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# Evaluation of *Echinococcus multilocularis* Tetraspanins as Vaccine Candidates against Primary Alveolar Echinococcosis

**Running title: Echinococcosis Tetraspanin Vaccination**

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**Abstract**

*Echinococcus multilocularis* causes an important zoonotic cestode disease. The metacestode stage proliferates in the liver of intermediate hosts including human and rodents and forms multiple cysts. Recently, members of a transmembrane protein tetraspanin (TSP) family have been used as vaccines against schistosomosis, or as diagnostic antigens for cysticercosis. In this study, seven tetraspanins of *E. multilocularis*, designated as TSP1 to TSP7, were evaluated for their protective potential against primary alveolar echinococcosis. The large extracellular loop (LEL) region of these tetraspanins was cloned from a full-length enriched cDNA library of *E. multilocularis* metacestodes and expressed in *Escherichia coli* as a fusion protein with thioredoxin. Recombinant TSPs were applied as vaccines against an *E. multilocularis* primary experimental infection in BALB/c mice. Cyst lesions in the livers of vaccinated and non-vaccinated mice were counted. The cyst lesion reduction rates induced by the seven tetraspanins in vaccinated vis-à-vis non-vaccinated mice were: 87.9%, 65.8%, 85.1%, 66.9%, 73.7%, 72.9% and 37.6%. Vaccination conferred protective rates to mice ranging from 0% (TSP5, 6, 7) to maximally 33% (TSP1, 3). The results indicated that recombinant tetraspanins have varying protective effects against primary alveolar echinococcosis and could be used in vaccine development.

Key words: *Echinococcus multilocularis*; Tetraspanin; Vaccine; Oncosphere; Primary Alveolar Echinococcosis

## 1. Introduction

*Echinococcus multilocularis* is a cestode parasite, whose larval stage causes hepatic alveolar echinococcosis in humans and rodents, and thus it represents an ecological and public health problem in many countries of the Northern Hemisphere [1]. Although prevention of this tumor-like disease is very important, most studies have concentrated on the parasite's morphology and immunology [2, 3]. The application of protective proteins as vaccines against other cestode parasites, especially *Taenia spp*. [4-10] and *E. granulosus* [11-14] suggests that vaccine development for *E. multilocularis* is also possible. Great success has been achieved in vaccinating against *E. granulosus* infection using the recombinant protein EG95 [12, 14]. In similar studies, it has been reported that recombinant proteins EMY162 [15], EM95 [15, 16] and 14-3-3 [17] protect against primary (egg) *E. multilocularis* infection.

Recently, many studies have focused on the tetraspanins, a transmembrane protein family, for their multiple functions involved in the coordination of intracellular and intercellular processes, including signal transduction, cell proliferation, adhesion, migration,

cell fusion, and host-parasite interactions [18]. The host-parasite interactions are thought to be associated with immune evasion [19], which has resulted in the use of tetraspanins as vaccines interfering with the schistosome survival strategy [19-22]. Several tetraspanins, TSP1, TSP2 [19] and Sm23 [20] of *Schistosoma mansoni,* and SJ23 of *S. japonicum* [21, 22] have been reported as potential vaccine candidates against schistosomosis. It is notable that these tetraspanins caused varying reductions in different parasitic stages: adult worms (TSP1, TSP2, Sm23 and SJ23), liver eggs/granulomas (TSP1, TSP2 and SJ23) and intestinal/fecal eggs (TSP2 and SJ23).

Although such successes have been achieved using tetraspanins to protect mice against different stages of schistosome infections, no studies have been made on tetraspanins in *Echinococcus*, especially with a view to exploiting their vaccine potential. As a preliminary study, we evaluated the immunogenicity and protective ability of immunization with seven tetraspanins against *E. multilocularis* infection in a murine model.

## 2. Materials and methods

### 2.1. Molecular analysis of TSP amino acid sequences by biological prediction servers

A full-length enriched cDNA library of *E. multilocularis* metacestodes (Hokkaido isolate)

was constructed using a vector-capping method (Hitachi Biotechnologies, Tokyo, Japan) and their 5' ends were sequenced. We identified members of the tetraspanin family by BLAST searching on-line using partial sequences, picked seven representative clones from the library and sequenced them in full. The conserved, crucial parts of these proteins-LEL domain (aa 1～aa 81 in figure 1) were compared with other known tetraspanins from helminthes using the Clustal W2 on-line service. Then we used the MegAlign component of DNAStar programme (Version 4.01 DNASTAR, Madison, Wis.) to construct a phylogenetic tree of transmembrane proteins, including the TSP1 to TSP7 tetraspanins of *E. multilocularis*; the Sm23 [20], Sm25 [23], Sm-TSP1 and Sm-TSP2 proteins of *S. mansoni* [19]; the Sj23 [21, 22] and Sj25 proteins of *S. japonicum* [24], the Sh23 protein of *S. haematobium* [25] and T24 protein of *Taenia solium* [26]. We also used the DNAStar program to determine the percentage identity of the several TSPs of *E. multilocularis*.

**2.2. Cloning, expression and purification of recombinant proteins**

For facilitating solubilization and purification of expressed proteins, the regions encoding the extracellular loop (LEL) were amplified by RT-PCR with Taq polymerase to fuse them in-frame with the N-terminal *E. coli* thioredoxin (TRX) and the C-terminal V5

and 6-His epitopes in the pBAD/Thio-TOPO plasmid (Invitrogen, USA). The primers used are listed in Table 1. The cDNAs were ligated into the plasmid and were transformed into *E. coli* TOP10 cells (Invitrogen, USA) with recombinant plasmids according to the manufacturer's instructions (pBAD/TOPO® ThioFusion™ Expression Kit, Invitrogen, USA). The recombinant bacteria were cultured in Lennox broth at 37°C for 16 hours, complemented with 0.05% arabinose as an inducer. Recombinant fusion proteins from *E. coli* lysates were purified with a HisTrap affinity column under nondenaturing conditions (HisTrap FF crude 1 ml, GE Healthcare, USA). The purified proteins were dialyzed in PBS with Snake Skin Pleated Dialysis Tubing (10,000 MWCO, PIERCE, USA) and stored at -80°C.

