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Research article

Molecular Characterization and Expression Analysis of Tumor Necrosis Factor Alpha-induced Protein 3 (TNFAIP3/A20) Gene from Japanese Pufferfish *Takifugu rubripes*

Jun-ichi Hikima[1*], Misaki Morita[1], Shunsuke Kinoshita[1], Madhubanti Basu[2], Gouranga Biswas[3], Tomoya Kono[1] and Masahiro Sakai[1]

[1]*Department of Biochemistry and Applied Biosciences, Faculty of Agriculture, University of Miyazaki, Miyazaki 889-2192, Japan*
[2]*Fish Health Management Division, ICAR-Central Institute of Freshwater Aquaculture, Odisha 751002, India*
[3]*Kakdwip Research Centre of ICAR-Central Institute of Brackishwater Aquaculture, West Bengal 743347, India*

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ABSTRACT—Tumor necrosis factor alpha (TNF-α)-induced protein 3 (TNFAIP3/A20) is an important deubiquitinating enzyme that takes part in homeostasis of immunity induced by TNF-α. During viral and bacterial infection, it plays a crucial role in negative regulation of innate immune responses in mammals. However, this molecule in fish is still poorly understood. In this study, we identified full-length A20 gene of Japanese pufferfish (Fugu) *Takifugu rubripes* and performed its expression analyses in various tissues and stimulated cells. Total length of the determined Fugu A20 gene spaned 6,229 bp and consisted of 9 exons and 8 introns. The Fugu A20 gene contained a 2,394 bp open reading frame (ORF) that encoded a 797-amino acid residue. In the exon-intron structure of Fugu A20 gene, the intron-boundary positions in the region having 7 zinc finger (Zf) domains were different from those of human. Expression analysis of Fugu A20 gene exhibited a higher transcription in thymus, head kidney (HK) and spleen tissues than the others. In HK and spleen cells stimulated with LPS, polyI:C and imiquimod, A20 gene was expressed after up-regulation of TNF-α gene expression. These results together suggest that expression of Fugu A20 gene is associated with bacterial and viral infections.

Key words: Tumor necrosis factor alpha-induced protein 3 (TNFAIP3/A20), TNF-α, Zinc finger domain, *Takifugu rubripes*, Japanese pufferfish, Fugu

Tumor necrosis factor alpha (TNF-α)-induced protein 3 (TNFAIP3) is a deubiquitinating enzyme, which is also called A20, belonging to the ovarian tumor family (Parvatiyar and Harhaj, 2011). A20 has an amino-terminal ovarian tumor ubiquitin (OTU) domain to cleave K63-linked ubiquitin residues and seven carboxy (C)-terminal Zinc finger (Zf) domains to conjugate K48-linked ubiquitin chains, and thus de-ubiquitination occurs through attenuating activation of specific E3-ubuqutin ligase such as the TNF receptor-associated factor 6 (TRAF6) (Frias-Staheli *et al*., 2007; Parvatiyar and Harhaj, 2011; Wertz *et al*., 2015). In human, A20 is induced by TNF-α to play a key role in the homeostasis of immune system (Dorronsoro and Lang, 2013). A20 can also suppress nuclear factor (NF)-κB activity. Bacterial or viral simulations are transferred to TRAF6 through Toll like receptor (TLR) 4 and TRAF6 activates NF-κB by phosphorylation (Kawai and Akira, 2009; Parvatiyar and Harhaj, 2011; Harhaj and Dixit, 2012). Activated NF-κB migrates into the cell nucleus and consequent inflammatory response occurs with the transcription of pro-inflammatory cytokines and A20 genes (Parvatiyar and Harhaj, 2011; Harhaj and Dixit, 2012). Due to the denaturation of TRAF6, inflammatory response is suppressed resulting in no activation of NF-κB (Parvatiyar and Harhaj, 2011; Harhaj and Dixit, 2012). Thus, A20 plays a role as negative feedback agent in the NF-κB pathway (Parvatiyar and Harhaj, 2011).

