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Transcriptional Expression Profiles of Olive Flounder (*Paralichthys olivaceus*) Toll-like Receptor 3 (TLR3) and TLR22 in Response to Extracellular Poly I:C Stimuli

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세포외 Poly (I:C) 자극에 대한 넙치(*Paralichthys olivaceus*) TLR3 및 TLR22의 전사발현 양상

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Abstract

Toll-like receptors (TLRs) recognize extracellular and intracellular double-stranded RNA (dsRNA) and play crucial roles in innate and adaptive immune systems. Here, we aimed to investigate the changes in the transcriptional profile of TLR3 and TLR22 genes of olive flounder (Paralichthys olivaceus) in response to polyinosinic:polycytidylic acid (poly I:C; a dsRNA analog) stimuli in vivo, using healthy olive flounders and in vitro, using Hirame natural embryonic (HINAE) cells. The role of JfTLR3 and *JfTLR22* in inducing type I interferon response in HINAE cells was analyzed through construction and transfection of short-hairpin RNA (shRNA)-producing vector targeting the *JfTLR22* and *JfTLR3*. Poly I:C stimuli upregulated the *JfTLR3* and *JfTLR22* genes both in vivo and in vitro, and *JfTLR22* showed an earlier (at 6 and 12 h post-stimulation) response than *JfTLR3*, suggesting that JfTLR22 is a more sensitive receptor and recognizes the extracellular dsRNA more quickly than *JfTLR3*. After 12 h of poly I:C stimulation, the expression of *ISG15* and *Mx* genes in HINAE cells harboring *TLR22*-targeting shRNA vector was markedly reduced, wherein the reduction in expression of these genes was weakly reduced in HINAE cells carrying *TLR3*-targeting shRNA vector. These results suggested that *JfTLR22* plays an important role, wherein *JfTLR3* partially induces type I interferon response to extracellular dsRNA at 12 h after poly I:C stimulation.

Key words : TLR3, TLR22, Poly I:C, *Paralichthys olivaceus*, RNAi, shRNA

Ⅰ. Introduction

Toll-like receptors (TLRs) are a type of pattern recognition receptor and a family of transmembrane sensor proteins that recognize pathogen-associated molecular patterns (*PAMPs*) and play crucial roles in both innate and adaptive immune systems as sentinel cells such as macrophages and dendritic cells as well as T and B lymphocytes (Schnare et al., 2001; Takeda et al., 2003; Iwasaki and

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Medzhitov, 2004). *TLRs* can detect intracellular and extracellular pathogenic factors, including the bacterial cell wall components and viral nucleotides, *TLRs* may play roles via. Several *TLRs* (from *TLR1* to *TLR13*) have been identified both vertebrata and invertebrates.

The *TLRs* in host cells bind specific factors of pathogens, including single-stranded and double-stranded (ds) RNA in viruses, lipoproteins, lipopeptides, and proteins such as flagellin in bacteria or the unmethylated CpG oligodeoxynucleotide DNA in both.

In mammals, *TLR3* recognizes dsRNA generated as a replication intermediate or by-product of many viruses (Alexopoulou et al., 2001; Wang et al., 2004; Schulz et al., 2005), one of the major *PAMP*, which trigger innate immune responses in vertebrates (Akira et al., 2006). It recruits the TIR domain-containing adaptor inducing interferon-β (*TRIF*) protein via interaction with its TIR domains (Yamamoto et al., 2002) and activates the *TRIF* dependent signaling pathway (Jacobs and Langland 1996; Sen and Sarkar 2005; Haller et al., 2006). TRIF then indirectly activates several transcription factors, such as interferon regulatory factor 3 (*IRF3*), nuclear factor kappa-light-chain-enhancer of activated B cells (*NF-*κ*B*), and activator protein 1 (*AP-1*), which modulate the transcription of interferon β (*IFN-*β) and inflammatory cytokines (Sharma et al., 2003; Yamamoto et al., 2003; Meylan et al., 2004; Matsuo et al., 2008).

