2805 showed higher autologous depletion activity than KM8188.

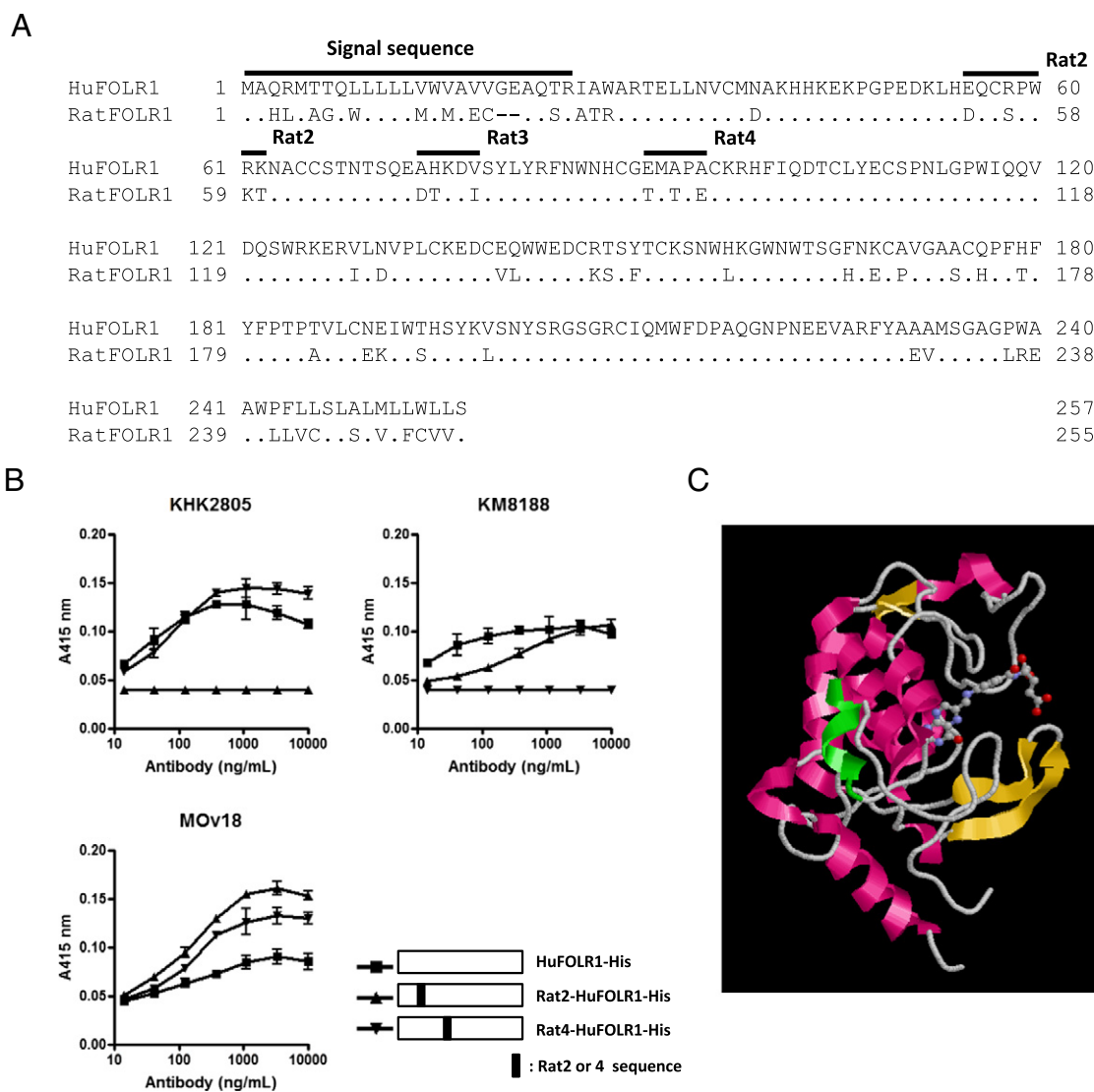
#### Induction of CDC In Vitro by KHK2805 with Healthy Donor's Sera

We next assessed whether the three anti-FOLR1 humanized antibodies induce CDC against IGROV1, OVMANA, SKOV3 or OVCAR3 cells with human complement sera *in vitro*. As shown in Figure 4A, CDC was observed when IGROV1 or OVMANA cells were used as target cells. In contrast, no CDC was detected when

SKOV3 or OVCAR3 cells were used, indicating that SKOV3 and OVCAR3 cells are CDC-resistant cells. Therefore, the expression of complement regulatory proteins (CRPs: CD46, CD55 and CD59) on these cells was evaluated by FCM. Although the expression of CD46, CD55 and CD59 on all four OC cells was observed, there were no clear differences in the expression patterns of these CRPs between CDC-sensitive (IGROV1 and OVMANA) and CDC-resistant (SKOV3 and OVCAR3) cells (Supplemental Figure S4). Regarding the CDC efficacy, KHK2805 and KM8188/113F(-f) showed comparable or higher CDC against CDC-sensitive cells than KM8188.

