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Developmental Expression Patterns of *ythdf* Genes in *Xenopus Tropicalis*

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Abstract

As the readers of N6-methyladenine(m6A), there are a lot of studies about *ythdf1* (YTH N6-methyladenosine RNA binding protein 1), *ythdf2* (YTH N6-methyladenosine RNA binding protein 2) and *ythdf3* (YTH N6-methyladenosine RNA binding protein 3). While in order to searching the function during embryonic development of *ythdf1*, *ythdf2* and *ythdf3*, we used *Xenopus tropicalis* as the experimental animal. To explore the conservatism of *ythdf* in evolution, we searched *Xenopus tropicalis* database with different species' YTHDF proteins sequence, making the evolutionary trees. And we studied both temporal and spatial expression patterns of *ythdf1*, *ythdf2* and *ythdf3* of *Xenopus tropicalis*. Real-time quantitative PCR results showed that the expression of the three genes was increased from stage 0 to stage 36. Whole-mount in situ hybridization showed that *ythdf1* was expressed in eyes, branchial arch, notochord, hindbrain and forebrain. Both *ythdf2* and *ythdf3* were expressed in eyes and hindbrain at stage 36, while *ythdf2* was expressed in branchial arch, too. Thus, the developmental expression patterns of these three *ythdf* genes are different in Xenopus embryos. These results provide a basis for further functional study of these three genes.

Keywords

ythdf1*; *ythdf2*; *ythdf3*; Developmental Expression; *Xenopus Tropicalis.

1. Introduction

Multiple modifications are present on RNA, such as N6-methyladenine (M6A), 5-Methylcytosine (M5C), N1-methyladenine (m1A) and pseudouracil, etc. M6A is the most abundant methylated modified form in eukaryotic mRNA, and it is also the most studied. It is a type of RNA modification that is thorough. The N6-methyladenosine modification is the most prevalent post-transcriptional mRNA modification, regulating mRNA decay and splicing. It plays a major role during normal development, differentiation, and disease progression. The modification is regulated by a set of writer, eraser, and reader proteins [1]. The proteins used to write, erase, and read are methyltransferase, demethylase, and binding proteins, respectively. The main components of the methyl transferase complex found so far include METTL3, METTL14, WTAP and KIAA1429, etc., while FTO and ALKBH5 as demethylases can remove the methylation. The biological function of m6A modification mainly plays a post-rna regulatory role through m6A binding proteins. Currently, the known binding proteins include YTH domain proteins (YTHDF1, YTHDF2, YTHDF3, YTHDC1 and YTHDC2) and nuclear heterogeneous ribonucleoprotein HNRNP family proteins (HNRNPA2B1, HNRNPC and HNRNPG). YTHDF1 can promote the ribosomal loading of M6A-modified mRNA molecules, and improve the translation efficiency of target mRNA by interacting with translation initiation factors [2]. YTHDF2 competitively binds methylated transcripts with rRNA that mediates mRNA translation, thereby affecting the half-life of RNA, accelerating RNA degradation, and affecting mRNA stability [3]. In zebrafish, YTHDF2 also accelerates the clearance of m6A-modified maternal mRNA and it is

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Involved in the endothelial-to-hematopoietic transition [2,4]. The interaction of YTHDF3 with YTHDF1 and YTHDF2 can enhance the binding of YTHDF3 and YTHDF1 to the substrate M6A-mRNA, thus promoting the protein translation efficiency or degradation of M6A-mRNA [5,6].

2. Experimental procedures

2.1 Blast searches and phylogenetic analysis

Percentages of identical amino acids of human YTHDF proteins sequence with different species were calculated by BLAST (BLASTP) searches in the Ref-Seq protein database at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Protein sequence alignments and phylogenetic tree were performed using the neighbor-joining method in MEGA-X software. Protein conserved domains were retrieved by protein database at NCBI (http://www.ncbi.nlm.nih.gov/protein/).

