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ORIGINAL ARTICLE

Inhibiting casein kinase 2 overcomes paclitaxel resistance in gastric cancer

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Abstract

Purpose Casein kinase (CK) 2 activation has been implicated in the proliferation of various tumor types and resistance to chemotherapy. We investigated the mechanistic basis for the association between CK2 activation and paclitaxel resistance in a gastric cancer (GC).

Experimental design CK2 expression was evaluated in 59 advanced GC patients treated with paclitaxel as the second-line therapy. The efficacy of a CK2 inhibitor, CX-4945, and paclitaxel was evaluated in GC cell lines and a xenograft model.

Results Patients with high CK2 expression (29/59, 39%) showed lower disease control rates (47.7% vs. 72.3%, $p=0.017$) and shorter progression-free survival (2.8 vs. 4.8 months, $p=0.009$) than patients with low CK2 expression. CK2 protein expression was associated with sensitivity to paclitaxel in 49 GC cell lines. Combination therapy with CX-4945 and paclitaxel exerted synergistic antiproliferative effects and inhibited the downregulation of phosphatidylinositol 3-kinase/AKT signaling in SNU-1 cells. In the SNU-1 xenograft model, the combination treatment was significantly superior to either single agent, suppressing tumor growth without notable toxicities.

Conclusions These results demonstrated that CK2 activation was related to paclitaxel resistance and that CX-4945 in combination with paclitaxel could be used as a potential treatment for paclitaxel resistance in GC.

Keywords Gastric cancer · Paclitaxel · Drug resistance · Casein kinase 2

Introduction

Gastric cancer (GC) is a major health problem worldwide, with high incidence and a poor prognosis [1]. Surgical resection in combination with adjuvant chemotherapy is the only curative treatment strategy for localized GC [2, 3]. However, recurrence is common, and for advanced or metastatic GC, chemotherapy is the first treatment option. Although clinical trials have sought to improve survival rates in GC patients, the median overall survival (OS) for metastatic disease is only about 15 months [4]. Therefore, there is an urgent need

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for new drug and innovative treatment strategies for improving treatment outcome and survival of metastatic GC.

Paclitaxel is effective for advanced GC treatment and has response rates in the range of 15–28% when used as monotherapy [5, 6]. It is most commonly used as a second-line therapy in GC and shows favorable toxicity profiles in GC [7]. However, the median duration of response of paclitaxel monotherapy and combined with ramucirumab in GC are only 4.4 and 2.8 months, respectively, and eventually, patients develop paclitaxel resistance [8]. The molecular mechanism underlying paclitaxel resistance is not well understood. The common mechanisms of drug resistance are the overexpression of P-glycoprotein and protein kinase C-α (PKC-α), as well as the upregulation of the mitogen-activated protein kinase and phosphatidylinositol-3-kinase (PI3K)/AKT signaling pathways [9-11]. The PI3K/AKT pathways are specifically activated following paclitaxel treatment, whereas PI3K inhibition sensitizes tumors to paclitaxel and induces cell death via mitotic arrest [11].

Recently, casein kinase (CK)2 has been proposed as a potential therapeutic target for several cancer types. CK2 is a constitutively active serine/threonine protein kinase that has pro-survival/anti-apoptotic functions [12]. Given that CK2 is overexpressed in multiple cancers and is implicated in many non-oncogenic processes required to sustain the cancer phenotype, its selective inhibition is an attractive strategy for cancer treatment [11]. CK2 has been shown to phosphorylate AKT1 at Ser129 to promote cell survival by generating a constitutively active form of the protein [13]. It also phosphorylates and stabilizes phosphatase and tensin homolog, thereby inhibiting its activity and inducing PI3K-mediated survival signaling and oncogenesis [14]. 5-[(3-Chlorophenyl)amino]-benzo[c]-2,6-naphthyridine-8-carboxylic acid (CX-4945, New Taipei City, Taiwan) is a novel small-molecule inhibitor of CK2, the biological activity of which has been investigated in vitro and in vivo [15]. The key attributes of CX-4945 include potent inhibition of CK2 enzymatic activity and a highly selective kinase profile [16].

We speculated that paclitaxel resistance is associated with CK2 activation and PI3K/AKT signaling and that combining CK2 inhibition with paclitaxel chemotherapy can improve GC treatment efficacy. To test this hypothesis, we investigated the association between CK2 expression and paclitaxel resistance and evaluated whether CX-4945 in combination with paclitaxel can overcome paclitaxel resistance in GC.

Methods

Clinical specimens and chemotherapy protocol

We used archived paraffin blocks of surgical specimens or endoscopic biopsied specimens of 59 patients from January 2009 to December 2016 with advanced GC, obtained before paclitaxel therapy at the Yonsei Cancer Center, Yonsei University Health System (Seoul, Korea). All patients received paclitaxel (175 mg/m^2 on day 1 every 3 weeks, or 70–80 mg/m^2 on days 1, 8, and 15 every 4 weeks) as a second-line treatment. Paclitaxel was administered until disease progression or the occurrence of intolerable toxicities. Tumor assessments were performed every two cycles, and disease response was categorized as complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD) according to the Response Evaluation Criteria in Solid Tumors (RECIST, v.1.1) [17]. Overall response rate (ORR) and disease control rate (DCR) are defined as the percentage of patients who have achieved CR and PR, and ORR plus SD, respectively. This study was approved by Institutional Review Board of Severance Hospital (IRB No.4–2017-0313).

Immunohistochemical analysis of CK2 and phosphorylated-AKT

CK2 and phosphorylated (p)-AKT expression was evaluated by immunohistochemistry using anti-CK2 (Abcam, Cambridge, UK; 1:50) and anti-p-AKT (Cell Signaling Technology, Danvers, MA, USA; 1:40) antibodies. The numbers of tumor cells with membrane and cytoplasmic staining of CK2 and cytoplasmic and nuclear staining of p-AKT were counted. Scoring was performed by an independent pathologist blinded to the patients' clinical information. Protein expression was interpreted by the weighted histoscore method (H score method) [18]. The intensity of protein expression was scored as 0 (negative), 1 (light brown), 2 (brown), or 3 (dark brown) (Fig. 1a). The final score was calculated as follows: (0 × % of negative cells) + (1 × % of light brown cells) + (2 × % of brown cells) + (3 × % of dark brown cells). For example, a specimen with 20% of cells staining 3, 20% of cells staining 2, 30% of cells staining 1, and 30% of cells unstained would have a histoscore of (3 × 20) + (2 × 20) + (1 × 30) = 130. Histoscores range from 0 to 300. Tumors with a staining H score of more than 100 were defined as having high CK2 or p-AKT expression, while tumors with a score of less than 100 were defined as having low CK2 or p-AKT expression.

