Up-Regulated Galectin-1 in *Angiostrongylus Cantonensis* L5 Reduces Body Fat and Increases Oxidative Stress Tolerance

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Research

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Abstract

Background: *Angiostrongylus cantonensis* L5, parasitizing in human cerebrospinal fluid, leads to eosinophilic meningitis, which is attributed to tissue inflammatory responses caused primarily by high percentage of eosinophils. Eosinophils are also involved in helminthic killing, using the peroxidative oxidation and hydrogen peroxide (H₂O₂) generated by dismutation of superoxide produced during respiratory burst. In contrast, helminthic worms have evolved to attenuate eosinophil-mediated tissue inflammatory responses for their survival. In previous study, we have demonstrated the extracellular function of *Acan*-Gal-1 in inducing the apoptosis of macrophages. And here, the intracellular functions of *Acan*-Gal-1 were investigated with the aim to further reveal the mechanism of *A. cantonensis* L5 worms surviving in the central nervous system of human from inflammatory responses.

Methods: Bioinformatics were used to analyse the structural characterisation of *Acan*-Gal-1; qRT-PCR and microinjection were performed to detect the expression patterns of *Acan-gal-1*; microinjection was performed to construct transgenic worms; oxidative stress assay and Oil Red O fat staining were used to determine the functions of *Acan*-Gal-1.

Results: The results showed that *Acan*-Gal-1 was expressed ubiquitously and mainly localized in cuticle, and it was up-regulated in both L5 and adult worm. N2 worms expressing *pCe-Acan-gal-1::Acan-gal-1::rfp*, with lipid deposition reduced, were significantly resistant to oxidative stress. *lec-1* mutant worms, with lipid deposition increased, showed susceptible to oxidative stress, and this phenotype could be rescued by expressing *pCe-Acan-gal-1::Acan-gal-1::rfp*. And *fat-6;fat-7* double-mutant worms expressing *pCe-Acan-gal-1::Acan-gal-1::rfp* showed no significant changes in oxidative stress tolerance.

Conclusion: In *C. elegans* worms, up-regulated *Acan*-Gal-1 plays a defensive role against damage due to oxidative stress for worm survival through reducing fat deposition. And this might indicate the mechanism of *A. cantonensis* L5 worms, with *Acan*-Gal-1 up-regulated, surviving in the central nervous system of human from immune attack of Eosinophil.

Background

Angiostrongylus cantonensis is considered as the primary causative pathogen of human eosinophilic meningitis or meningoencephalitis in China, Japan, Southeast Asia and the Pacific Islands [1–3]. The final host of this parasite is rat, and it lives in the rat pulmonary artery where it develops to sexual maturity [4–5]. Human, as an atypical host, mainly acquire this parasite by consuming raw terrestrial freshwater snails, such as the golden apple/channeled apple snail *P. canaliculata* in which the infective third-stage larvae (iL3) resides. After passage to the human small intestine, iL3 will infect the central nervous system through blood stream, and it will develop into the fifth-stage larvae (L5), causing eosinophilic meningitis [6–10].

High percentage of eosinophils, recruited from the circulation into the central nervous system [7], as the primary pathological change in eosinophilic meningitis, is thought to contribute to tissue inflammatory

responses and host defense in helminthic infections [11]. High level of the enzyme complex is expressed and released in the granule matrix of eosinophils, such as eosinophil peroxidase (EPO) [12, 13] which is associated with helminthic killing, using the peroxidative oxidation and hydrogen peroxide (H_2O_2) generated by dismutation of superoxide produced during respiratory burst [14–16]. In contrast, helminthic worms, parasitizing with high level of eosinophils, have evolved to attenuate eosinophil-mediated tissue inflammatory responses for their survival [11]. Therefore, *A. cantonensis* L5, residing in human cerebrospinal fluid (CSF) with high percentage of eosinophils, may take some measures to resist the oxidative stress damage from eosinophils.

In our previous study, proteomic analysis of *A. cantonensis* L5 and *A. cantonensis* iL3, using two-dimensional difference gel electrophoresis (2D-DIGE), showed that the expression level of *A. cantonensis* RPS-30 (*Acan*-RPS-30) was lower in L5 than that in iL3, and that of *A. cantonensis* Galectin-1 (*Acan*-Gal-1) was higher [8]. Recently, we have demonstrated that down-regulated *Acan*-RPS-30 in *A. cantonensis* L5 played a defensive role against damage due to oxidative stress for worm survival through inhibiting apoptosis by regulating *ced-3* down-regulated [17].

