**ORIGINAL ARTICLE**

# Propranolol suppresses gastric cancer cell growth by regulating proliferation and apoptosis

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## Abstract

**Background** Despite improvements in gastric cancer treatment, the mortality associated with advanced gastric cancer is still high. The activation of β-adrenergic receptors by stress has been shown to accelerate the progression of several cancers. Accordingly, increasing evidence suggests that the blockade of β-adrenergic signaling can inhibit tumor growth. However, the effect of β-blockers, which target several signaling pathways, on gastric cancer remains to be elucidated. This study aimed to investigate the anti-tumor effects of propranolol, a non-selective β-blocker, on gastric cancer.

**Methods** We explored the effect of propranolol on the MKN45 and NUGC3 gastric cancer cell lines. Its efficacy and the mechanism by which it exerts anti-tumor effects were examined using several assays (e.g., cell proliferation, cell cycle, apoptosis, and wound healing) and a xenograft mouse model.

**Results** We found that propranolol inhibited tumor growth and induced G1-phase cell cycle arrest and apoptosis in both cell lines. Propranolol also decreased the expression of phosphorylated CREB-ATF and MEK-ERK pathways; suppressed the expression of matrix metalloproteinase-2, 9 and vascular endothelial growth factor; and inhibited gastric cancer cell migration. In the xenograft mouse model, propranolol treatment significantly inhibited tumor growth, and immunohistochemistry revealed that propranolol led to the suppression of proliferation and induction of apoptosis.

**Conclusions** Propranolol inhibits the proliferation of gastric cancer cells by inducing G1-phase cell cycle arrest and apoptosis. These findings indicate that propranolol might have an opportunity as a new drug for gastric cancer.

**Keywords** Gastric cancer · β-Adrenergic receptor · β-Blocker · Propranolol · Repurposing drug

## Introduction

Gastric cancer is one of the most common malignancies and cause of cancer-related death worldwide, particularly in East Asian countries [1]. Despite recent advances in gastric cancer treatment, including surgical resection, chemotherapies, and molecular targeted therapies [2], the mortality associated with advanced gastric cancer remains

high [3]. Further, although there have been several novel findings about molecular and gene biomarkers that can be applied for new targeted therapies, only human epidermal growth factor receptor 2 (HER2) is used in clinical practice [4]. To improve treatment outcomes for gastric cancer, further research is required to investigate new biomarkers and develop novel treatments.

β-adrenergic receptors (β-ARs) are G-protein-coupled receptors activated by catecholamines, particularly norepinephrine and epinephrine [5]. There are four subtypes of the β-AR family: β1-AR, β2-AR, β3-AR, and β4-AR [6]. Both β1-AR and β2-AR are widely expressed in the majority of mammalian cell types [7]. Generally, β1-AR regulates myocardial stimulation and β2-AR regulates bronchodilation and vasodilation [8]. β3-AR is mainly expressed in adipose tissue and is associated with metabolic regulation [9]. β4-AR is thought to be a low-affinity state of β1-AR [10].

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Stimulation of β-AR by catecholamines leads to a coupling of Gs proteins and activation of adenylyl cyclase, resulting in increased levels of cyclic 3′-5′ adenosine monophosphate (cAMP) [11, 12]. cAMP is an intracellular second messenger that regulates various cellular processes [13, 14].

Several studies have reported that these β-ARs are expressed in various kinds of cancers, and β-adrenergic signaling is an important factor that promotes tumor progression [15–17]. Epidemiologic studies have also shown that the use of β-AR antagonists (β-blockers) results in lower recurrence, progression, or mortality of cancer [18–20]. Propranolol is a non-selective β-blocker widely used for the treatment of hypertension, arrhythmia, and angina. Recent investigations have demonstrated that propranolol has anti-proliferative, cytotoxic, and anti-angiogenic effects in various kinds of cancer [21–24]. Léauté-Labrèze et al. reported that oral administration of propranolol is safe and effective for the treatment of large infantile hemangiomas [25, 26]. De Giorgi et al. also reported that oral administration of propranolol improves progression-free survival without any adverse events in patients with melanoma [27].

Zhang et al. have previously reported that propranolol blocked stress-induced enhancement of tumor progression/metastasis in gastric cancer [28]. In this study, tumors transplanted in mice that were partially restricted in food and water showed activation of β2-adrenergic signals and these stresses could promote the growth of gastric cancer xenografts. Furthermore, propranolol could inhibit this effect. However, it remains unclear whether propranolol inhibits the progression of gastric cancer without chronic stress. We aimed to investigate the anti-tumor effects of propranolol and examine its mechanisms of action on gastric cancer.

## Materials and methods

### Cell lines

We used two gastric cancer cell lines, namely, MKN45 and NUGC3. Both cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) and 100 U/mL penicillin and 100 μg/mL streptomycin (Life Technologies, Carlsbad, CA, USA) at 37 °C, under a humidified atmosphere of 5% $CO_2$.

### Cell proliferation assay

Cell proliferation assays were performed using MKN45 and NUGC3 cell lines. Cells were seeded in 96-well plates at a density of $3 \times 10^3$ cells per well and incubated for 24 h. Then, cells were incubated with various concentrations of (1)

**Fig. 1** Effects of β2-AR signaling on proliferation of gastric cancer ▶ cells. **a** Western blot analysis of β1-AR and β2-AR in gastric cancer cell lines. **b** Cell proliferation was determined via WST-8 assays at 24 h after incubation with isoproterenol and 48 h after propranolol. Each value represents the mean ± SD. Statistical analysis was performed using the unpaired Student's *t* test (*p < 0.01). **c** Cell proliferation was determined using WST-8 assays at 48 h after incubation with bisoprolol and ICI-118, 551. Each value represents the mean ± SD. Statistical analysis was performed using the unpaired Student's *t* test (*p < 0.01). **d** Western blot analysis of β2-AR in MKN45 cell lines transfected with β2-AR siRNA (Si-β2-AR) and negative control (N.C.). **e** Cell proliferation was determined using WST-8 assays on MKN45 cell lines transfected with siRNAs at 24 h after incubation with isoproterenol. Each value represents the mean ± SD. Statistical analysis was performed using the unpaired Student's *t* test (*p < 0.01). **f** Western blot analysis of p-MEK/MEK, p-ERK/ERK, p-CREB/CREB, p-ATF2/ATF2 in MKN45 and NUGC3 cell lysates at 24 h after incubation with isoproterenol. **g** Western blot analysis of p-MEK/MEK, p-ERK/ERK, p-CREB/CREB, and p-ATF2/ATF2 in MKN45 and NUGC3 cell lysates at 24 h after incubation with propranolol

isoproterenol hydrochloride (β-AR agonist, Tokyo Chemical Industry, Tokyo, Japan) for 24 h, (2) propranolol hydrochloride (β-AR non-selective antagonist, Sigma-Aldrich, St. Louis, MO, USA) for 48 h, (3) bisoprolol hemifumarate (β1-AR selective antagonist, Tokyo Chemical Industry, Tokyo, Japan) for 48 h, and (4) ICI-118, 551 hydrochloride (β2-AR selective antagonist, Tocris Bioscience, Bristol, UK) for 48 h.

Cell proliferation was evaluated using WST-8 [2-(2-methoxy-4-nitro-phenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] assays (Cell Counting Kit-8; DOJINDO LABORATORIES, Kumamoto, Japan). The absorption of WST-8 was measured at a wavelength of 450 nm using a microplate reader (iMark; Bio-Rad Laboratories, Hercules, CA, USA). The growth rate was expressed as the percentage of absorbance for treated cells versus that for control cells. Experiments were performed with six replicate wells for each sample.

