

Bioinformatic Analyses of CCCH-Zinc Finger Family in Zebrafish (*Danio rerio*)

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Abstract.- CCCH-type zinc finger (ZF) proteins are RNA-binding proteins with regulatory functions at all stages of mRNA metabolism. In this study, a genome-wide survey of CCCH ZF genes in zebrafish was performed for the first time. A total of 51 CCCH genes were identified, but most have not been previously characterized. CCCH motifs and other functional domains in the CCCH protein family were classified and analyzed. Phylogenetic and alignment analyses revealed that the zebrafish CCCH family could be divided into seven groups. Unlike in mammals, CCCH proteins with six CCCH motifs were not found in zebrafish. The fraction of CCCH motifs with C-X₇₋₈-C-X₅-C-X₃-H type (63.5%) was smaller than that found in mouse CCCH proteins (79%).

Key words: CCCH motif, transcriptional regulation, RNA-binding protein (RBP), zebrafish.

INTRODUCTION

Zinc finger (ZF) motifs, which are classified based on the arrangement of the zinc-binding amino acids, are present in many transcription factors and have critical functions in interactions with other molecules. ZF proteins are generally considered as DNA-binding transcription factors, but certain classes of ZF proteins, including the common CCHH-type (Cys-Cys-His-His) motif, function as RNA-binding proteins (RBPs) (Netti *et al.*, 2013). One group of RBPs is defined by the presence of CCCH type ZF motif that directly binds to RNA.

The CCCH-type ZF is less common compared with other types of ZFs (CCHH and CCCC), and represents approximately 0.8% of all ZFs (Hall, 2005). After analysis of the entire CCCH family in *Arabidopsis* and rice, Wang *et al.* redefined that CCCH proteins are characterized by 1-6 C-X₄₋₁₅-C-X₄₋₆-C-X₃-H motifs, which are Gly- and Phe-rich sequences (Wang *et al.*, 2008). In general, they are RBPs with regulatory functions at all stages of mRNA metabolism. Different CCCH proteins regulate all stages of mRNA life. Among the most

studied CCCH proteins are the proteins of the TIS11 family, and the most characterized is the mammalian protein tristetraproline (TTP). TTP is an RBP that binds AU-rich elements located in the 3'-untranslated region of the mRNAs that encode for important proinflammatory cytokines, including tumor necrosis factor- α , granulocyte/macrophage colony-stimulating factor, and interleukin-2 (Morgen and Massi, 2010; Ogilvie *et al.*, 2005). U2af1 is a small subunit of the U2 small nuclear ribonucleoprotein auxiliary factor that contains CCCH domains, and has an important function in the regulation of pre-mRNA splicing (Kanadia *et al.*, 2003). The muscleblind-like protein family, which is also a member of CCCH proteins, is essential for muscle and eye differentiation by controlling proper pre-mRNA splicing of several important genes, including skeletal muscle chloride channels, cardiac troponin T, and insulin receptor (Ho *et al.*, 2004). Zfp3612, similar to its relative Zfp36, is also a mRNA-binding and destabilizing protein that functions in the physiological control of female fertility at the level of early embryonic development (Ramos *et al.*, 2004). Recent studies revealed the possibility of a functional module of CCCH ZF genes in the regulation of macrophage activation (Liang *et al.*, 2008). These studies implied that CCCH proteins may be critical regulators in basic biological processes, such as embryonic development and immunity and inflammatory

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response.

Zebrafish (*Danio rerio*) is an excellent vertebrate model for both embryologic analysis and functional genomics (Alestrom *et al.*, 2006; Koc and Akbulut). As of this writing, the molecular features of the CCCH family have not been documented in zebrafish. With the availability of complete genome and large EST database, large-scale analysis of CCCH ZF motifs may yield informative conclusions. In this study, we performed a bioinformatic survey and comparison of CCCH proteins in zebrafish to gain an overall insight into the entire CCCH ZF gene family, and evaluate their potential function in activation.