Acan-Gal-1, as the homologous protein of human Galectin-9 and *Caenorhabditis elegans* LEC-1, belongs to the family of Galectins that are glycan-binding proteins distributed among animals and fungi and are defined by their conserved carbohydrate recognition domains (CRDs) and affinity for β -galactoside structures [18–20]. Galectins are secreted by cells via an unconventional mechanism [21, 22], and function in various biological phenomena, such as development, immunity and tumourigenesis via recognition of cell surface or extracellular glycoconjugates [22]. Parasite Galectins have a sequence and structure similar to those of mammalian homologs and are presumed to participate in host-parasite interactions [23, 24]. Our previous studies showed that up-regulated *Acan*-Gal-1 in *A. cantonensis* L5 induced the apoptosis of macrophages by binding to Annexin A2 and activating the JNK apoptotic signaling pathway [24]. However, galectins are also found intracellularly, and are involved in RNA splicing, cell growth, apoptosis and other functions [25, 26]. In the nematode *C. elegans*, 11 galectins have been determined, and the endogenous ligands and some of the functions of LEC-6, LEC-8 and LEC-10 have been identified. LEC-6 has a role in growth regulation by affecting the localization and function of its ligands such as F57F4.4 [27, 28]; LEC-8 acts against bacterial infection by binding to glycolipids [29] and LEC-10 functions against oxidative stress by binding to glycoproteins [27, 30]. LEC-1, the homologs of *Acan*-Gal-1, is a tandem repeat-type galectin and its two CRDs have different sugar-binding properties [31]. As LEC-1 is mainly localized in the cuticle and pharynx of *C. elegans* [32], it is thought to have some functions as a component of the durable outer barrier via recognizing and cross-linking glycoconjugates by its two CRDs with different sugar-binding properties, and play a defensive role against damage due to oxidative stress in *C. elegans* [33]. Therefore, *Acan*-Gal-1, up-regulated in *A. cantonensis* L5, may have function of resisting immune attacks in human by not only inducing the apoptosis of its host's macrophages extracellularly [24], but also increasing oxidative stress tolerance of the worm itself intracellularly.

In this study, we further explored the intracellular functions of *Acan*-Gal-1 up-regulated in *A. cantonensis* L5, using the model organism *C. elegans* as a surrogate, for the lack of effective genetic manipulation in

parasitic nematode, with the aim of investigating its role in protecting the worm from its host's immune attacks.

Methods

Propagation of *A. cantonensis* and *C. elegans*

A. cantonensis ZJ strain was maintained and propagated in Wenzhou Medical University, China by cycling through the *Pomacea canaliculata* and Sprague-Dawley (SD) rats as described previously [8]. Intermediate hosts *P. canaliculata* were infected with *A. cantonensis* L1 through feeding on rat feces. L3 were collected at 20 days p.i. Infected snails were shelled and crushed. The intestines and other organs were removed and the remaining tissue was homogenized. The homogenates were filtered through a 40-mesh sieve, deposited for 5 min at 4°C, and precipitated 2–3 times at room temperature. The sediments were removed and L3 number and viability were determined by direct observation under a light microscope. Three-week-old Sprague-Dawley (SD) rats (weight 100–120 g, grade clean, Certificate SYXK (zhe2015-0009)), supplied by the Laboratory Animal Center of Wenzhou Medical University were orally infected with 50 L3/rat. The rats were housed in polypropylene cages with free access to food and water, and then sacrificed by anesthesia at 25 days and 45 days p.i., respectively. The L3 worms were collected from the intermediate hosts *P. canaliculata*; the L5 harvested from the brains of mice (C57BL/6J (B6), Certificate SYXK (zhe2015-0009)) (non-permissive host same as humans), which were orally infected with 30 L3/mouse; the adult worms were collected from the blood vessels of the hearts and lungs. Individuals of different sexes were separated using morphological criteria: Females are usually longer and thinner than males, and males exhibit typical copulatory bursa. L3, L5, and adults were washed three times with 0.01 mol/L PBS buffer, and stored at – 80°C. These rats were not used for any other part of the study.

C. elegans strains N2, *lec-1* (*tm1345*), *ced-3* (*ok2734*) and *fat-6;fat-7* (BX156) were maintained on Nematode Growth Media (NGM) agar plates at 20°C as described previously [34]. Worms were fed with *Escherichia coli* strain OP50 unless otherwise stated. The mutant strains *lec-1* (*tm1345*), *ced-3* (*ok2734*) and *fat-6;fat-7* (BX156) were obtained from the Caenorhabditis Genetic Center (CGC) (University of Minnesota, USA).

Isolation, purification, treatment and storage of nucleic acids

Total RNA was extracted from worms at different developmental stages employing Trizol reagent (Invitrogen, USA), followed by treatment with 2 U of DNase I (Takara Biotechnology Co., Ltd, Japan). First strand cDNA was obtained using the M-MLV RTase cDNA Synthesis Kit (Takara Biotechnology Co., Ltd, Japan). RNA samples were stored at – 80°C.

