SHORT COMMUNICATION

Immunostimulatory effect of DDX41 of olive flounder (Paralichthys olivaceus)

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ABSTRACT
The cellular DEAD-box helicase DDX41, which functions as an initial sensor for cytoplasmic DNA, is involved in the activation of type I interferon (IFN-1) immune response in olive flounder. A plasmid encoding DDX41 (pEF-D) was introduced into flounder cells \textit{in vitro} and \textit{in vivo}. Immune responses induced by DDX41 were evaluated by relative quantification value (\(\Delta\Delta_{Ct}\)) method of RT-qPCR using specific IFN-related gene primers. Results in \textit{in vitro}, transcript levels of IFN-1, IRF-3, ISG-15 and IL-1\(\beta\) were significantly higher in pEF-D- than pEF-A-transfected cells, with 15-, 4-, 10- and 32-fold changes, respectively. \textit{In vivo}, elevated expression of these genes was observed in the kidney of pEF-D group on days 1 and 3 post-treatment. The viral challenge test revealed higher survival rate in fish treated with pEF-D (67.5\%) than controls, PBS- and pEF-A-treated fish (45\%). Conclusively DDX41 elicits a robust IFN-mediated immune response, validating its adjuvant property.

1. Introduction
DEAD-box proteins represent the largest family of helicases that participate in the regulation of essentially all the processes involving RNA in cells, from transcription to degradation (Cordin, Banroques, Tanner, & Linder, 2006). Parvatiyar et al. (2012) showed that the cellular DEAD (aspartate-glutamate-alanine-aspartate)-box helicase, DDX41, binds to bacterial cyclic dinucleotides (CDN), and plays a role in activation of the signaling pathway that leads to induction of IFN-1. Specifically, DDX41 senses cytosolic double-stranded DNA (dsDNA), such as that from DNA viruses, which initiates the STING-TBK-IRF3 pathway (Zhang et al., 2011).

Some of the molecular sensors for cytosolic DNA identified to date include AIMS2, IFI16, DDX41 and cGAS (Burckstummer et al., 2009; Fernandes-Alnemri, Yu, Datta, Wu, & Alnemri, 2009; Hornung et al., 2009; Stetson & Medzhitov, 2006; Unterholzner et al., 2010). The interactions of these molecular sensors are orchestrated by a key molecule, ultimately leading to STING signaling (Bhat & Fitzgerald, 2014; Paludan & Bowie, 2013). Especially, DDX41 works together with the STING adaptor to activate
IFN-1 in addition to its role as a pathogen recognition receptor (PRR) that directly binds viral DNA or B-DNA.

When the IFN pathway was activated, a wide array of IFN effector genes, which play pivotal roles in the inhibition of viral replication and/or display combinatorial antiviral properties, will be stimulated thereafter. Activation of IFN is an important step in the immune response mechanism of any organism, since its regulation in the host system is crucial in the anti-pathogen response mediated by innate immune stimulation (Keating, Baran, & Bowie, 2011). This signaling mechanism acts as an initiator of the immune response and is fundamental for the effective function of DNA vaccines. And since the inflammatory signal triggered upon cytosolic DNA recognition, observed in DDX41, appears to have an adjuvant effect on DNA vaccination via activation of the two major pro-inflammatory pathways either by NF-κB or STING pathway (Grunwald & Ulbert, 2015), this study elucidated on this ability of the DDX41.

DDX41 from olive flounder functions in a similar manner as its mammalian counterpart, wherein its expression triggers activation of the IFN-mediated response. The full-length olive flounder DDX41 gene is 2267 bp long and encodes 614 putative amino acids, including the functional domains, DEAD box and helicase (Quynh et al., 2015). In Quynh et al.’s work, over expression of DDX41 in HINAE cells (flounder embryonic cells) stimulated via introduction of C-di-GMP (used as a CDN representative) enhanced the transcriptional activity of the IFN-1 promoter. Elevation of DDX41 levels triggered the expression of antiviral and inflammatory cytokines, such as IFN-1, Mx, IL-6 and IL-1β.

