

# $\beta$ -blockers inhibit the viability of breast cancer cells by regulating the ERK/COX-2 signaling pathway and the drug response is affected by *ADRB2* single-nucleotide polymorphisms

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**Abstract.** The  $\beta_2$ -adrenergic receptor ( $\beta_2$ -AR, encoded by the *ADRB2* gene) is a member of the G-protein-coupled receptor superfamily that can be stimulated by catecholamines. Studies *in vivo* and *in vitro* have confirmed that  $\beta$ -blockers ( $\beta$ -AR antagonists) exert antitumor effects on various tumors. Furthermore, *ADRB2* single-nucleotide polymorphisms (SNPs) have been identified to alter the expression and conformation of  $\beta_2$ -AR, which may alter the  $\beta$ -blocker drug response. The aim of the present study was to investigate the effect of  $\beta$ -blockers on triple-negative breast cancer cells and determine whether *ADRB2* SNPs affect the response to  $\beta$ -blocker drugs. Propranolol and ICI 118,551 significantly inhibited the viability of MDA-MB-231 cells, arrested cell cycle progression at G<sub>0</sub>/G<sub>1</sub> and S phase and induced cell apoptosis. Western blot analysis indicated that the phosphorylation levels of extracellular-signal-regulated kinase (ERK)1/2 and the expression levels of cyclo-oxygenase 2 (COX-2) were significantly decreased following  $\beta$ -blocker treatment. Four haplotypes, which comprised *ADRB2* SNPs rs1042713 and rs1042714, were transfected into 293 cells. After 24 and 48 h of transfection, *ADRB2* mRNA expression was significantly decreased

in mutant groups compared with the wild-type group. The *ADRB2* SNPs exerted no effect on cell viability, but did affect the drug response of ICI 118,551. Furthermore, *ADRB2* SNPs also affected the regulatory function of ICI 118,551 on the ERK/COX-2 signaling pathway. Collectively, propranolol and ICI 118,551 inhibited the viability of MDA-MB-231 cells by downregulating the ERK/COX-2 signaling pathway and inducing apoptosis. The results of the present study indicated that SNPs rs1042713 and rs1042714 of *ADRB2* affected the response to ICI 118,551, and the underlying molecular mechanism was elucidated.

## Introduction

Breast cancer is the most frequently diagnosed cancer among women worldwide, and between 12 and 17% of female patients are diagnosed with triple-negative breast cancer (TNBC) (1). TNBC is characterized by the absence of estrogen receptor (ER), progesterone receptor and human epidermal growth factor receptor type 2 (HER2) expression, which means that patients with TNBC cannot be treated with endocrine therapy or therapies targeting HER2 (2-4). Notably, conventional chemotherapy remains the principal treatment for TNBC, but is associated with a high recurrence rate (5,6).

Adrenergic receptors (ARs) are members of the G-protein-coupled receptor superfamily and are divided into two principal types:  $\alpha$  and  $\beta$  (7). According to function and tissue distribution,  $\alpha$ -ARs are divided into six subtypes ( $\alpha_{1A}$ ,  $\alpha_{1B}$ ,  $\alpha_{1D}$ ,  $\alpha_{2A}$ ,  $\alpha_{2B}$  and  $\alpha_{2C}$ ), whereas  $\beta$ -ARs are divided into three subtypes ( $\beta_1$ ,  $\beta_2$  and  $\beta_3$ ) (7). Catecholamines, such as epinephrine and norepinephrine (NE), stimulate  $\beta$ -ARs and activate adenylate cyclase, thereby promoting the release of the second messenger 3'-5'-cyclic adenosine monophosphate, which regulates various downstream signal transduction pathways (8). There is evidence that  $\beta$ -AR stimulation may promote the proliferation, metastasis, invasion and angiogenesis of tumors (9,10). Notably, a proof-of-principle study and two population studies have identified that patients with breast

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**Abbreviations:** TNBC, triple-negative breast cancer; ER, estrogen receptor; HER2, human epidermal growth factor receptor type 2; NE, norepinephrine;  $\beta$ -AR,  $\beta$ -adrenergic receptor; PI, propidium iodide

**Key words:** triple-negative breast cancer,  $\beta$ -blocker,  $\beta$ -adrenergic receptor, mitogen-activated protein kinase signaling pathway,  $\beta_2$ -adrenergic receptor single-nucleotide polymorphisms

cancer who were treated with  $\beta$ -blockers ( $\beta$ -AR antagonists) exhibited a significant decrease in the incidence of metastasis, cancer recurrence and cancer-specific mortality (11-13).  $\beta$ -blockers are commonly used to treat cardiovascular diseases, including angina pectoris and hypertension (14,15). However, propranolol (a non-selective  $\beta$ -blocker) was identified to be effective in treating severe infantile hemangiomas and has been approved as the first-line therapy for proliferating infantile hemangioma by the US Food and Drug Administration in 2014 (16). A number of *in vitro* and *in vivo* studies have also demonstrated the antitumor activity of  $\beta$ -blockers against various types of cancer, including breast (17,18), pancreatic (19) and prostate cancer (20), and ovarian carcinomas (21), melanoma (22) and neuroblastoma (23). İşeri *et al* (24) identified that propranolol and ICI 118,551 (selective  $\beta_2$ -blockers) inhibited the invasion and migration of non-stimulated breast cancer cells (MCF-7) *in vitro*. In addition, Choy *et al* (25) demonstrated that propranolol treatment of triple-negative brain-metastatic cells (MDA-MB-231 Br) decreased the development of brain metastases *in vivo*.  $\beta$ -blockers have been identified to be beneficial to the survival of patients with TNBC; however, the antitumor mechanism involved remains unclear.

*In vitro* studies have confirmed that  $\beta$ -ARs were markedly expressed in the human breast adenocarcinoma cell line MDA-MB-231, with  $\beta_2$ -AR being the most prominent (26). Pharmacogenomics studies have demonstrated that the polymorphisms of *ADRB2*, which encodes  $\beta_2$ -AR, may lead to the individual differences in the treatment of asthma and cardiovascular disease (27,28). rs1042713 (A46G) and rs1042714 (C79G) are two classic non-synonymous single-nucleotide polymorphisms (SNPs) of *ADRB2* (29). SNP rs1042713 of *ADRB2* has been identified to be associated with Tumor-Node-Metastasis grade, lymph node metastasis and 1-year survival rate of pancreatic carcinoma (30). With regard to SNP rs1042714, Wang *et al* (31) identified that this polymorphism was significantly associated with the risk of lung adenocarcinoma (AC) in young subjects (aged  $\leq 50$  years). Although the association between *ADRB2* SNPs and tumor development has been reported, it remains unclear whether such SNPs affect the anticancer effects of  $\beta$ -blockers.

