Cloning of an orange-spotted grouper *Epinephelus coioides* heat shock protein 90AB (HSP90AB) and characterization of its expression in response to nodavirus

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**A B S T R A C T**

The heat shock proteins (HSPs) family which consists of HSP90, HSP70, and low molecular mass HSPs are involved in chaperone activity. Here, we report the cloning and characterization of HSP90AB gene from orange-spotted grouper, *Epinephelus coioides*. The full-length of grouper HSP90AB was 727 amino acids and possessed an ATPase domain as well as an evolutionarily conserved molecular chaperone. The HSP90AB-green fluorescent protein fusion protein was evenly distributed in the cytoplasm. Immunohistochemistry (IHC) and real-time polymerase chain reaction (PCR) analyses indicated that the expression of grouper HSP90AB was marginally increased following nodavirus infection. Grouper *E. coioides* that received HSP90 inhibitor geldanamycin (GA) showed an increase in HSP90AB expression and growth of nodavirus supporting nodavirus replication.

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1. Introduction

Betanodavirus belongs to the family of nodaviridae that usually infects a wide variety of larval and juvenile marine fish, while *Alphamodavirus* infects insect species [1,2]. The molecular chaperone heat shock protein 90 (HSP90) has been identified as important factors involved in the regulation of the RNA polymerase synthesis of viral replication of *Alphamodavirus* [3], the genome of which contains two positive-sense RNA strands known as RNA1 and RNA2. The former encodes RNA-dependent RNA polymerase (RdRp), an enzyme responsible for viral replication that is located in mitochondria, whereas the latter encodes coat protein, which assembles to form the viral particles.

Recent studies demonstrated the possibility that betanodavirus requires cellular stress protein [4], grouper HSP90AB, a stimulatory host factor for its coat protein synthesis. The abundance of this protein may be advantageous to viral infections among animals, such as hepatitis C virus (HCV) [5] and influenza virus [6]. Temperature, an important parameter influences virus growth, regulate heat shock proteins (HSPs), and interrelate HSPs and virus growth.

References have pointed out that there are four main types of HSP90 isoforms of the vertebrates found in the expressing areas. One of the two types of the cytosolic isoforms is called HSP90AA (HSP90 alpha or inducible form) and the other is called HSP90AB (HSP90 beta or constitutive form). Another type is found in the endoplasmic reticulum region of all the eukaryotes, except the fungi, and is referred to as HSP90B (94 kDa glucose regulated protein, Grp94). Lastly, a HSP90 homologue is found in the mitochondrial region, the TRAP (tumor necrosis factor receptor-associated protein) [7]. Although cytosolic HSP90 isoform is involved in cell proliferation and differentiation, HSP90AA has also similarities with growth promotion, cell cycle regulation, and stress-induced cytoprotection, on the other hand, HSP90AB is also associated with early embryonic development, germ cell maturation, cytoskeletal stabilization, cellular transformation, signal transduction, and long-term cell adaptation [8].

Viruses induce a heat shock response indicative of a stressed cell state [9–13]. HSP90 is involved in the immune response especially in lipopolysaccharide (LPS) recognition [14–16], and is regulated by a range of stressors such as heat or cold shock [17–20], hyperosmotic stress [21], food-deprivation [22], reduced oxygen level.
and the presence of polychlorinated biphenyl (PCB) [24], arsenates [25], and heavy metals [26,27]. However, little is known on the function of HSPs in nodavirus infection.

HSP90 is an atypical member of the family of heat shock-induced chaperones that promote folding, cellular translocation and assembly of newly synthesized polyptides of viruses [28]. HSP90 participates in the growth of vaccinia virus [29], as well as in the function of translocation and the assembly of polyptides of polyomavirus and SV40 [30,31]. In the case of hepatitis B virus, HSP90 interacts with viral reverse transcriptase to facilitate the formation of a ribonucleoprotein (RNP) complex [32]. These activities of HSP90 centers on a specific set of client proteins that facilitate their folding into stable or active conformations, and are assisted by the proteins ATP-dependent chaperone capability.