**2.3. Animal experiments**

All animal experiments were performed in accordance with the guidelines of Hokkaido University and Hokkaido Prefecture.

**2.4. Antigen preparation from infected cotton rats**

Cotton rats were infected with the eggs of *E. multilocularis* (Hokkaido isolate). Nine weeks post-infection, the brood capsules were formed and the protoscolex appeared. Cotton rats were then dissected and cyst blocks were collected from the abdominal cavity,

removing as much host tissue as possible. After washing several times with 0.15M PBS (pH7.4), the cyst blocks were cut with scissors and treated to several cycles of freeze/thawing in liquid nitrogen. We then added 9 volumes of PBS (pH7.4), containing protease inhibitors (5 mM ethylenediaminetetraacetic acid, 5 mM iodoacetamide, 1 mM phenylmethanesulfonyl fluoride, 1 μM pepstatin) and 1% Triton X-100, and homogenized the cyst blocks with a glass-homogenizer and subsequently with a teflon-homogenizer. The tissues were further homogenized using the ultrasonic disruptor (UR-200P, Tomy Seiko, Tokyo, Japan) at 40 W for 3 min. The lysate was centrifuged at 10,000×g for 30 min at 4°C and the supernatant was dialyzed for 48 hrs in PBS (pH 7.4). Dialyzed proteins were finally filtered through a nitrocotton filter (1 μm pore size). The cyst-extracted antigen was stored at -80°C.

**2.5. Rabbit immunization and polyclonal antisera generation**

For the studies on specific antibody response and cross-reactivity of the seven tetraspanin proteins, polyclonal anti-sera directed against the seven TSPs were generated in seven 15 weeks old male rabbits. One rabbit for each recombinant TSP was immunized by three successive intramuscular injections of purified recombinant fusion TSP proteins at day 0, 14 and 28 separately at a dosage of 150 μg/rabbit. TSP proteins for primary immunization

were emulsified in Freund's complete adjuvant, while those for the second and third boosters were emulsified in Freund's incomplete adjuvant. Immunization was completed within six weeks. Pre-immune serum was collected as a negative control and all sera were aliquoted and stored at -80°C. For immunoblotting analysis and ELISA tests, the antisera to TSP proteins were absorbed by affinity chromatography with HiTrap affinity columns (HiTrap NHS-activated HP 1 ml, GE Healthcare, USA), coupled with purified TRX. This absorption was performed in order to deplete the antisera of antibodies directed to the fusion partner protein TRX.

**2.6. SDS-polyacrylamide gel electrophoresis and immunoblotting**

Recombinant and cyst-extracted proteins were treated with Laemmli sample buffer (BIO-RAD, USA), heated at 98°C for 5 min, and then separated on a 12% SDS-polyacrylamide gel. For immunoblotting analysis, proteins were transferred from the gel to a PVDF membrane (BIO-RAD, USA) using the Trans-Blot™ SD Semi-Dry Transfer Cell (Bio-Rad, USA). The membrane was blocked with 5% skimmed milk in TBS-T for 1 hour and was subsequently incubated with the anti-TSP antibody at a dilution of 1:5,000 in 3% bovine serum albumin/PBS for 1 hour. This was followed by incubation with horseradish peroxidase (HRP)-conjugated goat-anti-rabbit antibody at a dilution of

1:10,000 in 3% BSA/PBS for 1 hour. Finally, an HRP substrate Luminol Reagent (Millipore Corporation, USA) was added and the reaction was detected by chemiluminescence using the LAS1000 Mini Reader (FUJIFILM, Japan). Three washes with TBS-T were performed after every step. Immunoblotting was performed at room temperature.

**2.7. Vaccination procedure**

Seventy female BALB/c mice were divided into 10 groups at seven weeks old, each having seven mice. Groups One, Two and Three were non-immunized or immunized with PBS plus adjuvant, and TRX plus adjuvant, respectively as controls, while Groups Four to Ten were immunized with respective TSPs (TSP1 to TSP7) plus adjuvant. For Groups Four to Ten, each mouse received a subcutaneous injection of 20 µg of protein dissolved in 100 µl PBS, and emulsified in an equal volume of Freund's complete adjuvant (primary immunization) and Freund's incomplete adjuvant (booster). PBS and TRX controls were similarly treated by replacing TSP proteins with PBS or TRX. The primary immunization was done on day 0. Boosters were given twice on days 21 and 42. Sera were collected and tested for the antibody by ELISA on day 49 post-immunization. One mouse from Group One, Four, Five, Six and Nine died during the etherizing pre-challenge.

### 2.8. Evaluation of vaccine efficacy

Experimental infection was done using *E. multilocularis* eggs collected from the feces of an experimentally infected dog. The etherized mice were orally administrated with 0.5 ml of eggs suspension at a concentration of 400 eggs per ml in physiological saline 56 days post-immunization. One month post-infection, all mice were sacrificed and necropsies were performed. Livers were collected in plastic dishes and cut into approximately 0.5 mm thick slices, from which the numbers of all detectable lesions were counted. Based on these data, the cyst reduction rate of each tetraspanin was calculated as a percentage reduction in cyst lesion counts in vaccinated groups as compared to non-vaccinated ones.