In rainbow trout, a partial nucleotide sequence of A20 cDNA has been identified as a transcript up-regulated by LPS-stimulation, and this gene was significantly induced by LPS and zymosan, but not by polyI:C in the macrophages (Iliev *et al*., 2006). In zebrafish, a full-length nucleotide sequence of A20 cDNA encoding OTU

* Corresponding author
E-mail: jhikima@cc.miyazaki-u.ac.jp

and Zf domains has been identified (Oehlers *et al*., 2011). However, information on A20 gene in fish is still insufficient and it should additionally be discussed for expression pattern. In the present study, we have cloned the entire nucleotide sequence of the A20 gene from the Japanese pufferfish, Fugu, *Takifugu rubripes*, predicted the structure and examined a response to bacterial and viral mimic stimulations in the head kidney and spleen cells.

Materials and Methods

Fish maintenance and handling

The Japanese pufferfish, Fugu, *Takifugu rubripes* (mean mass: 100 ± 8 g) was obtained from Matsumoto Fisheries Farm in Miyazaki, Japan. The fish were firstly acclimatized in an aerated seawater tank at 20°C and fed with a commercial diet (Sango, Higashimaru Co. Ltd.) at 1% body weight per day for two weeks under a natural photoperiod prior to their use in the study. All animal experiments were 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.

cDNA synthesis

Fish was anesthetized with 2-phenoxyethanol (0.05%, Sigma-Aldrich) before being killed for tissue collection. Tissues from healthy Fugu [thymus, head kidney (HK), trunk kidney (TK), spleen, liver, intestine, brain, heart, gill, muscle and skin] were isolated under sterile condition from six individual fish for total RNA extraction.

Total RNA was isolated using ISOGEN (Nippon Gene) following the manufacturer's instructions and poly(A) mRNA was purified using a quick prep micro mRNA kit (Amersham Pharmacia Biotech). cDNA was synthesized from 2 μg of mRNA using ReverTra Ace RT kit (Toyobo) and used as a template for PCR.

Molecular cloning and sequencing

Initially, PCR was performed using the HK cDNA prepared above, with primers Fw/A20 F3 and Rv/A20 R1 (Table 1) specific to the region including open reading frame (ORF) and untranslated regions, to allow amplification of A20 gene. After amplification of this partial Fugu A20 sequence, the 5′ and 3′ end were obtained by RACE-PCR using SMARTer RACE cDNA amplification kit (Clontech Laboratory Inc.) according to the manufacturer's instruction. RACE PCR was performed with Rv/A20 GSP2, Fw/A20 GSP2 or Fw/A20 NGSP2 (Table 1). For sequencing of Fugu A20 gene, amplification was performed in a 50 μL reaction volume containing 5 μL of dNTP mixture (2.5 mm each dATP, dCTP, dGTP, dTTP) and 10× Gene *Taq* Universal buffer, 0.5 μL of Taq polymerase (5 units μL^{-1}; Nippon Gene), 5 μL of each primer set (F and R; 2.5 μM), 28.5 μL of distilled water and 1 μL of Fugu thymus cDNA. The cycling conditions for all conventional PCRs were: 1 cycle of 95°C for 1 min, 35 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 3 min, followed by 1 cycle of 72°C for 5 min. Genomic DNA was isolated from the head kidney of Fugu with a blood and cell culture DNA mini kit (Qiagen) according to the manufacturer's instructions. The Fugu A20 gene was PCR-amplified using the *Taq* polymerase with the specific primers in Table 1. The PCR profile was same

