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Potent inhibition of human gastric cancer by HER2-directed induction of apoptosis with anti-HER2 antibody and caspase-3 fusion protein

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ABSTRACT
Background and aims HER2, an oncogene, has been found to be over-expressed in 10–40% of human gastric carcinomas. The aims of this study were to investigate if a fusion protein consisting of anti-HER2 sFv and constitutively active caspase-3 was capable of inducing apoptosis in HER2-expressing human gastric cancer cells and blocking the growth of human gastric cancer xenografts in nude mice.

Methods NIH3T3 cells stably transduced with the pcDNA3.1-HER-PE-CP3 recombinant plasmid containing a secretion signal, a single-chain anti-HER2 monoclonal antibody fragment, a Pseudomonas exotoxin A translocation domain and a constitutively active caspase-3 molecule were used to induce apoptosis in human gastric cancer cells both in vitro and in vivo. Immunofluorescence staining and western blotting were used to examine the expression of the recombinant protein HER-PE-CP3. Apoptosis was determined by flow cytometry and TUNEL assay.

Results Co-cultivation of HER-PE-CP3/NIH3T3 with pcDNA3.1-HER-PE-CP3 recombinant plasmid containing a secretion signal, a single-chain anti-HER2 monoclonal antibody fragment, a Pseudomonas exotoxin A translocation domain and a constitutively active caspase-3 molecule were used to induce apoptosis in human gastric cancer cells both in vitro and in vivo. Immunofluorescence staining and western blotting were used to examine the expression of the recombinant protein HER-PE-CP3. Apoptosis was determined by flow cytometry and TUNEL assay.

Conclusions The HER-PE-CP3 chimeric molecule could induce selective apoptosis and potent growth inhibition of HER2-positive human gastric cancer cells and might represent a novel HER2-directed treatment option for human gastric cancer.

INTRODUCTION
Gastric cancer is one of the most common types of human malignancy and the second leading cause of cancer death in the world.1 In most countries the majority of gastric cancer cases are diagnosed at the advanced stage, when only around 50% of patients with gastric cancer could receive surgery as treatment.2 The efficacy of chemotherapy for gastric cancer is limited due to the relative insensitivity of gastric cancer to chemotherapy agents and the development of multidrug resistance.3 Other treatment options are even less optimal. The 5-year survival rates of gastric cancer patients are around 25%, which represents a serious health problem.2

Multiple genetic and epigenetic alterations have been found in gastric cancer and some have been shown to be associated with the development and progression of the cancer. Human epidermal growth factor receptor 2 (HER2), a member of the epidermal growth factor receptor family, is a cell membrane receptor-associated tyrosine kinase and is believed to be an oncogene. Amplification of the HER2 gene or over-expression of HER2 protein has been found in a variety of human tumours, including breast, ovary and stomach cancers. According to previous reports, over-expression of HER2 could be detected in around 10–40% of gastric cancer patients.4–8 Activation of HER2 has been suggested to play an important role in progression and metastasis of advanced gastric cancer.

Caspases are a family of cysteine proteases that cleave target proteins at specific aspartate residues.9,10 Caspase-3 is a key effector molecule in the caspase-dependent cell apoptosis pathway that cleaves a number of cellular proteins, leading to the apoptotic changes.11 Wild-type caspase-3 exists as a zymozyme in the cell and consists of an N-terminal prodomain, a large subunit and a small subunit at the C-terminus. Upon activation, caspase-3 is cleaved at the caspase processing sites (CPSs) between these domains, which results in the release of the prodomain and the subsequent re-association of the large and small subunit as a heterodimer. The reunification of the large and small subunits produces an active form of caspase-3, in which the small subunit is located at the N-terminus, opposite to the order in which the large and small subunits are positioned in the precursor molecule.

In the present study, we constructed an artificial chimeric molecule which consists of a secretion signal, a single-chain anti-HER2 monoclonal antibody fragment (c25sFv), a Pseudomonas exotoxin A-derived transportation domain (PEII) and a constitutively active caspase-3 component and tested its efficacy in inducing selective apoptosis in HER2-expressing gastric cancer cells and in suppressing the growth of human gastric cancer xenografts in a nude mouse model.

