Glycoprotein hormone α-subunit promotes cell proliferation and tumorigenesis in breast cancer

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Abstract. The glycoprotein hormone α-subunit (CGA) is implicated in the occurrence and progression of a number of solid tumors. However, its role in breast cancer remains unclear. The present study aimed to investigate the biological functions and mechanisms of action of CGA in breast cancer. CGA protein expression was evaluated in clinical breast cancer specimens using immunohistochemistry. The association between CGA expression and patient prognosis was determined using the Kaplan-Meier method and Mantel-Cox test. At the same time, CGA mRNA and protein expression was explored in a normal mammary epithelial cell line and breast cancer cell lines. Breast cancer cell lines overexpressing or deficient in CGA were established, and the effect of CGA on cell proliferation was evaluated in vitro, and in vivo using a mouse xenograft tumor model. Intracellular signaling pathway activities were evaluated using western blotting in CGA-overexpressing or -depleted cells. Increased CGA protein expression was significantly associated with a poor prognosis in patients with breast cancer. Furthermore, while CGA mRNA and protein expression level was negligible in normal mammary epithelial cells, it was elevated in breast cancer cell lines. In vitro and in vivo experiments showed that CGA overexpression enhanced breast cancer cell proliferation via activation of the epidermal growth factor receptor, extracellular signal-regulated kinase 1/2 and serine/threonine kinase Akt signaling cascades. The present results suggest that CGA is upregulated in breast cancer tissues and that it is associated with a poor prognosis. CGA may serve as a candidate for developing targeted therapies for breast cancer.

Introduction

Breast cancer (BC) has now surpassed lung cancer as the most common cancer worldwide, with ~2.3 million new cases in 2020 (1), which makes the disease a major public health burden (2). Despite recent advances in medical technology, the molecular basis of breast cancer progression remains unclear. Clarifying the mechanisms of breast cancer biology can help identify new therapeutic targets, thereby providing a basis for the development of more effective treatments.

The incidence of postmenopausal breast cancer is increasing with the aging world population (3). Follicle-stimulating hormone (FSH) levels remain high after menopause owing to the loss of estrogen negative feedback. However, the relationship between FSH levels and breast cancer onset and progression remains unclear. FSH is a glycoprotein hormone related to luteinizing hormone (LH), thyroid-stimulating hormone (TSH) and human chorionic gonadotropin (hCG). These hormones are secreted by the pituitary gland and consist of a heterodimer of two noncovalently linked α- and β-subunits. The former is identical in all these hormones (4) and is encoded by a single gene, the glycoprotein hormone α-subunit (CGA), whereas β-subunits in each hormone are encoded by a different gene (5). CGA has been implicated in the occurrence and progression of a number of solid tumors, including lung (6), prostate (7) and gastric (8) cancer. In addition, CGA is a candidate marker for predicting the response to tamoxifen treatment in breast cancer (9,10). However, the precise role and mechanisms of action of CGA in breast cancer are unclear.

The main function of the gonadotropin-releasing hormone (GnRH) is to control the release of FSH and LH. GnRHa is a synthetic GnRH derivative that inhibits the synthesis and release of LH and FSH, and reduces CGA levels in the body by continuous administration. There is still a debate about the clinical use of GnRHa in premenopausal women with breast cancer. Those who supported the use of GnRHa believed that it protected patient ovarian function during chemotherapy (11), objections were made over the use of GnRHa, as no apparent benefit and ovarian protection were observed in another study (12). Whether the benefits of GnRHa treatment are related to the decrease in CGA levels in the body remains to
be understood. Therefore, it is necessary to study the mechanism of action of CGA in breast cancer, which has practical significance for the clinical treatment of this disease.

The present study aimed to investigate the role and mechanism of action of CGA in breast cancer by comparing its expression between breast cancer and normal breast tissues, and the overexpression and depletion of CGA in different cancer cell lines.