Cell culture and reagents

A total of 49 GC cell lines were used in this study [19]. The YCC series was established by the Yonsei Cancer Center (Cancer Metastasis Research Center and Sondang Institute for Cancer Research, Yonsei University College of Medicine Seoul, Korea) from the ascites or peripheral blood of advanced GC patients. Other cell lines were obtained from

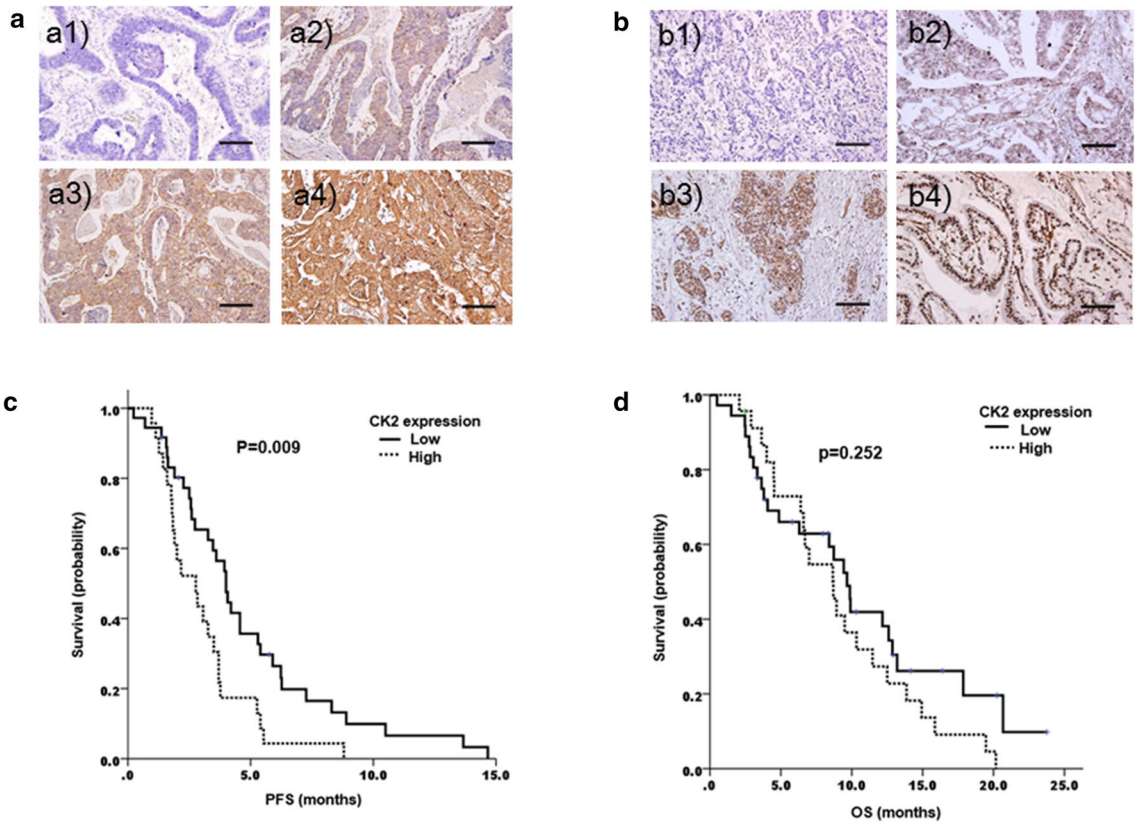

Fig. 1 Casein kinase (CK2) and phosphorylated (p)-AKT protein expression and Kaplan–Meier survival curve according to CK2 expression in gastric cancer patients. **a** CK2 and **b** p-AKT immunoreactivity was graded as **a** negative, **b** weak (light brown) **c** moderate (brown), or **d** strong (dark brown). Original magnification: 200X, Scale bar: 50 μm; **c** PFS in 59 patients; the median values for patients with low and high CK2 expression were 4.1 months versus 2.8 months ($p=0.009$). **d** There was no difference in OS according to CK2 level

the Korean Cell Line Bank (Seoul National University, Seoul, Korea) and the American Type Culture Collection (American Type Culture Collection, Manassas, VA, USA).

Cells were maintained in Roswell Park Memorial Institute-1640 medium (Lonza, Walkersville, MD, USA) or Eagle's minimal essential medium (Lonza, Walkersville, MD, USA) and supplemented with 10% fetal bovine serum (Omega Scientific, Tarzana, CA, USA), 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mmol/l glutamine in a humidified atmosphere at 37 °C with 5% CO_2. Cell lines were expanded and cryopreserved in liquid nitrogen in our laboratory.

Cell growth inhibition assay

Cell proliferation was evaluated by the 3-(4,5-dimethylthiazol-2yl)-2.5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded at a density of 5×10^3 cells into a 96-well plate and incubated at 37 °C for 24 h. CX-4945 was synthesized by and supplied by Senhwa Biosciences (New Taipei City, Taiwan, ROC). CX-4945 was prepared at concentration of 5 mmol/L in dimethyl sulfoxide and stored at -70̈C. For treatment it was diluted in serum-free media. Paclitaxel was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cells were treated with various concentration CX-4945 (range, 0.1–20 μM) or paclitaxel (range, 0.001–10 μM). After 72 h of incubation, 50 μl of MTT solution (400 μg/ml) was added to each well. Following an additional incubation of 4 h, the MTT reaction was terminated by adding 150 μL of DMSO (dimethyl sulfoxide, Sigma-Aldrich, St. Louis, MO, USA). Absorbance was measured with a multiwell ELISA automatic spectrometer recorder (Behring ELISA Processor II, Germany) at 570 nm. An IC50 (μM) of CX-4945 and paclitaxel in each cell line was calculated with Calcusyn software (Biosoft Inc. Cambridge, UK).

