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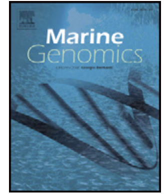

# Presence of two tumor necrosis factor (*tnf*)-α homologs on different chromosomes of zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*)

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ABSTRACT

Two or more isoforms of several cytokines including tumor necrosis factors (*tnf*s) have been reported from teleost fish. Although zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) possess two *tnf-α* genes, their genomic location and existence are yet to be described and confirmed. Therefore, we conducted *in silico* identification, synteny analysis of *tnf-α* and *tnf-n* from both the fish with that of human *TNF*/*lymphotoxin* loci and their expression analysis in zebrafish. We identified two homologs of *tnf-α* (named as *tnf-α1* and *tnf-α2*) and a *tnf-n* gene from zebrafish and medaka. Genomic location of these genes was found to be as: *tnf-α1*, and *tnf-n* and *tnf-α2* genes on zebrafish chromosome 19 and 15 and medaka chromosome 11 and 16, respectively. Several features such as existence of *TNF* family signature, conservation of genes in *TNF* loci with human chromosome, phylogenetic clustering and amino acid similarity with other teleost TNFs confirmed their identity as *tnf-α* and *tnf-n*. There were a constitutive expression of all three genes in different tissues, and an increased expression of *tnf-α1* and *-α2* and a varied expression of *tnf-n* ligand in zebrafish head kidney cells induced with 20 μg $mL^{-1}$ LPS *in vitro*. Our results suggest the presence of two *tnf-α* homologs on different chromosomes of zebrafish and medaka and correlate this incidence arising from the fish whole genome duplication event.

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## 1. Introduction

Cytokines are low molecular weight cell derived glycoproteins communicating the information between immunocompetent cells, such as lymphocytes and macrophages. Cytokine members of tumor necrosis factor super family (TNFSF) involve in different pathways, *viz.* inflammation, lymphocyte maturation, and apoptosis to lymphoid and epithelial tissue development (Bodmer et al., 2002; Ware, 2003). Especially, *TNF-α* and lymphotoxin-α (*LT-α*; also known as *TNF-β*) are important mediators of inflammatory and immune defense mechanisms (Kadowaki et al., 2009). *LT-β* is another TNFSF member received by the receptor different from that of *TNF-α* and *TNF-β* (Weins and Glenney, 2011). *LT-β* has important roles in development and homeostasis of secondary lymphoid organs, adaptive immune response and intestinal immunological homeostasis (Remouchamps et al., 2011). Location of the genes encoding *TNF-α*, *TNF-β*, and *LT-β* has been found to be on the short arm of human chromosome 6 (Spies et al., 1986) and mouse chromosome 17 (Lawton et al., 1995). The three *TNF* genes (*TNF-α*, *TNF-β*, and *LT-β*) exist on the major histocompatibility complex (MHC) class III region in humans and in this locus, *TNF-α* and *TNF-β* genes are arranged in tandem in the same transcriptional orientation.

Identification *of tnf-α* gene has been reported from several fish species, such as ayu fish *Plecoglossus altivelis* (Uenobe et al., 2007), bluefin tuna *Thunnus orientalis* (Kadowaki et al., 2009), channel catfish *Ictalurus punctatus* (Zou et al., 2003), common carp *Cyprinus carpio* (Saeij et al., 2003), goldfish *Carassius auratus* (Grayfer et al., 2008), grass carp *Ctenopharyngodon idella* (Zhang et al., 2012), Japanese flounder *Paralychthys olivaceus* (Hirono et al., 2000), rainbow trout *Oncorhynchus mykiss* (Laing et al., 2001), red seabream *Pagrus major* (Cai et al., 2003), tilapia *Oreochromis niloticus* (Praveen et al., 2006), turbot *Psetta maxima* (Ordás et al., 2007) and zebrafish *Danio rerio* (Eimon et al., 2006). From these reports, high divergence in *tnf-α* gene structures has been found among different species. The typical characteristics of the mammalian *TNF-α* gene are present in these cloned genes with a well-defined transmembrane domain, two conserved cysteines important for disulphide bond formation, a well-defined cleavage site for the release of the mature protein, high conservation of the *TNF* signature motifs, and a similar genomic structure. In several researches, it has been thought that there is only one *tnf-α* gene in fish. However, existence of two *tnf-α* genes was reported in Atlantic salmon (Morrison et al., 2007), bluefin tuna (Kadowaki et al., 2009), goldfish (Grayfer et al., 2008), rainbow trout (Laing et al., 2001; Zou et al., 2002) and zebrafish (Eimon et al., 2006). Four isoforms of *tnf-α* (*tnf-α1*, *α2*, *α3* and *α4*) have also been discovered from common carp (Saeij et al., 2003; Savan and Sakai, 2004; Zhao et al.,

*Abbreviations:* HK, head kidney; TNF/*tnf*, tumor necrosis factor; LT/*lt*, lymphotoxin; qPCR, quantitative polymerase chain reaction; LPS, lipopolysaccharide; Th, T helper; PBL, peripheral blood leukocyte.

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2012). Probable reason for existence of multiple isoforms of cytokines is the occurrence of genome duplication event in bony fish (Laing et al., 2000). A novel *tnf* gene (*tnf*-new or *tnf-n*), similar to *LT-α* was described for the first time, apart from mammals, from the Japanese pufferfish *Takifugu rubripes* and zebrafish and is present in tandem with the *tnf-α* on the same chromosome (Savan et al., 2005). Later, on identifying two orthologs of *lt-β* in rainbow trout, Kono et al. (2006) suggested the similarity of the proteins to *tnf-n*. Subsequently, Glenney and Wiens (2007) ruled out this conclusion with comprehensive phylogenetic analyses containing all available TNFSF members that indicated no relationship of *tnf-n* with *lt-β* due to the absence of the location of a conserved cysteine in *lt-β*. Although the existence of two fish *tnf-α* homologs has been reported, there is no information on their genomic position and structure. Therefore, in this effort to reveal the understanding on the evolution of TNF superfamily, we systematically searched genomic databases of zebrafish and medaka, *Oryzias latipes* for the purpose of analysis of the *tnf-α* gene loci on the genome. Thus, we could identify two *tnf-α* genes (designated as *tnf-α1* and *tnf-α2*) and a *tnf-n* gene located on different chromosomes, analyzed their synteny with human genome and investigated the expression patterns in zebrafish.