#### Induction of CDC In Vitro by KHK2805 with Patient-Derived Serum or Malignant Ascites

Ten serum samples from patients with OC (6 serous, 2 mucinous and 2 clear cell-type) were evaluated for their CDC against IGROV1



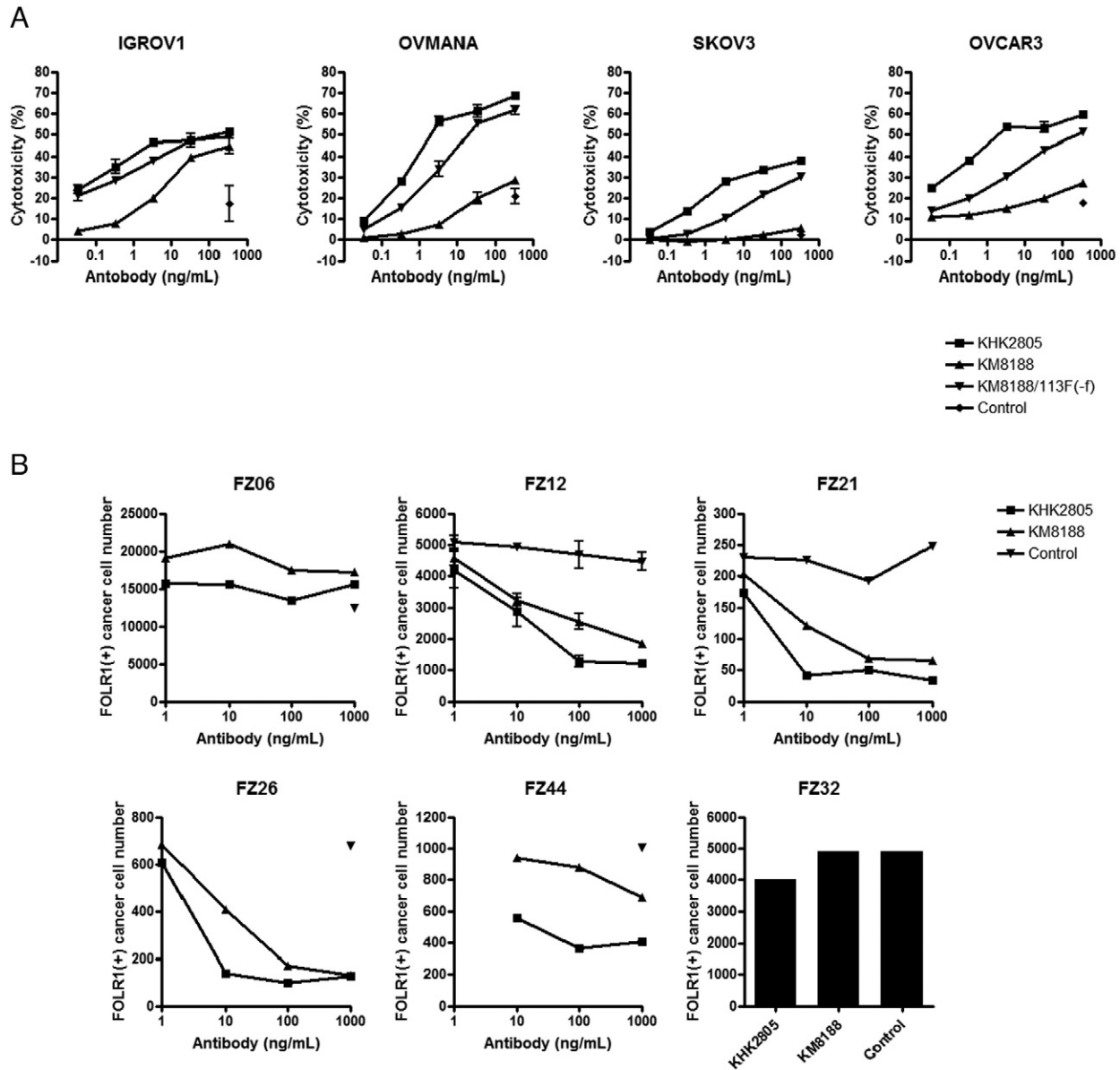
**Figure 2.** The identification of the KHK2805 epitope. (A) The amino acid differences between the N-terminal of human and rat FOLR1 (HuFOLR1 and RatFOLR1) were classified as Rat2, Rat3 and Rat4. (B) The binding activity of anti-FOLR1 antibodies (KHK2805, KM8188 and MOV18) against HuFOLR1, Rat2-FOLR1 or Rat4-FOLR1 was measured by ELISA. HuFOLR1-His and Rat2- or Rat4-HuFOLR1-His (a chimeric recombinant human FOLR1 substituted with a Rat2 or Rat4 sequence, respectively) were coated onto an ELISA plate at 10  $\mu$ g/mL. Various concentrations of anti-FOLR1 antibodies were then reacted for 1 h. Goat anti-human IgG-HRP or Rabbit anti-mouse IgG-HRP was used as a secondary antibody for anti-FOLR1 humanized (KHK2805, KM8188) or mouse antibody (MOV18), respectively. After ABTS substrate reaction, the A450 nm was measured. Each point represents the mean  $\pm$  SD of triplicate experiments. (C) The Rat2 region (green) is presented as the epitope of KHK2805 on the crystal structure of FOLR1 (26). Folic acid is presented as a ball and stick model. The structure was described using the RasMol software program (Windows Version 2.7.5.2).