The purpose of the present study was 1) to present cloning and molecular characterization of HSP90AB cDNA from orange-spotted grouper Epinephelus coioides, 2) to compare its sequences and conduct phylogenetic analyses with other HSP90, 3) to examine the expression of HSP90AB and protein level from different tissues, and 4) to evaluate the changes of HSP90AB expression whether or not the groupers were infected by nodavirus, and 5) to evaluate the changes of HSP90AB expression after geldanamycin (GA) treatment of the grouper cells (GF-1), whether viral coat protein expression will be affected or not.

2. Materials and methods

2.1. Cell culture and reagents

The grouper cell line GF-1 [33], derived from the fin tissue of orange-spotted grouper E. coioides, was grown at 28 °C in Leibovitz’s L-15 medium (GibcoBRL, Gaithersburg, MD, USA) supplemented with 5% fetal bovine serum (FBS). Grouper cells (GF-1), which are susceptible to nodavirus infection and nodavirus replication, obtained from the Bioresources Collection and Research Center in Taiwan BRC960094 was used. Transient transfection was performed by introducing 1–2 μg of plasmid encoding grouper HSP90-fused green fluorescent protein (GFP) into cells using Lipofectamine (Invitrogen). After transfection, cells were grown for 24–30 h. Intracellular localization of GFP-fused proteins was examined using an Olympus IX70 microscope (Olympus, Tokyo, Japan). GA was dissolved in 20% dimethylsulfoxide (DMSO; Sigma–Aldrich) in sterile 0.9% saline. An alkaline phosphatase-conjugated substrate Western blotting detection system kit was purchased from Bio-Rad (Hercules, CA, USA). Alkaline phosphatase-conjugated anti-mouse, anti-rabbit, and anti-goat IgG antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were diluted 1:5000 prior to use.

2.2. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from grouper at 40–45 days post-hatching using a previously described single-step acid guanidinium thiocyanate–phenol–chloroform extraction method [34]. Extracted cellular total RNA (5 μg) as template was incubated at 42 °C for 60 min in 20 μL of 1X reaction buffer containing 2 U Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Madison, WI, USA), 0.25 mM dNTP and 4 μM oligo(dT)15 primer, and lastly 0.4 U RNase (Boehringer Mannheim Biochemicals, Mannheim, Germany) was added. Putative HSP90AB sequences were examined for homogeneity by comparison with published HSP90AB sequences using Blast [http://www.ncbi.nlm.nih.gov/BLAST]. In the first experiment, the grouper, 40–45 days post-hatching nodavirus naturally-infected, were obtained from hatchery farms. We excised the eye from healthy and nodavirus naturally-infected grouper, and total RNA was extracted for HSP90AB expression. First-strand cDNA was synthesized using total RNA (5 μg) and cDNA (250 ng) as a template for the PCR. The grouper HSP90AB nucleotide sequences of forward and reverse primers were as follows: HSP90-RT-S 5'-ATGCCGTAAGAAATGCGCAAGGAC-3' (forward primer) and HSP90-RT-A, 5'-CCTTACGGTACCCCTGTC- GACTCTG-3' (reverse primer), were designed to specifically amplify a 523-bp PCR fragment. Grouper β-actin gene expression was analyzed as an internal marker using the following primers: β-ACTIN-RT-S 5'-ACCAAGAGGAAGATGACCC-3' (forward primer) and β-ACTIN-RT-A 5'-CTGATCCATCTGCTGAAG-3' (reverse primer). PCR was conducted under the following conditions: 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, with 25 cycles; the last cycle was followed by extension for 5 min at 72 °C. The total amount of cDNA was calibrated based on the amplification of β-ACTIN cDNA from the same template, and PCR products were analyzed by agarose gel electrophoresis. In the second experiment, real-time RT-PCR was used to quantify the expression of mRNA for HSP90AB with β-ACTIN as control. First-strand cDNA was synthesized using 2 μg total RNA and the SuperScript First Strand cDNA synthesis kit (Invitrogen). The amplification was performed using the qPCR core kit for SYBR Green (Qagen, Valencia, CA, USA) and Step-One™ Real Time PCR system (Applied Biosystems, Foster City, CA, USA). Typical profile times used were 95 °C for 15 s, followed by a second step at 94 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s for 40 cycles with melting curve analysis. After the PCR program, fluorescent real-time PCR data from three replicate samples were analyzed.