At least three replicates were prepared for each treatment, and the average of these data was used for data analyses. Synergy was assessed using the New Bliss Independence Model [20]. The combined percentage inhibition $Y_{ab,\,P}$ was predicted using the complete addition of probability theory as $Y_{ab,}p = Y_a + Y_b - Y_aY_b$ (drug A at dose a inhibits Y_a% of tumor growth, and drug B at dose b inhibits Y_b% of tumor

growth). The observed combined percentage inhibition $Y_{ab,O}$ was then compared as $Y_{ab,P}$, $Y_{ab,O} > Y_{ab,P}$, $Y_{ab,O} = Y_{ab,P}$, and $Y_{ab,O} < Y_{ab,P}$, which indicated that effects were synergistic, independent (additive), and antagonistic, respectively.

Detection of CK2 activity in cell lysates

CK2 activity was determined using a CK2 kinase assay kit (CycLex Co., Ltd., Nagano, Japan) according to the manufacturer's instructions. Briefly, cell lysates were incubated with the synthetic p53 peptide coated on a 96-well plate with the kinase reaction buffer. The amount of phosphorylated substrate was measured using a horseradish peroxidase-conjugated anti-phospho-p53 serine 46-specific antibody. Kinase activity was calculated by subtracting the mean of the background control samples without enzyme from the mean of samples with enzyme.

CK2 RNA expression by RT-PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First strand cDNA was synthesized from 2 μg total cellular RNA with oligo (dT) using a cDNA synthesis kit (Fermentas, Hanover, MD, USA). Quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed on a 7500 Fast real-time PCR system using the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The primers used were as follows: CK2a, 5′-TGTCCGAGTTGCTTCCCGATACTT-3′ and 5′-TTGCCAGCATACAACCCAAACTCC-3′, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5′-CCA TGGAGAAGGCTG GGG-3′ and 5′-CAAAGTTGTCAT GGATGACC-3′. Relative copy number was determined using the comparative *Ct* method. GAPDH served as the internal control for normalization.

Transfection of CK2 small interfering RNA (siRNA)

MKN-28 cells were plated at $2.5X10^5$ cells in 6-well plates and 24 h later, cells were transfected with 50 nM siGENOME SMARTpool scramble or CK2α using 5ul of DharmaFECT 1 (Dharmacon, Lafayette, CO, USA) per well according to the supplier's recommended protocol. 48 h after transfection, cells were collected and plated in 96-well plates for MTT assay. Cell lysates were also obtained for western blot analysis for checking knockdown efficiency.

Cell cycle analysis

Cell cycle distribution was evaluated by propidium iodide (PI) (BD Biosciences, San Jose, CA, USA) staining and flow cytometry. SNU-1 cells were left untreated or were treated for 36 h with paclitaxel (0.1 or 1 μM) and CX-4945 (5 or 1 μM) and then trypsinized, fixed, and permeabilized with 70% ethanol overnight at − 20 °C. This was followed by incubation for 30 min in the dark at room temperature with 200 μl PI solution. Cells were washed with PBS, resuspended in 500 μl PBS, and analyzed on a FACS Calibur system (BD Biosciences, San Jose, CA, USA).

Western blot analysis

Total protein (50 μg) extract of SNU-1 cells was prepared with cell lysis buffer (50 mM Tris HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM, Sodium orthovanadate, 1 mM NaF, 10 lg/ml aprotinin, 10 lg/ml, leupeptin, 1 mM PMSF). Then, 20 μg of proteins was separated on SDS polyacrylamide gels and transferred to PVDF membranes (Immobilon-P transfer membrane, Bedford, MA, USA). The primary antibodies used were the following proteins: p-CK2 (pS/pTDXE, #8738), AKT (Ser473, #9272), mammalian target of rapamycin (mTOR) (7C10, #2983), p-mTOR (Ser2448, #2971), p70S6K (#9202), p-p70S6K (Thr387, #9234), cleaved poly(ADP-ribose) polymerase (PARP) (Asp214, #9541) (all from Cell Signaling Technology, Danvers, MA, USA); anti-p-AKT (Ser129, ab133458, Abcam, Cambridge, UK); and CK2 (1AD9, sc-12738, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing, the blots were incubated with horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as secondary antibodies and visualized using super ECL detection (Amersham Pharmacia Biotech, Buckinghamshire, UK).

In vivo Xenograft experiment

For tumor cell implantation, 1×10^7 SNU-1 cells were trypsinized and resuspended in 100 μl serum-free medium prior to inoculation. Cells were implanted subcutaneously into the right flank of 6- to 8-week- old Balb/c female nu/nu mice. When tumors reached a volume of 150–200 mm^3, mice were randomly grouped into a control group, and paclitaxel-, CX-4945-, and paclitaxel plus CX-4945-treated groups (five mice each). The start day of drug treatment was defined as day 1. Tumor volume was measured every day, and the volumes were calculated using the following formula: tumor volume (mm^3) = (length × $width^2$)/2. CX-4945 was administered by oral gavage twice daily at 75 mg/kg per day, and paclitaxel was injected intraperitoneally once a week at 20 mg/kg for 3 weeks. The control group received saline according to the same schedule. Tumor volumes and body weights were measured twice weekly. Four hours after the final dose of treatment, the animals were euthanized, and tumors were resected and fixed in 10% buffered formalin for 24 h before being transferred to 70% ethanol.

Immunohistochemical analysis for CK2 α (ab15048, Abcam, Cambridge, UK; 1:50), p-AKT (#4060, Cell Signaling Technology, Danvers, MA, USA; 1:40), and Ki-67 (clone MIB-1; Dako no. M7240, Dako, Glostrup, Denmark; 1:50) was performed in xenograft tumor tissue with the same protocol as in the clinical samples.

Statistical analysis

Clinical characteristics and treatment outcomes of patients were compared according to CK2 expression with the chi-square (χ^2) test and Fisher's exact test. Survival estimates were calculated using the Kaplan–Meier method. Progression-fee survival (PFS) was calculated from the date chemotherapy was started to the earliest date of disease progression or death from any other cause. OS was defined as the time from the start of paclitaxel treatment to death from any cause. Differences in PFS and OS according to CK2 and p-AKT expression were compared by the log-rank test. Significant effects between treatment groups or between treatment and control groups were evaluated by the Mann–Whitney *U* test. Data were analyzed by SPSS, v.23.0 (SPSS Inc., Chicago, IL, USA), and plots and curves were generated with Prism v.5 software (GraphPad Inc., San Diego, CA, USA).

