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Molecular characterization and expression analysis of annexin B3 and B38 as secretory proteins in *Echinococcus granulosus*

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Abstract

Background: Cystic echinococcosis is a parasitic zoonotic disease, which poses a threat to public health and animal husbandry, and causes significant economic losses. Annexins are a family of phospholipid-binding proteins with calcium ion-binding activity, which have many functions.

Methods: Two annexin protein family genes [*Echinococcus granulosus* annexin B3 (*EgAnxB3*) and *EgAnxB38*] were cloned and molecularly characterized using bioinformatic analysis. The immunoreactivity of recombinant *EgAnxB3* (*rEgAnxB3*) and *rEgAnxB38* was investigated using western blotting. The distribution of *EgAnxB3* and *EgAnxB38* in protoscoleces (PSCs), the germinal layer, 18-day strobilated worms and 45-day adult worms was analyzed by immunofluorescence localization, and their secretory characteristics were analyzed preliminarily; in addition, quantitative real-time reverse transcription polymerase chain reaction was used to analyze their transcript levels in PSCs and 28-day strobilated worms stages. The phospholipid-binding activities of *rEgAnxB3* and *rEgAnxB38* were also analyzed.

Results: *EgAnxB3* and *EgAnxB38* are conserved and contain calcium-binding sites. Both *rEgAnxB3* and *rEgAnxB38* could be specifically recognized by the serum samples from *E. granulosus*-infected sheep, indicating that they had strong immunoreactivity. *EgAnxB3* and *EgAnxB38* were distributed in all stages of *E. granulosus*, and their transcript levels were high in the 28-day strobilated worms. They were found in liver tissues near the cysts. In addition, *rEgAnxB3* has Ca²⁺-dependent phospholipid-binding properties.

Conclusions: *EgAnxB3* and *EgAnxB38* contain calcium-binding sites, and *rEgAnxB3* has Ca²⁺-dependent phospholipid-binding properties. *EgAnxB3* and *EgAnxB38* were transcribed in PSCs and 28-day strobilated worms. They were expressed in all stages of *E. granulosus*, and distributed in the liver tissues near the hydatid cyst, indicating that they are secreted proteins that play a crucial role in the development of *E. granulosus*.

Keywords: *Echinococcus granulosus*, Annexin, Calcium ion, Western blotting, Immunofluorescence localization, Secretory protein

Background

Cystic echinococcosis (CE), which is caused by the larval stage of *Echinococcus granulosus*, is a parasitic zoonosis currently prevalent in Mediterranean countries, southern South America, Central Asia, China, Australia and parts of Africa [1, 2]. CE seriously threatens public health and impedes the development of animal husbandry, resulting in approximately US \$ 3 billion losses to the livestock

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industry each year [3]. It has been listed as one of the neglected tropical diseases by the World Health Organization [4, 5]. *E. granulosus* pathogenic research is of great significance, and studying the mechanism of the interaction between *E. granulosus* and its host is also necessary for the prevention and control of CE.

Proteomics analysis revealed that annexins were present in *E. granulosus* hydatid fluid, protoscoleces (PSCs) culture fluid and exosomes [6–8]. Annexins are a family of phospholipid-binding proteins with calcium ion-binding activity, which function in calcium signaling, and are widely distributed in eukaryotic cells [9]. According to differences in gene location and protein structure, annexins are divided into five categories [10]. Annexins have multiple functions, such as cell anti-inflammatory activity, membrane repair and membrane transport, and probably participate in cell proliferation, differentiation, and apoptosis [11–15].

Parasite annexins have been found to regulate the host immune response and maintain the structural integrity of the cell [16, 17]. Parasite annexins have also been considered as potential targets for the development of drug and vaccine candidates [16, 18]. *Leishmania* promastigotes could combine annexin V to ensure normal function in the absence of phosphatidylserine [19]. Annexin 2 of *Schistosoma mansoni* is involved in epidermal development [20]. Recombinant annexin B30 of *S. mansoni* gave no significant protection against the parasite, which suggested it may not be suitable as a vaccine candidate [21]. Annexin B1 of *Taenia solium* can downregulate the immune response of the host [22, 23]. In humans, *E. granulosus* annexins have only been found in the fluid of pulmonary hydatids, not in the fluid of vertebral or paravertebral hydatids, which suggests that they play an important role in the calcification process of cysts [7]. Up until now, only *E. granulosus* annexin B33 (*EgAnxB33*) has been studied [24]. In the present study, cloning, expression, bioinformatic analysis, western blotting, relative fluorescence quantitative polymerase chain reaction (PCR), immunofluorescence localization, and phospholipid-binding bioactivity analyses of *EgAnxB3* and *EgAnxB38* were performed to provide basic information and new research directions for the interaction between *E. granulosus* and its hosts.

Methods

Animals

Four female New Zealand rabbits (9 weeks old) were purchased from Dashuo Experimental Animal (Chengdu, China; experimental animal production license no. SYXK2019-189). Four male beagles (6 months old) were provided by the Dujiangyan Beagle Breeding Center of the Sichuan Institute of Musk Deer Breeding.

Albendazole and levamisole were used for deworming in the first 3 months.

Parasites

Cysts of *E. granulosus* were obtained from naturally infected sheep in Sichuan Province, China. PSCs and the germinal layer were separated aseptically as described previously [25, 26]. The 28-day strobilated worms were acquired by artificially infecting beagles. Each beagle was given 50,000 PSCs orally and then euthanized after 28 days. The 18-day strobilated worms and 45-day adult worms were provided by the Department of Parasitology of Sichuan Agricultural University.

Sera

Sera against *E. granulosus* were isolated from naturally infected sheep. Negative sera were collected from cestode-free sheep. Corresponding sera were obtained in Sichuan Province, China and infection was determined by autopsy.

The preparation process of the polyclonal antibodies of recombinant *EgAnxB3* (*rEgAnxB3*) and *rEgAnxB38* was the same as previously reported [26]. Briefly, rabbit sera were collected as the negative control before immunization. Then each rabbit was immunized with 200 µg recombinant protein emulsified with Freund's complete adjuvant (Sigma-Aldrich, St. Louis, MO), followed by three boosters using Freund's incomplete adjuvant. Two weeks after the final immunization, antisera were isolated and immunoglobulin G (IgG) was extracted.