The aim of the present study was to investigate the effect of  $\beta$ -blockers on MDA-MB-231 cells, and determine whether SNPs rs1042713 and rs1042714 of *ADRB2* affect the response to ICI 118,551.

## Materials and methods

**Cell lines and reagents.** The human breast adenocarcinoma cell line MDA-MB-231 was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and was authenticated by short tandem repeat profiling. 293 cells were obtained from the Molecular and Biological Laboratory, Department of Clinical Pharmacology, Central South University (Changsha, China). The MDA-MB-231 cell line was cultured in Leibovitz's L-15 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a tissue culture incubator with free gas exchange with atmospheric air. The 293 cell line

was cultured in Dulbecco's modified Eagle's medium (Gibco, Thermo Fisher Scientific, Inc.) supplemented with 10% FBS at 37°C in a tissue culture incubator in an atmosphere containing 5% CO<sub>2</sub>.

Propranolol hydrochloride (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and metoprolol tartrate (Sigma-Aldrich; Merck KGaA) were dissolved in water. ICI 118,551 hydrochloride (MedChemExpress, Monmouth Junction, NJ, USA) was dissolved in dimethylsulfoxide.

**Cell viability assay.** The viability of the MDA-MB-231 cells was determined using an Alamar Blue assay (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were plated in 96-well culture plates at a density of  $1 \times 10^4$  cells/well and treated with metoprolol (25–400  $\mu$ M), ICI 118,551 (25–200  $\mu$ M) and propranolol (25–400  $\mu$ M) for 24, 48 and 72 h. The cells were incubated with Alamar Blue (10% of total volume) at 37°C for at least 4 h. The optical density (OD) was determined at 570 nm using an Eon Microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

**Cell cycle analysis.** The cell cycle phase was analyzed using propidium iodide (PI) staining with a cell cycle and apoptosis analysis kit (Beyotime Institute of Biotechnology, Haimen, China). MDA-MB-231 cells were plated in 6-well plates and treated with propranolol (100 and 200  $\mu$ M) or ICI 118,551 (100 and 150  $\mu$ M) for 24 h when grown to 70% confluence. Cells were subsequently harvested, washed twice with PBS and fixed in 70% ethanol for 24 h at 4°C. Following fixation, the cells were washed with PBS and then stained with PI staining buffer (each sample contained 25  $\mu$ l PI and 10  $\mu$ l RNase A) for 30 min at 37°C in the dark. Analyses were performed on BD Accuri™ C6 (BD Biosciences, Franklin Lakes, NJ, USA).

**Apoptosis assays.** MDA-MB-231 cells were grown to 70% confluence in 6-well plates and treated with propranolol (100 and 200  $\mu$ M) or ICI 118,551 (100 and 150  $\mu$ M) for 24 h. The cells were then harvested, washed and resuspended in binding buffer. Subsequently, the cells were double-stained with Annexin V-fluorescein isothiocyanate (FITC) and PI staining solution for 10 min at room temperature in the dark using an Annexin V-FITC Apoptosis Detection kit (Beyotime Institute of Biotechnology). The proportions of viable cells (Annexin V<sup>-</sup> and PI<sup>-</sup>), early apoptotic cells (Annexin V<sup>+</sup> and PI<sup>-</sup>) and late apoptotic cells (Annexin V<sup>+</sup> and PI<sup>+</sup>) were determined using a Cytomics FC500 flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) in 1 h.

**Western blot analysis.** Cell extracts of MDA-MB-231 cells treated with propranolol (100 and 200  $\mu$ M) or ICI 118,551 (100 and 150  $\mu$ M) for 24 h were analyzed by western blotting. The cells were lysed in radioimmunoprecipitation buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) supplemented with phenylmethylsulfonyl fluoride (Sigma-Aldrich; Merck KGaA), and SigmaFAST™ Protease Inhibitor Cocktail (Sigma-Aldrich; Merck KGaA) on ice for 30 min. Total protein concentration was quantified using a Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology). Protein samples were boiled with loading buffer (SDS-PAGE Sample Loading Buffer; Beyotime Institute of Biotechnology)

for 10 min, and then equal quantities of total protein (20  $\mu$ g) were separated by SDS-PAGE (10% gel) and then transferred on to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline with 1% Tween-20 (TBST) for 2 h at room temperature and subsequently incubated overnight with primary antibodies at 4°C. The membranes were washed three times for 30 min with TBST at room temperature, and then incubated with secondary antibodies for 1 h at room temperature. Following washing with TBST three times, the protein bands were visualized using Enhanced Chemiluminescence Prime Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, UK) and semi-quantified analysis was carried out using ImageJ software (version k 1.45, National Institutes of Health, Bethesda, MD, USA).

293 cells were transfected with vector control, wild-type *ADRB2* and mutant *ADRB2* plasmids and treated with 80  $\mu$ M ICI 118,551 for 24 h. Protein extraction and western blot analysis were performed as aforementioned.

The following primary antibodies were used: Anti-extracellular-signal-regulated kinase (ERK)1/2 (dilution 1:1,000; cat. no. 9102; Cell Signaling Technology, Inc.), anti-phospho-ERK1/2 (Thr<sup>202</sup>/Tyr<sup>204</sup>) (dilution 1:2,000; cat. no. 9101; Cell Signaling Technology, Inc.), anti-p38 (dilution 1:1,000; cat. no. 9212; Cell Signaling Technology, Inc.), anti-phospho-p38-Thr<sup>180</sup>/Tyr<sup>182</sup> (dilution 1:1,000; cat. no. 4092; Cell Signaling Technology, Inc.), anti-cyclo-oxygenase 2 (COX-2; dilution 1:1,000; cat. no. ab62331; Abcam, Cambridge, UK) anti- $\beta_2$ -AR antibody (dilution 1:1,000; cat. no. ab61778; Abcam) and GAPDH (dilution 1:10,000; cat. no. AC002; ABclonal Biotech Co., Ltd., Woburn, MA, USA). The following secondary antibodies were used: Horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG; whole molecule) (dilution 1:10,000; cat. no. A9169; Sigma-Aldrich; Merck KGaA) and anti-mouse IgG (whole molecule) (dilution 1:10,000; cat. no. A9044; Sigma-Aldrich; Merck KGaA).