### Small-interfering RNA (siRNA) transfection

siRNAs (negative control siRNA and β2-AR siRNA) were purchased from Life Technologies (Carlsbad, CA, USA). MKN45 gastric cancer cells were seeded in a 6-well plate with antibiotic-free RPMI 1640 medium with 10% FBS. The next day, the cells were transfected with the siRNAs using Lipofectamine 3000 Reagent and Opti-MEM Reduced Serum Medium (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

### Western blot analysis

For Western blot analysis, cells were seeded into 6-well plates at a density of $2 \times 10^5$ cells per well and incubated for 48 h. We extracted proteins using protease and phosphatase

**a**



**b**



**c**
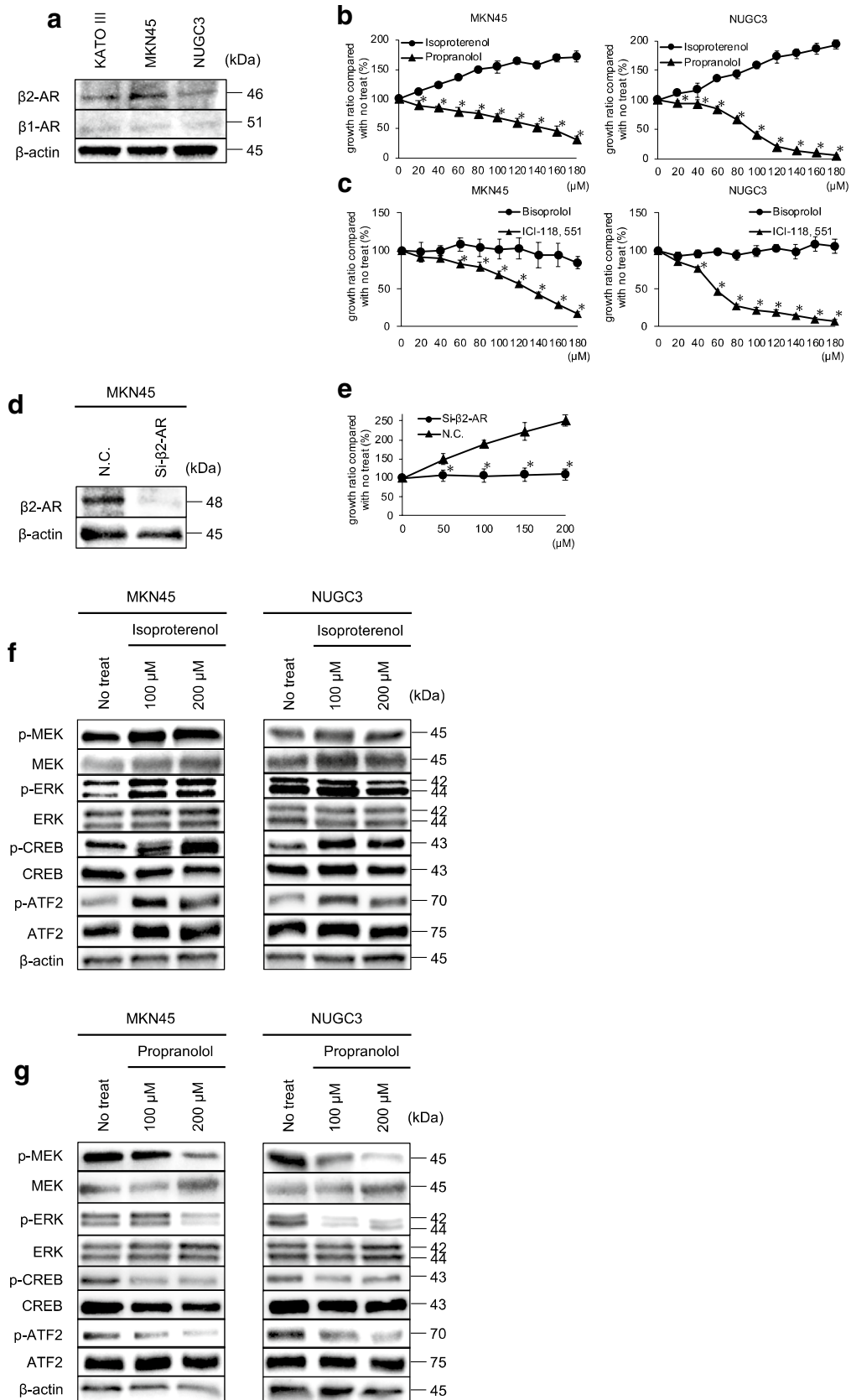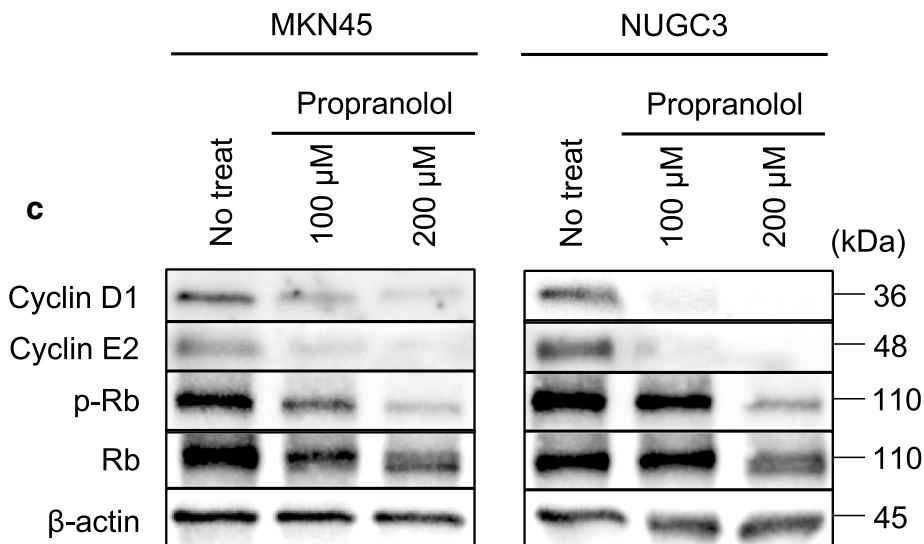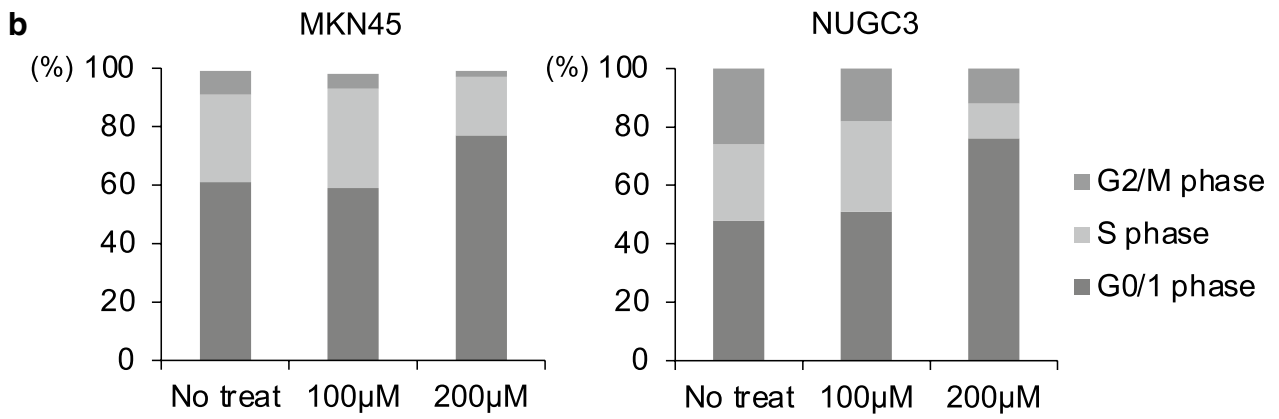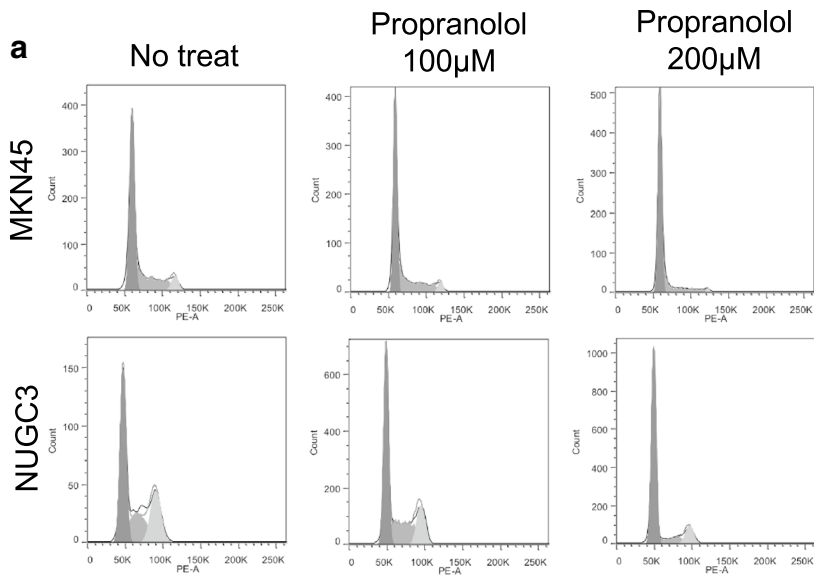
◄**Fig. 2** Effects of propranolol on cell cycle in gastric cancer cells. **a** Representative graphs of flow cytometry analysis of MKN45 and NUGC3 cell cycle using PI staining at 24 h after incubation with propranolol. **b** The relative proportion of cells in each of the G0/1, S, and G2/M phase from **a** is shown in this graph. **c** Western blot analysis of cyclin D1, cyclin E2, and p-Rb/Rb in MKN45 and NUGC3 cell lysates at 24 h after incubation with propranolol