Materials and methods

**Tissue microarrays and immunohistochemistry.** Tissue microarrays (TMAs) containing 160 breast cancer tissues were obtained from Shanghai Outdo Biotech, Co., Ltd. Immunohistochemistry (IHC) was performed on the TMA sections. The sections were first deparaffinized with xylene, rehydrated using a graded series of ethanol, and washed with water. Antigen retrieval was performed by heating the sections in citrate buffer for 20 min, followed by cooling to room temperature. The sections were incubated in 3% hydrogen peroxide for 10 min at room temperature to quench endogenous peroxidase. Subsequently, a sodium citrate buffer solution (0.01 M; pH 6.0) was added to incubate the sections for 3 min under boiling conditions. Bovine serum albumin (5%) was used to block the sections for 30 min at 37°C. The sections were incubated overnight at 4°C with a mouse anti-human monoclonal antibody against CGA (1:500; ab11232; Abcam), followed by incubation with a HRP-polymer conjugated anti-mouse IgG secondary antibody (Fuzhou Maixin Biotech Co., Ltd.) for 30 min at room temperature. After washing in phosphate-buffered saline (PBS), the sections were stained with diaminobenzidine at room temperature for 2 min and counterstained with hematoxylin at room temperature for 10 sec. Immunoreactivity was scored by experienced pathologists (who were blinded to patient identity) according to the immunoreactivity scoring system (IRS), which used the percentage of immunopositive cells (0%, 0; 1-10%, 1; 11-50%, 2; 51-80%, 3; and 81-100%, 4) and staining intensity (negative, 0; weak, 1; moderate, 2; and strong, 3). IRS score ranging from 0 to 12 was calculated by multiplication of the two scores. Patient consent was obtained and the use of TMAs containing human tissues was approved by the Ethics Committee of Shanghai Outdo Biotech, Co., Ltd. (approval no. SHYJS-CP-1804004). The study was approved by the Ethics Committee of the Women's Hospital, Zhejiang University School of Medicine (Hangzhou, China) and was in accordance with the Declaration of Helsinki.

**Cell lines and cell culture maintenance.** SK-BR-3, MDA-MB-231, MCF-7 and T-47D human breast cancer cell lines and MCF-10A normal mammary epithelial cells were obtained from the Cell Bank of the Shanghai Institute of Cell Biology. SK-BR-3 and MDA-MB-231 cells were cultured in McCoy’s 5a, RPMI-1640 and L-15 media supplemented with 10% fetal bovine serum (FBS) (all Gibco; Thermo Fisher Scientific, Inc.) and 1% antibiotics (penicillin-streptomycin, ST488; Shanghai Biyuntian Biotechnology Co., Ltd.). MCF-7 and T-47D cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS and 1% antibiotics. MCF-10A cells were grown in DMEM/F12 containing 5% horse serum (Thermo Fisher Scientific, Inc.), 0.5 µg/ml hydrocortisone, 100 ng/ml cholera toxin, 20 ng/ml EGF, 10 µg/ml insulin and 1% antibiotics. All cells were grown in a humidified atmosphere of 5% CO₂ at 37°C.

**RNA isolation and reverse transcription-quantitative PCR (RT-qPCR).** Total RNA was extracted from cultured cells (MCF-10A, MCF-7, T-47D, MDA-MB-231 and SK-BR-3) using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse-transcribed into cDNA using the PrimeScript RT-PCR kit (Takara Biotechnology Co., Ltd.) according to the manufacturer’s protocol. qPCR was performed using SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions on a StepOnePlus Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: 95°C for 10 min, followed by 38 cycles of 95°C for 15 sec and 60°C for 40 sec. The results are expressed as the fold-change in relative mRNA expression levels, calculated using the 2−ΔΔCq method (13). Each reaction was performed in triplicate. The following primers were used: CGA forward, 5'-TGCCCA GGCTGCTCTCAAACCT-3' and reverse, 5'-GCAA TAGGTA CCCTGAGGTCGAGC-3'; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; internal control) forward, 5'-GGTGTCGTAGGACTC-3' and reverse, 5'-GGGATGATG TTCTGGAGAG-3'.