Table 1. Oligonucleotide primers used in this study

Primers	Sequences (5′→3′)	bp	Application
Rv/A20 GSP2	CAGCATGTATTGAGAGGTGGCATGAAGC	28	5′- RACE PCR
Fw/A20 GSP2	GAGGGCTACTGTGACAAGTGCTATGTC	27	3′-RACE PCR
Fw/A20 NGSP2	TTAACCAGGTGGCACATCGCTCGACAC	27	
Fw/A20 F1	ACGTTGGAGCAGGAGAAGAA	20	Conventional PCR for gene sequencing
Fw/A20 F3	ATTTCTCCCCACAGTCTCCAC	21	
Fw/A20 F5	TATCCTGGACAGAGCCATGC	20	
Fw/A20 F6	ACATTCTTCGCAGACCCATC	20	
Fw/A20 F7	AGCTCGTCAACCACGAGTTC	20	
Fw/A20 F8	CTTTGTGAGCGCTGTTTCAA	20	
Rv/A20 R1	ATTGAGAGGTGGCATGAAGC	20	
Rv/A20 R6	GCCAGTGGAGTGGCAGATAA	20	
Rv/A20 R7	CCACGGAAACGTAAAAGGTG	20	
Rv/A20 R8	TTCTGGTTCACGACCAGTTG	20	
Rv/A20 R9	TAAGCCTCGCACTTTGCTC	19	
Fw/A20-qF2	CGTAAAGATGGCCTCTCCAG	20	qPCR
Rv/A20-qR2	GATGGGTCTGCGAAGAATGT	20	
Fw/TNFα-qF	CAGGCTTCTTTCCGAGTGAC	20	
Rv/TNFα-qR	TTCTGACACGCTGACCTCAC	20	
Fw/β-actin Fw2	CGTGCGTGACATCAAGGA	18	
Rv/β-actin Rv2	GCAGCGGTGCCCATCTC	17	

as above-mentioned.

Obtained PCR products were ligated into the pGEM-T Easy vector (Promega) and transfected into competent *Escherichia coli* TAM competent cells (Active Motif), where recombinants were identified through red-white color selection when grown on MacConkey agar (Sigma-Aldrich). Plasmid DNA from at least three clones was extracted using a QIAprep Spin Miniprep Kit (Qiagen) and sequenced using a CEQ8000 Automated Sequencer (Beckman Coulter). The sequences generated were analyzed for similarity with other known sequences using the FASTA (Pearson and Lipman, 1988) and BLAST (Altschul *et al*., 1990) programs.

Sequence and structural analysis

Multiple sequence alignments were generated using ClustalX v1.81 (Thompson *et al*., 1997) and homology analysis was performed using the MatGat software v2.02 (Campanella *et al*., 2003). Finally, the protein family signature was analyzed using the ExPASy PROSITE database of protein families and domains (http://prosite.expasy.org/; Falquet *et al*., 2002), and phylogenetic analysis was performed on the full-length amino acid sequences of the known A20 molecules using the neighbor-joining method (Saitou and Nei, 1987). MEGA6 (Tamura *et al*., 2013) was used to construct the tree with confidence limits (Felsenstein, 1985).

Quantification of Fugu A20 gene expression

HK and spleen cells were collected under sterile condition from freshly killed Fugu (three individuals) and gently pushed through a 100 μm nylon mesh (John Staniar and Co.) with RPMI 1,640 medium (Gibco BRL) supplemented with 5% fetal bovine serum (FBS; Gibco) and 1% Streptomycin/Penicillin (Gibco). After washing with the above medium, HK and spleen cells were stimulated with 20 μg mL^{-1} of lipopolysaccharide (LPS; Sigma-Aldrich), the viral mimic, polyinosinic acid-polycytidylic acid (polyI:C; Sigma-Aldrich) and Imiquimod (Wako Pure Chemical Industries, Ltd.) for 0, 4, 12, 24 and 48 h at 22°C (0 h was a non-treated control). cDNA was synthesized from stimulated HK and spleen cells, and unstimulated tissues (muscle, brain, TK, heart, skin, gill, intestine, spleen, HK, thymus and liver) as described above and diluted to 20 times with 10 mM Tris buffer (pH 8.0).

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 (CT) method ($2^{-\Delta\Delta CT}$ method) (Schmittgen and Livak, 2008) was used to analyze the expression levels of A20 and TNF-α genes using Fugu β-actin (GenBank Accn no. 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 under different stimulations at a given time point using SPSS for Windows v. 17.0 program (SPSS Inc.).

