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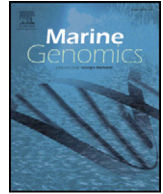

Evolutionary evidence of tumor necrosis factor super family members in the Japanese pufferfish (*Takifugu rubripes*): Comprehensive genomic identification and expression analysis

Gouranga Biswas [a,1], Shunsuke Kinoshita [b], Tomoya Kono [b], Jun-ichi Hikima [b], Masahiro Sakai [b,*]

[a] Interdisciplinary Graduate School of Agriculture and Engineering, University of Miyazaki, 1-1 Gakuenkibanadai-nishi, Miyazaki 889-2192, Japan
[b] Department of Biochemistry and Applied Biosciences, Faculty of Agriculture, University of Miyazaki, 1-1 Gakuenkibanadai-nishi, Miyazaki 889-2192, Japan

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ABSTRACT

Tumor necrosis factor (TNF) and its superfamily (TNFSF) members are important inflammatory cytokines. Although fish have fourteen *TNFSF* genes, their genomic location and existence are yet to be described and confirmed in the Japanese pufferfish (Fugu) (*Takifugu rubripes*). Therefore, we conducted *in silico* identification, synteny analysis of *TNFSF* genes from Fugu with that of zebrafish and human TNFSF loci and their expression analysis in various tissues. We identified ten novel *TNFSF* genes, *viz. TNFSF5* (*CD40L*), *TNFSF6* (*FasL*), three *TNFSF10* (*TRAIL*) (*−1, 2 and 3*), *TNFSF11* (*RANKlg*), *TNFSF12* (*TWEAK*), two *TNFSF13B* (*BAFF*) (*1* and *2*) and *TNFSF14* (*LIGHT*) belonging to seven TNFSFs in Fugu. Several features such as existence of TNF family signature, conservation of genes in TNF loci with human and zebrafish chromosomes and phylogenetic clustering with other teleost TNFSF orthologs confirmed their identity. Fugu *TNFSF* genes were constitutively expressed in all eight different tissues with most of them expressed highly in liver. Fugu *TNFSF10* gene has three homologs present on chromosomes 10 (*TNFSF10-1*), 8 (*TNFSF10-2*) and 2 (*TNFSF10-3*). Moreover, a phylogenetic analysis containing all available vertebrate (mammals, birds, reptiles, amphibians and fish) TNFSF10 orthologs showed that Fugu TNFSF10-1 and 10-3 are present in all vertebrates, whereas TNFSF10-2 was not related to any mammalian and avian orthologs. Viral double-stranded RNA mimic poly (I:C) caused an elevated expression of three Fugu *TNFSF10* genes in head kidney cells at 4 h indicating probable role of these genes to induce apoptosis in virus-infected cells. In conclusion, Fugu possesses genes belonging to nine TNFSFs including the newly identified seven and previously reported two, *TNFSF New* (*TNF-N*) and *TNFSF2* (*TNF-α*). Our findings would add up information to TNFSF evolution among vertebrates.

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1. Introduction

Cytokines are small protein mediators produced by immune cells to regulate and mediate immunity, inflammation and hematopoiesis (Savan and Sakai, 2006). Tumor necrosis factor (TNF) and its superfamily (TNFSF) members are important inflammatory cytokines (Aggarwal et al., 2012). In human, TNFSFs composed of 19 ligands and 29 TNF receptor superfamily (TNFRSF) members, play highly diversified roles (Aggarwal et al., 2012). TNFSF members bind to their cognate receptors to activate the assembly of receptor-associated signaling complexes, stimulate activation of multiple signaling pathways, viz. nuclear factor (NF)-κB, the c-Jun N-terminal kinase (JNK) and p38 mitogen activated protein kinases (MAPKs), and often cause cell death (apoptosis) (Li and Lin, 2008; Wagner and Nebreda, 2009). Some of the TNFSF members have also been reported to play a role in morphogenetic changes and differentiation (Aggarwal et al., 2012). Most members of the TNFSF have both beneficial and potentially harmful effects (Aggarwal, 2003). The family members involved in cell proliferation, TNFSF5 (CD40L), TNFSF4 (OX40L) and TNFSF7 (CD70) are expressed on activated T cells. On the other hand, representative molecules involved in apoptosis are TNFSF6 (FasL), TNFSF10 (TRAIL) and TNFSF12 (TWEAK) (Aggarwal et al., 2012). Although several studies on the function and clinical application of TNFSF were conducted, research in vertebrates other than mammals is meager. Furthermore, despite information available on function of the TNFSF members, a little work has been carried out on TNFSF–TNFRSF interactions.

The first invertebrate TNFSF ligand, Eiger (Igaki et al., 2002) and TNFRSF member, Wengen (Kanda et al., 2002) were identified from an arthropod, *Drosophila*. Through phylogenetic ancestry there is a high possibility that TNFSF and TNFRSF genes were evolved from invertebrates to vertebrates (Collette et al., 2003). On the basis of Eiger and

* Corresponding author. Tel./fax: +81 985 587219.
E-mail address: m.sakai@cc.miyazaki-u.ac.jp (M. Sakai).
[1] Present address: Kakdwip Research Centre of Central Institute of Brackishwater Aquaculture (ICAR), Kakdwip, South 24 Parganas, West Bengal, PIN-743347, India.

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Table 1
Primers used for Fugu *TNFSF* gene expression analysis (qPCR) in this study.

Gene	Primer sequence (5′ → 3′)	Product size (bp)	Amplification efficiency (%)
TNFSF5 Fw	TGATGCTGCTGCAGTTTCTC	127	108
TNFSF5 Rv	CTGGCCGTCATCTCTTTTTC		
TNFSF6 Fw	GGGTTCGAGGCGTACGAG	200	102
TNFSF6 Rv	GACTCCCACCTCAAAGTCTTG		
TNFSF10-1 Fw	CAGTTCCAATCCTGCTGATG	137	108
TNFSF10-1 Rv	GTTGGTCACAGTCACAAAGAGG		
TNFSF10-2 Fw	TGCGCGATCGAGTAAATACC	111	95
TNFSF10-2 Rv	ATGAGCAGAGCACGAAACG		
TNFSF10-3 Fw	TCTGCTCAGGTTTTGGAAGG	81	98
TNFSF10-3 Rv	CGTTGCTAACGGTGATGAAG		
TNFSF11 Fw	TCTGCCCATCACAACACATC	131	99
TNFSF11 Rv	ACTCCATCACCAACAGCTTG		
TNFSF12 Fw	GGCCATCGTTCAGTTGATTC	115	100
TNFSF12 Rv	ATGAGAGGCCGCTTTCTTTC		
TNFSF13B1 Fw	TGAATCTGCCACGAACTGAG	108	105
TNFSF13B1 Rv	AGGACTCTGAGCATTTGCAG		
TNFSF13B2 Fw	TGAATCTGCCACGAACTGAG	98	106
TNFSF13B2 Rv	AGGACTCTGAGCATTTGCAG		
TNFSF14 Fw	TCCTGTTGGGGAGGATAATG	180	101
TNFSF14 Rv	TTCATGACCTTGTGCTGGAC		
β-actin Fw	CCAGAAAGACACAGCTACGTTGG	147	108
β-actin Rv	GCAACTCTCAGCTCGTTGTAG		

Fw = forward; Rv = reverse.