MATERIALS AND METHODS
Cell culture
Human gastric cancer cell lines MKN28 and MKN45 were obtained from RIKEN Cell Bank.
(Tsukuba, Japan). Human gastric cancer cell lines AGS and KATO III, human hepatocellular carcinoma HepG2 cell line, human cervical carcinoma HeLa cell line and NIH3T3 mouse fibroblast cell line were purchased from American Type Culture Collection (ATCC, Rockville, Maryland, USA). Human gastric cancer cell lines SGC7901, MGC803, and BGC823 were obtained from Beijing Institute of Oncology (Beijing, China). NIH3T3 cells were grown in high-glucose Dulbecco’s medium and other cell lines were maintained in RPMI 1640 medium. All the cell culture media were supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. All assays were carried out in triplicate. Animal studies were repeated twice.

Western blotting
Cells were washed with cold phosphate-buffered saline (PBS) and resuspended in a lysis buffer containing 0.5% NP-40, 1 mM sodium vanadate, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 50 µg/ml aprotinin, and 20 mM Tris-HCl (pH 8.0) and were placed on ice for 60 min. The lysate was centrifuged at 12,000 g for 20 min to remove cellular debris and the protein concentration in the supernatant was measured using a Bradford protein assay kit (Bio-Rad, Richmond, California, USA). Samples containing 50–100 µg of protein were added to a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer and then loaded on an 8% denaturing polyacrylamide gel. Proteins were then transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes (Amersham–Pharmacia Biotech, Piscataway, New Jersey, USA). Membranes were blocked using 5% non-fat dry milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature, and then incubated for 2 h at room temperature with monoclonal antibodies against HER2 (1:100; Chemicon International, Temecula, California, USA) or rabbit anti-mouse cleaved caspase-3 antibodies (1:1000; Cell Signalling Technology, Danvers, Massachusetts, USA). Anti-β-actin (1:5000; Sigma, St Louis, Missouri, USA) was used as an internal control for protein loading. Blots were washed three times with TBST containing 0.1% Tween 20 for 15 min each and incubated with a secondary antibody supplied with an enhanced chemiluminescence (ECL) kit (Amersham–Pharmacia Biotech) for 1 h at room temperature. The signal was detected by chemiluminescence using the ECL detection kit. Scanning densitometry was conducted for protein quantification.

Chimeric molecule construction
The human caspase-3 cDNA was obtained from human lymphoma Jurkat cells using reverse-transcription PCR. A constitutively active form of caspase-3 (CCP3) was generated using overlapping PCR. The genes for a truncated Pseudomonas exotoxin A-derived transportation domain II fragment (aa 275–358), an anti-HER2 single chain monoclonal antibody fragment (ε23sFv) and a secretion signal peptide (Met-Lys-His-Leu-Arg) were added sequentially to CCP3 in the 3’→5’ direction by overlap extension strategy. The resultant chimeric molecule was cloned into the Hind III/Xba I site of pcDNA3.1/V5-His vector (Invitrogen, Carlsbad, California, USA) and designated HER-PE-CP3.

Stable transfection of NIH3T3 cells with recombinant plasmids
NIH3T3 cells (3×10^5 in 2 ml of RPMI 1640) were seeded in six-well Costar tissue culture plates. Twenty-four hours later, cells were transfected with vector alone or 2.5 µg HER-PE-CP3 recombinant plasmids using LipofectAmine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Positive transfectants were selected in high-glucose DMEM containing 800 µg/ml G418 for 5 weeks. Cell lines were established from individual colonies using cloning cylinders.

Detection of secreted fusion protein from culture medium
The stably transfected NIH3T3 cells were maintained in RPMI 1640 culture medium in 200 ml flasks. After 3 days of culture the medium was collected and cryoconcentrated. The concentrated medium was then mixed with an equal volume of 2 × protein sample buffer and was loaded onto an 8% denaturing SDS–PAGE for western blotting analysis as above.

