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Genome-wide organization, evolutionary diversification of the *COMMD* family genes of amphioxus (*Branchiostoma belcheri*) with the possible role in innate immunity

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ABSTRACT

The *COMMD* (COpper Metabolism gene MURR1 Domain) gene family with ten members participates in various biological processes, such as the regulation of copper and sodium transport, NF-κB activity and cell cycle progression. However, studies on the *COMMD* gene family in amphioxus (*Branchiostoma belcheri*) are yet largely unknown. In this study, we have identified and characterized the ten *COMMD* family members from amphioxus (designated as *AmphiCOMMDs*). Firstly, we clone the full length of *AmphiCOMMDs*, and all AmphiCOMMD proteins contain the conserved COMM domain with two NES (Nuclear Export Signal) motifs. Secondly, the genomic structure analysis demonstrates that genes of the *COMMD* family have undergone intron loss and gain during the process of divergence from amphioxus to vertebrates. Thirdly, phylogenetic analysis indicates that *AmphiCOMMDs* are more closely related to vertebrates, implying the *AmphiCOMMDs* may be the ancestor of the vertebrate *COMMDs*. Fourthly, *AmphiCOMMDs* are ubiquitously and differentially expressed in five investigated tissues (muscles, gills, intestine, heaptic cecum and notochord). Finally, our results show that expression levels of *AmphiCOMMD* genes are fluctuating after LPS stimulation to some different extent. Taken together, our studies have elaborated the evolutionary dynamic and the innate immune role of the *COMMD* family genes in amphioxus.

1. Introduction

The nuclear factor-κB (NF-κB) family, as ubiquitous transcription factors, have extensively participated in diverse biological processes such as immunity and inflammation [1]. The key role of NF-κB is to induce transcription of proinflammatory mediators and then recruit and activate various immune cells. However, the incorrect regulation of the NF-κB pathway could lead to cancer, inflammatory diseases and immune disorders [2]. Therefore, the NF-κB-mediated transcriptional response must be tightly regulated. So far, a few proteins, such as IκB, A20/TNFAIP3 and CYLD, could negatively regulate the NF-κB signaling by acting on the upstream of NF-κB [3–5]. However, little is known about the mechanism through which NF-κB activity is terminated within the nucleus.

Recently, the COMMD (COpper Metabolism gene MURR1 Domain) protein family has been identified as a new class of proteins with the role of inhibiting NF-κB activity [1]. The COMMD family contains ten members. The *COMMD1* is first identified in close proximity to the imprinted murine gene *U2af1-rs1* [6], which is initially designated as *MURR* [7]. The other members of this COMMD family are identified via biochemical screening for homology of COMMD1 [8,9]. Most COMMD proteins possess a COMM domain, which is a very conserved region with 70–85 amino acids and located at the extreme carboxyl-terminal end of COMMD proteins [8]. The COMM domain can offer an interface for protein-protein interactions, and mediate COMMD1–COMMD1 dimer formation, as well as bind to other COMMD proteins, Elongin B/C and Cul2, and SOCS1 (ECSSOCS1) [10]. Two highly conserved nuclear export signals (NESs) are also included in the COMM domain [11]. The hydrophobic residues forming the NES (NES consensus sequence of $\Phi X_{2-3}\Phi X_{2-3}\Phi X_{1-2}\Phi$) are conserved among the COMMD proteins. Besides the COMM domain, each COMMD protein contains unique amino terminal regions, which are divergent within all COMMD members.

These COMMD proteins are involved in NF-κB regulation, copper homeostasis, sodium transport and adaptation to hypoxia [12–15]. The COMMD1can act as a regulator of copper metabolism via copper transporter ATP7B and XIAP [16]. Many studies have demonstrated that the COMMD1 can interact with the human epithelial sodium channel to inhibit its activity [17], and the COMMD1 can also inhibit

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the NF-κB activity through the COMMD1-ECSSOCS1 complex [8,9,18]. Moreover, the COMMD1 is required for normal clearance of circulating LDL [19]. In recent years, studies about other COMMD proteins have been paid close attention. For example, a study has revealed that COMMD7 acts as a novel NEMO interacting protein to participate in the termination of NF-κB signaling [20]. The COMMD8 forms complex with coiled-coil domain-containing protein (CCDC22) and Cullin1 to mediate the degradation of IκBa protein, whilst the depletion of COMMD8 also impairs IκBa degradation leading to that NF-κB transcriptional activity is inhibited [21]. The COMMD4 as the target of PKA is able to interact with MMGL [22], and is also potentially involved in amphioxus innate immune response [23]. *AmphiCOMMD4* was ubiquitously and differentially expressed in muscles, gills, intestine, hepatic cecum and gonad. The highest expression was found in gonad [23]. The COMMD5 is found to participate in the regulation of cell proliferation and cell migration [24,25], and the COMMD6 can interact with creatine kinase to inhibit the activity of creatine kinase in amphioxus [26]. It's worth noting that the COMMD9 interacts with TFDP1 to promote TFDP1/E2F1 transcriptional activity in non-small cell lung cancer [27]. Specially, COMMD3 and COMMD9, as endogenous regulators of EnaC, can also regulate Na^+ transport through altering ENaC cell surface expression [28].