Results

Patient characteristics and the expression of CK2 and p-AKT

The clinicopathologic parameters of patients treated with paclitaxel as a second line were summarized in Table 1. The median age was 59 years (range 53–68 years), and 62.7% were men. About 60% patients underwent gastrectomy before paclitaxel treatment. Eight (13.6%) of them were positive for human epidermal growth factor receptor 2 (HER2). Median CK2 and p-AKT immunoreactivity scores were 50 (range, 0–210) and 110 (range, 0–270), respectively (Fig. 1a, b). The expression of CK2 was high in 23 (40%) of the 59 patients, and p-AKT expression was high in 40 (67.8%) of them. There were no differences of ICAM-1 expression between HER2 negative and positive tumors (data not shown).

Table 1 Patients' clinicopathological characteristics

Characteristics	Number	%
Age		
Median	59	
Range	34–80	
Sex		
Male	37	62.7
Female	22	37.3
WHO classification		
Adenocarcinoma, well differentiated	6	10.2
Adenocarcinoma, moderately differentiated	15	25.4
Adenocarcinoma, poorly differentiated	26	44.1
Signet ring cell	9	15.5
Others	3	5.1
Stage at diagnosis		
II–III	32	54.2
IV	27	45.8
Prior gastrectomy		
Yes	36	61
No	23	39
HER2 status		
Positive	8	13.6
Negative	39	66.1
Unknown	12	20.3
Disease		
Measurable	30	50.8
Non-measurable	22	37.3
Metastatic location		
Distant lymph node	16	27.1
Liver	13	22
Peritoneum	37	62.7
Lung	3	5.1
Bone	2	3.4

WHO World Health Organization, *HER2* human epidermal growth factor receptor 2

Association between CK2 and p-AKT expression and paclitaxel response

Response to paclitaxel was evaluated in 56 of 59 patients. Two patients could not undergo a second cycle of chemotherapy due to decreased performance status, and one patient refused chemotherapy after the first treatment cycle. The ORR was 10.2%, and the DCR was 62.7%. There was no association between ORR or DCR and p-AKT expression. However, the patients with high CK2 levels had lower DCRs (47.8% vs. 72.3%, $p = 0.017$) (Table 2). In all patients, the median PFS and OS were 3.5 months [95% confidential interval (CI), 2.7–4.3 months] and 8.9 months (95% CI, 6.3–10.8 months), respectively. There was no difference in PFS according to age and sex, and no difference in PFS and OS according to p-AKT expression (data not shown). However, the PFS was longer in patients with low CK2 expression than in those with high CK2 expression (median PFS, 4.1 vs. 2.8 months, $p = 0.009$), while OS was not associated with CK2 expression (Fig. 1c, d).

Table 2 Paclitaxel response according to the expression of CK2 and p-AKT

	All (%)	CK2			p-AKT		
		Positive ($n=23$)	Negative ($n=36$)	p value	Positive ($n=40$)	Negative ($n=19$)	p value
CR	0	0	0	0.004	0	0	0.295
PR	6 (10.2)	4 (17.4)	2 (5.6)		2 (5)	4 (21.1)	
SD	31 (52.5)	7 (30.4)	24 (66.7)		22 (55)	9 (47.4)	
PD	19 (32.2)	12 (52.2)	7 (19.4)		14 (35)	5 (26.3)	
NE	3 (5.1)	0	3 (8.3)		2 (5)	1 (5.3)	
ORR	6 (10.2)	4 (17.4)	2 (5.6)	0.181	2 (5)	4 (21.1)	0.077
DCR	37 (62.7)	11 (47.8)	26 (72.2)	0.017	24 (60)	13 (68.4)	0.503

CR complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease, *NE* not evaluable or not assessed, *ORR* overall response rate, *DCR* disease control rate, *p-AKT* phosphorylated AKT

Association between CK2 expression and paclitaxel sensitivity in 49 GC cell lines

Since CK2 protein expression was associated with paclitaxel resistance in the tumor tissue of GC patients, we investigated the association between paclitaxel sensitivity and CK2 expression in 49 GC cell lines. The YCC-30 cell line was the most sensitive to paclitaxel, with a half-maximal inhibitory concentration (IC_{50}) of 0.0003 μM; in contrast, YCC-25, YCC-28, and YCC-33 cells were highly resistant with $IC_{50} \geq 10$ μM. When the cut-off level of paclitaxel sensitivity/resistance was determined as 0.1 μM according to the Catalogue of Somatic Mutations in Cancer (https://cancer.sanger.ac.uk/cosmic), 18 cell lines (36.7%) were resistant to paclitaxel (data not shown).

When we compared CK2 mRNA, protein expression and CK2 activity in 49 GC cell lines with paclitaxel sensitivity, paclitaxel-resistant cell lines had higher CK2 protein expression than those that were sensitive ($p=0.041$, Fig. 2a). However, neither CK2 mRNA nor activity was correlated with paclitaxel sensitivity (Fig. 2b, c). Also, p-AKT expression was not associated with paclitaxel sensitivity (Fig. 2d).

