Identification and preliminary characterization of *Hc-clec-160*, a novel C-type lectin domain-containing gene of the strongylid nematode *Haemonchus contortus*

Ling Zhang, Lingyun Mou, Xueqiu Chen, Yi Yang, Min Hu, Xiangrui Li, Xun Suo, Xing-Quan Zhu and Aifang Du

**Abstract**

**Background:** The strongylid parasite *Haemonchus contortus* causes severe anemia in domestic animals worldwide. Effective preventive and therapeutical agents are lacking, because of drug resistance and that little is known about the molecular mechanism of the interaction between *H. contortus* and host cells.

**Methods:** A new gene, *Hc-clec-160*, was discovered with RT-PCR. Transcriptional levels of *Hc-clec-160* and *Ce-clec-160* throughout different growth phases of corresponding nematodes were assayed by qPCR. Immunofluorescence staining of paraffin section were performed to determine the protein localization in adult worms of *H. contortus*. To monitor the promoter capacity of the 5'-flanking region of *Ce-clec-160*, micro-injection was used. Overexpression and RNAi constructs was carried out in the N2 strain of *Caenorhabditis elegans* to find out the gene function of *Hc-clec-160*.

**Results:** The full-length cDNA of 1224 bp of *Hc-clec-160* was cloned by RT-PCR. The corresponding gene contained twelve exons. Its transcripts peaked in male adult worms. Hc-CLEC-160 was predicted to have a Willebrand factor type A (vWA) domain and a C-type lectin domain. The proteins were not detected by expression in *C. elegans* or paraffin section experiments in adult of *H. contortus*. Knockdown of *Ce-clec-160* expression in *C. elegans* by RNAi resulted in shortened body length and decreased brood size.

**Conclusions:** In this experiment, a new gene *Hc-clec-160* was obtained in *H. contortus* and its function was addressed using a model organism: *C. elegans*. Our study showed that *Hc-clec-160* possesses characteristics similar to those of *Ce-clec-160* and plays an important role in the growth and reproduction of this parasitic nematode.

**Keywords:** *Hc-clec-160, Haemonchus contortus, Caenorhabditis elegans, C-type lectin*

**Background**

C-type lectin (CTL) is a Ca\(^{2+}\)-dependent glycan-binding protein (GBP) that shares primary and secondary structural homology in its carbohydrate-recognition domain (CRD). The CRD of CTL is generally regarded as the CTL domain (CTLD), representing a ligand binding motif that binds to sugars, proteins, lipids and even inorganic ligands [1]. Not all CTLs with this domain bind either glycans or Ca\(^{2+}\). The CTLD is defined by amino acid sequences and Cys positions, as well as the folded structure. The latter is characterized by two highly conserved disulfide-stabilized bicyclic structures that participate in binding to sugars with at least four conserved cysteine residues, two to three hydrophobic cores, and up to four calcium binding sites. The main conserved residues that bind to sugars include the EPN (Glu--Pro-Asn) motif which enhances binding to Man, GlcNAc, Fuc, and Glc and the WND (Trp-Asp-Asn) motif which promotes binding to Gal and GalNAc, as seen in mouse L-selectin and rat mannose-binding
protein C [1, 2]. They often oligomerize, which increases their avidity for multivalent ligands. CTLs bind to various types of glycans with high affinity. CTLs include selectins, collectins, endocytic receptors and proteoglycans. Some CTLs are exocrine whereas others are transmembrane proteins. They play a role in adhesion and signaling receptors in many pathways, including homeostasis and innate immunity, and are crucial in inflammatory responses and leukocyte and platelet trafficking. It is difficult to predict the glycan structures that bind to a particular CTL due to the relatively shallow CRD of CTL with few contacts to sugars motifs.