Results

Structural analysis of Fugu A20 gene

Fugu A20 gene consists of 6,229 bp containing nine exons and eight introns, and the coding 2,394 bp mRNA (deposited in GenBank under the accession number LC076438) translates a protein of 797 amino acids (aa) in length (Fig. 1), which was located at Fugu genome scaffold_124 (791,414-797,300 bp) in Ensemble Genome Browser (FUGU 4.0) (www.asia.ensembl.org/Takifugu_rubripes/). Although, the splice donor/ acceptor sequence (GT/AG) was conserved at the 5′ and 3′ ends of the introns (Fig. 1), between exon 6 and exon 7 it was CT/TG. The exon-intron structure of Fugu A20 gene was very similar to that in human although the divided positions of exons in the region having Zf domains when compared to those of humans were different in number (Figs. 2 and 3). The results of PROSITE database analysis showed that Fugu A20 protein contains only five Zf DNA binding domains (*i.e.*, Zf1: E^{385}–E^{410}; Zf2: A^{487}–F^{511}; Zf4: A^{604}–Y^{631}; Zf5: N^{656}–K^{683}; Zf7: K^{765}–H^{790}) (the regions underlined in Fig. 3), comparing with the human A20 protein possessing seven Zf domains such as S^{380}-N^{416} (Zf1), S^{471}-S^{507} (Zf2), D^{514}-S^{548} (Zf3), D^{600}-H^{636} (Zf4), R^{650}-F^{686} (Zf5), S^{709}-P^{745} (Zf6) and D^{755}-G^{790} (Zf7) (the regions in the gray boxes in Fig. 3; Wertz *et al*., 2015). However, four cysteines (*i.e*., $C_{X4}C_{X11}C_{X2}C$ and $C_{X2}C_{X11}C_{X2}C$) in seven regions similar to the human Zf domains were well-conserved in fish. Interestingly, one common aa deletion among teleosts was observed in Zf6 (Fig. 3). This deletion is probably one of the features in the fish A20 aa sequences. One Fugu OTU domain (N^{102}–Q^{257}) from the result of PROSITE database analysis was also shortened from the OTU domain (M^{1}–S^{360}) known in human (the regions in the gray boxes; Wertz *et al*., 2015). The catalytic site forming the ubiquitin-binding site in the human OTU domain (D^{100}, C^{103} and H^{256}) (Accession no. P21580 in UniProt database: http://www.uniprot.org/uniprot/P21580) is highly conserved in the Fugu OTU domain (D^{104}, C^{107} and H^{258}). It was suggested that the aa sequence of Zf and OTU domain were conserved among vertebrates.

Multiple alignments and phylogenetic analysis of A20 sequence

Multiple alignments with known A20 molecules in other vertebrates revealed a high conservation between the mammalian, bird, reptile and fish aa sequences (Fig. 3). The high identities of A20 were shown between Fugu and zebrafish (75.0%) or tilapia (87.3%), but the low identities were shown between Fugu and coelacanth

Fig. 1. Hikima *et al*., submitted.

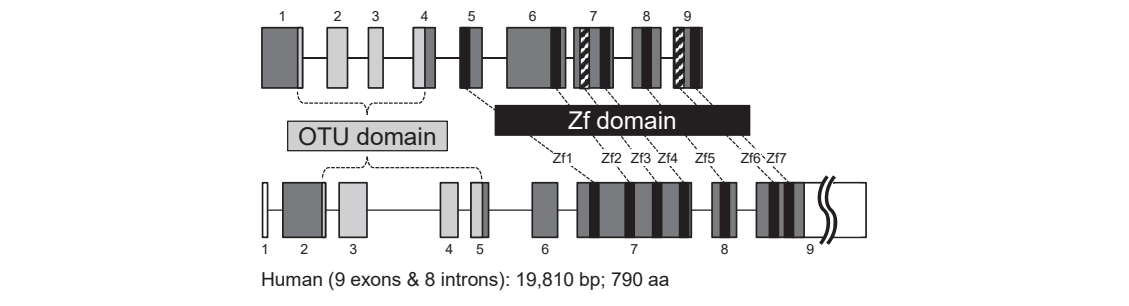

Fig. 2. Hikima *et al*., submitted.

