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Identification of host immune regulation candidate genes of *Toxascaris leonina* by expression sequenced tags (ESTs) analysis

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ABSTRACT

Toxascaris leonina adult worms live in the gastrointestinal tract of dog, cat, and fox, releasing eggs which enter the environment by the fecal route. Previously, we reported that *T. leonina* adult worm derived protein was able to inhibit OVA-specific Th2 responses, and in particular, immunization with parasite proteins exerts a more profound protective effect than allergen treatment. In order to gain greater insight into the relevant immune evasion mechanisms as well as basic scientific information, we have generated ESTs of *T. leonina* adult female worm and investigated their functions using euKaryotic Orthologous Groups (KOG) database analysis. From the randomly selected plasmids containing DNA inserts, a total of 487 reads were collected from the *T. leonina* adult worm cDNA library. The annotated ESTs were classified into 25 KOG categories; the most of ESTs (7.90%) were annotated with energy production and conversion, and the second highly annotated category is translation, ribosomal structure and biogenesis related ESTs (7.69%). We also identified many host–parasite immune related genes including C-type lectin, galectin, SXP, and cathepsin L-like cysteine protease coding genes. It is necessary to get more information regarding these genes for understanding about the mechanisms of immune evasion of *Toxascaris*.

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1. Introduction

Toxascaris leonina is a common nematode parasite of dogs, cat, and fox. Adult worms live in the gastrointestinal tract, releasing eggs which enter the environment by the fecal route. The infective stage is the egg containing a

second-stage larva. This stage, under optimal conditions outside the host, is reached in three to six days (Soulsby, 1982). Embryonated eggs are then infective if ingested by a new host, after hatching and second-stage larvae enter the wall of the intestine. In definitive host, larvae may mature to the adult worm in the mucosa of intestine without tracheal migration, which is a very common feature in most roundworm (Soulsby, 1982). *T. leonina* may infect the other non-specific host, including mouse, and human. In these animals, third-stage larvae are distributed in many tissues especially liver, lung, and intercostals muscle (Prokopic and Figallova, 1982a,b; Sprent, 1959). This disease was called as visceral larvae migration which

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was also developed by *Toxocara canis* and *T. cati*. If a human infected with larvae of these nematodes, it can evoke severe inflammation and actual mechanical damage to the organs. Signs of this disease include an enlarged liver, intermittent fever, loss of weight and appetite, and a persistent cough, asthma or pneumonia (Bratt and Tikasingh, 1992).

Recently, we reported that parasite-derived protein is able to inhibit OVA-specific Th2 responses, and in particular, immunization with parasite proteins exerts a more profound protective effect than allergen treatment (Lee et al., 2008). We applied excretory–secretory protein (TI-ES) or total protein (TI-TP) of the adult worm *Toxascaris leonina* to asthma model mouse prior to or simultaneously with OVA challenge. The group subjected to immunization with TI-ES and TI-TP evidenced quite reduced allergic response; a thinning of the bronchial epithelial and muscle layers, inhibited recruitment of inflammatory cells into the airway, and a significant reduction in IL-4 levels as compared to the OVA-induced asthma group. But there is no information about molecules related with host immune regulatory genes. Furthermore, the role of the parasite in regulating their host immune system remains largely unknown.

Also, *T. leonina* has some interesting biological features as compared with *Toxocara*. One is they have no tracheal migration period in their specific final host. Most of nematode belonging to the family of Ascarididae migrates to the lung and trachea for maturing as the adult worm (Soulsby, 1982). The other is that they can infect dogs and cats, beside *T. canis* and *T. cati* can only be their specific final host, respectively dog and cat. But a number of technical obstacles exist which impede progress in many of these issues, including a paucity of information regarding the genes that comprise *T. leonina*.

The characterization of the transcribed genes of a certain organism via expressed sequence tags (ESTs) analysis and the single-pass sequencing of randomly selected cDNA clones has been attempted with a host of organisms, including parasitic nematodes such as *Ancylostoma caninum* (Abubucker et al., 2008; Mitreva et al., 2005), *Brugia malayi* (Blaxter et al., 2002), *Toxocara canis* (Maizels et al., 2000), *Strongyloides ratti* (Thompson et al., 2005), and *Trichinella spiralis* (Park et al., 2008). In addition to genes which are candidates for pathogenic factors, immune evasion, stage-specific or cell cycle regulation has been identified in these parasites via EST analysis, as well as a great many cell signaling-associated genes. Recently, some ESTs were analyzed using a new database, referred to as euKaryotic Orthologous Groups (KOG) (Akao et al., 2007; Schwartz et al., 2007).

