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Characterization and immunolocalization of mutated ornithine decarboxylase antizyme from *Angiostrongylus cantonensis*

Jing Chen[a,b], Qian Liu[a,b], Xiao Yang[a,b], Xiansheng Wu[a,b], Dongjing Zhang[a,b], Ai He[a,b], Ximei Zhan[a,b,*]

[a] *Department of Parasitology, Zhongshan School of Medicine, Sun Yat-sen University, 74 Zhongshan 2 Road, Guangzhou, Guangdong 510080, People's Republic of China*
[b] *Key Laboratory for Tropical Disease Control, Ministry of Education, Sun Yat-sen University, 74 Zhongshan 2 Road, Guangzhou, Guangdong 510080, People's Republic of China*

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ABSTRACT

Ornithine decarboxylase antizyme (OAZ), a prominent regulator of cell proliferation, DNA/RNA transformation and tumorigenesis, can bind to ornithine decarboxylase (ODC) and facilitate its degradation. Expression of OAZ requires a unique ribosomal frame shift that is regulated by levels of polyamine in the cell. In this study, we cloned an OAZ gene with the +1 ribosomal frame-shift from a fourth-stage larvae cDNA library of *Angiostrongylus cantonensis*. We removed one nucleotide to express the gene without polyamine. The sequence analysis showed that the deleted-mutation ornithine decarboxylase antizyme (DM-AcOAZ) contained a conservative domain related to other species OAZ. Quantitative real-time PCR revealed that DM-AcOAZ was expressed in L3 and L4 stages and adult female worms. More notably the expression level is the highest in the adult female stage. Immunohistochemical studies indicated that DM-AcOAZ was specifically localized in the uterus, oocyte and intestine in adult female worms. MTT assays showed that in DM-AcOAZ transfected HeLa cells, cell proliferation is inhibited. In conclusion, DM-AcOAZ may be a female-enriched protein and may involved in the cell proliferation in *A. cantonensis*.

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1. Introduction

Human angiostrongyliasis, caused by *Angiostrongylus cantonensis*, is an important emerging disease. *A. cantonensis* has spread from its traditional endemic regions of the Pacific islands and Southeast Asia to the American continent including the USA, Caribbean islands and Brazil [1]. Major outbreaks have been reported several times during the last decade in mainland China, for example in Dali, Yunnan Province [2] and Guangning, Guangdong Province [3]. Eosinophilic meningitis and ocular angiostrongyliasis are the two main clinical manifestations of human angiostrongyliasis. Immunological tests are the primary means for angiostrongyliasis diagnosis. However, these parasites are usually not recoverable in fecal or cerebrospinal fluid [4], so research is increasingly focusing on biochemical characteristics of *A. cantonensis*.

Polyamines are known to play important roles in the differentiation and proliferation of many types of cells [5,6], in biosynthesis and function of proteins and nucleic acids [7], in modulation of enzyme activities, and in regulation of ion channels and apoptosis [8]. Ornithine decarboxylase (ODC), which is ubiquitously found in organisms ranging from bacteria to humans, is the principal enzyme involved in polyamine biosynthesis and catalyzes the decarboxylation of ornithine to putrescine [9]. Antizyme was originally found to be a suppressor of ornithine decarboxylase (ODC) activity. It binds ODC and facilitates proteasomal ODC degradation [10]. It has also been shown that antizyme induction can lead to degradation of the cell cycle regulatory protein cyclin D1 [11]. In recent years, ODC antizyme has also been reported to function as a tumor suppressor and to suppress tumor cell proliferation and transformation [12].

We infer that ornithine decarboxylase antizyme may play a crucial role in parasite development and growth. So in this study, we cloned and characterized DM-AcOAZ from *A. cantonensis*, analyzed its expression and localization in different life-stages, revealing its possible biological role in cell proliferation.

2. Materials and methods

2.1. Preparation of parasites

Third-stage larvae (L3) of *A. cantonensis* were obtained from naturally infected giant African snails (*Achatina fulica*) in Guangzhou,

* Corresponding author at: Department of Parasitology, Zhongshan School of Medicine, Sun Yat-sen University, 74 Zhongshan 2 Road, Guangzhou, Guangdong 510080, People's Republic of China. Tel.: +86 139 2226 6189.
E-mail address: zhanximei@yahoo.com.cn (X. Zhan).

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China. The L3s were digested in artificial gastric juice (pH 1.5, 1% pepsin-1%HCl, 10 mL/g tissue) at 37 °C for 2 h, then they were isolated under a microscope. The fourth-stage larvae (L4) were separated from the brains of BALB/c mice 20 days after infection (50 L3/mouse), and the adult worms were collected from the lung vessels of Sprague-Dawley (SD) rats 35 days after infection (60 L3/rat). All collected parasites were washed repeatedly to avoid host contamination and then stored at −80 °C. Rats and mice were purchased from the animal center in Sun Yat-sen University (Guangzhou, China). The maintenance and care of animals used in this study were approved by the Use Committee of Sun Yat-sen University and the Institutional Animal Care.