### 2.9. Cross-reaction among TSPs determined by ELISA

We used ELISA to analyze sera from immunized rabbits for their cross reactivity to heterologous TSPs. Prior to ELISA, the sera were absorbed with TRX as described above. Ninety-six well Costar® plates (Corning Inc., USA) were coated with recombinant TSPs at a concentration of 0.1μg/ml in carbonate buffer (pH 9.6) for 2 hrs at room temperature. This was followed by blocking with 5% skimmed milk overnight at 4°C and washing four times with PBS-Tween. Then serum samples, diluted 1: 5,000 in 3% BSA/PBS, were added

and the plates were incubated for 1 hour at 37°C before washing four times. Goat-anti-rabbit IgG conjugated with horseradish peroxidase (Invitrogen, USA), diluted 1:10,000 in 3% BSA/PBS, was then added and incubated at 37°C for 1 hour. This was followed by washing four times and the addition of 3, 3', 5, 5'-tetramethylbenzidine substrate before incubation for 20 min at room temperature. The reaction was stopped using 1N HCL and color development was measured at 450 nm with the Biotrack II plate reader (Amersham Biosciences, USA). Antisera to TSP proteins were absorbed with TRX-coupled HiTrap affinity columns (HiTrap NHS-activated HP 1 ml, GE Healthcare, USA) deplete the antibodies directed to the fusion partner protein TRX. Pre-immune serum was used as a negative control. The mean OD value of pre-immune sera was calculated and used as a cut-off point (threshold value) to discriminate between "positive" and "negative".

**2.10. Detection of TSPs transcripts in oncosphere by RT-PCR**

Eggs were treated with artificial gastric fluid for 1 hour at 37°C and centrifuged at 1,000×g for 5 min. The pellet was used to extract total RNA using a TRIzol plus RNA Purification Kit (Invitrogen, USA), and cDNA synthesis was carried out using the SuperScript III First-strand Synthesis System Kit (Invitrogen, USA) according to the manufacturer's instructions. Sense and anti-sense primers (Table 1) were designed to

amplify tetraspanin open reading frames (ORFs) and RT-PCR was performed with GoTaq Flexi DNA Polymerase (Promega, USA).

### 2.11. Data analysis

The protective effect obtained by immunization with each tetraspanin was calculated as the percentage reduction of the number of cyst lesions in vaccinated mice as compared to non-vaccinated ones. Multiple comparison analysis was performed with the Tukey-Kramer method using a computer package (Statcel 2, Japan) to determine differences between the vaccinated groups and control groups, which were considered significant at the 5% level.

## 3. Results

### 3.1. Molecular analysis of *E. multilocularis* tetraspanin

Molecular components analysis of LEL domain using the on-line Clustal W2 program revealed that TSP1 to TSP7, of *E. multilocularis,* share many conserved residues with each other, and with those of *Schistosoma* and *Taenia* (Fig. 1), which has been reported previously [27]. The amino acid sequence of these residues was as follows: E/Q···CCG···D···W/F/Y···P···SCC···C···GC. The percentage conservation of the amino acids CCG, the first C of SCC and the terminal C, of TSP1 to 7, was 100%. Very interestingly, we found that residues F/L at aa 5, G at aa 12 and P/Y at aa 13 are extremely conserved in

these helminths, while some residues only exist in *Taenia* and *Echinococcus*: A at aa 55, G at aa 69 and aa 81. Percentage identity analysis of the seven TSPs showed that the amino acid sequence identity ranged from 12.6% to 57.7% (Table 2). In addition, the phylogenetic relationship revealed that TSP3 and TSP5 are closely related to T24, a diagnostic antigen of *T. solium*; and TSP2 is related to the protective proteins Sm23, Sh23, Sj23 and TSP2 of *Schistosoma* (Fig. 2).

### 3.2 Amplified fragments of tetraspanins

Using the primers listed in Table 1, the LELs of the seven tetraspanins from cyst cDNA and the entire ORFs of the seven tetraspanins from oncosphere cDNA were amplified by PCR. The results showed that the amplified fragment sizes of the LELs ranged from 195 bp to 330 bp (Fig. 3A) and those of the tetraspanin ORFs ranged from 447 bp to 771 bp as expected (Fig. 3B). Only a very weak band of oncosphere TSP1 ORF could be detected (Fig. 3B).

### 3.3 SDS-PAGE analysis of recombinant tetraspanins

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the purified fusion proteins showed the expected molecular masses, which ranged from 24 to 26 kDa (Fig. 4). Purified recombinant proteins were soluble and pure enough for further immunological analysis and

vaccine trials in mice.

### 3.4 Anti-TSPs IgG antibody response

Sera from immunized rabbits were examined for the presence of anti-TSP antibodies against TSPs using immunoblotting. Western blot analysis showed that anti-TSP antibodies had very strong responses to cyst-extracted antigens (Fig. 5). Double bands can be found to TSP3. In contrast, pre-immune serum was not reactive with either recombinant or cyst-extracted proteins. The immunostained bands were approximately 23 to 29 kDa. Cross-reaction investigation using ELISA showed that TSP1 and TSP3 could induce highly specific antibody responses, while the antibody to TSP2 was strongly cross-reactive with other TSPs including TSP4 to 7. Antisera to TSP4, TSP5, TSP6 and TSP7 showed cross-reactivity with the others to varying extents. According to mean OD value to pre-immune sera (threshold value), all the OD value to anti-TSPs were determined as "positive" (Table 3).

ELISA results using individual sera collected from vaccinated mice showed that although there were many variations among mice within each group and between groups, relatively strong antibody responses were observed against recombinant tetraspanins and cyst-extracted proteins (Fig. 6).