2.2 Quantitative reverse transcription-PCR (qPCR) analysis

Total RNA was extracted from Xenopus embryos at indicated developmental stages using the TRI reagent (Molecular Research Center Inc., USA). cDNA was synthesized from total RNA (2 μg) and oligo (dT) 18 primers (0.5 μg) using the ReverTra Ace® qPCR RT Kit (Toyobo, Japan). Real-time PCR was performed using a Light Cycler 480 SYBR Green I Master (Roche, USA) and the MiniOpticon Real-Time PCR System (Bio-Rad, CA, USA). The sequences of the qPCR primers were listed as following, ythdf1 Fw, 5'-CTC AGC CTC CAC CAA TGT CTC-3'; ythdf1 Re, 5'-GTA AGC AGC GTT CCT GTT CC-3'; ythdf2 Fw, 5'-GTT ATT ACA GCC CAT CTA TCA G-3'; ythdf2 Re, 5'-AAT GTG GTT CTC CGT TGC TC-3'; ythdf3 Fw, 5'-TAC CTC CAG TTA GCA GTT CA-3'; ythdf3 Re, 5'-TTT ATC ATC CCA AGT TCC A-3'; odc Fw, 5'-TGT TCT GCG CAT AGC AAC TG-3'; odc Re, 5'-ACA TCG TGC ATC TGA GAC AGC-3'. Ornithine decarboxylase (odc) was used as the internal standard control. After denaturation for 10 min at 95°C, the reactions were subjected to 45 cycles of 95°C for 30s, 60°C for 30s, and 72°C for 30s odc was used as the internal standard control to normalize gene expression using the △△Ct method.

2.3 Probe preparation and whole-mount in situ hybridization

The whole ORFs of *X. tropicalis* ythdf1 (accession number: NM_204061.1), ythdf2 (accession number: XM_002934644.4) and ythdf3 (NM_001126961.1) were amplified using cDNA templates from st42 tadpoles. Primers used for RT-PCR are as follows: ythdf1 Fw (BamH1): 5'- CGG GAT CCCT AGC AAG AGT CGG GTT TAG CC -3'; ythdf1 Re (Xhol): 5'- CCC TCG AGG GGC GGG ACC ATA GTG GAC GGA C -3'; ythdf2 Fw (BamH1): 5'- CGG GAT CCC TAA TTC TTG ATC ACC TTT TTG A -3'; ythdf2 Re (Xhol): 5'- CCC TCG AGA TGT CCG CCA GTA GTC TTC TGG A -3'; ythdf3 Fw (Xhol): 5'- CCC TCG AGA TGT CTG CGA CCA GTG TTG ATC -3'; ythdf2 Re (BamH1): 5'- GAG AGG AAT AGA AAC AAA CAA TAA GGA TCC CG -3'. PCR fragments were then subcloned into pBlueScript II SK (+) plasmid and verified by sequencing. Plasmids were linearized and then used as templates for synthesis of digoxigenin-labeled antisense probes with T7 RNA polymerase (Roche, Indianapolis, IN).

Whole-mount in situ hybridization was performed using digoxigenin-labeled antisense RNA probe and anti-digoxigenin monoclonal antibody labeled with alkaline phosphatase. Probe signals were developed using NBT/BCIP (Roche, USA) as previously described [7]. Vibratome sectioning was performed as previously described [8-12].

3. Results

3.1 Phylogenetic analysis of YTHDF proteins from X. *tropicalis* and other species

To explore the conservatism of *ythdf* in evolution, protein sequences were aligned among different vertebrates. Phylogenetic trees illustrated the evolutionary distance of YTHDF proteins in human, mouse, rat, *X. tropicalis*, and *Danio rerio* (Figure.1). These results reveal that YTHDF proteins are conservative during the evolution of vertebrates.