Similar dsRNA recognizing mechanisms mediated via *TLR3* have also been reported in fish (Matsuo et al., 2008). The secreted *IFN* regulates the expression of numerous genes encoding antiviral proteins, including dsRNA-dependent eukaryotic initiation factor kinase as protein kinase R (*PKR*) and Mx proteins (de Veer et al., 2001). In other words, signaling through *TLR3* results in type I IFN responses in mammals (Hoebe et al., 2003). Mammalian *TLR3* homologs have been reported in several fish species, such as fugu (Takifugu rubripes; Oshiumi et al., 2003), zebrafish (Danio rerio; Meijer et al., 2004; Jault et al., 2004), channel catfish (Ictalurus punctatus; Bilodeau and Waldbieser 2005), rainbow trout (Oncorhynchus mykiss; Rodriguez et al., 2005), and grass carp (Ctenopharyngodon idella; Su et al., 2009).

In addition, studies have identified an additional *TLR, TLR22* in several fish species including goldfish (Stafford et al., 2003), zebrafish (Jault et al., 2004), olive flounder (Paralichthys olivaceus; Hirono et al., 2004), rainbow trout (Rebl et al., 2007), and fugu (Matsuo et al., 2008). Moreover, *TLR22* has been shown to be mainly expressed in olive flounder stimulated with polyinosinic:polycytidylic (poly I:C), a dsRNA analog used in in vivo experiments to model viral infections (Chakrapani et al., 2018; Qiu et al., 2019), indicating its possible involvement in immune response in olive flounder. However, unlike *TLR3,* the precise role of *TLR22* and the underlying mechanism in regulating the expression of immune-related genes in olive flounder has not been explored fully.

To gain a better insight into the response of immune-related genes to extracellular viral nucleotides, in the present study, we aimed to analyze the molecular patterns and changes in olive flounder *TLR3* (*JfTLR3*) and *JfTLR22* expression at the transcript level. For this purpose, the expression profiles of *JfTLR3* and *JfTLR22* in vivo using healthy olive flounders and in vitro using the HINAE cells stimulated with poly I:C were investigated.

Furthermore, we also investigated the functional

roles of *TLR3* and *TLR22* in the induction of type I interferon response in HINAE cells stimulated with poly I:C through construction and transfection of short-hairpin RNA (shRNA)-producing vectors targeting the *TLR22* and *TLR3*.

Ⅱ. Materials and methods

1. Fish and poly I:C stimulation

Healthy juvenile olive flounders (average body weight: approximately 10 g) were obtained from a local fish hatchery farm in South Korea. Fish were divided into 2 tanks (six fish/50L tank), and fed with commercial pellets (WOOSUNG, Zeus Premium 10 kg). The water in the tank was renewed once a day. Before the experiment, fish were acclimatized for at least two weeks. Fish from two tanks were used for the experiments in this study, and those in one tank were kept as a backup. Fish in one tank (n=6) were intraperitoneally injected with 1 mg of poly I:C (Sigma, USA) suspended in 100 μL PBS (pH 7.6), hereinafter referred to as poly I:C group. Those in the second tank (n=6) were intraperitoneally injected with 100 μL PBS only, hereinafter referred to as PBS group.