### 3.5 Protective efficacy of tetraspanin proteins

The number of counted cyst lesions in the liver and the reduction rate of cyst lesions for the seven TSPs (TSP1 to TSP7) are shown in Table 4. The recombinant proteins TSP1, TSP2, TSP3, TSP4, TSP5 and TSP6 gave statistically significant reductions of more than 65% ($P<0.05$) in the number of cyst lesions in vaccinated mice as compared to non-vaccinated ones. However, TSP7 protein did not produce a significant reduction in cyst lesions (37.6%) ($P>0.05$). PBS showed no vaccine efficacy, while TRX displayed a 27.8% reduction in cyst lesions ($P>0.05$). Among the vaccinated groups, two out of six in the TSP1-vaccinated group, one out of six in the TSP2-vaccinated group, two out six in TSP3-vaccinated group, and one out of seven TSP4-vaccinated group showed no cyst lesions in the liver (Table 4). In consequence, vaccination conferred an overall protection rate against infection ranging from 0% (TSP5, 6, 7) to maximally 33% (TSP1, 3).

## 4. Discussion

Despite high expression levels in the plasma membrane, and intracellular vesicles, tetraspanins remain among the most mysterious transmembrane molecules 20 years after their discovery [28]. To date, several tetraspanins have been identified in helminths, 20 in *Caenorhabditis elegans* and at least 25 in the parasite *Schistosoma* [29]. Although some

tetraspanins have been demonstrated to be potential vaccine candidates against different stages of schistosome infection [19-22], nothing is known about them in another important platyhelminth *E. multilocularis*. Recently, many studies have focused on the LEL domain of tetraspanin because of its important functions in mediating protein–protein interactions and homodimerization [30]. In our present study, LEL domain of seven tetraspanins were cloned, expressed and characterized in order to focus on the protective potentials of tetraspanins against *Echinococcus* infection.

Tetraspanins belong to a four transmembrane protein family, nearly all members of which share 26 conserved key residues. These conserved residues have been used as a key criterion to define whether a newly identified membrane protein is a member of tetraspanin family or not [27, 31]. Thirteen out of 26 conserved residues are within LEL domain. Molecular component analysis of LEL domain, using the Clustal W2 on-line biological service, indicated that these key residues were conserved in TSP proteins of *E. multilocularis*, *S. mansoni, S. japonicum* and *T. solium*. Most importantly, we found that among those proteins the crucial cysteine-cysteine-glycine (CCG) motif has no mutations as previously reported [27]. Three extra conserved sites, F/L at aa 5, G at aa 12 and P/Y at aa 13, were found in tetraspanins of these helminths (Figure 1), implying the possibly

closer relationship of these helminths than others. Moreover, residues A at aa 55, G at aa 69 and aa 81 appear to be more conserved in cestode *Echinococcus* and *Taenia*. The amino acid sequence analysis of the seven TSPs displayed a relatively high percentage identity ranging from 12.6% to 57.7%. This is mainly due to the existence of the largest, conserved domain, known as the large extracellular loop (LEL). Members of the tetraspanin family from different organisms are structurally and functionally related [18, 31]. Phylogenetic analysis indicated that TSP2 of *E. multilocularis* is phylogenetically related to TSP2 [15], Sm23 [20] and SJC23 [21, 22] of *Schistosoma*, the protective antigens against schistosomiasis, while TSP5 is closely related to the T24 antigen of *T. solium*, a diagnostic antigen for cysticercosis [26]. We suppose that among these proteins, some may be vaccine candidates, while others may be useful as diagnostic antigens.

Cyst-extracted antigens had strong reactivity with TRX-absorbed rabbit anti-TSP antibodies as determined by immunoblot assay (Fig. 5 and Fig 6) similar to that of recombinant proteins (Fig. 6), suggesting a well antigenicity of TSP proteins. The much closed double bands detected to TSP3 indicated possible differential glycosylation (Fig. 5).

Since tetraspanins in the same family share common crucial residues [27, 31], antigenic cross-reactivity would be expected between the different TSP molecules. The ELISA

results indicated that recombinant tetraspanin proteins showed varying degrees of cross-reactivity with each other. Although all the OD values to anti-TSPs sera were determined as "positive" referencing to threshold value, anti-TSP1 and anti-TSP3 antibodies exhibited the highest specific reactivities to homologous antigens but had low cross-reactivities with others (Table 3). On the other hand, TSP2 and TSP7 showed a broad range of specificity, while TSP4, TSP5 and TSP6 cross-reacted with other members of the family to varying extents.

Tetraspanin proteins regulate cell morphology, motility, invasion, fusion and signaling, in the brain, immune system, in tumors and elsewhere [32-37], and the most distinctive feature of the tetraspanin family is the ability of its members to form lateral associations with multiple partner proteins, and with each other, in a dynamic assembly, described as the 'tetraspanin web' [34, 38]. The fact that some tetraspanin proteins cross-reacted with several others implies that immunization with one tetraspanin antigen could block several tetraspanins functions or disrupt the lateral associations with multiple partner proteins and with each other in the tetraspanin web, thereby providing a more effective protection mechanism.

Primary infection, initiated through the oral *E. multilocularis* egg infection of mice,

followed by counting liver cyst lesions four weeks post-infection, revealed that six out of seven TSPs had a cyst lesion reduction rate of over 65% in this study. TSP1 and TSP3 exhibited reduction rates of 87.9% and 85.1%, respectively, while TSP7 had the poorest protective effect of 37.4%. The most encouraging results were found in the groups for TSP1 to 4, in which the number of liver cyst lesions in some mice was found to be zero, indicating that these mice were completely protected against *E. multilocularis.* Compared to the non-vaccinated group, the PBS plus adjuvant group showed no protective efficacy, while TRX plus adjuvant had a 27.8% reduction in cyst lesions.

