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Identification, sequence analysis and characterization of *Clonorchis sinensis* ubiquitin

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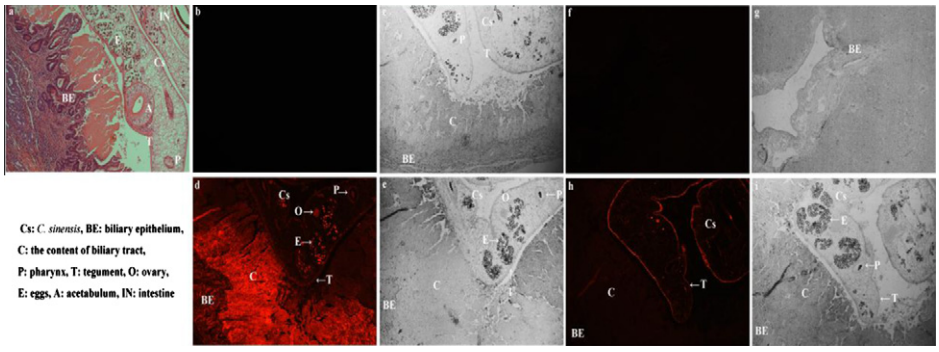
HIGHLIGHTS

- Ubiquitin loci and their transcribe isoforms of *Clonorchis sinensis* were identified from its genome.
- The transcript level of ubiquitin at life stages of *C. sinensis* were investigated.
- The distribution of *C. sinensis* ubiquitin or ubiquitination was observed.

GRAPHICAL ABSTRACT



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ABSTRACT

Ubiquitin is a functionally important protein expressed in eukaryotic cells usually encoded by multigenic families containing two types of genes, ubiquitin extension genes and polyubiquitin genes. One independent monomeric locus and two independent polyubiquitin loci were firstly identified from the genome of carcinogenic liver fluke, *Clonorchis sinensis* (*C. sinensis*). The nucleotide and amino acid sequence of *C. sinensis* polyubiquitin, especially polyubiquitin with five tandem ubiquitin repeats (CsPUB5), were analyzed. We obtained recombinant CsPUB5 (rCsPUB5) and anti-rCsPUB5 IgG. The ubiquitin transcripts in life cycle of *C. sinensis* were investigated. In addition, we found that ubiquitin or ubiquitination was ubiquitous in adult worm of *C. sinensis* and significantly observed in the content of biliary tract and intrahepatic biliary epithelium of liver from *C. sinensis* infected rat. We confirmed that rCsPUB5 could bind to human intrahepatic biliary epithelial cell by immunofluorescence in vitro. It was considered that ubiquitin family constitutively expressed in *C. sinensis* for variety of cellular processes and might be implicated in the genesis and progression of cholangiocarcinoma induced by the infection of *C. sinensis*.

1. Introduction

Human clonorchiasis, caused by infection of *Clonorchis sinensis* (*C. sinensis*) which inhabits in intrahepatic bile duct of piscivorous mammal, is endemic in China, Korea, Vietnam and other Southeast Asian countries (Lun et al., 2005). Clonorchiasis is a tropical disease once being neglected. Since some investigations showed that

Abbreviations: CsPUB5, polyubiquitin with five ubiquitin unit of *Clonorchis sinensis*; ORF, open reading frame; bp, base pair; aa, amino-acid; PBS, phosphate buffer saline; CsESP, excretory/secretory products from *C. sinensis*.

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C. sinensis infection was highly associated with cholangiocarcinoma (Choi et al., 2006; Papachristou et al., 2005; Schwartz, 1980), take together that World Health Organization (WHO) announced that *C. sinensis* infection was one of biological agents induced cholangiocarcinoma in 2009 (Bouvard et al., 2009), a large amount of fundamental researches on *C. sinensis* were performed to learn more about physiology of *C. sinensis* and pathogenesis of clonorchiasis.

The transcriptome data of different developmental stages of *C. sinensis* showed that genes coding ubiquitin family belonged to the 30 genes most abundantly expressed in adult worm, metacercaria and egg (Yoo et al., 2011). It demonstrated that ubiquitin family involved in basal development and physiology of the worm.

The strong cation exchanger-liquid chromatography–tandem mass spectrometry (SCX-LC–MS/MS) analysis of excretory/secretory proteins (ESPs) from *C. sinensis* adult illustrated that polyubiquitin was one of component molecules (Zheng et al., 2011). ESPs play important roles in the interaction between parasite and the host (Giacomin et al., 2008; Gonzalez-Miguel et al., 2011; Reinhardt et al., 2011). When attached to proteins, ubiquitin or polyubiquitin chains act as 'codes' to direct suitable protein–protein interactions to effect or regulate specific cellular functions (Wu et al., 2010). As a component of ESPs from *C. sinensis* adult, polyubiquitin might take a part in the interaction between parasite and host. Based on the above description, we take a keen interest in ubiquitin family of *C. sinensis*.