Wengen evolution, using molecular sequence information several TNFSF–TNFRSF pairs have been discovered and this provides useful insight into phylogenetic divergence and comparative genomics among vertebrates and invertebrates. In particular, by conducting comparative genome analysis, information on conservation of gene sequences can be obtained and there is a possibility to find evidences for genome evolution (Clark et al., 2001). Eukaryote genome sequencing has been endeavored through various international genome sequencing projects for more than 100 species with so far accomplished ones for whole genomes of human (IHGSC, 2004), chicken (ICGSC, 2004) and zebrafish (Howe et al., 2013). In addition, genome sequencing has been completed for the organism possessing the most compact genome among vertebrates, the Japanese pufferfish (Fugu) (*Takifugu rubripes*) (Aparicio et al., 2002). Whole genome of human is 3000 Mb with around 22,000 structural genes covering only about 1.5% of the entire genome. On the other hand, Fugu genome is 365 Mb that is about one-eighth of the human genome (Aparicio et al., 2002). However, Fugu genome contains 21,000 genes that are almost similar to human. Many of the functions of these compressed genes in Fugu genome are unknown. In fact, looking at the array of the Japanese pufferfish whole genome, the amount of repetitive sequences is only 15% or less compared to the human genome that contains 50% repetitive sequences. Furthermore, 75% of introns in human are 2609 bp or longer, whereas in Fugu it is 425 bp or less (Aparicio et al., 2002; Edwards et al., 1998; Watabe et al., 2008). After publication of the genome and chromosome map in 2011, Fugu has become an excellent model organism for comparative genomics (Kai et al., 2011). Therefore, Fugu genome can be used as a useful reference to discover genes and gene regulatory molecules to that of human genome for deriving novel understanding on vertebrate genome organization and evolution. In fish, a TNFSF2 member, *TNF-α* has been reported from several species (Eimon et al., 2006; Grayfer et al., 2008; Hirono et al., 2000; Laing et al., 2001; Saeij et al., 2003; Zhang et al., 2012; Zou et al., 2003a). Moreover, existence of two or more *TNF-α* genes in several teleost species occurred because of the fish whole genome duplication (FWGD) event (Laing et al., 2000). However, information on other TNFSF members is scarcely available in fish including Fugu. Previously, through bioinformatics searches of zebrafish, *Tetraodon* and Fugu genome, and other teleost expressed sequence tag databases, Glenney and Wiens (2007) identified a total of 14 distinct TNFSF members, indicating expansion of this superfamily before the divergence of bony fish and tetrapods, ~360–450 million years ago. However, the information on Fugu TNFSF members remains incomplete. Moreover, there are no diseases reported in Fugu associated with tumor necrosis. Although, Fugu displaying nodular lesions in the branchial chamber as well as on the inner surface of operculum had a moderate mortality caused by *Vibrio harveyi* (Mohi et al., 2010), no association of TNF was reported. Generally, in fish, TNFs act as pro-inflammatory cytokines (Zou et al., 2003b) and also take part in morphogenesis like formation of adipose tissue (Liu et al., 2015). However, comprehensive information on function of all TNFSF members is not available from lower vertebrates. Therefore, in this study, to reveal the understanding on the evolution of TNFSF, we systematically searched genome database of Fugu for the purpose of analysis of the *TNFSF* gene loci on the genome. Thus, we could identify a few novel TNFSF members, then analyzed their synteny with human and zebrafish genomes, and investigated the transcription patterns in tissues and stimulated cells.

2. Materials and methods

2.1. TNFSF gene search and identification from databases

Fugu *TNF* orthologs were determined by searching the EST and genomic databases of NCBI (http://www.ncbi.nlm.nih.gov/) and Ensembl (http://uswest.ensembl.org/index.html). The retrieved Fugu TNF amino acid sequences were subjected to blast search with that of

Table 2
Confirmation of newly identified *TNFSF* genes from Fugu by BLAST search corresponding to that of human and zebrafish.

Gene	Genomic location (chromosome no.)		
	Human	Zebrafish	Fugu
TNFSF5	X	14	14
TNFSF6	1	20	20
TNFSF10	3	24, 7, 5, 22	10, 8, 2
TNFSF11	13	9	Scaffold 437
TNFSF12	17	7	8
TNFSF13	17	5	NE
TNFSF13B	13	9	1, 14
TNFSF14	19	3	17

NE = non-existent.

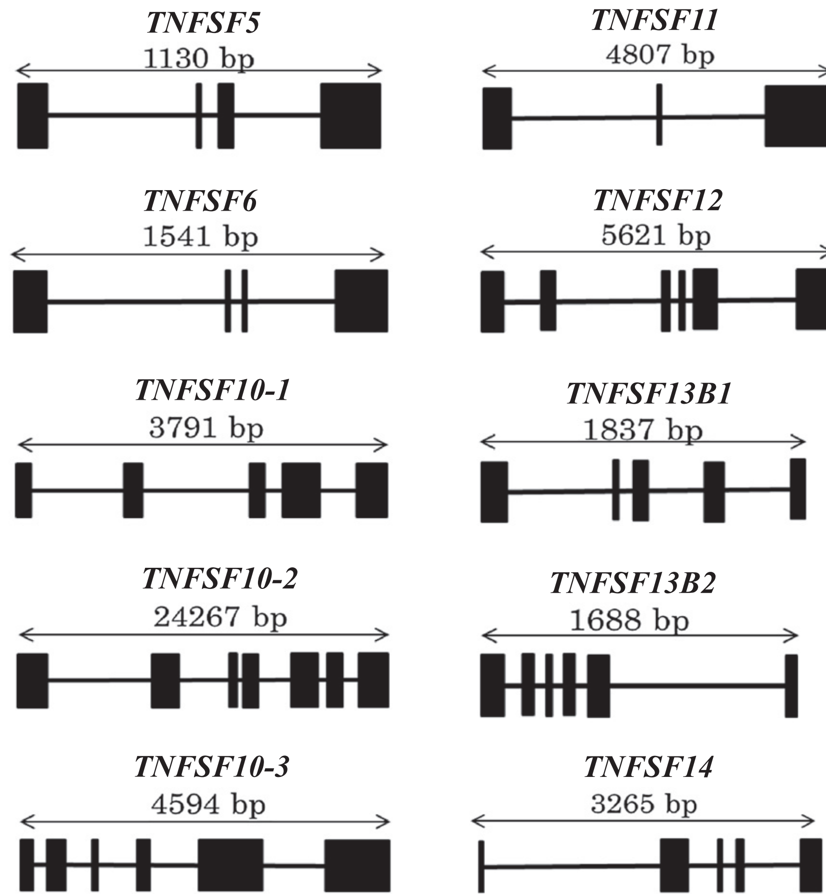


Fig. 1. Structural analysis of Japanese pufferfish *TNFSF* genes. Exons and introns are represented as closed boxes and lines, respectively.

human and zebrafish genomes using blastp (protein vs protein) and tblastn (protein *vs* DNA sequence). Nomenclature to Fugu TNFSF members was assigned based on the previous classifications (Aggarwal et al., 2012; Glenney and Wiens, 2007). Upon establishment of gene synteny in Fugu genome with human and zebrafish genomes, the gene/protein was designated with equivalent vertebrate category.

2.2. Sequence, structural and synteny analyses

Amino acids multiple sequence alignments of identified Fugu TNFSF ligands 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 Fugu, zebrafish and human harboring *TNFSF* ligands.

2.3. Phyolgenetic analysis of retrieved Fugu TNFSF genes

Phylogenetic analysis was conducted with related *TNFSF* orthologs from Fugu, other fish and vertebrates using the full-length amino acid sequences by the neighbor-joining (NJ) method (Saitou and Nei, 1987) using MEGA version 6 (Tamura et al., 2013). Node values which represent percent bootstrap confidence were derived from 1000 replications.

2.4. Expression analysis of TNFSF genes in unstimulated tissues

Fugu, *T. rubripes* (mean weight: 10.2 ± 0.5 g) were procured from Matsumoto Fisheries Farm, Miyazaki, Japan. Fish were first acclimatized in an aerated seawater tank at 22 ± 2 °C and fed a commercial diet (Sango, Higashimaru Co. Ltd., Kagoshima, Japan) at 1% body weight daily for two weeks under a natural photoperiod prior to their use in the study. The health status of experimental fish was checked following the method described earlier (Biswas et al., 2013). Individual fish was scooped out of holding tank and anesthetized with 2-phenoxyethanol (0.05%, Sigma-Aldrich, St. Louis, MO, USA) in a bucket containing aerated before being sacrificed for tissue collection. Eight different tissues (brain, gills, heart, head kidney, liver, spleen, intestine and skin) were excised aseptically from five 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 the 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 real-time quantitative PCR (qPCR) and their efficiency values are enlisted in Table 1. Amplification efficiency of these primer sets was determined according to the method of Pfaffl (2001). The qPCR reaction was conducted using THUNDERBIRD™ SYBR® qPCR Mix (Toyobo Co., Ltd.) for each sample run in triplicate as per the protocol described earlier (Kinoshita et al., 2014). The comparative threshold cycle (C_T) method ($2^{-\Delta\Delta CT}$ method) (Schmittgen and Livak, 2008) was used to analyze the

