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Molecular Characterization of a novel gene encoding an 8-kDa subunit of Antigen B from *Echinococcus granulosus* genotypes 1 and 6[§]

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[§] The nucleotide and amino acid sequences of *EgG1AgB8/5* and *EgG6AgB8/5* have been deposited in the Genbank database with Accession Nos. AB260973 and AB260974, respectively.

Abstract

Antigen B in hydatid cyst fluid of *Echinococcus granulosus* is a polymeric lipoprotein of 160 kDa, and is an aggregate of several different but homologous small proteins with approximately 8 kDa which are encoded by a multigene family. Four genes encoding 8-kDa-subunit monomers of the antigen B have been identified from *E. granulosus*. Recently, we have isolated another novel gene from *Echinococcus multilocularis* encoding a fifth 8-kDa-subunit of AgB (named *EmAgB8/5*), predominantly transcribed in the adult worm, but not in vesicles of metacestodes. In this study, we cloned and characterized two *EmAgB8/5* homologue genes from *E. granulosus* genotypes 1 and 6 by PCR, and named as *EgG1AgB8/5* and *EgG6AgB8/5*, respectively. The phylogenetic relationship of these genes with other genes encoding the antigen B 8-kDa-subunit monomers was also discussed.

Keywords: Echinococcus granulosus, genotypes 1 and 6, Antigen B, 8-kDa subunit,

EgG1AgB8/5, EgG6AgB8/5, molecular characterization.

Antigen B (AgB), initially identified in hydatid cyst fluid of *Echinococcus granulosus* causing cystic echinococcosis, is a major excretory-secretory antigenic component of the metacestode stage of the parasite [1], and may play an important role in host-parasite interaction during the echinococcal infections, since it is the major component of hydatid cyst fluid [2]. The *E. granulosus* AgB (EgAgB) is a thermostable polymeric lipoprotein of 160 kDa [1]. On SDS-PAGE, it disassociates to show a characteristic ladder-like pattern, consisting of regularly spaced subunits with apparent molecular sizes of 8, 16, 24 and 32 kDa, and the higher molecular mass subunits are supposed to be derived from the 8-kDa-subunit component [3-5]. Now it is known that the 8-kDa subunit component is encoded by a gene family (6), and at least four members (*EgAgB8/1*, EgAgB8/2, *EgAgB8/3* and *EgAgB8/4*) of this gene family have been identified from *E. granulosus* [7-10] and high degrees of sequence polymorphism of these genes have been demonstrated [10, 11]. The sequence variability of *EgAgB* genes is considered as the *EgAgB* genes belong to a family of contingency genes, which might benefit an immuno-evasion mechanism of parasite in host-parasite interaction [11,12]. Recently, we have isolated five members of this family (*EmAgB8/1*, EmAgB8/2, *EmAgB8/3*, *EmAgB8/4* and *EmAgB8/5*) from *Echinococcus multilocularis* causing alveolar echinococcosis, and demonstrated the developmental regulation of these genes during the *E. multilocularis* life cycle [13]. Four (*EmAgB8/1*, *EmAgB8/2*, *EmAgB8/3* and *EmAgB8/4*) of

them showed a high similarity to each gene isolated from *E. granulosus*, but *EmAgB/5* had less homology to known AgB genes.

RT-PCR analyses revealed that *EmAgB8/5* is transcribed predominantly in adult worms, but not in vesicles of the *E. multilocularis* metacestode [13]. However, the existence of *EmAgB8/5* homologue gene in *E. granulosus*, and its expression patterns in parasite developmental stages has been unclear. In order to better understand the genomic organization of EgAgB genes, herein we isolated two *EmAgB8/5* homologue genes from *E. granulosus* by PCR. PCR was carried out using genomic DNA isolated from a single worm of *E. granulosus* genotype 1 (G1) and 6 (G6), collected from small intestines of two naturally infected dogs in Xinjiang, China [14]. A forward primer (5'-CTGGCTCTCGTAGCCTTCGTG-3') and a reverse primer (5'-GCATAAATGAATCATAATTTCTACTCC -3') derived from nucleotide sequence of EmAgB8/5 (GenBank accession No. **AB202118)** were applied. The reverse primer was designed from protein coding region (underlined) and 3' untranslated region (UTR). Since the 3' UTRs in each known AgB genes were highly conserved between *E. multilocularis* and *E. granulosus*, it has been assumed that the 3' UTR in AgB5 gene is also conserved. PCR was carried out using high fidelity polymerase, PrimeSTAR (TaKaRa, Kyoto, Japan) in a final volume of 25 µl reaction mixture, containing 0.2 µM of each primer, 200 µM of dNTPs and 0.625 units of PrimeSTAR DNA polymerase. Amplification was performed with 35 cycles of 98°C for 10 s, 55°C for 15 s, and 72°C for 20 s, followed by a final extension at 72°C for 2 min. The amplicons were excised from agarose gels using NucleoSpin