2.3. Suppression subtractive hybridization

cDNA subtraction was performed to generate subtracted cDNA library between 40 and 45 days post-hatching nodavirus naturally-infected grouper (tester) and healthy grouper (driver) using PCR-Select cDNA subtraction Kit (Clontech, Mountain View, CA, USA) according to the manufacturer’s protocol. Briefly, poly(A+) RNA was extracted using the FastTrack™ mRNA isolation kit (Clontech). cDNA was synthesized using SMART™ PCR cDNA Synthesis Kit according to the manufacturer’s instruction. Tester and driver cDNAs were purified by ethanol precipitation, and then digested with RsaI at 37 °C overnight to obtain shorter blunt-ended molecules. Two different adaptors, adaptor 1 and adaptor 2, were ligated to the 5'-end of each strand of tester cDNA, both of which were separately hybridized at 68 °C for 8 h with an excess of driver cDNA after denaturation at 98 °C for 90 s. After the first hybridization, the two samples were mixed together without denaturation and hybridized again with freshly heat-denatured driver cDNAs for 18 h at 68 °C. The resulting mixture was diluted 1:50, and then amplified by two rounds of PCR to enrich desired cDNAs containing both adaptors by exponential amplification of these products [35]. Nested PCR amplicons were subcloned into a pGEM-T easy Cloning Kit (Promega). Finally, the efficiency was evaluated by PCR with β-actin forward and reverse primers performed on tester (unsubtracted) and subtracted cDNAs for 25 cycles.

2.4. Rapid amplification of cDNA ends polymerase chain reaction (RACE-PCR)

RACE-PCR was conducted following the previously described method [36]. Based on the verified sequence of the 2437 bps fragment of HSP90AB cDNA from the subtracted plasmid library, HSP90-5RACE and HSP90-3RACE primers (Table 1) were applied in PCR amplification and cloning of the cDNA 5’ end and 3’ end, respectively. Two adaptor primers for both ends were provided in the Marathon cDNA Amplification Kit (Clontech). The RACE-PCR thermal cycle profile was as follows: 94 °C for 1 min; 94 °C for 30 s, 72 °C for 4 min, 72 °C for 10 min with thirty cycles; extension at 72 °C for 10 min. The amplified fragment was verified with subcloning into
cDNA was synthesized using 2 primer pair used in this experiment was designed to cross-design to specific intron/exon boundaries as inferred from sequence of grouper QRT-A, 5’-GTCAGCTCCAGCCTGCAGGGC-3’/C14-0-2131 and 5’-GTCAGCTCCAGCCTGCAGGGC-3’/C14-0-2131 were designed to specifically amplify a 227-bp PCR fragment. Total RNA was separated by the MegAlign v7.2.1 program from the LASERGENE software suite (DNASTAR). The search matrix for the bootstrap analysis was generated from the aligned sequence data using SEQBOOT (200 replicates) in the PHYML package [37]. The phylogenetic tree based on maximum likelihood algorithm was constructed using PHYML v2.4.4 with non-parametric bootstrap analysis (200 replicates). The phylogenetic tree was subsequently modified using TreeView program v1.6.2 [38].

### 2.5. Phylogenetic analysis

The searches for amino acid sequence similarities were conducted with BLAST programs at the National Center for Biotechnology Information (http://www.ncbi.nlm.gov/BLAST/). Alignments of sequences were carried out and the sequence identities calculated by the MegAlign v7.2.1 program from the LASERGENE software suite (DNASTAR). The sequence matrix for the bootstrap analysis was generated from the aligned sequence data using SEQBOOT (200 replicates) in the PHYML package [37]. The phylogenetic tree based on maximum likelihood algorithm was constructed using PHYML v2.4.4 with non-parametric bootstrap analysis (200 replicates). The phylogenetic tree was subsequently modified using TreeView program v1.6.2 [38].