In order to gain greater insight into the relevant immune evasion mechanisms as well as basic scientific information, we have, in this study, generated ESTs of *T. leonina* adult female worm and investigated their functions using KOG database analysis. The characterization of the EST data should provide valuable insights into both the immune evasion and the life maintenance of this parasite.

2. Materials and methods

2.1. Isolation of *Toxascaris leonina* female adult worm

T. leonina was isolated from naturally infected dogs. The recovered worms were classified in accordance with the morphological features of the eggs and the adult worms, all of which were maintained in our laboratory (Bellamy and Olexson, 2000).

2.2. Preparation of total RNA

About 1 mg/ml of *T. leonina* adult female worm was homogenized by tissue homogenize. Total RNA was extracted from homogenized tissue solution using the Trizol reagent (Invitrogen Life Technologies, Milan, Italy). After the tissues were lysed with 10 ml of Trizol reagent, and a 1/5 volume of chloroform was added and vigorously mixed, the aqueous layer was collected via 15 min of centrifugation at 12,000 rpm and 4 °C, then precipitated with an equal volume of isopropanol followed by 10 min of incubation at room temperature and an additional 15 min of centrifugation at 12,000 rpm and 4 °C. The precipitated pellet was washed with 75% ethanol and air dried. The pellet was dissolved in DEPC-treated water. The integrity of the RNA was checked by measuring the absorbance of 260 nm and 280 nm with spectrophotometer. For the assessment of quality, 3 µg of total RNA was loaded onto denaturing 1% formaldehyde agarose gels.

2.3. Construction of cDNA library

For the construction of cDNA library, ZAP-cDNA[®] Synthesis Kit was used (Stratagene, San Diego, CA, USA). With 5 µg mRNA purified with mRNA isolation kit (Quiagen, Hilden, Germany) from the 1 mg total RNA, a cDNA library was constructed in ZAP II expression vector according to the manufacturer's instructions (Stratagene, San Diego, CA, USA).

2.4. Sequencing and analysis of ESTs

The chromatogram files obtained from the sequencer were initially submitted to Phred (Ewing and Green, 1998; Ewing et al., 1998) for base calling and quality assignment. The trace files were trimmed with trim-alt 0.05 (Phred score ≥ 20). Each vector sequence was identified and trimmed off using cross-match software (<http://www.phrap.org>). EST sequences shorter than 100 bp were discarded. The EST sequences were assembled and clustered using TGICL (Pertea et al., 2003) package and cap3 software. Finally, the clusters and singletons were analyzed for annotation using a homology search engine, local BLAST (Altschul, 1990; Altschul and Lipman, 1990) against Parablast database (<http://blast.inje.ac.kr/parablast>) (not released) and NCBI nr database, NLM, USA. The significant matches were determined when expectation value was less than 1×10^{-5} with previously report genes. Function of ESTs was predicted through KOG analysis.

Table 1Statistics of ESTs of *Toxascaris leonina* adult female worm.

Category	No. of clones (%)
Total clones sequenced	487
ESTs submitted for BLAST search	481
No. of EST clusters	57
Singletons	248
Annotated ESTs	266 (55.3)
ESTs with homology to Family Ascarididae	38 (7.9)
ESTs with homology to <i>Caenorhabditis elegans</i>	62 (12.9)
ESTs with homology to <i>Homo sapiens</i>	10 (2.1)

3. Results

3.1. Analysis of *T. leonina* adult worm gene expression profile by KOG classification

From the randomly selected plasmids containing DNA inserts, a total of 487 reads were collected from the *T. leonina* adult worm stage cDNA library. The length of ESTs ranged from 81 bp to 882 bp with an average of 690 bp. These ESTs were assembled into 57 clusters and 248 singletons. Of the annotated ESTs, 38 (7.9%) ESTs were homologous with Family Ascarididae gene transcripts and 62 (12.9%) ESTs matched with *C. elegans* genes (Table 1). The KOG system classifies genes on the basis of orthologous relationships between genes (Tatusov et al., 2003). The KOG classification of ESTs from the *Toxascaris* cDNA library utilized in this study is shown in Table 2. The annotated ESTs were classified into 25 KOG categories; the most of ESTs (7.90%) were annotated with energy production and conversion, and the second highly annotated category is translation, ribosomal structure and biogenesis related ESTs (7.69%). This means that many proteins were newly generated at egg production and immune evasion.

3.2. Identification of host immune regulatory genes in *T. leonina*

We also identified many host immune regulatory genes, which included C-type lectin, SXP1, galectin, and cysteine protease in this study (Table 3). Especially, C-type or Ca²⁺-dependent lectins (CTL) was the most frequently identified, which was a family of animal lectins that bind carbohydrates in a Ca²⁺-dependent fashion, ranging from simple monosaccharides to complex glycoconjugates (Weis et al., 1998). Five clones and four clones for SXP1 and RAL-2 antigen, respectively, were identified in *T. leonina* female adult worm cDNA library. Also, a cathepsin L-like cysteine protease enzyme was identified from the EST database.

