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Molecular characterization of *Schistosoma japonicum* tegument protein tetraspanin-2: Sequence variation and possible implications for immune evasion

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ABSTRACT

Members of tetraspanin family expressed on the tegument of *Schistosoma mansoni* have been regarded as potential protective antigens. In this work, we were surprised to identify seven tetraspanin-2 (TSP-2) homologs of the protective antigen Sm-TSP-2 in *Schistosoma japonicum* and found that the transcription profiles of *Sj-tsp-2* subclasses were highly variable in individual adult worms. RT-PCR revealed that *Sj-tsp-2* genes were transcribed in cercariae, schistosomula, adult worms, and eggs; however, Western blot analysis indicated that the Sj-TSP-2 proteins were not expressed in eggs. Immunolocalization assays showed that the Sj-TSP-2 proteins localized on the tegument of schistosomula and adult worms, but exposed only on the surface of adult worms. Mice immunized with the recombinant protein of a single TSP-2 subclass showed no protection, while immunized with a mixture of seven recombinant TSP-2 subclasses provided a moderate protection. Those data implicate that the tegument protein Sj-TSP-2 is involved in immune evasion and the high polymorphism of this molecule must affect its potential as a vaccine candidate.

Schistosomiasis, caused by blood fluke of the genus *Schistosoma*, remains a major global public health problem affecting more than 200 million people living in the endemic areas of 76 countries and territories [1,2]. Three major pathogenic schistosome species, *Schistosoma japonicum*, *Schistosoma mansoni*, and *Schistosoma haematobium* are known to contribute to the case load. In China, schistosomiasis japonica remains one of the most serious parasitic diseases today. It was estimated that 800,000 people were infected with *S. japonicum* in 2005 [3]. The resurgence of schistosomiasis japonica has been seen in the past years despite several controlling programs have been carried out including elimination of the intermediate host, snail, and large-scale application of the chemotherapy, using praziquantel (PZQ). Immigration, flood, extended infected snail habitats by returning lands back to the Dongting Lake in the purpose of flooding buffer, and global warming all increase the risk of schistosomiasis japonica transmission [4]. Therefore, vaccination, alone or in combination with anthelmintic drugs would provide a major boost in the disease control program [5].

So far, a handful of antigens have been characterized and tested in immunization trials against *S. japonicum*, including the homologs of six candidate antigens of *S. mansoni* recommend by the World Health Organization (WHO) in mid-1990s [6]. Unfortunately, none of the antigens tested has shown a consistent protection rate over 50% in model animals [4], which makes the identification of protective immunogen an urgent task.

Within several hours after skin penetration, the host-interactive tegumental outer membrane of the cercariae rapidly developed to an unusual architecture, which is a single syncytium covered by two closely apposed lipid bilayers [7]. The inner leaflet of the two lipid bilayers is a plasma membrane, and the outer leaflet is known as membranocalyx which protects the parasite against the host's defense system. Although the tegument of schistosome is the key structure for its survival within the host, the tegument or tegument-associated antigens could act as targets of protective immune responses [8]. In 2003, two novel tetraspanins, TSP-1 and TSP-2, were identified in *S. mansoni* [9]. By immunizing mice with the two antigens, respectively, Tran et al. further demonstrated that both of them were protective antigens, especially Sm-TSP-2, which provided a protection rate as high as around 60% in mouse model [10]. They also found an ortholog of Sm-TSP-2 in the ESTs database of *S. japonicum*, and predicted its value as a vaccine candidate against *S. japonicum* [10].

In this report, we identified several cDNA clones of *S. japonicum* parasite, further characterization showing that they were from the transcripts of the *Sj-tsp-2* genes. The encoded Sj-TSP-2 proteins were located in lung-stage schistosomula and adult worms.

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Vaccination with the recombinant Sj-TSP-2 proteins in mouse model has also been carried out.

Materials and methods

Parasite maintenance. The freshly shed cercariae of *S. japonicum* were obtained as previously described [11]. Adult worms were obtained by perfusion of New Zealand rabbit 6-weeks after infection [12]. Schistosome eggs were extracted from liver tissues of rabbit infected 6-weeks before [13]. The 72 h lung-stage schistosomula were collected from lung tissues of infected Kunming mice as previously described [14].