## 2. Materials and methods

### 2.1. TNF gene search and identification from zebrafish and medaka databases

Zebrafish *tnf-α*, and *-n* and medaka *tnf-α* homologs were retrieved from the NCBI database (GeneBank IDs: zebrafish *tnf-α1*, NM_212859; zebrafish *tnf-α2*, NM_001024447; zebrafish *tnf-n*, AB183468; medaka *tnf-α1*, XP_004074383; medaka *tnf-α2*, XP_004078539). For medaka *tnf-n*, the nucleic acid sequence was retrieved from medaka chromosome 16 (25694643-25698981) (Ensembl release 72) and amino acid (aa) sequence, exons and introns were predicted using FGENESH in SoftBerry (http://linux1.softberry.com/berry.phtml). *In silico* screening of zebrafish and medaka homologs for *tnf-α* and *tnf-n* genes was conducted using their respective genome databases, http://www.ensembl.org/Danio_rerio/Info/index and http://www.ensembl.org/Oryzias_latipes/Info/index and it resulted in the presence of *tnf-α1*, and *tnf-n* and *tnf-α2* genes on zebrafish chromosome 19 and 15 and medaka chromosome 11 and 16, respectively.

### 2.2. Sequence, structural, synteny and phylogenetic analyses

Sequences were compared with those in the database using the blastx algorithm (Altschul et al., 1990). Amino acids multiple sequence alignments were generated by ClustalW using BioEdit software (Hall, 1999) and homology analysis was performed using MatGat software version 2.02 (Campanella et al., 2003). The gene structure, orientation and synteny were analyzed using blastn (http://www.ensembl.org/Multi/blastview) on the respective genomic sequences of zebrafish, medaka and human harboring *TNF* ligands. Phylogenetic analysis was carried out for the full-length aa sequences of known *TNF*/*LT* homologs from various animals by the neighbor-joining (NJ) method (Saitou and Nei, 1987) using MEGA5 software (Tamura et al., 2011). Gaps or missing data were treated by complete deletion. Node values which represent percent bootstrap confidence were derived from 1000 replicates. The minimum evolution analysis was performed using ClustalX v1.81.

### 2.3. Expression analysis of *tnf* genes in zebrafish by real-time qPCR

#### 2.3.1. Expression in various unstimulated tissues

Zebrafish (mean weight: 2.8 ± 0.2 g) were obtained from a pet shop at Miyazaki, Japan. The fish were acclimatized in an aerated freshwater tank at 20 °C under a natural photoperiod and fed a commercial diet, floating type (TetraPlankton, Tetra Japan Co., Ltd., Tokyo, Japan) at 1% body weight daily for two weeks prior to their use in this study. Fish were scooped out of holding tank and anesthetized with 2-phenoxyethanol (0.05%, Sigma-Aldrich, St. Louis, MO, USA) in a bucket containing aerated freshwater before being sacrificed for tissue collection. Nine different tissues (brain, gills, heart, liver, spleen, head kidney, intestine, muscle and skin) were excised aseptically from 50 individual fish for total RNA extraction. The tissues were submerged immediately in RNAlater solution (Ambion, Austin, TX, USA) for overnight and finally stored at -80 °C until use. Total RNA was extracted from the stored tissues using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. To avoid presence of genomic DNA, RNA samples were treated with recombinant DNase I (RNase-free) as per the manufacturer's protocol (Takara Bio Inc., Shiga, Japan). Quantity and quality of all RNA samples were checked using a NanoDrop spectrophotometer, ND-1000 (Thermo Scientific, Wilmington, DE, USA). cDNA was synthesized *via* reverse transcription from 0.5 μg mRNA using ReverTra Ace (Toyobo Co., Ltd., Osaka, Japan). The primer sequences used in qPCR and their efficiency values are listed in Table 1. Amplification efficiency of these primer sets was determined according to the method of Pfaffl (2001). The qPCR reaction mixture contained 10 μL of THUNDERBIRD™ SYBR® qPCR Mix (Toyobo Co., Ltd.), 2 μL of each 5 μM primer, 2 μL of template cDNA (30 ng) and 4 μL nuclease-free water in a total volume of 20 μL. Thermal cycling and fluorescence detection was conducted using the 7300 Fast Real-Time PCR System (Applied Biosystems, CA, USA) under the following conditions: 95 °C for 1 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C, followed by a dissociation curve to ensure that a single PCR product was amplified in each reaction. Each sample was run in triplicate. The threshold cycle ($C_T$) represents the PCR cycle at which an increase in reporter fluorescence above a baseline signal was first detected. The comparative $C_T$ method ($2^{-\triangle\triangle CT}$ method) (Schmittgen and Livak, 2008) was used to analyze the expression level of each *tnf* gene using *β-actin* as internal control gene.

The animal experiment was conducted according to the relevant national and international guidelines, 'Act on Welfare and Management of Animals' (Ministry of the Environment, Japan). Ethics approval from the local IACUC was not sought since this law does not mandate protection of fish.

#### 2.3.2. Expression in LPS-stimulated head kidney (HK) cells

The aseptically excised HK tissues were gently pushed through a 100-μm nylon mesh (John Staniar, Whitefield, Manchester, UK) with RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS; Invitrogen) and a 1% solution of streptomycin + penicillin (Invitrogen). After washing with the above medium and depletion of erythrocytes, the cells were then pushed again through a 40-μm nylon mesh cell strainer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Finally, the number of prepared cells was adjusted to $1 \times 10^7$ cells $mL^{-1}$. The cells were seeded in a 24-well plate (Nunc A/S, Roskilde, Denmark), stimulated with the bacterial mimic lipopolysaccharide (LPS; Sigma, MO, USA) at 20 μg $mL^{-1}$ and incubated for 1, 4, 8, 12 and 24 h at 25 °C. Cells collected at 0 h without

**Table 1**
Gene specific primers used for zebrafish *tnf* gene expression analysis (Real-time qPCR) in this study.

| Gene | Primer sequence (5′ → 3′) | Length (mer) | Amplification efficiency (%) |
|---|---|---|---|
| *tnf-α1* Fw[a] | GGTCCTCTGTTGCGGAGGCG | 20 | 108 |
| *tnf-α1* Rv[b] | CGAGCTGGAAGTCGCCCTGAG | 21 | |
| *tnf-α2* Fw | GAGCAAATCCGTTATTCTCG | 20 | 108 |
| *tnf-α2* Rv | GAGGGTGATGTCTGATAGAAC | 21 | |
| *tnf-n* Fw | GGAGTCACGAGTCGGCCGAG | 20 | 95 |
| *tnf-n* Rv | ACACCAAGTGGTGGAAGGAGG | 21 | |
| *β-actin* Fw | ACCTCATGAAGATCCTGACC | 20 | 108 |
| *β-actin* Rv | TGCTAATCCACATCTGCTGG | 20 | |

[a] Fw = Forward; [b]Rv = Reverse.

stimulation served as a control for the experiment. RNA extraction, cDNA synthesis and qPCR were performed as per the protocols described in Section 2.3.1. Data on quantified relative expression of a particular gene in the stimulated and control HK cells at each time point were compared by an independent samples *t*-test for equality of means using SPSS for Windows v.17.0 program (SPSS Inc., Chicago, IL, USA).

