The NCK and ABI adaptor genes in catfish and their involvement in ESC disease responses

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Abstract

Adaptor proteins non-catalytic region of tyrosine kinase (NCK) and Abelson interactor (ABI) are crucial for disease response. NCK1 was identified to be a candidate gene for enteric septicemia of catfish (ESC) disease resistance, and was speculated to play similar roles during ESC and enteropathogenic *Escherichia coli* (EPEC) pathogenicity. ABI1 was reported as a positional candidate gene for bacterial cold water disease (BCWD) resistance in rainbow trout. In this study, three NCK genes and six ABI genes were identified in the channel catfish (*Ictalurus punctatus*) genome and blue catfish (*I. furcatus*) transcriptome, and annotated by domain structures, phylogenetic and syntenic analyses. Their expression patterns were examined in the intestine and liver of catfish after challenge with *Edwardsiella ictaluri*. In the intestine, NCK1, ABI2a, ABI2b, ABI3a were differentially expressed after *E. ictaluri* infection. In the liver, NCK2a, NCK2b, ABI1b, ABI2a, ABI2b were significantly upregulated in ESC susceptible fish. In general, the NCK and ABI genes, with exception of ABI3a gene and NCK1 gene, were expressed at higher levels in susceptible fish after infection than in control fish, but were expressed at lower levels in resistant fish than in the control fish. Taken together, these results support the notion that NCK and ABI genes are involved in disease processes facilitating pathogenesis of the *E. ictaluri* bacteria.

Keywords: adaptor gene, NCK, ABI, catfish, *Edwardsiella ictaluri*, immunity

1. Introduction

Adaptor proteins are major contributors to the control of cross-talk between signaling cascades (Flynn, 2001). They contain a variety of protein-binding modules that link protein-binding partners together and facilitate the creation of larger signaling complexes. Non-catalytic region of tyrosine kinase (NCK) and Abelson interactor (ABI) are important adaptor proteins that are involved in many signaling pathways (Cowan and Henkemeyer, 2001). In mammals, NCK protein family has two members, NCK1 and NCK2 (Chen et al., 1998), while ABI protein family has three members, ABI1, ABI2 and ABI3 (Hirao et al., 2006). NCK proteins contain one SRC homology 2 (SH2) domain and three SRC homology 3 (SH3) domains, while ABI proteins contain one SH3 domain and one target SNARE coiled-coil homology (T_SNARE) domain (www.uniprot.org).

NCK is known to be adopted by several microbial pathogens to mediate actin polymerization. NCK is essential for the actin-based motility of vaccinia virus (Frischknecht et al., 1999). NCK has also been implicated in the initiation of actin signaling, binding to a tyrosine-phosphorylated 12 amino acid sequences of enteropathogenic *Escherichia coli* (EPEC) translocated intimin receptor (TIR) in a Y474 phosphorylation-dependent manner (Nieto-Pelegrin et al., 2014). Recently, NCK1 was identified to be a candidate gene for enteric septicemia of catfish (ESC) disease resistance of catfish, and was speculated to play similar roles during ESC and EPEC pathogenicity (Zhou et al., 2016).

ABI proteins are key regulators for many molecular signaling pathways, which also play important roles in disease response. The ABI1 was reported as a positional candidate gene for

ESC disease resistance of catfish (Zhou et al., 2016), and bacterial cold water disease (BCWD) resistance in rainbow trout (Palti et al., 2015). ABI proteins are components of the ABI/Wave complex which regulates actin polymerization (Hirao et al., 2006). Overexpression of ABI genes negatively regulate cell growth and transformation by specifically targeting the extracellular signal regulated kinases (ERK) pathway (Fan and Goff, 2000). It was postulated that the binding of ABI1 to v-Abl might block activation of critical signal transduction pathways. Signaling molecules activated downstream of v-Abl include RAS (Sawyers et al., 1995), phosphatidylinositol 3-kinase (PI3K) (Varticovski et al., 1991), RAC (Renshaw et al., 1996), c-Jun N-terminal kinase (JNK) (Raitano et al., 1995; Renshaw et al., 1996), and ERKs (Raitano et al., 1995; Renshaw et al., 1996). RAS, RAC, JNK, ERKs signaling molecules are important targets of viral and bacterial virulence factors (Alto and Orth, 2012; Bangi et al., 2012; Krachler et al., 2011).