Cloning of Sj-tsp-2 cDNA. Total RNA from mixed-sex adult worms of *S. japonicum* was isolated using Trizol reagent (Invitrogen), and contaminating genomic DNA was removed by the Turbo DNA-free endonuclease (Ambion). First strand cDNA was synthesized using 500 ng RNA, SuperScript III Reverse Transcriptase (Invitrogen), and oligo (dT) 15 primer. The *Sj-tsp-2* open reading frame was amplified with two specific primers, TSP-U (5′-ATGGCACTCG GGTGTGGATA-3′) and TSP-D (5′-CTATTCATCATCGCCTCGTT-3′) designed based on the *S. japonicum tsp-2* EST sequences (GenBank Accession No. AY815387 and AY810722) using Phusion DNA polymerase (NEB). The PCR was performed with an initial denaturation for 1 min at 98 °C. Thirty PCR cycles were performed as follows: 98 °C for 8 s; 50 °C for 30 s and 72 °C for 15 s. The final extension was 10 min at 72 °C. The resulting amplicons were cloned into pGEM-T vector (Promaga) and sequenced.

Further, total RNAs from male and female adult worms of *S. japonicum* (8 each) were isolated individually for *Sj-tsp-2* subclasses transcription profile analysis. RT-PCR and molecular cloning were performed against each worm as described above. For every worm, 20 cDNA clones were sequenced.

Genomic sequence analysis. Genomic DNA was obtained from adult *S. japonicum* worms by a proteinase K-based extraction method [15]. Using the genomic DNA as template, we amplified *Sj-tsp-2* gene, from the translational start codon to stop codon, by PCR with Phusion DNA polymerase and two specific primers TFGF (5′-ATGGCACTCGGGTGTGGATACAAGTGTTTGC-3′) and TFGR (5′-CTATTCATCATCGCCTCGTTTTATAGCCATAAG-3′). The PCR was performed with an initial denaturation at 98 °C for 1 min. Thirty PCR cycles were performed as follows: 98 °C for 8 s; 55 °C for 30 s; and 72 °C for 90 s, followed by a final extension at 72 °C for 10 min. The amplicons were cloned into pGEM-T vector for sequencing. PCR reactions were also performed with genomic DNAs from 8 male and 13 female adult worms individually.

Detection of Sj-tsp-2 transcription at different developmental stages. Total RNAs were isolated from eggs, cercariae, lung-stage schistosomula, and adult worms, respectively. *Sj-tsp-2* mRNA levels were detected by a semi-quantitative RT-PCR analysis as previously described [9]. The *S. japonicum* TPI-specific primers (5′-GTCTGAGGCTCATTTTTGTGGAGAC-3′, and 5′-TGAACTTCTTGAGCTT GACCTGGTG-3′) were used to amplify a PCR product that served as a constitutively transcribed control.

Recombinant protein expression and purification. The gene fragments encoding large extracellular loop (Glu107-His180) of seven *Sj-tsp-2* subclasses were amplified by PCR with *Pfu* polymerase. Specific primers (bp 319–338 and the complementary sequence of bp 521–540 with introduction of a HindIII or XhoI site at their extremities, respectively) were used for each subclass. The amplicons were double-digested with these enzymes, and cloned into pET32b vector (Novagen). The recombinant plasmids were transformed into BL21 (DE3) *Escherichia coli.* Expression of thioredoxin or the recombinant proteins was induced with IPTG at 0.1 mM and the proteins were purified with His-Trap™ column (GE Healthcare Biosciences).

Western blot. The tegument proteins of adult worms were obtained by a freeze/thaw method as previously described [12]. The membrane proteins of cercariae, schistosomula, and eggs were extracted with Triton X-114 [16]. Membrane protein preparations were subjected to 12% SDS–PAGE and transferred to PVDF membrane. The PVDF membrane was blocked with 5% SMP in TBS for 90 min at room temperature, and then incubated with primary antibody (1:3000 dilution) at 4 °C overnight. After washing, the membrane was incubated with an HRP-conjugated goat anti-mouse IgG (H+L) antibody for 1 h at room temperature. Subsequently, the Immobilon™ Western chemiluminescence HRP substrate (Millipore) was used to detect the specific bands.