Amphioxus is a crucial organism for the understanding of chordate evolution [29]. However, the function and evolution of the *COMMD* gene family in amphioxus are yet largely unclear. To further understand the function and evolution of the *COMMD* family members, in this study, we have cloned and characterized the *COMMD* gene family members from Chinese amphioxus (*Branchiostoma belcheri*) (designated as *AmphiCOMMDs*), and analyzed all *AmphiCOMMD* gene structures, and protein primary structures, as well as domain architectures. In addition, quantitative RT-PCR analysis shows that all transcripts of *AmphiCOMMD* genes are extensively distributed in all tested amphioxus tissues, and the temporal expressions levels of all *AmphiCOMMD* genes after LPS stimulation are fluctuating. Our results have provided important insights into the innate immune role and the evolution dynamic of the *COMMD* gene family in amphioxus.

2. Material and methods

2.1. Cultivation and immune stimulation of amphioxus

Matured adults of amphioxus (*Branchiostoma belcheri*) were obtained from Zhanjiang (Guangdong province, China), and then cultured at 24–25 °C in a tank filled with air-pumped circulating artificial seawater and supplied with Chlorella daily. Fifteen microliters of LPS PBS suspension (1 mg/ml) was injected into the coelom of amphioxus and cultured in tanks at 28 °C. PBS was injected as the negative control. Six animals (3 LPS stimulated amphioxuses and 3 PBS stimulated amphioxuses) were separately collected and frozen by liquid nitrogen for RNA extraction at 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 24 h and 48 h after injection.

2.2. Isolation of full-length AmphiCOMMD cDNAs

Total RNA of the whole amphioxus was extracted using Trizol reagent (Invitrogen, Carlsbad, USA) as described in manufacturer's instruction. The first strand of cDNA was synthesized with Moloney Murine Leukemia Virus reverse transcriptase (M-MLV; TaKaRa, Dalian, China). Except for *COMMD4* and *COMMD6*, the fragments of the other eight *AmphiCOMMD* cDNAs were cloned by RT-PCR using primers in Table S1, which were designed based on the sequence of annotated information of *Branchiostoma belcheri*. To obtain complete cDNA sequences, 5′ and 3′ RACE procedures were performed using SMARTer™ RACE cDNA Amplification Kit (Clontech, CA, USA) and First Choice®RLM-RACE Kit (Ambion, Austin, TX, USA), respectively, as described in manufacturer's instruction. According to the partial sequence obtained, specific primers used for 5′ and 3′ RACE were listed in Table S1. Finally, the end to end primers (Table S1), were used to amplify the full-length cDNA. The amplified fragments were cloned into pMD®19-T Simple Vector (TaKaRa, Dalian, China) and sequenced.

2.3. Sequence analysis of AmphiCOMMDs

These predicted AmphiCOMMD protein sequences were analyzed using the BLAST network server at the NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The domains of AmphiCOMMD proteins were predicted with PROSITE (http://prosite.expasy.org/)). The COMM domains of all AmphiCOMMDs were aligned using the ClustalX program. For sequence analysis, ten COMMD protein sequences of *Homo sapiens* were acquired from Ensembl, next the BLASTP was used to search human COMMD1-10 homologous proteins in *Drosophila melanogaster*, *Strongylocentrotus purpuratus*, *Ciona intestinalis*, *Danio rerio*, *Oryzias latipes* and *Xenopus tropicalis* from Ensembl database. Gene structure and position were analyzed by hand. Conserved motifs were analyzed online using a MEME system Version 4.11.4 (http://meme-suite.org/tools/meme) with default settings. Except for the distribution of motif occurrences, the minimal and maximal motif widths and the number of different motifs were defined as any number of repetitions, 6, 50, and 15, respectively. The COMMD1-10 proteins from different evolutionary status were used for phylogenetic analysis. Multiple protein sequences were aligned using the MUSCLE program. The phylogenetic tree was constructed with the MrBayes using the full-length protein sequences.

2.4. Codon-based sequence analysis

The ratio of nonsynonymous/synonymous substitution rates ($\omega = dn/ds$) could provide a measurement for the change of selective pressures. Here, the CODEML program in the PAML4.4 software package [30] was used to analyze changes in selective pressure of COMMD genes. To test the presence of sites under positive selection, the presence of codons evolving under positive selection was tested by contrasting the M1a and M2a models, and the M7 and M8 models by likelihood ratio tests (LRTs). Afterwards, the nonsynonymous/synonymous substitution rate ratio of COMMD genes was computed by branch model. Finally, positive selection sites in the AmphiCOMMD sequences were detected by applying a branch-site model. The statistical analysis was carried out by Bayes empirical Bayes (BEB) methods [31].

2.5. Real-time RT-PCR analysis of AmphiCOMMD mRNAs

To test spatial distribution of the *AmphiCOMMDs* in different tissues, total RNA was extracted from gills, hepatic cecum, intestine, muscles and notochord of fifteen healthy adult amphioxuses. Due to limited tissue of notochord collected, two *COMMD* genes were not detected. In the temporal expression analysis of *AmphiCOMMDs*, RNA was extracted from the whole amphioxus collected from different time points after injection. Total RNA was extracted by the Trizol reagent (Invitrogen, Carlsbad, USA), and the cDNA was synthesized by PrimeScript® RT reagent kit (TaKaRa, Dalian, China). Primers for qRT-PCR of *AmphiCOMMDs* were designed using Beacon Designer 7.0 software (Table S1). In parallel, the *β-actin* gene of amphioxus was used as internal control for normalizing the expression level of *AmphiCOMMDs*. The RT-PCR was performed in the condition: 95 °C for 10 min, 40 cycles (95 °C for 10 s and 62 °C for 30 s). All samples were analyzed in three replicates, the results were shown in terms of relative mRNA level as mean ± SE (n = 3). The results were analyzed using the two tailed Student's t-test with statistical significance for $P < 0.05$.