Cloning of *EgAnxB3* and *EgAnxB38*

An RNA extraction kit (Tiangen, Beijing, China) was used to extract total RNA from PSCs, and the Reverse Transcription System Kit (TaKaRa, Dalian, China) was used to synthesize cDNA. Specific primers were designed at GenBank using the sequences of *EgAnxB3* and *EgAnxB38* (accession numbers XM_024495323.1 and XM_024494485.1, respectively). The sequence of *EgAnxB3* was amplified using the primers 5'-CGGA TCCATGGCGACTGTCAAGCCTTGCTG-3' (BamHI) and 5'-CCGGAATTCTTACTCCAGTATGGCAAGC-3' (EcoRI). The sequence encoding *EgAnxB38* was amplified using primers 5'-CGGATCCATGGCTGGCTAT CCTCCACC-3' (BamHI) and 5'-CGGAATTCTCAGGG TCCAACCAAGCCAC-3' (EcoRI). Target fragments were amplified and cloned. Single colonies were selected for PCR identification, and the plasmid from the bacterial solution that tested positive by PCR was sequenced.

Bioinformatic analysis

The physicochemical properties were predicted using the ExPasy proteomics server (<http://au.expasy.org>). The

open reading frames (ORFs) of *EgAnxB3* and *EgAnxB38* were analyzed using ORF Finder (<https://www.ncbi.nlm.nih.gov/orffinder/>). Signal peptides and transmembrane area were predicted using online software SignalP (<http://www.cbs.dtu.dk/services/SignalP-3.0/>) and TMHMM-2.0 (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>). Tertiary (three-dimensional) structures were modeled through SWISS-MODEL (<http://swissmodel.expasy.org/>). MEGA software (version 5.05) was used to construct the phylogenetic tree using the maximum likelihood method [27].

Expression and purification of recombinant *EgAnxB3* and *EgAnxB38*

The correctly sequenced *EgAnxB3* and *EgAnxB38* plasmids were digested with restriction enzymes, ligated into the pET32a(+) plasmid. The resulting recombinant plasmids were transformed into *Escherichia coli* BL21 (DE3) (Tiangen, Beijing). *E. coli* cells containing pET32a-*EgAnxB3* and pET32a-*EgAnxB38* were cultivated at 37 °C for 8 h. Then the transformants were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside. The recombinant proteins were purified using a Ni²⁺ affinity chromatography column (Bio-Rad, Hercules, CA).

Western blotting

Western blotting was performed as described previously [28]. Briefly, the crude protein extracts of PSCs and the purified recombinant protein were transferred onto a polyvinylidene difluoride membrane. The membrane was incubated with sera [1:200 volume/volume (v/v) dilution] from CE-infected sheep or polyclonal antibodies (1:200 v/v dilution) for 12 h at 4 °C. After four washes, horseradish peroxidase (HRP)-conjugated sheep anti-rabbit IgG or rabbit anti-sheep IgG (1:2000 v/v dilution; Boster, Wuhan, China) was added and incubated for 1 h at 37 °C. The immunoreactive protein signals were visualized using an Enhanced HRP-DAB Chromogenic Substrate Kit (Tiangen).

Quantitative real-time reverse transcription PCR

Total RNA and cDNA of PSCs and 28-day strobilated worms were obtained as described above. Quantitative real-time reverse transcription PCR was used to analyze expression profiles of *EgAnxB3* and *EgAnxB38* in PSCs and 28-day strobilated worms. The primers for *EgAnxB3* were 5'-TGCCAACACGGATGCCCAAAC-3' and 5'-CTGGTGCGGTGTGCGAGAAC-3'. The primers for *EgAnxB38* were 5'-CGCTACGCAGAGGACAAGAACG-3' and 5'-CTCGCATCTACCCAGCACAAAC-3'. Expression of the actin gene was detected for use as an internal control for normalization. Primers specific to *E. granulosus* actin were 5'-ATGGTTGGT

ATGGGACAAAAGG-3' and 5'-TTCGTCACAATACCGTGCTC-3'. The data were analyzed using the 2^{-ΔΔCT} method [29].

Immunofluorescence localization

Immunofluorescence localization was performed as described previously [26]. The sections (PSCs, germinal layer from fertile/infertile cysts, 18-day strobilated worms and 45-day adult worms) were incubated with purified anti-r*EgAnxB3*/anti-r*EgAnxB38* rabbit IgG or negative rabbit sera (1:200 v/v dilutions) for about 14 h at 4 °C. Fluorescein isothiocyanate-conjugated sheep anti-rabbit IgG (1:100 dilution in 0.1% Evans blue solution) was incubated with sections for 1 h at 37 °C in the dark. The fluorescence signals were observed under a fluorescence microscope. Meanwhile, to detect the possible secretion of *EgAnxB3* and *EgAnxB38*, the liver tissues away from the cysts and near the cyst wall were also evaluated [24].

Phospholipid-binding bioactivity assay

The preparation of liposomes was based on previous reports with some modifications [24, 30]. Soybean lecithin (0.9 g; Sangon, Shanghai, China) and 0.3 g cholesterol (Sangon) were mixed and dissolved in anhydrous ethanol in a small beaker; the beaker was placed in a 65–70 °C water bath with stirring in order that the contents could dissolve completely, after which the beaker was rotated to remove the ethanol. Thirty milliliters of preheated phosphate-buffered saline was added to the beaker containing lecithin and cholesterol lipid membrane, and the contents stirred and heated in a water bath at 65–70 °C for 10 min. Finally, the beaker was placed on a magnetic stirrer and the contents stirred for 30–60 min. The phospholipid-binding assay was performed as described previously [24, 31]. There were three experimental groups for each recombinant protein: A, B, and C. Twenty microliters of liposomes, 30 μl r*EgAnxB3*/r*EgAnxB38*, and 30 μl of 1 mM CaCl₂ (except for group C) was added to each group, and 50 mM Tris-HCl added as a supplement to a total volume of 100 μl. All the groups were incubated in ice water and centrifuged to separate the supernatant from the precipitate. The precipitate in group B was washed with Tris-HCl. Thirty microliters of 1 mM ethylenediaminetetraacetic acid (EDTA) and 70 μl of Tris-HCl was added to the precipitate of group B and the mixture incubated in ice water for 30 min. The supernatant and precipitate were separated by centrifugation. All the supernatant and precipitate samples were analyzed using 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

Results

Gene amplification and bioinformatics analysis

The results of the bioinformatics analysis of *EgAnxB3* and *EgAnxB38* are shown in Table 1. Both *EgAnxB3* and *EgAnxB38* were predicted to be extracellular proteins, but without a signal peptide.