Immunolocalization. Immunofluorescence assays on whole-mount live adult worms of *S. japonicum* were conducted as previously described [17]. Briefly, after blocking, live adult worms were incubated with primary antibodies (1:100 dilution) overnight at 4 °C. After washing, the samples were incubated with TRITC-conjugated goat anti-mouse IgG (1:200 dilution) for 1 h at room temperature. Lung-stage schistosomula were pelleted by a brief (2 s) centrifugation at 500*g*. The supernatant was removed and the parasites were resuspended and fixed in cold acetone for 5 min. Immunofluorescence assays on live or fixed lung-stage schistosomula were carried out as described above, and a FITC-conjugated secondary antibody was used. Serum raised against the thioredoxin-tag alone was used as negative controls. Images were acquired with a confocal microscope (adult worms) or a fluorescence microscope (schistosomula).

Vaccination and challenge. BALB/c mice (female 6–8-weeks old) were divided into four groups (PBS, Trx, Trx-TSP-2c, and mixture of all Trx-TSP-2 subclasses) with 10 mice per group. Each mouse was immunized subcutaneously with 25 μg recombinant protein or of 50 μl PBS in Freund's complete adjuvant, followed by two boosts in Freund's incomplete adjuvant at intervals of 3-weeks. Sera were collected 10 days after the third immunization. All mice were challenged with 40 ± 1 cercariae of *S. japonicum* by percutaneous infection 4-weeks after the final injection. The mice were sacrificed 6-weeks after challenge, and protective immunity evaluation was carried out. SPSS version 11 was used for all statistical analysis. A value of $P < 0.05$ was considered significant.

Results

Cloning and variation of Sj-tsp-2 sequences

The ORF of *Sj-tsp-2* is 645 bp in length, encoding 215 amino acids with a theoretical molecular weight ~23.5 kDa. Variations were observed among the Sj-TSP-2 protein sequences. Based on the variable region within the large extracellular loop [18], the Sj-TSP-2 molecules can be divided into seven subclasses, termed from Sj-TSP-2a to Sj-TSP-2g. The peptide sequences of Sj-TSP-2 subclasses were aligned with their homologous sequence of Sm-TSP-2 (Fig. 1A) and the homology tree was shown in Fig. 1B. The TSP-2 sequences between the two species shared low homology over the large extracellular loop, suggesting functional divergence may be present in this molecule along evolution. Meanwhile, chimeric hybrid cDNAs among different *Sj-tsp-2* subclasses were found with characterization that the 5′-portion of the molecule is originally from one subclass and the 3′-portion from another. These sequences are attractive to us with respect to whether they are true transcripts or artificial products resulted from RT-PCR.

Transcription profiles of Sj-tsp-2 subclasses in individual worms

In order to discern the transcription profile of *Sj-tsp-2* in single worm, we performed RT-PCR against adult worms individually.