**Plasmids and cell transfection.** pcDNA3.1-*ADRB2* (46AA/79CC; wild-type *ADRB2* plasmid), pcDNA3.1-*ADRB2* (46AA/79GG), pcDNA3.1-*ADRB2* (46GG/79CC) and pcDNA3.1-*ADRB2* (46GG/79GG) were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). pcDNA3.1-vector was obtained from the Molecular and Biological Laboratory, Department of Clinical Pharmacology, Central South University. 293 cells were transfected with plasmids in 6-well plates using Lipofectamine<sup>®</sup> 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay.** 293 cells were transfected with plasmids for 24, 48 and 72 h. Following transfection, total RNA was extracted from cells using TRIzol<sup>®</sup> reagent (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA was synthesized using a Transcriptor First Strand cDNA Synthesis kit (Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's protocol. qPCR was carried out using SYBR<sup>®</sup> Premix DimerEraser<sup>™</sup> (Takara Biotechnology Co., Ltd.) and a LightCycler<sup>®</sup> 480 Sequence Detection system (Roche

Diagnostics GmbH, Mannheim, Germany). Primers for target genes were as follows: *ADRB2*, 5'-TGGGCATCGTCATGTCTCTC-3' (forward) and 5'-GACGCTCGAACTTGGCAA TG-3' (reverse); and *GAPDH*, 5'-TTGATTTTGGAGGGATCTCGCTC-3' (forward) and 5'-GAGTCAACGGATTGTTGGTCGTATTG-3' (reverse). The thermocycling conditions were as follows: Pre-incubation at 95°C for 2 min; amplification at 95°C for 5 sec, 60°C for 30 sec, 72°C for 30 sec, 40 cycles; melting at 95°C for 5 sec, 65°C for 60 sec and 97°C for 1 sec. The results were calculated using the 2<sup>- $\Delta\Delta C_q$</sup>  method (32).

**MTS assay.** 293 cells were plated in 96-well culture plates and transfected with vector control, wild-type *ADRB2* and mutant *ADRB2* plasmids for 24, 48 and 72 h. Cell viability was assessed using CellTiter 96<sup>®</sup> AQueous One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI, USA). Culture medium was removed and replaced with 100  $\mu$ l FBS-free Dulbecco's modified Eagle's medium and 20  $\mu$ l MTS. Following incubation at 37°C for 1 h, the OD was determined at 490 nm using an Eon Microplate spectrophotometer. The effect of *ADRB2* SNPs on the anti-proliferative properties of  $\beta$ -blockers was also assessed using the MTS assay. Following transfection with different plasmids for 24 h, 293 cells were treated with ICI 118,551 (25-200  $\mu$ M) for 24, 48 and 72 h. The assay was performed as aforementioned.

**Statistical analysis.** Statistical analyses were performed using GraphPad Prism software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA). Differences between two groups were compared using the unpaired Student's t-test. Differences between more than two groups were compared using one-way analysis of variance followed by Tukey's multiple comparison test or Dunnett's multiple comparison test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Propranolol and ICI 118,551 inhibit MDA-MB-231 cell viability.** To determine the effects of different  $\beta$ -blockers on TNBC, MDA-MB-231 cells were treated with increasing concentrations of metoprolol, ICI 118,551 and propranolol for 24, 48 and 72 h. Cell viability was assessed using the Alamar Blue assay. Following  $\beta$ -blocker treatment, propranolol and ICI 118,551 were identified to exert concentration-dependent anti-viability effects against MDA-MB-231 cells, whereas the effect of metoprolol was not notable (Fig. 1A-C). The half-maximal inhibitory concentration (IC<sub>50</sub>) for propranolol was between 180 and 261.5  $\mu$ M; the IC<sub>50</sub> for ICI 118,551 was between 86.3 and 153.5  $\mu$ M; and the IC<sub>50</sub> for metoprolol was >400  $\mu$ M (Fig. 1D).

**Propranolol and ICI 118,551 inhibit the cell cycle and induce apoptosis.** The cell cycle was analyzed using flow cytometry following  $\beta$ -blocker treatment for 24 h. Following treatment of cells with 100 and 150  $\mu$ M ICI 118,551, and 100 and 200  $\mu$ M propranolol, an increased number of MDA-MB-231 cells in G<sub>0</sub>/G<sub>1</sub> phase was observed, from 43.07 to 61.04 (100  $\mu$ M ICI 118,551), 59.37 (150  $\mu$ M ICI 118,551), 61.83 (100  $\mu$ M propranolol) and 49.32% (200  $\mu$ M propranolol) ( $P < 0.05$ ; Fig. 2A and B), along with a decreasing distribution of cells in

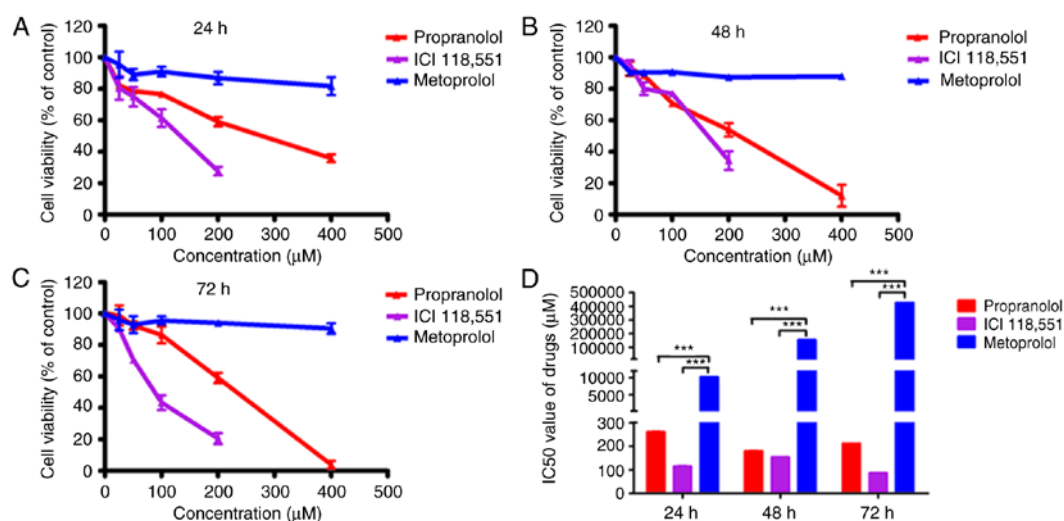


Figure 1. Effects of  $\beta$ -blockers on MDA-MB-231 cell viability. The viability of MDA-MB-231 cells was determined using Alamar Blue following treatment with increasing concentrations (25, 50, 100, 200 and 400  $\mu$ M) of metoprolol, ICI 118,551 and propranolol for (A) 24, (B) 48 and (C) 72 h. Cell viability was normalized to untreated controls at each dose for each replicate. (D)  $IC_{50}$  of metoprolol, ICI 118,551 and propranolol following incubation for 24, 48 and 72 h. All experiments were performed at least three times independently and results are expressed as the mean  $\pm$  standard error of the mean. \*\*\* $P < 0.001$ .  $IC_{50}$ , half-maximal inhibitory concentration.