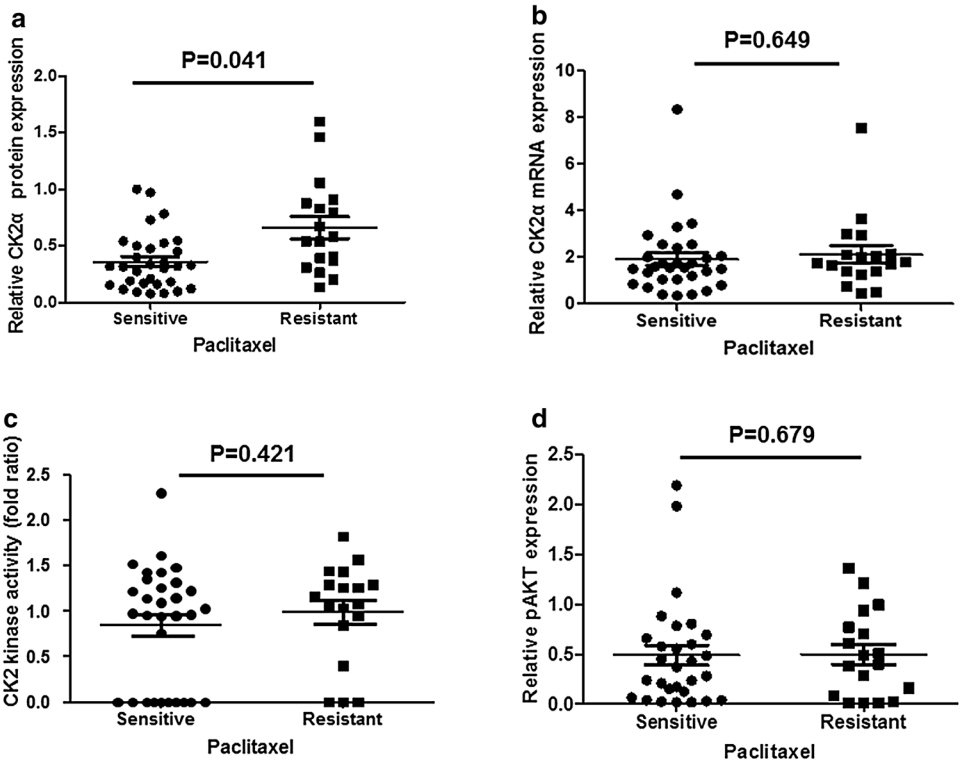

Fig. 2 Association between CK2 expression and paclitaxel sensitivity. **a** CK2 protein levels in 49 gastric cancer cell lines were evaluated by western blotting and compared between paclitaxel-sensitive and paclitaxel-resistant cells. **b** CK2 mRNA expression was assessed by quantitative reverse transcription PCR (qRT-PCR) and compared between paclitaxel-sensitive and paclitaxel-resistant lines. **c** CK2 kinase activity was evaluated by the CK2 kinase assay and compared between paclitaxel-sensitive and paclitaxel-resistant lines. **d** p-AKT expression was assessed by western blotting and compared between paclitaxel-sensitive and paclitaxel-resistant cell lines. The mRNA and protein levels were normalized to those of Jurkat cells

CK2 down-regulation and paclitaxel sensitivity

For down-regulation of CK2α, we selected the MKN-28 cell line, which highly expressed CK2 α. MKN-28 cells were transfected with scramble or CK2α small interfering RNA (siRNA). We performed Western blot analysis and MTT assay. The protein expression level of CK2α was markedly decreased in MKN-28 cells transfected with CK2α siRNA compared to those with scramble RNA (Fig. 3a). MKN-28 cells treated with CK2α siRNA were significantly more sensitive from paclitaxel than cells treated with scramble RNA (Fig. 1b, $p < 0.01$).

Synergistic effect of paclitaxel and CX-4945 on the proliferation of paclitaxel-resistant SNU-1 cells

The above results indicated that CK2 expression was associated with paclitaxel resistance; therefore, we speculated that combined treatment of paclitaxel and CX-4945 through inhibiting CK2 protein could overcome paclitaxel resistance. Among the 49 cell lines, we selected SNU-1, which was paclitaxel resistant ($IC_{50} = 1.122$ μM) and CX-4945 sensitive ($IC_{50} = 7.2$ μM) and had high CK2 expression (data not shown). SNU-1 cells were treated with paclitaxel (0.1 or 1 μM monotherapy), CX-4945 (5 or 10 μM monotherapy), or both (0.1 μM paclitaxel and 5 or 10 μM CX-4945). The combination of both drugs had synergistic inhibitory effects on cell proliferation according to the New Bliss Independence Model ($Y_{ab,O} = 0.767 > Y_{ab,}p = 0.478$) (Fig. 4a).

Using flow cytometry, we investigated whether paclitaxel, CX-4945, or the combination thereof inhibited the cell cycle progression of SNU-1. The proportion of cells in the G2/M phase was increased by concurrent treatment with both drugs. Notably, G2/M arrest was markedly increased in the cells when treated with paclitaxel combined with CX-4945 (Fig. 4b).

Effects of paclitaxel combined with CX-4945 on CK2 expression, PI3K/AKT signaling, and apoptosis

The effects of combined paclitaxel and CX-4945 treatment on CK2 expression, PI3K/AKT signaling, and apoptosis were analyzed by western blot analysis in SNU-1 cells. CX-4945 decreased the phosphorylation of CK2, AKT, and the downstream signaling protein p70S6K. Paclitaxel induced CK2 in SNU-1 cells resistant to paclitaxel (Fig. 4c). In contrast, paclitaxel did not increase CK2 in SNU-638 and SNU719 which are sensitive to paclitaxel (Supplementary Fig. 1). CX-4945 combined with paclitaxel decreased p-CK2, p-AKT, and p-p70S6K expression. When the effect of CX-4945 on apoptosis was also investigated by evaluating cleaved PARP protein expression, each CX-4945 and paclitaxel monotherapy induced cleaved PARP-1 expression and this effect was potentiated by combined treatment with both drugs.

Antitumor efficacy of paclitaxel combined with CX-4945 in SNU-1 xenograft model

We further evaluated whether CX-4945 plus paclitaxel increased the antitumor activity in SNU-1 xenograft models in vivo. There was a significant reduction in tumor growth with paclitaxel or CX-4945 treatment than with the vehicle (43.1% and 44.6%, respectively). In addition, the efficacy of paclitaxel combined with CX-4945 showed significantly decreased tumor growth than monotherapy (Fig. 5a). There was no noticeable toxicity or significant body weight reduction with either drug alone or the combined treatment (Supplementary Fig. 2).

