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Enhancement of glycoprotein-based DNA vaccine for viral hemorrhagic septicemia virus (VHSV) via addition of the molecular adjuvant, DDX41

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ABSTRACT

The use of molecular adjuvants to improve the immunogenicity of DNA vaccines has been thoroughly studied in recent years. Glycoprotein (G)-based DNA vaccines had been proven to be effective in combating infection against Rhabdovirus (especially infectious hematopoietic necrosis virus, IHNV) in salmonids. DDX41 is a helicase known to induce antiviral and inflammatory responses by inducing a type I IFN innate immune response. To gain more information regarding G-based DNA vaccines in olive flounder *(Paralicthys olivaceus)*, we tried to develop a more efficient G-based DNA vaccine by adding a molecular adjuvant, DDX41. We designed a DNA vaccine in which the VHSV glycoprotein (G-protein) and DDX41 were driven by the EF-1α and CMV promoters, respectively. Olive flounders were intramuscularly immunized with 1 μg of plasmids encoding the G-based DNA vaccine alone (pEF-G), the molecular adjuvant alone (pEF-D), or the vaccine-adjuvant construct (pEF-GD). At two different time points, 15 and 30 days later, the fish were intraperitoneally infected with VHSV (100 μL; 1×10^6 $TCID_{50}$/mL). Our assays revealed that the plasmid constructs showed up-regulated expression of IFN-1 and its associated genes at day 3 post-vaccination in both kidney and spleen samples. Specifically, pEF-GD showed statistically higher expression of immune response genes than pEF-G and pEF-D treated group ($p < 0.05/p < 0.001$). After VHSV challenge, the fish group treated with pEF-GD showed higher survival rate than the pEF-G treated group, though difference was not statistically significant in the 15 dpv challenged group however in the 30 dpv challenged group, the difference was statistically significant ($p < 0.05$). Together, these results clearly demonstrate that DDX41 is an effective adjuvant for the G-based DNA vaccine in olive flounder. Our novel findings could facilitate the development of more effective DNA vaccines for the aquaculture industry.

1. Introduction

The development of effective fish vaccines had been the subject of many recent studies aimed at helping the virus-plagued aquaculture industries of Asia, especially those in South Korea and Japan. Many of the in-development fish vaccines are DNA-based systems whereby fish are inoculated with plasmid DNA encoding a specific portion of a virus gene under the control of a eukaryotic promoter. This kind of vaccine resembles a virus in that it requires the same cellular machinery for replication and initiates an immune response normally observed after viral infection [1]. DNA vaccines offer numerous advantages over conventional fish vaccines (e.g., live-attenuated vaccines, subunit vaccines, killed vaccines, etc.). For example DNA vaccines: can induce both humoral and cellular immune responses [2]; are easier and safer to handle as they will never revert to an infectious state; and are characterized by fast and cost-effective production, as well as a high degree of stability during the preparation process (unlike other vaccine types) [3]. In contrast to other vaccines (e.g. attenuated vaccines) that were rampantly used in the past, the antigenic gene within the DNA vaccine is conserved the same way as its antigenicity therefore giving a more advanced initiation of immune response after its

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introduction into the fish cells.

DDX41 belongs to the DEXD/H-box (DDX) protein family. The members of this family are RNA and DNA helicases that are homologous to MDA5, RIG-I, and LGP2 and all contain a DEXD/H box domain [4]. These proteins are known to play vital roles in regulating gene induction, and are involved in processes such as signal transduction, gene promoter regulation, mRNA splicing, and translational regulation [5,6]. DDX41, which was recently identified in the teleost fish species, *Paralichthys olivaceus* (olive flounder), is represented by a 2267-bp gene that encodes a protein of 614 amino acids [7]. Its mRNA expression was found to increase significantly in monocyte-like cells following infection with the DNA virus, Lymphocystis Disease Virus (LCDV) [7]. Moreover a reporter assay showed that the transcriptional activity of the interferon (IFN)-1 promoter as well as antiviral and inflammatory cytokine gene expression was enhanced by overexpression of DDX41 in HINAE cells treated with c-di-GMP [7]. These observations suggest that olive flounder DDX41 acts as a cytosolic sensor. In this, its function is similar to what was observed in mammals, which can elicit antiviral and inflammatory responses by inducing a type I IFN innate immune response [7].

The efficacy of glycoprotein (G)-based DNA vaccines can be effective against salmonid Rhabdovirus as was proven from decades of studies [8–14]. Researchers are currently seeking to use molecular adjuvants to develop G-based DNA vaccines with improved immunogenicity. Given that DDX41 senses bacterial cyclic dinucleotides in the cytoplasm and responds by triggering the transcription of IFN stimulated genes (ISGs). We speculated that it could be a good candidate as a molecular adjuvant. The G-protein is known to be a protective antigen whose transcription stimulates IFN-mediated responses. We therefore hypothesized that combining G-protein with DDX41 could yield an enhanced DNA vaccine with improved immunogenicity in the olive flounder. We assume that an intensified IFN-mediated immune response would be triggered by the cellular transcription of these two proteins, giving immunized fish increased protection and/or resistance against viral challenge. Here, we present experimental evidence showing that DDX41 can be added to a G-based DNA vaccine, and that it can enhance the immunogenicity of such a vaccine in olive flounder.