2. Expression analysis of genes after poly I:C stimulation in olive flounder

Three fish, each from the poly I:C and PBS groups, were sampled at 12 and 24 h post-treatment and kidney tissues were collected and immediately frozen in liquid nitrogen until further use. Total RNA was extracted from the kidney tissues using RNAiso Plus reagent according to the manufacturer's instruction (Takara, USA). Total RNA (0.5 μg) was then reverse-transcribed to cDNA using HyperScriptTM kit (GeneAll Biotechnology, Seoul, Korea). Semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) was performed to determine the relative mRNA expression levels of *TLR3, TLR22, Interferon-stimulated gene* 15 (ISG15), *Mx*, and 18S rRNA (internal control) genes of olive flounder using the gene-specific primers shown in <Table 1>. Total reaction volume of 20 μL for PCR comprised 2×Prime Taq Premix (Genet Bio, Daejeon, Korea) and 1 μL of 10 times diluted cDNA template. The thermal cycling conditions for the semi-quantitative RT-PCR were 1 cycle of 4 min at 95°C (initial denaturation), followed by 17 cycles (for 18S rRNA), 26 cycles (for ISG15 and MX), or 32 cycles (for *TLR3* and *TLR22*) of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s with a final extension of 72°C for 7 min. PCR products were electrophoresed on 1% agarose gels and visualized with ethidium bromide (Et-Br) staining.

3. Expression analysis of genes after poly I:C stimulation in HINAE cell

HINAE cells were stimulated either with 100 μ g/mL poly I:C or the same volume of PBS (pH 7.6) and cultured in 6-well plates (2×106 cells/well) at 20°C in Leibovitz (L-15, Sigma) medium containing 10% fetal bovine serum (FBS; Sigma), 1% penicillin (100 U/mL; Gibco), and 1% streptomycin (mg/mL; Gibco). The cells were harvested at 6, 12, 24 and 48 h after poly I:C or PBS stimulation. Total RNA was extracted by RNeasy Mini kit (Qiagen) following the manufacturer's instructions. The cDNAs were synthesized using M-MLV reverse transcriptase, and Oligo(dT)15 Primer (Promega, USA) was used in semi-quantitative RT-PCR. The oligonucleotide

primer pairs used to amplify the *TLR3, TLR22, IRF3, IRF7*, and *ISG15* genes and internal control (18S rRNA) genes of olive flounder are shown in <Table 1>. The PCR was carried out the following thermal cycling conditions: 1 cycle of 4 min at 95°C (initial denaturation) followed by 17 cycles (for 18S rRNA), 30 cycles (for *IRF3, IRF7*, and *ISG15*), or 33 cycles (for *TLR3* and *TLR22*) at 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, with a final extension step at 72°C for 7 min. PCR products were electrophoresed on 1% agarose gel and visualized with ethidium bromide (Et-Br) staining.

4. Construction of shRNA-producing vector targeting the JfTLR22 and JfTLR3

Using Ambion's "siRNA Target Finder and Design Tool," siRNA target sequences for *JfTLR22* and *JfTLR3* genes were designed <Table 2>. The fugu U6 promoter-driven shRNA expression vector (Zenke and Kim, 2008) was used to construct plasmids producing shRNA targeting the J*fTLR22* and *JfTLR3* genes using the shRNA system used to silence target gene expression via RNA interference (RNAi). The amplification conditions were 5 cycle at 95°C for 3 min (initial denaturation), 5 cycles at 95°C for 30 s, 48°C for 30 s, 72°C for 30 s, followed by 30 cycles at 95°C for 30 s, 65°C for 30 s, 72°C for 30 s.