Bioinformatics analysis

A sequence alignment between *Acan-Gal-1*, *Toxascaris leonine* *TI-Gal-9* (ARM19984.1) and *C. elegans* *LEC-1* (NP_001370038.1) was generated using Clustal Omega. Homology models were built by SWISS-MODEL using *T. leonine* *TI-Gal-9* (PDB code 5glv.1) [35] as templates. Three-dimensional structural analysis was performed using the PyMOL program. All calculations were carried out under default conditions.

The amino acid sequence inferred for *Acan-Gal-1* and 11 selected other homologues sequences were subjected to phylogenetic analyses. The phylogenetic analysis was conducted using the neighbour-joining (NJ) and maximum parsimony (MP) methods, respectively, based on the Jones-Taylor-Thornton (JTT) model [36]. Confidence limits were assessed using a bootstrap procedure with 1000 pseudo-replicates for NJ and MP trees, and other settings were obtained using the default values in MEGA v.5.0. A 50% cut-off value was implemented for the consensus tree.

Quantitative real-time PCR (qRT-PCR) analysis

qRT-PCR was performed to determine the abundance of *Acan-gal-1* transcripts in different developmental stages (L3, L5 female, L5 male, Adult female, Adult male) of *A. cantonensis*.

Gene expression levels were determined by qRT-PCR using SYBR® Green PCR Master Mix and a 7500 Real-Time PCR System (Applied Biosystems, USA). Relative gene expression was compared with 18S ribosomal RNA gene (GenBank: AY295804) as an internal loading control. The target genes and the primers used are listed in Additional file 1: Table S1. Statistical analysis was conducted using a one-way ANOVA; $P < 0.05$ was set as the criterion for significance.

Transgenic worms

About 2000 bp sequence upstream of *Acan-gal-1* 5'-UTRs was used as putative promoter. To analyze promoter activity of *Acan-gal-1*, the promoter regions of *Acan-gal-1* and *Ce-lec-1* were amplified and cloned into plasmid pPD95.77 to construct *pAcan-gal-1::gfp* and *pCe-lec-1::rfp*, respectively (Fig. 1a).

To perform cross-species expression of *Acan-Gal-1* in *C. elegans* strains, cDNA sequence was amplified and cloned into pPD95.77 using the promoter of *Ce-lec-1* to construct plasmid *pCe-lec-1::Acan-gal-1::rfp* (Fig. 1b). All primers used are listed in Additional file 1: Table S1.

Recombinant plasmids were each microinjected into the gonad of young, adult *C. elegans* hermaphrodites as described [6], together with plasmid pRF4 containing a dominant mutant allele of *rol-6* gene, each at a final concentration of 50 µg/mL in the same mixture, using pPD95.77 (*pCe-lec-1::rfp*) and pRF4 plasmid mixture as a control. Plasmid pRF4 was included in all transformations as a behavioural marker, and transgenic worms showing the roller phenotype were selected. The F2 and subsequent generations were analyzed and selected to examine the expression patterns of GFP or RFP, using a fluorescent microscope (Olympus IX71). A minimum of three independent lines expressing each transgene were evaluated.

Oxidative stress assay

The oxidative stress assay was performed as described previously [30]. Briefly, adult hermaphrodites (30 worms/group) were transferred to a 96-well plate containing M9 buffer with 3mM H₂O₂. After incubation at 20°C for the specified durations, the number of dead worms was determined. Worms were scored as dead when they no longer responded with movement to light prodding of the head. Three (H₂O₂) independent experiments were performed. Statistical analysis was performed with Microsoft Excel 2010 software using an unpaired two-tailed *t*-test.

RNAi feeding experiments

To generate *lec-1* specific RNAi vectors, *lec-1* partial cDNA was cloned into the L4440 vector. Plasmids were transformed into *E. coli* strain HT115. Primers used for PCR analysis are listed in Additional file 1: Table S1. RNAi plates and media were prepared according to [17]. Gravid adult *ced-3* mutant worms and *fat-6;fat-7* double-mutant worms were allowed to lay eggs overnight on the RNAi plates and adult worms were picked off. Empty vector-containing *E. coli* were used on separate plates as negative controls.

Oil Red O staining and quantitation

Oil Red O staining was performed as described previously [37]. Worms were washed off the NGM or RNAi plates and incubated in PBS buffer for 30 min on a shaker at room temperature. The worms were then fixed in Modified Ruvkun's witches brew (MRWB) buffer containing 1% paraformaldehyde. After three rounds of freezing/thawing, the worms were dehydrated in 60% isopropanol followed by addition of saturated Oil-Red-O (Sigma, USA) solution. Fixed worms were incubated overnight on a shaker at room temperature, mounted on slides and viewed using a microscope with differential interference contrast optics (Nikon, Japan).