DNA vaccination against infectious diseases remains more advantageous than the classical immunization methods, owing to the possibility of rapid manufacturing, fast adaptation to newly emerging pathogens and high stability at ambient temperatures (Grunwald & Ulbert, 2015). Vaccination triggers the innate immune response of an organism which provides the first line of defense against viral pathogens (Schoggins & Rice, 2011). The activation of the innate immune response is antigen-independent and relies on the ability of the host to recognize pathogens through PRRs (ie toll-like receptors (TLRs), RIG-1-like receptors (RLRs) and NOD-like receptors (NLRs)) (Medzhitov, 2009; O’Neill & Bowie, 2010; Takeuchi & Akira, 2010). However, the immunogenicity of DNA vaccines has been its prevailing drawback, which has prompted for a comprehensive search of optimal adjuvants in recent years. Adjuvanticity as described by Guimaraes et al. (2008) is the capacity to facilitate and/or enhance an immune response of a molecule or a reagent.

Here, we looked into the adjuvanticity of olive flounder DDX41 and evaluated its protective effect after virus infection (Viral hemorrhagic septicemia virus, VHSV, which causes high mortality rate to infected olive flounders). In view of proving that DDX41 effectively induces IFN-1, we investigated the potential utility of olive flounder DDX41 as an immunoadjuvant as well as its potency as a plasmid vaccine.

2. Materials and methods

2.1. Preparation of cell and virus

HINAE (Hirame natural embryo) cells (This cell line was kindly provided by Professor Takashi Aoki, Tokyo University of Marine Science and Technology) derived from
flounder embryo were maintained at 20°C in Leibovitz’s L-15 medium (Life Technologies, USA) containing 10% FBS (Life Technologies, USA), 100 units/mL penicillin, 100 µg/mL streptomycin and 250 ng/mL amphotericin B. For preparation of virus stock, confluent epithelioma papulosum cyprinid (EPC) cells (ATCC®-CRL-2872™) were infected with VHSV (W-VHSV 150402-P2) suspended in 2% FBS at a multiplicity of infection (MOI) of 1 and the virus was allowed to absorb into cells at 14°C. After 1 h of absorption, the inoculum was removed and replaced with 2% FBS/L-15 medium (Life Technologies, USA). Cells were incubated at 14°C until cytopathic effect (CPE) was apparent. In cases where extensive CPE was observed, the medium was collected and cellular debris were removed via low-speed centrifugation. The virus concentration of the collected supernatant was determined based on the 50% Tissue Culture Infective Dose (TCID₅₀).

2.2. Plasmid construction

To obtain olive flounder DDX41, total RNA was extracted from the whole kidney of healthy fish using an R&A-Blue™ Total RNA Extraction Kit (iNtRon Biotechnology, Korea), according to the protocol provided by the manufacturer. Extracted RNA (1 µg) was treated with DNAsel I (Fermentas, ThermoScientific, Korea) to remove unwanted genomic DNA, and subjected to reverse transcription using the TOPscript™ cDNA Synthesis kit (Enzynomics, Korea) to synthesize cDNA. Gene-specific primers for DDX41 (designed from P. olivaceus DEAD-box protein 41 (DDX41) mRNA, accession number: KJ934880.1) flanked by Kpn I and Not I restriction sites were used to amplify the gene from the synthesized flounder cDNA. The following PCR conditions were used for amplifying the 1842bp DDX41 gene (without the stop codon): one cycle of initial denaturation for 3 min at 94°C, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 20 s, extension at 72°C for 90 s, one cycle of final elongation at 72°C for 5 min. The amplified DDX41 gene was inserted downstream of the EF-1α promoter within the KpnI/NotI restriction sites of pTracer™-EF/V5-His A (Invitrogen, Korea), and transformed into DH5α cells. The resulting construct was sent for sequencing (SolGent, Korea) and designated “pEF-D.” The vector, pTracer™-EF/V5-His A, without insert was used as the negative control. It was transformed into DH5α cells was named as pEF-A.