Each shRNA-producing cassette was prepared following three-step PCR reactions. Using cloned fugu U6 promoter as the template, the first-step PCR was performed with two primers, U6F-SphI and each of shRNA R1, containing the last 16 bps of fugu U6 promoter, shRNA sense and loop. Next, each amplified product from the first PCR was purified and used as the template for second-step PCR with U6F-SphI and each of shRNA R2 containing 10 bps of shRNA sense, loop and 4 bps shRNA antisense. One-tenth of the second PCR product was then used as the template for the third-step PCR with U6F-SphI and each of the shRNA R3-SacII containing 5 bps of shRNA sense, loop, shRNA antisense and termination signal. Intermediately after the U6 promoter sequence, one guanine residue, which is predicted to be a transcription start point of shRNA, was included in this construct. Finally, each PCR product was gel-purified and cloned into pGEM T-easy vector (Promega), resulting in vector pFuguU6shRNA vectors (pshTLR3·577, pshTLR3·939, pshTLR3·939cont, pshTLR22·903, pshTLR22·1726, and pshTLR22·1726cont). The pshTLR22·1726cont or pshTLR3·939cont vector differed from pshTLR22·1726 or pshTLR3·939 for two nucleotides, each. To select shRNA clones that effectively suppressed *JfTLR22* expression, a dual vector harboring each shRNA-producing cassette and *JfTLR22* gene expressing cassette was constructed. The *JfTLR22* or *JfTLR3* gene ORFs containing HindIII and BamHI sites were cloned into pGEM T-easy vector and then inserted into the pcDNA 3.1(+) vector (Invitrogen). Then, the cassette containing the CMV promoter, *JfTLR22* ORF and bovine growth hormone polyadenylation signal was digested using BglII and SalI restriction enzymes and inserted into each of the above-mentioned shRNA expression vectors, resulting in pshTLR3·577-fTLR3, pshTLR3·939-fTLR3, pshTLR3·939cont-fTLR3, pshTLR22·903-fTLR22, pshfTLR22·1726-fTLR22, and pshTLR22·1726cont-fTLR22, and control vector is p-fTLR3 or fTLR22. A neomycin-resistant cassette, including the SV40 early promoter and the SV40 polyadenylation signal in the pcDNA 3.1 vector, was inserted into the ApaI and AatII sites of each

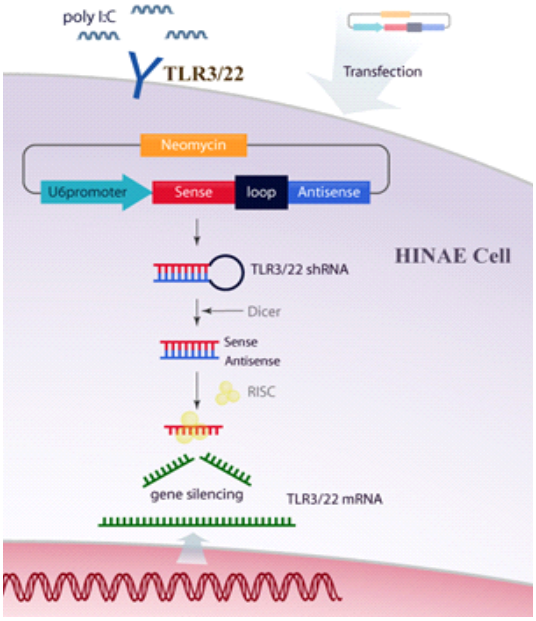
shRNA expression vector and the resulting vectors were named pNeoshTLR3·939, pNeoshTLR22·1726, pNeoshfTLR22·1726cont, and control vector was named pNeo. The integrity of the DNA sequences of the vectors was confirmed by sequencing using an automatic sequencer (Applied Biosystems).

5. Transfection of HINAE cell with shRNA producing vectors and interference analysis of the *JfTLR3* and *JfTLR22* genes

HINAE cells were cultured on 6-well plates (3×10^5 cells/well) at 28°C containing L-15 medium supplemented with 20 mM HEPES and 10% FBS. Upon reaching 80% confluence, the cells were transfected with pshTLR3·577-fTLR3, pshTLR3·939-fTLR3, pshTLR3·939cont-fTLR3, p-fTLR22, pshTLR22·903-fTLR22, pshTLR22·1726-fTLR22, and pshTLR22·1726cont-fTLR22 vectors using FuGENE 6 (Roche) according to the manufacturer's instructions.

6. Transfection of HINAE cells with shRNA expression vectors and selection

HINAE cells were cultured on 6-well plates (2×10^6 cells/well) at 20°C containing L-15 medium supplemented with 20 mM HEPES and 10% FBS. At 80% confluence, the cells were transfected with pNeo, pNeoshTLR3·939, pNeoshTLR22·1726, and pNeoshTLR22·1726cont vectors using FuGENE 6 (Roche) according to the manufacturer's instructions. To select HINAE cells harboring shRNA expressing vectors, 400 μg/mL G-418 (geneticin) antibiotic was added to the culture medium of cells transfected with each pFuguU6shRNA-neomycin plasmid. The surviving cells were then trypsinized using TrypLE Express (Gibco) and plated in new T25 and were grown in L-15 medium containing 10% FBS, antibiotics (penicillin 100 U/mL and streptomycin 100 μg/mL) and 400 μg/mL of G-418. The selected cells were maintained for six months.