4. Discussion

In this study, we generated ESTs of adult female *T. leonina* worms and investigated their functions via KOG database analysis. The most of ESTs (7.90%) were annotated with energy production and conversion, and the second highly annotated category is translation, ribosomal structure and biogenesis related ESTs (7.69%).

Table 2KOG classification of the ESTs from cDNA library of *T. leonina* female adult worm.

Categories	Gene distribution rates (%)
Information storage and processing	
Translation, ribosomal structure and biogenesis	7.69
RNA processing and modification	0.83
Transcription	0.00
Replication, recombination and repair	0.42
Chromatin structure and dynamics	0.00
Metabolism	
Energy production and conversion	7.90
Carbohydrate transport and metabolism	4.37
Amino acid transport and metabolism	4.16
Nucleotide transport and metabolism	0.42
Coenzyme transport and metabolism	0.21
Lipid transport and metabolism	0.00
Inorganic ion transport and metabolism	0.42
Secondary metabolites biosynthesis, transport and metabolism	0.62
Cellular processes and signaling	
Cell cycle control, cell division, chromosome partitioning	0.62
Nuclear structure	0.00
Defense mechanisms	0.21
Signal transduction mechanisms	4.78
Cell wall/membrane/envelope biogenesis	0.62
Cell motility	0.21
Cytoskeleton	5.82
Extracellular structures	1.04
Intracellular trafficking, secretion, and vesicular transport	2.08
Posttranslational modification, protein turnover, chaperones	5.20
Poorly characterized	
General function prediction only	2.91
Function unknown	1.21

These tendencies were observed in *T. spiralis* 3rd stage larva gene expression profile (Park et al., 2008). Although it is difficult to directly compare the results of KOG analyses of *T. spiralis* 3rd stage larva ESTs and those of *T. leonina* adult worm ESTs, *T. spiralis* 3rd stage larva expressed more genes for RNA and protein expression (respectively, 16.82% and 14.41%) than did those of *Toxascaris* (respectively, 7.90% and 7.69%) adult worms. Whereas structure and movement related genes (especially actin) were more expressed in *Toxascaris* adult worm (5.82%) than in *Trichinella* larva stage (1.8%) (Park et al., 2008). These results showed that *Trichinella* 3rd stage larva might need many genes for invasion to their host tissue and molting to next stage larva, whereas *Toxascaris* adult worm might need many genes for egg production and movement to escape from host intestine peristalsis. Fewer genes for posttranslational modification, protein turnover, chaperones (5.20%), and especially proteases in this study were identified than was the case in a study using *Trichinella* 3rd stage larva (12.61%). *Toxascaris* adult worm might not need protease genes for tissue invasion which should be necessary to *Trichinella* 3rd stage larva. But about half of ESTs (44.69%) was not classified within 25 KOG categories, because it is very difficult to find information about the gene of *T. leonina*.

Table 3

The ESTs were matched with known host–parasite immune response related genes.

Clone ID	Putative identification	No. of clone	E-value	Organisms
TI 136	C-type LECTin family member	5	3.00E–28	<i>Caenorhabditis elegans</i>
TI 025	SXP1 protein	5	6.00E–21	<i>Loa loa</i>
TI 100	RAL-2 homolog	4	1.00E–04	<i>Acanthocheilonema viteae</i>
TI 171	C-type lectin precursor	3	2.00E–12	<i>Necator americanus</i>
TI 066	Intracellular lectin family member	2	3.00E–45	<i>Caenorhabditis elegans</i>
TI 232	P-glycoprotein A	2	2.00E–25	<i>Caenorhabditis elegans</i>
TI 194	Putative glycine-rich protein	2	8.00E–16	<i>Arabidopsis thaliana</i>
TI 441	C-type lectin-like protein 2	1	8.00E–09	<i>Bungarus fasciatus</i>
TI 148	Galectin	1	1.00E–71	<i>Dirofilaria immitis</i>
TI 358	Trypsin inhibitor-like, cysteine-rich TIL	1	6.00E–12	<i>Strongylocentrotus purpuratus</i>
TI 192	Chymotrypsin/elastase inhibitor-1	1	2.00E–21	<i>Ascaris suum</i>
TI 394	Immunodominant antigen	1	1.00E–05	<i>Onchocerca volvulus</i>
TI 082	Immunosuppressive ovarian message protein	1	9.00E–79	<i>Ascaris suum</i>
TI 225	Ovt1 protein	1	6.00E–104	<i>Onchocerca volvulus</i>
TI 031	Polyprotein allergen/antigen	1	2.00E–59	<i>Ascaris suum</i>
TI 002	Cathepsin L cysteine protease	1	2.00E–78	<i>Haemonchus contortus</i>