For quantification of Oil Red O staining, using ImageJ, we separated out each color image into its RGB channel components. As it has been reported that Oil Red O absorbs light at 510 nm, we used the green channel for further analysis [38]. We measured the average pixel intensity for a 40 pixel radius immediately behind the pharynx of each animal. In addition, we measured a 40 pixel radius of the background, which was later subtracted from the values obtained from the staining. A minimum of 9 animals was measured for each strain and we repeated the experiments an additional 2 times. Significance was determined by Student's *t*-test.

Results

Structural characterisation of Acan-Gal-1

To characterize the structure of *Acan-Gal-1*, amino acids sequence alignment and structural analysis were performed. Full-length *Acan-Gal-1* was composed of 285 amino acids, containing N-terminal CRD (residues 1-150) and C-terminal CRD (residues 158–285) in the manner of tandem repeat, and a short linker (residues 151–157) held them together (Fig. 2a). The amino acids sequence was aligned with *Ce-Lec-1* and *Tl-Gal-9* (Fig. 2b). The results showed that *Acan-Gal-1* had a similarity of 84.4% and 71.6% to *Ce-Lec-1* and *Tl-Gal-9*, respectively. And the conserved motifs HXXXR and WGXEER, involved with

carbohydrate binding sites [35], were located in both NCRD and CCRD. The charged Arg⁶⁹/Arg²⁰³ and Glu⁸⁸/Glu²²² were conserved in the motifs, that are critical amino acids for recognizing carbohydrate binding, and affect protein folding and structure [35]. The shorter linker “GKYYPVP” than other tandem repeat-type galectins, such as galectin-9, that are flexible and susceptible to proteolysis [39–41], may indicate the structural stability of *Acan*-Gal-1. Structural analysis from homology models revealed that the NCRD of *Acan*-Gal-1 possessed 11 β -sheets and a small α -helix located between β 9 and β 10, whereas the CCRD contained 10 β -sheets and no α -helix (Fig. 2b, c). The conserved motifs HXXXR and WGXEER, the carbohydrate binding sites, were located in concave surface surrounded by β 4 and β 6 of NCRD, and those were also located in the surface formed by β 4' and the loop between β 5' and β 6' of CCRD (Fig. 2c).

Evolutionary relationship of *Acan*-Gal-1 with its orthologues from other nematode species

To determine the evolutionary relationship between *A. cantonensis* and other nematodes, the amino acid sequence of *Acan*-Gal-1 was aligned with its 11 orthologs selected from other 11 nematodes, and subjected to phylogenetic analyses (Fig. 3). *Acan*-Gal-1 clustered closely with *Ce*-Lec-1 (from *C. elegans*) and *Hc*-Galectin (from *Haemonchus contortus*) with similarity of 83% and 93%, respectively. Cladistic analysis showed that the *Acan*-Gal-1 and its homologues were mainly grouped into three clades. *C. elegans*, *H. contortus*, *Ancylostoma ceylanicum*, *T. leonine* and *A. cantonensis* were in Clade V; *Brugia malayi*, *Brugia pahangi*, *Wuchereria bancrofti*, *Loa loa*, *Enterobius vermicularis* and *Toxocara canis* were in Clade III; *Trichinella spiralis* was in Clade I. This result was in agreement with the modern phylogenetic analysis of nematodes [42].

The expression patterns of *Acan*-Gal-1

To determine the relative abundance of *Acan-gal-1* transcript in different developmental stages (L3, L5 and adult) and genders [females (F) and males (M)] of the life cycle of *A. cantonensis*, qRT-PCR was performed with the 18S ribosomal RNA gene as an internal loading control. The results showed that *Acan-gal-1* was transcribed in larval and adult developmental stages examined in different levels (Fig. 4; Additional file 2: Table S2). The expressions of *Acan-gal-1* were greatly up-regulated in both L5 and adult, compared with that in L3; whereas the expression levels were not significantly changed among L5, adult and different genders. This might indicate the important roles of *Acan*-Gal-1 in L5 and adult, which reside in mammals, such as humans and rats, where a full immune system exist.