### 2.6. Real time RT-PCR

Real time RT-PCR was used to quantify the expression of mRNA for *HSP90AB* with expression of elongation factor 1 α (*EF-1α*) as a reference gene. The grouper *HSP90AB* primers and reverse primers were as follows: *HSP90AB*-QRT-S, 5’-AACGCTTCTGATGTTGGAC-3’ (forward primer) and *HSP90AB*-QRT-A, 5’-GCAGCTCAGCTGACAGGCCG-3’ (reverse primer), were designed to specifically amplify a 227-bp PCR fragment. The *HSP90AB* primer pair used in this experiments was designed to cross-intron/exon boundaries as inferred from sequence of grouper *HSP90AB* structure. Grouper *EF-1α* gene expression was analyzed as an internal marker using the following primers: EF-1α-QRT-S 5’-ACCCCTCATTGGTGGC-3’ (forward primer) and EF-1α-QRT-A 5’-GCGGAGGAGGCCGAGTTG-3’ (reverse primer), were designed to specifically amplify a 184-bp PCR fragment. Total RNA was separated from healthy grouper, following the single-step acid guanidinium thiocyanate−phenol−chloroform extraction method. First-strand cDNA was synthesized using 2 μg total RNA and the SuperScript First Strand cDNA synthesis kit (Invitrogen). The amplification was performed using the qPCR core kit for SYBR Green (Qiagen, Valencia, CA, USA) and StepOne™ Real Time PCR system (Applied Biosystems, Foster City, CA, USA). Typical profile times used were initial step, 95 °C for 15 min, followed by a second step at 94 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s for 40 cycles with melting curve analysis. The level of target mRNA was normalized to the level of *EF-1α* and expressed as relative to controls (healthy grouper) by 2^−ΔΔCT method.

### 2.7. Production of polyclonal rabbit anti-grouper *HSP90AB* antiserum

The cDNA for the mature *HSP90AB* from amino acids 1–727 of an orange-spotted grouper was cloned into pGEM-Tvector (Promega) and subcloned into the pET-24a vector (Novagen, Madison, WI, USA) between the EcoRI and Xhol sites to obtain pET24a-*HSP90AB*. The resulting expression vector encoded *HSP90AB* with a (His)6 and several extra amino acids at the N-terminus. The expression vector pET24a-*HSP90AB* was transformed into the bacterial host, *Escherichia coli* BL21 (DE3), for expression driven by T7 polymerase. Induction by 0.5 mM isopropyl-β-thiogalactopyranoside was carried out 28 °C for 3 h. After undergoing freezing and thawing once, cells were sonicated on ice, and the cleared lysate was obtained by centrifugation at 12,000 rpm for 15 min. The (His)6-tagged *HSP90AB* was bound to a nickel-charged HisTrap column (HisTrap HP, 5 ml bed volume; Amersham Biosciences, Piscataway, NJ, USA) which was equilibrated with binding buffer and washed with binding buffer containing 50 mM imidazole. The bound *HSP90AB* eluted with binding buffer containing imidazole in a step-gradient manner (100–500 mM). The *HSP90AB* protein peaks, eluted with 250–350 mM imidazole, were combined and dialyzed against buffer A (50 mM Tris−HCl, pH 8.0, 1 mM dithiothreitol, 50 mM NaCl, 5 mM MgCl2, 10% glycerol), followed by freezing at −70 °C in a minimal aliquot. Protein concentrations were determined using a Bio-Rad protein assay kit with a bovine serum albumin standard. To obtain anti-grouper *HSP90AB* rabbit polyclonal antibody, (His)6-tagged *HSP90AB* was used to immunize two New Zealand white rabbits with a primary injection emulsified in Freund’s incomplete adjuvant at 1 mg ml−1 and 1 ml was injected subcutaneously into two rabbits. The rabbits were boosted after 4, 8, and 12 weeks with the same amount of antigen in the adjuvant. The *HSP90AB* antibody was obtained after clotting overnight at 4 °C followed by centrifugation at 1200 rpm [39].