Although adaptor proteins are important regulators of pathogen infection, their involvement in the ESC disease has not been reported. ESC, caused by the bacterium *Edwardsiella ictaluri*, is one of the most severe bacterial diseases in catfish. Although *E. ictaluri* pathogenesis is relatively well characterized, the knowledge about *E. ictaluri* intestinal interaction is limited (Santander et al., 2014). Besides, little is known about the molecular mechanism of pathogenicity of *E. ictaluri*. Intestinal tract was confirmed as a site of *E. ictaluri* entry by bacteriological and microscopic methods (Baldwin and Newton, 1993). It has been suggested that *E. ictaluri* survives in intestinal macrophages (Santander et al., 2013) and causes intestinal barrier disruption and immune suppression (Li et al., 2012). *E. ictaluri* traverses the epithelial lining with no epithelial cell damage by exploiting normal cellular transport systems

(Skirpstunas and Baldwin, 2002). Actin polymerization and receptor-mediated endocytosis are involved in the uptake of *E. ictaluri* by rat small intestinal epithelial cells (Skirpstunas and Baldwin, 2002). It was corroborated that *E. ictaluri* lipopolysaccharide oligo-polysaccharide (LPS O-PS) plays a major role during catfish intestinal infection and immune protective stimulation, since they influence the recognition of the LPS by the intestinal mucosal immune system of catfish (Santander et al., 2014). However, the internalization process of *E. ictaluri* into its natural host, channel catfish, together with the involved pathways, genes, and coordinators are still unknown.

Our recent QTL mapping studies revealed that the adaptor genes, including the NCK genes and the ABI genes, are located within the genomic regions harboring the most significant QTLs (Zhou et al., 2016), suggesting that these genes could be involved in the determination of resistance, and possibly through their roles as adaptors assisting the entry of the pathogen into the host. As a first step for the understanding of the roles of the adaptor genes in ESC pathogenesis, in this study, we identified the adaptor genes and determined their expression after ESC infection. Here we report the identification, annotation, and expression analysis of three NCK and six ABI genes.

2. Material and methods

2.1. Data mining and gene identification

To identify NCK and ABI genes of channel catfish, the amino acid sequences of NCK and ABI genes from human and zebrafish were retrieved from UniProt (www.uniprot.org) and

Ensembl (www.ensembl.org) databases. Then these sequences were used as queries to search against the channel catfish transcriptome (Li et al., 2012; Liu et al., 2012), with cutoff E-value of $1e^{-5}$ using TBLASTN. The identified candidate sequences from channel catfish transcriptome were submitted to ORF (opening reading frames) finder (<https://www.ncbi.nlm.nih.gov/orffinder/>) to predict coding sequences. The predicted coding sequences for NCK and ABI genes were further validated by BLASTP against Uniprot and NCBI non-redundant (nr) protein database. The validated sequences were used as queries to search against channel catfish whole genome database (Liu et al., 2016) to verify copy number and the sequence accuracy of NCK and ABI genes in channel catfish, with cutoff E-value of $1e^{-10}$ using BLASTN. The AUGUSTUS (Stanke et al., 2004) program was used to predict the genes from retrieved genomic sequences. The InterPro (www.ebi.ac.uk/interpro/) database was used to classify the families and domains of the predicted genes. The amino acid sequences of the identified genes were used in the following phylogenetic analysis.

For blue catfish, all the available RNA-Seq reads were downloaded from NCBI Sequence Read Archive (SRA) database (SRR799807, SRR799808, SRR799809, SRR799810, SRR799812, SRR799813, SRR070302). The raw reads were trimmed by using Trimmomatic (Bolger et al., 2014), and then were assembled by using Trinity (Grabherr et al., 2011). Similar with the procedures used in channel catfish, the blue catfish NCK and ABI mRNA were identified from the assembled blue catfish transcriptome. The amino acid sequences were predicted from the identified blue catfish NCK and ABI mRNA, and were used in the following phylogenetic analysis.