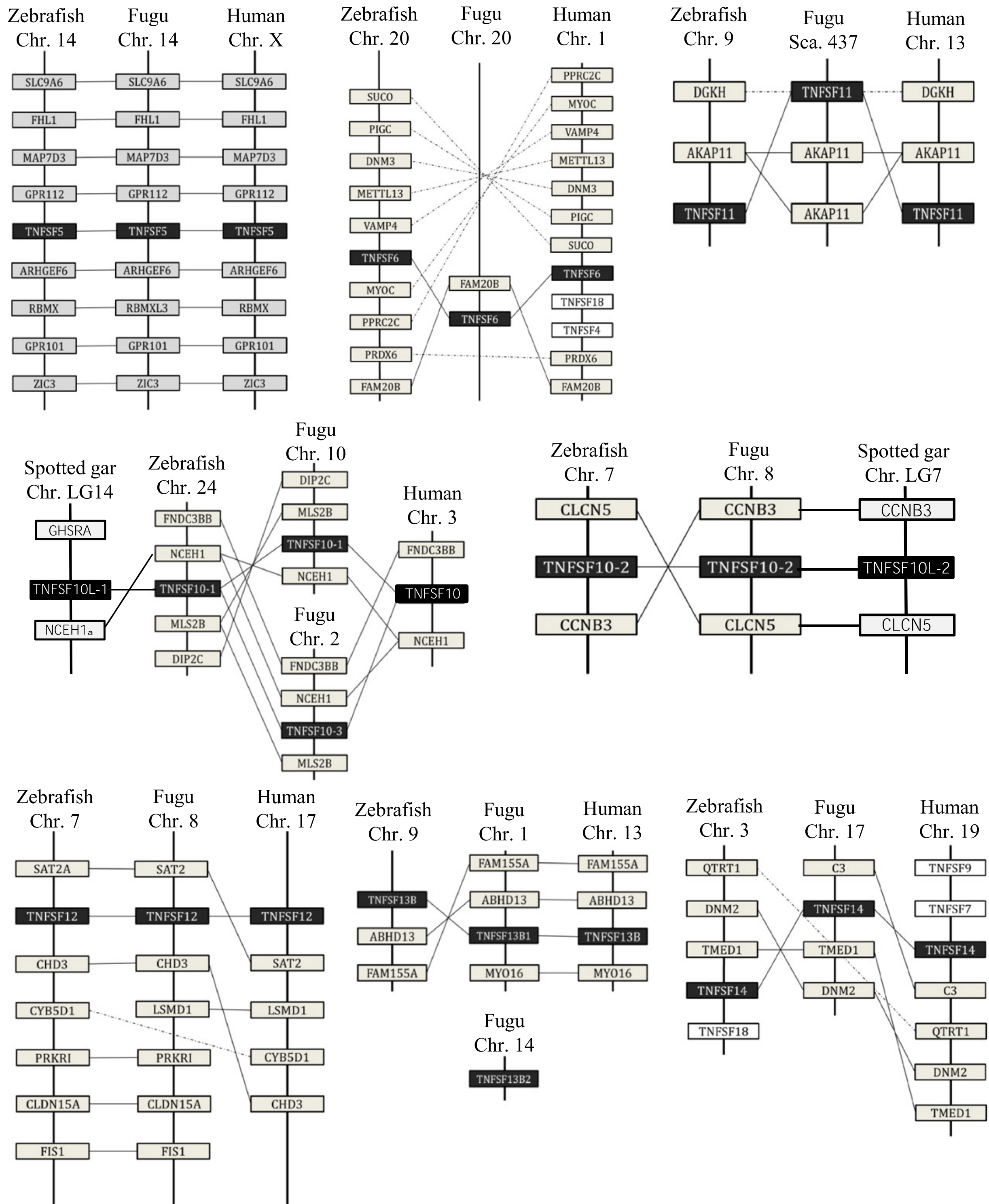


Fig. 2. Schematic diagram of gene synteny between Fugu, zebrafish and human chromosomes for *TNFSF* genes that are highlighted in black boxes.

expression level of ten *TNFSF* genes using β-*actin* (U37499) as an internal control gene. One-way analysis of variance was performed to find out any difference in quantified relative expression of a particular gene among various tissues using SPSS for Windows v. 17.0 program (SPSS Inc., Chicago, IL, USA).

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.

2.5. Phylogenetic and expression analysis of novel TNFSF10 (TRAIL) genes

Results of phylogenetic and synteny analyses indicated identification of three novel *TNFSF10* homologs from Fugu. Therefore,

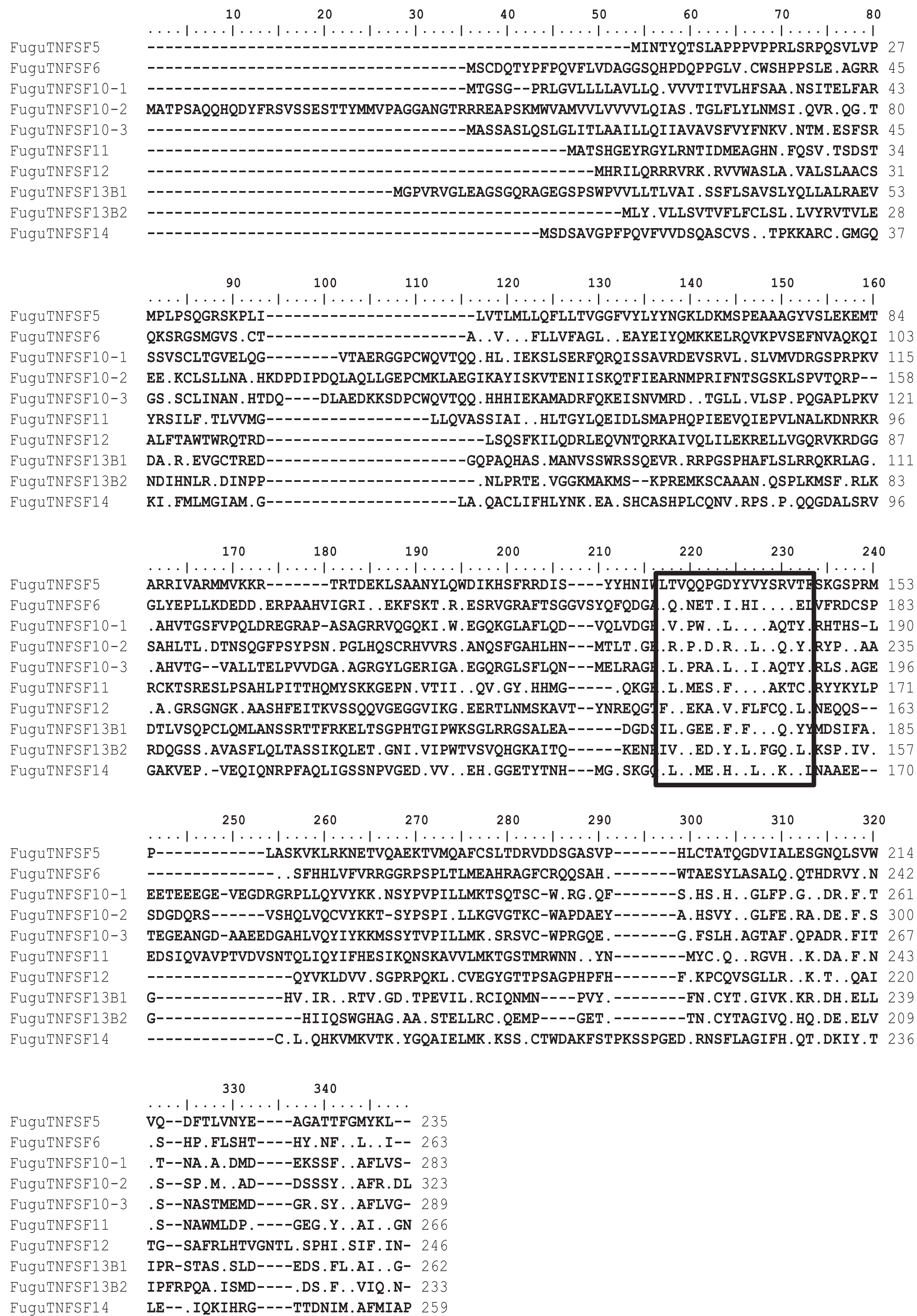


Fig. 3. Multiple alignment of newly identified Fugu TNFSF amino acid sequences. Identical amino acid residues are indicated by dots, and dashes are introduced to fill the gaps for optimal alignment. TNF family signature motifs are marked with an empty box.

further analysis of these genes was conducted for phylogeny and transcription.