cells. All of the tested sera induced higher CDC of KHK2805 than that of KM8188, regardless of the type of derived OC (Figure 4B). We next used two supernatant samples of malignant ascites from patients with serous OC. One ascites sample (donor 2) induced higher CDC of KHK2805 against IGROV1 cells than that of KM8188, although the other sample did not induce CDC for either antibody (Figure 4C).

#### Significant Reduction in the Intraperitoneal Tumor Burden and Amount of Malignant Ascites, and the Extension of the Survival of SCID Mice with Peritoneal Dissemination by KHK2805

To understand the *in vivo* efficacy of KHK2805, a peritoneal dissemination model was established by IGROV1 cell-inoculation

into SCID mice. KHK2805 treatment (10 mg/kg twice a week) significantly reduced the intraperitoneal tumor burden and amount of malignant ascites in these mice (Figure 5A). Furthermore, KHK2805 at lower doses (1 mg/kg per week) also showed significant suppression of both the intraperitoneal tumor burden and amount of malignant ascites (Figure 5B). KHK2805 at 0.1 mg/kg per week treatment also showed such suppression, but these effects were not statistically significant compared with the control. Weekly or twice weekly treatment of 10 mg/kg KHK2805 significantly prolonged the survival of SCID mice with peritoneal dissemination compared with the vehicle control (Figure 5C). The median survival of mice treated with vehicle control, 10 mg/kg per week KHK2805 and 10 mg/kg twice a week KHK2805 was 31.0 days (95% confidence interval [CI], 29 to 33 days), 56.5 days (95% CI, 45 to 62 days) and 55.5 days (95% CI,



**Figure 3.** The *in vitro* or *ex vivo* ADCC of KHK2805. (A) The ADCC of anti-FOLR1 humanized antibodies was evaluated. Four OC cell lines and PBMCs were used as the target and effector cells, respectively. <sup>51</sup>Cr-labeled target cells ( $1.0 \times 10^4$  cells) were incubated with various concentrations of anti-FOLR1 antibodies (KHK2805, KM8188 and KM8188/113F[-f]) and PBMCs at an E:T ratio of 25:1 for 4 h at 37 °C. The radioactivity of each supernatant was measured as the cytotoxicity. Representative data using single-donor-derived PBMCs as the effector cells for each OC cells are shown. Each point represents the mean  $\pm$  SD of triplicate experiments. (B) Primary cells from malignant ascites of patients with OC (FZ06, FZ12, FZ21, FZ26, FZ44, and FZ32) were seeded onto a 24-well assay plate ( $2 \times 10^6$  cells/well) with various concentrations of KHK2805 or KM8188. After 24-h incubation, FOLR1(+) cancer cells were counted as 7-AAD(-) FS(high)CD45(-)CD14(-)FOLR1(+) cells by FCM. FZ12: Each point represents the mean  $\pm$  SD of triplicate experiments. FZ32: The concentration of each antibody was 100 ng/mL. Anti-DNP human IgG1 was used as the control.