Homology modeling was carried out on *EgAnxB3* and *EgAnxB38* in the SWISS-MODEL database, and two proteins with high homology were found, whose Protein Data Bank numbers were 1ala.1.A and 1aii.1.A. Using 1ala.1.A and 1aii.1.A as templates, three-dimensional models of *EgAnxB3* and *EgAnxB38* were constructed, respectively. The similarity between *EgAnxB3* and the template sequence was 45.75%, while the similarity was 39.31% between *EgAnxB38* and the template sequence. Both *EgAnxB3* and *EgAnxB38* have calcium-binding sites, but *EgAnxB3* contains two types of calcium ion-binding domain, whereas *EgAnxB38* only contains one calcium ion-binding domain (Fig. 1).

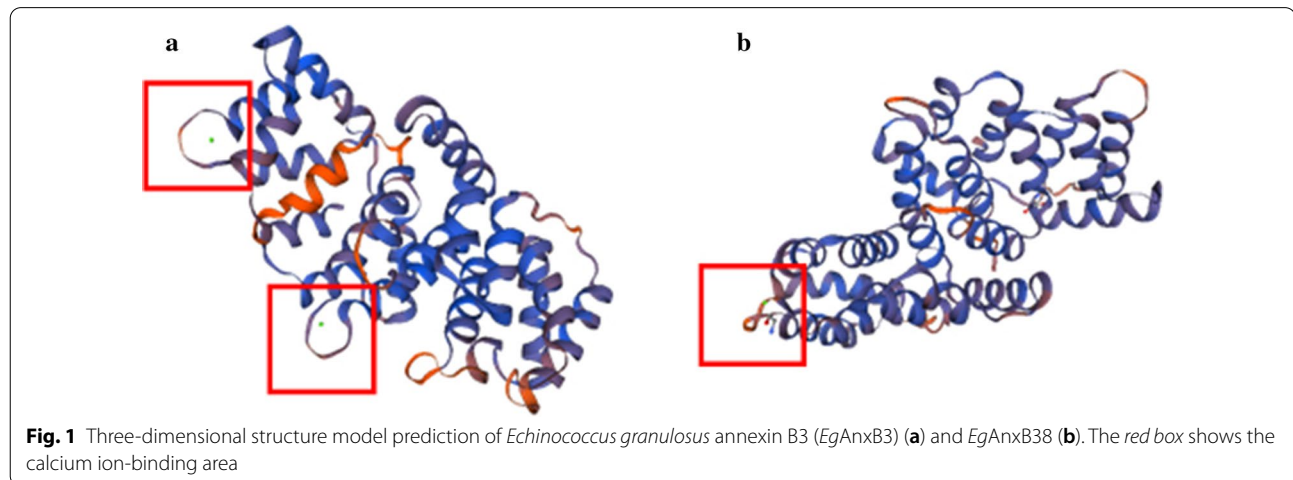
DNAMAN was used to compare the protein sequences of *EgAnxB3* and *EgAnxB38*, and it was found that the similarity was 32.15%. Multiple sequence alignment revealed that *EgAnxB3* shared 97.42% identity with *Echinococcus multilocularis* annexin, 83.23% with *T. solium* AnxB3, and 63.87% with *Hymenolepis microstoma* annexin (Fig. 2a). *EgAnxB38* shared 92.63% identity with *E. multilocularis* annexin, 64.13% with *H. microstoma* annexin, 40.17% with *Fasciola hepatica* annexin, and 39.88% with *Clonorchis sinensis* annexin (Fig. 2b). The maximum likelihood phylogenetic tree demonstrated that *EgAnxB3* and *EgAnxB38* were located on different branches, and had the closest genetic relationship with *E. multilocularis* (Fig. 3).

Expression, purification, and western blotting of r*EgAnxB3* and r*EgAnxB38*

The molecular mass of *EgAnxB3* was about 52 kDa, which was close to the expected value [note: the pET-32a(+) tag proteins weigh about 18 kDa]. Solubility