2.5.1. Phylogenetic analysis of Fugu TNFSF10 homologs

Phylogenetic tree was constructed using Fugu TNFSF10 with related TNFSF10 orthologs from other fish and vertebrates using the full-length amino acid sequences as described in Section 2.3.

2.5.2. Expression of TNFSF10 genes in LPS and poly (I:C)-stimulated head kidney (HK) cells

HK cells from the healthy fish were collected as per the protocol described previously (Biswas et al., 2013). The cells (1×10^7 cells mL^{-1}) were seeded in a 24-well plate (Nunc A/S, Roskilde, Denmark), stimulated with the bacterial mimic lipopolysaccharide (LPS; Sigma, MO, USA) and double-stranded (ds) RNA viral mimic poly (I:C) (Sigma) at

Table 3
Percentage of amino acid identity (%) among the newly identified Fugu *TNFSF* genes.

	1	2	3	4	5	6	7	8	9	10
1. TNFSF5										
2. TNFSF6	13.2									
3. TNFSF10-1	11.9	14.7								
4. TNFSF10-2	11.3	9.6	23.5							
5. TNFSF10-3	10.2	12.8	46.3[a]	22.9						
6. TNFSF11	13.4	12.7	15.9	15.3	14.6					
7. TNFSF12	9.0	10.4	12.7	9.6	10.1	10.2				
8. TNFSF13B1	11.4	8.0	14.8	13.1	13.6	10.4	10.1			
9. TNFSF13B2	13.4	9.7	11.4	10.6	9.8	10.0	9.9	23.1		
10. TNFSF14	14.3	17.5	15.6	12.2	15.7	10.3	9.4	11.0	9.0	

[a] Highest amino acid identity (%).

20 μg mL^{-1} and incubated for 4, 12 and 24 h at 25 °C. A control (cells without stimulation) was maintained for each time point. Each treatment and control had three replicates. Incubated cells were harvested at the time points mentioned above, submerged in RNAlater solution (Ambion, Austin, TX, USA) for overnight and finally stored at −80 °C prior to RNA extraction. RNA extraction, cDNA synthesis and qPCR were performed as per the protocols described in Section 2.4. 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.).

3. Results

3.1. Genome database search results

From Fugu genome database, 10 *TNFSF* genes were identified and through BLAST search they were confirmed to belong to seven TNFSFs (Table 2). The identified genes were *TNFSF5* (*CD40L*), *TNFSF6* (*FasL*), three *TNFSF10* (*TRAIL*) (*−1, 2* and *3*), *TNFSF11* (*RANKlg*), *TNFSF12* (*TWEAK*), two *TNFSF13B* (*BAFF*) (*1* and *2*) and *TNFSF14* (*LIGHT*).

3.2. Gene structural and chromosome synteny analyses

Fugu *TNFSF5* (*CD40L*) gene consists of 1130 bp containing four exons and three introns (Fig. 1). The gene order and orientation analysis demonstrated that like human X chromosome genes, several genes are located upstream and downstream of Fugu *TNFSF5* gene harbored on chromosome 14 same as in zebrafish (Fig. 2).

Fugu *TNFSF6* (*FasL*) gene has four exons and three introns spanning 1541 bp (Fig. 1). There was a presence of *FAM20B* gene upstream of *TNFSF6* gene on chromosome 20 of Fugu (Fig. 2). However, *FAM20B* gene was downstream of *TNFSF6* on zebrafish chromosome 20 and human chromosome 1. In addition, in the vicinity of *TNFSF6* gene, few genes such as *MYOC*, *VAMP4*, *METTL13*, *DNM3*, *PIGC*, *SUCO*, *PPRC2C* and *PRDX6* were common between human and zebrafish. However, these genes do not exist around *TNFSF6* gene on Fugu chromosome 20.

The structural analysis of Fugu *TNFSF10* (*TRAIL*)*-1* gene showed that it is composed of five exons and four introns with 3791 bp length (Fig. 1). However, Fugu *TNFSF10-2* gene is long consisting of seven exons and six introns with a longer second intron (9730 bp) in comparison to *TNFSF10-1* gene (Fig. 1). Fugu *TNFSF10-3* gene is 4594 bp long with six exons and five introns (Fig. 1). Fugu *TNFSF10-1* gene exists on chromosome 10 with two upstream *DIP2C* and *MLS2B* and a downstream *NCEH1* genes (Fig. 2). Fugu chromosome 2 contains *TNFSF10-3* gene that has upstream *FNDC3BB* and *NCEH1* genes and a downstream *MLS2B* gene (Fig. 2). Human chromosome 3 harbors *TNFSF10* gene. Zebrafish chromosome 24 has *TNFSF10-1* gene along with similar other genes in identical and opposite structural orientations compared to Fugu *TNFSF10-3* and *TNFSF10-1*, respectively. Moreover, spotted gar has *TNFSF10like-1* gene on chromosome LG14 that harbors a downstream *NCEH1a* gene similar to that of Fugu. Fugu *TNFSF10-2* gene exists on chromosome 8 that contains *CCNB3* on upstream and *CLCN5* on downstream. Similar to Fugu, spotted gar contains *TNFSF10like-2* gene on chromosome LG7 with identical upstream and downstream gene orientation. Although, zebrafish chromosome 7 contains *TNFSF10-2* along with *CCNB3* and *CLCN5* genes, the structural orientation of the latter two genes is completely opposite to that of Fugu (Fig. 2).

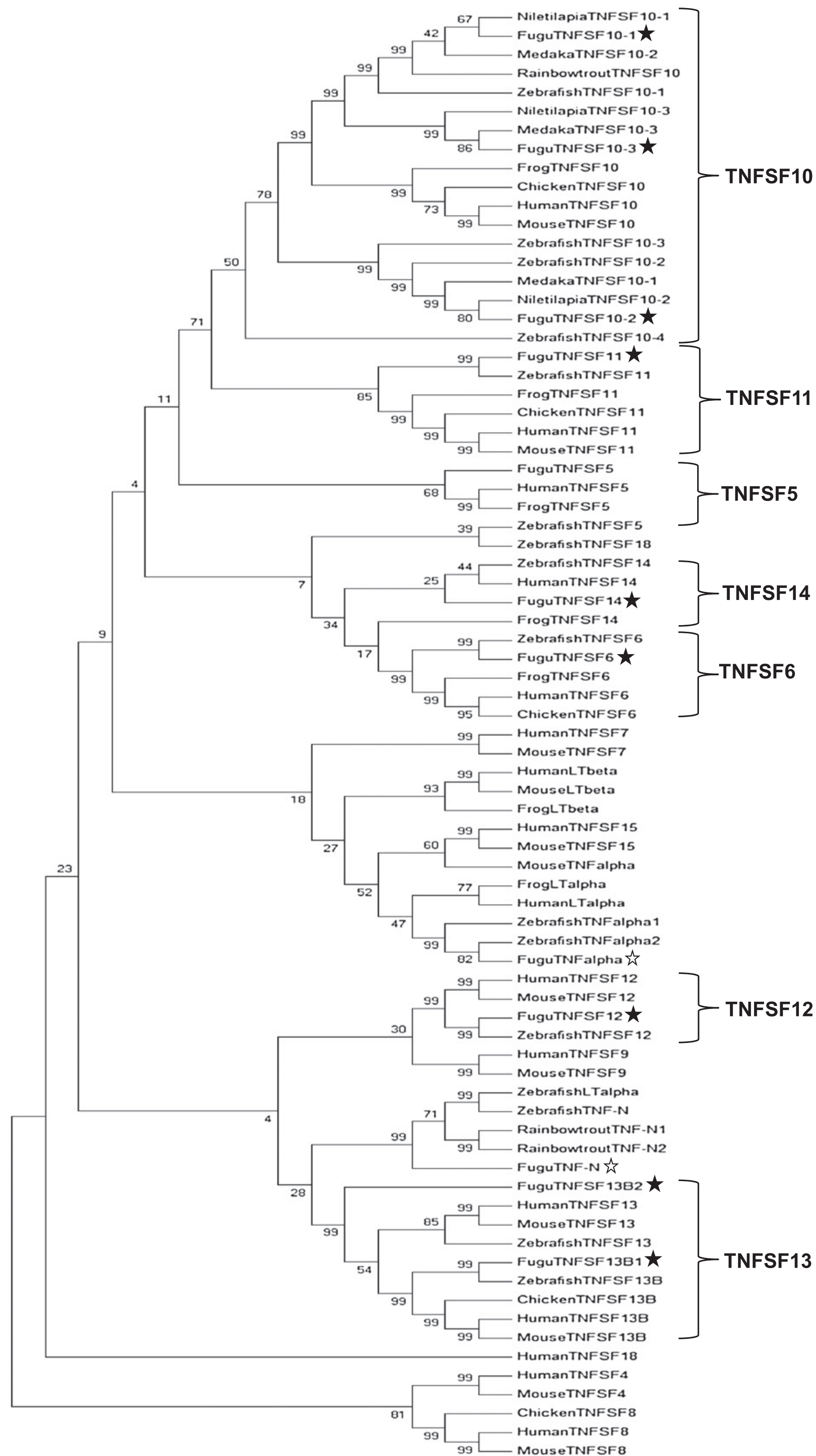
Fugu *TNFSF11* (*RANKlg*) gene is 4807 bp long with three exons and two introns (Fig. 1). This gene was found to be located on scaffold 437 where two *AKAP11* genes exist downstream of it (Fig. 2). However, zebrafish chromosome 9 and human chromosome 13 contain *TNFSF11* gene with upstream *AKAP11* and *DGKH* genes.