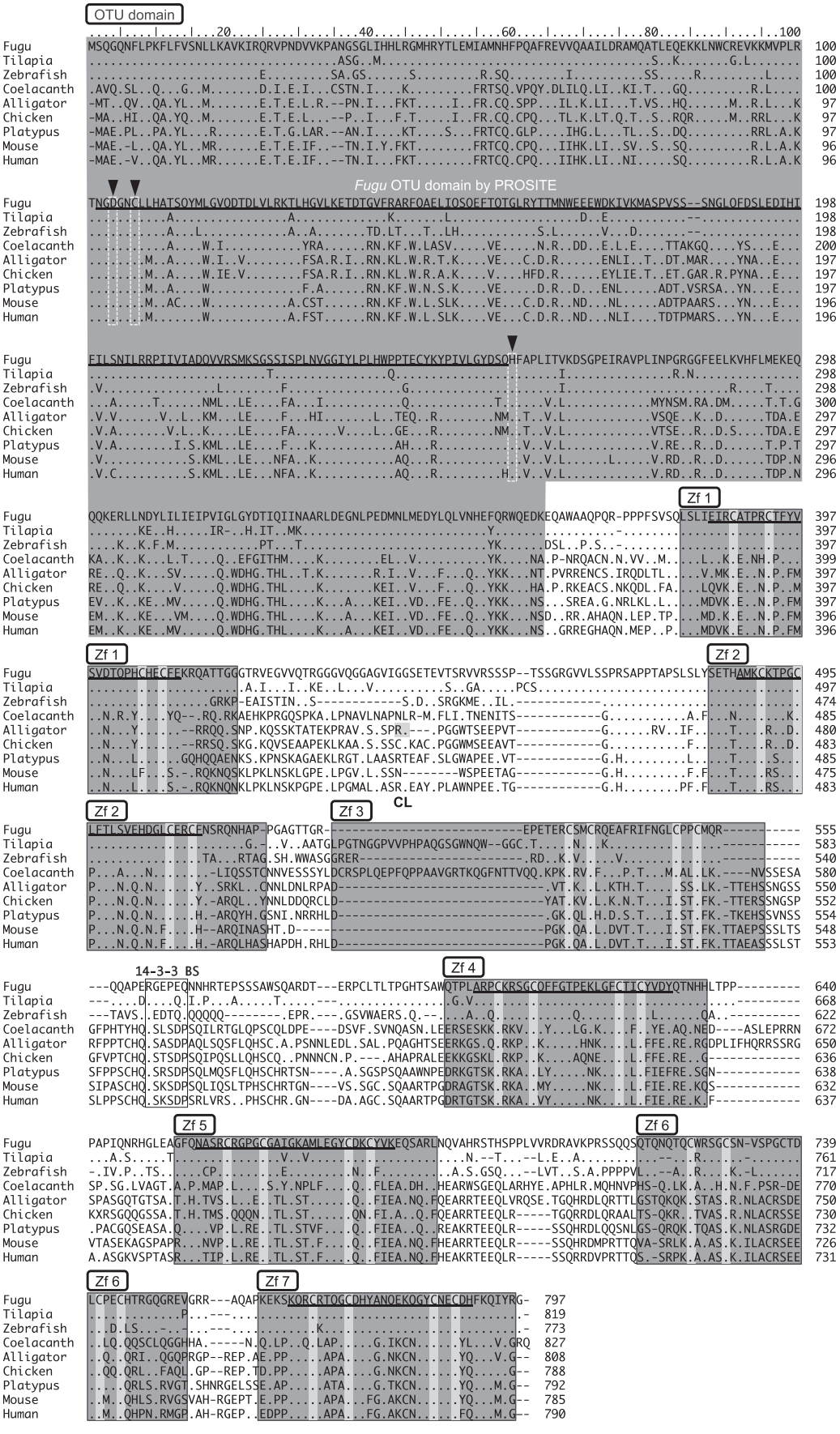

Fig. 3. Hikima *et al*., submitted.