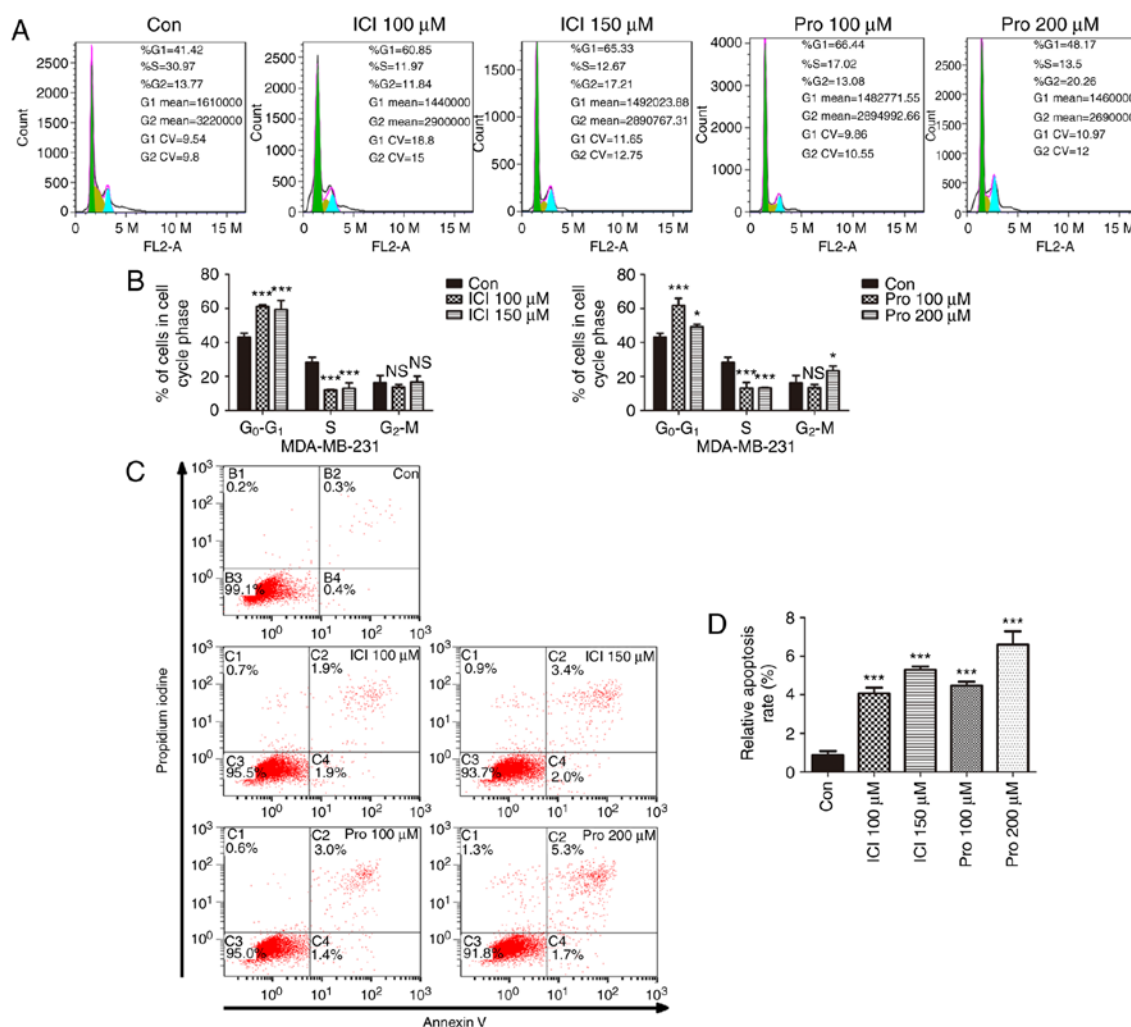


Figure 2. Propranolol and ICI 118,551 block the cell cycle and induce cell apoptosis in MDA-MB-231 cells. (A) MDA-MB-231 cells were treated with propranolol (100 and 200  $\mu$ M) and ICI 118,551 (100 and 150  $\mu$ M) for 24 h, and stained with PI to analyze the cell cycle using flow cytometry. (B) Proportions of cells in each phase were quantified. (C) Following treatment with propranolol and ICI 118,551 for 24 h, MDA-MB-231 cells were stained using Annexin V and PI, and cell apoptosis was analyzed using flow cytometry. (D) Relative apoptosis rates were quantified. All experiments were performed at least three times independently and results are expressed as the mean  $\pm$  standard deviation. \* $P < 0.05$ , \*\*\* $P < 0.001$  vs. control. PI, propidium iodide; ICI, ICI 118,551; Pro, propranolol; Con, control.