inhibitors in RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA) from MKN45 and NUGC3 cells lines and tumor tissues harvested from the xenograft mouse model. Proteins were resolved using Mini-PROTEAN TGX Precast Gel (Bio-Rad Laboratories, Hercules, CA, USA), transferred onto Immun-Blot PVDF Membrane (Bio-Rad Laboratories, Hercules, CA, USA), and incubated with primary antibodies at 4℃ overnight. After incubation with secondary antibodies, signals were detected with the ECL Prime Western Blotting Detection reagent (GE Healthcare Bioscience, Piscataway, NJ, USA). The following antibodies were used: anti-β1-AR and anti-β2-AR (1:1000 dilution) from Abcam (Cambridge, UK); anti-phospho-MEK1/2 (Ser217/221), anti-MEK1/2, anti-phospho-p44/42 MAPK (Thr202/Tyr204), anti-p44/42 MAPK, anti-phospho-CREB (Ser133), anti-CREB, anti-phospho-ATF-2 (Thr71), anti-ATF-2, anti-Cyclin D1, anti-Cyclin E2, anti-phospho-Rb (Ser807/811), anti-Rb, anti-cleaved PARP, anti-cleaved caspase-3, and anti-cleaved caspase-6 (1:1000 dilution) from Cell Signaling Technology (Danvers, MA, USA); and anti-matrix metalloproteinase (MMP)-2, anti-MMP-9, and anti-VEGF (1:100 dilution) from Santa Cruz Biotechnology (Santa Cruz, Dallas, TX, USA).

## Cell cycle assay

For cell cycle analysis, cells were incubated for 24 h with propranolol and fixed with 70% ethanol. After centrifugation, cells were stained with 50 mg/ml propidium iodide (PI) solution (Dojindo Laboratories, Kumamoto, Japan) and 0.1 mg/ml RNase A (Invitrogen, Carlsbad, CA, USA). The cells were analyzed using flow cytometry on BD FACSCanto II (BD, Franklin Lakes, NJ, USA). Each histogram was constructed using data from at least 5,000 events. Flow cytometry data were analyzed using FlowJo software (Digital Biology, Tokyo, Japan).

## Apoptosis assay

An annexin V-FITC apoptosis detection kit (BD, Franklin Lakes, NJ, USA) was used. First, cells were incubated for 24 h with propranolol. Cell suspension (100 μl) was mixed with 5 μl annexin V-FITC and 2.5 μl PI and left for 30 min at 37℃ in the dark. Samples were then analyzed using flow cytometry on BD FACSCanto II (BD, Franklin Lakes, NJ, USA). Cells stained with annexin V were considered as

apoptotic cells. Flow cytometry data were analyzed using FlowJo software (Digital Biology, Tokyo, Japan).

## Wound healing assay

A wound healing assay was performed using MKN45 gastric cancer cells. Cells were seeded in 6-well plates and incubated until confluent. Linear scratch wounds were made in the cell monolayer using a pipette tip. We added 200 μM propranolol into each well and incubated the cells in RPMI 1640 medium supplemented with 0.1% FBS. Every 24 h after scratching, we evaluated the percent reduction of the scratched area using the scientific image-analysis program ImageJ (National Institutes of Health, Bethesda, MD, USA). A total of five samples were used for each experiment.

## Animal experiments

All animal experiments were conducted in accordance with the guidelines approved by Osaka University. We purchased BALB/cAJcl-nu/nu nude mice from CLEA Japan Inc. (Tokyo, Japan) and implanted MKN45 gastric cancer cells. For cell inoculation, $1.0 \times 10^6$ cells were injected subcutaneously into the back of the mice. When the tumor volume reached approximately 50 mm$^3$, the mice were randomized to three groups. The no-treatment group was administered intraperitoneally (i.p.) with phosphate-buffered saline (PBS) whereas the other groups were administered with different doses of propranolol (20 mg/kg/day and 40 mg/kg/day) for 2 weeks (Fig. 4a). Tumor sizes and body weight were measured every 3 days throughout the study. We determined tumor volumes by measuring the length (L) and width (W) and calculated it as (W2×L)/2.

## Immunohistochemistry

Mice were sacrificed on day 14, and tumors were resected. The tumors were fixed in formalin and embedded in paraffin for immunohistochemical analysis using anti-Ki67 antibodies (Abcam, Cambridge, UK). Terminal dUTP nick-end labeling (TUNEL) assays (with DAPI Fluorescein In Situ Apoptosis Detection Kit [Chemicon International, Temecula, CA, USA]) were performed according to the manufacturer's instructions.

## Immunohistochemical staining of β2-AR

We prepared 3.5-μm-thick sections of the resected specimens from formalin-fixed paraffin-embedded blocks. These were deparaffinized with xylene and then rehydrated with multistep descending concentrations of ethanol. The sections were autoclaved in citrate buffer at 115℃ for 20 min, immersed in 0.3% hydrogen peroxide to block endogenous peroxidase,