MATERIALS AND METHODS

Sequence searches were made at NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>) using BLAST and PSI-BLAST in the non-redundant and EST database. Novel genes were searched from this database and the zebrafish genome database by tBLASTn. Blast algorithms at GenBank were used to search against the corresponding database with the defined CCCH proteins as the query. To verify the reliability of our results, we also searched several other databases, including EMBI-EBI, Pfam, and SMART, using the terms “CCCH zinc finger, *Danio*” or “C3H zinc finger, *Danio*.”

After checking the sequences and removing the redundancies, all sequences were edited and assembled in SeqMan 4.0 (DNASTAR Inc.). ClustalX 1.8 program (Thompson *et al.*, 1997) and services in EMBI-EBI (<http://www.ebi.ac.uk/service>) were used to generate a multiple sequence alignment for a given set of homologous sequences. Secondary structure predictions of the proteins were analyzed using ExPASy Proteomics Server (<http://prosite.expasy.org/>) (Gasteiger *et al.*, 2003).

Phylogenetic analyses were performed using maximum parsimony (MP) and Bayesian methods. MP analyses were carried out with PAUP 4.0b10 (Swofford, 2002). Bootstrap proportions (BP_{MP}) were obtained from 1000 replicates using 10 replicates of random stepwise addition of taxa. An additional program, MEGA 4.0 (Tamura *et al.*, 2007), was used to confirm the results.

RESULTS AND DISCUSSION

Identification of genes encoding CCCH ZF protein in zebrafish

We identified homologous proteins from NCBI in the protein and nucleotide databases to obtain a complete set of zebrafish CCCH-type ZF proteins. After checking the sequences and removing the redundancies, 51 proteins with typical CCCH motif encoded by 48 genes were identified. Two genes *viz.*, *zfp3611* and *mbnl2*, were found to contain two and three isoforms, respectively.

Among the 51 zebrafish CCCH proteins, 15 proteins were annotated as the predicted protein. Six proteins (*mbnl2b*, *mbnl2c*, *mbnl2d*, *mkrn1*, *zgc:154073*, and *si:ch211-244b2.4*) contained the CCCH motifs with confidence values but have not yet been defined. In addition, four proteins, *viz.*, *ccdc76* (GenBank accession no. NP_001014331), *traf7* (NP_001073654), *zc3h18* (NP_001037804), and *LOC100536074* (XP_003201692), respectively, contained a CCCH motif but could not be detected by ExPASy Proteomics Server, and they were eliminated from our analysis.

The complete list of genes is shown in Supplementary Data 1. The size of zebrafish CCCH proteins varied from 173 aa (*zmat5*) to 1860 aa (*zgc:77407*). The CCCH proteins were located at all the zebrafish chromosomes, except chromosomes 23 and 25. Among the 51 CCCH proteins, 12 proteins (*e.g.*, TTP family, muscleblind-like family, and U2af family) are involved in RNA metabolism, including pre-mRNA splicing, mRNA transportation, subcellular localization, and stability (Kanadia *et al.*, 2003).

Analysis and classification of CCCH ZF motifs

The CCCH ZF is a highly conserved motif that has been widely found in plants, invertebrates, and vertebrates. In this study, classification analysis of zebrafish CCCH protein was performed using the ExPASy Proteomics Server program. As shown in Figure 1, among the identified 51 CCCH proteins, 28 contained one to five copies of CCCH motifs, whereas 23 contained a CCCH motif and several other functional domains. Previous research revealed that CCCH proteins contain one to six copies of CCCH-type motifs characterized by three