44 to 57 days), respectively. In addition, the median survival of CBDCA-, CBDCA + PTX- and PTX-treated mice was 31.0 days (95% CI, 28 to 34 days), 40.0 days (95% CI, 38 to 44 days) and 42.0 days (95% CI, 38 to 43 days), respectively (Figure 5D). CBDCA did not show any treatment effects on the survival in either monotherapy or combination therapy with PTX compared with the control or PTX monotherapy. Thus, this model is indicated as platinum-resistant.

**Generation of an IHC Method for Companion Diagnostics**

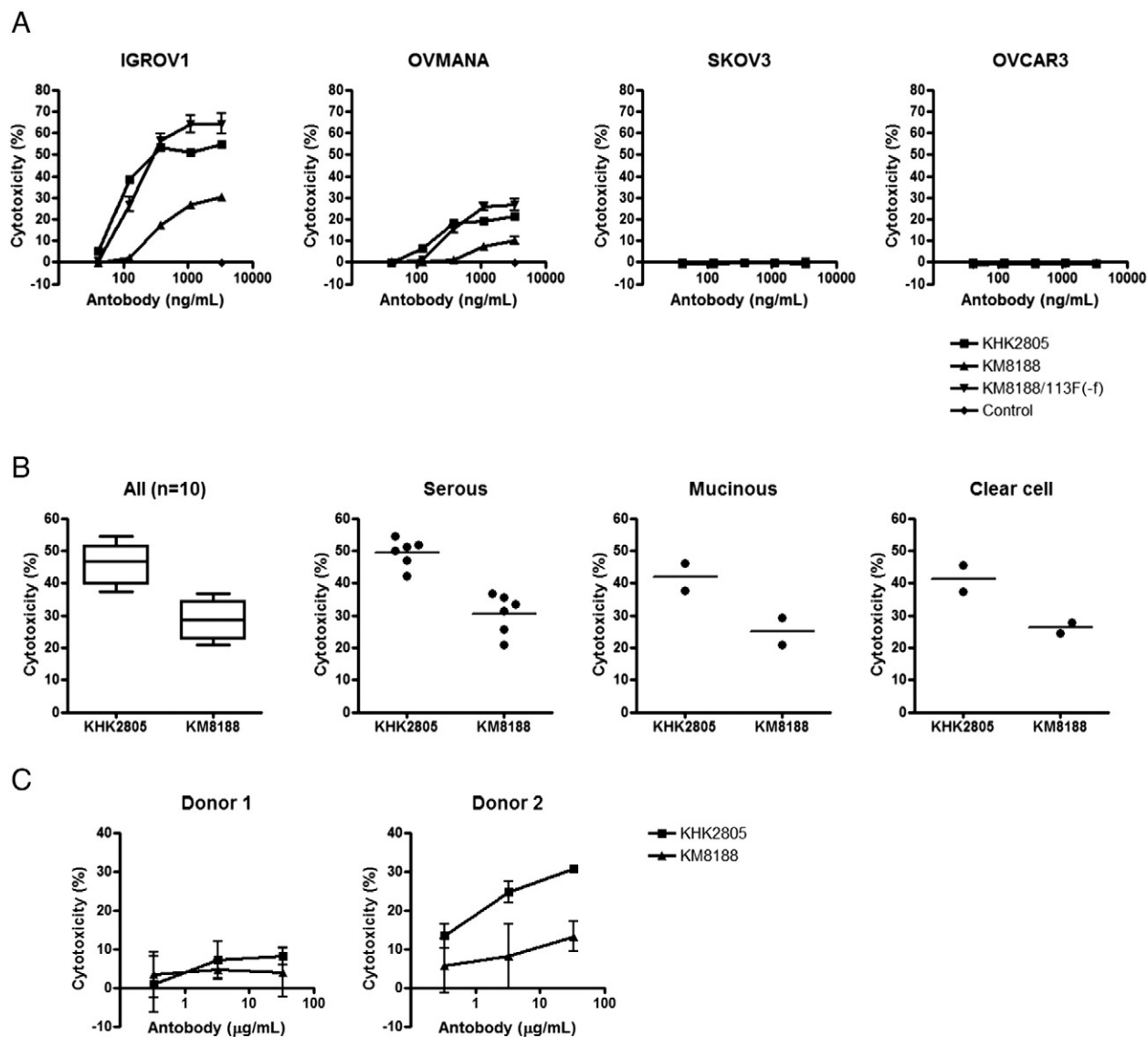
An IHC protocol was established using KM4193, the parental rat monoclonal antibody of KHK2805. Staining was observed on mouse

xenograft tissue of SKOV3 cells (Figure 6A), which was confirmed to be FOLR1-positive by FCM (Figure 1B). FFPE OC patient samples were then stained using this protocol. The immunoreactivity of KM4193 was detected in the membrane and cytoplasm of cancer cells but not in the surrounding stromal cells. Serous and clear cell-type OC specimens were strongly positive, and mucinous type was weakly positive for KM4193 (Figure 6B).

