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Characterization and enzymatic function of thioredoxin glutathione reductase in *Orientobilharzia turkestanicum* isolated from Xizang

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Abstract

Orientobilharziasis, caused by *Orientobilharzia turkestanicum*, is a zoonotic parasitic disease that leads to significant economic losses in livestock and cercarial dermatitis in humans. This study focuses on the molecular characterization and functional analysis of thioredoxin glutathione reductase (TGR) from *O. turkestanicum*, a key enzyme involved in the parasite's antioxidant defense system. The full-length *O. turkestanicum* thioredoxin glutathione reductase (OtTGR) cDNA and *O. turkestanicum* thioredoxin glutathione reductase with selenocysteine (OtTGRsec) were cloned and expressed as recombinant proteins in *E. coli*. Western blotting confirmed the specific immunoreactivity of rOtTGR with polyclonal antibodies, and immunohistochemistry revealed its predominant localization on the tegument of adult worms. Enzymatic activity assays demonstrated that rOtTGRsec possesses thioredoxin reductase, glutaredoxin, and glutathione reductase activities, with optimal activity under physiological pH and temperature conditions. RNA interference assays showed that siRNA3 effectively suppressed OtTGR expression in vitro, reducing mRNA levels by 46.1%. These findings highlight the critical role of OtTGR in parasite survival. Comparative with other trematodes, such as *Schistosoma japonicum* and *Fasciola hepatica*, suggests that OtTGR may serve as a promising target for vaccine or drug development. Although immune-protective studies were not feasible due to host incompatibility, the conserved role of TGR across trematodes underscores its potential for controlling orientobilharziasis. Future studies will explore the immunogenicity and protective efficacy of OtTGR to assess its candidacy as a therapeutic target.

Keywords *Orientobilharzia turkestanicum*, Thioredoxin glutathione reductase (TGR), Thioredoxin glutathione reductase with selenocysteine (TGRsec), RNA interference, Vaccine

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Background

Orientobilharzia turkestanicum parasitizes the portal and intestinal veins of various mammals, including cattle and sheep. The disease caused by *O. turkestanicum* is referred to as orientobilharziasis. This condition is primarily distributed across parts of Asia and Europe, posing significant challenges to livestock development [1, 2]. Additionally, the cercariae of *O. turkestanicum* can penetrate human skin, causing cercarial dermatitis and posing a serious threat to human health. Consequently, orientobilharziasis is recognized as a significant zoonotic parasitic disease [2]. Currently, the primary strategy for controlling orientobilharziasis involves treating infected domestic animals with praziquantel (PZQ) [3]. Although PZQ effectively reduces morbidity in domestic animals, its effects are not sustainable, as it does not prevent reinfection and may lead to the development of drug resistance with prolonged use [4]. The optimal approach to managing orientobilharziasis combines immunization through vaccine development [5]– [6] with drug treatment [7]. However, no effective vaccines have been developed for orientobilharziasis to date. Therefore, there is an urgent need to screen and identify effective vaccine candidates or novel drug targets to enhance the control of orientobilharziasis.

Worms require efficient mechanisms to sustain cellular redox homeostasis, particularly as they inhabit the oxidative environment of host veins. To survive, these parasites must effectively counteract reactive oxygen species (ROS) generated by the host's immune system [8], which acts as a defense mechanism to eliminate pathogens [9]. In most eukaryotes, two redox systems detoxify ROS: one is based on glutathione (GSH), and the other is based on the protein thioredoxin (Trx) [10–13]. In both systems, electrons are transferred from NADPH via the enzymes glutathione reductase (GR) and thioredoxin reductase (TrxR). Recently, a unique multifunctional enzyme, thioredoxin glutathione reductase (TGR), was discovered to replace these two enzymes in *Schistosoma mansoni* and *S. japonicum* [14, 15]. TGR contains a selenocysteine (Sec) residue, which features a carboxyl-terminal GCUG active site motif [16]. These findings suggest that TGR plays a crucial role in the development and survival of schistosomes within their host.

Based on these findings, it is reasonable to speculate that TGR may play a critical role in the development and survival of *O. turkestanicum* within the host. In this study, the full-length cDNA encoding *O. turkestanicum* TGR (OtTGR) was cloned and expressed in *E. coli*. The localization and transmembrane domain of OtTGR were analyzed. Polyclonal antibodies against OtTGR were prepared, and Western blotting, as well as enzymatic activity assays, were conducted to evaluate its characteristics. The immunolocalization of rOtTGR was observed using fluorescence microscopy. Biological functionality studies of the OtTGR gene were carried out, providing a foundation for evaluating OtTGR as a potential vaccine candidate or novel drug target for the control of orientobilharziasis in the future.

Materials and methods

Radix auricularia infection

Radix auricularia snails were collected from Xizang, and their egg masses were incubated in water tanks at room temperature. Upon reaching a shell diameter of approximately 1 cm, they are selected and individually exposed to 5–10 *O. turkestanicum* miracidia per snail for 30 min. Miracidia were harvested from the feces of infected goats using the miracidial hatching technique. Following exposure, the snails were maintained in complete darkness for one month to facilitate parasite development. Snail collection occurred on private land with landowner permission, and no specific collection permits were required under national or local regulations.

Worms and animals

The *O. turkestanicum* life cycle was maintained in *R. auricularia* and six-month-old female Chinese Yangtze river delta white goats ($n = 2$; 20–25 kg), obtained from a local farm. Additional laboratory animals included two clean-grade male New Zealand rabbits (6 weeks old, ~2.0 kg) and ten specific pathogen-free male BALB/c mice (4–6 weeks old, ~20 g; five mice per group with random allocation), sourced from the Shanghai Experimental Animal Centre, Chinese Academy of Sciences. Cercariae were harvested by exposing infected snails to light. For experimental infections, the abdominal fur of New Zealand rabbits was shaved (~5 × 10 cm area), and each rabbit was exposed to ~5000 cercariae via abdominal cutaneous patch for 20 min. Adult *O. turkestanicum* worms were collected at 120 days post-infection by portal perfusion with pre-warmed phosphate buffered saline (PBS, 37 °C). Approximately 1000 worms were preserved preserved in RNAlater (Sigma-Aldrich, USA) and stored at −80 °C for further experiments.

All animals were housed under standard laboratory conditions and handled in accordance with the guidelines of the Committee for Care and Use of Laboratory Animals of Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences. The experimental protocols were approved by the Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences Ethical Committee (Permit Number: SHVRI-SZ-20201020-02). A two-week acclimatization period was provided prior to experimentation. The number of animals used was minimised in accordance with the principles of reduction and refinement. All animals were euthanised in accordance with AVMA Guidelines

for the Euthanasia of Animals (2020) at the end of the experiments. Mice were euthanised by cervical dislocation performed by trained personnel. Rabbits and goats were euthanised via anesthetic overdose. Rabbits were euthanised via an overdose of pentobarbital sodium (100–150 mg/kg), administered via the marginal ear vein. Goats were euthanised via an overdose of pentobarbital sodium (100–150 mg/kg), administered via the jugular vein. All procedures adhered to the AVMA Guidelines for the Euthanasia of Animals (2020). Experimental outcomes were assessed in a blinded fashion to reduce bias.