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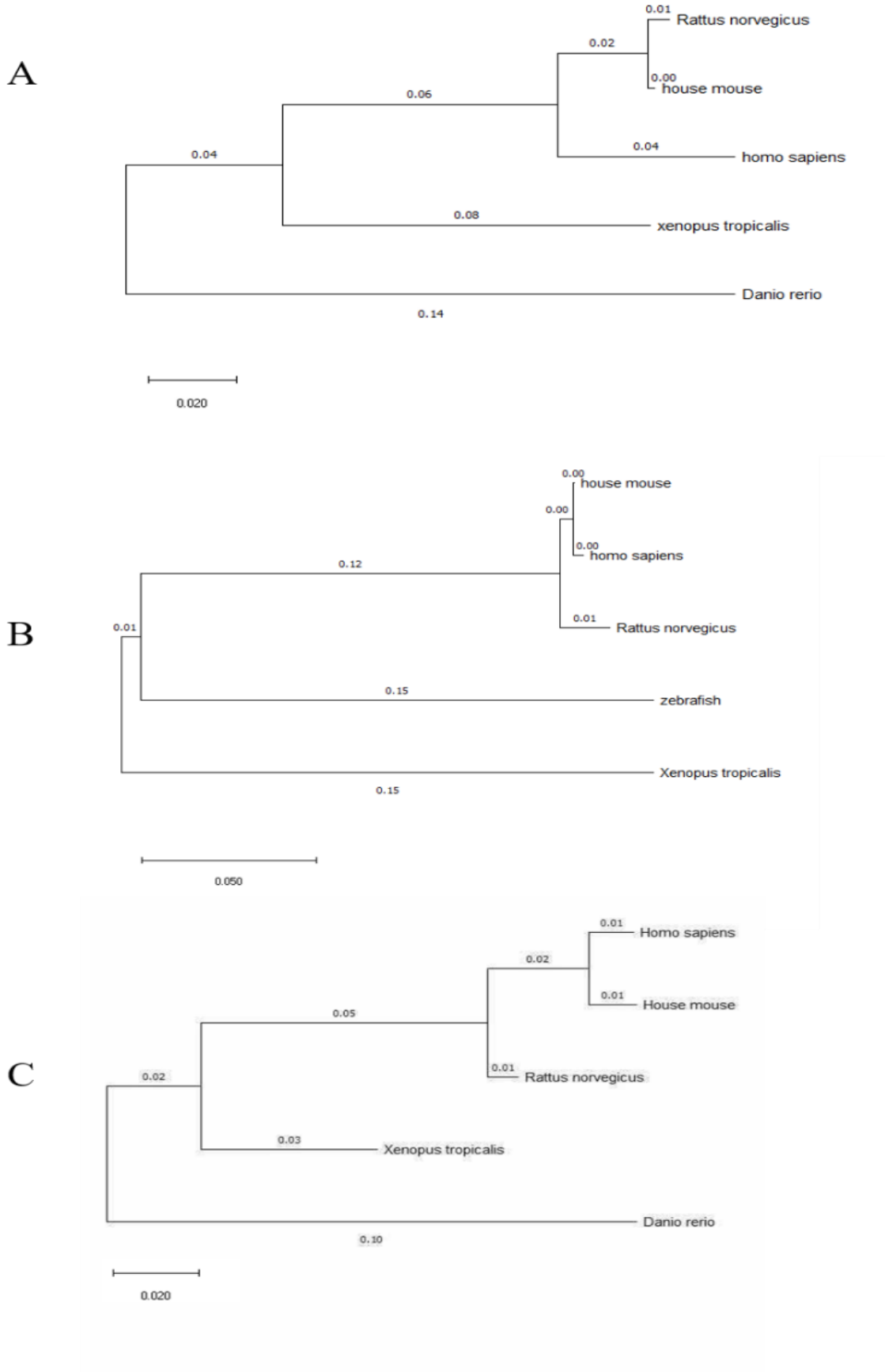

Figure 1. Identification of three ythdf genes from *Xenopus tropicalis*. Phylogenetic analysis of YTHDF1(A), YTHDF2(B) and YTHDF3(C) proteins from *Xenopus tropicalis*, human, mouse, rat and *Danio rerio*.