2.3. Transfection of pEF-D into HINAE cells

HINAE cells were seeded in 48-well plates (8.5 × 10⁵ cells/well) at 20°C, prior to transfection. In triplicate, cells were transfected with 700 ng plasmid DNA (pEF-A and pEF-D) mixed with 1 µL Lipofectamine 2000 (Invitrogen, Korea) and Opti-MEM (Life Technologies, USA). At 4 h after transfection, the DNA-Lipofectamine complex was replaced with 500 µL 10% FBS/L-15 medium. Cells were collected 48 h after transfection, and total RNA was extracted and processed for cDNA synthesis.

2.4. Immunization and viral challenge

Olive flounders weighing ∼10 g were distributed into six tanks and reared at 14°C (three tanks were for fish sampling and the other three were for mortality data). Fish were
intramuscularly injected with 1 µg plasmid construct (empty vector, pEF-A; adjuvant, pEF-D) diluted in 100 µL PBS. The group that served as the negative control was injected with 100 µL PBS. At three specific sampling days (days 1, 3 and 14), kidneys were isolated from three fish for RNA extraction. On day 15 post-treatment, 20 fish were injected intraperitoneally with 100 µL of 1 × 10⁶ virus/mL (W-VHSV 150402-P2). Mortality in each group was recorded for 14 days. Kidneys were collected from dead fish, and the presence of the virus was confirmed using RT (reverse transcriptase)-PCR. Efficacy of the plasmid DNA was determined by comparison of the cumulative percentage of mortality (CPM). Mortality data were obtained from two separate trials.

### 2.5. Analysis of IFN-related genes

Kidney samples collected on the sampling days were processed for RNA extraction with the R&A-Blue™ Total RNA Extraction Kit (iNtRon Biotechnology, Korea) following the manufacturer’s protocol, followed by treatment with DNase I (Fermentas, ThermoScientific, Korea). Total RNA (2 µg) was used for the synthesis of cDNA. The cDNA samples generated were diluted 10 times with RNase-free water and used as a template for RT-qPCR to evaluate the expression of several innate immune response genes, including IFN-1, IRF-3, ISG-15 and IL-1β. All primers (Table 1) employed have been reported and optimized in previous studies (Ohtani et al., 2010; Ohtani et al., 2012; Quynh et al., 2015). The specificity of amplification was verified based on analysis of dissociation curves. The ΔΔCt method was utilized to quantify fold changes of target immune gene transcripts in each sample. Expression levels of the target genes were normalized to those of β-actin and expressed as fold change relative to the average level in the PBS group, taken as 1.

### 2.6. Statistical analysis

Data are presented as means ± standard deviation (SD) from triplicate samples (in vitro) or three olive flounder fish (in vivo). The student’s t-test was used to determine the

| Table 1. Oligonucleotide sequences. |
|-------------------------------------|
| Target (GenBank accession number) | Purpose | Primer name | Oligonucleotide sequence (5’–>3’) |
|-------------------------------------|---------|-------------|----------------------------------|
| DDX41 (Degenerate) | PCR | WCUP-1892 | AARATCCAGAAGTTTGGCGAARAG |
| | | WCUP-1893 | CCWATYCTGTGGACRTAGTT |
| | Expression vector | Ddx41-KpnI | GGCCGGTGACCATGAAAAACACGCTTACCGCAGAAG |
| | (pTracer-EF-V5/His A) | Ddx41-NotI | GCTAGCGCCGGACGGCTGACCTGACTTCTGGAGAAGAAGG |
| B-actin (AU050773) | qPCR (amplicon: 85 bp) | WCUP-90 | TGGATGAGCCAGCAGCAAG |
| | (efficiency: 109%) | WCUP-91 | CTCCTATGTCACTCCGTG |
| IFN-1 (AU261156) | qPCR (amplicon: 100 bp) | WCUP-794 | ATGTCTAACGAGGCTTCTG |
| | (efficiency: 107%) | WCUP-795 | GTCTGAATTTATGATGTCATG |
| IL-1β (AB070835) | qPCR (amplicon: 96 bp) | WCUP377 | AATGCAACGTGAGCCAAGAGAGATG |
| | (efficiency: 104%) | WCUP378 | GGTGACACACACTTCTCATT |
| IRF-3 (GU017417) | qPCR (amplicon: 100 bp) | WCUP651 | AGCTGGTGGAGCCAGAGAAG |
| | (efficiency: 106%) | WCUP652 | CATCACCTTGCTCGGCAGAAG |
| ISG-15 (ABS19717) | qPCR (amplicon: 100 bp) | Jf-ISG15-Fwd | GAGAAGTCACGAGGAGCTTT |
| | (efficiency: 100%) | Jf-ISG15-Rev | CAGACGAGAAGAAGGAGCT |

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differences between control and vaccinated groups. Statistical analysis was performed with SPSS v.17.0, and differences were considered significant at $p < .05$. Survival data were analyzed via Kaplan–Meier analysis with the chi-square test using GraphPad Prism v.5 software. Differences were considered significant at $p < .05$.