Much success has been achieved in the vaccination campaign against *E. granulosus* infection using the recombinant protein EG95 [12, 14]. In similar studies, Kouguchi *et al.* [15] reported 74.3% protection against *E. multilocularis* infection in mice using the recombinant protein EMY162, and Gauci *et al.* [16] and Kouguchi *et al*. [15] reported 79% and 82.9% protection respectively, against *E. multilocularis* infection in mice using the recombinant protein EM95. In addition, the recombinant protein 14-3-3 was reported to have 97% protection against a primary infection of *E. multilocularis* [17]. It can be seen from the above results that the reduction in liver cysts for EMY162 and EM95 were lower than for TSP1 (87.9%) and TSP3 (85.1%). Moreover, secondary (intraperitoneal) infection

has not been performed to evaluate the vaccination efficacy with these proteins. Although the recombinant protein 14-3-3, showed a higher protection against primary infection than those used in this study, showed no protection against secondary infections in mice. As reported, except for erythrocytes, all cells seem to express several tetraspanins [39-41] and individual tetraspanin proteins are often expressed in large copy numbers per cell [33]. Additionally, as mentioned above, tetraspanins have showed their potential in protecting a host against different stages of *Schistosoma* infection [19-22]. We believe that the discovery of one, or more tetraspanins, which are expected to have 'broad-spectrum protection' against different stages of *E. multilocularis* infections, will be achieved in our future studies.

Our RT-PCR results showed that transcripts for these TSPs exist in both the metacestode and oncosphere stages of *E. multilocularis.* Moreover, our study on TSP localization revealed that most of these TSPs are expressed on the germinal layer of *E. multilocularis* metacestodes (in press). Usually, the tegument of *E. multilocularis* metacestodes is thought to be the outer part of the germinal layer [42, 43]; therefore, we speculate that TSPs on the *Echinococcus* germinal layer/surface play crucial roles in parasite survival and parasite-host interactions. Proteins expressed on the surface, as membrane-integrated proteins, have a

wide range of functions in parasite biology [44]. Loukas *et al*. [45] emphasized the importance of the schistosome tegument/body wall by describing it as 'the key to its success but also its Achilles heel', because the schistosome tegument has been described as being responsible for parasite survival through immune evasion, while from a vaccine perspective, it constitutes the host-parasite interface. Although we are unsure, in our experiments, whether the antibodies raised by these tetraspanins kill the metacestode or the oncosphere, or even a parasite stage in between, previous studies with schistosomes suggested that once the surface of the parasite is blocked by anti-TSP antibodies, they will lose their survival strategies and render the parasite surface vulnerable to the host defense mechanisms [19].

In this study, we are the first to identify and characterized a new tetraspanin family as vaccine candidates with high protection potential against primary *E. multilocularis* infection in mice. The numerous properties displayed by tetraspanins, and the results from our studies, call for in-depth analyses to explore their vaccine potential in protecting intermediate and final hosts against different stages of infections.

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Table 1. Oligonucleotide primers for amplification of the LEL domain and TSP ORFs.

| Primer[a] | Sequence[b] | | | |
|---|---|---|---|---|
| | LEL domain | Product (bp) | TSP ORF | Product (bp) |
| TSP1-s | CCTGATAACCTAAACAAAGC | 330 | AGCTATCTTCTCGGATGTGCTAGAC | 792 |
| TSP1-a | GAGGGTTTTGTTCTCTGCCAA | | TTCCGCTTTCCACTGCTTGTACAGC | |
| TSP2-s | TACAAGGATGAGTTGATGGAC | 195 | GGTGTTCTTCAAGGTGGAATGAAGTG | 621 |
| TSP2-a | CATGGAGGACTCAACTTTGCC | | CGCATAACTTCG AATTTTGGACTGC | |
| TSP3-s | CATGAGTTTGTCGGTCTTGTG | 264 | CTGAAGACGTTCGCGGTCATTGT | 447 |
| TSP3-a | CAGAGAGGAATCCTTAATTTG | | CTTCTCGTATTCGTTGACCTTCTTGGCC | |
| TSP4-s | TTCGCGCTGAAAAATCAGTCC | 267 | GCAATGCTGCGCTTTAGCGGTTGCT | 687 |
| TSP4-a | AGGTAGTCTATTTTCCGTCAA | | GGTTACAGATGCTGTCCTAGGGCCAA | |
| TSP5-a | CACGACTTCGTTCGTCTTGTT | 261 | GCTCTCTCTTGCGGTGGTAACTT | 678 |
| TSP5-s | CAGGGCAGAATCCTTTATCTC | | GACCTTCTCATATTCGCCGACCTTCTTG | |
| TSP6-s | GACAAGCAGGGTAAAGACTTT | 252 | GTTCTGACATGCGGAGAGCAGT | 669 |
| TSP6-a | GCTTCGAAGGTATTCGTAGAT | | GCTCCGCTTGCCGGCCAAACAGCA | |
| TSP7-s | GTTTTCAAAGATGAGGTGCGA | 249 | TCGAAAACGTGTGCTGGTTGTATCAG | 690 |
| TSP7-a | CAACGTATGTTTCTCAATCTC | | CACAGGTTCATAAGATTCAATGTCGG | |

[a] s, sense; a, antisense.

[b] Sequences are 5’ to 3’.

Table 2. Percentage identity analysis of the entire ORF of TSP by DNASTAR (Version 4.0)

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | | |
|---|---|---|---|---|---|---|---|---|
| 100 | 13.0 | 17.4 | 16.2 | 15.9 | 12.6 | 15.2 | 1 | TSP1 |
| | 100 | 23.5 | 18.4 | 29.0 | 23.7 | 18.4 | 2 | TSP2 |
| | | 100 | 24.2 | 57.7 | 26.8 | 16.8 | 3 | TSP3 |
| | | | 100 | 30.1 | 30.5 | 16.6 | 4 | TSP4 |
| | | | | 100 | 30.0 | 21.7 | 5 | TSP5 |
| | | | | | 100 | 19.3 | 6 | TSP6 |
| | | | | | | 100 | 7 | TSP7 |

Amino acid sequences were used for the percentage identity analysis of TSP proeins.