Next, we examined the effect of CX-4945 plus paclitaxel on proliferation and PI3K/AKT signaling by immunohistochemical staining in the tumor tissues (Fig. 5b). When compared to the control group, paclitaxel or CX-4945 monotherapy groups showed reductions in Ki-67, CK2, and p-AKT

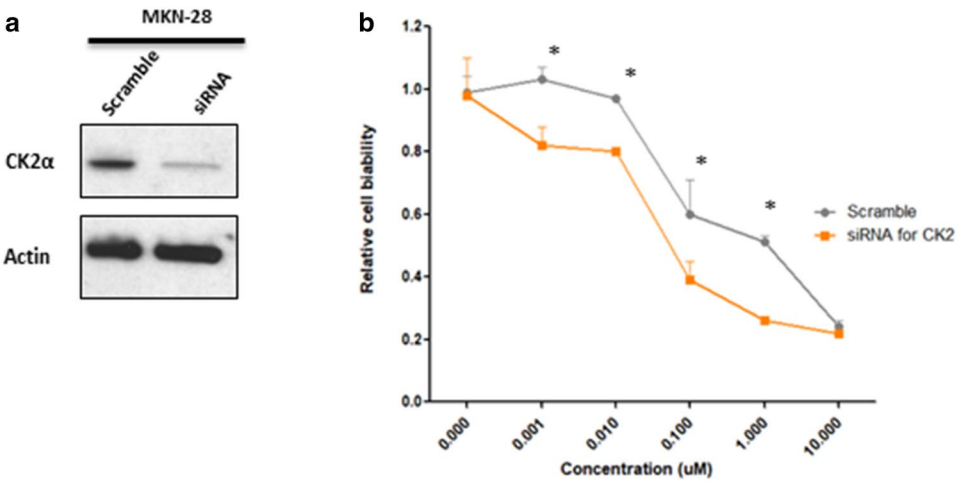

Fig. 3 CK2 knockdown and paclitaxel sensitivity. **a** CK2 protein levels were evaluated by western blotting in MKN-28 cell line which was transfected with scramble or CK2α small interfering RNA (siRNA). **b** After 24 h, cell viability assay was performed using a MTT assay. The group not treated with paclitaxel was presented as 100% viable cells and was used as an internal control for comparison. *$p < 0.05$

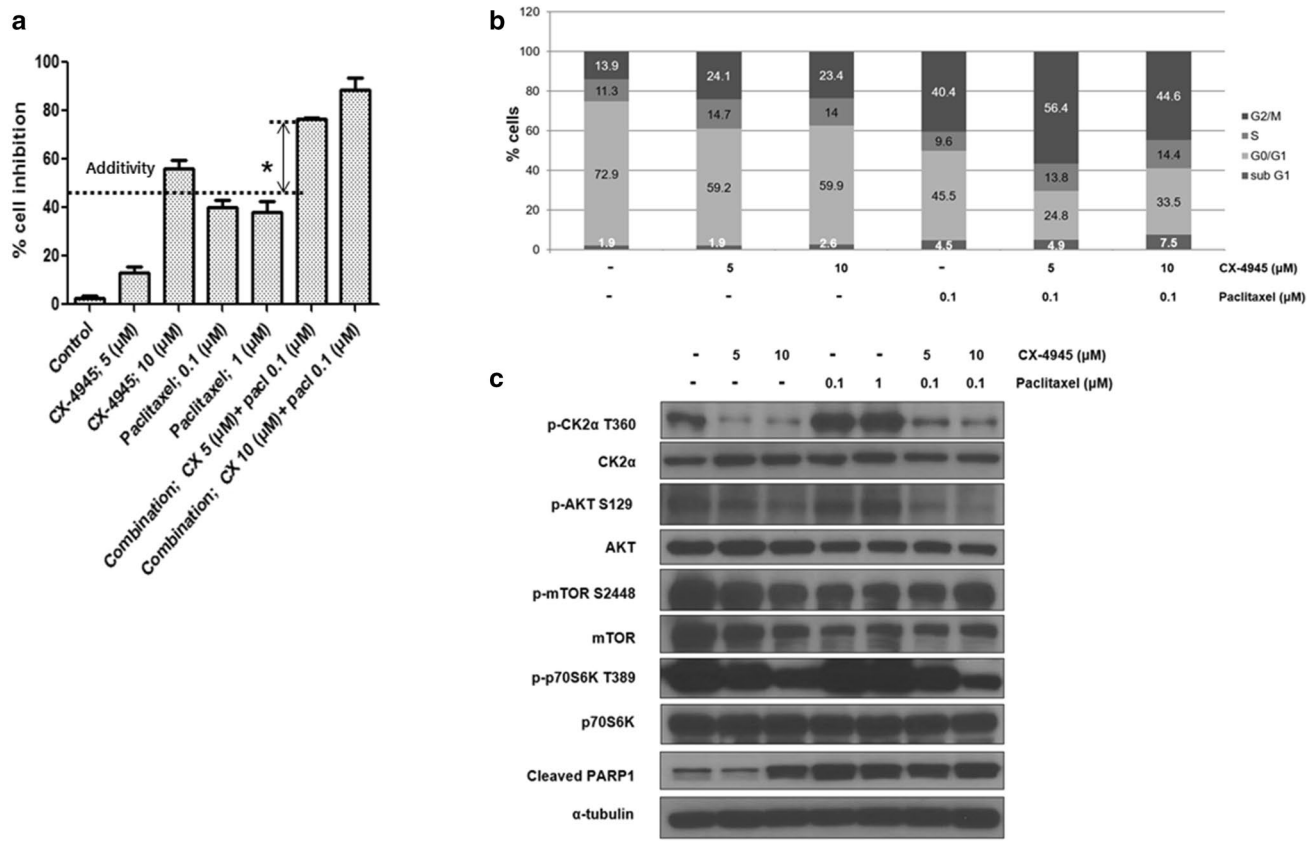

Fig. 4 Effect of CX-4945 combined with paclitaxel on antiproliferation, cell cycle, and CK2 expression, PI3K/AKT signaling, and apoptosis in SNU-1 cells. **a** The additivity line is equivalent to the theoretical combined antiproliferative effects of drugs A and B [%A + %B (100 − %A/100], wherein %A and %B are the percentages of cells killed by drugs A and B, respectively, at a given concentration (*28.9%). **b** SNU-1 cells were treated with CX-4945 (5 or 10 μM), paclitaxel (0.1 or 1 μM), and paclitaxel (0.1 μM) combined with CX-4949 (5 or 10 μM) for 36 h, and the cell cycle distribution was analyzed by flow cytometry. The fraction of cells in sub-G1, G0/G1, S, and G2/M phases of the cell cycle is shown. Combined treatment of paclitaxel and CX-4945 induced G2/M arrest. **c** effect of CX-4945 and paclitaxel on CK2, AKT, mTOR, p70S6K phosphorylation and PARP in SNU-1. Representative western blots using the indicated antibodies in SNU-1 treated with paclitaxel and CX-4945 as in **b**

expression levels (68.9%, 43.8%, and 58.3%, respectively, in paclitaxel; 67.4%, 60.4%, and, 56.1%, respectively, in CX-4945). In addition, the scores of Ki-67, CK2, and p-AKT were significantly decreased (82.8%, 65.6%, and 70%) in the combination group compared to the paclitaxel monotherapy group, which was consistent with the antitumor effects observed with these drugs and their combination (Fig. 5c).