3. Results and discussion

In this study, a DDX41-expressing plasmid vector was constructed and its immunostimulatory effects on HINAE cells in vitro and olive flounder in vivo were assessed. Since DDX41 is directly involved in the IFN pathway, several IFN-related genes can be used to gauge its capability in stimulating an immune response. As a preliminary experiment, the immunostimulatory effect of pEF-D was evaluated in HINAE cells. RT-qPCR results disclosed a significant increase in IFN-1 expression in pEF-D-transfected cells, which was 18- and 15-fold higher than that in cells transfected with the negative control and empty vector, respectively (Figure 1(A)). Other relevant IFN-related genes additionally showed significantly higher expression in pEF-D-transfected cells (4-, 10- and 32-fold increase

![Figure 1](image-url) RT-qPCR analysis of the expression of IFN-related genes after transfection of constructs in HINAE cells. Panels depict expression of (A) IFN-1, (B) IRF-3, (C) ISG-15, and (D) IL-1β. The error bars represent standard deviation of results obtained from three replicates, and the asterisk indicates a significant difference relative to pEF-A (*$p < 0.05$).

Notes: NC, negative control; pEF-A, empty vector; pEF-D, DDX41 adjuvant.
in IRF-3, ISG-15 and IL-1β levels, respectively; Figure 1(B–D)) \( (p < .05) \) than the empty vector, pEF-A. These results highlight the capacity of pEF-D to activate the IFN-mediated immune response in HINAE cells.

To further examine the immunostimulatory effect of pEF-D, the construct was introduced into olive flounder via intramuscular injection. Tissue distribution of DDX41 expression had been previously elucidated (data not shown). RT-qPCR evaluation of kidney samples revealed significant upregulation of all IFN-related genes specifically in pEF-D treated fish. IFN-1 was expressed 77-fold \( (p < .05) \) higher at day 1 post-treatment compared to the pEF-A control group and the level gradually decreased to 9-fold \( (p < .05) \) by day 3 (Figure 2(A)). IRF-3 was expressed 35-fold \( (p < .05) \) higher relative to the control groups on day 1, and showed a further significant increase on day 3 (50-fold, \( p < .05 \)) (Figure 2(B)). ISG-15 expression was markedly higher on day 1 and reduced by day 3 (23- and 8-fold increase, compared to the control, respectively, \( p < .05 \)) (Figure 2(C)). Similar to IFN-1 and ISG-15, IL-1β expression was significantly higher on day 1 with a 12-fold increase, compared to a minimal 4-fold change on day 3 (Figure 2(D), \( p < .05 \)). These findings implicate that pEF-D is capable of inducing IFN-mediated immune response in olive flounder.

Figure 2. RT-qPCR analysis of expression of IFN-related genes after i.m. injection of constructs in olive flounder. Panels show expression of (A) IFN-1, (B) IRF-3, (C) ISG-15, and (D) IL-1β in kidney. The error bars represent standard deviation of results obtained from three fish, and the asterisk indicates a significant difference relative to pEF-A \((* p < 0.05)\).

Notes: PBS, negative control; pEF-A, empty vector; pEF-D, DDX41 adjuvant.
Thus, data from both *in vitro* and *in vivo* experiments revealed remarkable upregulation of the majority of genes examined. The adjuvant was capable of triggering expression of IFN-1, as shown in Figures 1(A) and 2(A). This result was substantiated by the elevated expression of ISG-15 (Figures 1(C) and 2(C)), a type I-inducible gene. Following the STING-TBK-IRF3 pathway in the immune response, expression of IRF-3 was also evaluated (Figures 1(B) and 2(B)). Upregulation of IRF-3 was evident at the onset of plasmid administration, which is an indication that an innate immune response is stimulated at the early phase of plasmid injection. DDX41 is additionally reported to stimulate pro-inflammatory cytokine secretion. Our results disclosed significant expression of IL-1β (Figures 1(D) and 2(D)), further verifying our hypothesis that DDX41 is capable of mounting an immune response after administration into the organism’s system.