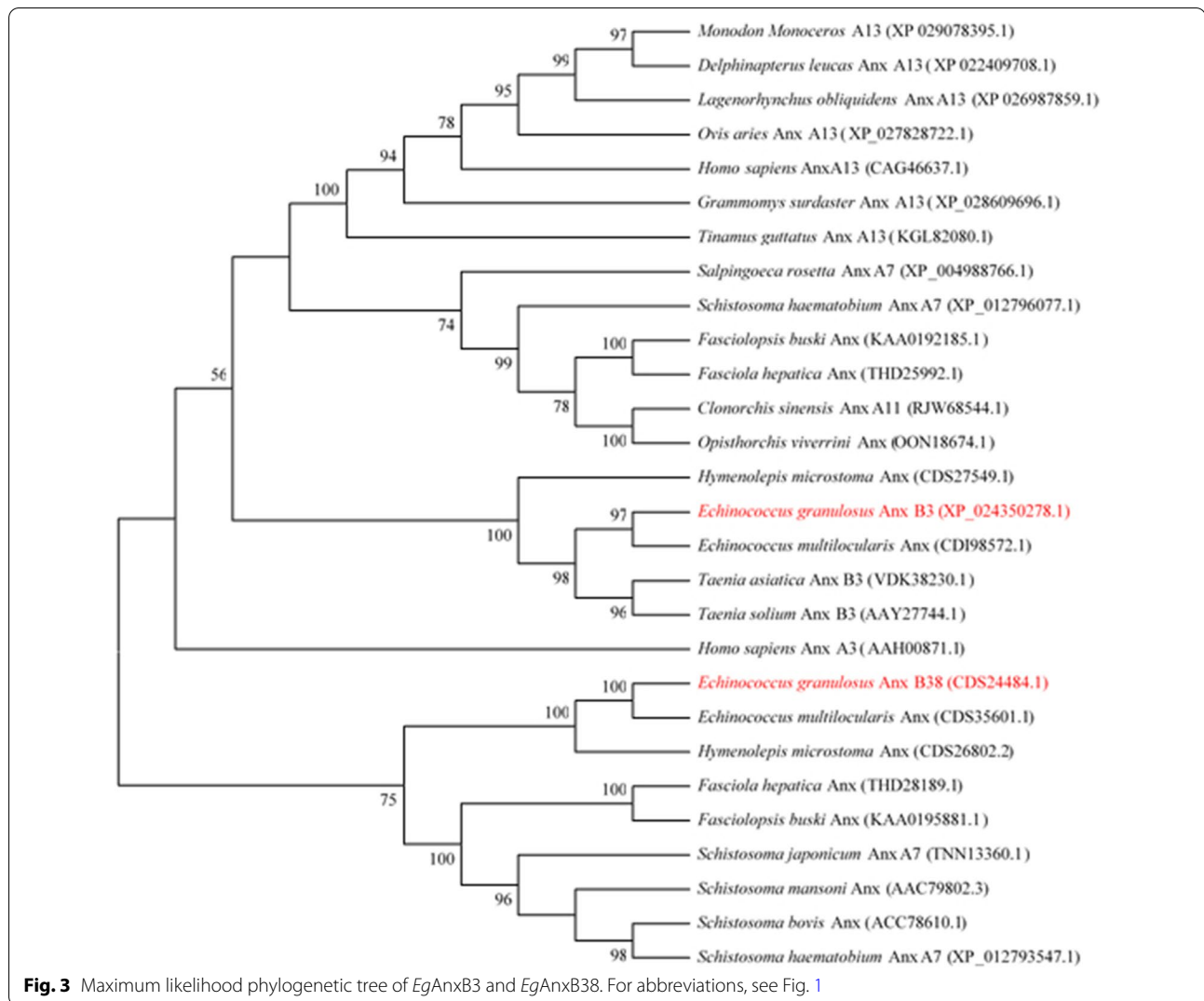
Table 1 Bioinformatics analysis of *Echinococcus granulosus* annexin B3 (*EgAnxB3*) and *EgAnxB38*

	Open reading frame	Amino acid	Molecular weight (kDa)	Isoelectric point (pI)	Lipid solubility coefficient	Instability coefficient
<i>EgAnxB3</i>	933	310	34.8	5.37	82.55	33.45
<i>EgAnxB38</i>	1356	451	48.5	6.66	68.63	35.46



(See figure on next page.)

Fig. 2 Sequence alignment of *EgAnxB3* (a) and *EgAnxB38* (b). Note the K-G-X-G-T sequence, indicated by yellow arrows. **a** *Eg Echinococcus granulosus* [GenBank (gb) XP_024350278.1], *Em Echinococcus multilocularis* (gb CDI98572.1), *Ts Taenia solium* (gb AAY27744.1), *Hm Hymenolepis microstoma* (gb CDS27549.1), *Hs Homo sapiens* (gb CAG46637.1), *Sm Schistosoma mansoni* (gb AAC79802.3), *Fh Fasciola hepatica* (gb THD28189.1), *Sj Schistosoma japonicum* (gb TNN13360.1). **b** *Eg E. granulosus* (gb CDS24484.1), *Em E. multilocularis* (gb CDS35601.1), *Hm Hymenolepis microstoma* (gb CDS26802.2), *Fh F. hepatica* (gb THD25992.1), *Cs Clonorchis sinensis* (gb RJW68544.1), *Ov Opisthorchis viverrini* (gb OON18674.1), *Sh Schistosoma haematobium* (gb XP_012796077.1), *Hs Homo sapiens* (gb AAH00871.1); for other abbreviations, see Fig. 1



analysis showed that *EgAnxB3* was largely expressed as a soluble form and partly in inclusion bodies (Fig. 4a). The molecular mass of *EgAnxB38* was about 67 kDa. Solubility analysis showed that *EgAnxB38* was largely expressed in inclusion bodies (Fig. 4b). Western blotting showed that *rEgAnxB3* and *rEgAnxB38* reacted with rabbit anti-*rEgAnxB3* antibody and rabbit anti-*rEgAnxB38* antibody, respectively, and with CE-positive sheep sera. Moreover, the native *EgAnxB3* protein and *EgAnxB38* protein in crude protein from PSCs were recognized using the rabbit anti-*rEgAnxB3* antibody and rabbit anti-*rEgAnxB38* antibody, respectively (Fig. 4).

Transcriptional profiles of *EgAnxB3* and *EgAnxB38*

EgAnxB3 and *EgAnxB38* genes were transcribed in both PSCs and 28-day strobilated worms, and the transcript levels of *EgAnxB3* and *EgAnxB38* in 28-day strobilated

worms were both significantly higher ($P < 0.05$) than those in the PSCs (Fig. 5).

Immunolocalization of *EgAnxB3* and *EgAnxB38*

EgAnxB3 and *EgAnxB38* were distributed in the germinal layer of fertile/infertile cysts, the parenchyma and tegument of 18-day strobilated worms and 45-day adult worms, and the calcareous corpuscles and hooks of PSCs. Compared with *EgAnxB3*, *EgAnxB38* was not only distributed in the calcareous corpuscles and hooks of PSCs, but also in the tegument of PSCs. Meanwhile, the distribution range of *EgAnxB38* in fertile/infertile cysts, adult worms, and PSCs was significantly wider than that of *EgAnxB3*, showing a strong fluorescence signal (Fig. 6).