## 3. Results

### 3.1. Chromosome synteny and gene structural analysis

The gene order and orientation was analyzed using blastn on the respective contigs of zebrafish and medaka harboring *tnf* ligands. It was demonstrated that like human chromosome genes, several genes are located between *tnf-α1* and *tnf-α2* gene in zebrafish and medaka genomes (Fig. 1). According to the zebrafish genome database, *bat2* and *flot1* genes were localized upstream of *tnf-α1* gene on chromosome 19. Furthermore, *bat1* and *gabbr1* genes were present downstream of *tnf-α1* gene. There were *bat3* and *tnf-n* genes upstream of *tnf-α2* and these are tandem repeat regions of zebrafish chromosome 15. Location of *gabbr1* gene was downstream of *tnf-α2* gene. In medaka genome, *gabbr1* and *flot1* genes were harbored upstream and downstream of *tnf-α1* gene on chromosome 11, respectively. Moreover, medaka *tnf-α1* gene has transcriptional orientation opposite to human *TNF-α* and zebrafish *tnf-α1* genes. Presence of *aif1*, *flot1* and *gabbr1* genes was downstream of the *tnf-α2* and *-n* genes on chromosome 16, whereas *apo-m* and *bat3* genes were placed upstream of them.

In zebrafish, the *tnf-α2* gene consists of 1220 bp containing four exons and three introns and translates a protein of 242 aa in length (Fig. 2A). The *tnf-n* gene located upstream of the *tnf-α2* gene on the same chromosome is composed of 1436 bp with five exons and four introns and it translates a 203 aa peptide. The *tnf-α1* harbored in different chromosome contains 1145 bp nucleotides spanned over four exons and three introns and produces a peptide of 234 aa. In medaka, similar to zebrafish there are four exons and three introns in the *tnf-α2* gene comprising of 768 bp and translating a protein of 255 aa (Fig. 2B). The *tnf-n* gene existed in tandem with *tnf-α2* gene on the same chromosome is composed of 603 bp with four exons and three introns and it translates a 200 aa peptide. The *tnf-α1* located on different chromosome contains 681 bp nucleotides extended over four exons and three introns and produces a protein of 226 aa.

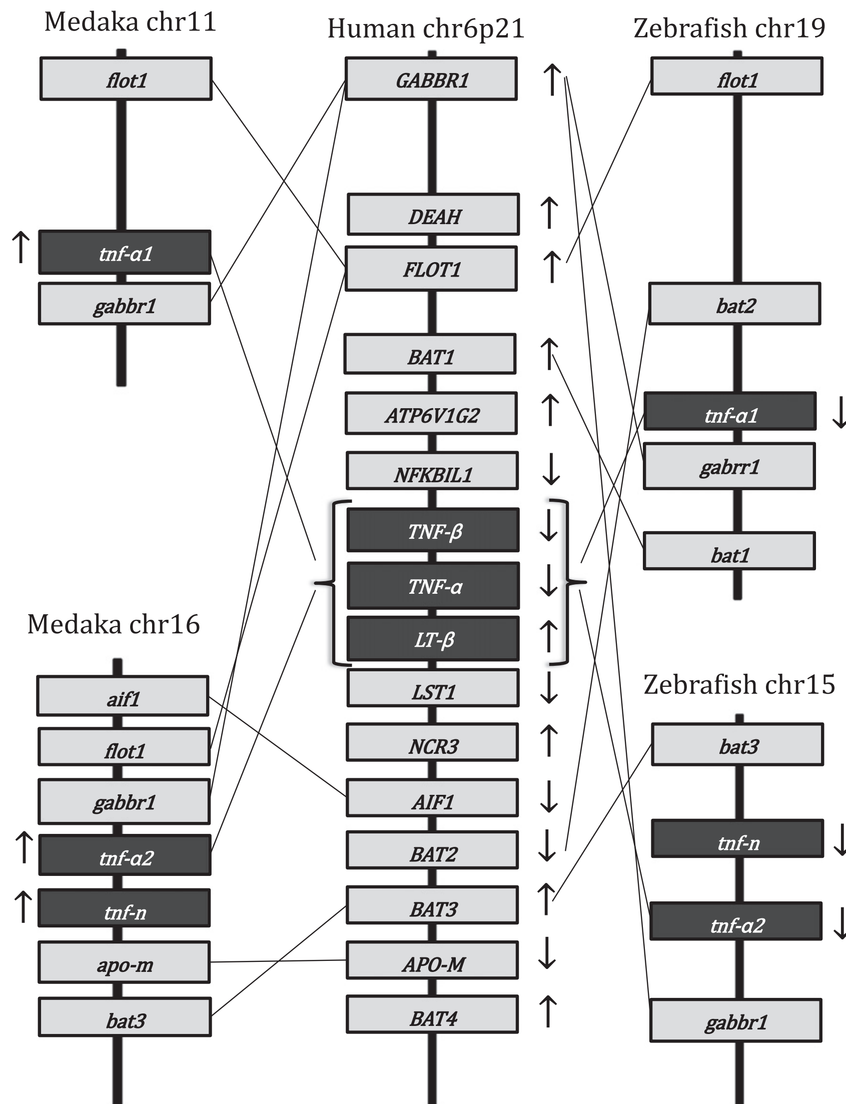


**Fig. 1.** Schematic diagram of gene synteny between zebrafish (chromosome 15 and 19), medaka (chromosome 11 and 16) and human (chromosome 6) *TNF/LT* locus. The *TNF* genes are highlighted as black boxes, and the transcription orientations of the genes are indicated by arrows.