RNA extraction and cDNA preparation of *O. turkestanicum*

The total RNAs were extracted from the 120-day-old *O. turkestanicum* worms using TRIzol reagent (Invitrogen, USA). Briefly, homogenized worms were lysed in 1 mL TRIzol, followed by addition of 0.2 mL chloroform, vigorous mixing, and a 3-minute incubation at room temperature. The mixture was centrifuged at 10,000×g for 15 min at 4 °C. The aqueous phase was transferred to a new tube, mixed with 0.5 mL isopropanol, and incubated for 10 min at room temperature to precipitate RNA. After centrifugation (10000×g, 10 min, 4 °C), the supernatant was discarded, and the RNA pellet was washed with 1 mL of 75% ethanol, followed by another centrifugation (7500×g, 5 min, 4 °C). The pellet was air-dried and dissolved in 50 µL of nuclease-free Water (10 min at 60 °C), then stored at −80 °C. First-strand cDNA synthesis was performed using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Japan), according to the manufacturer's instructions.

Cloning and molecular characterization of OtTGR

The cDNA of *O. turkestanicum* was used as a template to amplify the TGR gene. Primers were designed based on the *Ot*TGR sequences: forward primer (*Bam*H I, underlined: 5′-CCG*GGATCC*ATGTCTTGGTTTAGAAATCTCTG-3′ and reverse primer (*Xho* I, underlined):5′-CCG*CTCGAG*TCAGCAACCGGTCACCGCTGCA-3′. Additional primers targeting the selenocysteine-containing region (OtTGRsec) were also designed: The forward primer 2 (*Bam*H I, underlined): 5′-CCG*GGATCC*ATGTCTTGGTTTAGAAATCTCTG-3′ and reverse primer 2 (*Xho* I, underlined): 5′-CCG*CTCGAG*TTAGGCCGCATAGGCTAACGATTGGTGCAGACCTGCAACCGATTATTAGCCTCAGCAACCGGTCACCGCTGCA-3′. The PCR reaction consisted of an initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 63.5 °C for 30 s, and 72 °C for 2.5 min, with a final extension at 72 °C for 10 min. Amplified products were purified and subcloned into the pMD19-T vector (Takara, Japan). Positive clones were confirmed by sequencing.

Sequence analysis and bioinformatics

DNA and amino acid sequences were analyzed using BLAST against the NCBI nt and nr databases. The molecular weight (MW) and isoelectric point (pI) were predicted using the Compute pI/Mw tool (http://www.expasy.org/tools/pi_tool.html). Signal peptide were predicted with SignalP 4.0 [17]. Subcellular localization and transmembrane domain analyses were conducted using Euk-mPLoc 2.0 [18] and TMHMM2.0 [19], respectively. DNA sequence alignment were conducted using UniPro UGENE [20].

Recombinant protein expression and purification

The OtTGR and OtTGRsec ORFs were subcloned into the pET28a (+) vector (Invitrogen, USA) and transformed into *E. coli* BL21 (DE3) cells. The recombinant plasmid pET28a (+)-OtTGR was transformed into competent *E. coli* BL21 (DE3) cells. Additionally, the recombinant plasmid pET28a (+)-OtTGRsec and the pSUABC vector [21–23], which facilitates selenocysteine incorporation into selenoproteins, were co-transformed into *E. coli* BL21 (DE3) cells.

OtTGRsec expression required co-transformation with the pSUABC vector to incorporate selenocysteine. Protein expression was induced in LB medium with kanamycin (1 mg/mL) at 37 °C when OD_{600} reached 0.6, using IPTG for 8 h [24].

It is important to note that the induction of rOtTGRsec expression required the supplementation of sodium selenite and cysteine. Following induction, bacterial cells were harvested by centrifugation at 10,000 × g for 20 min. The resulting pellets were resuspended in 4 mL of ice-cold 1× binding buffer (Invitrogen, USA) per 100 mL of culture. Cell lysis was achieved through sonication on ice, followed by three freeze–thaw cycles to ensure complete disruption. The lysates were then centrifuged at 10,000 × g for 20 min, and the resulting pellets containing cellular debris and inclusion bodies were collected.

To solubilize the inclusion bodies, pellets were resuspended in 4 mL of 1× binding buffer supplemented with 6 M urea per 100 mL of culture and incubated on ice for 1 h. Expression of the recombinant His-tagged proteins, rOtTGR and rOtTGRsec, was verified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Protein purification was performed using His-tag magnetic beads according to the manufacturer's protocol (Novagen, USA). Refolding of purified proteins was carried out through stepwise dialysis in phosphate-buffered saline (PBS, pH 7.4) containing decreasing concentrations of urea (6, 4, 3, 2, and 1 M), with a final dialysis in PBS alone [24, 25].

Preparation of polyclonal antibodies against rOtTGR

The concentration of purified rOtTGR protein was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). The protein was emulsified with the adjuvant ISA 206 VG (Seppic, France) in a 46:54 (v/v) ratio, following the manufacturer's instructions. For immunization, 100 µL of the emulsion containing 20 µg of protein was administered subcutaneously at three dorsal sites per mouse. Polyclonal antibodies were raised in mice through three immunizations at two-week intervals. Control animals received the same volume of ISA 206 VG adjuvant diluted in PBS without antigen. Blood samples (500 µL per mouse) were collected from the retro-orbital sinus prior to the first immunization and one week after the final immunization. Sera were separated by centrifugation at 900 × g for 10 min at 4 °C and stored at – 20 °C until further use.

Antibody titers were determined by enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well microplates were coated overnight at 4 °C with 100 µL/well of rOtTGR protein. Plates were then blocked with 3% (w/v) bovine serum albumin (BSA) in PBST (PBS with 0.05% Tween-20) for 1 h at 37 °C. Gradient-diluted serum samples (100 µL/well) were added and incubated at 37 °C for 1 h. After washing, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma-Aldrich, USA) was added and incubated under the same conditions. Subsequently, 100 µL of TMB substrate solution (Tiangen, China) was added per well and incubated for 10 min at room temperature in the dark. The enzymatic reaction was stopped with 50 µL/well of 2 M H_2SO_4, and absorbance was measured at 450 nm using a microplate reader (BioTek, USA).

