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In silico characterization of *Echinococcus granulosus* paramyosin nucleotide sequence for the development of epitope vaccine against cystic echinococcosis

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Summary

The paramyosin (Pmy) protein has been presented as a potential vaccine candidate against *Schistosoma* spp. However, it remains elusive whether it works in controlling cystic echinococcosis (CE), which is caused by the larval stages of *Echinococcus granulosus* (*E. granulosus*). This study investigated the characteristics of *E. granulosus* Pmy (EgPmy) using *in silico* analysis and evaluated its potential as an epitope vaccine. The secondary structure was predicted by SOPMA software and linear B-cell epitopes were screened by the Kolaskar and Tongaonkar's method on IEBD while conformational B-cell epitopes were predicted by the Ellipro. Additionally, the epitopes of cytotoxic T lymphocyte (CTL) were analyzed by the NetCTL-1.2 server. The results showed that α-helices, extended strands, random coils and β-turns accounted for 84.82 %, 6.60 %, 5.56 % and 3.01 % in EgPmy's secondary structure, respectively. A total of 29 linear B-cell epitopes and 6 conformational epitopes were identified together with 25 CTL epitopes. The CTL epitope ^{709}KLEEAEAFA^{717} showed a high potential to elicit CTL response. These results suggested that EgPmy has a strong immunogenicity, which could serve as a reference for the development of EgPmy-based epitope vaccine against CE.

Keywords: *Echinococcus granulosus*; paramyosin; antigen epitope; vaccine; bioinformatics

Introduction

Cystic echinococcosis (CE) is a zoonotic disease caused by infection with the larval stages of *Echinococcosis granulosus* (*E. granulosus*). It has a worldwide distribution including Asia, Africa, Europe and North American (Eckert *et al*., 2000; Nunnari *et al*., 2012). In China, it is mainly endemic in pasture areas such as Tibet, Xinjiang, Gansu, Qinghai, Ningxia and semi-pasture areas. The disease does great harm to human health and the development of the local economy (Yang *et al*., 2015). At present, many actions have been taken to treat this disease, during which surgery in combination with medications remains the first choice. However, it inevitably entails surgical risk and requires considerable labour, material, and financial resources (Nasrieh *et al*., 2003). Moreover, surgery often causes inevitable damage to human body and the secondary infection rate is still high. Therefore, it is still necessary to seek high-efficiency treatment measures to control the disease. In recent years, immunoprophylaxis has become the dominant way to prevent and control echinococcosis and the advances in vaccine development have been documented. A study by Lightowlers *et al.* (1996) revealed that the Eg95 recombinant antigen induced a 95 – 100 % protective immune effect against oncosphere infection in sheep. Siles *et al.* (2003) used the Em14-3-3 recombinant protein to vaccinate mice infected by the eggs of *Echinococcus multilocularis* (*E. multilocularis*), reducing the number of liver lesions in vaccinated animals from 43 to 1 mean lesion per animal.

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Additionally, the Emy162 recombinant antigen showed a 74.3 % protective rate in rats against *E. multilocularis* (Kouguchi *et al*., 2011) and a vaccine against this parasite based on the Em95 protein, with a protective efficiency of 78.5 – 82.9 %, was developed by Katoh *et al* (2008). These results suggested that it is feasible and efficient to prevent echinococcosis by developing molecular vaccines.