**a**



**b**



**c**



**d**
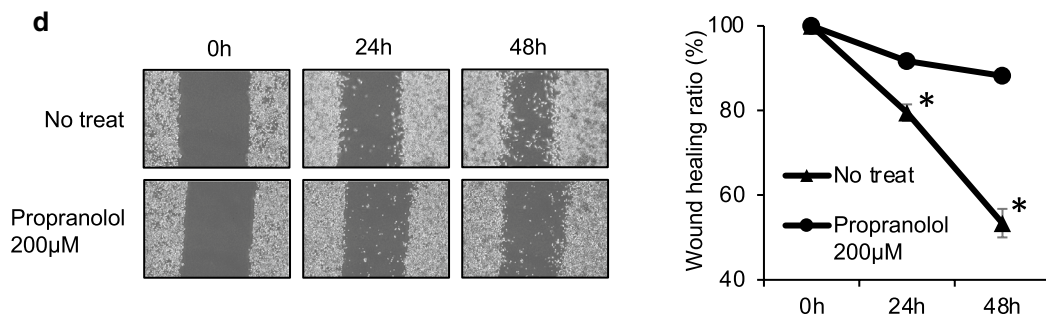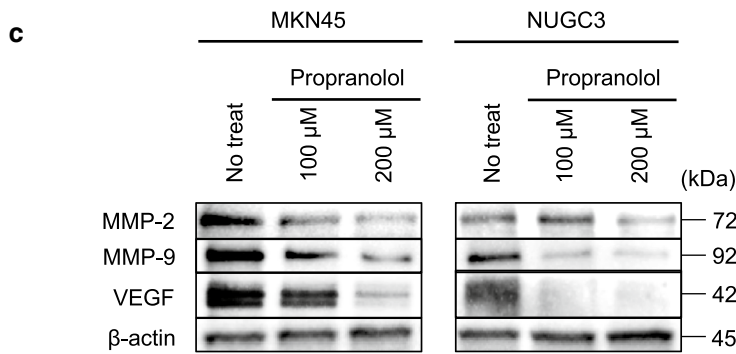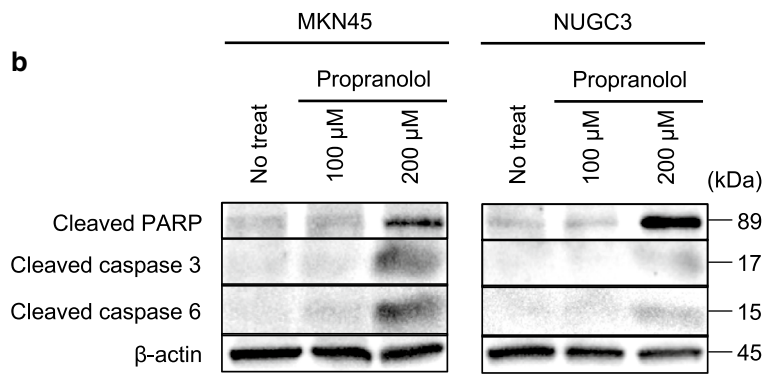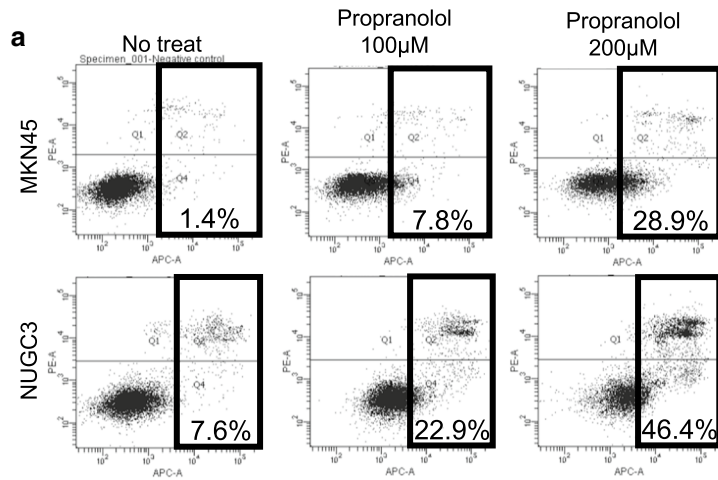
◄**Fig. 3** Effects of propranolol on apoptosis, cell migration, invasion, and angiogenesis in gastric cancer cells. **a** Representative graphs of flow cytometry analysis of MKN45 and NUGC3 apoptosis using annexin V-FITC and PI double staining at 24 h after incubation with propranolol. **b** Western blot analysis of cleaved PARP, cleaved caspase-3, and cleaved caspase-9 in MKN45 and NUGC3 cell lysates at 24 h after incubation with propranolol. **c** Western blot analysis of MMP-2, MMP-9, and VEGF in MKN45 cell lysates at 24 h after incubation with propranolol. **d** Wound healing assay was performed on MKN45 cell lines treated with propranolol to determine cell migration ability. Wounds were evaluated at 0, 24, and 48 h after treatment. Wound healing area was measured by calculating the scratched area in each period. **e** The percentage reduction of the scratched area by propranolol in comparison to that in the control is shown at the indicated times

and incubated in horse serum for 20 min to avoid nonspecific staining. The slides were incubated with anti-β2-AR (1:100 dilution, Abcam, Cambridge, UK) overnight at 4℃, with avidin–biotin-peroxidase complex (VECTASTAIN Elite ABC HRP Kit, Vector Laboratories, Burlingame, CA, USA) for 20 min, and with 3,3′-diaminobenzidine tetrahydrochloride (DAB Tablet, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) for 3.5 min to visualize the reactions with β2-AR. β2-AR expression was considered positive in each cell only if distinct cytoplasmic and plasma membrane staining was present. β2-AR expression was scored as follows: I, < 25% of cells in tumor area stained; II, 25–50% stained; III, 51–75% stained; and IV, > 75% stained. Tumors with staining score ≥ III were defined as high β2 expression tumors. Five fields (× 400) were analyzed to determine the frequency of β2-AR-positive cells, based on a previous study [29].

## Patients and tissue samples

We collected 162 consecutive patients with cStage I-III gastric cancer who underwent curative resection (R0) between January 2012 and December 2013. From all patients, written informed consent was obtained. We analyzed primary gastric cancer using by the resected specimens from formalin-fixed paraffin-embedded blocks. We used the 14th edition of the Japanese classification of gastric carcinoma to determine the pathological stage [30]. The present study was approved by the Institutional Review Board of Osaka University Hospital (approval number: 18227).

## Statistical analysis

Data are shown as the mean ± standard deviation (SD) for in vitro and in vivo experiments. Clinical data are shown as the median (range). Statistical analysis was performed using the unpaired Student's t-test for single comparisons