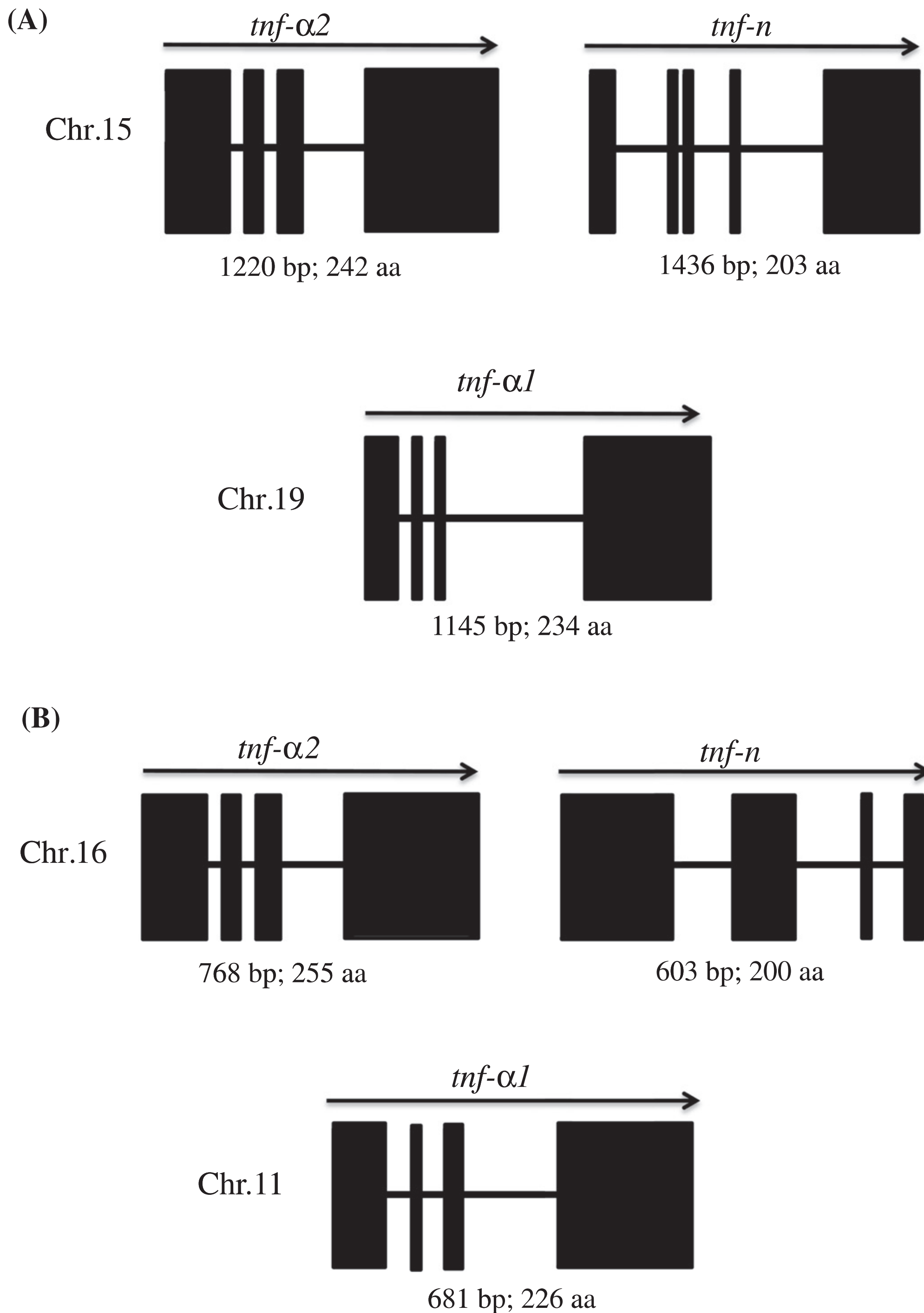


**Fig. 2.** Genomic organization of zebrafish (A) and medaka (B) *tnf* genes. An arrow indicates the orientation of the gene. Exons and introns are represented as closed boxes and lines, respectively. Length of gene (bp) and its translated amino acids (aa) are mentioned below the figure.

### *3.2. Alignment, homology and phylogenetic analysis*

Multiple alignments of the zebrafish and medaka *tnf* ligands with the human *TNF-α* (Fig. 3) showed high conservation of the *TNF* family signature motifs (LV)-x-(LIVM)-$x_3$-G-(LIVMF)-Y- (LIVMFY)$_2$-$x_2$-(QEKHL)-(LIVMGT)-x-(LIVMFY). TNF aa sequences of zebrafish and medaka were compared with other known fish *tnf* sequences (Tables 2 and 3). From the homology analysis, it was revealed that zebrafish *tnf* genes shared aa similarity (identity) ranging from 32.0% (18.8%) to 81.2% (66.9%) compared to other fish *tnf* genes (Table 2). Among the three *tnf* genes, the aa similarity of *tnf-α1* was found to be the highest with common carp *tnf-α1* (81.0%) and -α2 (81.2%). However, the aa similarity between the two zebrafish *tnf-α* was 55.0%. In medaka, *tnf* genes shared aa similarity (identity) ranging from 32.7% (20.2%) to 69.8% (54.2%) compared with that of other teleost *tnf* genes (Table 3). Contrary to zebrafish, among the three *tnf* genes, the aa similarity of *tnf-α2* was found to be the highest with bluefin tuna *tnf-α1* (69.8%). Medaka *tnf-α1* and *-α2* genes shared 55.3% similarity. However, *tnf-n* gene of both the fish species shared lower identity and similarity than that of *tnf-α1* and *-α2* genes with other fish *tnf*s. Phylogenetic tree analysis showed cluster formation of zebrafish TNF-α1 nearer to other cyprinid species such as common carp and goldfish (Fig. 4). However, zebrafish

**Fig. 3.** Multiple alignment of zebrafish and medaka TNF amino acid sequences with the known human *TNF-α* homolog. Identical amino acid residues are indicated by dots, and dashes are introduced to fill the gaps for optimal alignment. *TNF* family signature motifs are marked with an open box.

TNF-α2 was a lone member in a sub-cluster within the fish TNF-α peptides. Medaka TNF-α1 formed a distinct cluster with bluefin tuna TNF-α2 and ayu TNF, whereas there was a sub-cluster of medaka TNF-α2 within the teleost TNF ligands. Overall, teleost TNF-α sequences were in the same broad cluster distinctly away from the mammalian counterparts. However, TNF-N from both the fish was in an entirely separate cluster with fugu TNF-β and rainbow trout LT-β away from the mammalian and amphibian group.

### 3.3. Expression of tnf genes in zebrafish

#### 3.3.1. Tissue specific expressions

Zebrafish *tnf-α1*, *-α2* and *-n* genes were constitutively expressed in all the tested tissues with the highest expression levels recorded in intestine (Fig. 5A). Both *tnf-α1* and *-α2* genes showed similar expression patterns in most of the tissues. However, among the three genes, *tnf-n* was expressed in higher level in gill, liver and intestinal tissues.

#### 3.3.2. Expression in LPS-stimulated HK cells

The expression of zebrafish *tnf-α1* and *-α2* genes was significantly increased ($P < 0.01$) in the LPS-stimulated head kidney cells compared to unstimulated cells (0 h) at all the time points tested (Fig. 5B). Similar expression pattern of these two genes was observed except at 1 h post stimulation (hps) with lower expression level of the latter. The highest transcript level of *tnf-α1* and *-α2* was recorded at 24 hps. However, *tnf-n* gene was expressed in conspicuously lower level than *tnf-α1* and *-α2* but in significantly higher level ($P < 0.01$) compared to that of unstimulated cells at 12 and 24 hps only.