ExTract kit (MACHEREY-NAGEL, Düren, Germany) according to the instruction manual, and were cloned into pT7Blue T-Vector (Novagen, Madison, WI, USA) after the addition of adenine to the ends of PCR products. Nucleotide sequencing was carried out using vector derived primer (T7 promoter primer #69348-1) on an ABI PRSIM 377 Sequencer (Applied Biosystems, Foster City, CA., USA) with DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences Piscataway, NJ, USA). Multiple alignments were generated by Clustal W program using BioEdit software [15]. The phylogenic tree was constructed using nucleotide sequences corresponding to mature peptides of genes isolated in this study and other AgB genes obtained from GenBank by Neighbor Joining method, and tested by bootstrap with 1000 replicates using MEGA version 3.1 [16].

Primers specific for the AgB8/5 gene generated DNA fragments of 324 bp from both *E. granulosus* genotypes. Nucleotide sequence comparison revealed that the AgB8/5 gene from *E. granulosus* G1 and G6, named *EgG1AgB8/5* and EgG6AgB8/5, respectively, showed high identities to *EmAgB8/5* gene (Fig. 1A). In addition, genome structures with 2 exons and 1 intron were identical among three genes. Amino acid sequences of the predicted mature forms of EgG1AgB8/5 and EgG6AgB8/5 showed 96.9% homology with that of EmAgB8/5. Two amino acid substitutions at different positions were observed compared with the amino acid sequence of EmAgB8/5; serine at position 8 was substituted to alanine in EgG1AgB8/5 and EgG6AgB8/5, while the alanine at position 34 was substituted to serine in EgG1AgB8/5 and arginine at position 60 was substituted to lysine in EgG6AgB8/5 (Fig. 1B). As expected, a

phylogenic analysis revealed that the EgG1AgB8/5 and EgG6AgB8/5 together with EmAgB8/5 formed a new fifth cluster in comparison with AgB tree published previously [17].

The AgB genes were expressed in a developmentally regulated manner through different developmental stages of *E. multilocularis* [10] as mentioned above. This finding suggests that the differential expression of AgB genes might be relevant to different functions of the native AgB, which may composed of different 8-kDa-subunits of AgB at different developmental stages of the parasite, and may responsible to the different biological functions of this polymeric protein throughout the developmental stages of the parasite. In this study, we concentrated on to investigate the existence of *EmAgB8/5* homologue gene in *E. granulosus*. Further analysis on expression pattern of this gene at different developmental stages of *E. granulosus* should be carried out.

In vitro experiments have demonstrated that the EgAgB has a protease inhibitor-like activity to inhibit recruitment of neutrophils [4], and to exploit activation of helper T cells by eliciting a non-protective Th2 cell response [12, 18]. The AgB may also be involved in parasite detoxifying mechanism [19]. However, the exact biological function of this abundant protein in hydatid cyst fluid has not yet been completely understood. The findings in this study may facilitate our better understanding of protein structure as well as biological function of AgB in the host-parasite interactions during the echinococcal infection.

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Legends to figures:

Fig. 1. Alignments of nucleotide (A) and deduced amino acid sequences (B) of EgG1AgB8/5

and EgG6AgB8/5 with EmAgB8/5. The nucleotide sequences (A) are included partial exon I, intron (indicated by lower case) and partial exon II. Amino acid sequences (B) are corresponding to the mature form of the proteins deduced from exon II. The coding sequences of EmAgB8/5 are highlighted in bold. Primer regions used for cloning are indicated by dash. Multiple alignments were generated by Clustal W program using BioEdit software [12].

Fig. 2. The phylogenic tree inferred from nucleotide sequences corresponding to the mature peptides of EgG1AgB8/5, EgG6AgB8/5 and other AgB gene sequences obtained from GenBank (accession numbers are shown in the brackets). Phylogenic tree was constructed by Neighbor Joining method, and tested by bootstrap with 1000 replicates using MEGA version 3.1 [13].