**Discussion**

We herein report a novel anti-FOLR1 antibody KHK2805 that has potent activity against peritoneal dissemination and malignant ascites





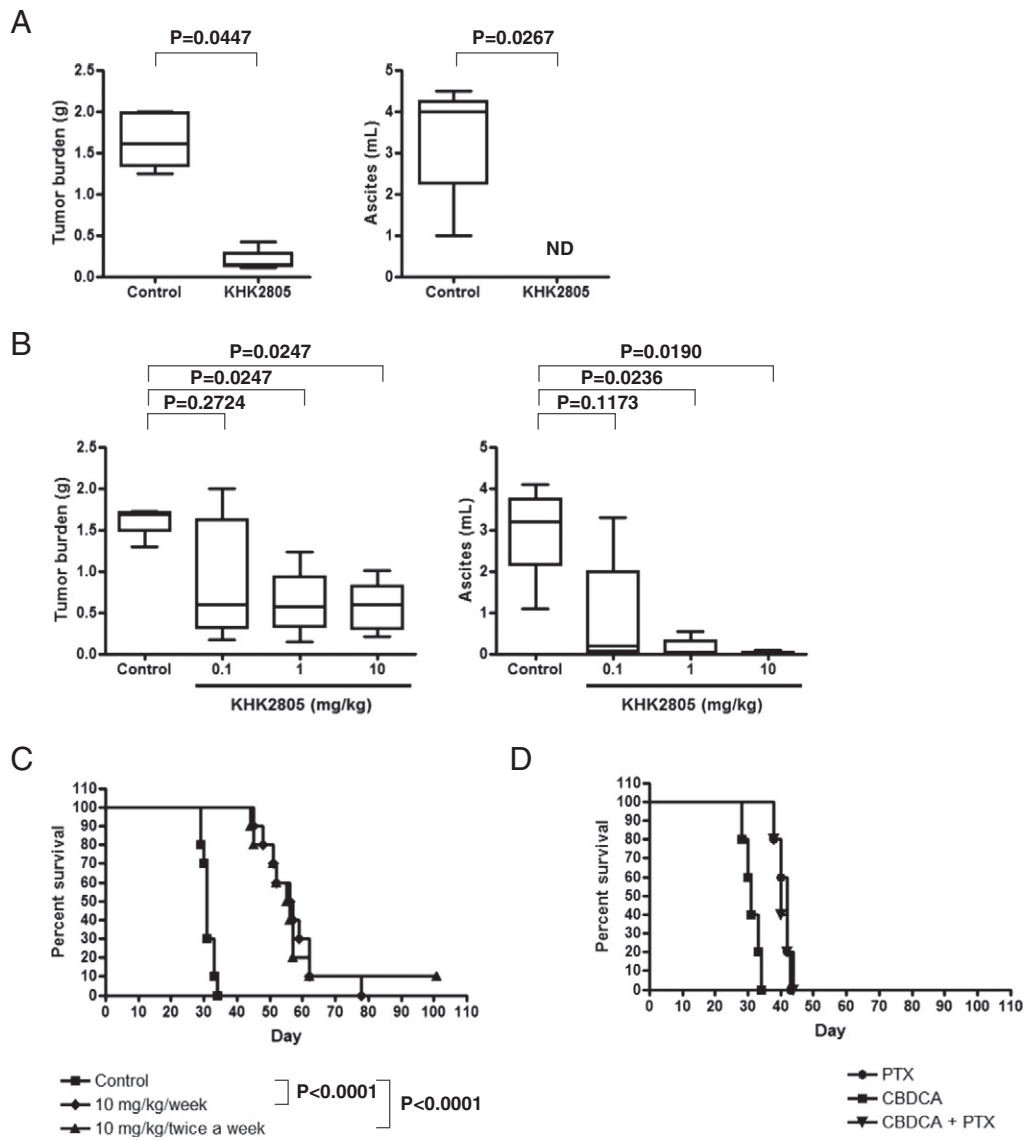
**Figure 4.** *In vitro* CDC of KHK2805. (A) The CDC of anti-FOLR1 antibodies was evaluated. Four OC cell lines and human sera were used as the target cells and the source of complement, respectively.  $^{51}\text{Cr}$ -labeled target cells ( $1.0 \times 10^4$  cells) were incubated with various concentrations of anti-FOLR1 antibodies (KHK2805, KM8188 and KM8188/113F[–f]) and human sera at a final dilution of 1/6 for 2 h at 37 °C. The radioactivity of each supernatant was measured as the cytotoxicity. Each point represents the mean  $\pm$  SD of triplicate experiments. Anti-DNP human IgG1 (3333 ng/mL) was used as the control. (B) Ten serum samples from patients with OC (6 from serous type, 2 from mucinous type and 2 from clear cell type) were used at a final dilution of 1/6 in the CDC assay against IGROV1 cells ( $1.0 \times 10^4$  cells) with 10  $\mu\text{g/mL}$  KHK2805 or KM8188. After incubation for 2 h at 37 °C, a cell viability assay was conducted. The CDC of the 10 serum samples is summarized as a box-and-whisker plot (left panel). The CDC using serum of each type of OC are represented as a scatter plot with a bar indicating the mean. Each point represents the mean of triplicate experiments. (C) Two supernatant samples of malignant ascites from patients with serous type OC were used at a final dilution of 1/3 in the assay, as described above. Each point represents the mean  $\pm$  SD of triplicate experiments.