**Table 2**
Percentage of amino acid identity and similarity of zebrafish *tnf-α* and *-n* with other known fish *tnf-α* (upper triangle: identity; lower triangle: similarity).

| Zf*tnf-α1* | 37.9 | 18.3 | 37.7 | 37.0 | 38.8 | 66.9 | 66.9 | 59.2 | 47.1 | 64.7 |
|---|---|---|---|---|---|---|---|---|---|---|
| 55.0 | Zf*tnf-α2* | 16.7 | 39.2 | 34.0 | 33.7 | 38.6 | 37.5 | 36.3 | 29.5 | 37.3 |
| 35.0 | 35.1 | Zf*tnf-n* | 19.4 | 19.3 | 18.4 | 20.0 | 18.8 | 19.4 | 18.8 | 16.7 |
| 55.1 | 55.5 | 36.4 | Bt*tnf-α1* | 39.0 | 35.2 | 39.0 | 39.0 | 34.8 | 31.9 | 39.7 |
| 59.2 | 52.7 | 34.7 | 57.1 | Bt*tnf-α2* | 44.0 | 37.5 | 38.3 | 36.0 | 30.6 | 38.8 |
| 63.0 | 54.1 | 34.9 | 54.7 | 61.6 | Ay*tnf* | 39.4 | 37.8 | 32.9 | 26.3 | 38.6 |
| 81.0 | 55.4 | 34.6 | 54.7 | 59.6 | 60.3 | Cc*tnf-α1* | 81.0 | 71.7 | 54.4 | 76.2 |
| 81.2 | 54.5 | 35.9 | 55.5 | 59.2 | 58.7 | 89.5 | Cc*tnf-α2* | 76.3 | 55.0 | 86.2 |
| 74.4 | 51.2 | 37.9 | 51.4 | 53.5 | 55.3 | 81.9 | 88.7 | Cc*tnf-α3* | 48.9 | 73.0 |
| 55.6 | 40.9 | 32.0 | 40.5 | 44.9 | 43.4 | 60.8 | 61.9 | 60.4 | Gf*tnf-α1* | 53.9 |
| 79.9 | 55.8 | 34.6 | 55.1 | 58.4 | 60.0 | 86.1 | 93.1 | 86.4 | 62.3 | Gf*tnf-α2* |

Zf, zebrafish (*Danio rerio*); Bt, bluefin tuna (*Thunnus orientalis*); Ay, ayu (*Plecoglossus altivelis*); Cc, common carp (*Cyprinus carpio*); Gf, goldfish (*Carassius auratus*).

**Table 3**
Percentage of amino acid identity and similarity of medaka *tnf-α* and *-n* with other known fish *tnf-α* (upper triangle: identity; lower triangle: similarity).

| Me*tnf-α1* | 38.1 | 20.8 | 35.0 | 53.5 | 39.7 | 38.6 | 38.3 | 33.1 | 29.6 | 39.2 |
|---|---|---|---|---|---|---|---|---|---|---|
| 55.3 | Me*tnf-α2* | 17.9 | 54.2 | 35.1 | 33.0 | 37.1 | 36.4 | 34.4 | 28.0 | 38.9 |
| 38.1 | 34.9 | Me*tnf-n* | 18.9 | 20.2 | 19.9 | 18.0 | 21.3 | 19.0 | 17.2 | 20.5 |
| 53.8 | 69.8 | 35.6 | Bt*tnf-α1* | 39.0 | 35.2 | 39.0 | 39.0 | 34.8 | 31.9 | 39.7 |
| 68.2 | 55.3 | 32.7 | 57.1 | Bt*tnf-α2* | 44.0 | 37.5 | 38.3 | 36.0 | 30.6 | 38.8 |
| 56.2 | 54.1 | 37.9 | 54.7 | 61.6 | Ay*tnf* | 39.4 | 37.8 | 32.9 | 26.3 | 38.6 |
| 58.6 | 52.9 | 34.2 | 54.7 | 59.6 | 60.3 | Cc*tnf-α1* | 81.0 | 71.7 | 54.4 | 76.1 |
| 61.0 | 52.9 | 38.5 | 55.5 | 59.2 | 58.7 | 89.5 | Cc*tnf-α2* | 76.3 | 55.0 | 86.2 |
| 55.5 | 48.6 | 38.8 | 51.4 | 53.5 | 55.3 | 81.9 | 88.7 | Cc*tnf-α3* | 48.9 | 73.0 |
| 44.7 | 38.4 | 33.5 | 40.5 | 44.9 | 43.4 | 60.8 | 61.9 | 60.4 | Gf*tnf-α1* | 53.9 |
| 61.0 | 54.5 | 36.0 | 55.1 | 58.4 | 60.0 | 86.1 | 93.1 | 86.4 | 62.3 | Gf*tnf-α2* |

Me, medaka (*Oryzias latipes*); Bt, bluefin tuna (*Thunnus orientalis*); Ay, ayu (*Plecoglossus altivelis*); Cc, common carp (*Cyprinus carpio*); Gf, goldfish (*Carassius auratus*).

## 4. Discussion

From evolutionary view point, teleost fish whole genome duplication (FWGD) event (Meyer and Van de Peer, 2005; Ravi and Venkatesh, 2008) is an important incidence which is responsible for the existence of more than one paralogs of a mammalian gene in fish including cytokines implicating immune diversity. FWGD caused origin of two or multiple isoforms of fish cytokines, e.g. two types of fish macrophage colony stimulating factors (Wang et al., 2008), multiple interleukin (*il*)-12p40 chains (Huising et al., 2006), multiple CC chemokines (Peatman and Liu, 2007) or two type of *il-1β* genes (Husain et al., 2012). Similar incidence has occurred in case of fish *tnf-α* gene that exists in two or more isoforms in several species (Grayfer et al., 2008; Kadowaki et al., 2009; Laing et al., 2001; Morrison et al., 2007; Saeij et al., 2003; Savan and Sakai, 2004; Zhao et al., 2012; Zou et al., 2002). However, all these genes share characteristics of mammalian *TNF-α* (TNFSF2), possessing a well-defined transmembrane region, two conserved cysteines involved in the formation of tertiary structure, a TNF-α converting enzyme (TACE) cleavage site to release the active form of *tnf-α* (Hirono et al., 2000; Laing et al., 2001; Zou et al., 2002, 2003; Cai et al., 2003; Saeij et al., 2003; Savan and Sakai, 2004). Typically, fish *tnf-α* encoding gene is composed of four exons interrupted by three introns. However, the splice sites and the position of introns are well conserved in human as well as in fish genes. The mammalian *TNF-α* and *LT-α* share more similarity to each other than to the teleost *tnf-α* sequences, indicating a recent, common evolutionary origin for mammalian *TNF-α* and *LT-α* before the fish and amphibian divergence (Glenney and Wiens, 2007). Although, two *tnf-α* genes have been reported from zebrafish (Eimon et al., 2006) and two predicted *tnf*-like homologs from medaka are found in NCBI data base, their structure or orientation, genomic position and synteny with mammalian counterparts need to be elucidated to confirm their existence arising from the FWGD. Based on the hypothesis of Th1 type responses such as complement fixing antibodies, macrophage activation, antibody-dependent cell-mediated cytotoxicity, and DH hypersensitivity observed in fish, the mammalian counterpart of *TNF-β* like gene, *tnf-n* was discovered from the Japanese pufferfish (fugu) and zebrafish (Savan et al., 2005) and through *in silico* cloning we identified this gene from medaka also. Since TNFSF is a critical role player in fish immune system, our results provide new information on the understanding of immune system evolution.