In other eukaryotic organisms, it is well known that ubiquitin family proteins function in wide variety of cellular processes including protein degradation, assembly of signaling complexes, and DNA damage repair by ubiquitination system (Wu et al., 2010). Functional ubiquitin is produced from two different types of ubiquitin genes, named polyubiquitin and ubiquitin extension genes. The polyubiquitin genes contain spacerless tandem repeats of 228 bp encoding ubiquitin protein (Jentsch, 1992; Schlesinger and Bond, 1987). The ubiquitin extension genes are comprised of a single ubiquitin repeat and sequences encoding one of two ribosomal proteins (Al-Khedhairy, 2004). For parasites, most researches on ubiquitin or ubiquitination system focused on apicomplexan parasites including trypanosome, plasmodium, amoeba and so on. It is revealed that the parasites contain dramatic differences in the numbers and sizes of ubiquitin genes and transcripts (Kirchhoff et al., 1988). *Giardia lamblia* has just one ubiquitin gene, which is not enhanced by heat shock (Krebber et al., 1994). *Entamoeba histolytica* contains at least five ubiquitin genes but lacks polyubiquitin gene, and the expression of its ubiquitin genes is not influenced by heat shock (Wostmann et al., 1996). *Trypanosoma cruzi* contains five genes coding for an ubiquitin fusion protein and five polyubiquitin genes. There are different transcript patterns of ubiquitin genes depending upon the nature of the stimulus the parasite experiences (Swindle et al., 1988). How about *C. sinensis* ubiquitin? In the present study, as abundantly expressed and important molecules, ubiquitin genes of *C. sinensis* were identified, sequence analyzed and cloned.

2. Materials and methods

2.1. Collection of C. sinensis adult worms, metacercariae and eggs

Intact living adult worms were collected from intrahepatic biliary tracts of infected cats and washed extensively and gently in physiological saline to remove any contamination from hosts. Eggs discharged from adult worms during the process were collected and washed too. *Pseudorasbora parva* naturally infected with *C. sinensis* were digested in artificial gastric juice (0.5% pepsin in 1% HCl, pH 2.0) at 37 °C for 3 h. The digestive juice was filtered with 80 meshes to remove debris, and metacercaria of *C. sinensis* were isolated under stereomicroscope and washed three times in physiological saline. They were respectively stored in sample protector (Takara) at −80 °C for RNA extraction. Species confirmation of the adult worms, metacercaria and eggs was verified by sequencing (ABI 3730) the second internal transcribed spacer (ITS-2) of nuclear ribosomal from their respective genomic DNA.

2.2. Preparation of serum and paraffin section of liver from Sprague–Dawley (SD) rat infected C. sinensis

A six-week-old male SD rat was infected with fifty genus identified *C. sinensis* metacercariae by intragastric administration. Forty five days after infection, the rat was executed. Its serum was collected, aliquoted and stored in −80 °C. Its liver was isolated and washed extensively in physiological saline in order to remove coagulated blood. Then the liver was fixed with 10% formaldehyde. Liver tissues closed to porta hepatis were embedded with paraffin wax and sliced into 5 μm sections for observation of the distribution of *C. sinensis* ubiquitin or ubiquitination in liver of infected rat.

2.3. Identification and sequence analysis of C. sinensis ubiquitin

At the beginning, the insert sequence of plasmid No.15C12 including open reading frame (ORF) of polyubiquitin homologue with five head-to-tail ubiquitin units (*Cs*PUB5) was screened out from cDNA plasmid library of *C. sinensis* adult we constructed previously (Yang et al., 2005; Zheng et al., 2005). Next, *C. sinensis* ubiquitin/polyubiquitin loci and their transcribed isoforms were identified when we mapped the cDNA sequence back to our database of *de novo* genome and transcriptome sequencing (http://fluke.sysu.edu.cn) (Wang et al., 2011) by BLAST.

We focused heavily on nucleotide polymorphisms of ubiquitin units in PUB5. A tree figure of Nearest Joining neighbor analysis was constructed using Vector NTI suite 8 program.

The amino acid sequences of polyubiquitin from *Homo sapiens* (Accession Number: NP_066289.2), *Rattus norvegicus* (Accession Number: BAA04129.1), *Mus musculus* (Accession Number: AAG00513.1), *Caenorhabditis elegans* (Accession Number: AAA28154.1), *Plasmodium falciparum* (Accession Number: CAB59728.1) and *Schistosoma japonicum* (Accession Number: CAX71214.1) were downloaded from NCBI Protein database. The alignment of their ubiquitin unit sequences was analyzed using Vector NTI suite 8 program.

2.4. Ubiquitin transcripts at life stages of C. sinensis

The adult worm, metacercariae, and eggs were respectively homogenized in Trizol (Invitrogen) with Tissue Ruptor (Qiagen) for 40 s on ice, and then their pure total RNA were extract using a commercial RNA extraction kit (OMEGA Bio-Tek) and Dnase (TaKaRa) according to the instructions. Their total cDNA were obtained by the method of reverse transcription PCR by using Reverse Transcriptase XL (TaKaRa) and Oligo $(dT)_{18}$ primer referred to the manuals. Quantitative real-time PCR based on SYBR-Green I fluorescence (TaKaRa) were performed on Roche LigtheCycler480. The primers (5′-GAAGACGCTGACTGGCAAGAC-3′ and 5′-GCCAGTCAGCGTCTTCACAAA-3′) were employed to amplify *C. sinensis* ubiquitin/polyubiquitin transcripts fragment. β actin of *C. sinensis* (GenBank Accession No. EU109284.1) was used as an internal control. Its forward and reverse primers were 5′-ACCGTGAGAAGATGACGCAGA-3′ and 5′-GCCAAGTCCAAACGAAGAATT-3′ respectively. After predegeneration at 95 °C for 30 s, the cycles were carried out for 40 cycles at 95 °C for 5 s and 60 °C for 20 s. All assays were tested in triplicate and repeated twice. The relative quantification analysis was carried out by calculating the values of $2^{-\blacktriangle\blacktriangle Ct}$ (Pfaffl, 2001). The data were