Western blotting analysis of rOtTGR

Recombinant rOtTGR protein was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a nitrocellulose membrane (0.45 µm pore size; Merck-Millipore, Germany) by electrophoresis at 250 mA for 90 min at 4 °C. The membrane was blocked overnight at 4 °C with 5% non-fat dry milk (CST, China) prepared in PBST (PBS containing 0.05% Tween-20). After three washes with PBST, the membrane was incubated with anti-OtTGR polyclonal antibody (1:1000 dilution in PBST) for 2 h at 37 °C. Following another three washes, the membrane was incubated for 2 h at room temperature with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:10,000 dilution; Beyotime, China) in PBST. The membrane was then washed three additional times [26]. Protein bands were visualized using diaminobenzidine (DAB) substrate solution (Sigma, USA), and images were captured using an ImageQuant 300 imaging system (GE Healthcare, USA).

Immunolocalization of rOtTGR in *O. turkestanicum* worms

Frozen sections of *O. turkestanicum* worms were prepared and fixed in cold acetone for 30 min at –20 °C. The sections were blocked with 5% bovine serum albumin (BSA) at room temperature (RT) for 2 h and then washed three times with PBST (0.05% Tween 20 in PBS). Subsequently, the sections were incubated with polyclonal antibodies against rOtTGR diluted 1:100 in PBST for 2 h at 37 °C, followed by three washes with PBST. Sera from non-immunized mice were used as a negative control. The sections were then incubated with Cy3-conjugated anti-mouse IgG (Beyotime, China), diluted 1:3000 in PBST, for 2 h at 37 °C, followed by three additional washes. The nuclei were stained with 10 µg/mL 4′,6-diamidino-2-phenylindole (DAPI) for 10 min at RT. Finally, immunofluorescence signals were visualized under a fluorescence microscope (Nikon Corporation, Japan).

Enzymatic activity assay of rOtTGRsec

The enzymatic activities of thioredoxin reductase (TrxR), glutaredoxin (Grx), and glutathione reductase (GR) for rOtTGRsec were assessed as previously described [27]. TrxR activity was measured using the 5, 5`-diothiobis (2-nitrobenzoic acid) (DTNB) reduction assay [22, 27]. Briefly, rOtTGRsec (0.5–2.5 µM) was added to a mixture of 2 mM NADPH, 2 mM DTNB, and 10 mM EDTA in 0.1 M potassium phosphate (pH 7.4). The increase in absorbance at 412 nm (ε_{412} = 13.6 mM^{-1} cm^{-1}) was monitored during the first 2 min. One enzyme unit was defined as the NADPH-dependent production of 2 µmol of 2-nitro-5-thiobenzoic acid per min at 25 °C [28]. Grx activity was evaluated by monitoring the NADPH consumption at 340 nm (ε_{340} = 13.6 mM^{-1} cm^{-1}) for 2 min. The assay mixture contained 100 mM GSH, 50% HED, 3.95 units of yeast GR, 2 mM NADPH in 0.1 M potassium phosphate (pH 7.4) with 10 mM EDTA. One unit of Grx activity was defined as the oxidation of 1 mmol of NADPH per min at 25℃. GR activity was measured using GSSG as a substrate. The reaction mixture consisted of100 µM NADPH, 100 µM GSSG in 0.1 M potassium phosphate (pH 7.4) containing 10 mM EDTA. NADPH oxidation was monitored at 340 nm (ε_{340} = 13.6 mM^{-1} cm^{-1}) during the first 2 min. One unit GR activity was defined as the oxidation of 1 mmol of NADPH per min at 25℃.

RNA interference assay

To investigate the significance of OtTGR in the survival of *O. turkestanicum*, RNA interference (RNAi) was performed on 90-day-old paired adult worms. Worms were collected by perfusion from New Zealand rabbits infected with approximately 1,000 cercariae of O. turkestanicum at 90 days post-infection. For in vitro culture,

15 pairs of worms were placed in each well of a 6-well flat-bottom plate (Corning, USA) containing 3 mL of culture medium and maintained at 37 °C in a humidified atmosphere of 5% CO_2 [29–31]. Worms were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Macklin, Sweden), 0.1% lactoalbumin hydrolysate (Sigma-Aldrich, USA), 0.2 U/mL insulin (Sigma-Aldrich), 1 μM hydrocortisone (Sigma-Aldrich), 0.5 μM hypoxanthine (Sigma-Aldrich), 1 μM 5-hydroxytryptamine (Sigma-Aldrich), and a double-antibiotic solution (Invitrogen, USA). Six specific small interfering RNAs (siRNAs) targeting different regions of the OtTGR gene, along with a non-targeting control siRNA (non-schistosome-specific; synthesized by HuaGen, Shanghai, China), were employed to knock down OtTGR expression (Table 1). siRNA delivery was performed via electroporation. After 24 h of treatment, the efficiency of gene silencing was evaluated by quantitative real-time PCR (qPCR).

Quantitative real-time PCR (qPCR)

Total RNA was extracted from worms 24 hours post-siRNA treatment using TRIzol reagent, and cDNA was synthesized using a reverse transcription kit according to the manufacturer's protocol. Specific primers for OtTGR were designed to amplify a 134 bp fragment (Forward primer: 5′-ACTATGGTTGATGGTGTTC-3′; Reverse primer: 5′-TCTGTTATCTGGACCTCAT-3′). Nicotinamide adenine dinucleotide (NADH)-ubiquinone reductase (forward: 5'-CGAGGACCTAACAGCAGAGG-3' and reverse: 5'- TCCGAACGAACTTTGAATCC-3'; product size. 174 bp), a house-keeping gene, was used as an internal reference for normalization [32, 33]. Quantitative PCR reactions were conducted in a 20 μL volume containing 10 μL of 2× ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech, China), 0.4 μL of each primer (10 μmol/L), 7.2 μL of nuclease-free water, and 2 μL of cDNA template. Amplification was carried out on an ABI 7900HT system (Applied Biosystems, USA) with the following thermal cycling conditions: initial denaturation at 94 °C for 30 s, followed by 40 cycles of 95 °C for 10 s and 58 °C for 30 s. A melting curve analysis was performed with steps of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 15 s to confirm amplification specificity.

Table 1 Primers sequences of SiRNAs and their positions

Name	Sense	Anti-sense	Position
siRNA1	GAUUGAACUAGAUCAGUUA	UAACUGAUCU-AGUUCAAUC	243–261
siRNA2	GAUGGUGUUCAAAGUCAUA	UAUGACUUUGAA-CACCAUC	697–715
siRNA3	GGUGGUGAUGUGAAAGUUA	UAACUUUCACAU-CACCACC	1027–1045
siRNA4	AGUGUGUCUUCCUUUGUUA	UAA-CAAAGGAAGACA-CACU	48–66
siRNA5	GCACCAAGCUGGACUCUUA	UAAGAGUC-CAGCUUGGUGC	597–615
siRNA6	GAUUACAAGUGAUGAUUUA	UAAAUCAUCA-CUUGUAAUC	921–939
siR-NANC	UUCUCCGAACGUGUCACGU	ACGUGACAC-GUUCGGAGAA	/

Statistical analysis

The experimental data were expressed as the mean ± standard deviation (S.D.). Statistical analyses were performed using SPSS Statistics software (Version 20). Differences between groups were evaluated, and a p-value < 0.05 was considered indicative of statistically significant.