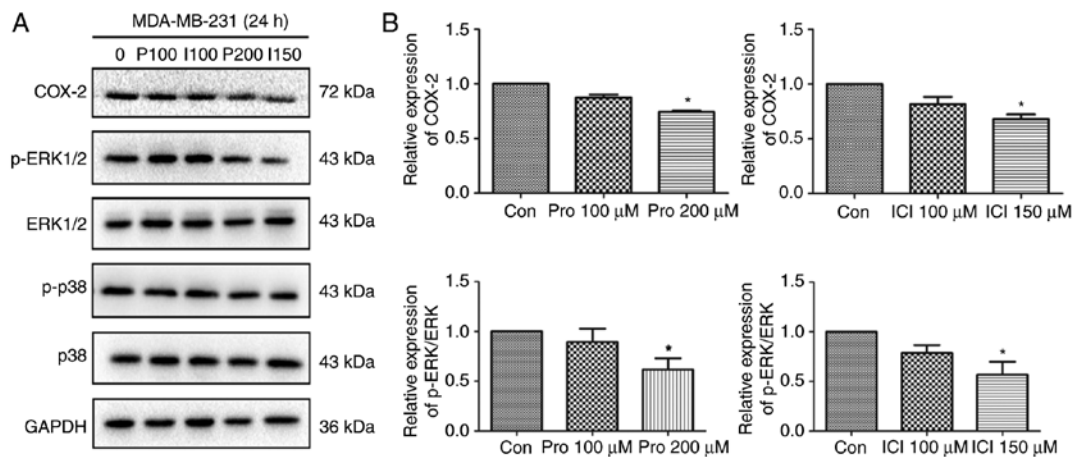


Figure 3. Propranolol and ICI 118,551 inhibit the ERK/COX-2 signaling pathway in MDA-MB-231 cells. (A) MDA-MB-231 cells were exposed to propranolol (100 and 200  $\mu$ M) and ICI 118,551 (100 and 150  $\mu$ M) for 24 h, and the expression levels of COX-2, p-ERK1/2 and p-p38 were analyzed using western blot analysis. (B) Quantification of protein expression. All experiments were performed at least three times independently and results are expressed as the mean  $\pm$  standard error of the mean. \* $P$ <0.05 vs. control. ERK, extracellular-signal-regulated kinase; COX-2, cyclo-oxygenase 2; p-, phospho-; Con, control; ICI/I, ICI 118,551; Pro/P, propranolol.

S phase from 28.16 to 11.94 (100  $\mu$ M ICI 118,551), 12.99 (150  $\mu$ M ICI 118,551), 13.17 (100  $\mu$ M propranolol) and 13.32% (200  $\mu$ M propranolol) ( $P$ <0.05; Fig. 2A and B). The number of cells in G<sub>2</sub>/M phase was increased following treatment with 200  $\mu$ M propranolol only (16.22 to 23.42%,  $P$ <0.05; Fig. 2A and B). Following cell cycle analysis, apoptosis was also analyzed using flow cytometry with Annexin V and PI staining. The proportion of apoptotic cells was determined as the sum of the ratios of early and late apoptotic cells. Following ICI 118,551 (100 and 150  $\mu$ M) and propranolol (100 and 200  $\mu$ M) treatment, the apoptosis rate of MDA-MB-231 cells increased from 0.9 to 4.1 (100  $\mu$ M ICI 118,551), 5.3 (150  $\mu$ M ICI 118,551), 4.5 (100  $\mu$ M propranolol) and 6.6% (200  $\mu$ M propranolol) ( $P$ <0.05; Fig. 2C and D). These results suggested that ICI 118,551 and propranolol inhibit the viability of MDA-MB-231 cells by arresting cell cycle progression at G<sub>0</sub>/G<sub>1</sub> and S phase and then inducing apoptosis. In addition, the effect on apoptosis induction was enhanced with increasing drug concentration.

*Propranolol and ICI 118,551 downregulate the phosphorylation levels of ERK1/2 and the expression levels of COX-2.* Previous studies indicate that the mitogen-activated protein kinase (MAPK) and COX-2 signaling pathways were associated with the proliferation and apoptosis of tumor cells (33,34). It was therefore hypothesized that propranolol and ICI 118,551 may exert antitumor effects by regulating the factors participating in the signaling pathways. The expression levels of several proteins in these pathways were analyzed using western blot analysis. Following treatment with 100  $\mu$ M propranolol and 100  $\mu$ M ICI 118,551, the expression levels of COX-2 and the phosphorylation levels of p38 and ERK1/2 were not altered markedly ( $P$ >0.05; Fig. 3A and B). Following an increase in the drug concentration (200  $\mu$ M propranolol and 150  $\mu$ M ICI 118,551), the expression levels of COX-2 and the phosphorylation levels of ERK1/2 decreased ( $P$ <0.05; Fig. 3A and B), whereas phosphorylated p38 remained unchanged. These results suggested that ICI 118,551 and propranolol may inhibit the viability of MDA-MB-231 cells by targeting ERK1/2 phosphorylation and COX-2 expression.

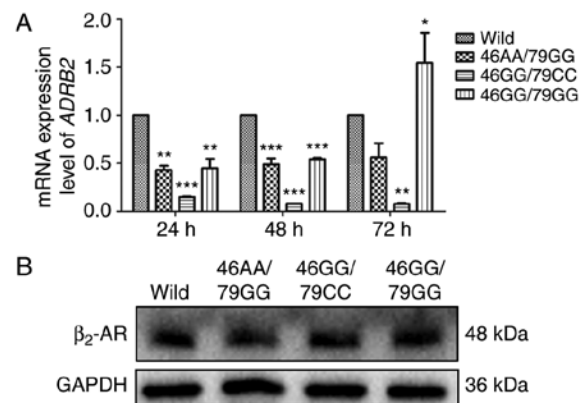


Figure 4. *ADRB2* single-nucleotide polymorphisms rs1042713 and rs1042714 affect  $\beta_2$ -AR expression. (A) 293 cells were transfected with wild-type *ADRB2* (46AA/79CC) and mutant *ADRB2* plasmids (46AA/79GG, 46GG/79CC and 46GG/79GG) for 24, 48 and 72 h. *ADRB2* mRNA expression was determined using the 2- $\Delta\Delta C_q$  method and normalized to the wild-type group. (B) Protein expression levels of  $\beta_2$ -AR were analyzed using western blot analysis following transfection for 24 h. All experiments were performed at least three times independently and results are expressed as the mean  $\pm$  standard deviation. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 vs. wild-type. *ADRB2*,  $\beta_2$ -adrenergic receptor gene;  $\beta_2$ -AR,  $\beta_2$ -adrenergic receptor.