Paramyosin (Pmy) is a structural protein in invertebrate muscles (Cohen, 1982), which involves in muscle physiological contraction and immune-regulation (Gobert & McManus, 2005). Interestingly, it has been demonstrated to be a promising vaccine candidate for controlling the infections of both *Schistosoma japonicum* (*S. japonicum*) and *S. mansoni* (Jiz *et al*., 2015). Also, a study by Nanduri *et al.* (1989) revealed that immunizing mice with the *Caenorhabditis elegans* paramyosin protects against *Brugia malayi* challenge with an immune protective effect of up to 60 %. Moreover, Vazquez *et al.* (2001) discovered that the parasite load in mice infected with *Taenia solium* (*T. solium*) larvae is reduced by approximately 52 % by immunizing mice with the total length of *T. solium* recombinant paramyosin. In addition, Ferrer *et al*. (2003) reported that the *T. saginata* paramyosin was highly recognized by antibodies in 100 % of the sera collected from patients with acute cysticercosis. However, little information is known about their protection efficiency as vaccines against CE. The finding attracts us to study the protection role of *E. granulosus* Pmy (EgPmy) against the parasite infection, since that EgPmy shares a high identity to that of *S. mansoni*, and is also expressed in the tegument of the larval cestode (Mühlschlegel *et al*., 1993). Previous studies have suggested that the protein was relevant to immuno-regulatory events at the host-parasite interface and the detection of its IgG4 was found to show a good diagnostic potential of human hydatidosis (Monteiro *et al*., 2010; Moghadam *et al*., 2013). What's more, EgPmy was reported to share some sequence elements and properties with EgA31, the latter protein was demonstrated to induce a high efficient protection against *E. granulosus* infection in dogs (Petavy *et al*., 2008). Overall, these results implied a potential of EgPmy to be an immunogen and vaccine target against CE.

With the advance of bioinformatics technology, the epitope vaccine has become a hot area in vaccine development (Ben & Arnon, 2005; Pfaff *et al*., 1988; Toussaint & Kohlbacher, 2009; Zieglmayer *et al*., 2016; Oyarzún & Kobe, 2016). Compared to traditional vaccines, epitope vaccines have the ability to stimulate an effective specific immune response with a minimal structure, while avoiding undesirable effects (Ben & Arnon, 2005). Considering that EgPmy is a macromolecular protein with 97-kDa (Cohen, 1982), a minimal molecular weight with the main immunogenic epitopes of EgPmy, should induce a more specific antibody response but less side effects. However, it is still unclear whether the EgPmy could be developed to design an epitope vaccine. In this study, we used *in silico* analysis to characterize the secondary structure, the B-cell and CTL epitopes in EgPmy, which would provide the theoretical foundation for developing EgPmy-based epitope vaccine.

Materials and Methods

Protein sequence retrieval

The nucleotide sequence of EgPmy was retrieved from GenBank (GenBank No. Z21787). A total of 863 amino acid residues were predicted in EgPmy protein, and were then used as an template for analyzing its antigenicity, secondary structure, as well as epitopes. The amino acid sequence of EgPmy was shown in Supplementary Fig. 1.

Structural analysis

Various parameters for EgPmy, including the molecular weight, theoretical pI, atomic composition, instability index, aliphatic index and grand average of hydropathicity (GRAVY), were predicted by the ProtParam (http://web.expasy.org/protparam/) (Gasteiger *et al*., 2005). To determine its antigenicity, the parameters of the secondary structure that include α-helices, extended strands, β-turns and random coils, were analyzed by the improved self-optimized prediction method (SOPMA) software (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html) (Geourjon & Deléage, 1995). The protein sequence was input, and the parameters of similarity threshold and window width were set to 8 and 17, respectively, while other parameters were not adjusted.

B-cell epitope prediction

Linear B-cell epitopes of EgPmy were predicted at the Immune Epitope Database (IEDB: http://tools.immuneepitope.org/bcell/) online software (Vita *et al*., 2010) using the method of Kolaskar and Tongaonkar (Kolaskar & Tongaonkar, 1990), which is based on the occurrence of amino acid residues in experimentally determined epitopes. The method was applied to predict antigenic determinants on a large number of proteins with about 75 % accuracy, which is much better than most of the known methods (Kolaskar & Tongaonkar, 1990). Moreover, Emini's method (Emini *et al*., 1985) was used for surface accessibility prediction while Karplus and Schulz's algorithm (Vihinen *et al*., 1994) for flexibility determination. Conformational B-cell epitopes based on the protein antigen's 3D structure were predicted by an online tool Ellipro (http://tools.immuneepitope.org/ellipro/) (Ponomarenko *et al*.,