[Fig. 1] Transfection of HINAE cells with shRNAproducing vectors, selection and expression analysis of genes after simulated poly I:C.

7. Expression analysis of *TLR3, TLR22, ISG15,* and *Mx* genes after poly I:C stimulation in selected HINAE cell

After 12 h of poly I:C stimulation, total RNA was extracted from the selected HINAE cells harboring shRNA expression vectors (pNeo, pNeoshTLR22·1726, pNeoshTLR22·1726cont, pNeoshTLR3·577, pNeoshTLR3·939, pNeoshTLR3·939cont) grown in culture medium containing G-418 (400 μg/mL, Sigma). Total RNA (0.5 μg) was then reverse-transcribed to cDNA, and semi-quantitative RT-PCR was carried out to

determine the relative mRNA expression level of TLR3, TLR22, ISG15, and Mx genes following the steps described in section II (2. Expression analysis of genes after poly I:C stimulation in olive flounder) and used for gene expression assay.

<Table 1> Oligonucleotide primer pairs used in this study

Name of primer		Sequence (5' to 3')	NCBI accession no.
Jf18S	F	AGTTGCTGCAGTTAAAAAGC	EF126037.1
	R	TGGCATCGTTTACGGTCGGAACTA	
JfTLR3	F	GGCTCATTTCAGTGGCTCACCAGACTTCAGACTC	AB675413.1
	R	GCTGCCCCTTTCAGAAACATCACCGGC	
JfTLR22	F	GACGATGCCTTCAGTTCTCTTCAGAGTCTT	AB109396.1
	R	CAAGCCGCGGAATTCTCCATGTTT	
JfIRF3	F	ACCCTGCTTTGTTTGAGAAGTGCG	GU017417
	R	TGTGCAGTTCGTTCAGACACTCCT	
JfIRF7	F	ATTTCACGGCCTTGGATCTGGGTA	GU017419
	R	TTGCCTCGTAGAAAGTGGGCTGAT	
JfMX	F	AACAGCCAAGGCAAAGATTG	AB110446
	R	AATGTCCAGCTCCTCCTTCA	
JfISG15	F	CTCCATGTAATCTGCAGCAA	our analysis gene
	R	CAGATCTAGTGCAGGTGTGA	