*Effect of ADRB2 SNPs on  $\beta_2$ -AR expression.*  $\beta_2$ -AR is the main target of ICI 118,551 and propranolol. Therefore, it was investigated further whether SNPs rs1042713 and rs1042714 of *ADRB2* affected the expression levels of the  $\beta_2$ -AR. 293 cells were transfected with wild-type *ADRB2* and mutant *ADRB2* plasmids for 24, 48 and 72 h. Subsequently, total RNA was extracted and mRNA expression levels in the cells were determined using qPCR. Furthermore, total protein was extracted and analyzed using western blotting following transfection for 24 h. Compared with the wild-type group, the *ADRB2* mRNA expression levels were significantly decreased in mutant groups (46AA/79GG, 46GG/79CC and 46GG/79GG) after 24 and 48 h of transfection ( $P$ <0.05; Fig. 4A). However, after 72 h of transfection, the *ADRB2* mRNA expression was

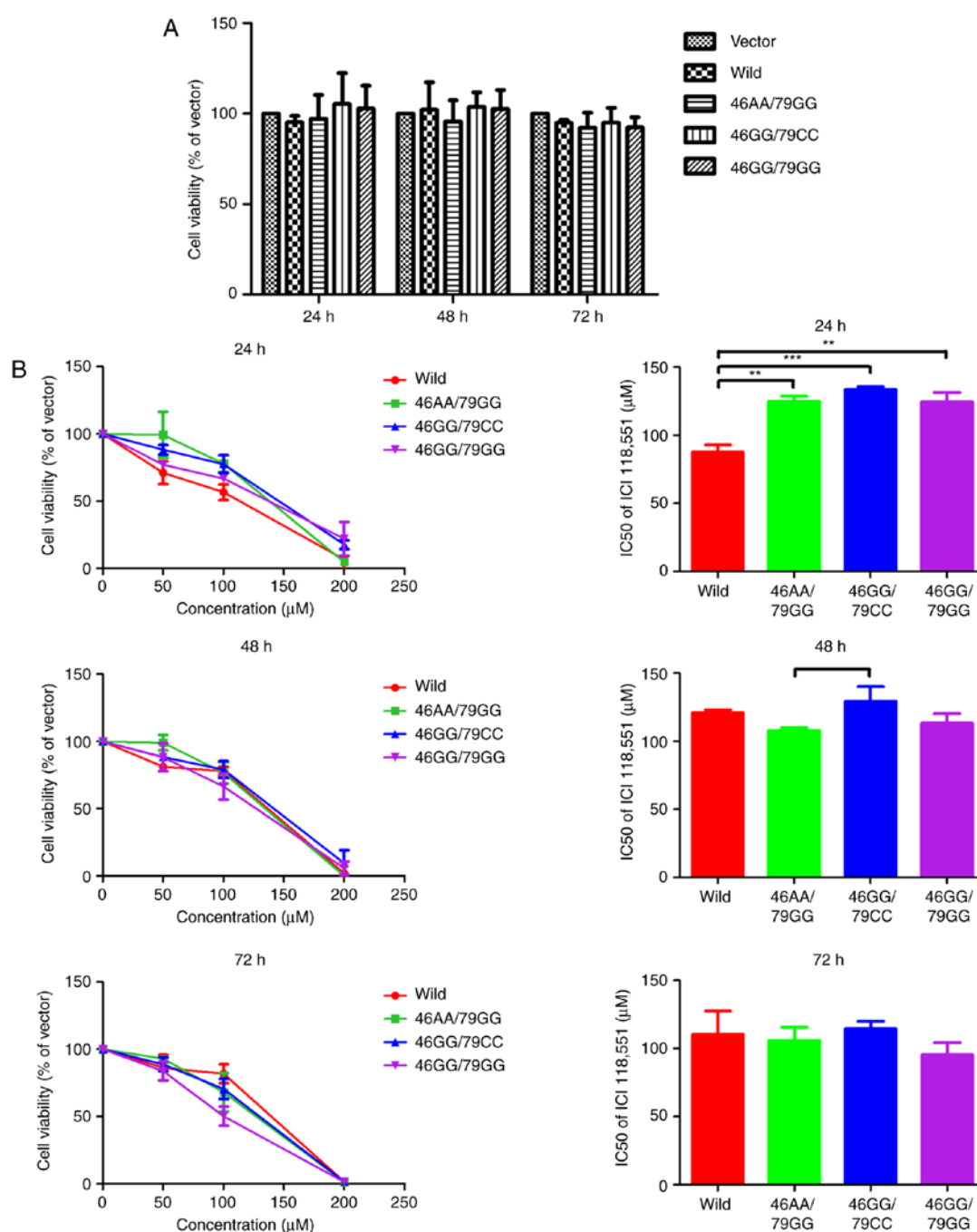


Figure 5. *ADRB2* single-nucleotide polymorphisms rs1042713 and rs1042714 did not affect cell viability, but did affect the anti-viability effect of ICI 118,551. (A) 293 cells were transfected with wild-type *ADRB2* (46AA/79CC) and mutant *ADRB2* plasmids (46AA/79GG, 46GG/79CC and 46GG/79GG) for 24, 48 and 72 h. Cell viability was determined using an MTS assay. Cell viability was normalized to vector controls. (B) Following transfection for 24 h, 293 cells were exposed to increasing concentrations (25, 50, 100 and 200  $\mu$ M) of ICI 118,551 for 24, 48 and 72 h. Cell viability was determined using an MTS assay. Cell viability was normalized to vector controls and the IC<sub>50</sub> values of ICI 118,551 after 24, 48 and 72 h of incubation are presented. All experiments were performed at least three times independently and results are expressed as the mean  $\pm$  standard deviation. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . *ADRB2*,  $\beta_2$ -adrenergic receptor gene; IC<sub>50</sub>, half-maximal inhibitory concentration.

significantly decreased only in the 46GG/79CC group ( $P < 0.05$ ; Fig. 4A). Interestingly, compared with the wild-type group, *ADRB2* mRNA expression was significantly increased in the 46GG/79GG group at 72 h. With regard to the  $\beta_2$ -AR protein expression levels, no significant difference was identified between the wild-type and the mutant groups ( $P > 0.05$ ; Fig. 4B). These results revealed that SNPs rs1042713 and rs1042714 of *ADRB2* may affect the mRNA expression levels, but not the protein expression levels of  $\beta_2$ -AR.