Immunofluorescence staining
Cells on coverslips were fixed in cold acetone, permeabilised with 0.25% Triton X-100 in PBS, blocked sequentially with 3% H2O2 and PBS-diluted normal rabbit serum (1:20), and then incubated overnight at 4°C with rabbit anti-cleaved caspase-3 polyclonal antibodies (Cell Signalling Technology) at a 1:100 dilution. Cells were extensively washed with PBS and incubated with fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit secondary antibodies (Sigma) at a 1:100 dilution for 2 h at room temperature followed by further rinsing. Then the cells were mounted and examined under a fluorescence microscope.

Conditioned culture of SGC7901 gastric cancer cells and FACS
SGC7901 cells and HepG2 cells were grown in 200 ml plastic flasks until log phase. The cells were then seeded in six-well plates and grown until 80% confluence. The supernatant was aspirated and replaced by freshly collected culture medium from HER-PE-CP3/NIH3T3 or pcDNA3.1/NIH3T3 cells every 24 h. Co-cultured cancer cells were subjected to indirect immunofluorescence staining using anti-caspase-3 as an antibody as above. The cells were also collected at various time and washed with cold PBS twice. The resuspended cells were mixed with annexin V–FITC and propidium iodide (PI) staining solution according to a standard protocol of BD Pharmingen (San Diego, California, USA). Cells were then analysed by flow cytometry.

Anti-tumour activity of HER-PE-CP3
Cancer cells were harvested and 4 × 10^6 cells in 200 µl PBS were injected subcutaneously into the right posterior flank of Balb/c nude mice. For treatment efficacy assessment, 10–12 mice were randomly divided into two groups with 5–6 mice in each group. The mice in the two groups received either HER-PE-CP3/NIH3T3 or pcDNA3.1/NIH3T3 (both with 5 × 10^6 cells in 200 µl PBS) immediately or 2 weeks after the seeding of SGC7901 cells at a site 2 cm apart. The growth of the tumours was monitored by measuring the largest and perpendicular diameters twice a week with a calibre and tumour volume (V) was calculated using the formula V=π/6LW^2, where L is the length and W is the width of the tumours. All mice were killed when the first mouse died and the measurement of tumour weight was made. In survival studies, eight mice in each group received the same inoculation regimen as before and the survival time of the mice was recorded.

Immunohistochemical staining
The xenograft tumours were excised, fixed in 10% (v/v) formalin and paraffin embedded. For immunocytochemistry, 0.5 µm tissue sections were prepared, deparaffinised and blocked
with 0.3% H₂O₂. The sections were incubated with trypsin and
normal goat serum, followed by rabbit anti-cleaved caspase-3
primary antibodies (1:50; Cell Signalling) or anti-HER2
monoclonal antibodies. The appropriate isotype antibodies were
used as negative controls. Secondary biotinylated goat anti-
rabbit IgG or goat anti-mouse IgG antibodies (Vector Labs,
Burlingame, California, USA) were added, followed by
streptavidin–horseradish peroxidase (Sigma). Slides were
stained with diaminobenzidine and counterstained in Gill’s
haematoxylin.

TUNEL assay
The TUNEL assay (terminal deoxynucleotidyl transferase-
mediated deoxyuridine triphosphate nick end labelling) was
performed on tumour sample sections as per the manufacturer’s
instructions (In Situ Cell Death Detection Kit, POD; Roche
Applied Science, Shanghai, China).

Statistical analysis
For all data, the effects of treatments between the two groups
were evaluated using the Student t test for repeated measures.
Survival function was analysed with the log-rank test. Analyses
were performed using the SPSS V.10.0 statistical software
package.

RESULTS
Expression of HER2 in gastric cancer
It has already been found that HER2 is over-expressed in
10–40% of gastric carcinomas. To gain further information on
the expression status of HER2 in human gastric cancer cell lines,
western blot analysis was used to determine the protein
expression of HER2/new in seven human cell lines. The results
showed that SGC7901, MKN45 and BGC823 had high levels of
HER2 expression. AGS and MGC803 cell lines showed low
expression of HER2/new (figure 1A). HepG2 and HeLa cell lines had
undetectable HER2 expression (figure 1B). In subsequent experiments,
the SGC7901 cell line was chosen for both in vitro and in vivo
studies and HepG2 was employed as a negative control.