Results

Molecular cloning and bioinformatic analysis of OtTGR

The full-length sequence encoding *O. turkestanicum* thioredoxin glutathione reductase (OtTGR) was successfully obtained through PCR amplification from prepared cDNAs of *O. turkestanicum* worms. Bioinformatic analysis revealed that the cDNA comprises a 1893 bp ORF, encoding a protein of 631 amino acids with a predicted molecular weight of 70 kDa and an isoelectric point (pI) of 6.8. Analysis using the SignalP 4.0 server suggested the absence of a signal peptide in the OtTGR protein. The multiple sequence alignment demonstrated that OtTGR protein was about 92% identity similar to SmTGR and 91% similar to SjTGR. Through alignment of TGR-related amino acid sequences from different species, conserved amino acid residues were shown in black backgrounds. Different grays and white backgrounds indicated varying conservation levels of amino acid residues (Fig. 1). The Grx contain an active site domain 'CPYC', followed by a pyridine nucleotide-disulphide active site domain 'CVNVGC'. Additionally, a Sec-containing redox center 'GCUG' was identified at the C-terminal of TGR sequences (Fig. 1).

Expression and purification of rOtTGR and rOtTGRsec

Recombinant OtTGR and OtTGRsec proteins were successfully expressed with the pET28a (+) expression vector. The rOtTGRsec protein was also co-expressed with the pSUABC vector to facilitate the incorporation of selenocysteine into the protein. Both of the two recombinant proteins were expressed as His-tagged fusion proteins with an approximate molecular weight of about 70 kDa, and they also existed in inclusion bodies (Fig. 2). Purification of rOtTGR and rOtTGRsec were respectively performed using His-tag magnetic beads. The two recombinant proteins were purified under the denaturing conditions. The purified rOtTGR and rOtTGRsec were confirmed by SDS–PAGE analysis and they were respectively revealed clear bands under the expected sizes

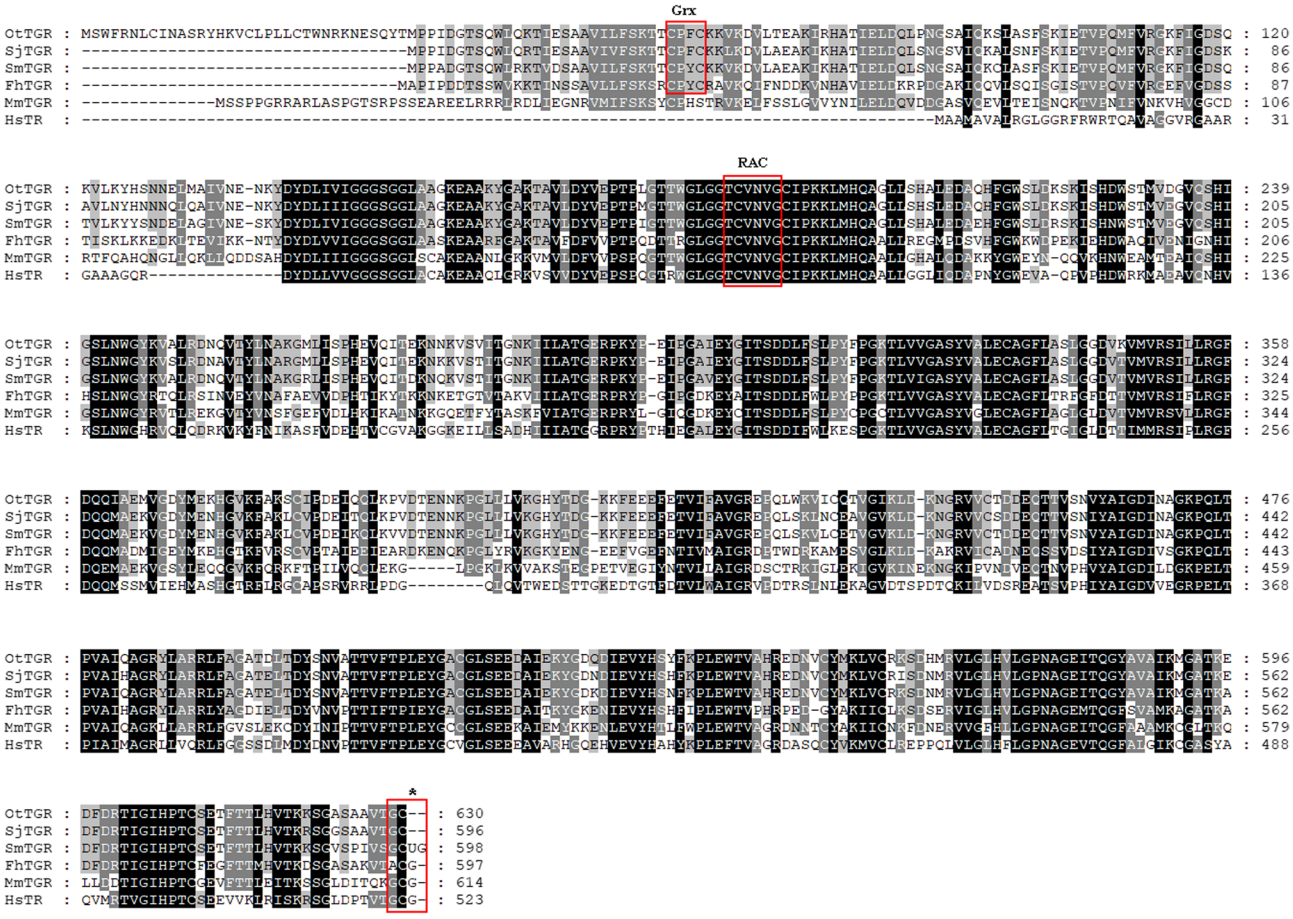

Fig. 1 Amino acid sequences of *O. turkestanicum* TGR and homologues proteins. Alignment of the deduced amino acid sequences of *O. turkestanicum* TGR with *S. mansoni* TGR (SmTGR, accession number AAK85233), *S. japonicum* TGR (SjTGR, accession number AEP33620), *F. hepatica* TGR (FhTGR, accession number CAM96615) *M. musculua* thioredoxin and glutathione reductase (MmTGR, accession number AAK31172), and *H. sapiens* thioredoxin reductase (HsTR, accession number AAD19597), respectively. The red boxes represented: glutaredoxin active site (Grx); pyridine-oxidoreductases redox active center (RAC); selenocysteine (U) residue (*)

(Fig. 2). The purified rOtTGR was subsequently used for mice immunization. The purified rOtTGRsec was dialyzed against PBS and then was used for further enzymatic activity analysis.