Table 1. Secondary structural characteristics of EgPmy (Genbank no.Z21787)

Criteria	Assessment
Number of amino acids	863
Molecular weight	98741.4 Da
Theoretical pI	5.21
Formula	$C_{4163}H_{6985}N_{1299}O_{1423}S_{23}$
Instability index	47.70
Aliphatic index	84.99
Grand average of hydropathicity (GRAVY)	-0.897

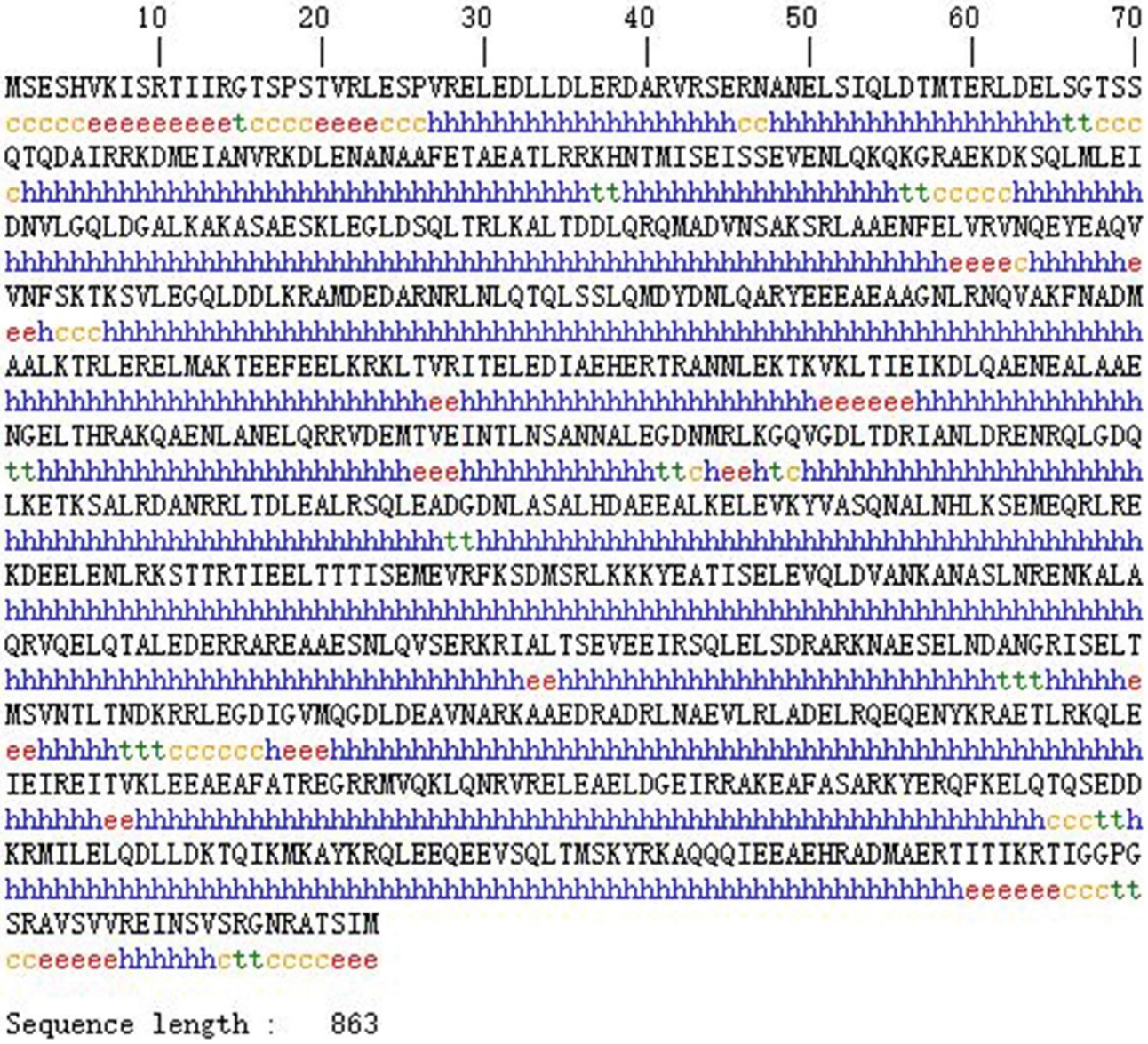

Fig. 1. Secondary structure of EgPmy using SOPMA analysis.