Construction of the chimeric molecule
By using overlapping extension and other molecular biology
techniques a recombinant plasmid containing the genes for
a secretion signal peptide, an anti-HER2 single chain monoclonal
antibody fragment (e23sFv), a Pseudomonas exotoxin A-derived
transportation domain fragment (aa275–358) and a constitutively
active caspase-3 component was constructed as shown in
figure 2. Sequencing confirmed the correct nucleotide sequence of
the recombinant molecule.

Expression and secretion of the recombinant protein in NIH3T3
cells
To obtain a cell line that could continuously synthesise and
secrete a recombinant protein in sufficient quantity, it is desir-
able to verify the expression of the recombinant plasmid in the
cells and the presence of the recombinant protein in cell
medium. To that end, western blotting was used to determine
protein expression of HER-PE-CP3 both in transected NIH3T3
cells and the cell culture supernatant (figure 3A, B). The positive
colonies showed high expression of HER-PE-CP3, and in control
colonies caspase-3 could hardly be detected. Indirect immuno-
fluorescent staining was also used to detect the expression of the
recombinant protein in HER-PE-CP3 transfectants. The results
showed the staining was strong in both the cytoplasm and
nucleus of HER-PE-CP3/NIH3T3 cells (figure 3C), which indi-
cated high efficiency of transcription and translation of the
chimera in the cells. The morphology of the NIH3T3 cells had
not noticeably changed.

Induction of apoptosis by HER-PE-CP3 recombinant protein
To test if secreted recombinant HER-PE-CP3 could specifically
target and induce apoptosis in HER2-expressing human gastric
cancer cells, conditioned medium from HER-PE-CP3 trans-
fecteds was added to human gastric cancer cells SGC7901 every
2 days. On day 2 of conditioned cultivation, some SGC7901 cells
were already positive for cleaved caspase-3 as shown by

immunofluorescent staining, indicating the uptake of the recombinant protein by the gastric cancer cells. Furthermore, during the first 3 days of co-culture, only a slightly increasing number of apoptotic SGC7901 cells were noted. However, by day 4 of co-culture, there was a sudden increase of the number of apoptotic SGC7901 cells. In three independent assays, the proportion of apoptotic cells from HER-PE-CP3-treated SGC7901 cells (19.5 ± 3.6%) was much higher than that of control

Figure 4  Induction of apoptosis in HER2-positive SGC7901 cells with HER-PE-CP3 recombinant fusion protein. SGC7901 cells and HepG2 cells were cultured in conditioned media from HER-PE-CP3- or pcDNA3.1 stably transfected NIH3T3 cells for various times. After 24 h of conditioned culture the cells were subjected to indirect immunofluorescent staining using anti-caspase-3 antibodies (A). The cells were also incubated with annexin V—FITC and PI staining solution and analysed by flow cytometry (B). pcDNA3.1/SGC7901, SGC7901 cells treated with supernatant from pcDNA3.1 stably transfected NIH3T3 cells; HER-PE-CP3/SGC7901, SGC7901 cells treated with HER-PE-CP3/NIH3T3 supernatant; pcDNA3.1/HepG2, HepG2 cells treated with supernatant from pcDNA3.1 stably transfected NIH3T3 cells. FITC, fluorescein isothiocyanate; PI, propidium iodide.
(1.8 ± 0.49%) by day 4 (figure 4A, B). However, the proportion of apoptotic cells of HepG2 remained comparable in the 4 days after exposure to conditioned medium from HER-PE-CP3 NIH3T3 transfectants (figure 4C).