2.2. Phylogenetic analysis

Phylogenetic analysis was conducted using NCK and ABI genes from human (*Homo sapiens*), mouse (*Mus musculus*), cow (*Bos taurus*), chicken (*Gallus gallus*), xenopus (*Xenopus tropicalis*), and several fish species including zebrafish (*Danio rerio*), cave fish (*Astyanax mexicanus*), medaka (*Oryzias latipes*), tilapia (*Oreochromis niloticus*), tetraodon (*Tetraodon nigroviridis*), fugu (*Takifugu rubripes*), channel catfish and blue catfish. Multiple amino acid sequences were aligned by ClustalW (Thompson et al., 2002). The phylogenetic analysis was conducted using MEGA 6 (Tamura et al., 2013) with the maximum likelihood method and Jones-Taylor-Thornton (JTT) model. The bootstrap with 1000 replications was performed to evaluate the phylogenetic tree.

2.3. Syntenic analysis

Syntenic analysis was conducted to provide additional evidence for identity of genes, when there is ambiguous annotation. The neighboring genes of catfish ABI3b genes were predicted from the catfish genomic sequences using AUGUSTUS program, and validated by BLASTP against Uniprot and nr databases. The NCK and ABI genes and their neighboring genes in zebrafish were obtained from Ensembl and Genomicus (Muffato et al., 2010) databases. For the nomenclatures of channel catfish NCK and ABI genes, we named them after zebrafish corresponding genes based on results from phylogenetic and syntenic analyses.

2.4. Expression analysis of NCK and ABI genes following *E. ictaluri* infection

Meta-analysis of RNA-Seq data generated from ESC challenge of catfish of previous studies (Li et al., 2012; Wang et al., 2013) was conducted to obtain the expression patterns of NCK and ABI genes in host immune response to *E. ictaluri* infection. The RNA-Seq reads sequenced from catfish intestine (SRP009069) and liver (SRP028159) were downloaded from NCBI SRA database. Channel catfish intestine tissues were sampled before the *E. ictaluri* infection (control) and 3 h, 24 h and 72 h after the *E. ictaluri* infection. Totally, four RNA samples from channel catfish intestine were sequenced. Hybrid catfish liver tissues were collected from control, ESC resistant and susceptible fish. Three RNA samples from catfish liver were sequenced. The gene expression analysis was performed using CLC genomics workbench software package. All the predicted genes (coding sequence) from channel catfish whole genome sequences (Liu et al., 2016) were used as reference for mapping. The trimmed high-quality reads were mapped to the reference with at least 95% sequence identity and a maximum of two mismatches being allowed. After mapping, the raw count of mapped reads for each gene was collected. The raw counts for each sample were normalized, and then used for the following differentially expression analysis. Fold change was calculated based on the comparison of each sample with control respectively. Kal's Z-test (Kal et al., 1999) was used to detect the statistical significance. The test relies on an approximation of the binomial distribution by the normal distribution (Kal et al., 1999), and allows to compare a single sample against another single

sample. Genes with a minimal number of 5 mapped reads and fold change greater than 1.5 were considered as significantly differentially expressed (Fu et al., 2016).

3. Results

3.1. Identification of NCK and ABI genes in channel catfish

Three NCK genes, NCK1, NCK2a, and NCK2b, and six ABI genes, ABI1a, ABI1b, ABI2a, ABI2b, ABI3a, ABI3b were identified from the channel catfish genome and blue catfish transcriptome respectively. The sequences of these genes have been deposited to GenBank, and their characteristics and accession numbers are summarized in Supplementary table 1. In channel catfish, all the three NCK genes encode proteins with three SH3 domains and one SH2 domain. All the channel catfish ABI genes except ABI3b encode proteins with one T_SNARE domain, one Abl-interactor, homeo-domain homologous domain (ABI_HHR) and one SH3 domain. The catfish ABI3b encodes only the ABI_HHR domain with no existence of the T_SNARE and SH3 domains.

The identified domains of NCK and ABI proteins in blue catfish were more divergent (Supplementary table 1). Blue catfish NCK1 and NCK2a possess three SH3 domain and one SH2 domain. However, only one SH3 and SH2 domain were identified in NCK2b. ABI1a and ABI2a harbor one T_SNARE domain, one ABI_HHR and one SH3 domain, while ABI2b and ABI3a lacked the SH3 domain. Similar with channel catfish, the blue catfish ABI3b encodes only the ABI_HHR domain. However, no domain was identified from ABI1b in blue catfish.