For the lack of functional genetic and in vitro culture methods, it is unable to detect the functions of *Acan*-Gal-1 directly in *A. cantonensis*. Here, *C. elegans*, proposed by numerous authors as a general model for many aspects of basic molecular, cellular and developmental biology in the less tractable parasitic nematodes [42–44], was used to investigate the anatomical expression patterns of *Acan*-Gal-1 for the closely evolutionary relationship between *A. cantonensis* and *C. elegans*, both belonging to Clade V according to Cladistic analysis [42]. Wild type *C. elegans* (N2 strain) were transformed with the construct *pAcan-gal-1::gfp* and *pCe-lec-1::rfp*, respectively (Fig. 1a). The results showed that GFP under the promoter *pAcan-gal-1* was only expressed in pharyngeal neurons of *C. elegans* (Fig. 5a–c), in contrast to

the situation in worms expressing *pCe-lec-1::rfp*, where RFP was mainly localized in cuticle, and less in intestine, nervous system and pharynx (Fig. 5d–f). This result was in agreement with the previous report [32].

Cross-species expressions of Acan-Gal-1 in *C. elegans* worms

The different activity of *pAcan-gal-1* and *pCe-lec-1* might be due to the heterologous expression, with the low promoter sequences similarity (data not shown). Therefore, *pCe-lec-1* was used as the promoter in this research on the functions of *Acan-Gal-1* in *C. elegans*.

To clarify the role of *Acan-Gal-1*, cross-species expression in *C. elegans* was performed. The expressing constructs containing *Acan-gal-1::rfp* coding sequences driven by *Ce-lec-1* promoter (Fig. 1b), were used to transform *C. elegans* strains N2, *lec-1* (*tm1345*), *ced-3* (*ok2734*) and *fat-6;fat-7* (BX156) respectively, and *pCe-lec-1::rfp* transforming was used as control. In worms, transformed with *pCe-lec-1::Acan-gal-1::rfp*, RFP was expressed widely, and mainly in cuticle (Fig. 6), consistent with the *pCe-lec-1::rfp* expression pattern (Fig. 5e, f). The level of lipid storage in *lec-1* mutant worms expressing *pCe-lec-1::Acan-gal-1::rfp* was lower (Fig. 6d) than that in *lec-1* mutant worms expressing *pCe-lec-1::rfp* (Fig. 6g). This might suggest the function of *Acan-Gal-1* in reducing lipid deposition in *C. elegans*, and this morphological change would be investigated further in the following research.

Functional role of Acan-Gal-1 in oxidative stress

To investigate the role of *Acan-Gal-1* in regulating oxidative stress resistance, we performed oxidative stress assays using H₂O₂. We found that the incidence of rapid death among *lec-1* deletion mutants was significantly higher than that among the N2 worms; and this oxidative stress susceptibility phenotype could be rescued by expressing *pCe-lec-1::Acan-gal-1::rfp* in *lec-1* mutant worms. The N2 worms expressing *pCe-lec-1::Acan-gal-1::rfp* were significantly more resistant to H₂O₂ than the N2 worms expressing *pCe-lec-1::rfp* (Fig. 7a; Additional file 2: Table S2). This might suggest the regulating role of *Acan-Gal-1* in increasing oxidative stress tolerance.

As we have demonstrated that down-regulated *Acan-RPS-30* in *A. cantonensis* L5 could resist oxidative stress damage through inhibiting worm apoptosis [17], and oxidative stress is thought to be one of the major factors that promote apoptosis [45], we next determined whether *Acan-Gal-1* increased oxidative stress tolerance via inhibiting worm apoptosis. Then, the expression levels of apoptosis genes were detected in *C. elegans*. The results showed that all the apoptosis genes were not significantly changed in *lec-1* mutant worms expressing *pCe-lec-1::Acan-gal-1::rfp*, compared with those in *lec-1* mutant worms expressing *pCe-lec-1::rfp* (Fig. 7b). Furthermore, oxidative stress assays were performed to detect the effects of expressing *Acan-Gal-1* on the oxidative stress damage in *ced-3* mutant worms (*ced-3* is the core apoptosis executive genes [46]). The results showed that the incidence of rapid death among *ced-3* mutant worms expressing *pCe-lec-1::Acan-gal-1::rfp* was significantly lower than that among that among the *ced-3* mutant worms (Fig. 7c; Additional file 2: Table S2). And *ced-3* mutant worms, with *lec-1* RNAi, exhibited greatly more susceptibility to oxidative stress than *ced-3* mutant worms (Fig. 7d; Additional file

2: Table S2). This might indicate that *Acan*-Gal-1 could not regulate worm cell apoptosis intracellularly, and the oxidative stress resistance function of *Acan*-Gal-1 was not via regulating apoptosis in *C. elegans*.