EgAnxB3 and *EgAnxB38* were distributed in hepatic sinusoids of liver tissue close to hydatid cysts. In addition, *EgAnxB38* was also found in hepatic sinusoids in the

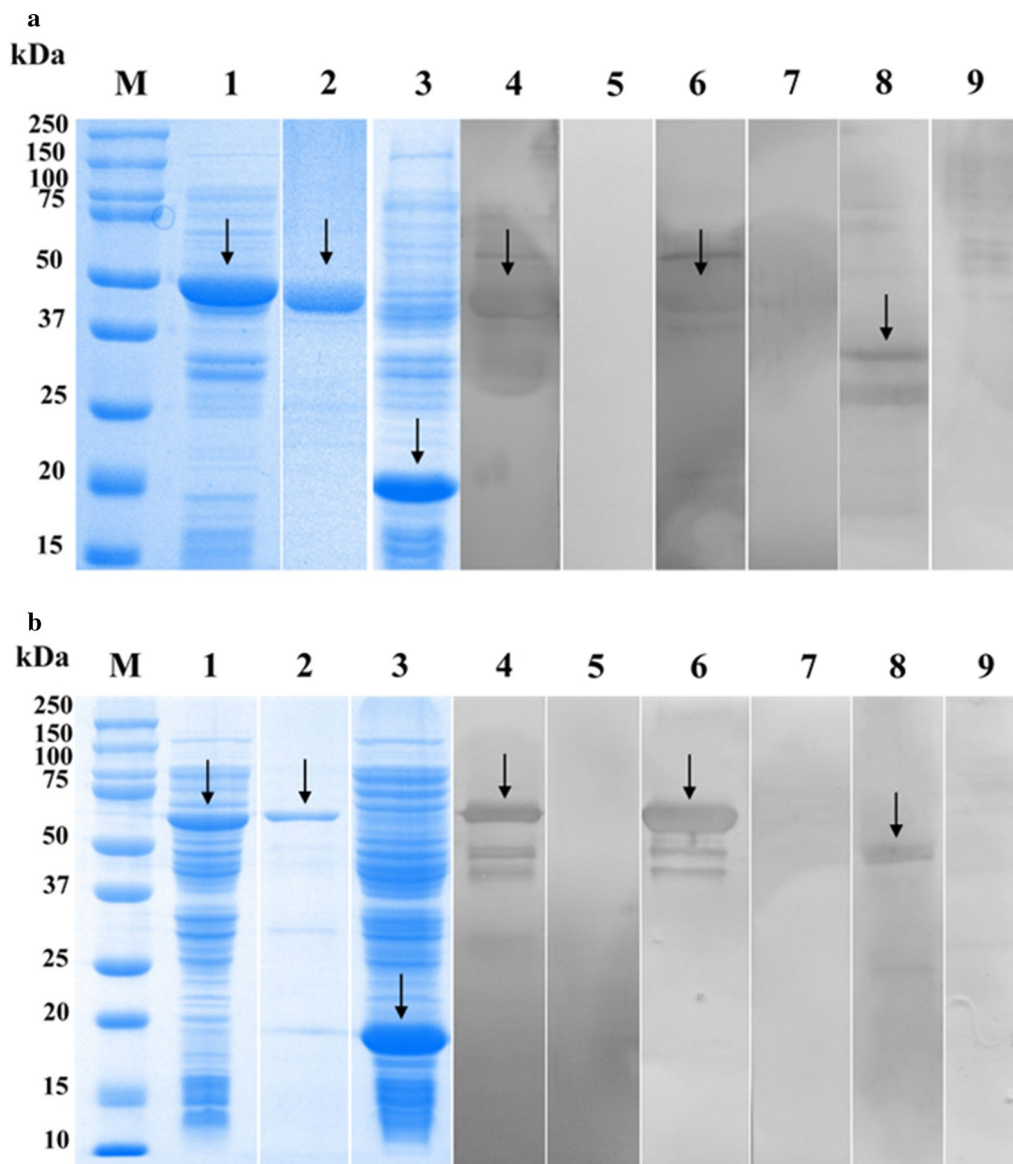


Fig. 4 Purification and western blotting of recombinant *EgAnxB3* (*rEgAnxB3*) (a) and *rEgAnxB38* (b). Lane M Protein marker, lane 1 *rEgAnxB3*/*rEgAnxB38* crude protein, lane 2 purified *rEgAnxB3*/*rEgAnxB38*, lane 3 pET32a(+) induced by isopropyl β -D-1-thiogalactopyranoside, lane 4 *rEgAnxB3*/*rEgAnxB38* incubated with rabbit anti-*rEgAnxB3*/*rEgAnxB38*-immunoglobulin G (IgG), lane 5 *rEgAnxB3*/*rEgAnxB38* incubated with negative rabbit serum, lane 6 *rEgAnxB3*/*rEgAnxB38* incubated with serum from a sheep infected with *E. granulosus*, lane 7 *rEgAnxB3*/*rEgAnxB38* incubated with serum of a healthy sheep, lane 8 crude protein of protoscoleces (PSCs) incubated with rabbit anti-*rEgAnxB3*/*rEgAnxB38*-IgG, lane 9 crude protein of PSCs incubated with negative rabbit serum IgG. For other abbreviations, see Fig. 1

liver distant from the hydatid cysts, whereas *EgAnxB3* was not found in this area (Fig. 7).

Phospholipid-binding bioactivity analysis

Recombinant *EgAnxB3* and *EgAnxB38* were reacted with liposomes in the presence or absence of Ca^{2+} to examine their calcium-dependent phospholipid-binding properties. The mixtures were centrifuged, and the

bound protein was pelleted with the precipitate and the unbound protein remained in the supernatant. In the presence of Ca^{2+} , *rEgAnxB3* was observed in the precipitate rather than in the supernatant (group A). When Ca^{2+} was removed from the recombinant protein using EDTA, *rEgAnxB3* largely remained in the supernatant (group B). *rEgAnxB3* was mainly observed in the supernatant in the absence of Ca^{2+} (group C). However, *rEgAnxB38* was