CTLs have been identified in some parasitic nematodes such as Trichostrongylus colubriformis, Onchocerca volvulus, Haemonchus contortus and Teladorsagia circumcincta [3]. They can significantly regulate host immune response [4–6]; however, their functions in H. contortus are poorly understood. Haemonchus contortus is one of the most economically important parasites of small ruminants (sheep and goats) worldwide, and can lead to anemia, weight loss and death of the host (sheep and goats) [7]. It can also cause immunosuppression and reduce the level of immunity in the host [8]. Caenorhabditis elegans is the most widely used model nematode in drug research [9], vaccine discovery [10] and helminth resistance studies [11–13].

In our study, we identified and characterized a novel gene of Hc-clec-160 in H. contortus, which is an ortholog of C. elegans and possesses one CTLD. Understanding the structure and function of Hc-clec-160 may provide useful information for deciphering how this parasite interacts with its hosts and crucial elements influencing its growth, development and reproduction.

**Methods**

**Parasites and animals**

Two female sheep, 4–5 months-old and maintained under helminth-free conditions for 30 days, were infected orally with 8000 L3s of H. contortus ZJ strain each, after being dewormed twice. Three weeks later, sheep feces were collected and helminth eggs detected by microscopy. First- (L1), second- (L2) and third-stage larvae (L3) were harvested at the 1st, 3rd and 7th day, respectively, after incubation of the collected eggs at 28 °C. Adults of H. contortus (ZJ strain) were obtained from abomasum of sheep in a Hu Zhou Slaughterhouse. They were washed several times in phosphate-buffered saline (PBS), pH 7.4. The clean adult worms were stored in li-

**Isolation of the Hc-clec-160 gene**

A homolog (>HCISE02108900.t1) was identified by using the Ce-clec-160 gene by searching the Sanger Institute genomic database for H. contortus (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/h_contortus). Specific primers (Hc-clec-160 1F and Hc-clec-160 1R) (Table 1) were designed according to the CDs sequence. The PCR reaction program included: one cycle at 94 °C for 3 min; 30 cycles at 94 °C for 30 s, 57 °C for 30 s and 72 °C for 80 s; and finally one cycle at 72 °C for 10 min. The amplified fragments were cloned into the pMD18-T vector (Takara Biotechnology Co., Ltd., Dalian, China) and sent to Shanghai BioSune Co., Ltd. (Shanghai, China) for sequencing.

**Bioinformatics analyses**

Function domains of Hc-clec-160 gene were analyzed by submitting its amino acid sequence to the NCBI conserved domains (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Homologues of Hc-CLEC-160 in other species: model nematodes (Caenorhabditis elegans, Pristionchus pacificus), animal parasite (Ascaris suum), zoonotic parasites (Toxocara canis, Oesophagostomum dentatum, Ancylostoma ceylanicum) and human parasites (Necator americanus, Necator americanus duodenale) were obtained by NCBI Protein BLAST alignment (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and all amino acid sequences were analyzed using Clustal W software [15]. Using evolutionary genetic analysis software MEGA v.7, based on the Jones-Taylor-Thornton (JTT) model, phylogenetic tree analysis was performed using Neighbor joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) methods [16].

**Table 1 Primers used in the study. The restriction sites are underlined.**

| Primer ID | Primer sequence 5'-3' |
|-----------|-----------------------|
| Hc-clec-160 1F | ATGACAACCCCTGATACCTT |
| Hc-clec-160 1R | TTGATGACGAGCATGTACG |
| Hc-clec-160 2F | GGATGCAACACCTCCTGTACCTT |
| Hc-clec-160 2R | AAGCTTTTAGAAGCGACAGATGTG |
| Hc-clec-160 3F | TCTAGATGCAAACCCCTGACCTT |
| Hc-clec-160 3R | GGATACATGACAGACAGATGTG |
| Hc-clec-160 4F | TCTAGATGCAAACCCCTGACCTT |
| Hc-clec-160 4R | AAGCTTTTAGAAGCGACAGATGTG |
| Ce-clec-160 pF | CCGGACCATTGACATTTGACATTA |
| Ce-clec-160 pR | CTGAAATCTCTGGATATTTAAAAAAAGA |
| Ce-clec-160 pF1 | GTACCGCCGACCATTCAATATGATTGAT |
| Ce-clec-160 pR1 | TCTAGATGCAAACCCCTGACCTT |
| Hc-clec-160 QF | AGTATGCGCTTGGATG |
| Hc-clec-160 QR | TGAATGACCTGTCCTTT |
| Ce-clec-160 F | CCCGGGATGATTTAAAATGGATGTTTAA |
| Ce-clec-160 R | AAGCTCTGAAATGAGGATTTGATTTA |
| Ce-clec-160 QF | AGTCCACCCTAACATCC |
| Ce-clec-160 QR | AAGTCCACCCTTGCCATTT |
Transcriptional levels of Hc-clec-160 in different developmental stages of H. contortus