Functional role of *Acan*-Gal-1 in lipid storage

Morphological changes were further detected under a light microscope, and we found the level of lipid storage in *lec-1* mutant worms expressing *pCe-lec-1::Acan-gal-1::rfp* was lower than that in *lec-1* mutant worms expressing *pCe-lec-1::rfp* (Fig. 6d, g). Then, Oil Red O fat staining was performed to detect the lipid storage in worms. The results showed that *lec-1* mutant worms stored significantly more lipid than N2 worms, and this lipid accumulation phenotype could be rescued by expressing *pCe-lec-1::Acan-gal-1::rfp* in *lec-1* mutant worms. The N2 worms, expressing *pCe-lec-1::Acan-gal-1::rfp* exhibited greatly less lipid storage than the N2 worms expressing *pCe-lec-1::rfp* (Fig. 8a, b; Additional file 2: Table S2). This might suggest the function of *Acan*-Gal-1 in reducing lipid deposition in *C. elegans*.

Fatty-acid metabolism is involved in the oxidative stress resistance in *C. elegans* [47, 48]. Therefore, we next investigated whether *Acan*-Gal-1 increased oxidative stress tolerance via reducing lipid deposition in *C. elegans*. *fat-6;fat-7* double-mutant worm was selected. Because *fat-6* and *fat-7* genes encode stearoyl-CoA desaturases (SCDs), that are key lipogenic enzymes, and the *fat-6;fat-7* double-mutant worms have decreased fat stores [49]. Then, oxidative stress assays were performed to determine the effects of expressing *Acan*-Gal-1 on the oxidative stress damage in *fat-6; fat-7* double-mutant worms. The results showed that the incidence of rapid death was not significantly changed among *fat-6;fat-7* double-mutant worms expressing *pCe-lec-1::Acan-gal-1::rfp*, compared with that among *fat-6;fat-7* double-mutant worms expressing *pCe-lec-1::rfp* (Fig. 8c). And the susceptibility to oxidative stress was not influenced by *lec-1*RNAi in *fat-6;fat-7* double-mutant worms (Fig. 8c). This might suggest the regulating role of *Acan*-Gal-1 in increasing oxidative stress tolerance was played via reducing lipid deposition in *C. elegans*.

Discussion

The parasitic nematode *A. cantonensis* L5, residing in human cerebrospinal fluid, can cause eosinophilic meningitis, that is primarily due to Eosinophils-inducing tissue inflammatory responses. Eosinophils, as a well-equipped immune cell, can be recruited from the circulation into inflammatory site in response to helminthic infections to play a role in protecting host against the infection, and make some tissue damages at the same time, when inflammatory response is serious. Eosinophils mainly contain four granules: crystalloid granules, primary granules, small granules, and secretory vesicles [50], and there are four kinds of cytotoxic granular proteins in crystalloid granules, including major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN) [12, 13]. Among them, EPO plays the crucial role in killing helminth [51]. It can catalyze the peroxidative oxidation of halides and thiocyanate present in the plasma together with hydrogen peroxide (H₂O₂) generated by dismutation of superoxide produced during respiratory burst [14–16]. High level of the enzyme complex that generates superoxide is expressed in Eosinophils, and superoxide anions are produced in response to helminth-derived immunomodulating agents. Therefore, Eosinophils are the

robust producers of extracellular superoxide. In contrast, helminthic worms have evolved to attenuate the oxidative stress damage from eosinophils for their survival in hosts, such as inducing apoptosis of eosinophils [52, 53] and increasing oxidative stress resistance of worm itself [17]. We have demonstrated that *Acan-Gal-1*, up-regulated in *A. cantonensis* L5, that resides in human with the senior immune system, could induced the apoptosis of macrophages extracellular, when it was secreted by cells via an unconventional mechanism [21, 22]. And *Acan-Gal-1* could also function intracellularly. In this study, we investigated the intracellular functions of *Acan-Gal-1*. Our results showed that *Acan-Gal-1* could increase the oxidative stress tolerance in *C. elegans*. This might indicate the regulating function of *Acan-Gal-1* in attenuating eosinophil-mediated immune attack upon *A. cantonensis* L5 worms in the central nervous system of human by its up-regulated expression.

Whereas, L3 worms, with low level of *Acan-gal-1*, reside in intermediate hosts (e.g., *P. canaliculata*), in which the immune system are lower than that in mammals, and the immune attack may be weaker, or even there may be no eosinophil-mediated superoxide attack in snails. Furthermore, the expression levels were significantly up-regulated in both L5 and adult, both of which reside in mammalian, such as humans and rats respectively, where there are the senior immune systems. Up-regulated *Acan-Gal-1* might protect L5 and adult from attacks of inflammatory response by both promoting immune cells apoptosis and increasing oxidative stress tolerance of worm itself.