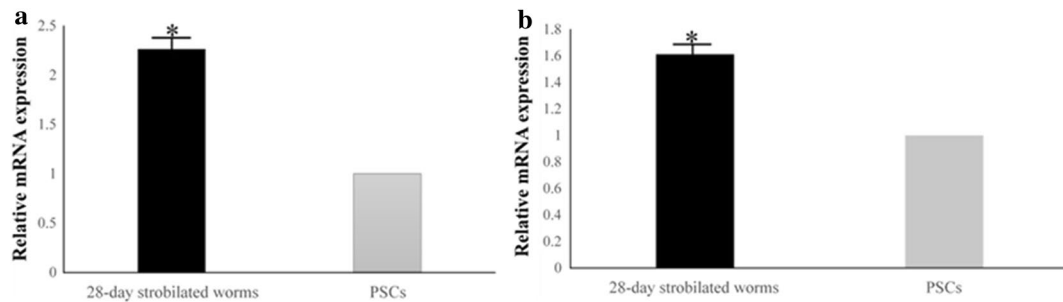


Fig. 5 Comparison of transcript levels of *EgAnxB3* (a) and *EgAnxB38* (b) genes in the PSCs and 28-day strobilated worms. Data are presented as the mean \pm SD of triplicate experiments. Statistically significant differences between PSCs (as the control) and 28-day strobilated worms were determined using Student's *t*-test [*EgAnxB3*, $t_{(4)} = 14.577$, $P = 0.00013$; *EgAnxB38*, $t_{(4)} = 20.744$, $P = 0.00003$] (* $P < 0.05$). For abbreviations, see Figs. 1 and 4

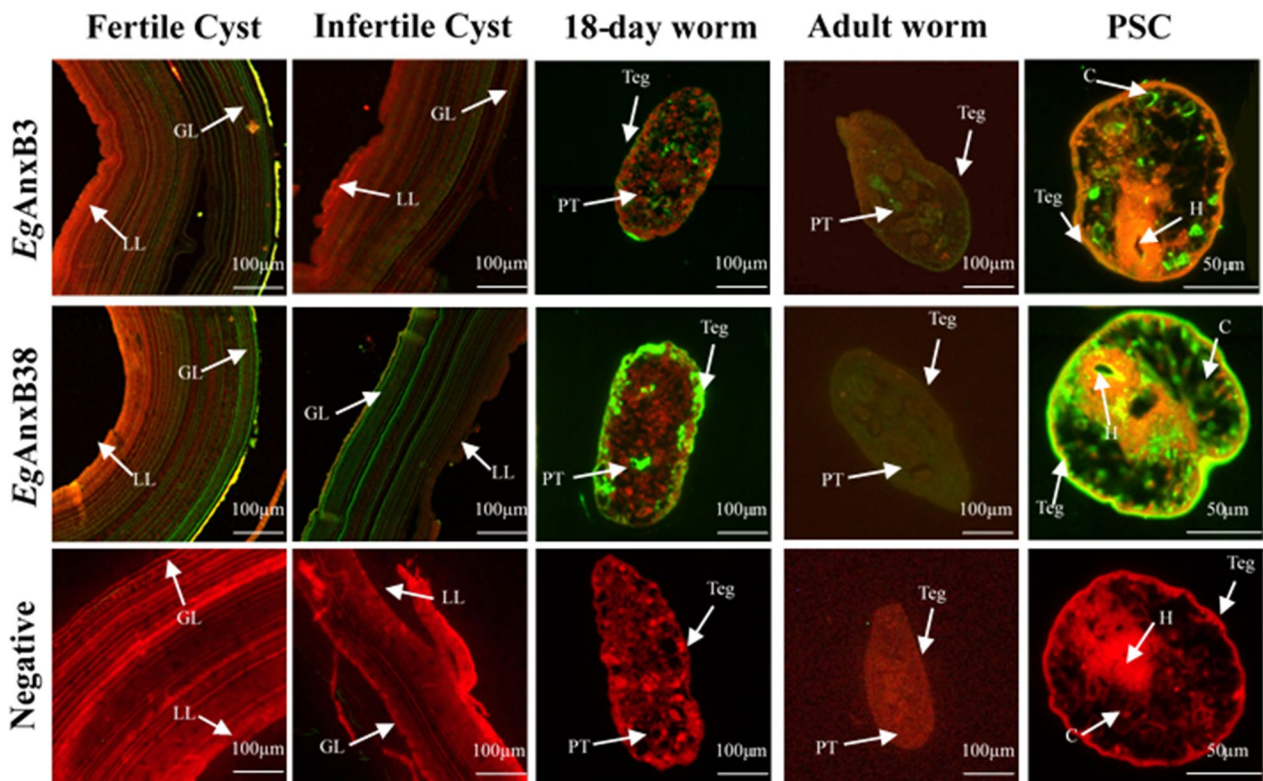


Fig. 6 Immunolocalization of *EgAnxB3* and *EgAnxB38* in different life cycle stages of *E. granulosus*. The green fluorescent region is the protein distribution region. GL germinal layer, LL laminated layer, Teg tegument, PT parenchyma, H hooks, C calcareous corpuscles; for other abbreviations, see Fig. 1

observed in both the precipitate and supernatant, regardless of the presence of Ca^{2+} (Fig. 8).

Discussion

The classification and nomenclature of annexin B group are confusing. Generally, parasites are invertebrates, and their annexins should be named “annexin B.” However,

when we screened *EgAnxB3* and *EgAnxB38* at the National Center for Biotechnology Information based on Zheng’s research in 2013 [32], they were both called “AnxA7,” which is obviously incorrect. We found that *EgAnxB3* had high similarity with *T. solium* annexin B3 via sequence alignment, so we named it “*EgAnxB3*.” However, we did not find an appropriate named parasite