**Protein extraction and western blot analysis.** Breast cells (MCF-10A, MCF-7, T-47D, MDA-MB-231 and SK-BR-3) were washed twice with ice-cold PBS and lysed in a lysis buffer (cat. no. 87787; Pierce; Thermo Fisher Scientific, Inc.) containing a protease inhibitor cocktail (1:100 dilution; MilliporeSigma) for 30 min at 4°C. Protein concentrations were determined by the bicinchoninic acid assay (cat. no. 23227; Thermo Fisher Scientific, Inc.). Protein samples (20-40 µg) were separated on a 10% gel using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. After being blocked with 5% skimmed milk for 2 h at room temperature, the membranes were incubated overnight at 4°C with the following primary antibodies: GAPDH antibody (1:2,500; cat. no. ab9485; Abcam), CGA antibody (1:1,000; cat. no. ab11232; Abcam), His-tag antibody (1:1,000; cat. no. 9991s; Cell Signaling Technology, Inc.), Phospho-EGF Receptor Pathway Antibody Sampler Kit (1:1,000; cat. no. 9789; Cell Signaling Technology, Inc.), Akt antibody (1:1,000; cat. no. 4685; Cell Signaling Technology, Inc.), EGFR antibody (1:1,000; cat. no. 4695; Cell Signaling Technology, Inc.), Erk1/2 antibody (1:1,000; cat. no. 4068; Cell Signaling Technology, Inc.) and EGFR antibody (1:1,000; cat. no. 4267; Cell Signaling Technology, Inc.). Next, the membranes were incubated with goat anti-mouse secondary antibodies (1:2,000; cat. no. A0216; Shanghai Biyuntian Biotechnology Co., Ltd.) for 1 h at room temperature. The protein bands were visualized with ECL detection reagents (cat. no. P0018FM; Shanghai Biyuntian Biotechnology Co., Ltd.) and analyzed with ImageJ software (version 1.47; National Institute of Health).

**Small interfering (si)RNA and plasmid transfection.** Using standard plasmid construction procedures, CGA cDNA (Shenggong Biotechnology) was cloned into the pcDNA3.1-His plasmid. CGA siRNAs and control siRNAs were designed by
Genepharm Co., Ltd. For siRNA transfection, MDA-MB-231 cells were seeded at 70-80% confluence in 6-well plates 1 day before transfection. Transfections of siRNA (at 100 pmol) were performed using the Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 20 min, according to the manufacturer’s protocols. Controls included nonspecific siRNA (negative control) and GAPDH siRNA (positive control). Similarly, pcDNA3.1-His-CGA was transfected into T-47D cells according to the standard plasmid transfection procedure, with the transfection of empty vector as the negative control. Breast cancer cell lines without any treatment were used as normal controls. The cells were evaluated for CGA expression and biological effects 72 h after transfection. The following forward and reverse primers were used to generate the siRNAs for the knockdown experiment: GAPDH (positive control), 5'-UGAGCUCAACUAUAGGUCGU UTT-3' and 5'-AAGACUGUAUUGAGGUCCAUTT-3'; negative control scrambled siRNA, 5'-UUUCGCGAAGUCGUCA CGAGUGTT-3' and 5'-ACUGAUAACGUUCCGAGAATT-3'; CGA-554 (no. 1), 5'-CUGCAUAUCUUUAUAUTT-3' and 5'-AUAAUAACAGUACGATTT-3'; CGA-490 (no. 2), 5'-CUAAUAUACAUUACGGGUUTT-3' and 5'-ACCUGUUUAUGAUUAGTT-3'; and CGA-404 (no. 3), 5'-UCUG UAGCGCAUAUCCCACUTT-3' and 5'-AGUUGGGAUUGC UCUAGAGTT-3'.

Cell proliferation assay. Breast cancer cells were cultured in 96-well plates seeded at a concentration of 5x10^4 cells/ml for 72, 120 and 168 h. When the cells were in the exponential growth phase, 20 µl of 5 mg/ml stock solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma-Aldrich; Merck KGaA) was added to each well and incubated for 4 h at 37°C. The medium was replaced with 150 µl dimethyl sulfoxide and the plate was incubated with gentle agitation for 10 min. The absorbance was measured at 490 nm using a microplate reader.