The evolutionary relationship between *A. cantonensis* and other nematode showed that *A. cantonensis* was relatively closely related to the model organism *C. elegans*, both belonging to clade V [42], and the homologous gene of *Acan-gal-1* is *lec-1*. Therefore, here, we used *C. elegans* as a surrogate to explore the in vivo functions of the homologous gene, *Acan-gal-1*, for the lack of effective genetic manipulation in parasitic nematode.

In *C. elegans*, fatty acid metabolism is involved in stress-resistance mechanisms [47, 48]. Unsaturated fatty acids are readily oxidized by intercellular reactive oxygen species [54] and it can act as intracellular scavengers [47]. *fat-6* and *fat-7* genes regulate the first step of the fatty acid desaturation pathway, encoding stearoyl-CoA desaturases that are key lipogenic enzymes that catalyze the biosynthesis of monounsaturated fatty acids from saturated fatty acids. The *fat-6;fat-7* double mutants have reduced fat stores and increased expression of genes involved in fatty acid oxidation, and this worm exhibits strongly sensitivity to oxidative stress, because of the reduction in the proportion of unsaturated fatty acids [47]. In this study, *fat-6;fat-7* double mutants were used to explore the possible mechanism of *Acan-Gal-1* increasing oxidative stress tolerance in *C. elegans*. And the results showed that the mutants, expressing *pCe-lec-1::Acan-gal-1::rfp* exhibited no significantly changes in oxidative stress tolerance. This might suggest *Acan-Gal-1* functioned in increasing oxidative stress tolerance via reducing lipid deposition in *C. elegans*, though we could not, here, determine whether *Acan-Gal-1* functioned in the process of inhibiting fat synthesis or promoting fat decomposition.

Conclusions

In this study, we determined the structural characterisation and functions of *Acan*-Gal-1 from *A. cantonensis*. *Acan*-Gal-1 could promote worms to be resistant to oxidative stress by reducing fat deposition. Our findings may further reveal the mechanism of *A. cantonensis* L5 worms surviving in the central nervous system of human from inflammatory responses.

Abbreviations

ZJ: Zhejiang, Gly: glycine, p.i: post infection, PBS: phosphate-buffered saline, PDB: protein data bank, GFP: green fluorescent protein, RFP: red fluorescent protein, bp: base pair, UTR: untranslated region, CEP-1: *C. Elegans* P-53-like protein, CED: Cell Death abnormality, EGL: EGg Laying defective, ZJ: Zhejiang, RT-PCR, real-time PCR, RGB: red, green and blue, RNAi, RNA interference, CoA: coenzyme A.

Declarations

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Ethics approval and consent to participate

All the experimental animals used were treated strictly in accordance with the recommendations in the Guide for the Regulation for the Administration of Affairs Concerning Experimental Animal of the People's Republic of China. The protocol employed was approved by Laboratory Animal Ethics Committee of Wenzhou Medical College & Laboratory Animal Centre of Wenzhou Medical College (Permit Number: SYXK (zhe2015-0009)). The care and maintenance of animals followed this institution's guidelines.

Consent for publication

Not applicable.

Availability of data and materials

Data supporting the conclusions of this article are included within the article and its additional files. The datasets used in the present study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no conflicts of interests.

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Authors' contributions

BLY, HCH and HFS conceived and designed the experiments. WWS, XMY, AJQ and BLY wrote the manuscript. WWS, XMY, YJZ and LY performed the experiments. WWS and XMY collected and analyzed the data. HCH, BLY and HFS participated in technological guidance and coordination. All authors read and approved the final manuscript.

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Figures

Figure 1

Cloning strategy for reporter and rescuing constructs. a About 2000 bp sequence upstream of *Acan-gal-1* 5'-UTRs was used as putative promoters. The promoter regions of *Acan-gal-1* and *Ce-lec-1*, fused with green fluorescent protein (GFP) or red fluorescent protein (RFP) downstream were cloned into plasmid pPD95.77 to construct *pAcan-gal-1::gfp* and *pCe-lec-1::rfp*, respectively. b *Acan-gal-1* cDNA sequence, fused with red fluorescent protein (RFP) downstream was cloned into plasmid pPD95.77, using *pCe-lec-1* as promoter, to construct *pCe-lec-1::Acan-gal-1::rfp*.

Figure 2

Structures and sequence analysis of Acan-Gal-1. a schematic diagram showing the domains of Acan-Gal-1 (residues 1-285). b Alignment of amino acid sequences of Acan-Gal-1 from *A. cantonensis* with those from *C. elegans* and *T. leonine* and the secondary structures. The accession numbers of sequences available from current databases are: NP_001370038.1 (Ce-Lec-1) and ARM19984.1 (Tl-Gal-9). Identical and similar residues are shown in black and grey blocks, respectively. Secondary elements are shown by coil (α -helix) and arrows (green indicates β -sheets of NCRD and blue indicates those of CCRD). The short linker between NCRD and CCRD is indicated by a red line. The conserved motifs HXXXR and WGXEER are indicated by blue boxes. The critical amino acids for recognizing carbohydrate binding are indicated by red stars. c Predicted tertiary structure of Acan-Gal-1, HXXXR motif and WGXEER motif in NCRD and CCRD, respectively.