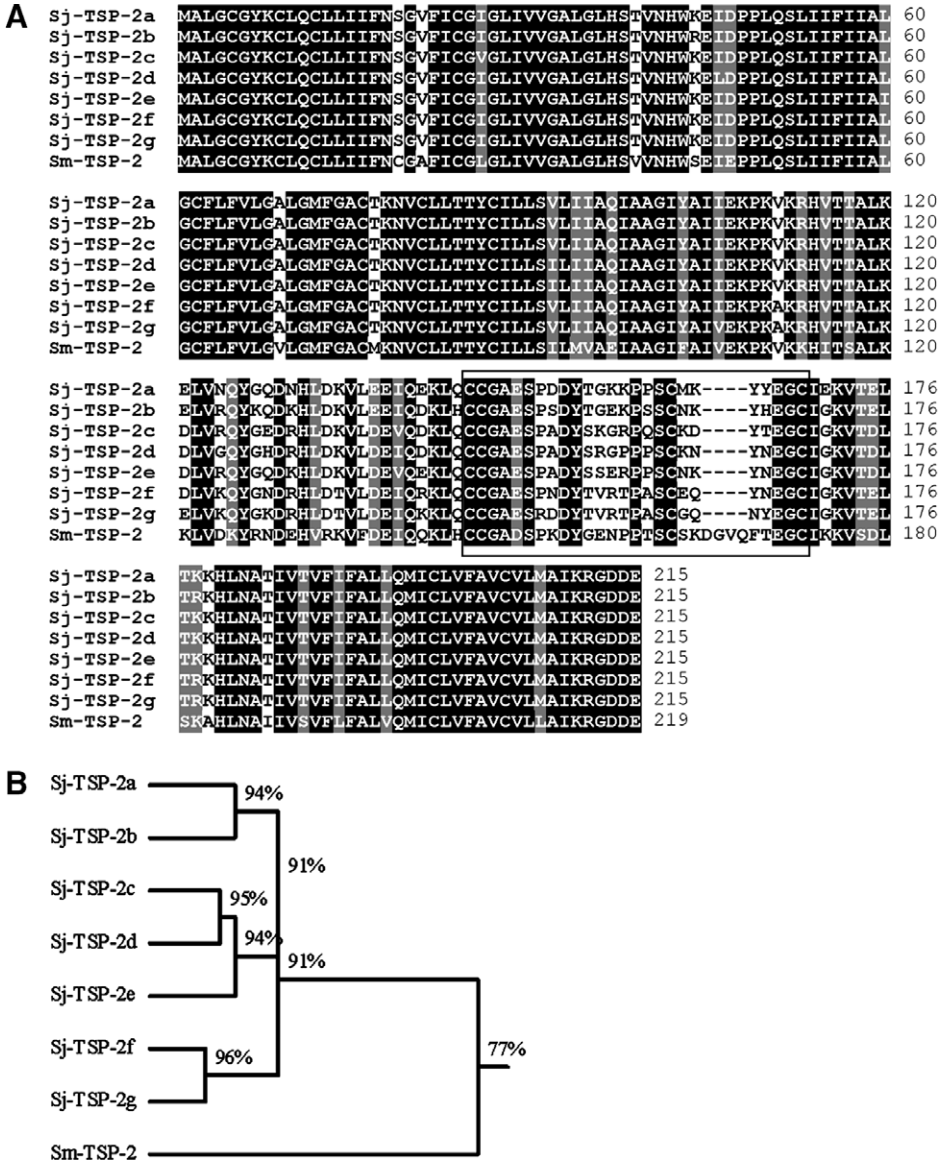

Fig. 1. Analysis of peptide sequences of Sj-TSP-2 subclasses and Sm-TSP-2 (GeneDB Accession No. Sm12366). (A) The alignment was obtained according to ClustalW algorithm with the use of the BioEdit v7.0.1 package. The variable region is marked in box. (B) Homology tree of peptide sequences of Sj-TSP-2 subclasses and Sm-TSP-2 created with the use of the DNAMAN version 6.

Intriguingly, one, two or three *tsp-2* mRNA subclasses could be expressed in one worm. As a whole, the transcription profiles among the individual worms were variable, although identical in some worms (Table 1).

Analysis of Sj-tsp-2 genes

Two fragments about 2.9 kb and 5.0 kb were amplified by PCR with genomic DNA from mixed-sex adult worms (data not shown). Sequencing revealed that the ~2.9 kb fragment contained genes encoding *Sj-tsp-2c* (2908 bp), *Sj-tsp-2d* (2907 bp), and *Sj-tsp-2e* (2915 bp); while the ~5.0 kb fragment contained genes encoding *Sj-tsp-2a* (4868 bp), *Sj-tsp-2f* (5158 bp), and *Sj-tsp-2g* (5148 bp). Splice sites were determined by alignment of the cDNA sequences with the genomic sequences of *Sj-tsp-2*. Results indicated that the *Sj-tsp-2* genes were composed of at least six exons and five introns (Fig. 2A). From sequencing of the two amplified fragments, we also found some *Sj-tsp-2* genes which were generated by recombination of *Sj-tsp-2* variants, confirming the hybrid sequences obtained in RT-PCR.