<Table 2 > Target sequences used for the production of pFuguU6shRNA vectors

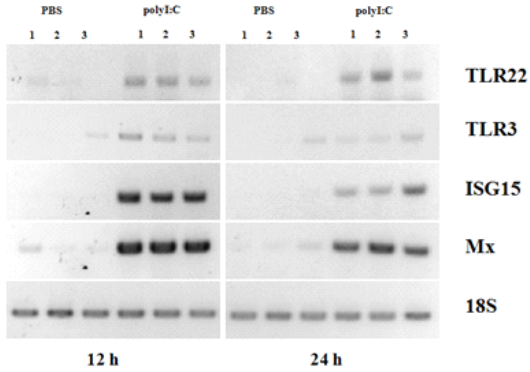
Name of primer		Sequence (5' to 3')	Restriction enzyme
U6	F	GCATGCCACTGGATCCATCTGACACTAA	*SphI*
shfTLR3 577	R1	TCTCTTGAAAAGGAAATAGAAGTCGTCTCAACAGCTAGCAACATC	
	R2	TTTCCTTTCTCTTGAAAAGGAAATAG	
	R3	**CCGCGG**AAAAAAAGACGACTTCTATTTCCTTTCTCTTGAAAAGGAA	*SacII*
shfTLR3 939	R1	TCTCTTGAACTTGGTCAGGTGCTTGATACAACAGCTAGCAACATC	
	R2	GACCAAGTCTCTTGAACTTGGTCAGG	
	R3	**CCGCGG**AAAAAATATCAAGCACCTGACCAAGTCTCTTGAACTTGG	*SacII*
shfTLR22 903	R1	TCTCTTGAAGTTAGCGCTGTGATATTGTCAACAGCTAGCAACATC	
	R2	GCTAACTCTCTTGAAGTTAGCGCTG	
	R3	**CCGCGG**AAAAAAACAATATCACAGCGCTAACTCTCTTGAAGTTAG	*SacII*
shfTLR22 1726	R1	TCTCTTGAAGTTACGAGGCAATTGAGACCAACAGCTAGCAACATC	
	R2	CGTAACTCTCTTGAAGTTACGAGGC	
	R3	**CCGCGG**AAAAAAGTCTCAATTGCCTCGTAACTCTCTTGAAGTTAC	*SacII*
shfTLR22 1726 control	R1	TCTCTTGAAGTTACGAGGCAtaTGAGACCAACAGCTAGCAACATC	
	R2	CGTAACTCTCTTGAAGTTACGAGGC	
	R3	**CCGCGG**AAAAAAGTCTCAtaTGCCTCGTAACTCTCTTGAAGTTAC	*SacII*

The U6 promoter 3' regions are underlined; the enzyme restriction sites are shown in boldface; the nucleotides shown in lowercase alphabets in the sequence of shfTLR22 1726 control R1 and R2 represent the two substituted nucleotides

Ⅲ. Results

1. Expression of *TLR3, TLR22, ISG15*, and *Mx* genes in poly I:C injected olive flounder

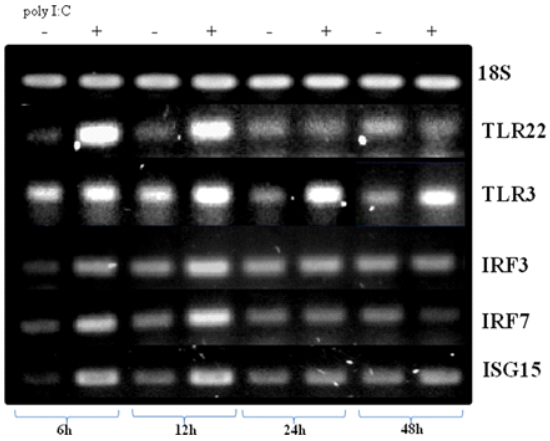
Expression analysis of *JfTLR3, JfTLR22, JfISG15*, and JfMx genes using semi-quantitative RT-PCR revealed no or very weak expression of these genes in the PBS group throughout the experimental period. However, poly I:C stimulation upregulated the expression of *JfTLR22* and *JfTLR3* genes at 12 and 24 h, wherein *JfTLR22* showed a higher expression than *JfTLR3*. In addition, the expression of *ISG15* and *Mx* genes was highly elevated by poly I:C stimulation [Fig. 2].



[Fig. 2] Effect of poly I:C injection on the expression of *TLR3*, *TLR22*, *ISG15*, and *Mx* genes in olive flounder kidney. At 12 and 24 h post-injection with poly I:C or PBS, the expression of each gene in the kidney was analyzed by semi-quantitative RT-PCR.

2. Expression of *TLR3, TLR22, IRF3, IRF7* and *ISG15* genes in poly I:C stimulated HINAE cells

Semi-quantitative RT-PCR results of the *JfTLR3, JfTLR22, JfIRF3, JfIRF7* and *JfISG15* genes in HINAE cells stimulated with poly I:C are shown in Fig. 2. The results revealed that the expression of *JfTLR22, IRF3, IRF7,* and *ISG15* genes was upregulated at 6 and 12 h post-stimulation, and that of the *TLR3* gene was approximately increased 5 times compared with the *TLR22* gene at 24 and 48 h post-stimulation [Fig. 3].