Table 3. Cross-reaction of anti-TSP-sera with TSPs by ELISA

| Protein[a] / Antibody[b] | TSP1 | TSP2 | TSP3 | TSP4 | TSP5 | TSP6 | TSP7 |
|---|---|---|---|---|---|---|---|
| *Anti-TSP1* | **1.903** | **0.573** | **0.237** | **0.172** | **0.224** | **0.154** | **0.154** |
| *Anti-TSP2* | **1.878** | **2.345** | **2.115** | **0.311** | **2.011** | **1.730** | **1.649** |
| *Anti-TSP3* | **0.182** | **0.293** | **2.301** | **0.147** | **0.731** | **0.149** | **0.155** |
| *Anti-TSP4* | **0.656** | **2.713** | **2.257** | **2.559** | **0.720** | **1.393** | **0.887** |
| *Anti-TSP5* | **0.568** | **1.023** | **0.594** | **0.531** | **1.367** | **0.432** | **0.440** |
| *Anti-TSP6* | **0.524** | **1.717** | **0.848** | **0.268** | **0.543** | **1.908** | **0.181** |
| *Anti-TSP7* | **0.463** | **1.265** | **0.801** | **0.344** | **0.375** | **0.359** | **1.293** |
| *PIS*[c] | 0.147 | 0.140 | 0.113 | 0.143 | 0.127 | 0.119 | 0.122 |

[a] Purified recombinant TSPs were used at a concentration of 0.1 μg/ml.

[b] Serum samples were diluted 1:5,000 in 3% BSA/PBS.

[c]PIS, Pre-immune sera, mean±SE=0.130±0.013. This is used as a cut-off point (threshold value) to discriminate between "positive" and "negative". Positive values were presented in bold letters.

OD value was measured at A450nm.

TSP, tetraspanin; TRX, thioredoxin.

Table 4. Number of cyst lesions in mouse liver and percentage reduction in cyst lesions in vaccinated groups compared with non-vaccinated control.

| Group | Number of cyst lesions | | | Vaccine efficacy (%) | |
|---|---|---|---|---|---|
| | Mean ± S.E | Range | Ratio of mice without lesions | Cyst lesion reduction rate | Protective rate |
| Non-vaccinated control (n=6)[d] | 19.00 ±3.1 | 7-25 | 0/6 | – | 0 |
| PBS (n=7) | 20.28 ±1.2[c] | 16-26 | 0/7 | -6.8 | 0 |
| TRX (n=7) | 13.71 ±3.5[c] | 7-34 | 0/7 | 27.8 | 0 |
| TSP1 (n=6) | 2.33 ± 1.1[a] | 0-7 | 2/6 | 87.9 | 33.3 |
| TSP2 (n=6) | 6.50 ± 2.7[b] | 0-18 | 1/6 | 65.8 | 16.7 |
| TSP3 (n=6) | 2.83 ± 1.5[a] | 0-8 | 2/6 | 85.1 | 33.3 |
| TSP4 (n=7) | 6.29 ± 1.7[a] | 0-14 | 1/7 | 66.9 | 14.3 |
| TSP5 (n=7) | 5.00 ± 0.9[a] | 2-8 | 0/7 | 73.7 | 0 |
| TSP6 (n=6) | 5.50 ± 1.8[a] | 1-13 | 0/6 | 72.9 | 0 |
| TSP7 (n=7) | 11.85 ± 2.9[c] | 1-27 | 0/7 | 37.6 | 0 |

[a] Reduction of cyst numbers was significant at $P<0.01$.

[b] Reduction of cyst numbers was significant at $P<0.05$.

[c] Reduction of cyst numbers was not significant ($P>0.05$).

[d] n represents the number of mice in each group.

**Figure legends**

**Fig. 1.** Comparison of the LEL domain amino acid sequences for the seven cloned transmembrane proteins (Em-TSP1 to TSP7) and those of Sm-TSP1, Sm-TSP2, Sm23, Sj23 and Ts-T24. Alignment of these amino acid sequences was performed with the Clustal W2 on-line service. Identical amino acid residues are marked with colored shadow. Key residues are marked with a red frame. Extra conserved residues in *Schistosoma*, *Echinococcus* and *Taenia* are marked with a black frame. *Echinococcus* and *Taenia* specifically conserved amino acids are in shorter black frames. Em, *Echinococcus multilocularis;* Sm, *Schistosoma mansoni;* Sj, *Schistosoma japonicum*; Ts, *Taenia solium;* The GenBank (http://www.ncbi.nlm.nih.gov/Genbank) accession numbers for *E. multilocularis* tetraspanin 1 to 7 and Sm-TSP1, Sm-TSP2, Sm23, Sj23 and Ts-T24 are **FJ384717**, **FJ384718**, **FJ384719**, **FJ384720**, **FJ384721**, **FJ384722**, **FJ384716**, **AF521093**, **AF521091**, **M34453**, **M63706** and **AY211879** respectively.

**Fig. 2.** Phylogenetic tree of transmembrane proteins constructed by the MegAlign component of the DNAStar program (Version 4.01 DNASTAR, Madison, Wis.). TSP1 to TSP7: tetraspanins of *E. multilocularis* (in marked frame); Sm: *Schistosoma mansoni*; Sj:

*Schistosoma japonicum*; Ts: *Taenia solium*; Sh: *Schistosoma haematobium*. GenBank accession numbers for Sm25, Sh23 and Sj25 are AF028730, U23771 and U77941 respectively.