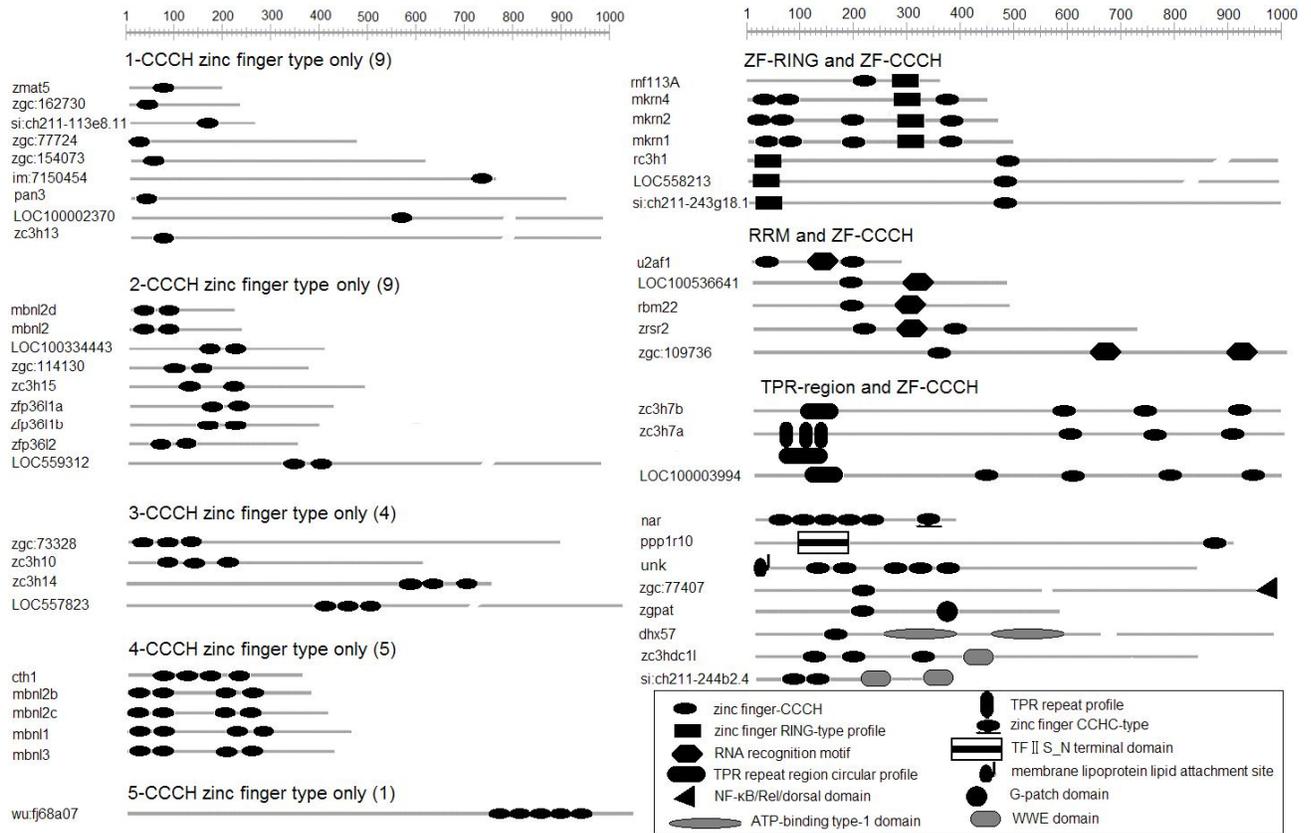


Fig. 1. Schematic structures of the identified CCCH proteins. The proteins were grouped according to the number of CCCH ZF motifs. The CCCH motifs are shown by oval boxes. The other conserved domains are also indicated by different boxes denoted at the right-bottom corner.

Cys and one His. For example, proteins noted as BC19429 and Zc3h5 in mice contain six CCCH ZFs (Liang *et al.*, 2008). Compared with previous studies, we did not obtain any protein with six copies of the CCCH motif. A protein of interest is nar, which contains a single CCHC-type motif and five copies of the CCCH motif, and is quite similar to the six tandem CCCH motifs in structure.