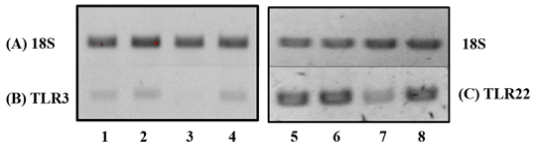


[Fig. 3] Effect of poly I:C on the expression of *TLR3*, *TLR22*, *IRF3, IRF7*, and *ISG15* genes in HINAE cells. At 6, 12, 24, 48 h post-stimulation, expression of the above genes and 18s rRNA gene (control) was analyzed by RT-PCR. '- sign' indicates PBS-stimulation and '+ sign' indicates poly I:C stimulation.

3. Selection of an effective shRNA clone targeting *JfTLR3* or *JfTLR22* in HINAE cell

The expression analysis of the *JfTLR22* or *JfTLR3* genes in HINAE cells transfected with p-fTLR3 or *fTLR22*, pshTLR3·577-fTLR3, pshTLR3·939-fTLR3, pshTLR3·939cont-fTLR3, pshTLR22·903-fTLR22, pshTLR22·1726-fTLR22, and pshTLR22·1726cont-fTLR22 revealed that both *JfTLR3* or *JfTLR22* genes were downregulated in HINAE cell transfected with pshTLR3·939-fTLR3 or pshTLR22 ·1726-fTLR22. However, cells transfected

with pshTLR3·577-fTLR3, pshTLR3·939cont-fTLR3, pshTLR22·939-fTLR22, and pshTLR22·1726cont-fTLR22 vectors showed no reduction in expression of the *JfTLR22* gene [Fig. 4].

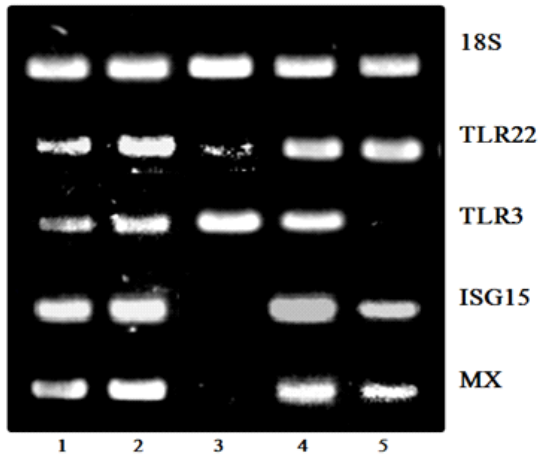


[Fig. 4] Interference effects of *JfTLR3* and *JfTLR22* genes in each transfected HINAE cells. HINAE cells transfected with p-fTLR3 (lane 1), pshTLR3·577-fTLR3 (lane 2), pshTLR3·939-fTLR3 (lane 3), pshTLR3·939cont-fTLR3 (lane 4), p-fTLR22 (lane 5), pshTLR22·903-fTLR22 (lane 6), pshfTLR22 ·1726-fTLR22 (lane 7) and pshTLR22·1726cont-fTLR22 (lane 8) vectors. Expression of (A) 18S rRNA, (B) *JfTLR3*, and (C) *JfTLR22* genes analyzed by semi-quantitative RT-PCR.

4. Effect of shRNAs targeting *JfTLR22* and *JfTLR3* on the expression of type I IFN-induced genes in HINAE cells

The poly I:C stimulation for 12 h of HINAE cells harboring pNeo, pNeoshTLR22·1726, pNeoshTLR22·1726cont, and pNeoshTLR3·939 selected using G-418 supplementation demonstrated that the expression of *JfTLR22* gene was reduced in HINAE cells harboring pNeoshTLR22·1726, whereas the cells harboring pNeoshTLR22 ·1726cont showed no reduction in *JfTLR22* expression compared with that of 18S rRNA [Fig. 5].