From the comparative gene content and orientation analysis carried out on zebrafish and medaka *tnf* genes harboring contigs against the human *TNF*/*LT* locus, it was revealed that upstream of *tnf-α1* and *-α2* genes, only a few exons were common. The *tnf-n* gene located in tandem with *tnf-α2* on the same chromosome in zebrafish (chromosome 15) and medaka (chromosome 16) was transcribed in the same and opposite direction, respectively to human *TNF-α* and *-β*. In human chromosome 6 p21, the *TNF-α* and *-β* genes are transcribed in the same orientation, whereas *LT-β* is transcribed in the opposite direction. Compared to human *TNF-α* and *-β* genes, zebrafish *tnf-α1* gene (chromosome 19) had similar and medaka *tnf-α1* gene (chromosome 11) had opposite transcriptional orientation. There was a presence of *apo-m* and *bat3* gene upstream of *tnf-n* and *-α2* in medaka and only *bat3* gene upstream of *tnf-α2* and *-n* genes in zebrafish. *APO-M* and *BAT3* genes which are specifically linked to the MHC class III region in humans existed in medaka, whereas *APO-M* was absent in zebrafish chromosome 15. However, previously it was revealed that *apo-m* and *txl2* located upstream of *tnf-α2* and *-n* genes in zebrafish without specifying their genomic location (Savan et al., 2005). Some genes like *LT-β*, *LST1*, *NCR3*, *AIF1* and *BAT4* are absent in the *tnf* locus in both zebrafish and medaka genomes. The gene order is not conserved, but the transcription orientation is well conserved between human and zebrafish but not with medaka. In a comprehensive synteny analysis, similar pattern of incomplete conservation of TNFSF genes and their orientation has already been reported in some loci of fish with that of human chromosome (Glenney and Wiens, 2007). Therefore, the observed systeny in the content of genes but not in gene order indicates the origin of two *tnf-α* genes due to FWGD. Since TNFSF family genes exist in the main MHC class III in mammals, from our analysis, it is interesting to suggest that the MHC class III has been overlapped in fish.

Zebrafish and medaka *tnf-α1* and *-α2* genes are composed of four exons and three introns measuring 1145, and 1220 bp (zebrafish) and 681, and 768 bp (medaka), respectively. Their genomic organization seemed to be well conserved as seen in other teleost *tnf-α* gene (Savan and Sakai, 2004; Zhao et al., 2012). Interestingly, both the *tnf-α* genes in zebrafish span over a wider genomic area than in medaka. This longer coverage of gene might be due to variations in the length of introns as seen in common carp, wherein the third intron was unusually longer (1003 bp) with a total size of 1800 bp (Savan and Sakai, 2004). The *tnf-n* gene from medaka is composed of four exons and three introns, whereas the genomic structure of zebrafish *tnf-n* gene differs with five exons and four introns. In zebrafish, occurrence of five exons is attributed to the split of third exon into two smaller ones as reported previously (Savan et al., 2005). Similar to medaka *tnf-n*, fugu also possesses four exons and three introns in *tnf-n* gene which covers a longer genomic area of 2034 bp (Savan et al., 2005) than that of medaka. Zebrafish *tnf-α1* and *-α2* genes translate aa sequences (234 and 242 aa, respectively) similar to human sequence (233 aa). In common carp, four different homologs of *tnf-α* (1-4) translate sequences ranging from 227-255 aa (Saeij et al., 2003; Savan and Sakai, 2004; Zhao et al., 2012). However, the translated medaka TNF-α2 sequence measures 255 aa, similar to that of the fugu (250 aa) (Savan et al., 2005) and Japanese flounder (256 aa) (Hirono et al., 2000) orthologs, but medaka TNF-α1 is 226 aa in length similar to the 227 aa long TNF-α3 of common carp (Savan and Sakai, 2004). The TNF-N peptide sequences from zebrafish and medaka measuring 203 and 200 aa, respectively are similar in length compared to human *TNF-β* of 205 aa but longer than fugu TNF-N (194 aa) (Savan et al., 2005).

Alignment analysis of the TNF aa sequences from zebrafish, medaka and human revealed some distinguishable features; TNF-α1 and TNF-α2 from both the fish contain isoleucine at the first position of the *TNF*