*Effect of ADRB2 SNPs on cell viability and anti-viability effect of ICI 118,551.* The SNPs of *ADRB2* were shown to affect the mRNA expression of  $\beta_2$ -AR. Further study was performed to assess whether *ADRB2* SNPs were able to affect cell viability and the anti-viability effect of  $\beta$ -blockers. 293 cells were transfected with the different plasmids for 24, 48 and 72 h. Subsequently, cell viability was detected by MTS. Following transfection, the cell viability of the five groups exhibited no significant difference ( $P > 0.05$ ; Fig. 5A). In order to investigate

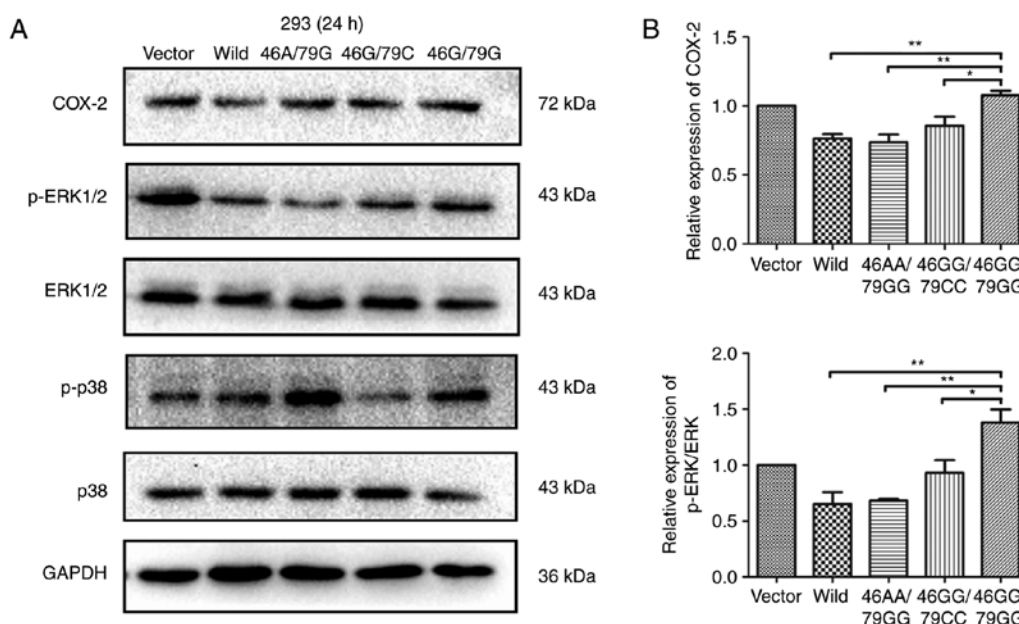


Figure 6. *ADRB2* single-nucleotide polymorphisms rs1042713 and rs1042714 affected the regulatory function of ICI 118,551 on the ERK/COX-2 signaling pathway. (A) 293 cells were transfected with wild-type *ADRB2* (46AA/79CC) and mutant *ADRB2* plasmids (46AA/79GG, 46GG/79CC and 46GG/79GG) and then treated with 80  $\mu$ M ICI 118,551 for 24 h. The expression levels of COX-2 and p-ERK1/2 were analyzed using western blot analysis. (B) Quantification of protein expression. All experiments were performed at least three times independently and results are expressed as the mean  $\pm$  standard error of the mean. \* $P < 0.05$ , \*\* $P < 0.01$ . *ADRB2*,  $\beta_2$ -adrenergic receptor gene; ERK, extracellular-signal-regulated kinase; COX-2, cyclo-oxygenase 2; p-, phospho-.

the effect of *ADRB2* SNPs on the anti-viability effect of  $\beta$ -blockers, 293 cells were treated with different concentrations of ICI 118,551 following transfection for 24 h. The results of the MTS assay demonstrated that ICI 118,551 significantly decreased the viability of 293 cells in a concentration-dependent manner ( $P < 0.05$ ; Fig. 5B). *ADRB2* SNPs significantly affected the  $IC_{50}$  value of ICI 118,551 when 293 cells were treated with the drug for 24 h ( $P < 0.05$ ; Fig. 5B). The  $IC_{50}$  value in the wild-type group ( $IC_{50}$ ,  $87.6 \pm 9.5 \mu$ M) was significantly lower compared with that in the mutant groups (46AA/79GG,  $125.1 \pm 6.1$ ; 46GG/79CC,  $133.7 \pm 3.7$ ; and 46GG/79GG,  $124.7 \pm 11.6 \mu$ M). However, after 48 h of ICI 118,551 treatment, the  $IC_{50}$  value was significantly different only in two mutant groups (46AA/79GG vs. 46GG/79CC,  $107.7 \pm 2.2$  vs.  $129.4 \pm 10.8 \mu$ M). Notably, after 72 h of ICI 118,551 treatment, there was no significant difference in the  $IC_{50}$  value between the four groups ( $P > 0.05$ ; Fig. 5B). These results suggested that *ADRB2* SNPs did not affect cell viability, but did affect the anti-viability effect of ICI 118,551.

*ADRB2* SNPs may affect the regulatory function of ICI 118,551 on the ERK/COX-2 signaling pathway. It was investigated whether  $\beta$ -blockers could affect ERK1/2 phosphorylation and COX-2 expression and the effect of *ADRB2* SNPs. 293 cells were transfected with different plasmids and treated with 80  $\mu$ M ICI 118,551 for 24 h, and the concentration of ICI 118,551 was determined on the basis of the results of previous MTS assays. The expression levels of COX-2 and p-ERK1/2 in the 46GG/79GG group were significantly increased compared with the wild-type, 46AA/79GG and 46GG/79CC groups ( $P < 0.05$ ; Fig. 6A and B). Although the protein expression levels were different between the wild-type, 46AA/79GG and 46GG/79CC groups, the differences were

not statistically significant ( $P > 0.05$ ; Fig. 6A and B). These results revealed that the regulatory function of ICI 118,551 on COX-2 and p-ERK1/2 expression was affected by *ADRB2* SNPs.

## Discussion

The results of the present study indicated that propranolol and ICI 118,551 inhibited the viability of MDA-MB-231 cells by downregulating ERK1/2 phosphorylation and COX-2 expression and inducing apoptosis. Furthermore, SNPs rs1042713 and rs1042714 of *ADRB2* were identified to affect the anti-viability effect of ICI 118,551, and the underlying molecular mechanism was investigated.