In vivo xenograft tumor model. A total of 20 female BALB/c nude mice (4-5 weeks old; weight, 16-20 g) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. The mice were housed in Makroten cages (5 per cage) in an airflow cabinet under pathogen-free conditions at 23°C on a 12/12 h day/night cycle, with water and food ad libitum. Animal health was regularly monitored by professional laboratory managers, including dietary intake, respiration, body weight, activity and tumor burden. Different groups of breast cancer cells (T-47D, T-47D/CGA, MDA-MB-231 and MDA-MB-231/CGA) in the exponential growth phase were collected, washed and resuspended in a sterile DMEM. The nude mice (4-5 weeks old; weight, 16-20 g) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. The mice were anesthetized by inhalation of isoflurane at a dose range of 2-6% for induction and 1-3% for maintenance. Next, tumors were dissected and weighed, and images were captured. All animal experiments were performed in compliance with the guidelines of the Institute of Experimental Animal Sciences (Zhejiang University Laboratory Animal Center, Hangzhou, China). The protocol was approved by the Animal Care and Use Committee of Zhejiang University (approval no. ZJU2015-339-01).

Bioinformatics analysis. CGA expression and associated proteins were analyzed using published databases. First, the cbioportal platform (http://www.cbioportal.org), an open data analysis platform was used, entering the query genes in the webpage according to the requirements, selecting different tumor databases and obtaining the CGA expression levels in various tumors. Second, CGA expression and survival data was obtained for patients with CGA upregulation in different breast cancer databases. By using GENEMANIA (https://genemania.org), the interaction of the analyzed proteins was predicted.

Statistical analysis. GraphPad Prism version 6.0 (GraphPad Software, Inc.) was used to perform all statistical analyses. Differences between groups were evaluated using either a two-tailed unpaired Student's t-test or a one-way analysis of variance and Tukey's post hoc test. A statistically significant difference was indicated by P<0.05. Each experiment was performed independently three times.

Results

High expression level of CGA in breast cancer is associated with a poor prognosis. CGA expression was examined in TMAs obtained from 160 patients with ductal carcinoma using IHC. The baseline characteristics of the patients are presented in Table I. Some cores in the TMAs were lost during sample processing; therefore, 141 of the 160 breast cancer specimens were available for the IHC analysis of CGA expression. Representative images of CGA expression in breast cancer tissues and staining intensity scores are shown in Fig. 1A and B. CGA expression was mainly cytoplasmic in the tumor cells and negative in the normal ductal epithelial tissue.

Patients with breast cancer who were positive for CGA expression had a shorter overall survival time compared with those who were negative for CGA expression (Fig. IC). Subgroup analysis according to estrogen receptor (ER) status confirmed that CGA expression was associated with a shorter overall survival time (Fig. 1D). In the progesterone receptor (PR)-negative group, the CGA-positive patients had longer survival times (Fig. 1E). No differences in survival based on CGA levels were found in the human epidermal growth factor receptor (Her)-2 subgroup (Fig. 1F).

CGA is highly expressed in breast cancer cell lines. CGA mRNA and protein expression levels were compared between a normal mammary epithelial cell line and breast cancer cell lines, and it was found that MCF-10A cells expressed very low levels of CGA transcripts and CGA protein (Fig. 2A and B). By contrast, breast cancer cell lines showed variable expression
of CGA: MDA-MB-231, a triple-negative (ER/PR/Her-2-) cell line, had the highest levels of CGA, whereas T-47D (ER+/PR+/Her-2), a moderately malignant cell line, showed relatively low CGA expression.

**CGA overexpression enhances breast cancer cell proliferation.** To investigate the role of CGA in breast cancer, a plasmid containing CGA coding sequence (Gene ID, 1081) fused with a His tag was first constructed for CGA overexpression (T-47D/CGA+), and knockdown MDA-MB-231/CGA- cell lines with siRNA were established. Successful transfection of pcDNA3.1-His-CGA into T-47D cells was confirmed (Fig. 3A). T-47D/CGA- cells showed a higher rate of cell proliferation than the control cells (Fig. 3B). Of the three siRNAs tested, no. 2 showed the highest knockdown efficiency (Fig. 3C). MDA-MB-231/CGA- cells showed decreased proliferative capacity compared with control cells (Fig. 3D).