Western blotting and immunolocalization analysis

Sera from mice immunized with rOtTGR were verified by ELISA. The ratio of positive/negative value was still over 2.1 after the immunized mice sera in a dilution of 1:8000 (data not shown). The rOtTGRsec was analyzed by Western blotting using sera from mice immunized with rOtTGR. Results showed that two positive bands appeared in size about 70 kDa and 60 kDa (Fig. 3), and a light band also existed in the SDS-PAGE of purified rOtTGRsec (Fig. 2B). Then, the band about 60 kDa was cut from SDS-PAGE for LC-MS/MS analysis and the result showed that it also the rOtTGRsec (Cover percent was about 65%). Moreover, the soluble whole worm proteins were also analyzed by Western blotting and OtTGR protein in worms could also be recognized successfully (Fig. 3).

The distribution of rOtTGR in 90-day-old adult *O. turkestanicum* worms was examined using immunofluorescence assays. Specific fluorescent staining was observed in sections probed with the rOtTGR-specific sera as the primary antibodies, while the negative control treated with sera from non-immunized mice. In the section of adult worm, a positive signal for OtTGR was detected. The specific red fluoresecence could be observed all over the worm. The results revealed that the OtTGR protein was not only localized in the tegument of the worms, but also widely distributed in the parenchyma of the worms (Fig. 4).

Enzymatic activity analysis

The enzyme activities of rOtTGRsec were detected using a broad range of substrates according to the different enzymatic characteristics of three enzymes. For the activity of TrxR, the increased absorbance of 412 nm was

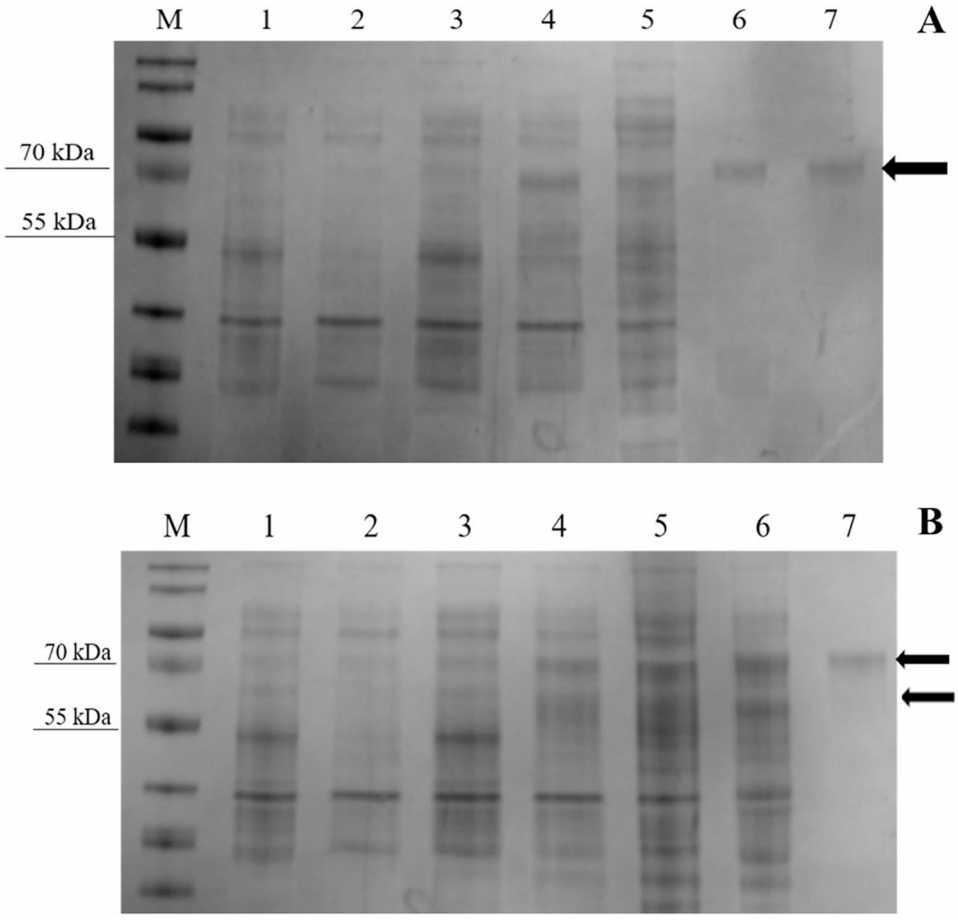

Fig. 2 Expression analysis of the rOtTGR (**A**) and rOtTGRsec (**B**) protein using SDS–PAGE (10%). Lane 1 and 2 total extract from bacteria containing pET28a (+) before and after induction with 1 mM IPTG, lanes 3 and 4 total extract from bacteria containing pET28a (+)-OtTGR (**A**), and pET28a (+)-OtTGRsec and pSUABC (**B**), lane 5 and 6 total extract from supernatant and inclusion bodies, line 7 from purified recombinant rOtTGR (**A**) and rOtTGRsec (**B**) protein, line M: Protein molecular weight marker. A predominant single band at ~70 kDa (black arrow) was observed in both purified recombinant rOtTGR (**A**) and rOtTGRsec (**B**), consistent with the predicted molecular weight

observed. For the GR and Grx activities of rOtTGRsec, a decrease at 340 nm was observed in GSSG and HED reduction, respectively. The results indicated that rOtTGRsec possessed the characteristic activities of TrxR, GR and Grx. The specific activities of TrxR, GR and Grx were 12.30 ± 1.63 U/mL, 8.83 ± 3.15 U/mL and, 0.31 ± 0.07 U/mL, respectively (Fig. 5).

RNA interference and real-time PCR assay analysis

Using qPCR, we analyzed the in vitro interference effect of siRNA on OtTGR expression (Fig. 6). The experimental results demonstrated that each siRNA had different interference effects compared to the blank control group. Specifically, siRNA3 achieved a suppression rate of 46.1%, while siRNA1, siRNA5, and siRNA6 exhibited suppression rates of 35.9%, 40.3%, and 34.0%, respectively. In contrast, siRNA2 and siRNA4 showed suppression rates of 22.9% and 10.3%. These findings indicate that, at the transcriptional level, siRNA3 exhibits the most effective interference with OtTGR expression.

Discussion

Orientobilharziasis is a zoonotic disease with a wide geographic distribution, particularly endemic in over 20 provinces in China [34]. This disease not only causes severe infections and significant economic losses in animals but also leads to cercarial dermatitis in humans, triggering acute inflammatory responses [35]. Therefore, identifying effective vaccine candidates or novel drug targets is critical for controlling orientobilharziasis.