in preclinical models. KHK2805 was established as an ADCC and CDC dual-enhanced antibody against FOLR1-expressing cancer cells for application in cancer therapy. The activities of KHK2805 fall into two categories: Fab- and Fc-mediated mechanisms. The Fab region of KHK2805 was established by humanization and CDR-optimization of KM4193 obtained by immunization of FOLR1-Fc to SD rats (Japan SLC, Shizuoka, Japan). In addition to humanization by a conventional CDR-grafting method, the cysteine located at CDR3 of the KM4193 heavy-chain was converted to threonine. This CDR substitution increased the affinity and CDC compared with the

antibody without CDR-optimization (Supplemental Figure S5). In the Fc region, the antibody was subjected to defucosylated IgG1/IgG3 chimerization (previously reported as 113F[–f]-engineering [11]) for the further enhancement of both the ADCC and CDC.

Such antibody engineering for both the Fab- and Fc-regions has made KHK2805 a potent cytotoxic anti-FOLR1 antibody. Indeed, KHK2805 showed higher ADCC against OC cells than both KM8188 and KM8188/113F(–f) (Figure 3A). In contrast, KHK2805 and KM8188/113F(–f) showed comparable CDC (Figure 4A). These results indicate that 1) the ADCC of KHK2805 exceeds that of a