There are six exons and five introns spanning 5621 bp in Fugu *TNFSF12* (*TWEAK*) gene (Fig. 1). *TNFSF12* gene presents on Fugu chromosome 8 that also contains upstream *SAT2* and downstream *CHD3*, *LSMD1*, *PRKRI*, *CLDN15A* and *FIS1* genes with similar structural orientation of zebrafish chromosome 7 except *LSMD1* gene that is replaced by *CYB5D1* gene (Fig. 2). Moreover, human chromosome 17 harbors *TNFSF12* gene with downstream *SAT2*, *LSMD1*, *CYB5D1* and *CHD3* genes of which *CYB5D1* gene is absent in Fugu but present in zebrafish.

The structural analysis showed that Fugu *TNFSF13B* (*BAFF*) *1* gene is composed of five exons and four introns extended over 1837 bp (Fig. 1). This gene is located on Fugu chromosome 1 that also harbors upstream *FAM155A*, *ABHD13* genes and downstream *MYO16* gene similar to that of human chromosome 13 (Fig. 2). On the contrary, zebrafish chromosome 9 has *TNFSF13B* gene with downstream *ABHD13* and *FAM155A* genes. Fugu *TNFSF13B2* is 1688 bp long containing six exons and five introns (Fig. 1). This gene was identified only on Fugu chromosome 14 (Fig. 2).

Fig. 4. Phylogenetic tree analysis of Fugu TNFSF with the known TNFSF homologs from other teleosts and vertebrates. The phylogenetic tree was constructed by neighbor-joining (NJ) method using MEGA v.6. The numbers indicate the bootstrap confidence values obtained for each node after 1000 replications. The newly identified TNFSF molecules of Fugu are marked with a solid asterisk and other Fugu TNFs are highlighted with an empty asterisk. The GenBank accession numbers of the TNF amino acid sequences used are as follows: Nile tilapia TNFSF10-1, XP_003439360.1; medaka TNFSF10-2, XP_004081235; rainbow trout TNFSF10, NP_001118037.1; Nile tilapia TNFSF10-3, XP_005477488.1; medaka TNFSF10-3, XP_004079990; medaka TNFSF10-1, XP_004079990; Nile tilapia TNFSF10-2, XP_003453980.2; zebrafish TNF-α1, NP_998024.2; zebrafish TNF-α2, NP_001019618.1; Fugu TNF-α, NP_001033074.1; frog LT-α, NP_001107150.1; human LT-α, NP_000586.2; mouse LT-α, NP_034865.1; frog TNF-α, NP_001108250.1; human TNF-α, NP_000585.2; mouse TNF-α, NP_038721.1; human TNFSF15, AAI04464.1; mouse TNFSF15, NP_796345.3; zebrafish TNFSF6, NP_001036166.1; Fugu TNFSF6, XP_003974146.1; frog TNFSF6, NP_001131050.1; human TNFSF6, NP_000630.1; chicken TNFSF6, NP_001026730.1; zebrafish TNFSF14, NP_001268924.1; frog TNFSF14, XP_002940097.2; human TNFSF14, NP_003798.2; Fugu TNFSF14, XP_003972137.1; human LT-β, NP_002332.1; mouse LT-β, NP_032544.1; frog LT-β, NP_001107163.1; human TNFSF11, NP_003692.1; mouse TNFSF11, NP_035743.2; chicken TNFSF11, NP_001076830.1; frog TNFSF11, NP_001088945.1; zebrafish TNFSF10-3, NP_001036178.1; zebrafish TNFSF10-2, NP_571918.2; Fugu TNFSF10-2, XP_003966894.1; human TNFSF5, NP_000065.1; frog TNFSF5, XP_002932670.1; zebrafish TNFSF10-1, NP_001002593.1; Fugu TNFSF10-1, XP_003968206.1; Fugu TNFSF10-3, NP_001233230.1; frog TNFSF10, NP_001163915.1; chicken TNFSF10, NP_989710.1; human TNFSF10, NP_003801.1; mouse TNFSF10, NP_033451.1; zebrafish TNFSF5, NP_001138281.1; Fugu TNFSF5, XP_003970229.1; Fugu TNFSF11, XP_003976078.1; zebrafish TNFSF11, XP_002663397.2; zebrafish TNFSF10-4, NP_001013301.1; human TNFSF9, NP_003802.1; mouse TNFSF9, NP_033430.1; human TNFSF12, NP_003800.1; mouse TNFSF12, NP_035744.1; Fugu TNFSF12, XP_003966992.1; zebrafish TNFSF12, NP_001070075.1; human TNFSF13, NP_003799.1; mouse TNFSF13, NP_076006.2; zebrafish TNFSF13, NP_001161936.1; Fugu TNFSF13B2, XP_003970507.1; Fugu TNFSF13B1, XP_003961760.1; zebrafish TNFSF13B, NP_001107062.1; chicken TNFSF13B, NP_989658.1; human TNFSF13B, NP_006564.1; mouse TNFSF13B, NP_296371.1; zebrafish LT-α, NP_001019992.1; human TNFSF18, NP_005083.2; human TNFSF7, NP_001243.1; mouse TNFSF7, EDL38239.1; Fugu TNF-N, NP_001033075.1; zebrafish TNFSF18, NP_001122290.1; chicken TNFSF8, NP_989740.1; human TNFSF8, NP_001235.1; mouse TNFSF8, NP_033429.1; human TNFSF4, NP_003317.1; mouse TNFSF4, NP_033478.1; zebrafish TNF-N, BAD98731; rainbow trout TNF-N1, ABC84587; rainbow trout TNF-N2, ABC84588.

Fugu *TNFSF14* (*LIGHT*) contains five exons and four introns stretched over 3265 bp (Fig. 1). This gene is located on Fugu chromosome 17 that harbors an upstream *C3* and downstream *TMED1* and *DNM2* genes (Fig. 2). However, all these three genes (*C3*, *TMED1* and *DNM2*) were found to be downstream of *TNFSF14* gene on human chromosome 19. Zebrafish chromosome 3 has *TNFSF14* gene with upstream *TMED1* and *DNM2* genes.



3.3. Alignment, homology and phylogenetic analyses

Multiple alignments of ten Fugu TNFSF amino acid (aa) sequences (Fig. 3) showed a high conservation of the TNF family signature motifs (LV)-x-(LIVM)-x_3-G-(LIVMF)-Y-$(LIVMFY)_2$-x_2-(QEKHL)-(LIVMGT)-x-(LIVMFY). Fugu TNFSF aa sequences were compared among each other (Table 3). From the homology analysis, the highest aa identity (46.3%) was observed between TNFSF10-1 and TNFSF10-3 followed by TNFSF10-1 and TNFSF10-2 (23.5%). Overall, there was a low aa identity (9–16%) among other TNFSF sequences.