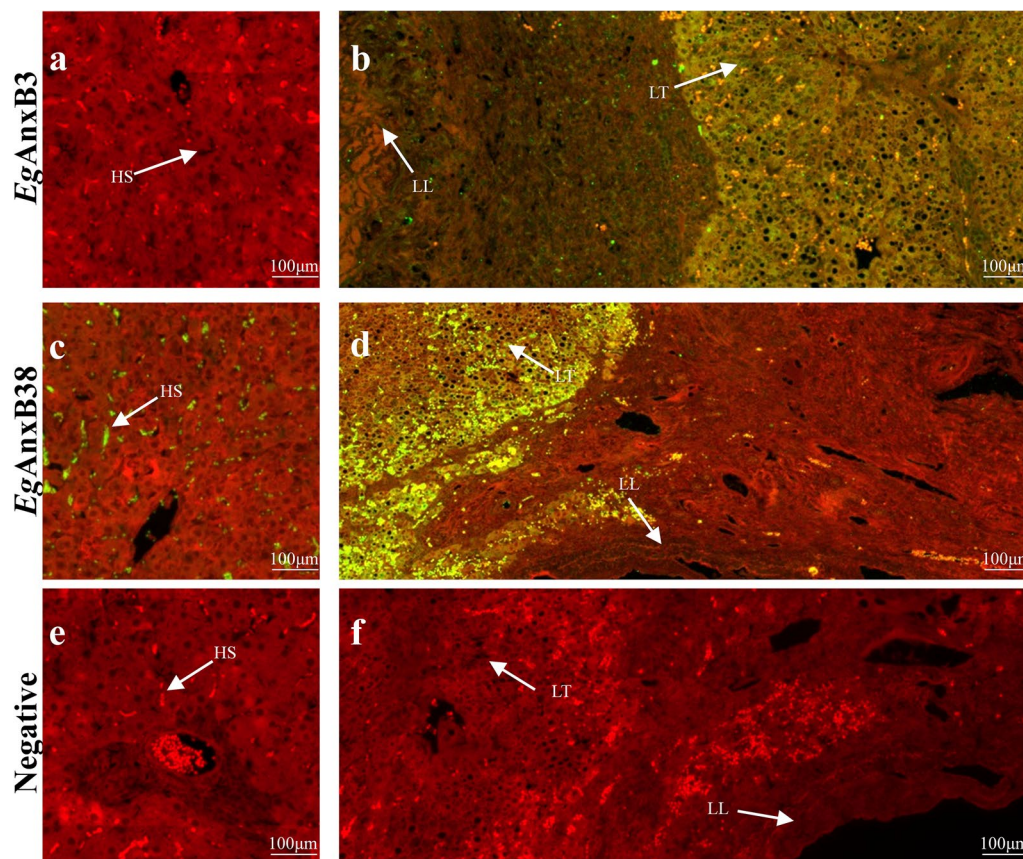


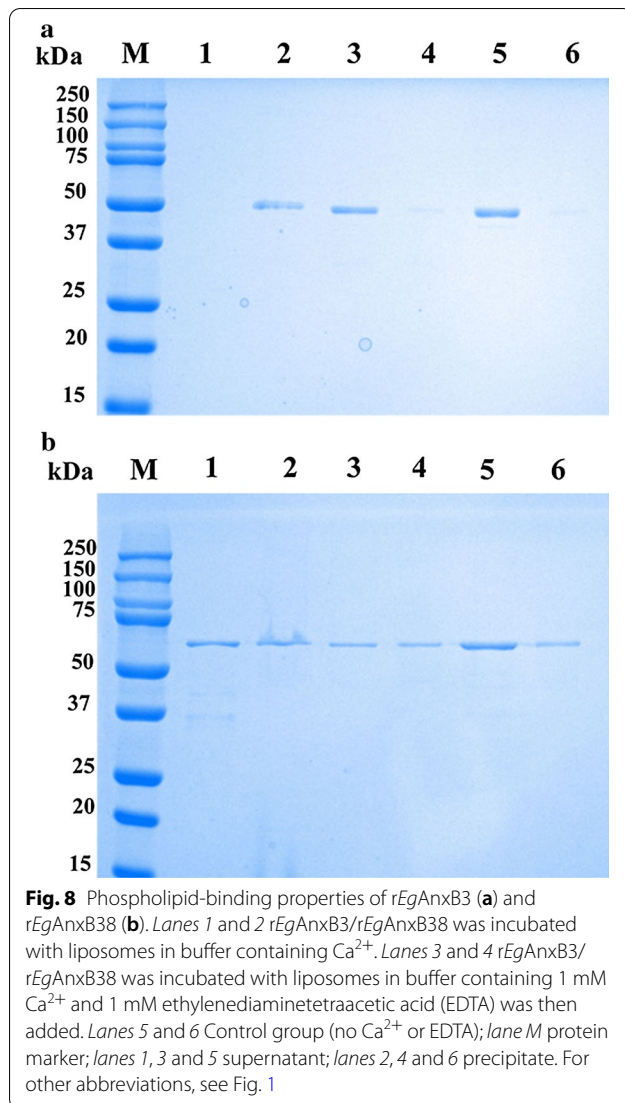
Fig. 7 a–f Immunolocalization of *EgAnxB3* and *EgAnxB38* in different regions of sheep liver infected with *E. granulosus*. **a, c, e** Liver tissue distant from the hydatid cysts. **b, d, f** Liver tissue close to hydatid cysts. The green fluorescent region is the protein distribution region. HS hepatic sinusoid, LL laminated layer, LT liver tissue; for other abbreviations, see Fig. 1

sequence with high homology to *EgAnxB38*. However, we found an article that named the annexins of many parasites by sequence alignment and phylogenetic tree analysis, including the annexins of *E. granulosus* [33]. We searched for *E. granulosus* annexins on the Hofmann Laboratory website (<http://www.structuralchemistry.org/annexins/seq/search.php>), which showed that our naming of *EgAnxB3* is correct, and that the name *EgAnxB33* is also consistent with that previously published [24]. Therefore, we confirm here that, for *E. granulosus*, “*EgAnxB38*” should be used rather than “AnxA7.”

Annexin proteins generally have four homologous repeat domains, and most of the domains contain a typical K–G–X–G–T sequence. It has been reported that the calcium-binding capacity of annexin proteins is based on their K–G–X–G–T sequence [33–35]. The amino acid sequence analysis showed that both *EgAnxB3* and *EgAnxB38* have four repeat domains and contain a K–X–G–T sequence. The tertiary structure prediction showed that both *EgAnxB3* and *EgAnxB38* have Ca^{2+} -binding sites. To verify the calcium-binding ability of *rEgAnxB3*

and *rEgAnxB38*, we conducted a Ca^{2+} -dependent phospholipid-binding assay and found that, in the absence of Ca^{2+} , *rEgAnxB3* could not bind to liposomes and was found in the supernatant, while in the presence of Ca^{2+} , *rEgAnxB3* bound with liposomes and was found in the precipitate. When EDTA was added to combine with calcium ions, *EgAnxB3* was again found in the supernatant, indicating that *rEgAnxB3* has Ca^{2+} -dependent phospholipid-binding properties. However, the Ca^{2+} -dependent phospholipid-binding properties of *rEgAnxB38* were relatively weak. *rEgAnxB38* was observed in both the precipitate and supernatant, regardless of the presence of Ca^{2+} . There are two possible reasons for this. First, in this study, *EgAnxB38* was expressed as an inclusion body protein, and thus possibly did not fold correctly, resulting in a lack of calcium-binding capacity. Second, regardless of the amino acid structure analysis or tertiary structure prediction, *EgAnxB38* had fewer Ca^{2+} -binding sites, resulting in an insufficient calcium-binding capacity.