Genomic PCR reactions were further performed to investigate the gene diversity in adult worms (Fig. 2B). Strikingly, a ~4.4 kb fragment was amplified in one female worm (Lane 3), and sequencing revealed that it contained a gene encoding another subclass, *Sj-tsp-2b* (4327 bp). In regard to some worms, both ~2.9 kb and ~5.0 kb fragments were amplified, indicating the existence of multiple *Sj-tsp-2* subclasses within one parasite.

Detection of Sj-tsp-2 mRNAs and proteins at different S. japonicum developmental stages

A semi-quantitative RT-PCR analysis revealed that the *Sj-tsp-2* transcripts were expressed in eggs, cercariae, schistosomula, and adult worms (Fig. 3A). However, the transcription levels varied: being up-regulated in lung-stage schistosomula and considerably down-regulated in eggs. Differential transcription levels were also observed in different sexual adult worm as high levels in male worms contrasting to that in female worms.

The expression of Sj-TSP-2 proteins was also examined by Western blot analysis. Sj-TSP-2 proteins were detected in cercariae,

Table 1
Transcription profiles of *Sj-tsp-2* subclasses in individual adult worms

Worms	*Sj-tsp-2a*	*Sj-tsp-2b*	*Sj-tsp-2c*	*Sj-tsp-2d*	*Sj-tsp-2e*	*Sj-tsp-2f*	*Sj-tsp-2g*
M1						√	
M2			√			√	
M3	√					√	
M4			√				
M5			√		√	√	
M6			√			√	
M7	√	√					
M8						√	
F1				√	√		
F2			√				√
F3					√	√	√
F4					√		√
F5			√				
F6			√				
F7				√	√		
F8				√	√		

M1–M8, male adult worms; F1–F8, female adult worms.

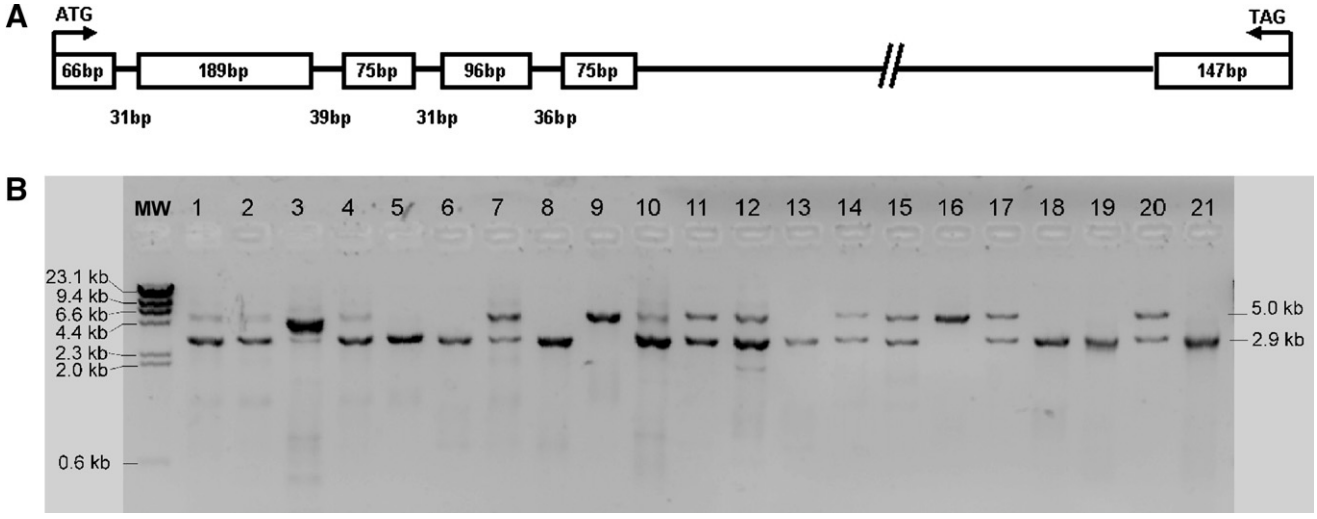

Fig. 2. Analysis of *Sj-tsp-2* genomic sequences. (A) Schematic structure of exon/intron of *Sj-tsp-2* genes. The thin rods represent the exons and the lines between them represent introns. The numbers refer to the length of exons and introns. The length of the fifth intron varies among different subclasses. (B) Analysis of the products of genomic PCR against individual adult worms on 1% agarose gel. MW: λHindIII; Lanes 1–8, female adult worms; Lanes 9–21, male adult worms.