2008). The input file of EgPmy to the online tool was provided in PDB format and the minimum score value was set at 0.7 while the maximum distance was selected as 6 Å.

CTL epitopes prediction

CTL epitope prediction was carried out using the NetCTL.1.2 server (http://www.cbs.dtu.dk/services/NetCTL) (Larsen *et al*., 2007). The method combines the prediction of peptide major histocompatibility complex (MHC) class I binding, proteasomal C terminal cleavage and T cell activating protein (TAP) transport efficiency. MHC class I binding and proteasomal cleavage are performed using artificial neural networks and TAP transport efficiency is predicted using weight matrix (Larsen *et al*., 2007). The input file for EgPmy was provided with the protein sequence and the parameter of MHC class I supertype was selected as A2. The remaining parameters were not altered. Therefore, the IEDB online prediction software (http://tools.immuneepitope.org/Immunogenicity/) (Calis *et al*., 2013) was applied to predict the immunogenicity of the candidate epitopes. This tool uses amino acid properties as well as their position within the peptide to predict the immunogenicity of a peptide-MHC (pMHC) complex (Calis *et al*., 2013).

Results

Structure characteristics

To understand the antigenic features, the secondary structural

characteristics of EgPmy were analyzed firstly. It included total length of 863aa, with a molecular weight of 98741.4 Da, theoretical pI of 5.21, formula of $C_{4163}H_{6985}N_{1299}O_{1423}S_{23}$, 732 α-helices, 57 extended strands, 26 β-turns and 48 random coils (shown in Table 1 and Fig. 1). The grand average of GRAVY was predicted to be negative (-0.897). This showed that the protein has a good hydrophilicity and most of the residues are located on the surface, which are likely to bind residues when interacting with other proteins (Kyte & Doolittle, 1982).

B-cell epitopes identification

The identification of B-cell epitope is one of the key steps in the development of an epitope vaccine. This study predicted the B-cell epitopes of EgPmy using the IEDB software, and EgPmy was found to contain 29 linear B-cell epitopes. Their length ranged from 6 to 20 amino acids with 6 octapeptides and 7 heptapeptides. The epitope length, sequences and locations, were shown in Table 2. The antigenic propensity varied along the sequence length, and the average antigenic propensity value of these epitopes was 0.992 with a minimum of 0.868 and a maximum of 1.159 (Supplementary Fig. 2).

Moreover, surface accessibility and fragment flexibility, the important features of B-cell eptitopes, were also analyzed by IEDB database. According to Emini's method, the maximum surface accessibility value of the predicted epitopes was 5.287 from amino acid position 754 to 759 for EgPmy. The sequence of the hexapeptide was ^{754}RKYERQ^{759}, where 756Y was the surface residue. The minimum surface accessibility value was 0.123 from amino

Table 2. Predicted linear B-cell epitopes of EgPmy using the Kolaskar and Tongaonkar's method on IEDB software.