Besides containing CCCH motifs, some CCCH proteins also carry a series of other functional domains, which can direct the CCCH proteins to certain complexes and mediate specific activities. In this study, seven proteins (rnf113A, mkrn4, mkrn2, mkrn1, rc3h1, LOC558213, and si:ch211-243g18.1) simultaneously contained a CCCH motif and RING-type ZF domains. RING-type ZF (C3HC4 type) is possibly involved in mediating protein-protein interactions and

transcription regulators (Vinuesa *et al.*, 2005). Many proteins containing a “RING finger” have an important function in the ubiquitination pathway (Serrano and Guzman, 2004). Moreover, five proteins (U2af1, LOC100536641, Rbm22, Zrsr2, and Zgc: 109736) contain CCCH motifs and eukaryotic RNA recognition motifs (RRMs). RRM, also known as RNA-binding domain (RBD) or ribonucleoprotein domain, is the most abundant RBD in higher vertebrates (present in about 0.5% to 1% of human genes) (Venter *et al.*, 2001) and the most extensively studied RBD. Some RRM and ZFs recognize RNA sequence specifically with multiple copies. In mice, some members in the Rbm, Zrsr, and U2af subfamilies display the same motif (Liang *et al.*, 2008).

Some proteins in the same family display similar domain architecture. For example, the mkrn

family members (mkrn1, mkrn2, and mkrn4) contain a RING-finger motif at the middle region and more than one CCCH ZF at the side. Moreover, all mbnl family members (mbnl1, mbnl2b, mbnl2c, and mbnl3) contain four tandem CCCH domains. As shown in Figure 1, other conserved domains, including tetratricopeptide repeat region, transcription factor IIS, N-terminal domain, NF- κ B/Rel/dorsal domain, and ATP-binding type domain, were also found in this study.

Phylogenetic analysis and sequence comparison of zebrafish CCCH proteins

Phylogenetic analysis was performed based on the alignment of 51 full-length CCCH protein amino acid sequences by the maximum likelihood method to evaluate the evolutionary relationship within the zebrafish CCCH protein family. An alignment of full-length amino acid sequences of CCCH proteins was constructed using ClustalW, and then refined manually (Fig. 2). Bootstrap analysis with 1000 replicates was performed for statistical reliability. The strong amino acid sequence conservation within each subfamily was evident from the short branch lengths at the tip of the trees, which indicates strong evolutionary relationships among subfamily members.

Based on the statistical support of each branch, we divided the total studied proteins into seven groups, which were designated as group I to VII. Different methods were utilized to construct the tree, and similar results were consistently reproduced.

As shown in Figure 2, some proteins belonging to the same subfamily were clustered into the same groups. Other proteins previously not recognized as subfamilies were clustered together, which suggests that they may belong to the same subfamily. For example, zc3h15 may form a subfamily with u2af1 and zrsr2 rather than with zc3h13 and zc3h14, whereas zc3h10 and zc3h7a may form another subfamily.

Among 51 proteins, 115 CCCH motifs were identified and analyzed to uncover the CCCH motif sequence features in zebrafish. Approximately 63.5% CCCH motifs analyzed were C-X₇₋₈-C-X₅-C-X₃-H type (73 motifs in 115). The fraction of this type of CCCH motif was smaller than that found

among mouse CCCH proteins, which reached 79%. Moreover, 13 CCCH motifs in other proteins were C-X₇₋₈-C-X₆-C-X₃-H type.