qPCR with specific primers (Hc-clec-160 QF-Hc-clec-160 QR and Ce-clec-160 QF-Ce-clec-160 QR) (Table 1) was carried out to determine the mRNA levels in different stages of growth and development of H. contortus and C. elegans including L1, L2, L3, L4 and adults. Total RNA was isolated with Trizol reagents (Invitrogen, Shanghai, China) according to the manufacturer’s instructions and treated with DNase I (Toyobo, Shanghai, China) to remove the DNA. The qPCR reaction program included: one cycle at 50 °C for 2 min and 95 °C for 1 min; 40 cycles at 95 °C for 15 s, 60 °C for 15 s and 72 °C for 30 s; with a dissolution curve being produced in the last cycle. Each sample was repeated three times using the β-tubulin of H. contortus and actin-1 of C. elegans as internal reference genes and an average threshold (Ct) was taken for data analysis.

Polyclonal antibody preparation

Specific primers with double restriction sites (Hc-clec-160 2F and Hc-clec-160 2R) (Table 1) were designed according to the full-length cDNA of the Hc-clec-160 gene sequence. Hc-clec-160 was cloned into the prokaryotic expression vector pET-30a to construct the plasmid pET-30a-Hc-clec-160. The vector was transformed into BL21 (DE3) cells of E. coli to produce recombinant Hc-CLEC-160 (rHc-CLEC-160), followed by treatment with 1 mM isopropyl Β-D-1-thiogalactopyranoside (IPTG) at 37 °C for 2 h and purification by affinity chromatography using a Ni-NTA agarose column (Qiagen, Shanghai, China) as per the manufacturer’s instructions. Anti-rHc-CLEC-160 polyclonal antibodies were produced by immunizing a New Zealand white rabbit and their titer and specificity were determined by enzyme-linked immunosorbent assay (ELISA).

Immunofluorescence staining of H. contortus adult paraffin sections

Male and female adults of H. contortus were fixed in 4% paraformaldehyde (PFA) at 4 °C for 2 days. After being washed with tap water for 12 h, they were consecutively dehydrated in an ethanol series [50%, 75%, 80%, 95%, 100% (twice), 10 min at each step]. The dehydrated worms were incubated in xylene:absolute ethanol (1:1) solution and xylene and then embedded in paraffin. Paraffin sections of 5 μm were stained with hematoxylin and eosin (H&E) and analyzed histologically. The remaining sections were subjected to immunofluorescence staining. Slices were treated with 0.01 M citrate buffer at 100 °C for 20 min for antigen repair. After overnight blocking with 2% BSA at 4 °C, polyclonal antibody and goat anti-rabbit IgG H&L (Alexa Fluor® 488 diluted 1:100 in PBS were sequentially added and incubated at 37 °C for 1 h each. The sections were stained with 4',6-diamidino-2-phenylindole (DAPI) for 40 min at 37 °C and then observed by fluorescence microscopy.

Ce-clec-160 promoter transformation of C. elegans

The Ce-clec-160 promoter was amplified by PCR using primers Ce-clec-160 pF1 and Ce-clec-160 pR1 (Table 1) containing restriction sites SalI or XbaI. The primers were designed according to the 2000 bp upstream of the start codon of Ce-clec-160. The PCR products were cloned into the multiple cloning sites upstream of the gfp gene of pPD95.77 eukaryotic expression vector, resulting in the plasmid pPD95.77-Ce-clec-160-prom. The latter was microinjected into the gonads of C. elegans along with another plasmid pRF4 carrying the rol-6 gene at a final concentration of 50 μg/ml each. The GFP expression of F2 progeny with the phenotype of roller was observed by fluorescence microscopy.