It was identified that the non-selective  $\beta$ -blocker propranolol and the selective  $\beta_2$ -blocker ICI 118,551 significantly inhibited the viability of MDA-MB-231 cells, whereas the selective  $\beta_1$ -blocker metoprolol did not. Similar results were reported by Chin *et al* (35), who identified that colorectal cancer cells were only sensitive to ICI 118,551 and propranolol, but not atenolol (a selective  $\beta_1$ -blocker), and the effects of ICI 118,551 were the most potent. However, Liu *et al* (36) identified that atenolol and ICI 118,551 were able to attenuate the function of epinephrine in promoting esophageal cancer cell proliferation. Different expression levels of three  $\beta$ -AR subtypes in various types of tumor cells may account for these conflicting results. Consistent with an *in vitro* study, which reported the sensitivity of MDA-MB-231 cells to propranolol ( $IC_{50}$  value of 200  $\mu$ M) (18), it was identified in the present study that propranolol exerted its anti-viability effect at a concentration that was significantly higher than the peak serum concentration (0.77-1.5  $\mu$ M *in vivo*) (37). Notably, there is a large discrepancy of drug concentration between *in vitro* and *in vivo*



models. This may be because propranolol inhibits the growth of the tumor itself *in vivo*, and also inhibits angiogenesis (38) and regulates immune system function (39,40). This multifaceted antitumor effect may be beneficial in decreasing the drug concentration in *in vivo* models.

The activation of the MAPK signaling pathway has been associated with the resistance of TNBC to chemotherapy (33). Huang *et al* (41) identified that NE stimulated the proliferation, migration and invasion of pancreatic cancer cells via the  $\beta$ -AR/P38/MAPK signaling pathway. Additionally, the overexpression of COX-2 has been associated with shorter relapse-free survival in stage III patients who were ER-negative (42). Furthermore, Liu *et al* (36) demonstrated that epinephrine was able to stimulate the proliferation of esophageal squamous cell carcinoma cells via the  $\beta$ -AR/ERK/COX-2 signaling pathway. Owing to the aforementioned results mentioned, the effect of  $\beta$ -blockers on p38, ERK1/2 phosphorylation and COX-2 expression was investigated in MDA-MB-231 cells. The results suggested that propranolol and ICI 118,551 inhibited the activation of the ERK/COX-2 signaling pathway, but had no effect on p38 phosphorylation.

Feigelson *et al* (43) identified that *ADRB2* SNPs were not associated with the risk of post-menopausal breast cancer; however, in the present study, it was identified that *ADRB2* SNPs rs1042713 and rs1042714 may affect the response to  $\beta$ -blockers in the treatment of breast cancer. The results of the present study revealed that *ADRB2* mRNA expression was significantly decreased in mutant haplotypes (46AA/79GG, 46GG/79CC and 46GG/79GG) after 24 and 48 h of transfection. However, there were no significant differences in  $\beta_2$ -AR protein expression levels between wild-type and mutant haplotypes. It has been identified previously that codon optimality may affect the degradation rate of mRNA and the elongation rate of ribosomes, and may ultimately affect the folding rate, stability and activity of proteins (44). SNPs rs1042713 and rs1042714 of *ADRB2* may alter the degradation rate of mRNA, thereby altering the function of  $\beta_2$ -AR and the response to  $\beta$ -blocker drugs. Marson *et al* (45) reported that SNPs rs1042713 and rs1042714 of *ADRB2* were associated with the bronchodilator response in cystic fibrosis. In patients with chronic heart failure, Metra *et al* (46) identified that SNP rs1042714 determined the left ventricular ejection fraction response to carvedilol, whereas SNP rs1042713 had no such effect. Similarly, Martin *et al* (27) identified an association only between SNP rs1042714 and the response to  $\beta_2$ -agonists in children with acute asthma. The association between the two SNPs, which may result in amino acid changes and altered response to drugs in different diseases, has been widely investigated. However, the effects of the two SNPs on tumors and on the response to  $\beta$ -blockers requires further investigation.

In the present study, the effect of *ADRB2* SNPs on the molecular mechanism underlying the antitumor effect of  $\beta$ -blockers was investigated and it was identified that only the 46GG/79GG haplotype significantly impaired the inhibitory effect of ICI 118,551 on the ERK/COX-2 signaling pathway. This phenomenon may explain why the  $IC_{50}$  value of ICI 118,551 was higher in the 46GG/79GG group. However, for the 46AA/79GG and 46GG/79CC haplotypes, the inhibitory effect of ICI 118,551 on the ERK/COX-2 signaling pathway did not change significantly, suggesting that other mechanisms

may be involved in the effect of *ADRB2* SNPs on the antitumor properties of  $\beta$ -blockers. Ahles *et al* (47) identified that variants carrying Gly<sup>16</sup> (46GG/79CC and 46GG/79GG) exhibited significant acceleration in response to repetitive stimulation compared with the Arg<sup>16</sup>Gln<sup>27</sup> (46AA/79CC) variant, suggesting that *ADRB2* SNPs altered the activation kinetics of  $\beta_2$ -AR during repetitive stimulation. The difference in activation kinetics may reflect distinct  $\beta_2$ -AR conformations, and the change in activation speed may result in altered downstream signaling.

The present study had certain limitations. With regard to *ADRB2* mRNA expression, it was identified that mRNA expression in the 46GG/79GG haplotype increased inexplicably, particularly following transfection for 72 h. Owing to the multifaceted antitumor effect of  $\beta$ -blockers, *in vitro* experiments may not suffice, and further verification of the antitumor effect of  $\beta$ -blockers on TNBC in *in vivo* experiments and clinical trials is required.

In summary, the results of the present study revealed that ICI 118,551 and propranolol, but not metoprolol, significantly inhibited the growth of TNBC by inhibiting cell viability, blocking cell cycle progression and inducing cell apoptosis. Furthermore, the molecular mechanism behind this effect may involve the inhibition of the ERK/COX-2 signaling pathway. It was also identified that SNPs rs1042713 and rs1042714 of *ADRB2* affected the anti-viability effect of ICI 118,551. *ADRB2* SNPs may alter the mRNA expression levels of  $\beta_2$ -AR and the activation of the downstream signaling pathway. Additionally, *ADRB2* SNPs may be involved in the response to ICI 118,551 treatment. Therefore,  $\beta$ -blockers may be a viable option for the treatment of TNBC, and SNPs rs1042713 and rs1042714 of *ADRB2* may result in individual responses to  $\beta$ -blockers.

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## Availability of data and materials

The datasets used/or analyzed during the present study are available from the corresponding author on reasonable request.

## Authors' contributions

WYX was involved in the molecular biology experiments, and was a major contributor to writing the manuscript. RHH was involved in the molecular biology experiments and the drafting of the manuscript. ZW and CFZ were involved in the molecular biology experiments and the analysis of data. JZ, YJT and ZL were involved in the interpretation of the data and revision of the manuscript. YJH, HLM and JL made substantial contributions to the conception and design of the study.



All authors have read and approved the final version of this manuscript.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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