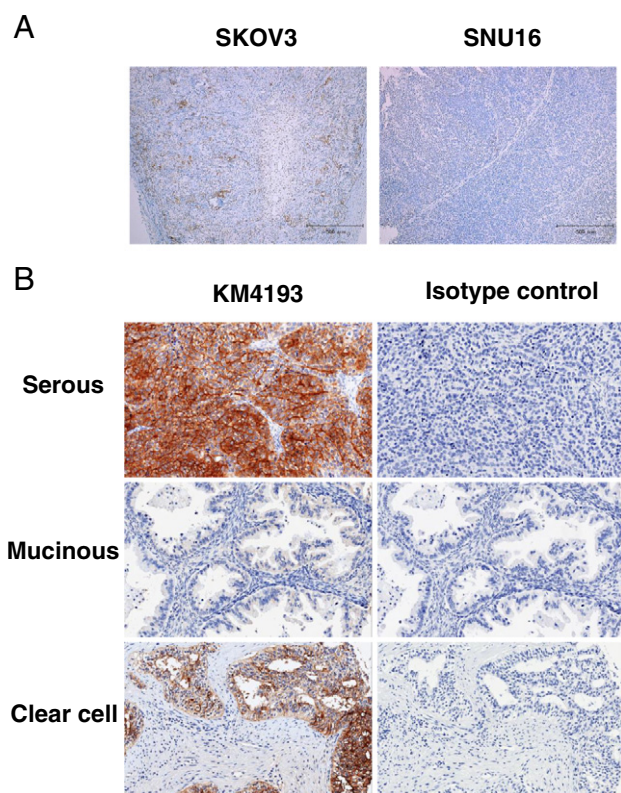


**Figure 5.** Therapeutic effect of KHK2805 in a peritoneal dissemination model. The effect of KHK2805 treatment was evaluated in a model of peritoneal dissemination induced by the inoculation of IGROV1 cells to SCID mice. Five days after the inoculation, treatment of KHK2805 was started and defined as Day 0. Vehicle was injected into the control mice. (A) The intraperitoneal tumor burden and amount of malignant ascites were measured on Day 24. KHK2805 (10 mg/kg twice a week) was intravenously injected (n = 5). The data are described as a box-and-whisker plot. ND: Not detected. The P values in each panel were assessed by the Kruskal-Wallis test plus Steel-Dwass test. (B) The intraperitoneal tumor burden and amount of malignant ascites were measured on Day 21. Various doses of KHK2805 were intravenously injected weekly (n = 5). The data are described as a box-and-whisker plot. The P values in each panel were assessed by the Steel test. (C) Kaplan–Meier survival curves of vehicle- or KHK2805 (10 mg/kg per week or twice a week)-treated mice. Treatments were intravenously injected (n = 10) until the endpoint (hypoactivity and/or abdominal distention was defined as the ethical endpoint). The median survival of control mice or those treated with 10 mg/kg per week KHK2805 or 10 mg/kg twice a week KHK2805 was 31.0 days (95% CI, 29 to 33 days), 56.5 days (95% CI, 45 to 62 days) or 55.5 days (95% CI, 44 to 57 days), respectively. The P values were assessed by the log rank test with Bonferroni correction. (D) Kaplan–Meier survival curves of PTX- (10 mg/kg at day 0, n = 5), CBDCA- (30 mg/kg at day 0, n = 5) or combination- (CBDCA plus PTX, n = 5) treated mice with peritoneal dissemination. The median survival of PTX-, CBDCA- or CBDCA + PTX-treated mice (n = 5) was 42.0 days (95% CI, 38 to 43 days), 31.0 days (95% CI, 28 to 34 days) or 40.0 days (95% CI, 38 to 44 days), respectively.

combination of the existing anti-FOLR1 humanized antibody (farletuzumab) and ADCC-enhancing technology (defucosylation), and 2) the CDC of KHK2805 is largely dependent on CDC-enhancing technology (113F).

This potent ADCC of KHK2805 may be caused by its higher affinity against FOLR1 (Figure 1C). Tang et al. showed that high-affinity binding of antibodies led to the most efficient and

powerful ADCC [27]. Alternatively, the epitope-related unique cytotoxicity of KHK2805 may be involved, since KHK2805 has been shown to recognize a novel epitope on FOLR1 distinct from those recognized by other reported antibodies [28] (Figure 2). Indeed, several characteristic biological effects of anti-FOLR1 antibodies have been reported. For example, farletuzumab played a role in autophagy in induced cell death but had no significant effect on apoptosis [29],



**Figure 6.** The FOLR1 expression in OC tissue. OC tissues were immunohistochemically stained by KM4193 (parental rat monoclonal antibody of KHK2805). (A) Mouse xenograft tissue of SKOV3 (FOLR1-positive OC) or SNU16 (FOLR1-negative gastric cancer) cells was immunohistochemically stained by KM4193. (B) FFPE OC patient samples (serous, mucinous and clear cell type) were stained by KM4193 or the isotype control antibody. Representative data are shown.

while MOv18 precipitated phosphorylated-lyn tyrosine kinase in lipid raft fraction [30]. Therefore, further studies are needed to clarify the epitope-related activity of KH2805.

We then evaluated whether anti-FOLR1 antibodies deplete primary cancer cells by autologous effector cells, since translational research using patient-derived sample is important for making value judgments regarding drug candidates. Malignant ascites are a major clinical issue in the management of OC and represent a promising target for translational research [3]. Malignant ascites include a number of different cellular populations, such as tumor cells, mesothelial cells, fibroblasts, macrophages and white and red blood cells [3]. According to our FCM study of malignant ascites cells (platinum-resistant, Supplemental Table S1) isolated from 6 patients with OC, the median lymphocyte/cancer cell ratio was 0.33 (range, 0.10 to 1.85) (Supplemental Table S2). These numbers are close to those of a previous report, which found that 1% to 60% of cells were tumor cells in ascites from patients with epithelial ovarian tumor [31].