Table 2. Identities of A20 amino acid sequence between Fugu and the other vertebrates

	Tilapia	Zebrafish	Coelacanth	Alligator	Chicken	Platypus	Mouse	Human
Fugu	87.3%	75.0%	46.7%	45.1%	45.6%	46.2%	43.5%	44.7%
	Tilapia	72.7%	46.1%	42.8%	43.1%	44.1%	41.8%	42.7%
		Zebrafish	47.0%	45.0%	45.8%	46.9%	44.9%	44.9%
			Coelacanth	54.9%	53.6%	55.5%	54.5%	54.8%
				Alligator	78.0%	68.0%	65.1%	66.2%
					Chicken	67.5%	64.9%	66.0%
						Platypus	76.1%	77.0%
							Mouse	87.9%

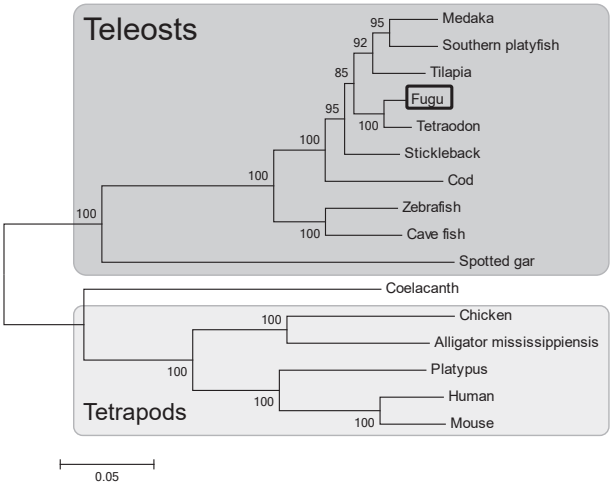

Fig. 4. Hikima *et al*., submitted.

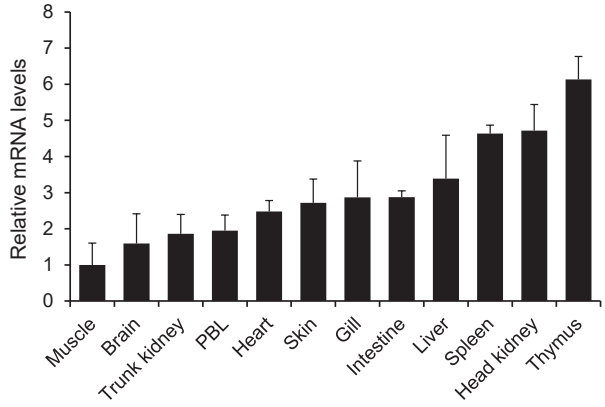

Fig. 5. Hikima *et al*., submitted.

(46.7%), chicken (45.6%), alligator (45.1%) or mammals (43.5–46.2%).

Phylogenetic analysis revealed that A20 identified from Fugu formed a cluster with A20s identified from other teleosts distinctly away from the clades of coelacanth, chicken, alligator and mammalian species (Fig. 4).

Expression analysis of Fugu A20 gene

A constitutive expression of the A20 gene was observed in all the tissues examined, with the highest expression seen in spleen, HK and thymus of healthy Fugu (Fig. 5).