In phylogenetic analysis, most of the Fugu TNFSF sequences formed clusters with the respective zebrafish and human sequences (Fig. 4). However, Fugu TNFSF5 was in the same cluster with that of human and frog away from zebrafish TNFSF5 sequence. Furthermore, Fugu TNFSF10-1 and 10-3 formed a sub-cluster with zebrafish TNFSF10-1 and this cluster bifurcated from the other vertebrates' TNFSF10 sub-cluster with a high bootstrap value (99%). However, Fugu TNFSF10-2 was within a cluster with zebrafish TNFSF10-2 and 10-3, and other teleost TNFSF10-1 and 10-2 orthologs.

3.4. Tissue level expression of Fugu TNFSF genes

Fugu *TNFSF* genes were constitutively expressed in all eight tissue types (Fig. 5). However, most of the genes (6 out of 10) had higher expression in liver. Moreover, HK had higher expression of 4 genes (*TNFSF10-3*, *TNFSF13B1*, *TNFSF13B2* and *TNFSF14*). Expression of *TNFSF13B1* and *13B2* genes was the highest in liver followed by head kidney and gill.

3.5. Phylogenetic analysis of Fugu TNFSF10 homologs

Phylogenetic tree constructed using fish and other vertebrate TNFSF10 aa sequences had three distinct clusters, *viz.* upper non-fish TNFSF10, middle fish TNFSF10 and lower mixed fish-reptile TNFSF10 clusters (Fig. 6). However, within the middle fish cluster, Fugu TNFSF10-1 and TNFSF10-3 formed two different sub-clusters. There were two sub-clusters in the lower broad cluster and Fugu TNFSF10-2 was within the fish sub-cluster.

3.6. Expression of Fugu TNFSF10 genes in stimulated HK cells

LPS stimulation did not cause any elevation in expression of all three Fugu *TNFSF10* genes (Fig. 7). However, in polyI:C stimulated HK cells, all three Fugu *TNFSF10* gene expressions were significantly increased ($P < 0.01$) compared to unstimulated cells only at 4 h post stimulation (hps).

4. Discussion

In this study, genome database search resulted in identification of ten Fugu *TNFSF* genes which were further confirmed based on the conservation of TNF family signature motifs among these genes. Moreover, when these genes were analyzed through BLAST search, they were found to be orthologs of human *TNFSF5* gene. However, from further phylogenetic analysis, they were classified into seven TNFSF members. In addition, a comprehensive syntenic analysis with that of zebrafish and human demonstrated periphery of the ten genes and contents, and supported results of the phylogenetic classification. However, compared to human *TNFSF* genes some of the Fugu genes have more than one homologs, *viz.* three *TNFSF10* (−1, 2 and 3) and two *TNF13B* (1 and 2). This may be due to the teleost FWGD event (Meyer and Van de Peer, 2005; Ravi and Venkatesh, 2008) that caused existence of more than one orthologs of a mammalian gene in fish including *TNF*s (Eimon et al., 2006; Kinoshita et al., 2014; Savan and Sakai, 2004; Glenney and Wiens, 2007). As comprehensive information on Fugu *TNFSF* genes, except *TNFSF2* (*TNF-α*) and *TNFSF New* (*TNF-N*) is not available and *TNFSF* genes play several critical roles in immunity, we have restricted our analysis to *TNFSF* members other than *TNFSF1* and *TNF-N*. Our results demonstrate the presence of some of the mammalian TNFSF orthologs in Fugu and provide a new insight into understanding on fish immune system diversity and evolution.

Fugu *TNFSF5*, *TNFSF10-2*, *TNFSF10-3*, *TNFSF11*, *TNFSF12* and *TNFSF13B1* genes have different exon and intron numbers compared to that of the human counterparts suggesting their structural diversity. Moreover, Fugu *TNFSF10-2* gene is unusually long (24,267 bp) with a 9730 bp second intron. However, this needs further confirmation based on molecular characterization. Fugu chromosome 14 contains *TNFSF5* gene that was found on scaffold 465 in the previous analysis by Glenney and Wiens, 2007. Our analysis exhibited that Fugu chromosome 8 harbors *TNFSF10-2* and *TNFSF12* genes, which is consistent with the genomic organization of these two genes on zebrafish chromosome 7 (Glenney and Wiens, 2007). Although, *TNFSF11* and *TNFSF13B* genes locate on the same chromosome in human (chromosome 13), their location is different in Fugu with the former on scaffold 437, and the latter with two homologs on chromosome 1 and 14. However, similar to human these two genes exist on a single chromosome in zebrafish (chromosome 9). In summary, there was conservation of gene organization for some of the TNFSF members with putative gene translocation, gene loss and duplication in Fugu as previously reported in other teleosts (Glenney and Wiens, 2007).

Alignment analysis of the newly identified Fugu TNF aa sequences revealed some distinguishable features; most of them contain leucine at the first position of the TNF signature similar to that in the human TNF-α (Kinoshita et al., 2014), whereas TNFSF12 has phenylalanine, and TNFSF13B1 and TNFSF13B2 contain isoleucine. Therefore, Fugu TNFSF peptides identified in this study comply the consensus pattern of family signature proposed by Kono et al. (2006) with a slight modification marked in bold: (L**FI**)-x-V-x_3-G-(**D**IL**R**FV**YH**)-Y-(LIVF**HY**)$_2$-x_2-(**R**QK)-V**T**-x-(LFY).

Expression of *TNFSF10-1*, *TNFSF10-2*, *TNFSF13B1*, *TNFSF13B2* and *TNFSF14* genes was apparent in eight different organs with relatively higher level in liver. Liver contains unique lymphocyte populations (Crispe and Mehal, 1996). Liver-associated lymphocytes, *viz.* NK T cells signal for apoptosis *via* TNFSF10 (TRAIL) to the target cells like tumor cells in human (Nakayama et al., 2003). Therefore, the highly expressed *TNFSF10* genes in the liver of Fugu may possibly have similar function as in human. Likewise, the TNFSF10 member, *TRAIL* gene was highly expressed in grass carp liver (Chang et al., 2006) and chicken lymphoid organs including liver (Abdalla et al., 2004). TNFSF13B (BAFF) plays a major function in B cell survival, proliferation, differentiation and antibody secretion (Gross et al., 2000; Rolink et al., 2002). In mammals, B cells mature in secondary lymphoid organ like spleen after their origin from bone marrow and *TNFSF13B* gene expresses highly in spleen to stimulate B cells (Mackay and Mackay, 2002). However, *TNFSF13B1* and *TNFSF13B2* gene expressions were at higher level in Fugu liver in this study. Similarly, *TNFSF13B* gene was up-regulated in liver of *Aeromonas hydrophila* infected grass carp (Pandit et al., 2013) indicating its immune function in liver of fish. TNFSF14 (LIGHT) is mainly expressed on activated T cells, NK cells and immature dendritic cells (DCs) (Tamada et al., 2000). This gene was constitutively expressed in all tissues with relatively higher level observed in Fugu liver followed by HK implying its important role in fish immunity.

Broad phylogenetic and synteny analyses of TNFSF10 indicated a different evolution from human, especially for TNFSF10-2 in fish. Therefore, a more elaborative phylogenetic analysis was conducted using all available vertebrate (mammals, birds, reptiles, amphibians and fish) TNFSF10 orthologs. Results showed that orthologs of Fugu TNFSF10-1 and 10-3 are present in all vertebrates, whereas TNFSF10-2 was not related to any mammalian and avian orthologs. From synteny analysis it was evident that genes located near Fugu *TNFSF10-1* and *TNFSF10-3* genes are not conserved near *TNFSF10-2* gene on chromosome 8.