Taking into account the finding that pEF-D induces IFN-related gene expression, we further investigated whether this construct is capable of protecting against VHSV infection in fish. To this end, at 15 days post-treatment, olive flounder were infected with 100 µl of 1 × 10⁶ virus/ml VHSV, and mortality was recorded for 14 days. Survival data showed an average of 67.5% of the fish administered with pEF-D survived after the challenge test, compared to 45% in both PBS and pEF-A groups, respectively (Figure 3(A,B), Table 2). This result concurred with qPCR data indicating that pEF-D is capable of protecting against VHSV infection in olive flounder by inducing IFN-related genes and stimulating an immune response.

Previous studies on fish vaccines had been dealing in the search of possible ways to alleviate the effect of several fish viruses that plague the aquaculture industry. One method that showed the most potential was the development of vaccines that could work with adjuvants. It had been observed that humoral response to weak immunogens can be enhanced by an admixture of the immunogen along with an adjuvant (Suryakala, Maiti, Sujatha, & Sashidhar, 2000). The use of adjuvants is considered one of the most powerful techniques to enhance immunogenicity of a vaccine, not only for humans (Quirk et al., 2014) and their mammalian counterparts (Chuang, Monie, Hung, & Wu, 2010; Jayakumar et al., 2011; Jin et al., 2004), but also other veterinary species, such as birds (Chang, Davis, Stringfield, & Lutz, 2007; Redig et al., 2011) and dogs (Quijano-Hernandez, Bolio-Gonzalez, Rodriguez-Buenfil, Ramirez-Sierra, & Dumonteil, 2008). Two major groups of adjuvants have been tested together with DNA vaccines: (1) traditional adjuvants, that is, chemical compounds used with established vaccine technologies, including killed bacteria, bacterial components, aluminum salts, oil emulsions, polysaccharide particles and biopolymers (Saade & Petrovsky, 2012) and (2) genetic adjuvants, that is, proteins such as cytokines, chemokines, co-stimulatory and immune signaling molecules that can be delivered directly with the DNA vaccine, either on the same or a separate expression plasmid (Grunwald & Ulbert, 2015). Although the use of genetic adjuvants is relatively new compared to traditional adjuvants (such as Alum, which has been utilized since 1926), numerous studies with murine models have confirmed their efficacy. The cytokines IL-2 (Kim et al., 1998), IFN-γ (Chow et al., 1998), GM-CSF (Qing et al., 2010) and IL-15 (Kutzler et al., 2005), when introduced together with a DNA vaccine, stimulate a more enhanced cellular and/or humoral immune response.

Genetic adjuvants together with DNA vaccines administrated in fish have additionally been being examined in recent years. Recombinant IL-1β has been shown to increase the antibody titer against antigens of fish pathogens in carp (Yin & Kwang, 2000, Asian
seabass (Bridle, Crosbie, Morrison, Kwang, & Nowak, 2002), barramundi (Buonocore et al., 2004) and olive flounder as both a DNA and subunit vaccine (Taechavasonyoo, Hirono, & Kondo, 2013). Another immune mechanism currently under exploration for

**Figure 3.** Viral challenge. Olive flounders were i.m. injected with 1 μg of the indicated constructs for two trials (panels A and B). At 15 days post-treatment, fish were challenged i.p. with 100 μL of 1x10^6 virus particles/ml VHSV per fish. Fish mortality was recorded for 14 days and survival data were subjected to Kaplan-Meier analysis using GraphPad Prism 5. Statistical significance between pEF-A and pEF-D were calculated by X^2 at p < 0.05.

Notes: PBS, negative control; pEF-A, empty vector; pEF-D, DDX41 adjuvant.