### 2.8. Immunohistochemical studies of *HSP90AB* tissue distribution

To correlate with study *HSP90AB* distribution on grouper tissues, immunohistochemical studies were performed as follows. Grouper were 40–45 days post-hatching were fixed in 10% formalin and embedded in paraffin following a routine procedure. Each 5-μm thick section was mounted on a polylysine-coated slide, deparaffinized in xylene, and rehydrated in descending grades (100–70%) of ethanol. Endogenous peroxidase activity was blocked by a 10 min incubation at room temperature with absolute methanol containing 3% H2O2. The sections were sequentially blocked with power block solution (Bio Genex, San Ramon, CA, USA), then washed with phosphate-buffered saline.
saline (PBS) and incubated with polyclonal rabbit anti-grouper HSP90AB or polyclonal rabbit anti-coat protein antibody (1:500 dilution) at 4 °C overnight. The sections were washed twice with PBS, incubated with secondary antibody (Super Sensitive™ Polymer-HRP IHC, Bio Genex) for 30 min at room temperature. The peroxidase activity was developed with 3,3'-diaminobenzidine (used as chromogen) for 10 min. The sections were counterstained with Harris hematoxylin for nuclei, dehydrated, and mounted. Negative controls were performed with preimmune rabbit serum and incubation with PBS instead of the anti-grouper HSP90AB antibodies. The sections were observed under Axiovert 40 microscope (Carl Zei, Gottingen, Germany). The images were obtained with an SPOT RTS™ camera (Diagnostic, Sterling Heights, MI, USA).

2.9. Western blot analyses of HSP90AB and nodavirus coat protein (CP)

After rinsing with phosphate-buffered saline (PBS), grouper cells (GF-1) were cultured by seeding 10⁵ cells ml⁻¹ in a Petri dish for 24 h. Cells were infected at a multiplicity of infection (m.o.i.) of 0.5 with nodavirus (previously propagated and titrated in GF-1 cells where the virus was found to have a TCID₅₀ per ml of 10⁴) for 1.5 h and treated with GA (1.5 μM) for 24 h. At the end of incubation time, the culture medium was aspirated and the cells were washed with cold PBS, lysed with 50 μl of sodium dodecyl sulfate (SDS) gel sample buffer (0.1 M Tris—HCl [pH 6.8], 0.2 M dithiothreitol, 2% glycerol, 0.1% bromophenol blue), boiled for 10 min, and centrifuged for 10 min at

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**Fig. 1.** Characterization of a cytosolic grouper HSP90AB. (A) The complete nucleotide and deduced amino acid sequences of the grouper *E. coioides* HSP90AB gene. The putative ATPase domain and geldanamycin-binding motif that binds to the ATP-binding pocket on putative region is boxed. The shaded boxes indicate highly charged regions of HSP90AB as analyzed by comparison of all known HSP90 sequences. The stop (TAA) codon is indicated with a dot. The positions of deduced amino acids are indicated in bold numbers. This nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide sequence databases and have been assigned the accession no. FJ644278. (B) Detection of the transiently expressed grouper HSP90AB protein in grouper cells. Visualized fluorescence of cells after transfection with pcDNA3/HSP90AB-EGFP expression plasmid. Intracellular localization of GFP-fused proteins was examined by microscopy.

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13,000 rpm. Cell extract (25 μg) was applied to each lane and subjected to SDS-10% polyacrylamide gel electrophoresis. Prestained protein marker Standard was included on each gel for molecular mass estimation. Briefly, blotting, blocking, and antibody incubation were performed as described previously [40]. Polyclonal rabbit anti-HSP90AB antibody and polyclonal rabbit anti-coat protein antibody were used for the detection of HSP90AB and nodavirus CP proteins, respectively. Alkaline phosphatase-conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.) diluted 1:2000 was used as the secondary antibody. To ensure equal protein loading, anti-β-ACTIN monoclonal antibody (Biogenesis, Kingston, NH, USA), which was diluted to 1:5000 dilutions of anti-mouse, anti-rabbit, or anti-goat IgG antibodies was conjugated to alkaline phosphatase (Santa Cruz Biotechnology). The expression of β-ACTIN was used as the control for equal protein loading.