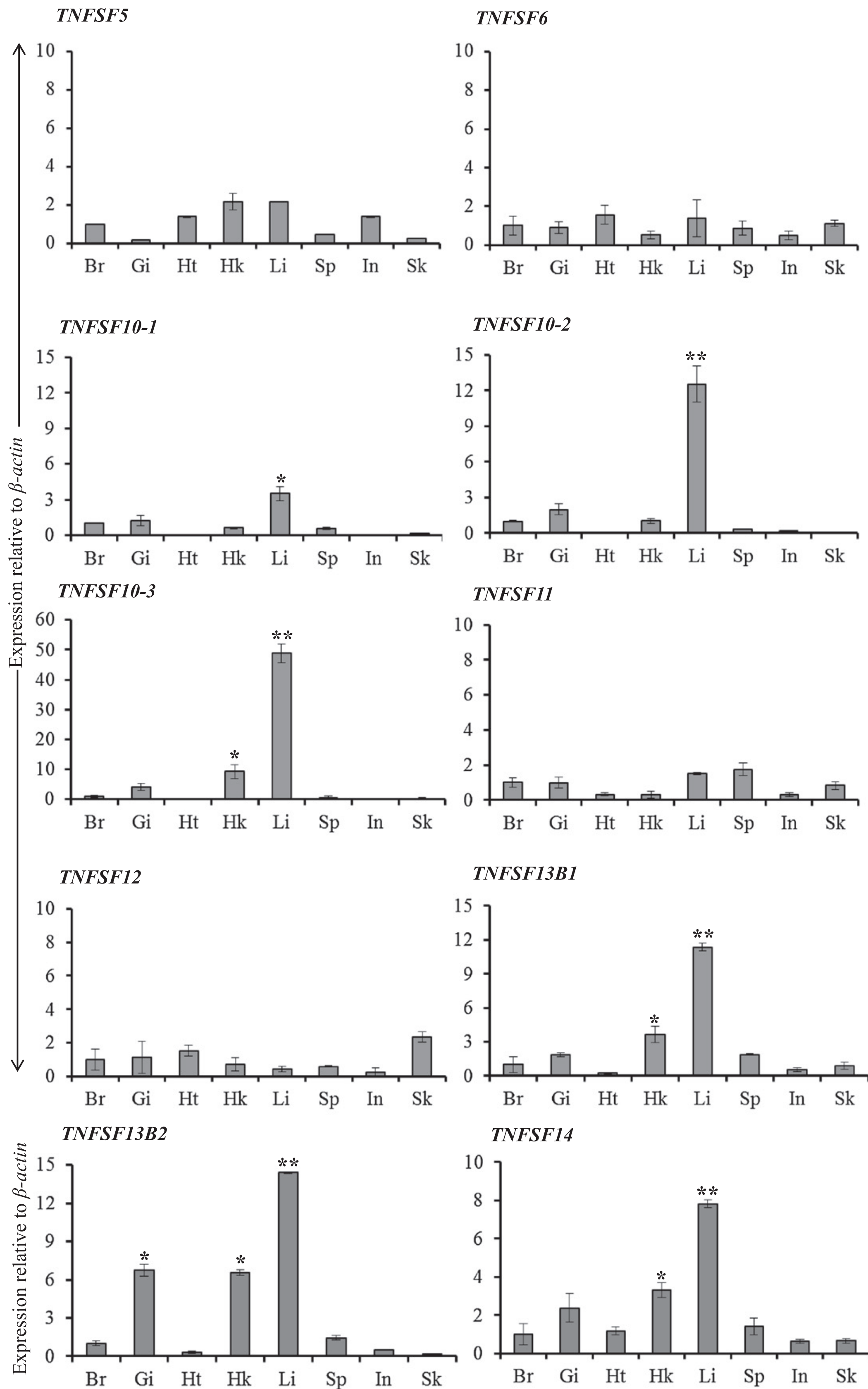


Fig. 5. Quantitative real-time PCR analysis of Fugu *TNFSF* genes in various tissues (Br, brain; Gi, gill; Ht, heart; Hk, head kidney; Li, liver; Sp, spleen; In, intestine; Sk, skin). Data were $2^{-\triangle\triangle CT}$ levels calculated and normalized against the *β-actin* mRNA levels. Data were presented as mean ± S.D. in 5 fish. Significantly higher expression level in tissue was indicated with asterisk(s) (*$P < 0.05$; **$P < 0.01$).

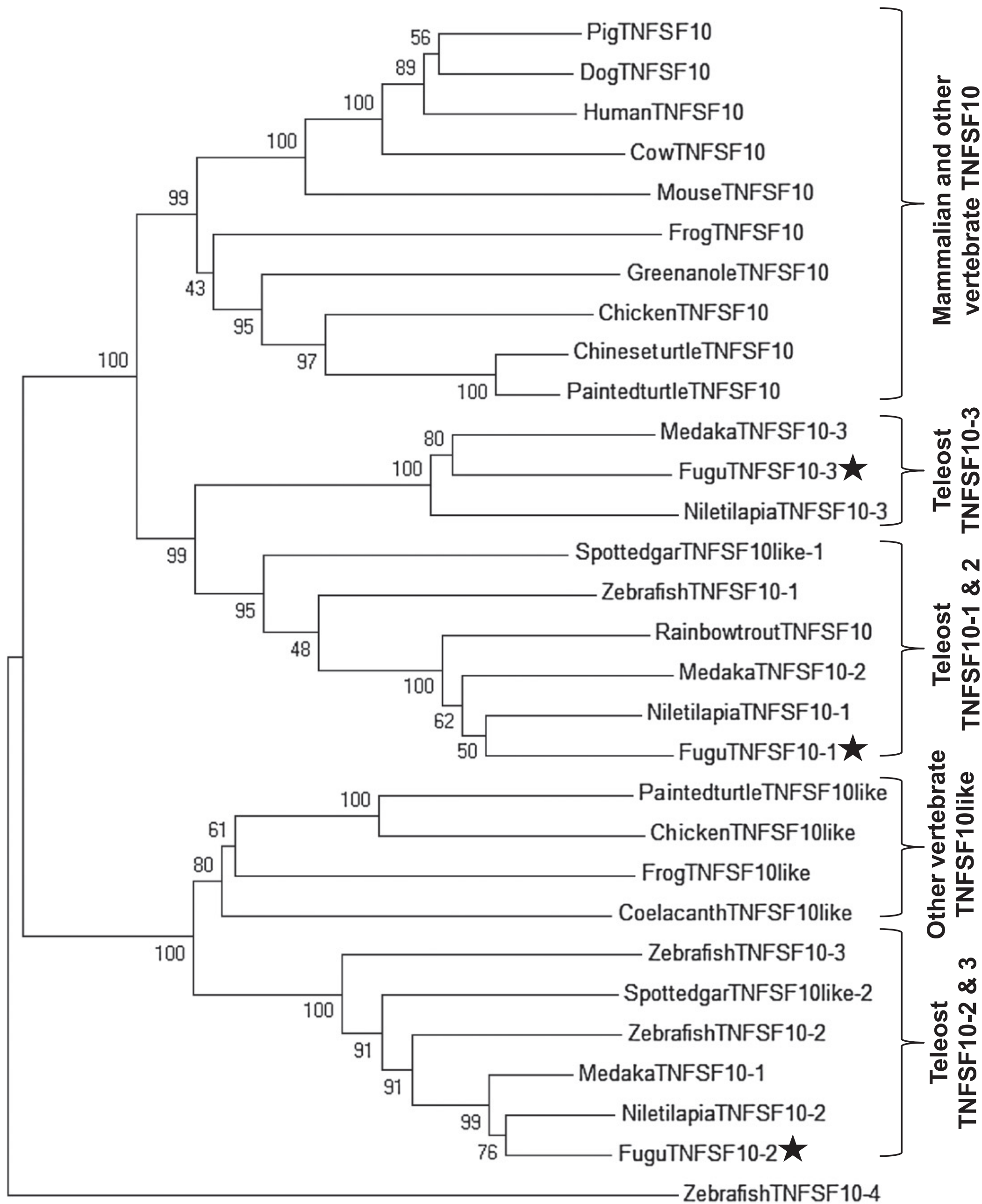


Fig. 6. Phylogenetic tree analysis of Fugu TNFSF10 with the known TNFSF10 homologs from other teleosts and vertebrates. The phylogenetic tree was constructed by neighbor-joining (NJ) method using MEGA v.6. The numbers indicate the bootstrap confidence values obtained for each node after 1000 replications. Fugu TNFSF10 molecules are marked with an asterisk. The GenBank accession numbers of used TNFSF10 amino acid sequences other than those of Fig. 4 are as follows: pig TNFSF10, NP_001019867.1; dog TNFSF10, NP_001124308.1; cow TNFSF10, XP_583785; green anole TNFSF10, XP_003218179; chicken TNFSF10, NP_989710.1; Chinese turtle TNFSF10, XP_006134688; painted turtle TNFSF10, XP_005315693; spotted gar TNFSF10like-1, XP_006637867; painted turtle TNFSF10like, XP_005279217; chicken TNFSF10like, NP_989922; coelacanth TNFSF10like, XP_005992946; frog TNFSF10like, NP_001163916; spotted gar TNFSF10like-2, XP_006632788.