No.	Start Position	End Position	Peptide	Peptide Length
1	4	11	SHVKISRT	8
2	18	37	PSTVRLESPVRELEDLLDLE	20
3	114	122	EISSEVENL	9
4	135	154	QLMLEIDNVLGQLDGALKAK	20
5	161	177	LEGLDSQLTRLKALTDD	17
6	194	203	AENFELVRVN	10
7	207	214	EAQVVNFS	8
8	216	227	TKSVLEGQLDDL	12
9	241	250	LQTQLSSLQM	10
10	270	276	RNQVAKF	7
11	303	310	RKLTVRIT	8
12	328	340	KTKVKLTIEIKDL	13
13	397	403	KGQVGDL	7
14	437	444	DLEALRSQ	8
15	453	460	ASALHDAE	8
16	463	480	LKELEVKYVASQNALNHL	18
17	532	545	EATISELEVQLDVA	14
18	558	569	ALAQRVQELQTA	12
19	582	587	SNLQVS	6
20	591	608	RIALTSEVEEIRSQLELS	18
21	629	635	LTMSVNT	7
22	672	681	NAEVLRLADE	10
23	705	711	EITVKLE	7
24	725	732	VQKLQNRV	8
25	749	754	AFASAR	6
26	774	784	ILELQDLLDKT	11
27	800	807	EVSQLTMS	8
28	810	816	RKAQQQI	7
29	841	853	SRAVSVVREINSV	13

acid position 645 to 650. The sequence of the hexapeptide was ^{645}GDIGVM^{650}, where 647I was the surface residue (Supplementary Fig. 3). On the other hand, the result of the flexibility prediction showed that the maximum value of the flexibility was 1.142 from amino acid position 67 to 72 for EgPmy. The sequence of the heptapeptide was ^{67}GTSSQTQ^{72}, where 70S was the flexibility residue. The minimum flexibility value was 0.917 from amino acid position 277 to 283 and its sequence of the heptapeptide was ^{277}NADMAAL^{283}, where 280M was the flexibility residue (Supplementary Fig. 4).

In addition, 6 conformational B-cell epitopes were obtained by the ElliPro with a score defined as a Protrusion Index (PI) value over 0.7. The highest probability of a conformational epitope was calculated at 98.7 % (PI score: 0.987). Conformational epitopes' residues, their sequence location, number of residues and scores were given in Table 3.

Table 3. Predicted conformational B-cell epitopes of EgPmy using the Ellipro.

No.	Residues and their Positions	Number of Residues	Score
1	R848, E849, I850, N851, S852, V853, S854, R855, G856, N857, R858, A859, T860, S861, I862, M863	16	0.987
2	M1, S2, E3, S4, H5, V6, K7, I8, S9, R10, T11, I12, I13, R14	14	0.813
3	R22, L23, E24, S25, P26, V27, R28, E29, L30, E31, D32, L33, L34, D35, L36, E37, R38, D39, A40, R41, V42, R43, S44, E45, R46, N47, A48, N49, E50, L51, S52, I53, Q54, L55, D56, T57, M58, T59, E60, R61, L62, D63, E64, L65, S66, G67, T68, S69, S70, T72, Q73, D74, I76, R78, K79, D80, M81, E82, I83, A84, N85, V86, R87, K88, D89, L90, E91, N92, A93, N94, A95, A96, F97, E98, T99, A100, E101, A102, T103, L104, R105, R106, K107, H108, N109, T110, M111, I112, S113, E114, I115, S116, S117, E118, E130	95	0.786
4	V732, R733, E734, L735, E736, A737, E738, L739, D740, G741, E742, I743, R745, A746, K747, E768, D769, D770, K771, R772, M773, I774, L775, E776, L777, Q778, D779, L780, L781, D782, K783, T784, Q785, I786, K787, M788, K789, A790, Y791, K792, R793, Q794, L795, E796, E797, Q798, E799, E800, V801, S802, Q803, L804, T805, M806, S807, K808, Y809, R810, K811, A812, Q813, Q814, Q815, I816, E817, E818, A819, E820, H821, R822, A823, D824, M825, A826, E827, R828, T829, I830, T831, I832, K833, T835, I836, G837, G838, P839, G840, S841, R842, A843, V844, S845, V846, V847	94	0.753
5	G398, Q399, V400, G401, D402, L403, T404, D405, R406, I407, A408, N409, L410, D411, R412, E413, N414, R415, A431, N432, R433, R434, L435, T436, D437, E439, A440, L441, R442, S443, Q444, L445, E446, A447, D448, G449, D450, N451, L452, A453, S454, A455, L456, H457, D458, A459, E460, E461, A462, L463, K464, E465, L466, E467, V468, K469, Y470, V471, A472, S473, Q474, N475, A476	63	0.741
6	S214, K215, T216, K217, S218, V219, L220, E221, G222, Q223, L224, D225, D226, L227, K228, K613, N614, A615, E616, S617, E618, L619, N620, D621, A622, N623, G624, R625, I626, S627, E628, L629, T630, M631, A679, D680, E681, L682, R683, Q684, E685, Q686, E687, N688, Y689, K690	46	0.707