**Inhibition of human gastric cancer xenografts in nude mice**

To validate if HER-PE-CP3 recombinant protein secreted by NIH3T3 cells could inhibit the growth of HER2-positive human gastric cancer cell transplants and induce apoptosis in vivo, HER-PE-CP3-expressing NIH3T3 cells were injected subcutaneously into nude mice bearing human gastric cancer xenografts established by inoculation of SGC7901 cells into the right posterior flank of Balb/c nude mice. In experiment 1, a single dose of the HER-PE-CP3/NIH3T3 transfectants or the pcDNA3.1/NIH3T3 control transfectants was injected into the mice in the two groups, respectively, immediately after inoculation of SGC7901 cells; As shown in figure 5A, B and C the average size of xenografts in mice receiving HER-PE-CP3/NIH3T3 transfectants at the time they were killed was significantly smaller than that of control mice (figure 5A, B and C 38.64±14.69 mm³ vs 1147.63±253.74 mm³, p<0.01) and the average weight of tumours was also much less than those of control mice (figure 5B 45±15.52 mg vs 598±100.19 mg, p<0.01). The survival time was much longer in mice receiving HER-PE-CP3/NIH3T3 transfectants than that of control mice (figure 5D 86.25±9.80 days vs 50.4±5.56 days, p<0.01). In experiment 2, the two types of transfectants were injected into the two groups of mice 12 days after the inoculation of SGC7901 cells. The results showed the implanted gastric cancer xenograft in the fusion protein-secreting NIH3T3 group also grew at a much slower rate compared with the control group. At the time when the mice were killed, the average volume of the tumours from the treatment group was 185.64±44.69 mm³ vs 1258.23±267.52 mm³ (figure 5E, F) of the tumours from the control group (p<0.01). The average survival time was also significantly different between the two groups (figure 5G 75.07±8.92 days of the HER-PE-CP3/NIH3T3 group vs 29.81±6.39 days of the control group, p<0.01).

**Immunohistochemical examination of xenografts**

When mice were killed, the xenografts were immunohistochemically examined for expression of caspase-3. In control mice, almost all cells were negative for caspase-3 (figure 6A1), while the xenografts from the mice which had received HER-PE-CP3/NIH3T3 showed large areas of positive staining of caspase-3 (figure 6A2–6). In some tumour samples, the cancer cells in the centre of a tumour were strongly stained for caspase-3 compared with the cancer cell nests in the periphery of the tumour (figure 6A2, 3). These positively stained cells were scattered and not well defined, implicating cell degradation or damage. In some other samples the positive and negative staining areas were clearly distinguished (figure 6A4–6). In both
treatment and control groups most tumour cells in xenografts were uniformly positive for HER2 (data not shown), indicating that tumour cells were homogenous in regard to HER2 expression. The distribution of Ki67, a marker for cell proliferation was also similar in both groups of mice (data not shown). As shown in the TUNEL assay, large areas of positive staining were seen in HER-PE-CP3/NIH3T3 xenografts and the staining pattern was almost the same as that of caspase-3-positive-staining tumours (figure 6B). When the mice were examined for tissue integrity in other important organs, such as liver, lung and kidney by haematoxylin–eosin staining, no signs of massive cell injury were found, indicating that the fusion protein had not caused any significant damage to these normal organs (figure 6C).

DISCUSSION

Targeted tumour cell killing or inhibition has been pursued for many years for successful tumour treatment. HER2 has been considered as one of the promising targets in the anti-tumour therapy in a variety of tumours. A number of HER2-targeting strategies have been employed to attack HER2-expressing tumours. Among them is the development of a humanised antibody, trastuzumab and the results of the use of trastuzumab in breast cancer12,13 have encouraged the development of other important organs, such as liver, lung and kidney by haematoxylin–eosin staining, no signs of massive cell injury were found, indicating that the fusion protein had not caused any significant damage to these normal organs. The truncated PE II domain is situated between e23sFv monoclonal antibody and caspase-3 and could facilitate the transportation of the whole recombinant protein into the cytosol.29 Once in the cytosol of tumour cells the recombinant protein would be cut between A279 and A280 of PE II by specific enzymes in the endocytoplasm, thus releasing the C-terminal fragment caspase-3. Thus the killing of human gastric cancer cells is facilitated by the uptake of the chimeric molecule mediated by single chain anti-HER2 monoclonal antibody and the subsequent release of a key apoptosis executioner caspase-3 mediated by PE II breakage. Other cells that lack the membrane receptor HER2, including some tumours cells, are thus spared by the attack of the HER-PE-CP3 fusion protein. Although there were reports that in HER2-negative cells such as colon cancers, HER2 antibody and the associated signalling pathway might work, in our experiments no obvious toxic effects were observed on HER2-negative colon cancer cell HepG2 line or other major organs, such as liver, lung and kidney from nude mice receiving HER-PE-CP3-secreting NIH3T3 cells. The avoidance of apoptosis in these cells might be related to the inability of these cells to internalise the fusion protein, which is a more complicated process and is highly dependent on the interaction of the single chain antibody and the HER2-receptor.