Moreover, *CCNB3* and *CLCN5* genes located in the vicinity of *TNFSF10-2* in Fugu also exist on human chromosome X (Supplementary Fig. 1). However, chicken chromosome 4 harbors an additional *TNFSF10like* gene with a downstream *CLCN5* gene (Supplementary Fig. 1) and this sequence also comes under the broad cluster containing Fugu TNFSF10-2. Therefore, these results suggest that overlapping and duplication of *TNFSF10* gene occurred in fish prior to branching in higher vertebrates.

Poly(I:C), a synthetic analogue of dsRNA, is recognized by Toll-like receptor (TLR) 3 (Alexopoulou et al., 2001). During replication most viruses produce dsRNA that is a molecular pattern associated with viral infection (Jacobs and Langland, 1996). Virus infection promotes expression and release of TNFSF10 (TRAIL) leading to apoptosis of infected cells (Clarke et al., 2000). Expression level of Fugu *TNFSF10* genes was enhanced by poly (I:C) stimulation at 4 hps in this study, which may suggest the role of these genes to induce apoptosis in virus-infected cells in fish also. However, no difference in the enhanced expression levels of three *TNFSF10* genes was observed at later time points. We propose that mammalian TNFSF10 orthologs are present in lower vertebrate like fish with multiple copies. However, it is difficult to conclude on the role of a particular homolog on viral-induced apoptosis and necessitates further investigation.

Fourteen *TNFSF* genes have so far been identified from fish, they are *TNFSF New* (*TNF-N*), *TNFSF2* (*TNF-α*), *TNFSF3* (*LT-β*), *TNFSF5* (*CD40L*),

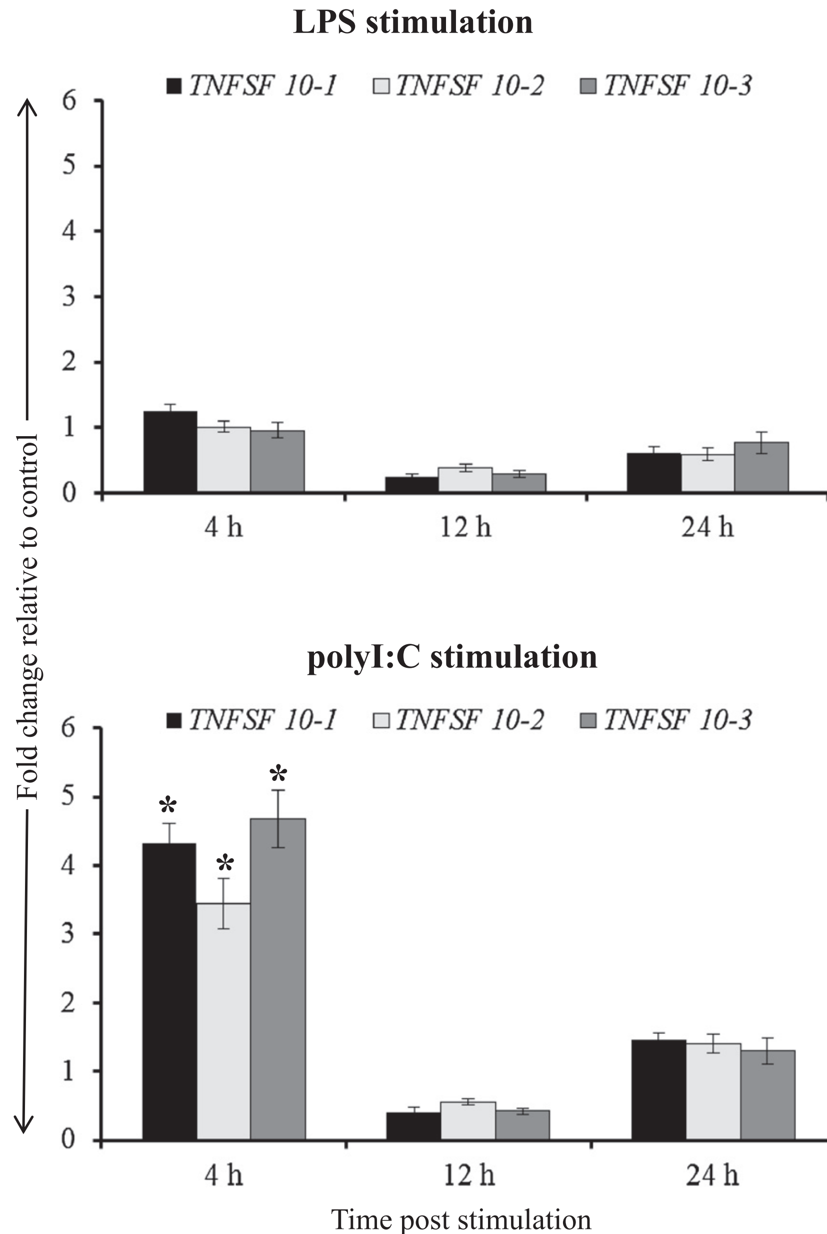


Fig. 7. Fugu *TNFSF10* gene expressions in head kidney cells stimulated with LPS and polyI:C (20 μg mL^{-1}) for 4, 12 and 24 h. Total RNA was extracted and quantitative real-time PCR was carried out. All data were calculated as a ratio relative to *β-actin* gene (internal control) in the same samples and data were expressed as fold change relative to the control (=1) at each time point. The results were expressed as mean ± S.D. (bar in the graph) from triplicate cell culture wells from three fish ($n = 3$). Significantly increased expression in the treated group was indicated with an asterisk (*$P < 0.01$).

TNFSF6 (*FasL*), *TNFSF9* (*4-1BBlg*), *TNFSF10* (*TRAIL*), *TNFSF11* (*RANKlg*), *TNFSF12* (*TWEAK*), *TNFSF13* (*APRIL*), *TNFSF13B* (*BAFF*), *TNFSF14* (*LIGHT*), *TNFSF15* (*TL1A*) and *TNFSF18* (*GITRlg*) (Glenney and Wiens, 2007; Weins and Glenney, 2011). In Fugu, *TNFSF New* (*TNF-N*) and *TNFSF2* (*TNF-α*) genes have been reported previously (Savan et al., 2005). Including these two and seven identified in the present study, the number of *TNFSF*s becomes nine in Fugu. In our analysis, some of the *TNFSF* genes such as *TNFSF9* (*4-1BBlg*), *TNFSF13* (*APRIL*), *TNFSF15* (*TL1A*) and *TNFSF18* (*GITRlg*) could not be confirmed from Fugu in spite of their existence in other teleost species. However, during *in silico* analysis, we have also found a sequence located on Fugu scaffold 139 and it appeared to be related to human and zebrafish TNFSF18 (GITRlg). Multiple alignment of this sequence with human and zebrafish TNFSF18 exhibited difference in TNF family motif (Supplementary Fig. 2). Although, phylogenetic analysis displayed cluster formation of this sequence with zebrafish TNFSF18, bootstrap value was low (22%). However, in syntenic analysis, some of the genes upstream of Fugu *TNFSF18* were similar to that of zebrafish chromosome 3 which contains *TNFSF18* gene, but there was no similarity in gene synteny with human chromosome 1 that harbors *TNFSF18* (Supplementary Fig. 2). Therefore, existence of *TNFSF18* gene in Fugu remains inconclusive and further elaborative analysis is required for confirmation. Absence of some of the *TNFSF* genes in fish and Fugu in particular, may be explained to be associated with an artifact of incomplete genome assemblies or their loss during teleost evolution (Glenney and Wiens, 2007). With the progressive genome sequencing and annotated data covering human to lower vertebrates including bony and cartilaginous fish (recently released one for elephant shark by Venkatesh et al., 2014), it will be further easier and reliable to perform genomic analysis for providing comparative evidences on TNFSF evolution.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.margen.2015.03.003.

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