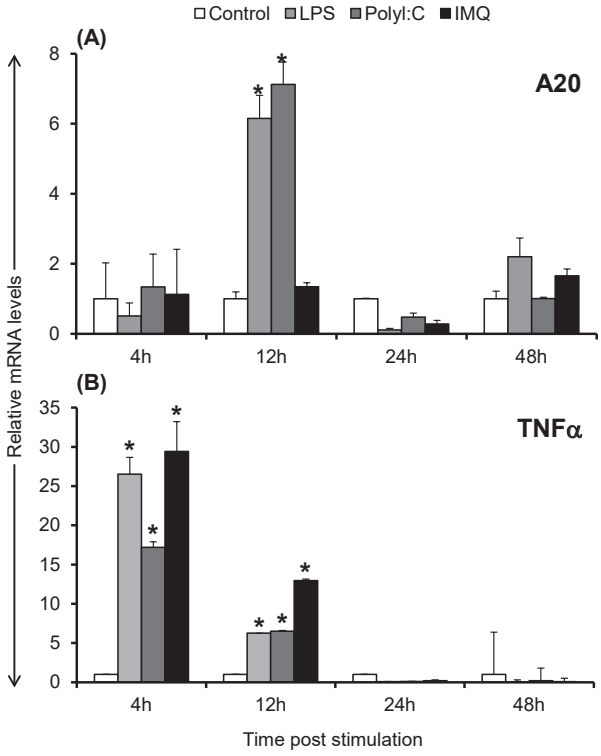

Fig. 6. Hikima *et al*., submitted.

LPS and polyI:C stimulations caused a significant increase ($P < 0.01$) in A20 gene expression at 12 h after treatment in HK cells (Fig. 6A). In the same samples, expression of TNF-α was up-regulated ($P < 0.01$) at 4 and 12 h post LPS, polyI:C and imiquimod treatment (Fig. 6B). In contrast, no change in the expression of A20 gene was noticed in HK cells treated with imiquimod (Fig. 6A). Furthermore, expression of A20 and TNF-α genes were sufficiently down-regulated in HK cells at 24 h post-stimulation by LPS, polyI:C and imiquimod (Fig. 6).

In spleen cells, the expression of A20 gene was significantly increased ($P < 0.01$; 0.05) at 4 and 12 h after polyI:C and imiquimod treatments (Fig. 7A). An increased expression of TNF-α was only ($P < 0.01$; 0.05) at 4 h post LPS, polyI:C and imiquimod treatments (Fig.

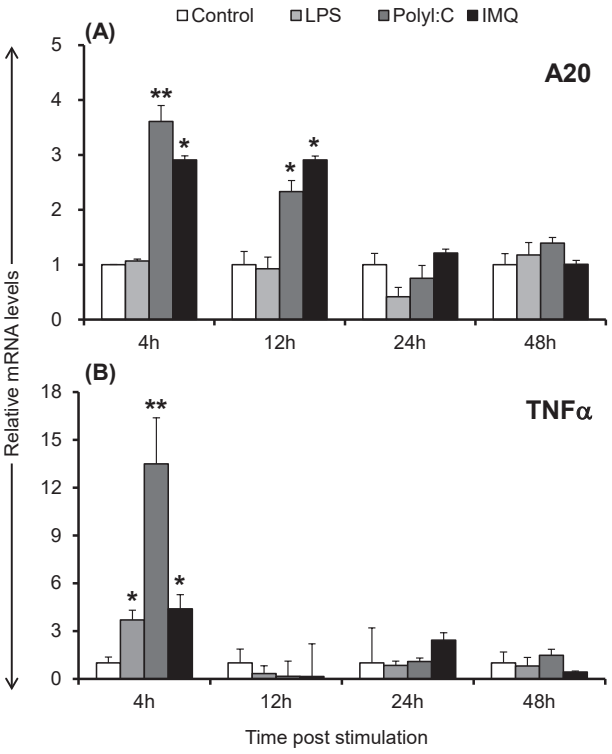

Fig. 7. Hikima *et al*., submitted.

7B).