and the Tukey–Kramer HSD test for multiple comparisons. Overall survival (OS) was defined as the time from the date of surgery to the date of death from any cause. Recurrence-free survival (RFS) was defined as the time from the date of surgery to either the date of recurrence or death from any cause. OS and RFS were estimated using the Kaplan–Meier method and compared using the log-rank test. Cox proportional hazards models were used for both univariate and multivariate analyses to identify independent predictors of OS and RFS. A univariate analysis was first performed to identify any potential predictor variables (sex, age, T factor, N factor, Histology and β2-AR IHC expression). Variables with a p value < 0.05 according to a univariate analysis were included in the multivariate analysis. The hazard ratios and corresponding 95% confidence intervals (CIs) were calculated to show the effect of factors on OS and RFS. Two-sided P values < 0.05 were considered significant. All analyses were performed using the JMP software, version 13.0 (SAS Institute, Cary, NC, USA).
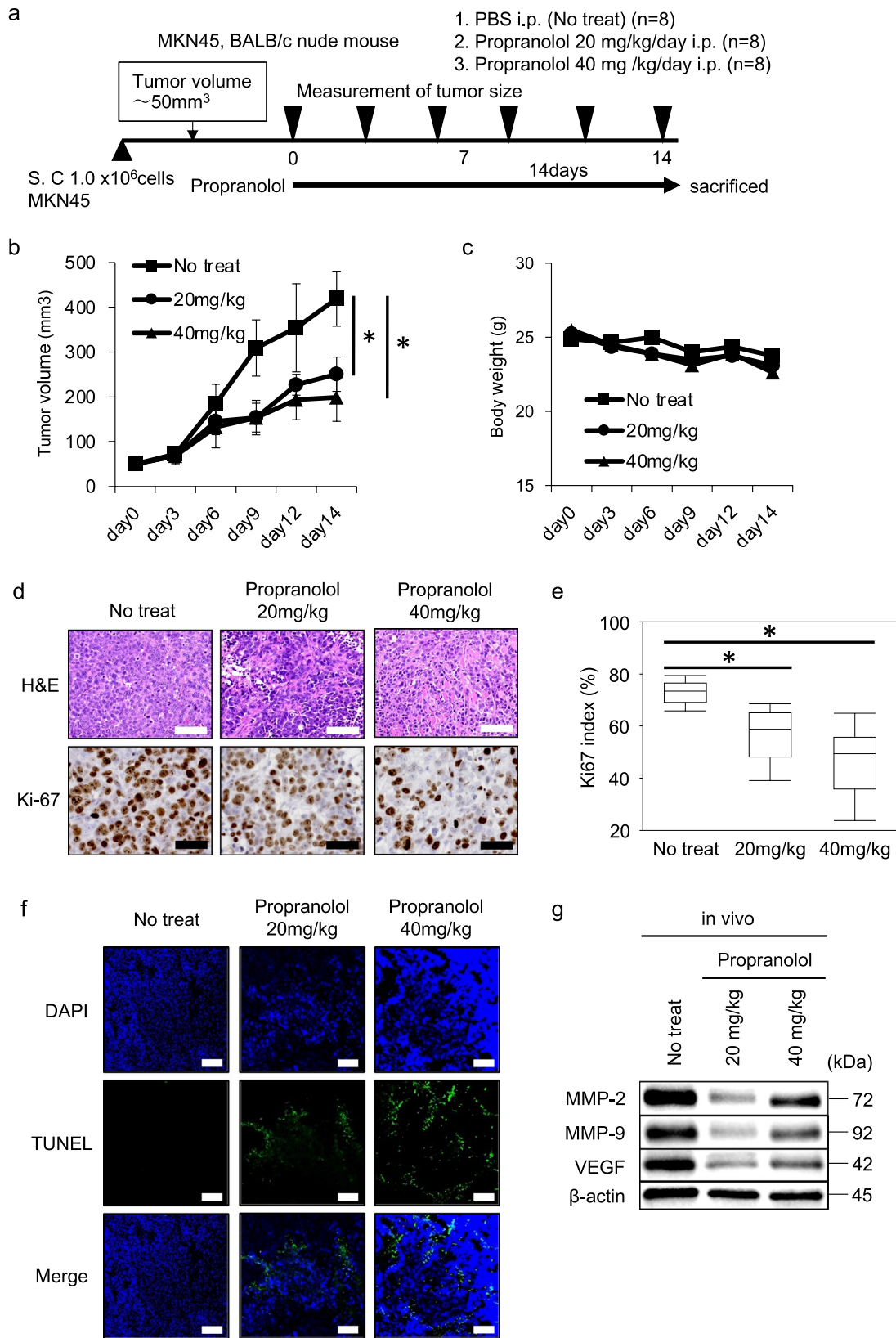
## Results

### *Effects of β2-AR signaling on proliferation of gastric cancer* in vitro

In all cell lines, we confirmed β2-AR expression by western blot analysis (Fig. 1a). Propranolol inhibited the proliferation of MKN45 and NUGC3 cell lines in a dose-dependent manner; isoproterenol stimulated the proliferation of the same cell lines (Fig. 1b). ICI-118, 551 suppressed the viability of MKN45 and NUGC3 cell lines, whereas bisoprolol had no significant effect, even at relatively high concentrations (Fig. 1c).

Next, we used siRNA-mediated RNAi, which suppresses β2-AR expression, to determine the role of β2-AR in gastric cancer in MKN45 cell lines. β2-AR expression was reduced at the protein levels by siRNA-mediated knockdown of β2-AR (Fig. 1d). Compared with the negative control, isoproterenol did not stimulate the proliferation of β2-AR suppressed cell lines (Fig. 1e).

We examined the expression of phosphorylated MEK-ERK and CREB-ATF pathways in MKN45 and NUGC3 cell lines (Fig. 1f, g). Isoproterenol increased phosphorylated MEK (pMEK) and ERK (pERK) levels. In contrast, propranolol decreased pMEK and pERK levels. Moreover, phosphorylated CREB and ATF2 levels, as well as the expression of phosphorylated MEK-ERK pathways, were increased by isoproterenol and decreased by propranolol.

a

MKN45, BALB/c nude mouse

1. PBS i.p. (No treat) (n=8)
2. Propranolol 20 mg/kg/day i.p. (n=8)
3. Propranolol 40 mg /kg/day i.p. (n=8)

Tumor volume
∼50mm³          Measurement of tumor size

▼     ▼     ▼     ▼     ▼     ▼

0              7          14days          14

S. C 1.0 ×10⁶cells     Propranolol                                    sacrificed
MKN45

b



c



d



e



f



g

Propranolol had anti-tumor effects in vivo. **a** Gastric cancer cell line xenograft mouse model; BALB/cAJcl-nu/nu mice were injected with $1.0 \times 10^6$ cells of MKN45. When the tumor volume reached approximately 50 mm³, the mice were administered intraperitoneally with PBS or propranolol at different doses (20 mg/kg and 40 mg/kg) for 2 weeks. **b** Tumor sizes were measured every 3 days. Statistical analysis was performed using the Tukey–Kramer HSD test (*$p < 0.01$). **c** Body weight was measured every 3 days. Statistical analysis was performed using the Tukey–Kramer HSD test. **d** Immunohistochemical analysis of H&E and Ki-67 staining in MKN45 xenograft mouse-derived tissue from PBS and propranolol-administered mice. **e** Ki-67 index was recorded as the ratio of positively stained cells to all tumor cells in five fields ($\times 200$). Statistical analysis was performed using the Tukey–Kramer HSD test (*$p < 0.01$). **f** Analysis of apoptosis using TUNEL staining (blue fluorescence, DAPI staining; green fluorescence, TUNEL staining) in MKN45 xenograft mouse-derived tissue from PBS and propranolol-administered mice (scale bar: black = 50 μm, white = 100 μm). **g** Western blot analysis of MMP-2, MMP-9, and VEGF in MKN45 xenograft mouse-derived tissue from PBS and propranolol-administered mice

## Mechanism of propranolol suppressing gastric cancer growth

To evaluate the effect of the cell cycle of propranolol in gastric cancer, we used flow cytometry and PI staining. Propranolol at 200 μM increased the proportion of cells in G0/1 phase in MKN45 and NUGC3 cell lines, indicating G1-phase cell cycle arrest (Fig. 2a, b). Propranolol downregulated the expression of cyclin D1, E2, and phosphorylated Rb, which regulate cell cycle G1 checkpoint (Fig. 2c).

Next, we used flow cytometry and V-FITC/PI double staining to quantitatively measure the rate of apoptosis. We found that 100 and 200 μM propranolol increased the rate of apoptosis in MKN45 and NUGC3 cell lines (Fig. 3a). In addition, the expression of cleaved PARP, cleaved caspase-3, and cleaved caspase-6 were increased by propranolol (Fig. 3b). Furthermore, propranolol suppressed the expression of MMP-2, MMP-9, and VEGF (Fig. 3c). Wound healing assay showed that propranolol inhibited gastric cancer cell migration in MKN45 cell lines (Fig. 3d, e).

### *Anti-tumor effects of propranolol* in vivo

To evaluate the anti-tumor effects of propranolol, we used a MKN45 xenograft mouse model (Fig. 4a). Propranolol significantly inhibited tumor growth via i.p. at 20 mg/kg/day and 40 mg/kg/day ($p < 0.01$ and $< 0.01$, respectively) (Fig. 4b). There was no significant difference in therapeutic effect between the doses of 40 mg/kg/day and 20 mg/kg/day.

To analyze the side effect of propranolol in this model, we checked body weight. Body weight loss after propranolol treatment was not observed (Fig. 4c).

Ki-67, a proliferation marker, was significantly decreased in the treatment groups compared with the nontreatment PBS group ($p < 0.01$ and $< 0.01$, respectively) (Fig. 4d and 4e). Moreover, compared with the nontreatment PBS group, TUNEL staining, which showed cellular apoptosis, was induced in the treatment groups ($p < 0.01$ and $< 0.01$, respectively) (Fig. 4f). As shown in Fig. 4g, propranolol suppressed the expression of MMP-2, MMP-9, and VEGF.