Thioredoxin glutathione reductase (TGR) is a multifunctional enzyme essential for the survival of various parasitic trematodes, including *Schistosoma mansoni* and *S. japonicum*, as it plays a critical role in their unique enzymatic antioxidant pathways [16, 36]. Studies on other trematodes, such as *Fasciola hepatica* and *F. gigantica*, indicate that TGR is a promising vaccine candidate or drug target due to its critical biological roles and species-specific functionality [10, 15, 37, 38]. Interestingly, in mammals, TGR is primarily expressed in the testis, highlighting its specialized role in reproductive biology [39,

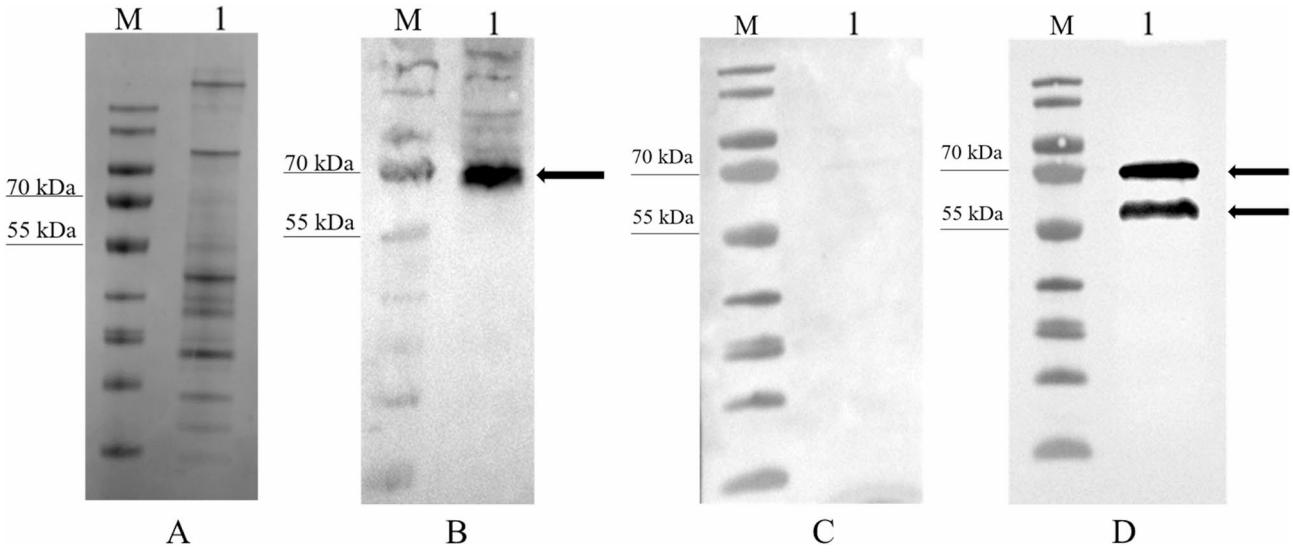

Fig. 3 Whole worm protein electrophoresis and Western blotting analysis of the antigenicity of rOtTGRsec. M (**A**, **B**, **C**, **D**): molecular mass markers; Lane 1 (**A**): Whole worm protein electrophoresis; Lane 1 (**B**): Whole worm protein was probed with anti-rOtTGR mice sera by Western blotting; Lane 1 (**C**, **D**): Purified rOtTGRsec was probed with the pre-immunized mice sera (negative control) (**C**) and anti-rOtTGR mice sera by Western blotting (the experiment group) (**D**). Both of two bands in Fig. 3**D** were confirmed as rOtTGRsec protein through LC-MS/MS analysis

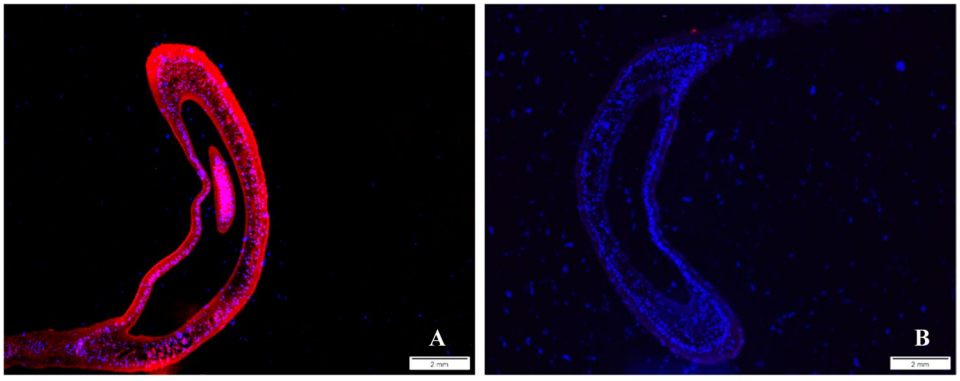

Fig. 4 Immunolocalization of rOtTGR in male adult worm of *O. turkestanicum*. Secondary antibody CY3-conjugated anti-mouse IgG (red) was used for fluorescence detection of rOtTGR on adult worm sections. DAPI (blue) was used to stain parasite nuclei. **A**: the section was probed with anti- rOtTGR mice serum. **B**: the section was probed with naive mice serum (the negative control). The scale bar is 2 mm

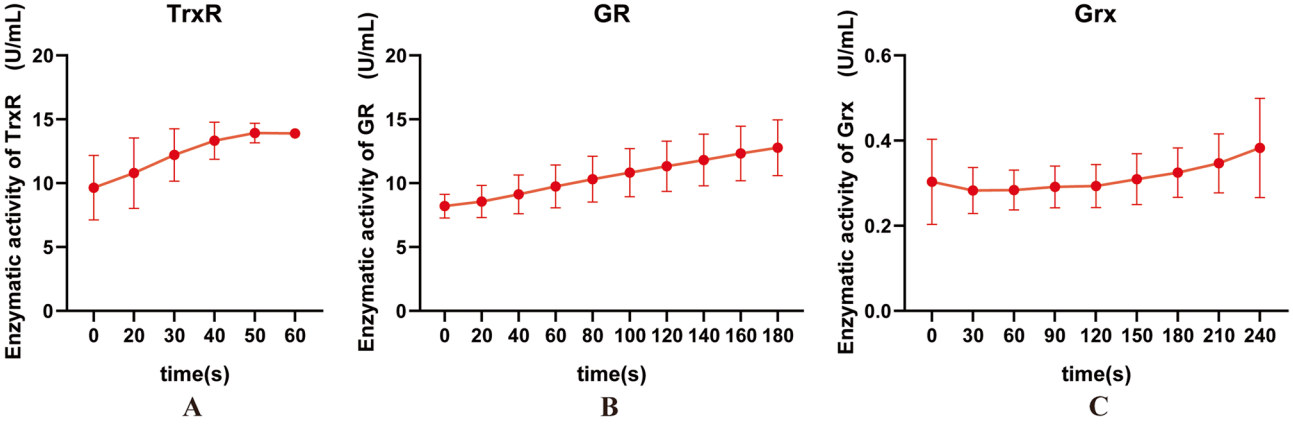

Fig. 5 The enzyme activity of TrxR (**A**), GR (**B**) and Grx (**C**) of rOtTGRsec. Each reaction was repeated three times and data were presented as means ± S.D