2.2. Sequence analysis of DM-AcOAZ

A fourth-stage larvae cDNA library of *A. cantonensis* was constructed and sequenced as previously reported [13]. In our previous work, we deleted the "T" base at the site of the translational frameshift by site-directed mutagenesis, therefore, OAZ can be expressed without induction by polyamine. DNA sequence of a deleted-mutation clone encoding the homologue of ornithine decarboxylase antizyme was analyzed using BLASTx and Open Reading Frame (ORF) Finder program in NCBI (http://www.ncbi.nlm.nih.gov). The conserved domain, physicochemical properties and secretory signal peptide were predicted using ExPASY (http://www.expasy.org/). Full-length protein sequences of OAZ homologues were aligned using Align X tool of NTI Advance 10 software.

2.3. Cloning, expression, and purification of the recombinant DM-AcOAZ

The full-length *DM-AcOAZ* was amplified by PCR using forward and reverse primers containing BamH I and Xho I restriction enzyme sites respectively. Forward primer: 5′-GTAGGATCCATGTTGTCCAATAAGTC-3′, BamH I, and reverse primer: 5′-CGCTCGAGTTAGACAGCATAACTC-3′, XhoI. The specific PCR product was digested by BamHI and XhoI, and cloned into pET30a vector. The correct recombinant plasmid was confirmed by DNA sequencing, and then transformed into *Escherichia coli* BL21 (DE3). The recombinant his-tagged protein was expressed by induction with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) at 37 °C for 4 h. The bacteria were harvested by centrifugation, resuspended in PBS and then sonicated. Recombinant protein was purified using His-band resin (Novagen) chromatography. Finally, the purified protein was defined by 12% SDS-PAGE, and the concentration was measured using a BCA protein assay kit (Sangon Biotech). The protein was stored at −80 °C.

2.4. Preparation of rDM-AcOAZ antiserum

100 μg of rDM-AcOAZ was mixed with an equal volume of complete Freund's adjuvant and then injected subcutaneously into BALB/c mice. Boosts of 50 μg of rDM-AcOAZ mixed with incomplete Freund's adjuvant were given at 2 and 4 weeks after first injection. The mice were bled and the titer of anti-rDM-AcOAZ IgG was determined by ELISA.

2.5. mRNA expression level of rDM-AcOAZ in different stages

Total RNA was isolated from different stages (L3, L4, adult male and adult female) of *A. cantonensis* using TRIzol Reagent (Invitrogen) following the manufacturer's instructions. The RNA yield was measured by spectrophotometer. The cDNAs were obtained by reverse transcription. Quantitative real-time PCR based on SYBR-Green I fluorescence (TaKaRa) was performed in Roche LightCycler480. The specific primers were designed as: forward 5′-ACCCGTAGAACAAGACCCAA-3′ and reverse5′-GAAGCACTCTGAAACCGACAT-3′. Primers for β-actin (internal control)[14] were designed as: forward 5′-TCATCACTGTGGGAAACGAA-3′ and reverse 5′-GTGTTGGCGTACCAATCCTT-3′. The qRT-PCR program was as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 56 °C for 20 s. A melting curve analysis was performed at 95 °C for 30 s, then 65 °C for 15 s after amplification. The relative quantification analysis was carried out by calculating the value of $2^{-\Delta\Delta Ct}$.

2.6. Immunolocalization of DM-AcOAZ in adult worms

Adult worms were fixed in 10% (v/v) formaldehyde, embedded in paraffin, and sliced into 5-μm thickness. Slides were baked at 60 °C for 30 min and saturated in goat serum overnight. The sections were then incubated for 2 h in anti-DM-AcOAZ sera diluted (1:200) in phosphate buffered saline-Tween 20 (PBS-T) containing 5% (v/v) bovine serum albumin. Preimmune mouse serum was used as the negative control. Slides were washed in PBST and then incubated with secondary antibodies (Alexa Fluorlabeled goat anti-mouse IgG, 1:400 in 0.1% PBS-T) for 1 h. After 20-min incubation with organization spontaneous fluorescence quench reagent, slides were observed under fluorescence microscope (Olympus IX61).

2.7. Cell culture, plasmid preparation and transfection

HeLa cells were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum with penicillin (100 U/mL) and streptomycin (100 μg/mL).The cell line was cultured in a humidified incubator supplied with 5% CO_2. Full-length of *Ac.* OAZ was amplified by PCR using the forward primer (5′-CGCTCGAGATGTTGTCCAATAAGT-3′) and reverse primer (5′-CGCGAATTCTTAGACAGCATAACT-3′). PCR products were then cloned into the pEGFP-N1 vector. HeLa cells were seeded in a 24-well plate at a density of 2×10^5 per well one day before transfection to obtain 80% confluence. The growth medium was removed and 400 μL of opti-MEM reduced serum medium (Invitrogen Corp.) was added before transfection. Lipofectamine2000 (Invitrogen Corp.) was used to transfect the cell line with the recombinant plasmid pEGFP-N1-DM-AcOAZ, based on the manufacturer's manual. Meanwhile, empty vector and blank control groups were also established. Stable sub-clonal cells were screened using G418 selection (Gibco; 800 μg/mL).

2.8. MTT assay

A stable sub-clonal population of HeLa cells was seeded into a 96-well plate at a concentration of 1000 cells/well. MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays were performed at different time points (24 hs, 48 hs, 72 hs, 96 hs after incubation) to determine the proliferation of cells at those time points. After the specified incubation time, 20 μL of MTT solution (1 mg/mL) was added to each well and incubated at 37 °C for 4 h. Then the medium and MTT in the wells were removed and 100 μL of DMSO was added to each well. A microplate reader was used to measure the optical density at 490 nm. Experiments were performed in triplicate.

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