### 3. Results

#### 3.1. Molecular cloning and nucleotide sequencing of grouper HSP90AB gene

From the PCR-based subtracted library, recombinant clones were randomly selected and sequenced. A partial cDNA fragment (2.4 kb) was identified by sequence analysis and considered likely to be HSP90AB. The deduced amino acid sequence showed homology with that of HSP90AB of Atlantic salmon in the C-terminal [21]. The HSP90AB full-length cDNA was achieved by RACE, which was conducted to determine the nucleotide sequence of the grouper HSP90AB cDNA. The complete HSP90AB cDNA is shown in Fig. 1A. Based on the putative HSP90AB cDNA sequences obtained by the PCR-based subtracted library, primers for 5’ and 3’ RACE were designed.

![Fig. 2. Phylogenetic relationships among HSP90 family of different organisms using the maximum likelihood method.](image)

The tree is based on an alignment corresponding to the open-reading frames of the HSP90AB sequence. The numbers on the branches are bootstrap values. The GenBank accession codes of the sequence designations. Homo sapiens (Hsa), NP_0031381, NP_005339, and NP_003290; Mus musculus (Mmu), NP_032328, NP_034610, and NP_035761; Rattus norvegicus (Rno), AAH20099, AA223609, NP_786937, and EDI71052; Bos taurus (Bta), BAC32448, and NP_00102668; Gallus gallus (Gga), CA40704, and CA30255; Danio rerio (Dre), AA99169, and NP_571403; Oncorhynchus mykiss (Omy), BAD90024, and BAD90023; Salmo salar (Ssa), AAD20278; Dicentrarchus labrax (Dla), AAQ55393; and Mitochondrial HSP90 homologue from Homo sapiens (Hsa), NP_057376 was indicated an outgroup to root tree. Only bootstrap values higher than 75% was used on each branch. The scale for branch length (0.1 substitutions/site) is shown below the tree.

![Fig. 3. Expression levels of HSP90AB transcript in different organs of grouper.](image)

(A) Relative expression levels of HSP90AB in different tissues from orange-spotted grouper, Epinephelus coioides. (B) Representative immunohistochemical (IHC) image of the orange-spotted grouper, 40–45 days post-hatching healthy grouper section following IHC staining using an anti-grouper HSP90AB antibody (original magnification × 10). IHC showed for HSP90AB tissue distribution. Strong labeling (grey brown colour) for HSP90AB expression is present in the liver and intestine tissues (arrows). B = brain; G = gill; L = liver; S = spleen; I = intestine; M = muscle. (C) Control used identical tissue sections with preimmune rabbit serum replacing rabbit anti-HSP90AB antibody.
The PCR-amplified for 5' and 3' UTR cDNAs were used for direct DNA sequencing and/or cloned into a pCR2.1 vector. Several 5'-termini were found, corresponding to alternative transcription sites. A comparison of the cDNA sequence of grouper HSP90AB with those of the fish HSP90 indicated that there were several motifs in grouper HSP90AB. They are SLINT (residues 25–30), NNLTIA (99–105), SMICQFGVGYS (113–124), AGG (160–162), RGT (176–178), KHFSVEGQLEF (319–329), VKK (409–411), HED (442–444), IDEY (517–520), QALRD (609–613), and MEEVD (723–727). The deduced amino acid sequences of grouper HSP90AB also revealed the amino acid signature sequences of endoplasmic reticulum (ER) HSP90 proteins. They are FLREL (38–42); IGGFVGVEYS (115–124), and LPLNISRE (386–393) [7]. Transient transfections were performed by introducing 1–2 μg of plasmid encoding grouper HSP90AB-GFP fusion protein into cells. Strong fluorescence probably resulting from overexpression was observed in the cytoplasm of some cells (Fig. 1B). The result was consistent with a common ancestor of all vertebrate HSP90AB isoforms.

