Molecular characterization and expression analysis of *Hsp90* in *Schizothorax prenanti*

Yundan Pu¹ · Jieyao Zhu¹ · Hong Wang¹ · Xin Zhang¹ · Jin Hao¹ · Yuanbin Wu¹ ·
Yi Geng² · Kaiyu Wang² · Zhiqiong Li¹ · Jian Zhou^{3,4} · Defang Chen^{1,3,4}

Received: 11 April 2016 / Revised: 11 July 2016 / Accepted: 17 July 2016 / Published online: 15 August 2016
© Cell Stress Society International 2016

Abstract Aquatic animals suffer from various environmental stresses because the aquatic environment is a very complex system. To monitor the health status of fish, *Hsp90* a potential early warning marker was determined in *Schizothorax prenanti* after infection with a bacterium. In this study, we cloned *Hsp90* from *S. prenanti* for the first time. The full-length cDNA sequence of *SpHsp90* was 2663 bp, contains an open reading frame of 2181 bp, and has a gene encoding 726 amino acids, an estimated molecular mass of 83.38 kDa, and a theoretical isoelectric point of 4.91. The *SpHsp90* amino acid sequence has five conserved HSP90 family signatures and shares 87.0–95.5 % identity with other vertebrates. Phylogenetic analysis and structure comparison indicated that *SpHsp90* should be a β isoform of the HSP90 family. *SpHsp90* was ubiquitously expressed in all examined tissues,

and the highest level of expression was in the kidney. After *Streptococcus agalactiae* infection, the level of *SpHsp90* expression had significant changes ($P < 0.05$) in the hepatopancreas, spleen, kidney, and blood. The expression increased to the highest level at 6 h in the blood and at 24 h in the hepatopancreas, spleen, and kidney. The results suggested that the *SpHsp90* gene could be induced by *S. agalactiae* in *S. prenanti* and that *SpHsp90* may be involved in resistance to bacterial infection and provide an early warning information. The kidney is the most suitable for detecting *SpHsp90* after bacterial infection.

Keywords *Hsp90* · *Schizothorax prenanti* · Gene expression · Molecular characterization · Bacterial infection

✉ Jian Zhou
zhoujian980@126.com

✉ Defang Chen
chendf_sicau@126.com

Yundan Pu
yundanpu@163.com

Jieyao Zhu
jieyaozhu@yahoo.com

Hong Wang
13880464407@163.com

Xin Zhang
zhangxinscn@163.com

Jin Hao
haojin0402@163.com

Yuanbin Wu
louisleotung@gmail.com

Yi Geng
gengyisicau@126.com

Kaiyu Wang
kywangsicau@126.com

Zhiqiong Li
lizhiqiong454@163.com

¹ Department of Aquaculture, College of Animal Science and Technology, Sichuan Agricultural University, WenJiang District Huimin Road No. 211, ChengDu, Sichuan Province 611130, China

² College of Veterinary Medicine, Sichuan Agricultural University, WenJiang District Huimin Road No. 211, ChengDu, Sichuan Province 611130, China

³ Fisheries Research Institute, Sichuan Academy of Agricultural Sciences, ChengDu, China

⁴ Sichuan Agricultural University, WenJiang District Huimin Road No. 211, ChengDu, China

Introduction

Schizothorax prenanti, commonly known as Ya-fish, is a valuable endemic fish in Western Sichuan Plateau, China. With the rapid development of intensive aquaculture, streptococcus disease has frequently emerged in *S. prenanti* farms in recent years (Geng et al. 2012). Therefore, monitoring the health status of fish is crucial for aquaculture management. An interesting focus of research involves finding a warning information marker of infection status. Heat shock proteins (HSPs) are ubiquitous and highly conserved stress proteins and play a critical role in the health of finfish, in relation to the host response to environmental pollutants, food toxins, and microbiologic infections (Roberts et al. 2010; Ryan and Hightower 1996; Ryan and Hightower 1994). According to molecular mass, HSPs are grouped into several families, as follows: HSP110, HSP90, HSP70, HSP60, and low molecular weight HSP (Basu et al. 2002).