We confirmed the existence of FOLR1 expressing cells with a variety of the expression levels throughout the cell population of ascites (Supplemental Figure S6 and Supplemental Table S2). As shown in Figure 3B, the proportion of FOLR1(+) cancer cells were reduced by simply adding KHK2805 to the cell population, indicating two points of note. First, KHK2805 had potent cytotoxicity against platinum-resistant cancer cells. Second, the

immune cells in malignant ascites were working as effector cells with KHK2805. This result is supported by the finding of a previous report that cytotoxic NK cells ( $CD56^{\text{dim}}CD16^{\text{bright}}$ ) were infrequently detected as tumor-infiltrating cells but were detected in the pleural and peritoneal fluids in cancer patients [32]. We found that samples with a higher percentage of NK cells tended to show clearer cancer-cell reduction, regardless of the FOLR1 expression or percentage of T cells, than those with a lower percentage of NK cells (Figure 3B and Supplemental Table S2). These findings suggest that NK cells in malignant ascites may act as the primary effector cells of the ADCC antibody KHK2805 in patients.

Two issues concerning the CDC of KHK2805 in patients need to be clarified: the CDC sensitivity of OC and the complement activity in serum or ascites. Regarding the CDC sensitivity, the overexpression of CRPs is known to inhibit the CDC of rituximab [33]. However, in the present study, there were no clear differences in the expression of CRPs (CD46, CD55 and CD59) and the CDC sensitivity among the four OC cell lines tested (Figure 4A and Supplemental Figure S4), although a trend towards correlation was noted between the CDC sensitivity and the FOLR1 expression (Figures 1B and 4A). Regarding the complement activity of patient-derived samples, elevated CDC of KHK2805 was observed in all tested patient-derived sera as well as in the healthy volunteers' sera (Figure 4B). In contrast, only one of the two supernatant samples of malignant ascites showed CDC for anti-FOLR1 antibodies. Bjorge et al. reported that most ascites fluid samples contain functional complements, but ovarian tumor cells are protected by CRPs [34]. Consequently, fundamental studies using patient-derived cancer cells and malignant ascites samples are still needed to assess both the CDC sensitivity of the cells and the complement activity in patients.

KHK2805 showed significant efficacy against CBDCA-resistant peritoneal dissemination model compared with not only the vehicle control but also PTX (Figure 5). In addition to this, KHK2805 treatment was well-tolerated in this model. In the preclinical toxicology studies, KHK2805 did not produce a toxicological response even at doses of 100 mg/kg per week for 4 weeks in female cynomolgus monkeys (data not shown), indicating that KHK2805 has an acceptable tolerability profile *in vivo*. These findings support several implications concerning the mechanisms of the superior efficacy of KHK2805 over chemotherapies *in vivo*. In SCID mice, the activities of NK cells and macrophages are unimpaired, and the number of neutrophils is within the normal range. Furthermore, increased complement activation was seen in SCID mice compared with Nude or BALB/c mice [35]. Consequently, the complement-enhanced ADCC [12,13] in peritoneal cavity may be a mechanism underlying the effects of KHK2805 *in vivo*. Regarding ADCC mechanisms, Srivastava et al. reported that the anti-EGFR antibody cetuximab markedly enhanced the induction of Th1-polarizing cytokines such as CXCL10 (IP-10) in cancer-NK-DC cells co-culture [36]. We similarly observed IP-10 induction after ADCC of KHK2805 (IGROV1 cells targeted; Supplemental Figure S7). Interestingly, the intratumor injection of IP-10 in human adenocarcinoma tumors in SCID mice reduced the angiogenic activity, indicating that IP-10 is an angiostatic factor [37]. Angiogenesis is known to be associated with increased vessel permeability, resulting in the generation of malignant ascites [3]. In this context, therefore, the suppression of angiogenesis by IP-10 induction after ADCC of KHK2805 may—at least in part—underlie its superior efficacy to

chemotherapies in suppressing the fatal malignant ascites induced by peritoneal dissemination. Although the current SCID mouse model is limited in evaluating adaptive immunity and further studies are needed, these Th1-polarizing innate immune responses enhanced by KHK2805 are expected to facilitate tumor-specific T cell immunity, as previously discussed [38,39].

## Conclusion

In summary, we established the novel anti-FOLR1 antibody KHK2805. This antibody showed remarkable cytotoxicity against not only OC cell lines but also patient-derived cells. Platinum-resistant peritoneal dissemination and malignant ascites were clearly inhibited by KHK2805 monotherapy. A candidate companion diagnostic method for the selection of FOLR1-positive tumors was also established. The findings from these translational studies strongly suggest that KHK2805 may be promising as a novel therapeutic antibody for platinum-resistant OC with peritoneal dissemination and malignant ascites.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.tranon.2017.06.007>.

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