3.2. Phylogenetic tree analysis

HSP90AB is an ATP-dependent chaperone that plays important roles in signal transduction networks, cell cycle control, transport of protein, and protein degradation [28]. HSP90AB expression is also associated with infecting virus. To elucidate the evolutionary history of HSP90, we identified novel HSP90 sequences of 30 species across all kingdoms of organisms and all sequences available in GenBank, and explored the phylogenetic relationships of the HSP90AB gene family. Phylogenetic analysis was constructed by the maximum likelihood method from a multiple sequence alignment of orange-spotted grouper cytosolic HSP90AB gene, and alignment of the amino acid sequences of the open-reading frames along with all known complete sequences of HSP90 cDNA (Fig. 2). As expected, the results indicated the presence of two families, HSP90AA and HSP90AB, of teleost HSP90 forming a well-supported clade and separating clades [7]. It was apparent that orange-spotted grouper HSP90 gene grouped into their orthologous teleost belonging to HSP90AB.

3.3. Grouper HSP90AB transcript expression in different organs

Real time quantitative RT-PCR using SYBR Green dye demonstrated levels of HSP90AB expression at similar levels in all tissues (Fig. 3A). HSP90AB transcript was ubiquitously expressed in all organs. Staining was visible as a brown granular reaction product with 3,3'-diaminobenzidine in chromogen solution (DAB) in the liver. The intestinal epithelium produced a patchy, focal pattern of...
HSP90AB expression (Fig. 3B). However, an immunohistochemical study indicated that grouper HSP90AB was highly expressed in liver and intestine (Fig. 3B), compared to preimmune rabbit serum (Fig. 3C) replacing rabbit anti-HSP90AB antibody.

3.4. Influence of HSP90AB expression in response to nodavirus

To analyze HSP90AB expression during natural nodavirus infection, total RNA from grouper eye was reverse transcribed and amplified for 25 cycles with the grouper HSP90AB primers HSP90-RT-S and HSP90-RT-A. Amplification of total eye RNA produced a PCR product of approximately 520 bp (Fig. 4A). The differential expression of HSP90AB was also examined by real-time qRT-PCR during nodavirus infection (Fig. 4B). Grouper HSP90AB mRNA levels increased about 3 times that of control levels in eye tissue. It is likely that grouper HSP90AB functions as a co-chaperone of HSP70, HSP90AB levels increased after the cells were infected with nodavirus (Fig. 5). The expression of coat protein (CP) was monitored in a nodavirus-infected sample using Western blot analysis. In contrast to grouper HSP90AB levels, the levels of HSP70 did not change significantly in response to nodavirus infection. In immunohistochemical image, HSP90AB detection occurred to a significantly

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higher degree in the brain region of nodavirus-infected than the 
brain of healthy grouper (Fig. 6A, B). Nodavirus CP was demonstrated 
by immunohistochemistry (IHC) in the brain from nodavirus natu-
really-infected grouper, but not observed in healthy grouper (Fig. 6C, 
D). Vacuolization was found in the brain area, when nodavirus-
infected, during nodavirus-infected, during the examination of H&E 
stain (Fig. 6E, F).

3.5. GA upregulates HSP90AB and coat protein of nodavirus 
expression in grouper cells after nodavirus infection

The expression of HSP90AB may provide a feasible host-derived 
factor that regulates growth of nodavirus. There was a close link 
between HSP90AB expression and stress-increase in nodavirus-
infected grouper. Presently, GA was used to assay whether HSP90AB 
chaperone function was required during nodavirus replication. Cells 
were infected with nodavirus at a MOI of 0.1 and incubated in 
medium lacking GA or containing 1.5 μM GA. Cells were infected 
with nodavirus for 1.5 h prior to exposure to GA for 24 h. Grouper 
HSP90AB was induced by GA and facilitated considerably the level of 
nodavirus coat protein (Fig. 7).

4. Discussion

In the present study, HSP90AB gene was cloned from orange-
spotted grouper Epinephelus coioides and demonstrate that it codes a protein 
727 amino acids in length. This protein has ability to bind GA, 
interference with chaperone functions, and multiple and highly 
conserved sequence alignment of grouper HSP90AB with other 
known HSP90 proteins, especially in the regions of HSP90 family 
signatures [7]. In addition, sequence analysis indicated that the 
deduced amino acid sequence of grouper HSP90AB has 92.4% iden-
tity with zebrafish HSP90α, it was placed far away from zebrafish 
HSP90. Interestingly, grouper HSP90AB-GFP fusion protein was 
distributed throughout the cytoplasm but was excluded from the 
N-terminus (Fig. 1B). Moreover, homology analysis revealed that it is 
possible to indicate potential structural and functional features of 
grouper HSP90s depending on conserved features of the extant 
HSP90 types. For example, like all vertebrate β-isofoms, there was 
no QTQQ sequence at the grouper HSP90AB N-terminus. The 
present study indicated that grouper HSP90AB is more closely 
related to the vertebrate HSP90AB isoforms.