2. Materials and methods

2.1. Cell lines and viruses

Hirame natural embryo (HINAE) cells derived from flounder embryo were maintained at 25 °C in Leibovitz's L-15 medium (Life Technologies, Carlsbad, CA, USA) containing 10% FBS (Life Technologies), 100 units/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B. For preparation of the virus stock, confluent Epithelioma papulosum cyprinid (EPC) cells were infected with W-VHSV 150402-P2 at a multiplicity of infection (MOI) of 1. After 1 h, the inoculum was replaced with L-15 medium containing 2% FBS, and the cells were incubated at 14 °C until a cytopathic effect (CPE) was apparent. When an extensive CPE was observed, the medium was collected, the cellular debris was removed by low-speed centrifugation, and the concentration of the virus in the collected supernatant was determined by calculating the 50% Tissue Culture Infective Dose ($TCID_{50}$).

2.2. Preparation of vaccine and adjuvant

The cDNA encoding the G-protein was obtained from naturally VHSV-infected olive flounders obtained from a fish farm in Namhae, South Korea. Total RNA was extracted from the spleens of infected flounders using an R&A-BLUE™ Total RNA Extraction kit (Intron Biotechnology, South Korea) according to the provided protocol. The extracted RNA was treated with DNAse I (Fermentas), and 1 μg of total RNA was reverse transcribed using a SuperScript III FirstStrand kit (Life Technologies). The G gene was amplified using specific primers that were designed using the VHSV strain KR-CJA G gene (GenBank Accession Number: JQ651388.1) and with flanking *Kpn I* and *Not I* recognition sequences. The PCR conditions were as follows: 3 min at 94 °C followed by 30 cycles of 94 °C for 30 sec, 55 °C for 20 sec and 72 °C for 90 sec, and a final elongation at 72 °C for 5 min. The amplified G-protein gene (1521 bp, lacking the Stop codon) was inserted downstream of the EF-1α promoter using the *Kpn I*/*Not I* restriction sites of pTracer™ EF/V5-His A (Invitrogen). The resulting construct was transformed to and grown in DH5α competent cells. Isolated plasmids were sent for sequencing verification, and the confirmed plasmid was designated as pEF-G (Fig. 1B). To obtain the olive flounder DDX41 sequence, total RNA was extracted from whole kidneys of healthy fish using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol, and cDNA was generated as described above. Gene-specific primers with flanking *Kpn I* and *Not I* sites were designed from the mRNA sequence of the DDX41 mRNA (GenBank Accession Number: KJ934880.1), and PCR amplification of the 1842-bp DDX41 (lacking a stop codon) was performed as described above. The amplified DDX41 gene was inserted into pTracer™-EF/V5-His A and verified as described above, and the confirmed plasmid was designated as pEF-D (Fig. 1C). To generate the DNA vaccine-adjuvant construct, pEF-G was subjected to site-directed mutagenesis to remove the GFP-Zeocin cassette from the plasmid and insert *Avr II* and *Spe I* sites into the backbone vector downstream of the CMV promoter. DDX41 was amplified using a new set of primers bearing *Avr II* and *Spe I* sites plus a sequence encoding the c-myc epitope, and inserted into the mutated pEF-G. The resulting plasmid was transformed to DH5α competent cells, verified by sequencing, and designated as pEF-GD (Fig. 1D).

2.3. Western blot analysis

pEF-G, pEF-D, pEF-GD, and pEF-A (empty vector) were purified using a Maxi-Prep Plasmid Extraction kit (Intron). Duplicate HINAE cell cultures in 12-well plates (3×10^5 cells/well) were transfected using total volumes of 250 μL containing Opti-MEM (Life Technologies), 700 ng plasmid DNA and 1 μL Lipofectamine 2000 (Life Technologies). Four hours after transfection, the DNA-Lipofectamine complex was replaced with 500 μL L-15 medium containing 10% FBS. The transfected cells were incubated at 20 °C throughout the rest of the experiment. Three days after transfection, the cells were collected and lysed using 1x Passive Lysis Buffer (Promega, USA). The protein content was measured using the bicinchoninic acid (BCA) assay, and equal amounts of protein were subjected to 10% SDS-PAGE. The resolved proteins were transferred to PVDF (polyvinylidene difluoride) membranes, which were blocked with 5% skim milk for 1 h on a rotary shaker at slow speed, and then incubated with antibodies against v5 (1:20,000; ThermoScientific) or myc (1:3000; Abcam) for 1 h with shaking. After three washes with 1x PBST (10 min each), the blots were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IGG (1:3000; ThermoScientific) for 1 h with shaking. Finally, the membranes were washed three times with 1x PBST, and the results were detected using enhanced chemiluminescence (ECL) and visual assessment for proteins of the expected size (68 kDa and 56 kDa for DDX41and G protein, respectively).