Table 4. Predicted CTL epitopes of EgPmy using NetCTL-1.2 software.

Residue number	Peptide sequence	Predicted MHC binding affinity	Rescale binding affinity	C terminal cleavage affinity	TAP transport efficiency	Prediction score	Identified MHC ligands	Immunogenicity
54	QLDTMTERL	0.5577	0.8314	0.9648	0.8200	1.0171	E	0.02654
57	TMTERLDEL	0.5850	0.8720	0.9557	0.8800	1.0594	E	0.23053
111	MISEISSEV	0.6817	1.0162	0.9733	0.4960	1.1870	E	-0.0602
135	QLMLEIDNV	0.6664	0.9934	0.6069	0.4660	1.1078	E	0.16956
136	LMLEIDNVL	0.6159	0.9181	0.9539	1.0490	1.1137	E	0.26629
163	GLDSQLTRL	0.5966	0.8894	0.9753	0.4520	1.0583	E	-0.21951
191	RLAAENFEL	0.7843	1.1692	0.8693	1.2890	1.3640	E	0.30078
240	NLQTQLSSL	0.4309	0.6424	0.9605	1.0270	0.8378	E	-0.35806
247	SLQMDYDNL	0.4410	0.6574	0.8503	1.0490	0.8374	E	-0.18124
304	KLTVRITEL	0.6162	0.9185	0.9743	1.1770	1.1235	E	0.32108
346	ALAAENGEL	0.4226	0.6299	0.8136	0.9680	0.8003	E	0.23058
371	RVDEMTVEI	0.5403	0.8054	0.8784	0.8730	0.9808	E	0.06683
381	TLNSANNAL	0.4165	0.6209	0.9516	1.0000	0.8137	E	-0.11916
388	ALEGDNMRL	0.3918	0.5840	0.9679	1.1150	0.7849	E	-0.03585
451	NLASALHDA	0.4866	0.7253	0.7043	-0.6010	0.8009	E	-0.08585
455	ALHDAEEAL	0.5672	0.8455	0.9084	1.2020	1.0419	E	0.27253
558	ALAQRVQEL	0.6097	0.9088	0.9757	1.2360	1.1170	E	-0.05386
593	ALTSEVEEI	0.6419	0.9568	0.8837	0.6710	1.1229	E	0.12549
625	RISELTMSV	0.7049	1.0508	0.9595	0.6530	1.2274	E	-0.17207
670	RLNAEVLRL	0.6396	0.9534	0.9521	0.9850	1.1455	E	0.19451
709	KLEEAEAFA	0.5067	0.7553	0.3189	-0.5060	0.7779	E	0.36702
727	KLQNRVREL	0.4619	0.6885	0.9753	1.0000	0.8848	E	0.14733
773	MILELQDLL	0.6008	0.8956	0.7384	1.2800	1.0703	E	-0.01045
793	RQLEEQEEV	0.5005	0.7461	0.4037	0.7530	0.8443	E	0.22861
845	SVVREINSV	0.3876	0.5778	0.9681	0.6310	0.7546	E	0.18614

CTL epitopes identification

CTL epitopes were predicted using NetCTL.1.2 server prediction tool. Protein sequence was predicted based on their MHC binding affinity, proteasomal C-terminal cleavage, and transport affinity. Prediction was performed using MHC supertype A2. Twenty-five peptides with 9-mer sequence, whose prediction scores were over 0.75000, were identified as CTL epitopes (Shown in Table 4). Then, these peptide sequences were submitted for MHC Class I immunogenicity prediction. Most of them showed high score of immunogenicity to activate and elicit CTL's effector functions (Table 4).