Expression of Hc-clec-160 in C. elegans

Microinjection was performed as previously described [17]. Hc-clec-160 was amplified based on the specific PCR primers Hc-clec-160 3F and Hc-clec-160 3R listed in Table 1 with XbaI/KpnI restriction enzyme sites. The amplified fragments were cloned into the plasmid pPD95.77 vector between the Ce-clec-160 promoter region and the gfp region. The recombinant plasmid CeP-pPD95.77-Hc-clec-160 with CeP-pPD95.77-Ce-clec-160 as a control was microinjected into N2 strains as described above. The F2 progeny with the roller phenotype were collected for further analysis.

Hc-clec-160 RNA interference

Ce-clec-160 and Hc-clec-160 genes were amplified by PCR with specific primers Hc-clec-160 4F-Hc-clec-160 4R and Ce-clec-160 F-Ce-clec-160 R, respectively (Table 1). The primer pairs contained the restriction sites for XbaI/HindIII and Smal/HindIII, respectively. The amplified fragments were cloned into the L4440 vector, resulting in the recombinant plasmids L4440-Ce-clec-160 and L4440-Hc-clec-160. The latter were transformed into E. coli HT115 (DE3) strain with the empty L4440 vector as a negative control. A feeding RNAi experiment was performed according to the classic bacterial feeding manuals [18] with three repeats. The transcriptional levels of RNAi worms were detected by qPCR and the RNAi worms were selected for further phenotype analysis.

Statistical analyses

Statistical analysis for Hc-clec-160 and Ce-clec-160 transcriptional levels and parameters were carried out in Excel (v.10.1.0.7022). Graphs were performed using the
of the sections showed that, regardless of the sex of adults *H. contortus*, parasite morphology was well maintained after sectioning and the tissue boundaries were clear (Additional file 1: Figure S1). Immunofluorescence staining of paraffin sections to detect protein localization was carried out, but no fluorescence staining was observed.

**Expression of Hc-clec-160 in *C. elegans***

The entire coding regions of *Hc-clec-160* fused in frame with the *gfp* gene was expressed in transgenic *C. elegans* to detect its expression *in vivo*. *Ce-clec-160* 5’-flanking region was used as a promoter. Activity analysis of the promoter showed that GFP was mainly localized in the distal and anterior part of the intestine (Fig. 3). The reconstructed plasmid Cep-PD95.77-*Hc-clec-160* and Cep-pPD95.77-*Ce-clec-160* was transformed into the gonads of *C. elegans* by microinjecting as described above. However, neither *Hc-clec-160* nor *Ce-clec-160* expression worms showed GFP signal in any stage of *C. elegans*.

**RNAi in *C. elegans***

Transcriptional levels of the RNAi worms were detected using qPCR to testify whether *Ce-clec-160* in *C. elegans* was successfully interfered. It was confirmed that the transcriptional levels of *Ce-clec-160* of the worms fed with the *Hc-clec-160* and *Ce-clec-160* interference vector were significantly decreased in *C. elegans* by 45% (t-test: $t_{(4)} = 2.846$, $P = 0.0466$) and 65% (t-test: $t_{(4)} = 4.436$, $P = 0.0114$), respectively (Fig. 4). The RNAi worms revealed significant reduction in brood size (t-test: $t_{(19)} = 2.428$, $P = 0.0253$;...
t-test: $t_{(19)} = 9.878, P < 0.0001$) (Fig. 5a) and shortened body length (t-test: $t_{(19)} = 2.765, P = 0.0123$; t-test: $t_{(19)} = 5.986, P < 0.0001$) (Fig. 5b) compared with the control group; however, the body width of Ce-clec-160 RNAi worms showed no significant differences from the control (Fig. 5c).