Furthermore, despite the stimulation of poly I:C both *ISG15* and Mx genes were highly down-regulated by knock-down of *JfTLR22* (in cells harboring pNeoshTLR22·1726); however, the *JfTLR3* knock-down (in cells harboring pNeoshTLR3·939) could only reduce the expression of these genes by 0.5 times compared with those in the control cells (in cells harboring pNeo).



[Fig. 5] Effects of shRNAs targeting *JfTLR22* or *JfTLR3* on the expression of *JfTLR22*, *JfTLR3*, ISG15, and Mx genes in HINAE cells analyzed by semi-quantitative RT-PCR at 12 h post-stimulation of HINAE cells with poly I:C. Lane 1, HINAE cells (no handling); lane 2, HINAE cells harboring pNeo; lane 3, HINAE cells harboring pNeoshTLR22·1726; lane 4, HINAE cells harboring pNeoshTLR22 ·1726cont; lane 5, HINAE cells harboring pNeoshTLR3·939.

Ⅳ. Conclusion and Suggestions

In mammals, including humans, TLR3 is located in the endosomal membrane or the cell surface and recognizes dsRNA (Matsumoto et al., 2014). However, in fish, an additional TLR, TLR22, is also known to respond to dsRNA. Recently, Matsuo et al. (2008) have reported that fish TLR22 might play a similar role in fish as that played by TLR3 in humans. Although the precise locations of proteins derived from these genes vary among

vertebrates (Paria et al.,2018), they are mostly located in the intracellular vesicle or cell membrane surface (Li et al., 2017; Hu et al., 2015). And we were analysis gene information and performed new cloning of the olive flounder TLR22 gene, it was mostly consistent with TLR3 of NCBI accession no. AB109396.1. It seems that the genetic information of TLR22 was incorrectly labeled as TLR3 in the NCBI gene bank. In the present study, we analyzed the changes in expression of the JfTLR3 and JfTLR22 genes at transcription level after poly I:C stimulation in vivo in olive flounders and in vitro in HINAE cells. The findings demonstrated that the mRNA levels of JfTLR3 and JfTLR22 were upregulated by extrinsically administered poly I:C; however, the increase was lower for JfTLR3 at 6 and 12 h post-stimulation, which became stronger at 24 and 48 h post-stimulation. These results suggest that JfTLR22 is a more sensitive receptor and recognizes extracellular earlier than JfTLR3. Therefore, it can be inferred that JfTLR3 might be located on the inner surface of the endosomal membrane in the cytoplasm, whereas JfTLR22 might be located on the outer surface of cells.

And plasmid vector-based shRNAs widely used to induce RNAi in vertebrate cells provide a useful tool to develop continuous immortalized cell lines in which the suppression of a target gene is stably maintained by RNAi (Taxman et al., 2010). Previously, we have demonstrated successful transcription of shRNA by fugu U6 promoter in bluegill fry (BF-2) cells and efficient knock-down of a target gene by the shRNA in BF-2, grunt fin (GF), and Chinook salmon embryo (CHSE) cells (Zenke and Kim, 2008). In the present study, we established HINAE cells that stably express shRNAs by transfection with the fugu U6 promoter-driven shRNA expressing vector and selection with G-418 The present study employing RNAi demonstrated the important role of TLR22 in recognizing extracellular poly I:C and induction of type I IFN response. HINAE cells harboring the JfTLR22-targeting shRNA vector showed markedly reduced JfTLR22 expression, which resulted in a large reduction of ISG15 and Mx genes expression, suggesting that JfTLR22 plays an important role in the induction of type I IFN response to extracellular dsRNA (poly I:C) stimulation for 12 h. On the contrary, at 12h after poly I:C stimulation, inhibition of TLR3 expression in HINAE cells with the JfTLR3-targeting shRNA vector weakly reduced the expression of ISG15 and Mx genes, suggesting that TLR3 partially participate in the induction of type I IFN response in cells.

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