**CGA increases EGFR, ERK1/2 and Akt phosphorylation.** Signaling pathways such as those of EGFR, mitogen-activated protein kinase (MAPK) and Akt play important roles in the proliferation of breast cancer cells and are mainly activated by phosphorylation (14). To clarify the mechanism by which CGA stimulates breast cancer cell proliferation, the total and phosphorylated protein levels of EGFR, ERK1/2 and Akt in CGA-overexpressing and CGA-depleted cells were evaluated. The phosphorylation of EGFR (Y1173), ERK1/2 and Akt (Y473) was increased in T-47D/CGA+ cells, whereas total protein levels were largely unchanged compared with those in control cells (Fig. 4). Conversely, MDA-MB-231/CGA- cells exhibited decreased levels of phosphorylated (p-)EGFR (Y1173), p-ERK1/2 and p-Akt (Y473). These results suggest that CGA enhances breast cancer cell proliferation via the activation of EGFR, ERK1/2 and Akt signaling cascades.

**CGA induces breast cancer growth in vivo.** To evaluate the role of CGA in tumor growth in vivo, a xenograft mouse model was established by subcutaneous injection of T-47D/CGA+, T-47D, MDA-MB-231/CGA- or MDA-MB-231 cells into BALB/c nude mice. Tumors in the T-47D/CGA+ and MDA-MB-231/CGA- groups grew more rapidly than those in the control groups, as assessed by measuring the diameter of the tumor (Fig. 5A and B). As MDA-MB-231 cells grew faster and T-47D cells grew relatively slowly, the cutoff times for observations were therefore different. At the end of observation, the largest tumor diameter in the T-47D cell group was 1.1 cm and the largest volume was 617 mm³, while those values in the T-47D/CGA+ group were 1.3 cm and 750 mm³, respectively. The maximum tumor diameter and volume of the MDA-MB-231 and MDA-MB-231/CGA- groups were 1.5 cm and 772 mm³, and 1.3 cm and 534 mm³, respectively. The xenografts of the T-47D/CGA+ group were relatively larger and heavier, while the low CGA expression in the MDA-MB-231 group led to relatively smaller and lighter xenografts (Fig. 5C-F).

**Discussion**

The present study found that increased CGA expression was significantly associated with a poor prognosis in patients with breast cancer. Consistent with the aforementioned findings, CGA was undetectable in a normal mammary epithelial cell line but was upregulated in breast cancer cell lines at both the mRNA and protein levels. The upregulated expression of CGA promotes the proliferation of breast cancer cells both in vitro and in vivo. Although further research is needed to understand the exact mechanism of CGA action, the present results indicated that it promotes breast cancer cell proliferation through the activation of the EGFR, ERK1/2 and Akt signaling cascades.

CGA is the α-subunit of glycoprotein hormones, including FSH, LH and TSH, and is widely present in all parts of the body. In recent years, it has been found to be closely associated with some malignant tumors, such as those of prostate (7), gastric (8) and breast cancer (15). Additionally, lung cancer cells lose their tumor phenotypes and show decreased proliferative capacity and tumorigenicity in mice upon depletion of α-hCG (6). In breast cancer, expression of the α-subunit of hCG has been linked to lymph node metastasis and a worse prognosis (15). In our previous study, it was found that FSH levels were associated with Her-2 and Ki67 expression in postmenopausal women with breast cancer (16). In the present study, it was further confirmed that a subunit of FSH was overexpressed in breast cancer cells and promoted their proliferation. This suggests that glycoprotein hormones may play a role in the tumorigenesis of breast cancer, and are worthy of further study.