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3.2 Temporal expression of xenopus *ythdf* genes

The temporal expression patterns of *ythdf* genes during early embryonic development of *X. tropicalis* were analyzed by quantitative RT-PCR. As shown in Figure. 2, basic expression of *ythdf* mRNA was detected from stage 0 to stage 36. However, the expression of *ythdf* at stage 15 to stage 36 was significantly increased. Especially at stage 36, the expression of *ythdf1* was upregulated by~10 folds (Figure. 2A), the expression of *ythdf2* was dramatically upregulated by ~250 folds (Figure. 2B), the expression of *ythdf3* was significantly upregulated by~400 folds (Figure. 2C). Consistent with *ythdf3*, a basic expression of *ythdf2* and *ythdf1* were detected from stage 0 to stage 36, followed by a significant increase at stage 15 and stage 36. Notably, the expression of *ythdf1* and *ythdf2* are significantly lower compared with *ythdf3* at stage 36. These results indicate the different temporal expression patterns between *ythdf1*, *ythdf2* and *ythdf3* during embryonic development of *X. tropicalis*, implying the potentially different functions of these three genes.

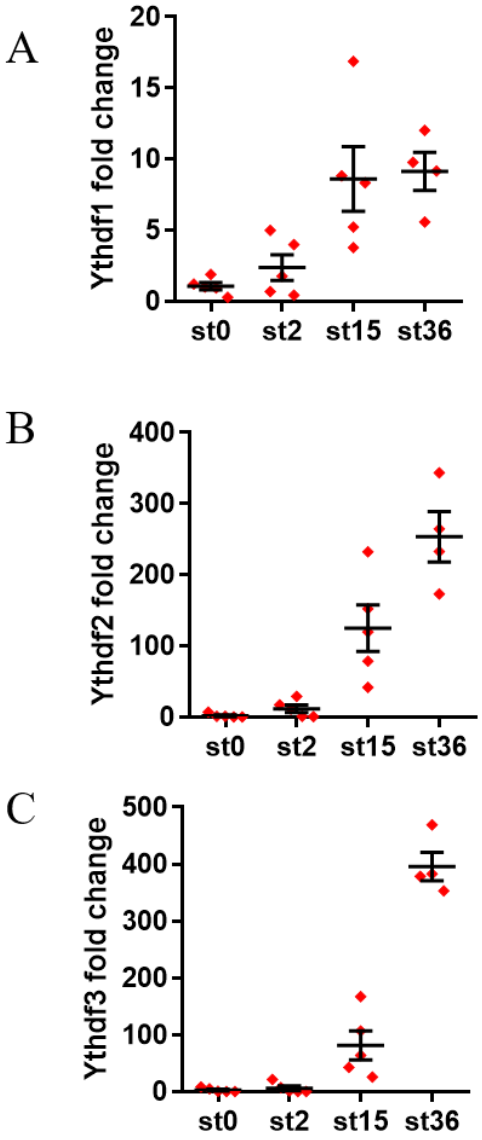

Figure 2. Temporal expression of *ythdf* genes in early development of *X. tropicalis*. The mRNA expression of *ythdf1*(A), *ythdf2*(B) and *ythdf3*(C) genes was analyzed by qPCR using total RNA isolated from *X. tropicalis* embryos at indicated developmental stages. Ornithine decarboxylase (odc) was used as the internal standard control. Results are presented as mean ± SEM (n = 5 experiments), ***p < 0.001. St, stage.

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3.3 Spatial expression patterns of xenopus *ythdf* genes

To further determine the spatial expression patterns of *ythdf* genes, whole-mount in situ hybridization (WISH) analysis was performed by using *X. tropicalis* embryos at different stages. As shown in Figure. 3, the expression of *ythdf1* was shown in Figure. 3 A2 to D2, *ythdf2* was shown in Figure. 3 A3 to D3, *ythdf3* was shown in Figure. 3 A4 to D4. Intense expression of *ythdf* was detected from stage 0 to stage 15. *ythdf* signals was detected observably at stage 36(Figure. 3). The intense expression of *ythdf1* at stage 36 was mainly detected in eyes, branchial arch and notochord in addition to moderate expression in somite (Figure.3, B4). The expression of *ythdf2* at stage 36 was mainly detected in the same regions as *ythdf1* but weaker. The expression of *ythdf3* was detected in eyes and branchial arch, too (Figure. 3, D4).