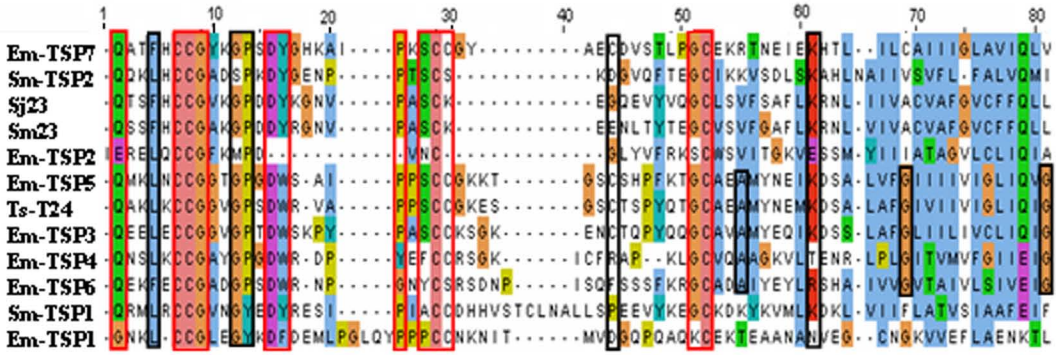
**Fig. 3.** Amplification of tetraspanins by RT-PCR. (A) LELs of tetraspanins from the cDNA library of *E. multilocularis* metacestodes with sense and antisense primers designed from the large extracellular loop domain. (B) Investigation of tetraspanins from cDNA of oncosphere cDNA with forward and antisense primers designed from ORFs of full-length tetraspanins. In both (A) and (B), Lane M, molecular marker. Lanes 1 to 7, TSP1 to TSP7.

**Fig. 4.** SDS-PAGE analysis of purified recombinant TSPs. Purified recombinant TSPs were loaded at 5-10 μg/well and separated on a 12% SDS-polyacrylamide gel. CBB staining was performed. Lane M, molecular marker. Lanes 1 to 7, TSP1 to TSP7. Lane 8, purified thioredoxin (TRX).

**Fig. 5.** Immunoblotting analysis of anti-TSP sera (TRX-removed) to cyst-extracted antigens. Serum samples were diluted 500-fold and the anti-rabbit IgG-HRP conjugate was diluted 5,000-fold with blocking buffer. Pre-immune serum was used as the negative control. Lane M, molecular marker. Lanes 1 to 7, reactivity of cyst-extracted antigens to anti-TSP1 to

TSP7 antibodies. Lane 8, negative control.