HSP90 is a highly conserved, extensively studied protein in the aforementioned families, which plays key roles in the cell as a molecular chaperone, including newly synthesized proteins, refolding denatured proteins, and signal transduction. *Hsp90* has been cloned from several aquatic animals, such as bay scallops (*Argopecten irradians*) (Gao et al., 2008), zhikong scallop (*Chlamys farreri*) (Gao et al., 2007), grass carp (*Ctenopharyngodon idella*) (Wu et al., 2012), common carp (*Cyprinus carpio*) (Hermesz et al. 2001), miiuy croaker (*Miichthys miiuy*) (Wei et al., 2013), and channel catfish (*Ictalurus punctatus*) (Xie et al., 2015). The expression of *Hsp90* is regulated by various environmental stressors, including temperature (Jiravanichpaisal et al. 2004; Tedeschi et al. 2015), benzo (a) pyrene (Liu et al. 2015), pH (Zhou et al. 2015), cadmium (Choi et al. 2008), crowding (Ni et al. 2014), hypoxia (Mohindra et al. 2015), and salinity (Peng et al. 2015). Thus, *Hsp90* could be a biomarker of aquatic animals for responding to water quality; however, the aquatic environment is a complex system. In addition to chemical and physical factors, the aquatic environment still has many biological factors, including pathogen microbiology. Recent study has indicated that *Hsp90* appears to be the dominant and critical HSP in olive flounder kidney infected with *Streptococcus parauberis* (Cha et al. 2013). *Hsp90* messenger RNA (mRNA) from *Crassostrea hongkongensis* is constitutively expressed in various tissues and is apparently inducible in hemocytes under *Vibrio alginolyticus*, suggesting an important role in response to bacterial invasion. Therefore, as a sensitive biomarker for environmental stressors, *Hsp90* may provide a warning of bacterial infection in aquaculture.

To better understand the mRNA level of *Hsp90* expression after *S. prenanti* infection with *Streptococcus agalactiae* infection, *Hsp90* from *S. prenanti* (named

SpHsp90) was cloned at the first time, and the patterns of tissue expression in response to *S. agalactiae* challenge were analyzed by quantitative real-time PCR (qPCR) in the study.

Material and methods

Animal treatment

Healthy *S. prenanti*, weighing 45 ± 3 g, were obtained from a commercial farm in Ya'an (Sichuan, China) in August 2015. *S. prenanti* were maintained in tanks (100 cm $\times$ 40 cm $\times$ 60 cm) with aerated water at a temperature of 23 ± 1 °C and fed regularly with commercial food at a ratio of 2 % of the total biomass for 1 week during acclimation. One half of the water was exchanged for fresh water daily. For full-length complementary DNA (cDNA) cloning and expression pattern analysis of the *Hsp90* gene, the following tissues were obtained from five healthy *S. prenanti*: brain, muscle, kidney, hepatopancreas, skin, spleen, heart, gill, and intestine. The tissues were immediately conserved in an RNA/DNA sample protector (TaKaRa, Dalian, China), then stored at -80 °C. Blood samples (approximately 100–200 μ l/fish) were taken from the caudal vein using a 1-ml syringe, and the blood was added to Cell Lysis Buffer (BIKW, Beijing, China), then stored at -80 °C. Before dissection of tissues, fish were anesthetized in 0.02 % tricaine methane sulfonate (MS-222). All experimental protocols using fish were approved by the Regulations for the Administration of Affairs Concerning Experimental Animals and approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University (Sichuan, China).

RNA extraction and cDNA synthesis

Total tissue RNA was isolated using the RNAiso Plus kit (TaKaRa). Total blood RNA was isolated using the Blood Total Protein, Total RNA, DNA, and microRNA-kit (BIKW). The RNA concentration and purity were determined by RNA electrophoresis and the optical density absorption ratio (A260/280) in the Nanodrop 2000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Samples with an absorption ratio of 1.8–2.0 were used for subsequent cDNA synthesis. cDNAs were synthesized using the PrimeScript™ RT reagent kit with gDNA Eraser (Perfect Real Time; TaKaRa). First-strand cDNA of hepatopancreas was synthesized using the SMART RACE cDNA Construction Kit for rapid amplification of cDNA ends (RACE; TaKaRa), referring to the method recommended by the manufacturer.

Full-length cDNA cloning of *Hsp90*

The cDNA was used as a template for the reverse transcription-polymerase chain reaction (RT-PCR) with a pair of specific primers (*Hsp90*-1F and *Hsp90*-1R) designed to amplify fragments of *Hsp90* in *S. prenanti*. The PCR primers are listed in Table 1. The PCR program was as follows: 5 min at 94 °C; 30 cycles at 94 °C for 30 s, 55.7 °C for 30 s, and 72 °C for 1.5 min; a final extension for 8 min at 72 °C; and then cooling to 4 °C. Purified PCR products were ligated into a pMD19-T vector (TaKaRa), transformed into competent *Escherichia coli* DH5 α cells, then sequenced.

To obtain the entire *Hsp90* cDNA sequence, the partial *Hsp90* cDNA was fully extended using 5' RACE and 3' RACE, respectively. The gene-specific primers used for 5' RACE and 3' RACE are listed in Table 1. The first round of PCR amplification was performed with primers UPM and *Hsp90*-5' R1 or *Hsp90*-3' F1, and the second round of PCR amplification was performed with primers UPM and *Hsp90*-5' R2 or *Hsp90*-3' F2, using methods described by Lin et al. (2014). PCR products were cloned and sequenced as described above. Finally, sequences were assembled and the full-length cDNA of *Hsp90* was obtained.