## Immunohistochemical analysis of β2-AR expression in gastric cancer patients

β2-AR immunostaining was evident in gastric cancer cells and was localized predominantly on the cytoplasmic and plasma membranes (Fig. 5a). β2-AR was highly expressed in 58.6% (95/162) patients. The patient's demographic characteristics by β2-AR expression are shown in Table 1. High β2-AR expression was significantly associated with T factor, N factor, venous invasion, and tumor stage. Kaplan–Meier analysis of OS and RFS according to β2-AR expression is shown in Fig. 5b. There were significant differences in both OS and RFS between patients with high and low β2-AR expression. In univariate analysis, T factor, N factor, and β2-AR expression were significantly related to poor RFS (Table 2). In addition, age, T factor, N factor, and β2-AR expression were significantly related to poor OS (Supplementary Table S1). Multivariate analysis confirmed that T factor, N factor, and β2-AR expression were independent prognostic factors of poor RFS. Meanwhile, age and N factor were a significant predictor of poor OS.

## Discussion

The anti-tumor effects of β-blockers in gastric cancer are still unclear. We found that β-blockers, particularly propranolol, inhibited the proliferation of gastric cancer cell lines. In MKN45 and NUGC3 cell lines, isoproterenol exhibited a concentration-dependent growth-facilitative effect. Propranolol and ICI-118, 551 exhibited a concentration-dependent growth-inhibitory effect, while bisoprolol had no anti-tumor effect.

The localization and function of β-AR vary depending on β-AR subtypes. β1-AR and β2-AR are highly expressed in cardiac tissue, but they play different roles in cardiac physiology and pathology [31]. Several studies have reported that both β1-AR and β2-AR are expressed in gastric cancer cell lines [32, 33]. β2-AR, but not β1-AR, signaling pathways are important for cancer development and progression [34–36]. Thaker PH et al. showed that chronic stress results in higher levels of tissue catecholamines, greater tumor growth of ovarian carcinoma cells. These effects are mediated primarily through activation of the tumor cell cAMP signaling pathway by β2-AR [37]. The present study found that β-AR was expressed in

**a**



I: <25%   II: 25-50%   III: 51-75%   IV: >75%

**b**
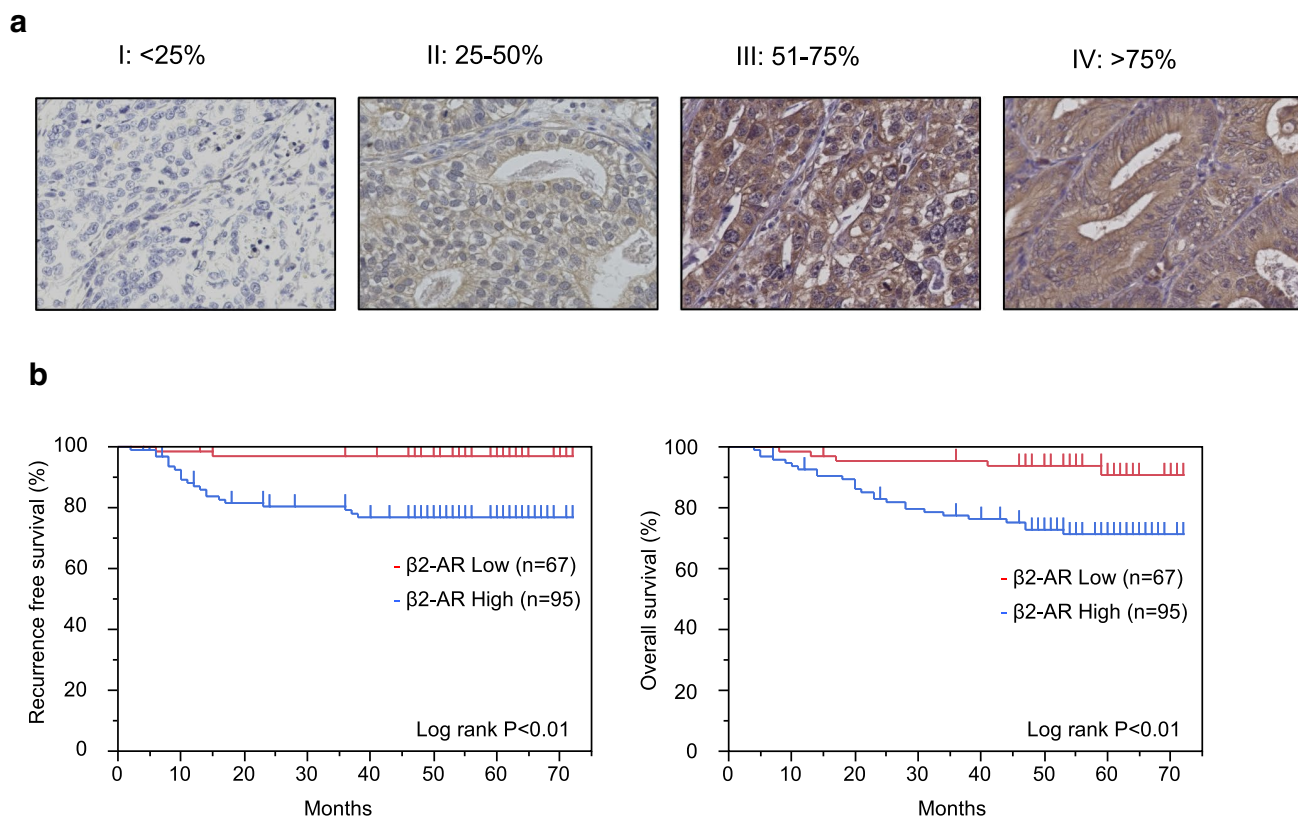


Fig. 5 Expression and significance of β2-AR in gastric cancer lesions. **a** Immunohistochemical staining of β2-AR in gastric cancer. Positive staining is evident on the cytoplasmic and plasma membranes of cancer cells. β2-AR immunostaining expression was scored I, II, III, and IV by evaluating the tumor area stained. **b** Survival outcomes after surgical resection according to β2-AR expression as shown using Kaplan–Meier analysis of overall survival (OS) and recurrence-free survival (RFS). There were significant differences in both OS and RFS between patients with high and low β2-AR expression

**Table 1** Clinicodemographic patient characteristics according to β2-AR expression

|  | β2-AR Low (n=67) | β2-AR High (n=95) | p value |
|---|---|---|---|
| Sex<br>Male/female | 46/21 | 62/33 | 0.736 |
| Age<br><70 years/≥70 years | 56/11 | 68/27 | 0.749 |
| Stage<br>I/II/III/IV | 48/13/6/0 | 50/19/26/0 | 0.010 |
| T factor<br>T1/T2/T3/T4 | 48/4/12/3 | 48/8/20/19 | 0.015 |
| N factor<br>N0/N1/N2/N3 | 55/7/2/3 | 53/20/10/12 | <0.01 |
| Histology<br>por, sig/others | 32/35 | 55/40 | 0.263 |
| Venous invasion<br>V0/V1 | 60/7 | 67/28 | <0.01 |
| Lymphatic invasion<br>Ly0/Ly1 | 39/28 | 44/51 | 0.153 |

gastric cancer cell lines, and β2-AR knockdown weakened isoproterenol`s growth-facilitative effect in MKN45 cell lines. These results show that β2-AR signaling pathways are important for gastric cancer proliferation.

In clinical practice, propranolol is a β-blocker used for hypertension, arrhythmia, and other illness. Thus, we used propranolol for in vitro and in vivo assays. We found that propranolol inhibited the β-adrenergic signaling pathway.