A

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EmAgB8/5     CTGGCTCTCGTAGCCTTCGTGGCCATCGCTTTGGCgtaaattgcattataacctcccattgtgtgccaat  70
EgG1AgB8/5   ----------------------.........G........g.............................  70
EgG6AgB8/5   ----------------------...G.....G........g.c.................c.........  70

EmAgB8/5     gcacttaaatttctcacttacccttttccagGGAAGACGACATCGATTCGAAATCGAAGAAGGGTGTCA 140
EgG1AgB8/5   a.....................................T...............G.............. 140
EgG6AgB8/5   a.....................................T...............G.............. 140

EmAgB8/5     TGAAAAGTGTAGCCGAATTAAAAGAATTCTTTGCAAGTGATCCAATGGGGCAAAAATTGGCTGCTATTTG 210
EgG1AgB8/5   ............................................................T....... 210
EgG6AgB8/5   ............................................................G........ 210

EmAgB8/5     CAAGGAGCTGAAAGATTTCTTCCTTTTGGCCAGGACAAAAGCTCGCTCGGCTTTGAGAGATTATGTCAAA 280
EgG1AgB8/5   .............................................A........A.............. 280
EgG6AgB8/5   ...........................................................A.....G 280

EmAgB8/5     AGGTTGATGGATGAAGGGGAGTAGAAATTATGATTCATTTATGC 324
EgG1AgB8/5   ................---------------------------- 324
EgG6AgB8/5   .A.C............---------------------------- 324
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B

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EmAgB8/5     EDDIDSKSKKGVMKSVAELKEFFASDPMGQKLAAICKELKDFFLLARTKARSALRDYVKRLMDEGE  66
EgG1AgB8/5   .......A.......................S.............................--  66
EgG6AgB8/5   .......A.....................................................K....--  66
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Fig. 1. Mamuti et al

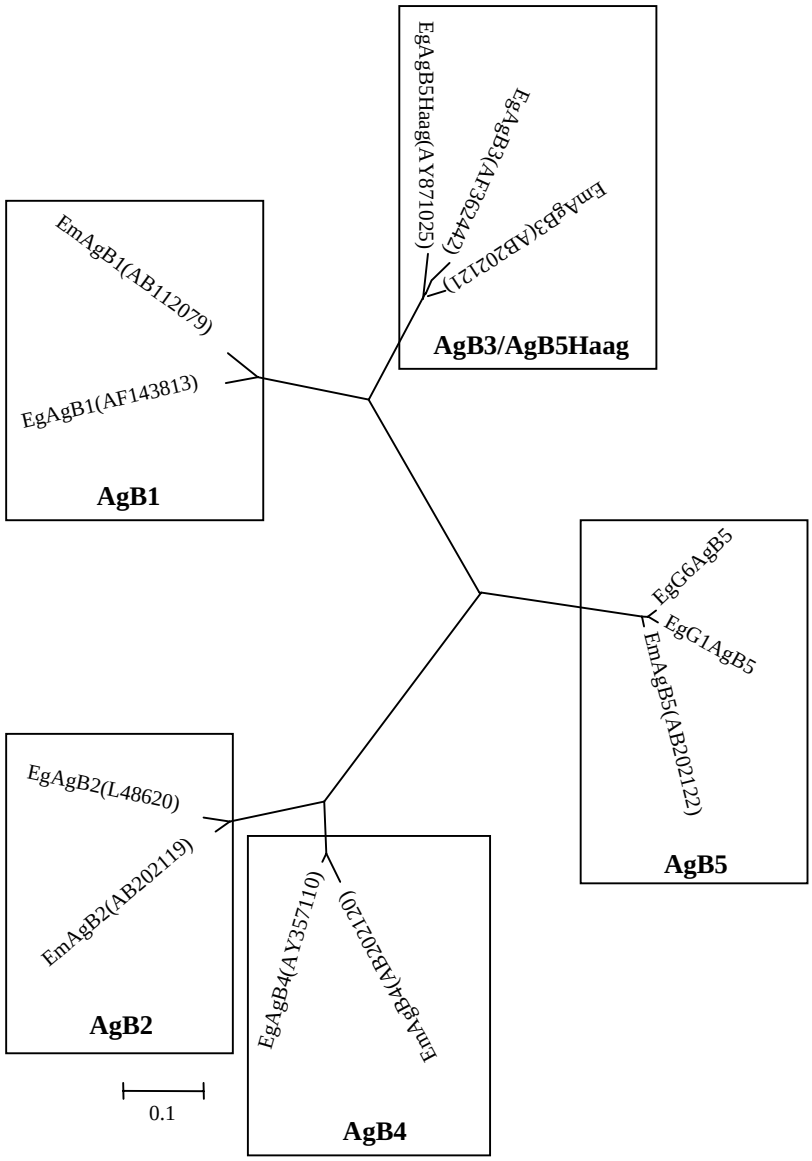

Fig. 2. Mamuti et al