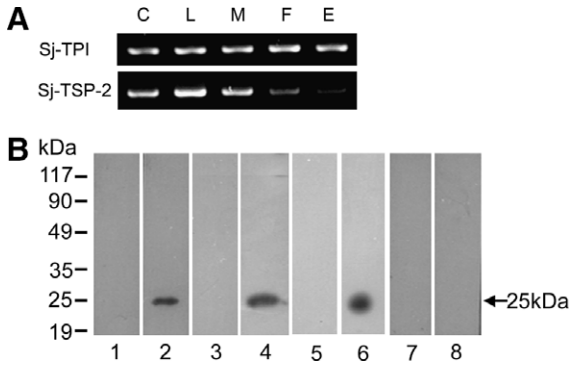

Fig. 3. Detection of transcription levels and expression of *Sj-tsp-2* at various developmental stages. (A) RT-PCR analysis of *Sj-tsp-2* transcription. Lane C, cercariae; lane L, lung-stage schistosomula; lane M, male adult worms; lane F, female adult worms; lane E, eggs. (B) Western blot analysis of Sj-TSP-2 expression. Lanes 1–2, lanes 3–4, lanes 5–6, and lanes 7–8 were subjected with membrane protein preparation from cercariae, schistosomula, adult worms, and eggs, respectively. Lanes 2, 4, 6, and 8 were incubated with anti-TSP-2 serum. Lanes 1, 3, 5, and 7 were incubated with anti-Trx serum.

schistosomula, and adult worms but not in eggs (Fig. 3B). The band detected was about 25 kDa, which is slightly larger than its theoretical molecular weight, indicating that the native Sj-TSP-2 may be glycosylated like most mammalian tetraspanins as reported perversely [19].

Localization of Sj-TSP-2 proteins in schistosomula and adult worms

In immunofluorescence assays, Sj-TSP-2 proteins were detectable in the fixed lung-stage schistosomula (Fig. 4B) but not in live parasites (Fig. 4F). However, they were obviously distributed over the surface of live adult worms, both male (Fig. 4I) and female (Fig. 4K).

Vaccination and protection

No protection was shown in mice immunized with a single recombinant protein of Sj-TSP-2c subclass, while in mice immunized with a mixture of recombinant proteins of all subclasses, the worm reduction was 14.9% ($P < 0.05$) and the egg reduction was 21.1% ($P < 0.05$), comparing to controls (Table 2).