**Table 2** Univariate and multivariate analyses of influencing factors of recurrence-free survival of the gastric cancer patients

| | Univariate analysis | | Multivariate analysis | |
| --- | --- | --- | --- | --- |
| | Hazard ratio (95% CI) | *p* value | Hazard ratio (95% CI) | *p* value |
| Sex (male) | 0.64 (0.28–1.51) | 0.303 | | |
| Age ($\geq$ 70 years) | 1.36 (0.59–3.10) | 0.464 | | |
| T factor (T2-4) | 11.21 (3.84 – 47.60) | < 0.001 | 4.08 (1.12–20.06) | 0.031 |
| N factor (N1-3) | 11.21 (4.21 – 38.68) | < 0.001 | 3.70 (1.17–15.16) | 0.024 |
| Histology (por, sig) | 1.22 (0.54 – 2.82) | 0.628 | | |
| β2-AR (High) | 8.20 (2.41–51.28) | < 0.001 | 4.71 (1.35–29.72) | 0.011 |

Ligation of β-AR by norepinephrine and epinephrine stimulates adenylyl cyclase synthesis of cAMP [38]. Creed et al. reported that isoproterenol increases cAMP accumulation, and this effect is blocked by propranolol [39]. Two major downstream signaling molecule of cAMP are protein kinase A (PKA) and exchange protein directly activated by cAMP (EPAC). PKA induces the phosphorylation of transcription factors such as CREB-ATF that enhance cell proliferation, angiogenesis, and migration [40]. Meanwhile, EPAC induces the phosphorylation of MEK-ERK pathway that is related to cell proliferation, apoptosis, and immune escape [41].

Propranolol induced G1-phase cell cycle arrest and caused apoptosis in MKN45 and NUGC3 cell lines. Liao et al. previously reported that propranolol might induce gastric cancer cell apoptosis and cell cycle arrest [32]. We found that propranolol decreased the expression of cyclin D1 and cyclin E2, and phosphorylated Rb, which is involved in cell cycle G1/S-phase transition. Propranolol also increased the expression of cleaved PARP and caspase-3 and caspase-6, which are key proteins in apoptosis [42]. In this study, we confirmed that propranolol induced G1-phase cell cycle arrest and apoptosis in gastric cancer cell lines.

Cancer invasion, migration, and angiogenesis are important processes for both cancer growth and metastasis. MMPs and VEGF play a significant role in these processes [43, 44]. We found that propranolol suppressed the expression of MMP-2 and MMP-9 and VEGF, inducing inhibition of cell invasion, migration, and angiogenesis. Collectively, these data suggest that propranolol has a therapeutic significance for targeting metastasis.

Moreover, we found that propranolol had significant antitumor effects against the MKN45 xenograft mouse model. In both treatment groups, propranolol significantly inhibited tumor growth. Ki-67 and TUNEL staining revealed that propranolol inhibited proliferation and induced apoptosis in vivo. Optimal inhibition of tumor growth was obtained by intraperitoneal injection of 20 mg/kg/day of propranolol, and there was no difference in tumor growth between the dose of 20 mg/kg/day and 40 mg/kg/day. Maccari et al. showed that propranolol inhibited tumor growth in a U-shaped biphasic manner in the melanoma xenograft mouse model. They speculated that it caused systemic vasoconstriction or vasodilation depending on the dose and alters tumor perfusion and growth. And they also reported that 20 mg/kg/day of propranolol had no effect on mean arterial blood pressure (from $72 \pm 2$ to $71 \pm 2$ mmHg), but it reduced the heart rate (from $417 \pm 16$ to $356 \pm 12$ mmHg) [45]. In our study, we did not observe any symptomatic adverse events associated with propranolol treatment, and no significant body weight loss was noted.

Finally, we showed that β2-AR expression was significantly related to poor RFS and OS. Significant relationships were found between high β2-AR expression and venous invasion, while there was no correlation between high β2-AR expression and lymph invasion or histological type. That might indicate that High β2-AR expression was an independent prognostic factor of recurrence by the vessel invasion. This poor prognostic group may be a good candidate for β-blocker therapy. Collectively, these findings indicate that to assess the therapeutic effect of β-blockers for human cancer, we should consider the expression of β2-AR in the primary cancer lesion.

There are several limitations to this study. First, we did not investigate the combined effect of propranolol and other therapies. Although Liao et al. reported that propranolol has a definite radiotherapy sensitization effect on gastric cancer [33, 46], few reports are available on the combined effect of propranolol and chemotherapies. Because some anti-cancer agents and molecule-targeting therapeutic agents have mechanisms of action similar to that of propranolol, it is necessary to evaluate the synergistic effect of propranolol and anti-cancer agents or molecule-targeting therapeutic agents. Second, we did not investigate the human equivalent dose of propranolol in gastric cancer patients. We only showed that intraperitoneal injection of propranolol at 20 mg/kg/day significantly inhibited tumor growth in a MKN45 xenograft mouse model, and thus, the human equivalent dose is still unclear. Third, we could not analyze the relationship between the administration of β-blockers and patient outcomes, because the

number of patients receiving β-blocker was too small in this retrospective population. Propranolol has anti-tumor effects as a repurposed drug, and the cost and risk of drug development are lower than that of new drug development [47]. Thus, prospective studies with large patient cohorts are required to further investigate the anti-tumor effects of propranolol for gastric cancer.

In conclusion, our results showed that propranolol significantly inhibited the proliferation of gastric cancer cells and reduced the growth of gastric cancer tumors in a xenograft mouse model. Propranolol induced G1-phase cell cycle arrest and apoptosis. These findings indicate that propranolol might have an opportunity as a new drug for gastric cancer.

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## Declarations

## References

1. Ferlay J, Colombet M, Soerjomataram I, Mathers C, Parkin DM, Piñeros M, et al. Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods. Int J Cancer. 2019;144(8):1941–53.
2. Colvin H, Mizushima T, Eguchi H, Takiguchi S, Doki Y, Mori M. Gastroenterological surgery in Japan: the past, the present and the future. Ann Gastroenterol Surg. 2017;1(1):5–10.
3. Irino T, Takeuchi H, Terashima M, Wakai T, Kitagawa Y. Gastric cancer in Asia: unique features and management. Am Soc Clin Oncol Educ Book. 2017;37:279–91.
4. Matsuoka T, Yashiro M. Biomarkers of gastric cancer: current topics and future perspective. World J Gastroenterol. 2018;24(26):2818–32.
5. Madamanchi A. Beta-adrenergic receptor signaling in cardiac function and heart failure. Mcgill J Med. 2007;10(2):99–104.
6. Xiao RP. Beta-adrenergic signaling in the heart: dual coupling of the beta2-adrenergic receptor to G(s) and G(i) proteins. Sci STKE. 2001;2001(104):re15.
7. He JJ, Zhang WH, Liu SL, Chen YF, Liao CX, Shen QQ, et al. Activation of β-adrenergic receptor promotes cellular proliferation in human glioblastoma. Oncol Lett. 2017;14(3):3846–52.
8. Minneman KP, Pittman RN, Molinoff PB. Beta-adrenergic receptor subtypes: properties, distribution, and regulation. Annu Rev Neurosci. 1981;4:419–61.
9. Singh K, Zaw AM, Sekar R, Palak A, Allam AA, Ajarem J, et al. Glycyrrhizic acid reduces heart rate and blood pressure by a dual mechanism. Molecules. 2016;21(10):1291.
10. Granneman JG. The putative beta4-adrenergic receptor is a novel state of the beta1-adrenergic receptor. Am J Physiol Endocrinol Metab. 2001;280(2):E199-202.
11. Lorton D, Bellinger DL. Molecular mechanisms underlying β-adrenergic receptor-mediated cross-talk between sympathetic neurons and immune cells. Int J Mol Sci. 2015;16(3):5635–65.
12. Kolmus K, Tavernier J, Gerlo S. β2-Adrenergic receptors in immunity and inflammation: stressing NF-κB. Brain Behav Immun. 2015;45:297–310.
13. Kim TJ, Sun J, Lu S, Zhang J, Wang Y. The regulation of β-adrenergic receptor-mediated PKA activation by substrate stiffness via microtubule dynamics in human MSCs. Biomaterials. 2014;35(29):8348–56.
14. Wallukat G. The beta-adrenergic receptors. Herz. 2002;27(7):683–90.
15. Shang ZJ, Liu K, Liang DF. Expression of beta2-adrenergic receptor in oral squamous cell carcinoma. J Oral Pathol Med. 2009;38(4):371–6.
16. Gargiulo L, May M, Rivero EM, Copsel S, Lamb C, Lydon J, et al. A Novel effect of β-adrenergic receptor on mammary branching morphogenesis and its possible implications in breast cancer. J Mammary Gland Biol Neoplasia. 2017;22(1):43–57.
17. Cole SW, Sood AK. Molecular pathways: beta-adrenergic signaling in cancer. Clin Cancer Res. 2012;18(5):1201–6.
18. Powe DG, Voss MJ, Zänker KS, Habashy HO, Green AR, Ellis IO, et al. Beta-blocker drug therapy reduces secondary cancer formation in breast cancer and improves cancer specific survival. Oncotarget. 2010;1(7):628–38.
19. Barron TI, Connolly RM, Sharp L, Bennett K, Visvanathan K. Beta blockers and breast cancer mortality: a population- based study. J Clin Oncol. 2011;29(19):2635–44.
20. De Giorgi V, Grazzini M, Gandini S, Benemei S, Lotti T, Marchionni N, et al. Treatment with β-blockers and reduced disease progression in patients with thick melanoma. Arch Intern Med. 2011;171(8):779–81.
21. Coelho M, Moz M, Correia G, Teixeira A, Medeiros R, Ribeiro L, et al. Antiproliferative effects of β-blockers on human colorectal cancer cells. Oncol Rep. 2015;33(5):2513–20.
22. Jean Wrobel L, Bod L, Lengagne R, Kato M, Prévost-Blondel A, Le Gal FA, et al. Propranolol induces a favourable shift of anti-tumor immunity in a murine spontaneous model of melanoma. Oncotarget. 2016;7(47):77825–37.
23. Pasquier E, Ciccolini J, Carre M, Giacometti S, Fanciullino R, Pouchy C, et al. Propranolol potentiates the anti-angiogenic effects and anti-tumor efficacy of chemotherapy