A major finding of this study is that a single injection of HER-PE-CP3/NIH3T3 cells could inhibit the growth and maintain the sustained dominant state of the inoculated SGC7901 transplants in nude mice for a long period of time. The results could be attributed to the ability of implanted NIH3T3 cells to continuously secrete HER-PE-CP3 chimeric proteins in sufficient amount. This cell-based molecular targeting strategy avoids the complicated protein purification procedure as used in traditional recombinant protein drug preparation and the repeated administration of the drug. As a result of the combination of specific targeting of the anti-HER2 monoclonal antibody with the pro-apoptotic potency of caspase-3 and continuous availability of the recombinant protein, both in the groups of mice which received inoculation of SGC7901 and transduced NIH3T3 cells almost simultaneously and in the groups of mice which received transduced NIH3T3 cells at a later time, the sizes of tumours from HER-PE-CP3-treated mice were shown to be significantly smaller than those of control mice. The survival time of the group of mice which had received the HER-PE-CP3-secreting NIH3T3 cells was also much longer than that of the control mice. The most significant results were observed in mice which received simultaneous inoculation of SGC7901 and NIH3T3 cells, when the baseline number of SGC7901 gastric cancer cells was small.

These models may represent different clinical settings for gastric cancer patients and have important clinical implications. In those patients whose tumour mass has been almost completely removed by surgery, the therapeutic effects might be more optimal. For those patients in whom the tumour is unresectable or relapsing, the therapeutic effects still might be significant, albeit to lesser extents. In these patients the residual tumour cells could be targeted readily by HER-PE-CP3 chimeras, which are continuously secreted from the engineered cells, leading to long-term survival of the patients. Given the potency shown with the chimeric molecule in the present study and combined with the exciting preliminary results from the ongoing ToGA trial, more promising treatment modalities might be expected from the application of the HER-PE-CP3 chimeras in gastric cancer.

However, these experimental results must be carefully translated into clinical strategies against human gastric cancer.
Figure 6  Immunohistochemical and histological examination of xenograft tumours. The tissue sections of the resected human gastric cancer xenografts were immunohistochemically stained for expression of caspase-3 and assayed by TUNEL for the detection of apoptosis. Caspase-3 could only be detected in tumour sections from mice which received HER-PE-CP3/NIH3T3 (A2–6). The TUNEL assay shows substantially increased apoptosis in xenograft tumours from mice which received HER-PE-CP3/NIH3T3 (B). pcDNA3.1, control tumours from the group of mice which received pcDNA3.1/HIN3T3 cells; HER-PE-CP3, xenograft tumours from the group of mice which were injected with HER-PE-CP3/NIH3T3.

Haematoxylin–eosin staining of the lung, liver and kidney of the mice which received HER-PE-CP3-secreting fusion protein shows no significant indications of cell injury or damage (C). TUNEL, terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick end labelling.
Not only would species differences account for the differential therapeutic and side effects of the treatment, but the source of HER-PE-CP3 would also be a problem considering the body weights of humans and the immunological rejection of foreign cells. Thus, other methods might be worth trying which do not involve foreign cells or which only induce minimal immunological rejection of injected foreign cells. Furthermore, multiple injections might even have better therapeutic effects and should be considered in the future.

In conclusion, an artificial chimeric fusion protein HER-PE-CP3, which provides both the specificity of the anti-HER2 antibody and the apoptosis-inducing ability of caspase-3, has been generated. The fusion protein can be produced and secreted by stably transfected NIH3T3 cells. The secreted fusion could be taken by and induce apoptosis in HER2-positive gastric cancer cells but not in HER2-negative cancer cells. The recombinant protein has shown potent growth inhibitory effect on human gastric cancer xenografts in a nude mouse model and could be considered a potential therapeutic tool for human gastric cancer treatment.

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**Competing interests** None.

**Ethics approval** All animals used in this study were housed, cared for and used in accordance with the institutional guidelines for the care and use of laboratory animals. Approval was given by Xijing Hospital on the 20 December 2005.

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