Discussion

Pmy has been demonstrated to be a promising vaccine candidate for control of *S. japonicum*, *S. mansoni* (Jiz *et al*., 2015), *Brugia malayi* (Nanduri *et al*., 1989), *T. solium* (Vazquez *et al.*2001) and *T. saginata* (Ferrer *et al*., 2003) infections. These results implied a potential of EgPmy to be an effective vaccine against CE. In order to lay the basis for the development of the EgPmy-based epitope vaccine to control and prevent cystic echinococcosis, the in silico analysis was performed to probe the secondary structures, B-cell and CTL epitopes of the EgPmy protein.

The α-helices and extended strands are very common structures and located inside the secondary structure of proteins, which cannot be changed easily. The α-helices and extended strands accounted for 84.82 % and 6.60 % in EgPmy, indicating a good stability of the protein. On the contrary, the random coils and β-turns often appear on the protein surface and have the potential to form epitopes (Li *et al*., 2013). The proportions of the random coils and β-turns were 5.56 % and 3.01 % in EgPmy, respectively. These regions are likely to form epitopes.

A key process of epitope vaccine preparation is to obtain useful epitopes (Li *et al*., 2013). Epitopes can be divided into B- and T-cell epitopes. B-cell epitope is a binding site of an antibody on an antigen, and accurate epitope identification is essential to developing epitope vaccines (Ren *et al*., 2014). To improve the accuracy of the B-cell epitope prediction, multi-parameter analysis was utilized in this study. The surface accessibility area analysis showed that the residues on the surface of EgPmy, might contact with the solvent molecules. The flexibility parameter prediction reflected the ability of the protein to fold and bend and the antigenic propensity analysis demonstrated the immunogenic regions of the antigen protein (Kolaskar & Tongaonkar, 1990; Emini *et al*., 1985; Vihinen *et al*., 1994). In this study, 29 linear B-cell epitopes were identified for EgPmy with the high antigenic property. Notably, ^{754}RKYERQ^{759} and ^{67}GTSSQTQ^{72} showed a high value of surface accessibility and fragment flexibility, respectively, which might suggested that the residues were the most likely vital epitopes that can be used to develop EgPmy-based epitope vaccine. However, it is worth noting that some epitopes, due to the restriction of complicated 3D structure, are shielded by the outer layer of the protein, which is called conformation epitope. This study also predicted 6 conformational epitopes in EgPmy. Overall, a total of 35 B-cell epitopes were found in EgPmy, which indicated a strong immunogenicity of this protein.

CTLs can destroy infected cells, and their activation takes place on the surface of antigen-presenting MHC molecules (Larsen *et al*., 2007). Hence, reliable prediction of CTL epitopes is a vital step for designing epitope-driven vaccine. In T-cell epitope prediction, the accuracy rate of the MHC-I epitope prediction has been demonstrated to be as high as 90 % (Testa *et al*., 2012). However, it is restricted by human leukocyte antigen (HLA) proteins which is highly polymorphic in diverse ethnic populations (Maenaka & Jones, 1999; Stern & Wiley, 1994). Yan *et al* (2003) found that the MHC-I HLA-A*0201-restricted T-cell epitopes are common in Chinese Han people. Therefore, this study analyzed the HLA-A*0201-restricted CTL epitopes in EgPmy, and 25 high score CTL epitopes were identified, which might be of great use to develop epitope vaccine.

In conclusion, this study analyzed the secondary structure, B-cell and CTL epitopes in EgPmy. Twenty-nine linear B-cell epitopes, 6 conformation epitopes and 25 CTL epitopes were identified as the potential dominant epitopes. Moreover, the CTL epitope ^{709}KLEEAEAFA^{717} showed a high potential to elicit a CTL response. Overall, the results would provide the basis for the preparation of the EgPmy-based epitope vaccine against CE.

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