agents: implication in breast cancer treatment. Oncotarget. 2011;2(10):797–809.

24. Landen CN Jr, Lin YG, Armaiz Pena GN, Das PD, Arevalo JM, Kamat AA, et al. Neuroendocrine modulation of signal transducer and activator of transcription-3 in ovarian cancer. Cancer Res. 2007;67(21):10389–96.

25. Léauté-Labrèze C, Hoeger P, Mazereeuw-Hautier J, Guibaud L, Baselga E, Posiunas G, et al. A randomized, controlled trial of oral propranolol in infantile hemangioma. N Engl J Med. 2015;372(8):735–46.

26. Léauté-Labrèze C, Dumas de la Roque E, Hubiche T, Boralevi F, Thambo JB, Taïeb A, et al. Propranolol for severe hemangiomas of infancy. N Engl J Med. 2008;358(24):2649–51.

27. De Giorgi V, Grazzini M, Benemei S, Marchionni N, Botteri E, Pennacchioli E, et al. Propranolol for off-label treatment of patients with melanoma: results from a cohort study. JAMA Oncol. 2018;4(2):e172908.

28. Zhang X, Zhang Y, He Z, Yin K, Li B, Zhang L, et al. Chronic stress promotes gastric cancer progression and metastasis: an essential role for ADRB2. Cell Death Dis. 2019;10(11):788.

29. Takahashi K, Kaira K, Shimizu A, Sato T, Takahashi N, Ogawa H, et al. Clinical significance of β2-adrenergic receptor expression in patients with surgically resected gastric adenocarcinoma. Tumour Biol. 2016;37(10):13885–92.

30. Japanese Gastric Cancer Association. Japanese classification of gastric carcinoma: 3rd English edition. Gastric Cancer. 2011;14:101–12.

31. Richter W, Day P, Agrawal R, Bruss MD, Granier S, Wang YL, et al. Signaling from beta1- and beta2-adrenergic receptors is defined by differential interactions with PDE4. EMBO J. 2008;27(2):384–93.

32. Liao X, Che X, Zhao W, Zhang D, Bi T, Wang G. The β-adrenoceptor antagonist, propranolol, induces human gastric cancer cell apoptosis and cell cycle arrest via inhibiting nuclear factor κB signaling. Oncol Rep. 2010;24(6):1669–76.

33. Liao X, Che X, Zhao W, Zhang D, Long H, Chaudhary P, et al. Effects of propranolol in combination with radiation on apoptosis and survival of gastric cancer cells in vitro. Radiat Oncol. 2010;5:98.

34. Zhang B, Wu C, Chen W, Qiu L, Li S, et al. The stress hormone norepinephrine promotes tumor progression through β2-adrenoreceptors in oral cancer. Arch Oral Biol. 2020;113:104712.

35. Kurozumi S, Kaira K, Matsumoto H, Hirakata T, Yokobori T, Inoue K, et al. β2-Adrenergic receptor expression is associated with biomarkers of tumor immunity and predicts poor prognosis in estrogen receptor-negative breast cancer. Breast Cancer Res Treat. 2019;177(3):603–10.

36. Yazawa T, Kaira K, Shimizu K, Shimizu A, Mori K, Nagashima T, et al. Prognostic significance of beta2-adrenergic receptor expression in non-small cell lung cancer. Am J Transl Res. 2016;8(11):5059–70.

37. Thaker PH, Han LY, Kamat AA, Avevalo JM, Takahashi R, Lu C, et al. Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma. Nat Med. 2006;12(8):939–44.

38. McGraw DW, Liggett SB. Molecular mechanisms of beta2-adrenergic receptor function and regulation. Proc Am Thorac Soc. 2005;2(4):292–6.

39. Creed SJ, Le CP, Hassan M, Pon CK, Albold S, Chan KT, et al. β2-adrenoceptor signaling regulates invadopodia formation to enhance tumor cell invasion. Breast Cancer Res. 2015;17(1):145.

40. Steven A, Seliger B. Control of CREB expression in tumors: from molecular mechanisms and signal transduction pathways to therapeutic target. Oncotarget. 2016;7(23):35454–65.

41. Peluso I, Yarla NS, Ambra R, Pastore G, Perry G. MAPK signalling pathway in cancers: Olive products as cancer preventive and therapeutic agents. Semin Cancer Biol. 2019;56:185–95.

42. Green DR. Apoptotic pathways: paper wraps stone blunts scissors. Cell. 2000;102(1):1–4.

43. Lyu Y, Xiao Q, Yin L, Yang L, He W. Potent delivery of an MMP inhibitor to the tumor microenvironment with thermosensitive liposomes for the suppression of metastasis and angiogenesis. Signal Transduct Target Ther. 2019;4:26.

44. Mahecha AM, Wang H. The influence of vascular endothelial growth factor-A and matrix metalloproteinase-2 and -9 in angiogenesis, metastasis, and prognosis of endometrial cancer. Onco Targets Ther. 2017;10:4617–24.

45. Maccari S, Buoncervello M, Rampin A, Spada M, Macchia D, Giordani L, et al. Biphasic effects of propranolol on tumour growth in B16F10 melanoma-bearing mice. Br J Pharmacol. 2017;174(2):139–49.

46. Liao X, Chaudhary P, Qiu G, Che X, Fan L. The role of propranolol as a radiosensitizer in gastric cancer treatment. Drug Des Devel Ther. 2018;12:639–45.

47. Pushpakom S, Iorio F, Eyers PA, Escott KJ, Hopper S, Wells A, et al. Drug repurposing: progress, challenges and recommendations. Nat Rev Drug Discov. 2019;18(1):41–58.