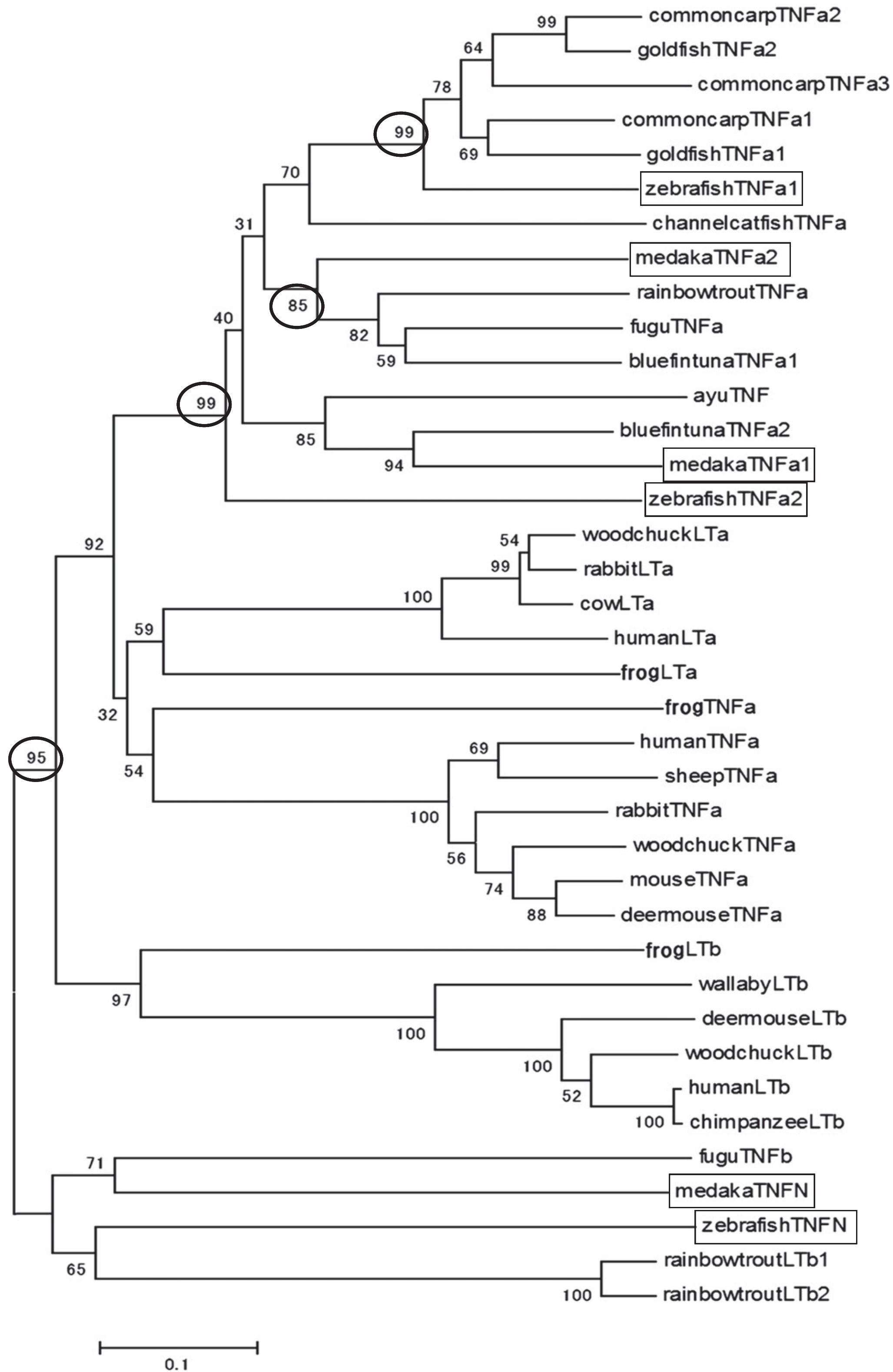


**Fig. 4.** Phylogenetic tree analysis of zebrafish and medaka TNF-α and -N with the known TNF/LT homologs from other teleosts and vertebrates. The phylogenetic tree was constructed by neighbor-joining (NJ) method using ClustulX software (v. 1.81). The numbers indicate the bootstrap confidence values obtained for each node after 1000 replications. The TNF molecules of zebrafish and medaka are highlighted in empty boxes. The bootstrap values at the root of family clades from different species and different TNF groups of interest are also highlighted with a circle. The scale at the bottom represents genetic distances in substitutions per amino acid. The GeneBank accession numbers of the TNF/LT sequences used are as follows: common carp TNF-α2, CAC84642; goldfish TNF-α2, EU069817; common carp TNF-α3, BAC77690; common carp TNF-α1, CAC84641; goldfish TNF-α1, EU069818; zebrafish T NF-α1, NM_212859; channel catfish TNF-α, CAD10389; medaka TNF-α2, XP_004078539; rainbow trout TNF-α, CAB92316; fugu TNF-α, AB183465; bluefin tuna TNF-α1, AB354733; ayu TNF, DD019003; bluefin tuna TNF-α2, AB354734; medaka TNF-α1, XP_004074383; zebrafish TNF-α2, NM_001024447; woodchuck LT-α, AAF34864; rabbit LT-α, CAA39275; cow LT-α, CAA78510; human LT-α, AAA61199; frog LT-α, AM041994; frog TNF-α, NM_001113671; human TNF-α, z15026; sheep TNF-α, NM_001024860; rabbit TNF-α, NM_001082263; woodchuck TNF-α, CAA74569; mouse TNF-α, NM_013693; deermouse TNF-α, AAG30264; frog LT-β, AM041995; wallaby LT-β, AAD41774; deermouse LT-β, AAP34710; woodchuck LT-β, AAF34865; human LT-β, Q06643; chimpanzee LT-β, BAB83881; fugu TNF-β, AB183466; zebrafish TNF-N, AB183468; rainbow trout LT-β1, AJ971433; rainbow trout LT-β2, AJ971434.

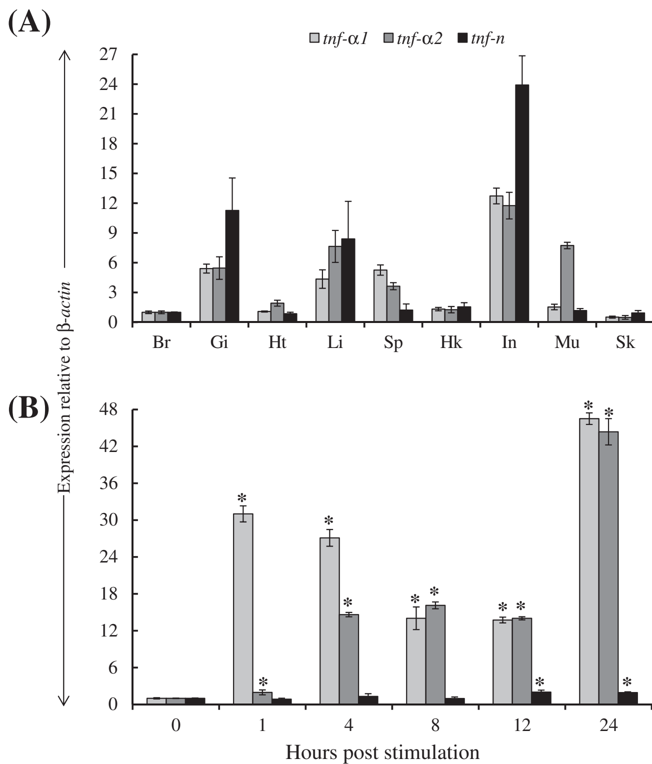


**Fig. 5.** Quantitative real-time PCR analysis of three zebrafish *tnf* genes in various tissues (Br, brain; Gi, gill; Ht, heart; Li, liver; Sp, spleen; Hk, head kidney; In, intestine; Mu, muscle; Sk, skin) (A) and head kidney cells treated with bacterial mimic lipopolysaccharide (LPS) (B). Data are $2^{-\triangle\triangle CT}$ levels calculated and normalized against the *β-actin* mRNA levels. Data are presented as mean ± S.D. in triplicates. Data are relative to the unstimulated cells (0 h) set to 1 and significant increase to the control (0 h) is indicated with $^*P < 0.01$ (B).