In humans, HSP90 is constitutively expressed in all organs [41]. 
When groupers were infected by nodavirus, the virus accumulated 
in the brain and eyes, and underwent replication at these two sites 
[33]. These two sites are, therefore, very important in the study of 
virus–host interactions. The eye is a very important tissue in the 
study of virus-induced stress. Our real-time PCR analysis indicated that when groupers were naturally-infected with nodavirus a faint 
increase in HSP90AB transcripts could be discerned in eye tissue. 
The HSP90AB expression was consistent with the significant increase in the expression level of grouper HSP90AB at 6, 12, 24, 48, 
and 72 h post nodavirus-infections when compared to the control 
results (Fig. 5). In contrast to HSP90AB, the levels of HSP70 which 
was monitored as a control, and a β-Actin did not change signifi-
cantly in response to nodavirus-induced stress.

The molecular mechanism of HSP90 induction and the role of 
grouper HSP90AB during nodavirus infection remain to be eluci-
dated. In humans, the ability of virus to generate reactive oxygen 
species (ROS) or reactive nitrogen species from phagocytes are 
evidence of the considerable host cell stress inflicted by viral 
fection [42]. It is possible that generation of ROS has an antiviral 
effect on cells, but that this is also damaging to the host cells, giving 
rise to a large amount of denatured protein. In view of nodavirus 
infected and subsequent ROS production, aggregation of 

misfolding proteins in the host cell may lead to expression of HSPs. 
The increased expression is considered to be associated with a 
protective mechanism, whereby HSPs possess the necessary 
chaperone machinery to refold into their original structure under 
physiologically relevant stress conditions.

Our purpose of this research is to investigate the role of grouper 
HSP90AB in nodavirus growth. The temperature will influence 
nodavirus growth, as it was shown that as the temperature increases, 
nodavirus growth rate increases consistently. The expressed protein 
amount of HSP90 will increase consistently with the increase of the 
temperature. As the references have indicated that HSP90 facilitated 
nodavirus replication, and on the account of the correlation factors 
between the temperature and nodavirus growth, the objective of the 
entire experiment is to interpret that the expression of the endoge-
nous HSP90AB will promote nodavirus growth. First of all, the cDNA 
of Epinephelus coioides HSP90AB was cloned, characterized and 

![Fig. 7. Effect of grouper HSP90AB protein expression on replication of nodavirus. Cells were infected with nodavirus (MOI = 0.1) for 1.5 h and the cells were treated with GA (1.5 μM) for 24 h. After cell lysis, protein extracts were extracted and a protein sample (25 μg) was used for Western blot analysis using the anti-grouper HSP90AB, anti-
nodavirus CP, anti-mouse actin antibody for grouper HSP90AB, β-Actin, and nodavirus CP, respectively.](image)
expressed, which indicated that grouper HSP90AB should be a cytosolic member of the HSP90AB family. Secondly, we examined the relationship between HSP90AB expression and nodavirus coat protein (CP) synthesis. The results suggested that the one important function of grouper HSP90AB is to facilitate virus growth. Lastly, the grouper is a model system for studying nodavirus growth in relation with HSP90 expression and CP level. Geldanamycin (GA), a specific inhibitor of the chaperone function of HSP90 protein, has been shown to mimic heat shock in the induction expression of HSP90 in mammalian cells. Unexpectedly, when grouper cells were treated with a standard amount of GA, they are able to mimic the situations of heat shock, thus will increase grouper HSP90AB protein expression. In the present study, treatment of grouper cells with GA increased HSP90AB level, as assayed 24 h after treatment, and facilitated heat shock, thus will increase grouper HSP90AB protein expression.

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