The transcript levels of annexins are different at various stages of parasite development. *Taenia multiceps*



AnxB2 and *AnxB12* show high levels of transcription in the oncosphere. The transcript levels of *AnxB2* and *AnxB12* decreased from oncosphere to adult, while that of *AnxB3* increased [34]. The transcript level of *C. sinensis AnxB30* is higher in the metacercaria stage, and lower in adult worms and eggs [36]. The transcript levels of *EgAnxB3* and *EgAnxB38* in the 28-day strobilated worms were higher than those of PSCs in this study. Considering that annexins can protect the parasite from the host immune system during parasitism, and that PSCs develop into adult worms in the small intestine of the definitive host [16, 17], it is suggested that *EgAnxB3* and *EgAnxB38* might play a crucial role in the process of PSC invasion of the definitive host.

The parasite communicates with the host through the tegument, and molecules distributed in the tegument participate in the host-parasite interaction, which

includes excretion, nutrient absorption, and interaction with the host immune system [37, 38]. In the present study, both *EgAnxB3* and *EgAnxB38* were found to be located in the tegument and parenchyma of immature and gravid proglottids on the basis of immunofluorescence localization analysis, but *EgAnxB38* had a wider distribution and stronger fluorescence intensity. These results indicate that both these proteins might have a paramount role in the process of PSC invasion of the definitive host and development of the parasite, but that *EgAnxB38* may possibly play a larger role than *EgAnxB3* in this process. *EgAnxB38* was distributed in the hooks and tegument of the PSCs, while *EgAnxB3* was either not distributed in the hooks and tegument at all, or at a much lower level. Considering that PSCs require the participation of the hook during development in the definitive host's intestines, it is speculated that *EgAnxB38* participates in the interaction between the PSCs and the definitive host. PSCs form and mature in the germinal layer of *E. granulosus* [39]. *EgAnxB3* and *EgAnxB38* were distributed in the germinal layer, and their distribution range in fertile cysts was larger than that in infertile cysts, suggesting that they might be involved in the growth and development of PSCs.

Most annexins are cytoplasmic proteins or cytoskeletal proteins [40]; however, traces of annexins have also been found in the extracellular regions, although these annexins lack the signal peptide sequences required for extracellular secretion [41–43]. This interesting phenomenon suggests that some annexins are secreted. *C. sinensis AnxB30* is a secretory protein that is involved in the interaction between the parasite and host, and affects the host's autoimmune response [36]. As a secreted protein, *T. solium AnxB1* is involved in the interaction with the host inflammatory cells [16, 22, 23]. Annexins were also found to be present in the excretory–secretory product and hydatid cyst fluid of *E. granulosus* [6, 44]. *EgAnxB33* could be detected in the hydatid fluid of *E. granulosus* and was located on the inflammatory cells and fibroblasts of the host-derived layer, suggesting that it might play a paramount role in the interaction between *E. granulosus* and the host [24]. Although *EgAnxB3* and *EgAnxB38* have no signal peptide, in this study, they were found to be located in the hepatic sinusoids of liver tissue near hydatid cysts, indicating that they can be secreted into the extracellular areas to exert their physiological effects through non-classical secretion pathways, such as transfer via extracellular vesicles [45]. *EgAnxB38* was also found to be distributed in hepatic sinusoids in the liver, which were far away from the cysts. The fact that *EgAnxB38* had a wider distribution in different life cycle stages of *E. granulosus* suggests that it plays a more important role in the development and interaction with

the host. Certain excretory or secretory products of the parasite can regulate the host immune system through interaction with the host [46, 47]. The results of this study indicate that further investigation is needed to show if *EgAnxB3* and *EgAnxB38* play a role as secreted proteins in the immune evasion of *E. granulosus*. In addition, parasite annexins have potential as drug and vaccine targets, so it is also worth exploring if *EgAnxB3* and *EgAnxB38* can be used as drug targets and vaccine candidates.

Conclusions

In conclusion, we found that both *EgAnxB3* and *EgAnxB38* contain calcium-binding sites, and *rEgAnxB3* has Ca^{2+} -dependent phospholipid-binding properties. Both *rEgAnxB3* and *rEgAnxB38* could be specifically recognized by CE-positive sheep sera, indicating that they had strong immunoreactivity. The transcription level of *EgAnxB3* and *EgAnxB38* in 28-day strobilated worms was higher than that in PSCs. *EgAnxB3* and *EgAnxB38* were distributed in all stages of *E. granulosus*, and were also distributed in the liver tissues near hydatid cysts, indicating that they are secreted proteins that might play a crucial role in the development of *E. granulosus*.

Abbreviations

rEgAnxB3: Recombinant *Echinococcus granulosus* annexin B3; *rEgAnxB38*: Recombinant *Echinococcus granulosus* annexin B38; PSCs: Protoscoleces; CE: Cystic echinococcosis; ORF: Open reading frame; HRP: Horseradish peroxidase; PCR: Polymerase chain reaction; IgG: Immunoglobulin G; EDTA: Ethylenediaminetetraacetic acid.

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Authors' contributions

SHY participated in the design of the study, the feeding of the experimental animals, the experiments, the statistical analysis and manuscript writing. HX and DXD performed the experiments. HRQ contributed to sample collection and performed the experiments. XJ, HR and YGY participated in the design of the study. GXB, XY and PXR helped with the study design. All the authors read and approved the final manuscript.

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article.

Ethics approval and consent to participate

The animal study was reviewed and approved by the Animal Care and Use Committee of Sichuan Agricultural University (SYXK2019-187). All animal procedures used in this study were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, Bethesda, MD) and recommendations of the ARRIVE guidelines (<https://www.nc3rs.org>).

[uk/arrive-guidelines](#)). All the methods were carried out in accordance with the relevant guidelines and regulations.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

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