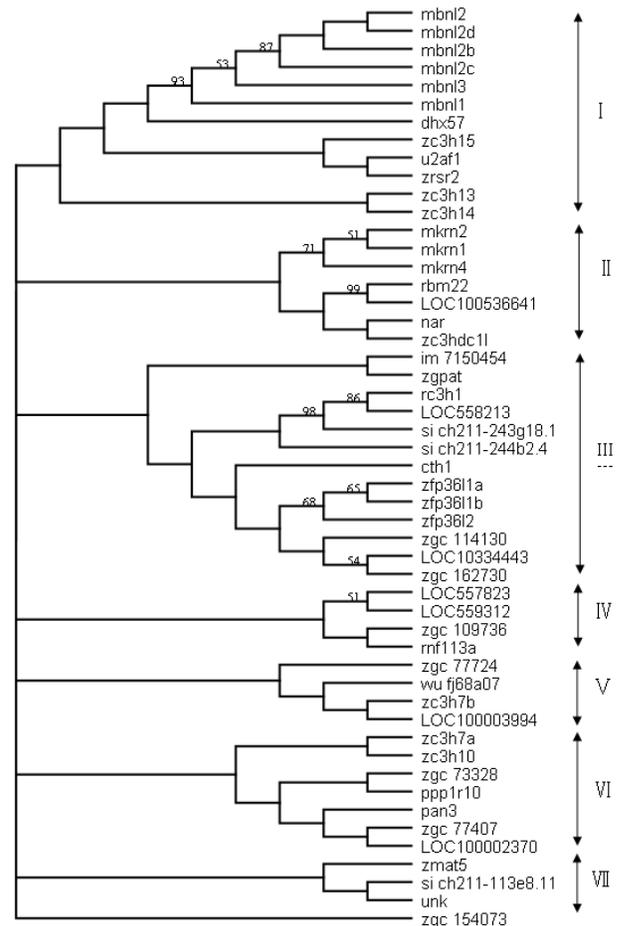


Fig. 2. Phylogenetic analysis of 51 zebrafish CCCH genes. The unrooted neighbor-joining tree was constructed based on the alignment of the full-length amino acid sequences of 51 zebrafish CCCH proteins using ClustalW from DNASTAR. The zebrafish CCCH family was mainly divided into seven groups based on their evolutionary relationship, which is denoted by the grey vertical bars on the right of the figure. The proteins were named according to their gene name.

According to motif classification analysis and without considering different kinds of motifs, 20 CCCH proteins contained a single CCCH motif, whereas the other 31 proteins contained more than one CCCH motif. The first CCCH motif in these

proteins was selected for amino acid sequence alignment analysis because multiple motifs in the same protein are usually similar and have redundant functions. As shown in Supplementary data 2, three Cys and one His residues were completely conserved in all CCCH motifs. Besides the CCCH residues, Gly and Phe were also highly conserved. However, the space and amino acids between CCCH residues varied, which may determine their substrate specificity.

As a consequence of whole genome searching, 51 CCCH proteins were identified in zebrafish in this study. The family size of zebrafish CCCH genes was smaller than that of the CCCH gene family in human and mice, which contained 55 and 58 members, respectively. CCCH gene family members in *Arabidopsis* and rice can even reach up to 68 and 67 members, respectively (Wang *et al.*, 2008). Although most CCCH proteins are less characterized, some CCCH proteins have important functions in many cellular processes, especially RNA metabolism. For example, we identified several muscleblind-like family genes in zebrafish (*e.g.*, *mbn1*, *mbn2*, and *mbn3*). The muscleblind (*mb1*) gene was initially identified in *Drosophila* (Begemann *et al.*, 1997), in which four alternatively spliced forms of the transcripts were described (*mb1A–D*). Compared with *Drosophila*, which only has a single *mb1* gene, three muscleblind-like genes (*mbn1*, *mbn2*, and *mbn3*) have been identified in humans and mice (Kanadia *et al.*, 2003). Unlike *mbn2* in mammals that have 4-CCCH ZFs, we found that homologous proteins in zebrafish carried 2-CCCH ZFs. The expression of zebrafish *mbn1* was consistent with mammalian *mbn1* in some aspects, which indicates that zebrafish *mbn1*s have overlapping but distinct functions than their mammalian orthologs. The CCCH protein with six tandem ZF motifs was not found in our study.

The zebrafish, with transparent embryos, external fertilization, clutch size, moderate generation time, and accessibility for genetic operation, has become an attractive vertebrate model organism in many research areas that range from basic developmental biology to applied toxicology (Pauli *et al.*, 2012). Research on zebrafish has provided important contributions to our understanding of development and diseases.

Upon working in the zebrafish genome, we were able to non-redundantly and comprehensively identify the CCCH ZF proteins, thereby providing a complete complement of these coding sequences in zebrafish on a whole-genome scale. Subsequently, we obtained a global perspective of this protein family in zebrafish. Although CCCH motifs are highly conserved, CCCH proteins of zebrafish may possibly reveal differences to other vertebrates in mRNA metabolism.

Our results provide an overall feature of the CCCH family in zebrafish. A total of 51 CCCH genes were identified, and most have not been previously characterized. Experimental approaches are necessary to examine the functions of the many uncharacterized genes.

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