Discussion

As other cancer therapeutic agents, the resistance of paclitaxel is a major factor in treatment failure. Several potential mechanisms for the resistance of paclitaxel have been proposed. The most well-known mechanism of paclitaxel resistance is drug efflux in relationship with P-glycoprotein (P-gp). P-gp, also known as ABCB1 gene or multidrug-resistance associated-protein (MRP), plays a role as an efflux pump and it correlated to chemosensitivity to paclitaxel [21]. Second, the paclitaxel resistance is mediated by selective alterations in expression of tubulin isotypes, such as Class III beta-tubulin [22]. Third mechanism is inhibition of apoptosis, activation of mitogen-activated protein kinase through the intracellular signaling pathway of PI3K [11]. The molecular analysis of gastric cancer revealed high rates of somatic alterations in PI3K/AKT/mTOR pathway, suggesting potential therapeutic targets with restoring paclitaxel resistance [23]. Although several inhibitors have been developed and tested in clinical trials in gastric cancer, nothing could confirm the clinical efficacy until now [24]. Inhibitors of PI3K pathway could activate multiple receptor tyrosine kinase, such as increasing insulin receptor substrate after mTOR inhibitors and loss of transcription factor FOXO3 after AKT inhibitors [25, 26]. Therefore, PI3K inhibitor alone is insufficient to overcome the resistance of paclitaxel through PI3K/AKT pathway in cancer [26].

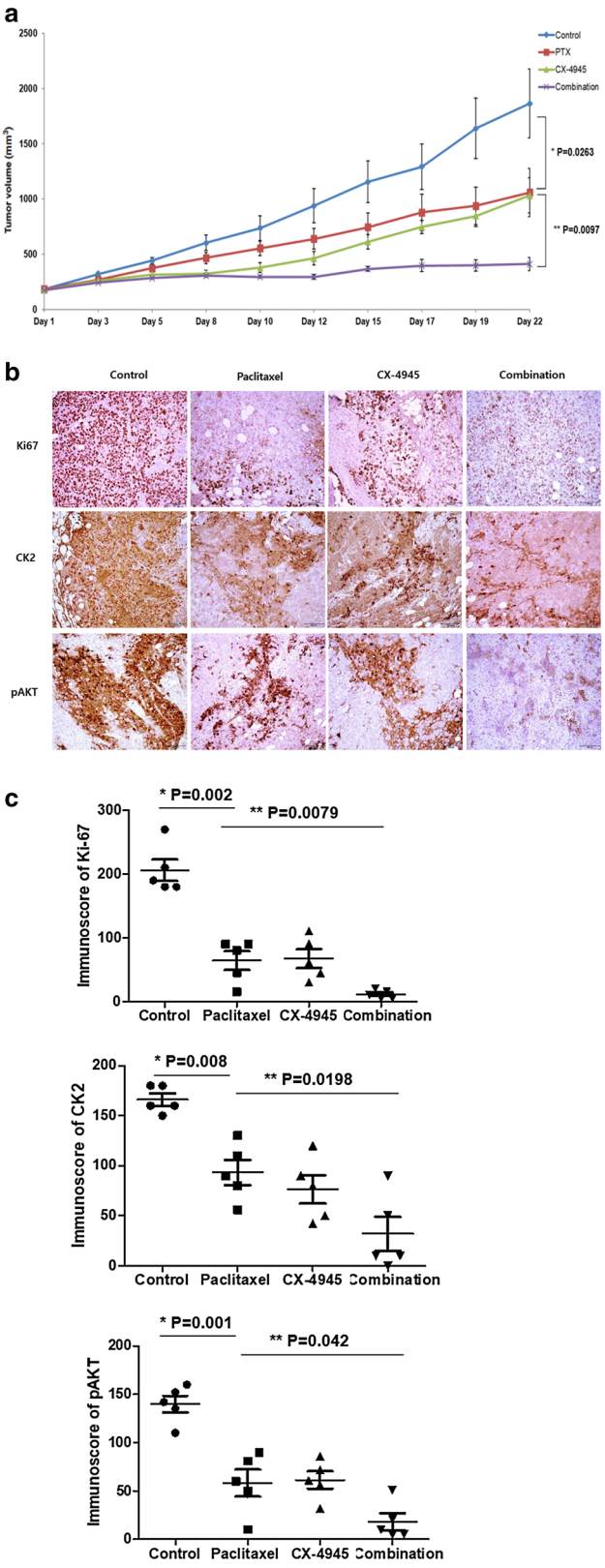

Fig. 5 Antitumor efficacy of CX-3945 and paclitaxel in SNU-1 xenograft model. The xenografts were established utilizing 5 × 107 SNU-1 cells implanted into the flank of Balb/c female nu/nu mice ($n = 5$ mice per treatment groups) Treatment were given 25 mg/kg of CX-4945 and by oral gavage twice daily at 75 mg/kg per day, and paclitaxel was injected intraperitoneally (i.p.) once a week at 20 mg/kg for 3 weeks. **a** The tumor volume according to treatment. Compared with vehicle treatment, treatment with paclitaxel or CX-4945 significantly inhibited the growth of the SNU-1 xenograft in nude mice ($p = 0.0263$). Addition of CX-4945 to paclitaxel resulted in significant tumor inhibition compared with paclitaxel monotherapy ($p = 0.0097$). **b** Representative image of Ki-67, CK2 and pAKT expression in xenografts. Original magnification: 200X, Scale bar: 50 μm. **c** Quantitation of immunohisto-score of Ki-67, CK2 and pAKT according to treatment. *p and **p values were compared with control and paclitaxel monotherapy, respectively

CK2 is a ubiquitous serine/threonine kinase that regulates a variety of cellular processes, including cell cycling, proliferation, and apoptosis [27]. CK2 overexpression was correlated with tumorigenesis in various types of cancers, as well as with the degree of tumor invasion; moreover, patients with elevated levels of CK2 had lower OS in surgically resected GC [12, 28]. Interestingly, CK2 was shown to exert anti-apoptotic effects via the upregulation of AKT [13]. Given that PI3K/AKT activation is associated with paclitaxel resistance, we speculated that CK2 is also related to paclitaxel resistance via the PI3K/AKT pathway in GC.