EGFR is a receptor tyrosine kinase that is usually upregulated in cancer and functions as a proto-oncogene by promoting proliferation and suppressing the apoptosis in cancer cells (17). Activation of EGFR induces downstream signaling molecules, including Akt and ERK1/2, which are the components of two major pathways regulating cell proliferation and survival (18).
The present study demonstrated that the increased proliferation of breast cancer cells induced by CGA overexpression was associated with the upregulation of p-EGFR (Y1173), p-ERK1/2 and p-Akt, implying that CGA acts via these pathways to promote cell proliferation. However, the direct target of CGA is not clear at present, and this mechanism requires continued study.

To investigate the molecular basis for the activation of intracellular signaling cascades by CGA in greater detail, a gene association analysis of breast cancer samples from TCGA database was performed using the CBioPortal platform (http://www.cbioportal.org) (19,20). Big data analysis also confirmed the expression variation of CGA in various tumors, such as downregulated expression in some blood tumors and high expression in lung, ovarian and breast cancer (Fig. S1A). The upregulated expression rate of CGA in breast cancer is nearly 1% according to research data from five different databases (Fig. S1B). Patients with CGA-positive breast cancer exhibited a lower overall survival rate, but the difference was not statistically significant due to the small amount of samples (Fig. S1C). Pathway Mapper shows that CGA is associated with a number of breast cancer proliferation-related signaling pathways.

Figure 1. CGA overexpression in patients with breast cancer is associated with a poor prognosis. CGA expression in (A) tissue microarrays and (B) the immunoreactivity scoring system based on the extent and intensity of immunoreactivity. CGA is localized in the nucleus as well as in the cytoplasm of breast cancer cells. (C) Survival analysis based on CGA-positive and -negative groupings showing lower survival rates in CGA-positive patients. Subgroup survival analysis based on (D) ER, (E) PR and (F) Her-2 expression. Irrespective of the ER status, CGA-positive patients have a worse prognosis. The Mantel-Cox test was used for statistical analysis. CGA, glycoprotein hormone α-subunit; ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor 2.

Figure 2. mRNA and protein expression levels of CGA in breast cancer and normal mammary epithelial cells. CGA (A) mRNA (A) and protein (B) levels in SK-BR-3, MDA-MB-231, MCF-7 and T-47D human breast cancer cell lines, and MCF-10A normal mammary epithelial cells. CGA, glycoprotein hormone α-subunit; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
pathways, such as those of WNT, TP53, PI3K and NOTCH. According to the results of GENEMANIA analysis, one of the direct interaction partner proteins of CGA is protein tyrosine phosphatase non-receptor type 12 (PTPN12) (Fig. S1D) (21). PTPN12 is a member of the PTP family and was recently identified as a tumor suppressor (22); it is downregulated in a variety of human malignancies, including colon (23), breast (24) and ovarian (25). Inactivation of PTPN12 resulted in HER-2/EGFR
hyperactivation and stimulation of downstream MAPK signaling in human mammary epithelial cells (24). In ovarian cancer cells, PTPN silencing activated phosphatidylinositide 3-kinase/AKT signaling (26). In the present study, T-47D cells with high CGA expression were also evaluated with regard to PTPN12, and it was found that PTPN12 was downregulated at the mRNA level (Fig. S1E). According to the experimental results, CGA can activate MAPK and AKT signaling pathways and regulate the expression of PTPN12, indicating that CGA may act through PTPN12, which is worthy of further study.

Based on the aforementioned observations and the present study findings, it was indicated that CGA promoted breast cancer progression via EGFR, ERK1/2 and Akt signaling. Although additional studies are required to elucidate the underlying mechanisms, these results indicate that therapeutic strategies targeting CGA may be an effective treatment for breast cancer.

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Availability of data and materials

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

Authors’ contributions

JZ and XZ participated in the design and data acquisition of the study, and wrote the original manuscript. YC, HC, and SW participated in the data analysis and interpretation of the study. All authors have read and approved the final manuscript. JZ and XZ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Women's Hospital, Zhejiang University School of Medicine.
(Hangzhou, China) and was in accordance with the Declaration of Helsinki. The protocol was approved by the Animal Care and Use Committee of Zhejiang University (approval no. ZJU2015-339-01).

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References