**Table 2.** Mortality data.

| Fish group | n | Number of fish survived | Survival percentage (%) | Cumulative percent mortality (%) | Significance (5%) |
|------------|---|-------------------------|-------------------------|----------------------------------|------------------|
| 1st trial  |   |                         |                         |                                  |                  |
| PBS        | 20| 9                       | 45                      | 55                               | –                |
| pEF-A      | 20| 11                      | 55                      | 45                               | –                |
| pEF-D      | 20| 12                      | 60                      | 40                               | +                |
| 2nd trial  |   |                         |                         |                                  |                  |
| PBS        | 20| 9                       | 45                      | 55                               | –                |
| pEF-A      | 23| 8                       | 35                      | 65                               | –                |
| pEF-D      | 24| 18                      | 75                      | 25                               | +                |

Note: Significance was calculated using Kaplan–Meier analysis (x^2 test) at 5% in relation to negative pEF-A.
the enhancement of DNA vaccination is the sensing of viral infections via the two PRRs, RIG-1 and MDA-5. Co-delivery of the coding sequence of MDA-5 on a vaccine plasmid against influenza in chickens resulted in significantly higher antibody titers that led to the increased protection against H5N1 challenge (Liniger, Summerfield, & Ruggli, 2012). In another study, co-expression of an RNA-based RIG-1 agonist led to improved antibody avidity after DNA vaccination with an influenza hemagglutinin-coding plasmid (Luke et al., 2011). More recently, the helicase DDX41 was identified as a novel intracellular DNA sensor in myeloid DC, and knockdown of its expression blocked the activation of TBK1, NF-κB and IRF-3 by B-form DNA (Zhang et al., 2011). These previously mentioned studies all regarded the use of genetic adjuvants as enhancers of the efficacy of fish DNA vaccines.

To validate that DDX41 could elicit IFN-mediated immune response, Zhang et al., showed that knockdown of DDX41 expression by a short hairpin RNA (shRNA) blocked the ability of myeloid dendritic cells (mDCs) to mount IFN-1 and cytokine responses to DNA and DNA viruses. In another study, knockdown of DDX41 via shRNA in murine or human cells inhibited the induction of innate immune genes, thereby leading to defects in the activation of STING, TBK1 and IRF-3 in response to cyclic-di-GMP (c-di-GMP) or c-di-AMP. Data obtained from these studies support a mechanism in which c-di-GMP or c-di-AMP is sensed by DDX41 that forms a complex with STING to signal to TBK1-IRF-3 and activate the interferon response (Parvatiyar et al., 2012). In teleost fish, olive flounder DDX41 has been the only PRR investigated so far. Earlier studies have reported significantly enhanced expression of DDX41 in adherent (monocyte-like) cells after stimulation with a DNA virus, implying a role in sensing cytosolic double-stranded DNA (dsDNA). In addition, DDX41 overexpression induced antiviral and inflammatory cytokine gene expression after c-di-GMP treatment (Quynh et al., 2015). In view of the ability of DDX41 to stimulate the IFN-mediated immune response, here we showed the adjuvant effect of DDX41 which can be used in conjunction with fish DNA vaccines.

Ultimately, the information presented in this study can be valuable in the development of DNA vaccines especially for VHSV. In the recent years, several studies dealt on the DNA vaccine designed with the VHSV G glycoprotein (Lorenzen & LaPatra, 2005), however, the problem on the immunogenicity of this DNA vaccine had been its primary setback. It is therefore imperative to develop this DNA vaccine and the use of immunoadjuvant is one of the methods to resolve the issue on its immunogenicity.

4. Conclusion

In conclusion, we have elucidated the potency of pEF-D to induce an innate immune response in vitro and in vivo, as evident from the expression patterns of critical IFN-related genes and pro-inflammatory cytokines. The strength of DDX41 as plasmid DNA vaccine was not as high as expected, but an ability to protect VHSV-infected fish was demonstrated. Our collective findings validate the utility of DDX41 as an immunoadjuvant, which may be further applied for the development of more effective DNA vaccines, not only for VHSV, but also other aquatic viruses deleterious to the aquaculture industry.
Disclosure statement

No potential conflict of interest was reported by the authors.

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