signature as compared to leucine in the human. Although, medaka TNF-N gene has also an isoleucine residue, zebrafish TNF-N contains a valine residue at the first position as described earlier (Savan et al., 2005). However, the molecular and functional characterization of medaka TNF-N needs to be confirmed in future. Fugu TNF-N also conformed to the signature at position 1 having isoleucine similar to that of the TNFs except zebrafish TNF-N (Savan et al., 2005). In consistence with the zebrafish TNF-N, rainbow trout LT-β1 and -β2 have valine at the first position of the family signature (Kono et al., 2006). Therefore, zebrafish and medaka TNF peptides identified in this study follow the consensus pattern of family signature proposed by Kono et al. (2006) with a slight modification: (LV*I*)-x-(LIVM*S*)-$x_3$-G*S*-(LIVMF*H*)-Y-(LIVMF*H*)$_2$-*x*$_2$-(QEKHL)-(LIVMGT*A*)-x-(LIVMFY).

In phylogenetic analysis, fish *tnf-α* genes constitute a distinct cluster away from their mammalian counterpart and *LT* genes. Zebrafish *tnf-α1* was more closely related than *tnf-α2* to common carp and goldfish *tnf-α* genes with a high bootstrap value (99%). This is because of the fact that zebrafish along with common carp and goldfish has similar phylogenetic origin as they belong to the same systematic family cyprinidae. However, zebrafish TNF-α2 and medaka TNF-α1, and -α2 were within the teleost TNF-α cluster with a high bootstrap support (99%). Similarly, in previous reports also, teleost TNF-α genes formed a distinct cluster unrelated to mammalian group (Grayfer et al., 2008; Kono et al., 2006; Savan et al., 2005; Zhao et al., 2012). Among the teleost TNF-α peptides, low bootstrap value (31%) diverting zebrafish TNF-α1 and medaka TNF-α2 indicated a high sequence divergence. Formation of a distinct cluster by the zebrafish and medaka TNF-N along with rainbow trout LT-β1 and -β2 with a high bootstrap support (95%) reconfirmed their close evolutionary proximity (Kono et al., 2006). Zebrafish TNF-α1 and -α2 isoforms showed aa similarity of 55% to each other, whereas for medaka it was 55.3%. Furthermore, phylogenetic clustering of zebrafish TNF-α1 with TNF-α from cyprinid group fish was supported by their high aa similarity ranged from 55.6 to 81.2%. However, aa similarity between TNF-α1, and -α2 to TNF-N in both the fish was similar and low (34.9–38.1%), and it indicates their probable functional difference. Similarly, TNF-N had low aa similarity with TNF-α in zebrafish and fugu (Kono et al., 2006; Savan et al., 2005).

Real-time qPCR analysis showed constitutive expression of zebrafish *tnf-α1*, *-α2* and *-n* genes in nine different tissues with higher level in intestine, liver and gills. Expression of *tnf-α1* and *-α2* was in almost similar level in all the tissues, whereas *tnf-n* exhibited higher expression in the three tissue types mentioned earlier. Previously, the expression of fugu *tnf-n* was confirmed in various tissues such as kidney, spleen, liver, intestine, skin and muscle, but zebrafish *tnf-n* was detected only in the kidney (Savan et al., 2005). However, three isoforms of *tnf-α* (1, 2 and 3) were expressed in common carp unstimulated intestine, gill, HK, muscle and spleen (Savan and Sakai, 2004). The differences in site and type of transcription factor might have caused the varied tissue level expression. As it is difficult to draw firm conclusions from the expression data in normal tissues, we conducted further expression analysis of these genes in lymphoid cells like HK cells stimulated with LPS.

The pleiotropic cytokine, *tnf-α* plays a critical role in early inflammation (Savan and Sakai, 2006) and is elicit in response to bacterial component. LPS induced expression of *tnf-α1* and *-α2* genes in zebrafish HK cells during 1 ~ 24 hps with the highest level at 24 hps. Similar to our observation, grass carp HK leukocytes treated with LPS showed increased expression of *tnf-α* at 1, 4 and 6 hps (Zhang et al., 2012), whereas LPS caused enhanced *tnf-α1* and *-α2* expression in common carp HK leukocytes after 4 h with higher expression of the latter (Saeij et al., 2003). Moreover, enhanced expression of common carp *tnf-α4* was noticed in *Aeromonas hydrophila* infected fish blood leukocytes (Zhao et al., 2012). In bluefin tuna peripheral blood leukocytes (PBLs), *tnf-α2* expression was induced at 1 hps, but *tnf-α1* expression was not elevated by different immunostimulating agents (Kadowaki et al., 2009). Similarly, LPS induced expression of *tnf-α2* at 2 hps and *tnf-α1* at 24 hps in PBLs of orange-spotted grouper (Lam et al., 2011). In cultured macrophages of gilthead seabream, *tnf-α* gene was not expressed after 4 h stimulation (García-Castillo et al., 2004). However, there was an increased expression of *tnf-α* in the HK and blood of large yellow croaker at 2–4 days after pathogen injection (Xie et al., 2008). Our results coupled with these observations indicate that the expression of zebrafish *tnf-α1* was induced at an early stage (1 hps) in response to an immune-stimulating agent, and the expression of *tnf-α2* was not induced immediately or induced over a longer time period along with *tnf-α1* (more than 24 h). Therefore, it is suggested that zebrafish *tnf-α1* is an acute and longer response-type *tnf-α* gene and *tnf-α2* is a delayed response-type *tnf-α* gene due to their probable differential regulation. However, *tnf-n* was less constitutively expressed with significantly higher level only at 12 and 24 hps. The reason of its lower expression compared to *tnf-α* cannot be inferred as the function is yet to be ascertained.

In conclusion, two *tnf-α* and a *tnf-n* ligands adjacent to each other have been identified from zebrafish and medaka through *in silico* approach. Several features suggest that these are homologs of *tnf-α* and *tnf-n* genes. Genomic location of these genes was found to be as: *tnf-α1*, and *tnf-n* and *tnf-α2* genes on zebrafish chromosome 19 and 15 and medaka chromosome 11 and 16, respectively. The phylogenetic and identity analyses support that both zebrafish and medaka *tnf-α* genes are similar to other teleost *tnf-α*. Existence of two isoforms of *tnf-α* and a *tnf-n* was further confirmed by tissue level and immunostimulant induced expression analyses. Further cloning and analysis of these genes in medaka are needed for classification and understanding the function.

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