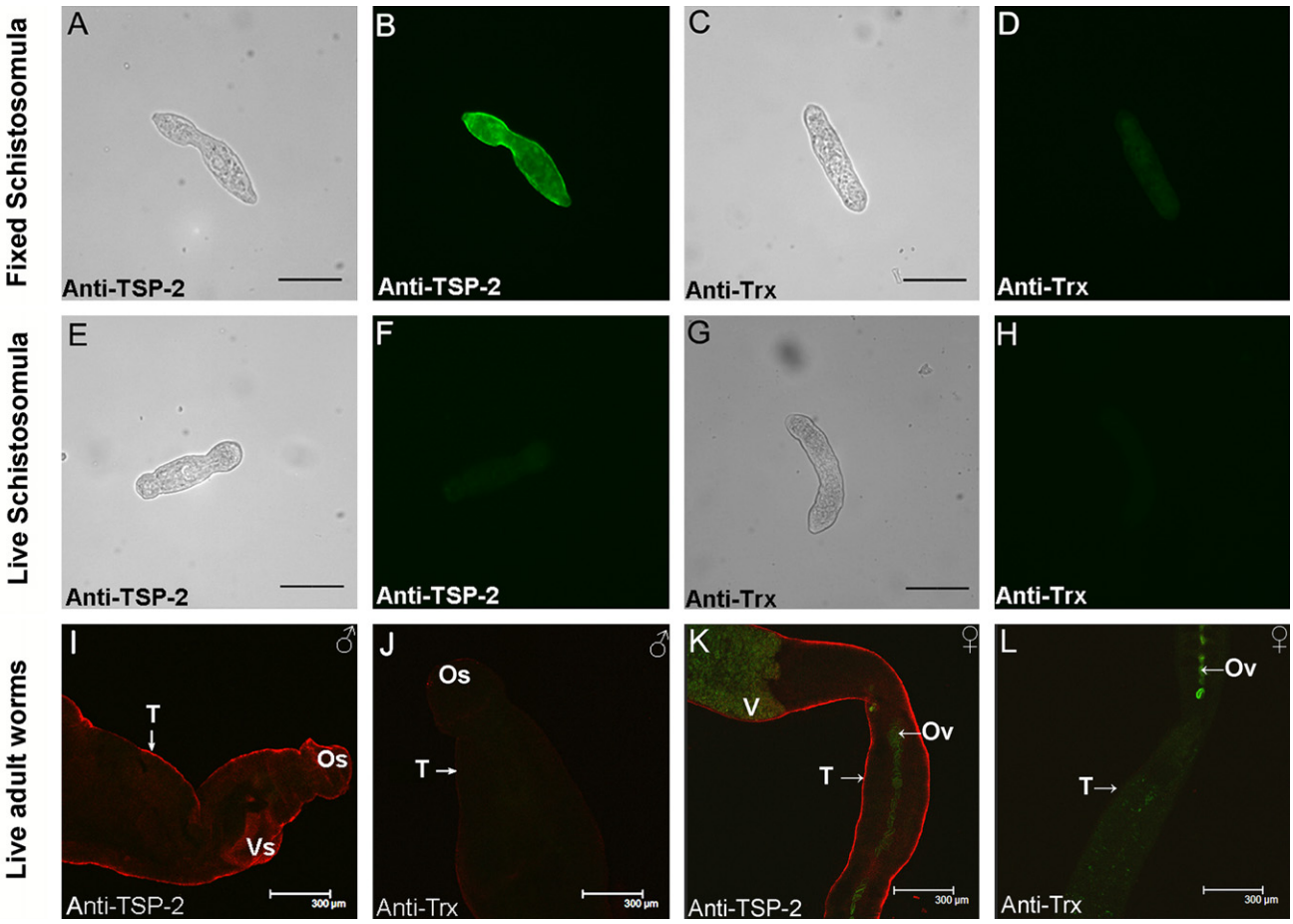

Fig. 4. Immunolocalization of Sj-TSP-2 proteins on tegument of lung-stage schistosomula and adult worms. Panels A–H, fluorescence microscopy images of lung-stage schistosomula. Differential interference contrast images (A, C, E, and G) and corresponding fluorescence microscopy images of fixed lung-stage schistosomula (B,D) and live lung-stage schistosomula (F,H). Magnification bars in panels A–H = 75 μm. Panels I–L, fluorescence confocal microscopy images of live adult worms. Magnification bars in panels I–L = 300 μm. No specific fluorescence could be visualized when anti-Trx antibody was used (D, H, J, and L). Some nonspecific green auto-fluorescence was shown in vitellaria (V) and ova (Ov) of female worms (K,L). T, tegument of adult worm; Gy, Gynecophoral canal; Os, oral sucker; Vs, ventral sucker. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
A summary of protective immunity induced in Balb/c mice vaccinated with the recombinant proteins of Sj-TSP-2, thioredoxin or PBS

Adjuvanted immunogen	Adult worms range	Adult worms mean ± SD (% reduction)	Adult worms median (% reduction) *P* values	Liver eggs mean ± SD (% reduction)	Liver eggs median (% reduction) *P* values
PBS (n = 10)	26–39	31.10 ± 3.90	30.50	88,750 ± 16,973	87,050
Trx (n = 10)	24–35	31.50 ± 3.44	32.50	91,670 ± 18,747	90,050
Trx-TSP-2c (n = 10)	23–38	29.40 ± 4.48 (6.67%)	29.50 (9.23%) P = 0.2548	78,400 ± 16,180 (14.48%)	80,950 (10.11%) P = 0.1074
Trx-TSP-2 mixture (n = 10)	22–32	26.80 ± 3.01 (14.92%)	27.00 (16.92%) P = 0.0044	72,320 ± 15,856 (21.11%)	65,700 (27.04%) P = 0.0227