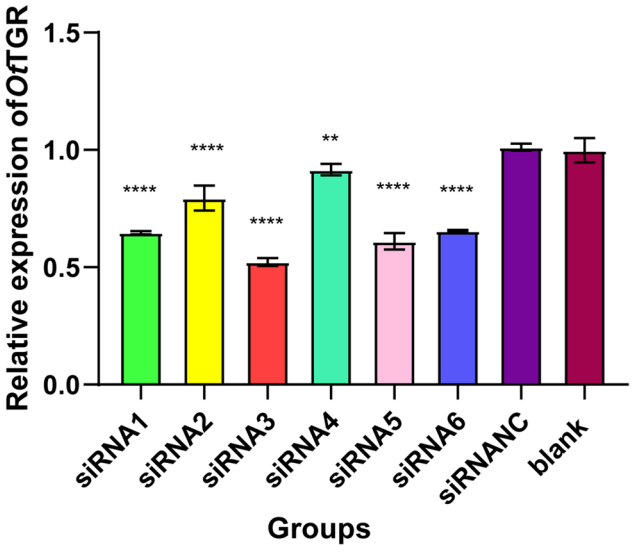

Fig. 6 Effects of siRNA on OtTGR gene transcription in vitro analyzed by qPCR. Six siRNAs were respectively used in three independent biological replicates, and siRNANC was a negative control. All data were normalized against the internal housekeeping gene and the asterisks (*) indicate significantly decreased of OtTGR expression compared with the NC group ($^{**}p < 0.01$ and $^{****}p < 0.0001$)

40]. Given its conserved and indispensable functions in trematodes, TGR from *O. turkestanicum* (OtTGR) could serve as a viable target for vaccine development or therapeutic intervention.

In this study, rOtTGR and rOtTGRsec were successfully expressed in *E. coli* using both single and dual-plasmid systems, with the latter enhancing protein yield. The proteins were purified using His-tag magnetic beads and subsequently characterized. Western blotting demonstrated that polyclonal antibodies raised against rOtTGR specifically recognized about 70 kDa protein, consistent with the expected size of natural TGR in *O. turkestanicum*. It is noteworthy that anti-rOtTGR sera could specific recognized two distinct bands in purified rOtTGRsec by Western blotting analysis. Subsequent LC-MS/MS analysis confirmed that both of the two bands were rOtTGRsec. This phenomenon also appeared in recombinant *S. japonicum* and *F. gigantica* TGRsec in our lab. This might be because the selenoprotein alters the conformation of the protein, thereby affecting its mobility in SDS-PAGE.

Immunohistochemical analysis revealed that OtTGR was primarily distributed on the surface and within the adult worms, with a more pronounced presence on the surface. This localization pattern aligns with findings from other trematodes, such as *S. japonicum* and *F. gigantica*, where TGR is distributed in the tegument and underlying tissues [15, 37].

The enzymatic activity assays confirmed that rOtTGRsec retained the characteristic activities of thioredoxin reductase (TrxR), glutaredoxin (Grx), and glutathione reductase (GR), albeit with slightly lower activity compared to TGR from *S. mansoni*. The optimal enzymatic activity of rOtTGRsec was observed at pH 8.0–9.0 and 30–35 °C. These findings suggest that the recombinant protein folded correctly and maintained functional activity, supporting its potential utility in functional and structural studies.

RNA interference (RNAi) assays demonstrated that siRNAs targeting OtTGR could effectively suppress its expression. Among the four siRNA sequences tested, siRNA2 and siRNA3 showed significant knockdown effects, reducing OtTGR mRNA levels by 39.05% and 33.93%, respectively. These results are consistent with previous studies on *S. mansoni* and *S. japonicum*, where TGR knockdown led to reduced enzymatic activity and worm viability [22, 30]. The effectiveness of siRNA-mediated gene silencing further highlights the critical role of TGR in the survival of *O. turkestanicum*.

Immune protection studies in other trematodes also underscore the potential of TGR as a vaccine candidate. For instance, immunization with recombinant TGR from *S. japonicum* reduced adult worm burdens by 33.5–36.5% and egg counts by 33.7–43.4% in mice [15], while immunization with recombinant TGR from *F. hepatica* in rabbits led to a 96.7% reduction in worm burdens [38]. Although immune protection studies could not be performed for *O. turkestanicum* due to host incompatibility with laboratory mice, the phylogenetic proximity of *O. turkestanicum* to *S. japonicum* and *F. hepatica* suggests that OtTGR might elicit a similar protective immune response.

Conclusions

The current study demonstrates that rOtTGR is a functional and immunogenic protein localized on the surface and within *O. turkestanicum* adult worms. It retains enzymatic activity and is susceptible to RNAi-mediated suppression, supporting its potential as a target for vaccine or drug development. Future research should focus on evaluating the immune-protective effects of rOtTGR in suitable animal models and further exploring its biological function to establish its role as a candidate for controlling orientobilharziasis.

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

Supplementary Material 4.

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Authors' contributions
T.W. and Y.Y. wrote the main manuscript text; S.B. and Z.X. revise the manuscript and Y.H. proposed the idea for the manuscripts and revised the manuscript. All authors reviewed the manuscript.

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Data availability
Data are available upon request to the corresponding author.

Declarations

Ethics approval and consent to participate
All animals were fed under standard conditions following the guidelines of the Committee for Care and Use of Laboratory Animals of Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences. The experimental protocols were reviewed and approved by the Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences Ethical Committee (Permit Number: SHVRI-SZ-20201020-02). Consent to participate is not applicable to this study.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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References