**Fig. 6.** Reactivity of recombinant TSP-immunized-mice sera (TRX-absorbed) to recombinant TSPs and cyst-extracted antigens measured by ELISA. Serum samples were diluted 5,000-fold and the goat anti-mouse-IgG-HRP conjugate was diluted 10,000-fold in blocking buffer. For each group, a, b represents reaction with recombinant TSP (symbol●) and cyst-extracted antigen respectively (symbol ✪). The mean OD value of control reaction where TRX had been used as antigen (range of the mean values among TSP1-TSP7: 0.05-0.12) was subtracted from each group. Vertical bars represent mean OD value of each group. N, number of mice in each group.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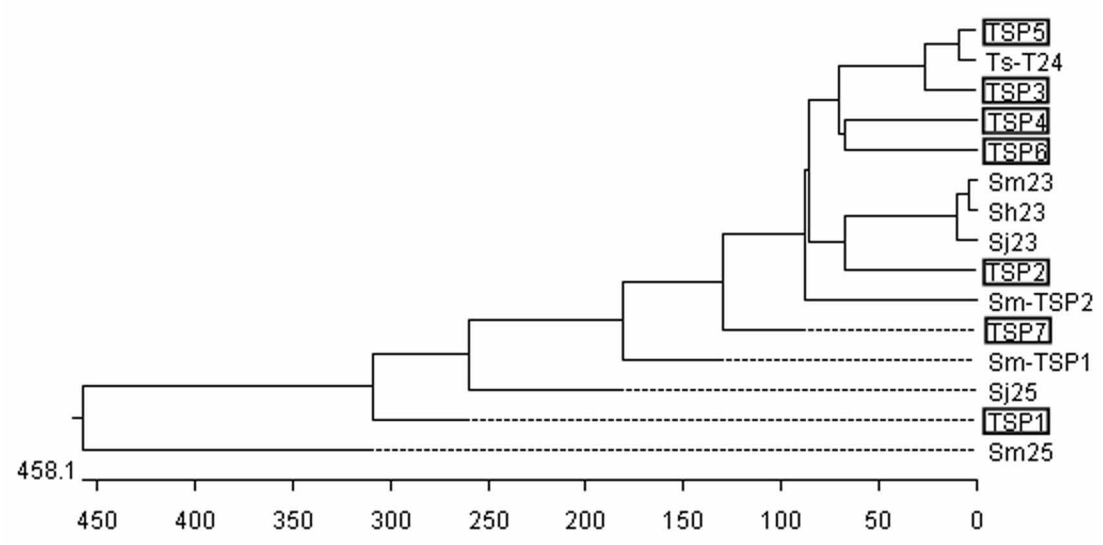
Em-TSP1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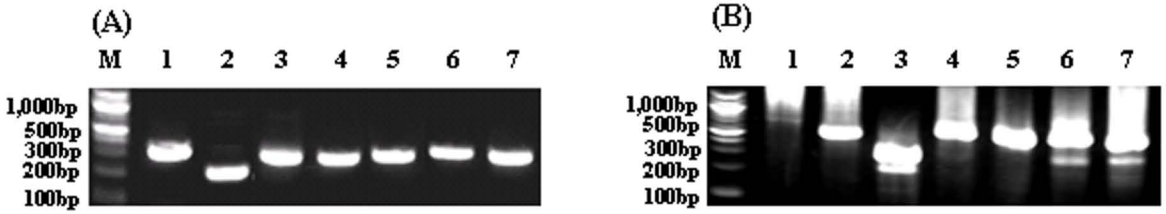
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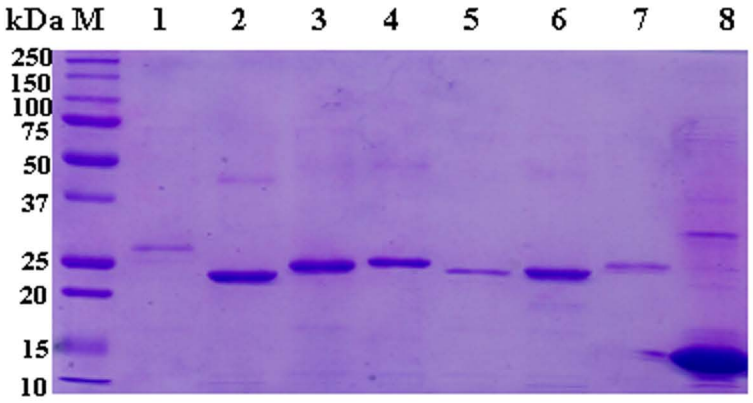
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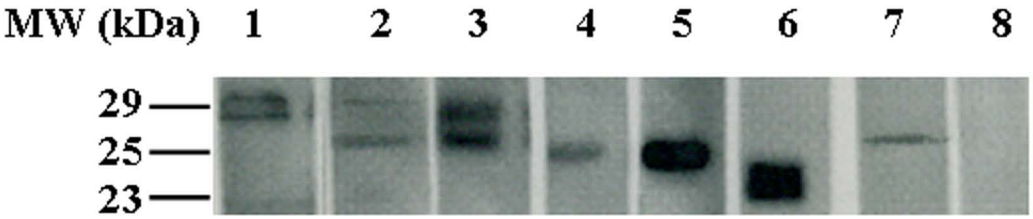
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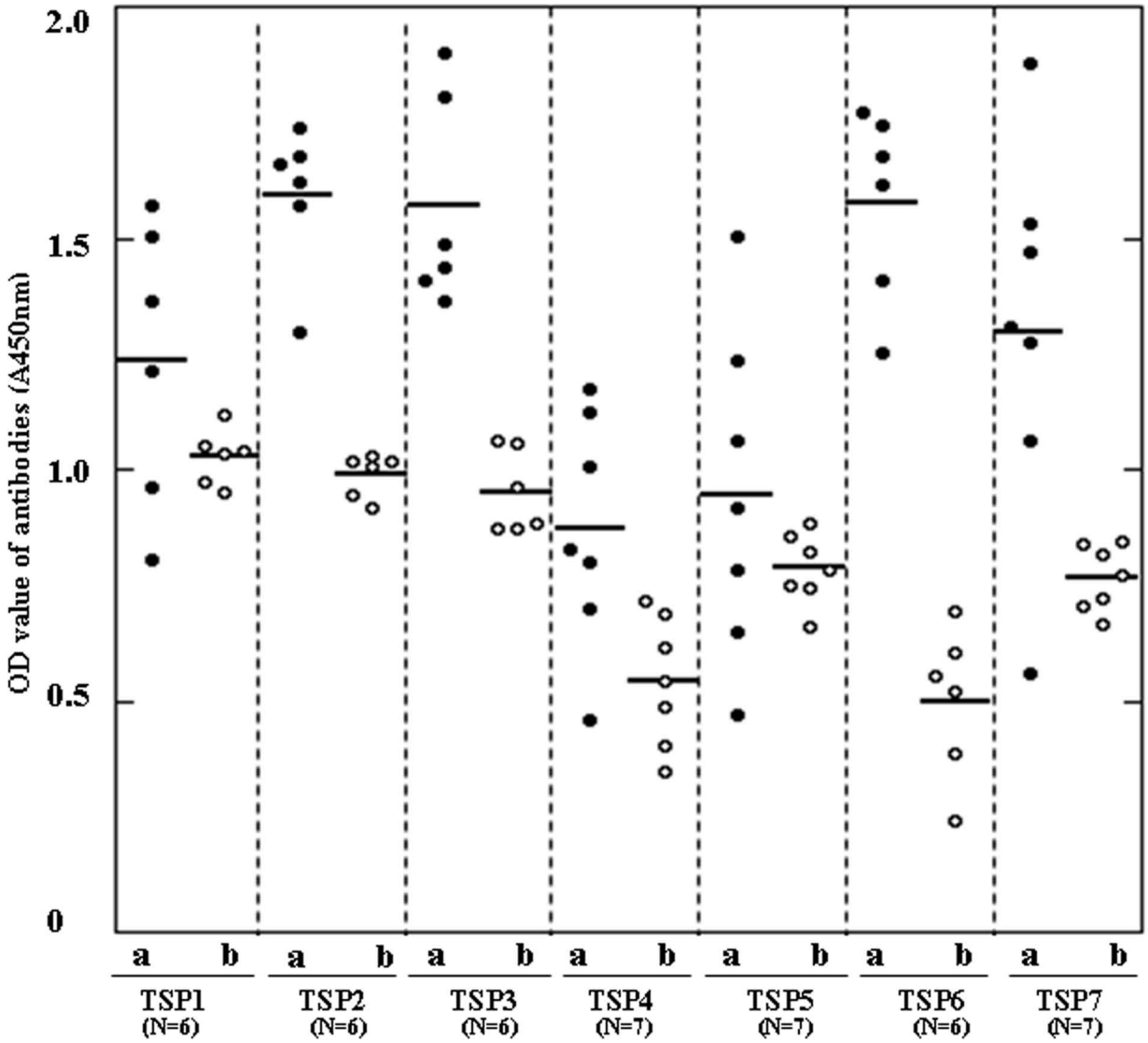
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