Figure 3

Neighbour-joining phylogenetic tree of Acan-Gal-1 and its orthologues from other nematode species. The tree is calculated using the Jones–Taylor–Thornton model in the MEGA program version 5.0. Bootstrap values above the branches (1000 iterations) are shown for robust clades (> 70%). Abbreviation for species names are as follows: Bm: *Brugia malayi*, Bp: *Brugia pahangi*, Wb: *Wuchereria bancrofti*, Ll: *Loa loa*, Ev: *Enterobius vermicularis*, Tc: *Toxocara canis*, Ce: *C. elegans*, Acan: *A. cantonensis*, Hc: *H. contortus*, Ace: *Ancylostoma ceylanicum*, Tl: *T. leonine*, Ts: *Trichinella spiralis*. The corresponding accession numbers are listed on the right of each species. Clade numbers were noted in Roman numerals.

Figure 4

Transcriptional profile of Acan-gal-1 in different developmental stages (L3, L5 and adult) and genders [females (F) and males (M)] of *A. cantonensis*, determined by real-time PCR analysis. Data shown are mean $\pm$ S.E.M derived from three technical replicates with two biological replicates. Relative transcription of the Acan-gal-1 gene in each sample was calculated by normalisation of the raw data, followed by the determination of abundance relative to the 18S ribosomal RNA gene (GenBank: AY295804), which was served as an internal loading control. Statistical analysis was conducted using a one-way ANOVA. * $P < 0.05$, ** $P < 0.01$.

Figure 5

Expression pattern of *A. cantonensis* *Acan-gal-1* promoter in *C. elegans*. a-c The promoter activity of *Acan-gal-1* in *C. elegans*. *pAcan-gal-1::gfp* is only expressed in pharyngeal nerves of *C. elegans*. d-f The promoter activity of *Ce-lec-1* in *C. elegans*. *pCe-lec-1::rfp* is expressed widely. Arrows indicate the following tissues: c, cuticle, i, intestine, n, neuron, p, pharynx.

Figure 6

Cross-species expressions of Acan-Gal-1 in N2 and the *lec-1* deletion mutant worms. a-c Expression of *pCe-lec-1::Acan-gal-1::rfp* in N2 worm. d-f Expression of *pCe-lec-1::Acan-gal-1::rfp* in *lec-1* deletion mutant worm. g-i Expression of *pCe-lec-1::rfp* in *lec-1* deletion mutant worm. White arrow heads indicate the lipid drops.

Figure 7

Up-regulated Acan-Gal-1 plays a defensive role against oxidative stress. a Oxidative stress assays using H₂O₂ in N2 and *lec-1* mutant worms expressing pCe-*lec-1*::Acan-gal-1::rfp. b The relative expression levels of apoptosis genes in *lec-1* mutant worms expressing pCe-*lec-1*::rfp and in *lec-1* mutant worms expressing pCe-*lec-1*::Acan-gal-1::rfp. c Oxidative stress assays using H₂O₂ in *ced-3* mutant worms expressing pCe-*lec-1*::Acan-gal-1::rfp or pCe-*lec-1*::rfp. (D) Oxidative stress assays using H₂O₂ in *ced-3* mutant worms with *lec-1* RNAi. The worms were counted as described in the “Methods” section. The error bars indicate standard deviation. *P<0.05, **P<0.01, *** P<0.001.

Figure 8

Up-regulated Acan-Gal-1 functioned in regulating lipid deposition and oxidative stress resistance. a Oil Red O fat staining of *C. elegans* strains N2, N2 expressing pCe-*lec-1::rfp*, N2 expressing pCe-*lec-1::Acan-gal-1::rfp*, *lec-1(tm1345)* mutant, *lec-1(tm1345)* mutant expressing pCe-*lec-1::rfp*, *lec-1(tm1345)* mutant expressing pCe-*lec-1::Acan-gal-1::rfp*. b Quantification of Oil Red O staining. c Oxidative stress assays using H₂O₂ in *fat-6;fat-7* double-mutant worms expressing pCe-*lec-1::Acan-gal-1::rfp* or with *lec-1* RNAi. The worms were counted as described in the “Methods” section. The error bars indicate standard deviation. **P*<0.05, ***P*<0.01.

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