**Discussion**

In this study, the full-length cDNA sequences of two genes (Hc-clec-160 and Ce-clec-160) were determined. CTLS are highly representative of all studied metazoan phyla to date. Many proteins in the CTL superfamily contain multiple CRDs, along with additional non-lectin domains. CTLDs were classified to seventeen groups; Hc-clec-160 was similar to the group XIII DGCR2 (DGCR2/ DD/ Sez 12), which is localized in the DiGeorge syndrome (OMIM 188400) critical region [1]. In von Willebrand factor, the vWF is the archetype for a protein superfamily. The structure of vWF was discovered in various plasma proteins: complement
factors B, C2, CR3 and CR4; the integrins (I-domains); collagen types VI, VII, XII and XIV; and other extracellular proteins [19, 20]. Most of the proteins containing vWF domains are exocrine. The vWF-containing proteins are involved in many biological functions such as cell adhesion, pattern formation, migration, homing, and signal transduction, concerning interactions with a large number of ligands. It is plausible that the interaction between lectins of gastrointestinal nematodes and complex mucin oligosaccharides can affect worm infection [21]. It has been reported recently that a CTL named cplec from Cryptosporidium parvum plays an important role in binding sulfated proteoglycans of host cells [22, 23]. These findings suggest that Hc-CLEC-160 may have the ability to adhere and bind to host cells.

The relative abundance of Hc-clec-160 transcripts throughout different developmental stages determined by qPCR showed that they had generally low abundance in all life-cycle stages except for adult males (Fig. 2a). High transcripational levels of Hc-clec-160 in the male adult stage suggests that Hc-clec-160 may be related to the mating and reproduction of H. contortus. Furthermore, higher Hc-clec-160 transcriptional levels in L3s and L4s than in eggs, L1s and L2s, indicate that Hc-clec-160 is likely to play a crucial part in the parasitic stages, i.e. L3s and L4s, for adhesion to the host cells. The microinjection of Ce-clec-160 promoter results indicated that this promoter was capable of promoting the GFP expression in C. elegans with GFP proteins being mostly located in the anterior and distal part of the intestine.

As for paraffin section immunofluorescence staining and microinjection transgene assays, the possible causes for the lack of fluorescence in H. contortus or GFP in C. elegans could be summarized as follows. First, the polyclonal antibodies can be detected by western blot rather than the immunofluorescence staining of sections, which may be because the structure of the parasite antigen immobilized by paraformaldehyde is different from the linear structure in the western blot experiment. Secondly, it is possible that the antigen of CTL was damaged during the production of the slices, resulting in the fact that the antibody cannot specifically recognize and bind to it. Thirdly, the GFP expressed in the worms microinjected with Ce-clec-160 promoter, indicating that the promoter was able to efficiently initiate the expression of GFP. However, the ability to drive the expression of clec-160 gene was weak. The most likely reason for the failures of these two experiments is due to the rather low expression levels of clec-160 in H. contortus and C. elegans.

The mechanism of RNAi is divided into two procedures. First, dsRNA (double stranded RNA) is processed into small ds RNA (siRNA) of 21 to 23 nt. Then, the antisense strands of siRNA induce a ribosome called RNA-induced...
sirNA: Small interfering RNA; PFA: Paraformaldehyde; qPCR: Real-time quantitative PCR; rHc-CLEC-160: Recombinant Hc-CLEC-160; RNA: Ribonucleic acid; RNAi: RNA interference; RT-PCR: Reverse transcription PCR; siRNA: Small interfering RNA; vWA: Von Willebrand factor type A; vWF: Von Willebrand factor; WND: Trp-D-Asn

Additional file

Additional file 1: Figure S1. Morphology and histology of male (a) and female (b) adults of H. contortus. Panels a and b represent H&E staining of worm paraffin sections at 10×. Scale-bars: 10 mm. (TIF 1691 kb)

Acknowledgments
We are grateful to Dr Yang CL (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences), Dr Wang XC (the National Institute of Biological Science, Beijing) and Dr Ren CH (Academy of Military Medical Science, Beijing) for their help in the microinjection technology of Caenorhabditis elegans and for donating the vector.