Discussion

For survival in the complex immunological environment of vascular system of the definitive host, schistosomal parasites have evolved numerous strategies to confront host defense mechanisms, including antigenic mimicry, membrane turnover, essential function of immunomodulatory molecules and proteases, unique biophysical properties of the tegument and modulation of expression of surface antigens [20]. Among field samples of *S. japonicum* from five provinces of southern China, genetic polymorphisms were found in some putative secretory and membrane antigens, suggesting that some genetic variants were included in certain genes involving in immune evasion and natural selection [21]. Here, we provided evidences showing the variable tegument protein of *S. japonicum*, Sj-TSP-2, may involve in immune evasion.

First, the distinct sequence diversity was shown in the variable region, which is on the surface of Sj-TSP-2 proteins, predicted by Robetta (http://robetta.bakerlab.org) (data not shown), resulting as much as seven subclasses. Variations in three different *Sj-tsp-2* large extracellular loop sequences were also found by another group [22]. Such sequence variation may modify the affinity or avidity of host's antibody–antigen reactions. Second, the hybrid *tsp-2* cDNA sequences among the different *Sj-tsp-2* subclasses were detected. Previously, it has been documented that RNA template could be switched by reverse transcriptase in either a homology-dependent manner or a temperature-dependent manner during cDNA synthesis [23,24]. Here, we used a SuperScript III reverse transcriptase in RT-PCR to prevent the outcome of false transcripts. In addition, genes encoding hybrid *Sj-tsp-2* cDNAs were found. Thus, we confirmed that at least some of those chimeric cDNAs should be the true transcripts rather than yielded

by RNA template switch in vitro. Genetic recombination, however, may count for this type of variation. Also some processed pseudogenes encoding hybrid cDNAs of *Sj-tsp-2* were detected (data not shown), and they can be another source for the hybrid cDNAs if they were transcribed in some situations. Third, not more than three subclasses of *Sj-tsp-2* were expressed in single worm, but the transcription profile varied among individual worms as a whole (Table 1); even in worms showing identical transcription profile such as F1, F7, and F8, the ratios of the two subclasses being expressed also varied widely. These tricks can be the key point for a variable protein to play a role in immune evasion. Because the polymorphisms of the molecule are obviously increased according to those strategies, and the host immune responses against each Sj-TSP-2 subclass become less effective, which has been proved by our immune protection experiments in mice model. A similar situation has also been observed in other parasites such as the PfEMP1 variants found in the malaria parasite *Plasmodium falciparum*, which could switch by differential expression of up to 60 *var* genes [25].

Although the *tsp-2* transcription level is highly up-regulated after parasite invasion of the definitive host, the Sj-TSP-2 proteins could only be detected in the fixed lung-stage schistosomula but not in live parasites, which remains a puzzle. The acquisition of host molecules, such as major histocompatibility complex [26], and being masked from its foreign status could be the reason for the fact. Interestingly, the MHC molecules have been suggested to be host ligands of Sm-TSP-2 [10]. Since the variable region within large extracellular loop of tetraspanins has been delineated to mediate specific interactions with other proteins [27], we suggest that the variation in this region of the Sj-TSP-2 is an adaptation evolved by *S. japonicum* in order to overcome polymorphisms of MHC molecules from more than 40 reservoirs.

In conclusion, our work explored the characterization of a tegument protein, Sj-TSP-2. The diversity of this molecule which includes the distinct variation on the surface of the molecule, the recombination among different subclasses and the differential transcription profiles in individual worms, together with the potential function of binding with the host MHC molecules to mask itself must help this genius parasite to survive in the complex immunological environment. The data also underline that such a highly polymorphic molecule can hardly be used as a candidate for development of a vaccine against *S. japonicum* infection.

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