3.2. Phylogenetic and syntenic analyses of channel catfish NCK and ABI genes

Phylogenetic analyses were conducted to annotate NCK and ABI genes in channel and blue catfish. As shown in Supplementary Fig. 1, The NCK genes were clustered into two subclades, NCK1 and NCK2. Zebrafish NCK1a was derived from the two subclades. For the three catfish NCK genes, one was placed in the clade of NCK1, and two were placed into the clade containing NCK2. For each NCK gene, members from fish were clustered together, and members from the other species formed a separate cluster (Supplementary Fig. 2). Similar to NCK genes, ABI genes from catfish, cavefish and zebrafish formed a cluster. To confirm the annotation of ABI3b, syntenic analysis was conducted. As shown in Supplementary Fig. 3, syntenic block containing ABI3b was conserved between channel catfish and zebrafish, providing additional evidence for the annotation.

3.3. Copy numbers of NCK and ABI genes in channel and blue catfish

The copy numbers of NCK and ABI genes in channel catfish genome were determined by sequence analysis from the whole genome sequences of channel catfish (Liu et al., 2016). In channel catfish genome, only one copy of NCK1 gene was identified. However, two copies were present for NCK2, ABI1, ABI2 and ABI3 (Supplementary table 2). The duplicates of NCK2 and ABI genes were all found in two different chromosomes. Copy numbers of NCK and ABI genes in blue catfish were same with channel catfish.

In zebrafish genome, all the NCK and ABI genes have two copies. Among all the species, only zebrafish genome has two copies of NCK1 gene, while birds (chicken, duck) do not possess NCK1 gene. Mammals, birds and amphibian genome contain one copy of NCK2 gene, while fish genome contain single or double copies of NCK2 gene. Similar results were observed for ABI genes: a single copy of ABI genes was found in mammals, birds and amphibian genome, while the copy number of ABI genes varies from one to three copies in fish genomes (Supplementary table 2).

3.4. Expression analysis of NCK and ABI genes after ESC infection

Expression of NCK and ABI genes after ESC challenge was determined to gain insights into their involvement in the disease responses. The fold change for each gene was calculated based on the comparison of expression values in each sample with control. In the intestine, NCK1 was significantly upregulated 3 hours after ESC challenge. For the ABI genes, ABI2a was significantly upregulated 3 hours, 24 hours and 72 hours after ESC challenge. ABI2b was significantly upregulated 24 hours after ESC challenge. However, ABI3a was significantly downregulated after ESC challenge (Fig. 1a).

In the catfish liver, most of the NCK and ABI genes were significantly upregulated in ESC susceptible fish, however slightly downregulated (insignificant) in ESC resistant fish. The NCK2a, NCK2b, ABI2a, ABI2b were significantly upregulated in ESC susceptible fish. The expression of most of the NCK and ABI genes in ESC resistant fish were insignificantly regulated, except for ABI3a which was significantly upregulated. Compared with the ESC

resistant fish, most of the NCK and ABI genes were significantly upregulated in ESC susceptible fish (Figure 1b).

4. Discussion

Adaptor genes NCK and ABI are important regulators for disease response. ABI1 was reported as a positional candidate gene for ESC disease resistance in catfish (Zhou et al., 2016), and bacterial cold water disease (BCWD) resistance in rainbow trout (Palti et al., 2015). NCK1 was reported to be a candidate gene for ESC disease resistance of catfish by using genome wide association analysis (Zhou et al., 2016). Blue catfish is more resistant to ESC than channel catfish (William and Terrence, 2004). Identification of NCK and ABI genes in channel and blue catfish and the expression analysis will provide insight for the research of molecular mechanism of ESC resistance.

Three NCK and six ABI genes were identified both in channel and blue catfish. The annotations of the NCK and ABI genes were explicit based on the phylogenetic, syntenic and protein domain structure analysis. NCK and ABI genes from channel and blue catfish showed closest phylogenetic relationship. Domain structures were well conserved for all the NCK and ABI genes in channel catfish. The domain of channel and blue catfish ABI3b protein was similar to zebrafish ABI3b protein, which contains only ABI_HHR domain but lacks the T_SNARE and SH3 domains. However, some domains were not observed in blue catfish NCK and ABI proteins. Future studies with more tissue source of RNA samples and increased sequence depth are needed to reassembly and validate the NCK and ABI genes in blue catfish. The predicted amino acid

sequences of NCK1 from channel and blue catfish were exactly same with each other. Three base variations were found in the shared 3' untranslated region (UTR) between channel and blue catfish NCK1 mRNA. Genetic variants in 3' UTR have been implicated in disease susceptibility because of the change in RNA structure and protein translation (Lu et al., 2015). Future studies of NCK protein expression level in channel and blue catfish are needed to figure out the molecular mechanism of ESC disease resistance difference between channel and blue catfish.