Discussion

In Fugu A20, this catalytic site forming the ubiquitin-binding site was also well-conserved (D^{100}, C^{103} and H^{256}). One common aa deletion in Zf6 was conserved in teleosts and coelacanth (Fig. 3), suggesting that their common ancestor molecules could be the one-aa-deletion type and that one aa could be obtained during the diversion from coelacanth to reptiles. In mammals, 14-3-3 protein binding sequence (BS) (*i.e*., RSKSDP) between Zf3 and Zf4 of A20 was functionally important to a number of signaling pathways and interaction of A20 with 14-3-3 protein alters localization of the former from a punctuated cytoplasmic staining to a more diffused cytoplasmic pattern, which is also associated with decreased amount of A20 in the insoluble cell fraction (Vincenz and Dixit, 1996). Lademann *et al*. (2001) reported that the binding to 14-3-3 protein protects A20 from ubiquitin degradation. The 14-3-3 BS was conserved in the mammals and coelacanth, but not in teleost fish species (Fig. 3). Therefore, teleost A20 may not have the 14-3-3 protein binding function, suggesting that teleost A20 may not conjugate K48-linked ubiquitin chains, and also the amount of A20 in the cytoplasm may not be affected by the interaction with 14-3-3 protein.

Fugu A20 gene was expressed in all the tissues examined with higher expression level observed in the immune-competent organs such as spleen, HK and thymus. In mice, A20 mRNAs were detected in lymphoid tissues like thymus, spleen and gut-associated lymphoid tissue (GALT), especially in thymus, meaning that A20 mRNAs are expressed in T- and B-cells and dendritic cells (Tewari *et al*., 1995; Hong *et al*., 2011). It suggests that Fugu A20 mRNAs are probably expressed in various leukocyte types.

In this study, we conducted an expression analysis of A20 gene using LPS as a representative ligand of bacterial infection, polyI:C (double strand RNA virus) as representatives of virus infection, and imiquimod as a TLR7 ligand. In case of LPS and polyI:C stimulation, an increased expression of A20 was observed in HK cells after 12 h (Fig. 5), suggesting that at 12 h post-stimulation, NF-κB or IRF3 activities could be reduced by de-ubiqutination of TRAF6 or TRAF3 mediated through the increased A20 (Kawai and Akira, 2009; Parvatiyar and Harhaj, 2011). In rainbow trout macrophages, A20 gene is also up-regulated by LPS between 6 and 24 h of stimulation but not by polyI:C (Iliev *et al*., 2006). It indicates that other type of immune cells excluding macrophages in Fugu HK cells might have induced A20 gene expression by polyI:C. Fugu spleen cells also revealed induction of A20 gene by polyI:C. As described above, mammalian A20 is highly expressed in lymphoid cells and dendritic cells, suggesting that polyI:C could induce A20 gene expression in fish lymphocytes and dendritic cells. In contrast, in stimulation by imiquimod, an increased expression of A20 gene was observed in spleen but not in HK cells. Furthermore, since the expression of TLR7 gene in the carp HK cells is also induced by imiquimod (Tanekhy *et al*., 2010), it seems that the HK cells are stimulated by imiquimod. These all suggest that the transcriptional regulation of A20 gene could vary depending on the cell-type.

In mammals, TNF-α and IL-1β proteins also induce expression of A20 gene as like LPS (Dixit *et al*., 1990; Dorronsoro and Lang, 2013) and in other words, A20 transcription is controlled by NF-κB (Dixit *et al*., 1990). In Fugu HK cells, following LPS and polyI:C stimulations, an elevated expression of TNF-α gene was observed at 4 h and expression of A20 gene was up-regulated at 12 h. The timing of A20 gene induction seems that TNF-α induced A20 expression after stimulation. On the other hand, induction timing of A20 gene in the spleen by polyI:C and imiquimod was earlier than that in HK cells. In this study, the expression of A20 gene is increased in Fugu organs stimulated by the LPS, polyI:C and imiquimod, although there are differences in the expression patterns between HK and spleen, suggesting that each different cell types (*i.e*., lymphocytes and dendritic cells) in these tissues would show different expression pattern of A20 mRNAs after stimulation.

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