To test our hypothesis, we investigated CK2 expression in the tumor tissue of GC patients treated with paclitaxel as second-line chemotherapy. As predicted, CK2 overexpression was correlated with paclitaxel resistance. Patients with higher CK2 expression levels had significantly shorter survival and lower DCR from paclitaxel therapy as compared to those with low CK2 expression. Then, the association between CK2 expression and paclitaxel resistance was confirmed in vitro and in vivo using GC cell lines.

In this study, CK2α protein expression in GC cell lines correlated with paclitaxel sensitivity, however, CK2 mRNA and CK2 activity were not associated with paclitaxel sensitivity. The level of mRNA expression does not always correlate with protein expression because additional post-transcriptional mechanisms, such as protein translation, post-translational modification and degradation, can all influence the level of a protein present in a given cell or tissue [29]. Since protein, not mRNA, is responsible for the biological activity, this could be the reason why we can see the correlation of CK2 protein expression with paclitaxel resistance and not with CK2 mRNA. Marshak reported that CK2 activity was decreased as cells progressed from the G1 to G2/M stages of the cell cycle [30]. In addition, another study has demonstrated that CK2α is a microtubule-associated protein that confers microtubule stability in a phosphorylation-independent manner; thus we can speculate that CK2α can affect paclitaxel resistance by two modes of action, one of which is kinase activity dependent that involves regulation of PI3K/AKT pathway and a kinase-independent function that regulates through stabilization of microtubules [31].

Therefore, CK2 activity could not be associated with paclitaxel resistance.

CX-4945 is a potent and selective small-molecule inhibitor of CK2 that is active against a broad range of malignancies, including breast, colon, pancreas, prostate, ovarian, and lung cancers [32].The efficacy of CX-4945 against GC has not been reported before. Here, we first identified gastric cancer showed modest activity to CX-4945 monotherapy with 20 sensitive cell lines among 49 GC cell lines (41%), with a CX-4945 IC_{50} cut-off level of 10 μM. Cell lines with high CK2 protein expression tended to be sensitive to CX-4945.

To prove the direct relation of paclitaxel resistance and CK2 activation, we downregulated CK2α in MKN-28 using siRNA. Interestingly, CK2α was decreased in MKN-28 cells transfected with siRNA and these cell lines changed to be sensitive to paclitaxel. In addition, paclitaxel monotherapy induced CK2 in SNU-1 cells resistant to paclitaxel; however, it did not increase CK2 in SNU-638 and SNU-719, which are sensitive to paclitaxel. Moreover, the combination treatment of paclitaxel and CX-4945 synergistically suppressed proliferation and decreased the phosphorylation of CK2, AKT, and the downstream factor p70S6K in SNU-1 cells. Therefore, CK2 and PI3K/AKT activation by paclitaxel may contribute to paclitaxel resistance.

The in vitro observations of synergistic growth inhibition of GC lines treated with a CK2 inhibitor and paclitaxel were evaluated in vivo using SNU-1 tumor xenografts. Our in vivo data demonstrated that the combined treatment of CX-4945 plus paclitaxel synergistically decreased tumor growth compared with paclitaxel alone. Furthermore, consistent with the antitumor activity, the CX-4945 treatment and combined treatment with paclitaxel also inhibited the proliferation marker Ki-67 and inhibited the p-AKT pathway in xenograft tissue. Thus, on the basis of in vitro and in vivo studies, we identified the potential mechanism for paclitaxel resistance to be related to the activation of PI3K/AKT signaling.

Until now, paclitaxel-combined ramucirumab is regarded as a standard second-line treatment for advanced gastric cancer [8]. However, there is no clear evidence of the association between angiogenesis and paclitaxel [33]. In addition, there is also no reproducible predictive biomarker for antiagniogenic therapy.[34] On the contrary, our preclinical and clinical data indicated that CK2 is associated with paclitaxel resistance and the patient with high expression of CK2 could be a good candidate for paclitaxel plus CK2 inhibitor. Therefore, the treatment of combined with CK 2 inhibitor with paclitaxel is likely to yield better result than paclitaxel plus ramucirumab.

This study has several limitation. The clinical data were retrospectively analyzed with a small sample size. The PFS in gastric cancer patients treated with paclitaxel was significantly different in patients with high and low CK2 expression; however, we did not find any significant difference in OS. This discrepancy of association between PFS and OS with CK2 expression might be the impact of subsequent therapies on OS after patients had disease progression after paclitaxel treatment. Another possible reason is the sample size in the present study was too small to detect a difference in OS time.

In conclusion, high CK2 expression is a negative predictive marker for paclitaxel response in GC. Paclitaxel resistance was related to CK2 expression through the PI3K/AKT signaling pathway. Our findings suggested that CK2 inhibition with CX-4945, which induces G2/M cell cycle arrest and increases apoptosis by suppressing PI3K/AKT signaling in combination with paclitaxel, can be effective against paclitaxel-resistant GC.

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Authors' contributions Conception and design: M Jung, SY Rha; Development of methodology: M Jung, KH Park, HM Kim. Acquisition of data: M Jung, KH Park, HM Kim, HC, Chung, SY Rha, SH Beom, HS Kim, Sun-Mi Park. Analysis and interpretation of data: M Jung, KH Park, HM Kim, SY Rha, JH Cheong, Sun-Mi Park. Writing, review, and/or revision of the manuscript: M Jung, SY Rha. Administrative, technical, or material support: Xianglan Zhang, KH Park, HM Kim, Tae Soo Kim, John Soong, Shu-chuan Lin. Study supervision: HC, Chung, SY Rha.

Compliance with ethical standards

Ethics approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The protocol was reviewed and approved by the local Institutional Review Board of Severance Hospital (IRB approval no. 4-2017-0313). The institutional IRB decided to waive the informed consent of this study because all patients died at the analysis.

Conflict of interest CX-4945 was synthesized by and supplied without cost by Senhwa Biosciences, Inc. (New Taipei City, Taiwan, ROC).

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