1. Arfaa F, Sabaghian H, Ale-Dawood H. Studies on *Ornithobilharzia Turkestanicum* (skrjabin, 1913), price, 1929 in Iran. Ann Parasitol Hum Comp. 1965;40:45–50.
2. Wang CR, Chen J, Zhao JP, Chen AH, Zhai YQ, Li L, et al. *Orientobilharzia* species: neglected parasitic zoonotic agents. Acta Trop. 2009;109:171–5.
3. Bergquist NR. Schistosomiasis: from risk assessment to control. Trends Parasitol. 2002;18:309–14.
4. Loukas A, Tran M, Pearson MS. *Schistosome* membrane proteins as vaccines. Int J Parasitol. 2007;37:257–63.
5. Bergquist NR. Schistosomiasis vaccine development: progress and prospects. Mem Inst Oswaldo Cruz. 1998;93(Suppl 1):95–101.
6. Bergquist NR, Leonardo LR, Mitchell GF. Vaccine-linked chemotherapy: can schistosomiasis control benefit from an integrated approach? Trends Parasitol. 2005;21:112–7.
7. Fenwick A, Savioli L, Engels D, Robert Bergquist N, Todd MH. Drugs for the control of parasitic diseases: current status and development in schistosomiasis. Trends Parasitol. 2003;19:509–15.
8. Badwey JA, Karnovsky ML. Active oxygen species and the functions of phagocytic leukocytes. Annu Rev Biochem. 1980;49:695–726.
9. Schafer FQ, Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic Biol Med. 2001;30:1191–212.
10. Alger HM, Williams DL. The disulfide redox system of *Schistosoma mansoni* and the importance of a multifunctional enzyme, thioredoxin glutathione reductase. Mol Biochem Parasitol. 2002;121:129–39.
11. Arnér ES, Holmgren A. Physiological functions of thioredoxin and thioredoxin reductase. Eur J Biochem. 2000;267:6102–9.
12. Gromer S, Urig S, Becker K. The thioredoxin system–from science to clinic. Med Res Rev. 2004;24:40–89.
13. Townsend DM, Tew KD, Tapiero H. The importance of glutathione in human disease. Biomed Pharmacother. 2003;57:145–55.
14. Alger HM, Sayed AA, Stadecker MJ, Williams DL. Molecular and enzymatic characterisation of *Schistosoma mansoni* thioredoxin. Int J Parasitol. 2002;32:1285–92.
15. Han Y, Zhang M, Hong Y, Zhu Z, Li D, Li X, et al. Characterization of thioredoxin glutathione reductase in *Schiotosoma Japonicum*. Parasitol Int. 2012;61:475–80.
16. Sun QA, Kirnarsky L, Sherman S, Gladyshev VN. Selenoprotein oxidoreductase with specificity for thioredoxin and glutathione systems. Proc Natl Acad Sci U S A. 2001;98:3673–8.
17. Petersen TN, Brunak S, von Heijne G, Nielsen H. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods. 2011;8:785–6.
18. Chou K-C, Shen H-B. A new method for predicting the subcellular localization of eukaryotic proteins with both single and multiple sites: Euk-mPLoc 2.0. PLoS ONE. 2010;5:e9931.
19. Krogh A, Larsson B, von Heijne G, Sonnhammer EL. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol. 2001;305:567–80.
20. Okonechnikov K, Golosova O, Fursov M. The UGENE team. Unipro UGENE: a unified bioinformatics toolkit. Bioinformatics. 2012;28:1166–7.
21. Rengby O, Johansson L, Carlson LA, Serini E, Vlamis-Gardikas A, Kårsnäs P, et al. Assessment of production conditions for efficient use of *Escherichia coli* in high-yield heterologous Recombinant Selenoprotein synthesis. Appl Environ Microbiol. 2004;70:5159–67.
22. Kuntz AN, Davioud-Charvet E, Sayed AA, Califf LL, Dessolin J, Arnér ESJ, et al. Thioredoxin glutathione reductase from *Schistosoma mansoni*: an essential parasite enzyme and a key drug target. PLoS Med. 2007;4:e206.
23. Li G, Guo Q, Feng C, Chen H, Zhao W, Li S, et al. Synthesis of oxadiazole-2-oxide derivatives as potential drug candidates for schistosomiasis targeting SjTGR. Parasit Vectors. 2021;14:225.
24. Hong Y, Han H, Peng J, Li Y, Shi Y, Fu Z, et al. *Schistosoma japonicum*: cloning, expression and characterization of a gene encoding the α5-subunit of the proteasome. Exp Parasitol. 2010;126:517–25.
25. Zhang M, Han Y, Zhu Z, Li D, Hong Y, Wu X, et al. Cloning, expression, and characterization of *Schistosoma Japonicum* tegument protein phosphodiesterase-5. Parasitol Res. 2012;110:775–86.
26. Cao X, Hong Y, Zhang M, Han Y, Wu M, Wang X, et al. Cloning, expression and characterization of protein disulfide isomerase of *Schistosoma Japonicum*. Exp Parasitol. 2014;146:43–51.
27. Song L, Li J, Xie S, Qian C, Wang J, Zhang W, et al. Thioredoxin glutathione reductase as a novel drug target: evidence from *Schistosoma Japonicum*. PLoS ONE. 2012;7:e31456.
28. Angelucci F, Miele AE, Boumis G, Dimastrogiovanni D, Brunori M, Bellelli A. Glutathione reductase and thioredoxin reductase at the crossroad: the structure of *Schistosoma mansoni* thioredoxin glutathione reductase. Proteins. 2008;72:936–45.
29. Han Q, Jia B, Hong Y, Cao X, Zhai Q, Lu K, et al. Suppression of VAMP2 alters morphology of the tegument and affects glucose uptake, development and reproduction of *Schistosoma Japonicum*. Sci Rep. 2017;7:5212.
30. Han Y, Fu Z, Hong Y, Zhang M, Han H, Lu K, et al. Inhibitory effects and analysis of RNA interference on thioredoxin glutathione reductase expression in *Schistosoma Japonicum*. J Parasitol. 2014;100:463–9.
31. Liu X, Liu J-M, Song Z, Xing R-H, Jin Y-M, Guo Y, et al. The molecular characterization and RNAi Silencing of SjZFP1 in *Schistosoma Japonicum*. Parasitol Res. 2015;114:903–11.
32. Gobert GN, Moertel L, Brindley PJ, McManus DP. Developmental gene expression profiles of the human pathogen *Schistosoma Japonicum*. BMC Genomics. 2009;10:128.
33. Hong Y, Huang L, Yang J, Cao X, Han Q, Zhang M, et al. Cloning, expression and enzymatic characterization of 3-phosphoglycerate kinase from *Schistosoma Japonicum*. Exp Parasitol. 2015;159:37–45.
34. Wang CR. Pi baoan, song zhuo. Investigation on the epidemic situation and the distribution feature of orientobilharziasis in cattle and sheep in Heilongjiang. Progress Veterinary Med. 2002;23:91–3.
35. Wang C-R, Xu M-J, Fu J-H, Nisbet AJ, Chang Q-C, Zhou D-H, et al. Characterization of MicroRNAs from *Orientobilharzia turkestanicum*, a neglected blood fluke of human and animal health significance. PLoS ONE. 2012;7:e47001.
36. Loverde PT. Do antioxidants play a role in *schistosome* host-parasite interactions? Parasitol Today. 1998;14:284–9.

37. Changklungmoa N, Kueakhai P, Sangpairoj K, Chaichanasak P, Jaikua W, Riengrojpitak S, et al. Molecular cloning and characterization of *Fasciola gigantica* thioredoxin-glutathione reductase. Parasitol Res. 2015;114:2119–27.
38. Maggioli G, Silveira F, Martín-Alonso JM, Salinas G, Carmona C, Parra F. A Recombinant thioredoxin-glutathione reductase from *Fasciola hepatica* induces a protective response in rabbits. Exp Parasitol. 2011;129:323–30.
39. Su D, Novoselov SV, Sun Q-A, Moustafa ME, Zhou Y, Oko R, et al. Mammalian Selenoprotein thioredoxin-glutathione reductase. Roles in disulfide bond formation and sperm maturation. J Biol Chem. 2005;280:26491–8.
40. Sun Q-A, Su D, Novoselov SV, Carlson BA, Hatfield DL, Gladyshev VN. Reaction mechanism and regulation of mammalian thioredoxin/glutathione reductase. Biochemistry. 2005;44:14528–37.

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