Many of the well-known CTL and CTL-like proteins are cell surface receptors with pivotal roles in the activation of the vertebrate immune system (Weis et al., 1998). Additionally, helminths have CTLs like their mammalian hosts, which are comprised of a tandem repeat of the proto-type lectins, but the two repeats have diverged considerably and therefore may have different functions, including cuticle formation, regulation of the immune response to the parasite (Loukas and Maizels, 2000). Also helminth C-type lectins are sharing sequence and structural similarity with mammalian immune cell lectins have recently been identified from nematode parasites, suggesting clear roles for these proteins at the host–parasite interface, notably in immune evasion candidate genes (Loukas and Maizels, 2000; Loukas et al., 1999). The one clone for galectin was identified which have high affinity of IgE and down-regulate IL-5 expression in a number of different cell types, suggesting these proteins involved in allergic inflammation (Cortegano et al., 1998, 2000; del Pozo et al., 2002). Therefore, these lectins were thought as novel anti-inflammatory agents or targets for anti-inflammatory drugs.

SXP1 have already been identified as potential anti-parasite vaccines based on findings that gerbils immunized with recombinant *Brugia malayi* SXP protein showed significantly reduced worm burden following challenge (Wang et al., 1997, 1999). In addition, SXP proteins have also been exploited as antigens for the development of serological diagnostic assays (Chandrashekar et al., 1994; Lalitha et al., 2002; Ramachandran et al., 2004). Recently, SXP1 protein from *Wuchereria bancrofti* (WbSXP-1) as a recombinant protein was evaluated for its immunological characterization and has shown that it preferentially induces IL-10 secretion (Sasisekhar et al., 2005).

One cathepsin L-like cysteine protease enzyme was identified in this study. Cysteine proteases of the papain superfamily are particularly prominent among parasitic helminths and represent the largest class of proteases described so far. Cysteine protease, which is very important for parasite, was able to invade host tissue and to evade immune systems. Of these enzymes, cathepsin B-like proteases have been found in many

species of nematodes (Harrop et al., 1995; Kamata et al., 1995; Pratt et al., 1992). While cathepsin B-like proteases are prominent among the nematodes, genes encoding cathepsin L-like enzymes are less common and have largely been found in protozoa (Leon-Sicaire et al., 2004; Mundodi et al., 2005; Ruszczky et al., 2008; Solano-Gonzalez et al., 2006), trematodes (Cancela et al., 2008; Cornelissen et al., 2001; Kaewpitoon et al., 2008) and cestodes (Kong et al., 2000; Li et al., 2006; Sako et al., 2007), where they are thought to play vital roles in immune evasion and feeding. However, a few gene sequences encoding for cathepsin L-like proteases of the parasitic nematode have been previously reported (Loukas et al., 1998). Once summarized previous reports, cathepsin B-like protein coding genes were more abundant than those of cathepsin L-like protein in nematode parasites. Notwithstanding, we could not find any cathepsin B-like protein coding gene and could find only one clone for cathepsin L-like protease. As mentioned before, the *T. leonina* adult worm should not need protease gene for tissue invasion or molting. Interestingly, some cathepsin L-like proteases of nematode parasite did not match between structure characters and their specific cleaving ability. In *Toxocara*, Tc-cpl-1 was similar to cathepsin L-like protease, but it could cleave as cathepsin B-specific substrate as cathepsin L-specific substrate. In the future, structure and function of the cathepsin L-like protease of *T. leonina* would be studied and compared to those of reported cysteine proteases (Loukas et al., 1998). Also, the cathepsin B-like genes encoding secreted proteases of *Ancylostoma caninum* show potent Z-Phe-Arg-AMC-cleaving ability but very little Z-Arg-Arg-AMC-cleaving ability (Brinkworth et al., 1996).

In this study, we evaluated gene expression profiles using KOG analysis and identified some host parasite immune response related genes of *T. leonina* female adult worm. We are able to reveal host–parasite interaction mechanism easily by the study of several immune related genes including identified genes in this study. In addition, 45% of ESTs were located in the category of unknown proteins while 55% of the *T. leonina* EST clusters evidenced homology with proteins from other species. Although we

could not determine their correct function, these genes might be centrally involved in immune evasion or worm life maintenance. We should get more information regarding these genes for understanding about the mechanisms of immune evasion of *Toxascaris*.

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