With the publication of channel catfish genome (Liu et al., 2016), the annotation is available in NCBI genome database. The three NCK genes were annotated by NCBI (GI: 108272450, 108266513, 108273233), however, only five ABI genes (GI: 108272842, 108267643, 108266499, 108272690, 108276098) were identified in NCBI annotation, with absence of ABI3b. The genomic region encode ABI3b gene in channel catfish was predicted to be “formin-like protein 5” (GI: 108273397) in NCBI annotation. However, phylogenetic (Supplementary Fig. 2) and syntenic analysis (Supplementary Fig. 3) supported our annotation that this region encodes ABI3b.

The copy numbers of NCK and ABI genes in teleost fish and tetrapods should provide insights to the evolution of NCK and ABI genes. Mammals and amphibian genome contain single copy of NCK and ABI genes, while teleost fish genome contains one to three copies of NCK and ABI genes. Most of the teleost fish contain one or two copies of NCK and ABI genes. The duplicated catfish NCK and ABI genes were likely derived from the teleost-specific whole genome duplication as well as lineage-specific tandem duplications because they were found on separate chromosomes (Hoegg et al., 2004).

The expression profiles of NCK and ABI genes after ESC bacterial infection were determined. As shown in Fig. 1a, NCK1, ABI2a and ABI2b were significantly induced after ESC infection. While, ABI3a was significantly down-regulated. Intestine was confirmed as a site of *E. ictaluri* entry by bacteriologic and microscopic methods (Baldwin and Newton, 1993). It has been reported that *E. ictaluri* crosses the intestinal mucosa of channel catfish in 15 minutes after oral inoculation (Baldwin and Newton, 1993). The upregulation of NCK1, ABI2a and ABI2b in intestine after ESC challenge indicated its involvement in the disease processes.

The most interesting findings were the observation of differential expression of the NCK and ABI genes between resistant and susceptible hybrid catfish (Fig. 1b). In general, the NCK and ABI genes, with exception of ABI3a gene and NCK1 gene, were expressed at higher levels in susceptible fish after infection than in control fish, but were expressed at lower levels in resistant fish than in the control fish. It appeared that the expression of most NCK and ABI genes were correlated with susceptibility. At present, we do not know why the genes were expressed at higher in susceptible fish and how the higher expression may be related to pathogenicity.

However, NCK1 and NCK2 were reported to play an important role in linking cell surface receptor signaling to the actin-based cytoskeleton as a binding partner in other disease system such as infantile diarrhea (Gruenheid et al., 2001; Li et al., 2001; Todd, 1997). In humans, it was reported that the host protein NCK binds to a 12 residue amino acid sequences of EPEC TIR in a phosphotyrosine-dependent manner and is recruited to EPEC to initiate actin remodeling and pedestal formation (Campellone et al., 2002; Gruenheid et al., 2001; Nieto-Pelegrin et al., 2014). NCK1, ABI1 and ABI2 involved in regulation of actin dynamics for phagocytic cup formation, which is a crucial stage for phagocytosis (www.reactome.org). We do not know if this similar

mechanism existed in the ESC situation, but this is certainly worthwhile of future studies. In our QTL mapping study, we mapped the ESC resistance QTL to a genomic region containing the NCK gene, which led us to speculate the involvement of catfish NCK in the pathogenesis of ESC (Zhou et al., 2016). The significant upregulation of NCK genes in catfish intestine and liver after ESC challenge supported the notion that NCK genes are involved in disease processes facilitating pathogenesis of the ESC bacteria.

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Figure legends

Fig. 1 Differential expression analysis of NCK and ABI genes after ESC challenge. (a) Fold change of gene expression in channel catfish intestine tissue at different time points after ESC infection; (b) Fold change of gene expression in catfish liver tissue in ESC susceptible and resistant fish. con, control sample; sus, ESC susceptible fish; res, ESC resistant fish. Asterisks indicated statistical significant difference ($p < 0.05$, $|\text{fold change}| > 1.5$).