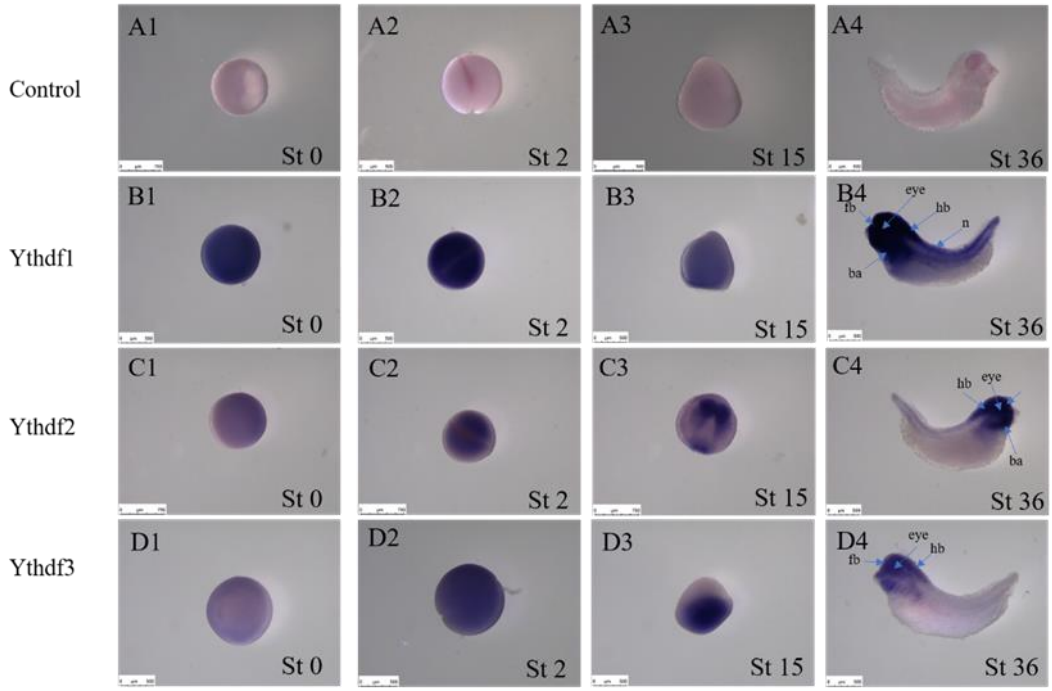

Figure 3. Spatial pattern of *ythdf1*, *ythdf2* and *ythdf3* analyzed by whole mount in situ hybridization. (A1, B1, C1 and D1) Stage 0, animal view. (A2, B2, C2 and D2) Stage 2, animal view. (A3, B3, C3 and D3) Stage 15, dorsal view. (A4, B4, C4 and D4) Stage 36, dorsal view. Ba, branchial arches; fb, forebrain; hb, hindbrain; n, notochord. B1-D4 indicates the sense probe control.

4. Discussion

In recent years, more and more studies have shown that RNA methylation modification plays an important role in regulating animal reproduction, growth and development. m6A is the most prevalent internal mRNA modification and is known to control transcript splicing, turnover, and translation to dictate gene expression changes. As binding proteins that play the role of m6A methylation modification, the YTHDF family has great significance in animal body. Some proteins can specifically bind to sequences containing m6A modification, which are related to mRNA shearing, degradation and translation. Most of the previous studies on m6A binding protein were based on a mouse model.

In this study, we used *X. tropicalis* as the experimental model and searched that YTHDF proteins are conservative during biological evolution (Figure. 1), although the detailed functions of these proteins are different in individual cell type or species. For example, in the cytosol, YTHDF1 enhances translation of its targets by interacting with initiation factors and facilitating ribosome loading, YTHDF2 promotes mRNA degradation by localizing m6A-modified mRNA to processing bodies in

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the cytoplasm, and could change its own cellular localization in response to heat-shock stress. Moreover, we examined the spatial and temporal expression pattern of *ythdf1*, *ythdf2* and *ythdf3* genes during early embryonic development of *X. tropicalis*, and found the different expression patterns for these three genes at different stages.

Acknowledgments

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