Funding
The project was supported by a grant from the National Key Basic Research Program (973 Program) of China (No. 2015CB15030X), the National Key Research and Development Program of China (No. 2017YFD0501200), the National Natural Science Foundation of China (No. 31602041), the Opening Fund of State Key Laboratory of Veterinary Etiological Biology (No. SKLVEB2016KFT018) and the Science and Technology Plan Project of Jiaxing (No. 2016AY23025).

Availability of data and materials
The data supporting the conclusions of this article are included within the article and its additional files.

Authors’ contributions
LZ completed the isolation of Hc-clec-160, microinjection and defined the transcriptional levels of clec-160. LM preformed promoter activity analysis and relative quantification PCR of RNAi. XC carried out the RNAi experiments and performed the prokaryotic expression of Hc-clec-160. YY showed the characteristics of Hc-clec-160 and prepared the polyclone antibody. MH, XL, JS, X-QZ and AD critically revised the manuscript. All authors read and approved the final manuscript.

Conclusions
In the present study, a novel gene Hc-clec-160 encoding a cDNA of 1224 bp in H. contortus was identified. The gene possesses one conserved domain of carbohydrate-recognition domains (CRDs) and contains 12 exons. A possible promoter in the 2000 bp sequence upstream of the 5′-flanking region was demonstrated by microinjection experiments. Furthermore, partially silencing the Ce-clec-160 in N2 worms of C. elegans achieved by RNAi with Hc-clec-160 showed that Hc-clec-160 shared similar characteristics and functions with Ce-clec-160 and plays a crucial part in the development and reproduction of H. contortus.
Ethics approval and consent to participate
This study was approved by the Zhejiang University Experimental Animals Ethics Committee (ZJU2013038-1-10-072). All animals were treated in accordance with relevant guidelines and regulations.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Author details
1 College of Animal Sciences, Zhejiang Provincial Key Laboratory of Preventive Veterinary Medicine, Zhejiang University, Hangzhou, China. 2 State Key Laboratory of Agricultural Microbiology, College of Veterinary Medicine, Laboratory of Agricultural Microbiology, College of Veterinary Medicine, Zhejiang University, Hangzhou, China. 3 College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, China. 4 State Key Laboratory of Agrobiotechnology, Key Laboratory of Zoology of Ministry of Agriculture, National Animal Proteoza Laboratory and College of Veterinary Medicine, China Agricultural University, Beijing, China. 5 State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Veterinary Parasitology of Gansu Province, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou 730046, Gansu Province, China.

Received: 7 April 2018 Accepted: 9 July 2018
Published online: 20 July 2018

References
1. Zelensky AN, Gready JE. The C-type lectin-like domain superfamily. FEBS. 2005;272:617–27.
2. Dickerman W. C-type lectin-like domains. Curr Opin Struct Biol. 1999;9:385–90.
3. Vasta GR. Roles of galectins in infection. Nat Rev Microbiol. 2009;7:424–38.
4. Young AR, Meuseen EN. Galectins in parasite infection and allergic inflammation. Glycobiol J. 2002;12:601–6.
5. Rabinovich GA, Gruppi A. Galectins as immunoregulators during infectious diseases. Prog Allergy. 2008;82:65–88.
6. Ortolani EL, Leal ML, Minervino AH, Aires AR, Coop RL, Jackson F, et al. Alteration of the expression profiles of acidic mucin, sialyltransferase, and sulfotransferases in the intestinal epithelium of rats infected with the nematode Nippostrongylus brasiliensis. Parasitol Res. 2008;103:1427–34.
7. Chernin J. A call for collaboration. Parasitol Today. 2000;16:83.
8. Ortolani EL, Leal ML, Minervino AH, Aires AR, Coop RL, Jackson F, et al. Modification of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994;22:4673–80.
9. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016;33:1870–4.