Sequence analysis

The open reading frame (ORF) of *S. prenanti Hsp90* (*SpHsp90*) terminated using the ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). The calculated molecular

weight and theoretical isoelectric point of the deduced amino acid sequences were obtained using online ProtParam software (<http://web.expasy.org/protparam/>). Nucleotide and amino acid sequence identity and the prediction of conserved domains in the peptide were performed using the BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Multiple sequence alignments were performed using the ClustalW program (<http://www.ebi.ac.uk/clustalw/>) (Thompson et al. 1994). A phylogenetic tree of different vertebrate *Hsp90s* based on amino acid sequences was constructed by the neighbor-joining method in the MEGA 6.0 program (<http://www.megasoftware.net/index.html>).

Tissue distribution of *SpHsp90* mRNA

Total RNA was extracted and cDNA was synthesized, as described previously ("RNA extraction and cDNA synthesis"). RT-PCR analysis was performed using the applied Biosystems Step One Plus (Life Technologies, Hercules, CA, USA). The gene-specific primers are listed in Table 1. *S. prenanti*-specific β -actin and 18S-rRNA primers served as internal controls to normalize the cDNA quantity for each tissue sample (Lin et al. 2014). Quantification of *SpHsp90*, β -actin, and 18S-rRNA was performed in triplicate on all samples using SYBR Premix Ex Taq II (Tli RNaseH Plus; TaKaRa), according to the manufacturer's instructions and methods according to Wang et al. (2015). The qPCR data were obtained as Ct values and analyzed using the comparative Ct method ($2^{-\Delta\Delta C_t}$; Livak and Schmittgen 2001).

Table 1 The primers used for *Hsp90* clone and qPCR

Primer	Sequence (5'-3')	Annealing temperature (°C)	Size (bp)
Primers for RT-PCR analysis			
Hsp90-1F	ATY G GK CAG TTY GGY GTSG	55.7	1472
Hsp90-1R	GCC TGR GCY TTC ATG ATCC		
5' RACE primers			
UPM-Long	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	70.0 → 68.0 → 66.0	
UPM-Short	CTAATACGACTCACTATAGGGC		
Hsp90-5' R1	TCTCCTCCTCTGCCTCGTCGTCACT	70.0 → 68.0 → 66.0	
Hsp90-5' R2	TGAAAGAGCCACCAGCGGATGACTC	66.0	
3' RACE primers			
Hsp90-3' F1	GCGACGAGATGACCTCCCTCACAGAAT	70.0 → 68.0 → 66.0	
Hsp90-3' F2	GCTGCCTGAGGATGAAGACGAGAAG	64.0	
Primers for quantitative real-time PCR analysis			
Hsp90-F	AAAGACCAGGTCGCCCCACTC	57.0	91
Hsp90-R	AGTACTCGTCGATGGGCTCG		
β-actin-F	CGAGCTGTCTTCCCATCCA	60.0	86
β-actin-R	TCACCAACGTAGCTGTCTTTCTG		
18s rRNA-F	ACCACCCACAGAATCGAGAAA	58.0	99
18s rRNA-R	GCCTGCGGCTTAATTTGACT		

Results

Characterization of the SpHsp90 gene cDNA

The full-length *SpHsp90* cDNA was 2663 bp, including an ORF of 2181 bp, an 84 bp 5'-untranslated region (UTR), and a 398 bp 3'-UTR with a typical polyadenylation signal

sequence (ATTAAA) and a poly-(A) tail (Fig. 1). The predicted ORF encoded a protein of 726 amino acids with a calculated molecular mass of 83.38 kDa and a theoretical isoelectric point of 4.91. BLASTP analysis showed that SpHsp90 was most similar with *C. carpio* Hsp90 (GenBank accession no. AJG39407), and the identity was 96 %. As expected, *SpHsp90* had five conserved HSP90 family signatures in the

Fig. 2 Multiple sequence alignment of SpHsp90. Analysis was performed by ClustalW, using representatives of Hsp90 from different previously known vertebrates. Five conserved HSP90 family signatures and the MEEVD appear were underlined

deduced amino acid sequence (Fig. 2). A conserved motif (G-X-G) and C-terminal MEEVD motif were identified in *SpHsp90*. The *SpHsp90* protein has a conserved ATP binding/ Mg^{2+} binding site and a typical histidine kinase-like ATPase domain. Multiple polypeptide sequence alignment revealed a high degree of identity for *Hsp90*.

Phylogenetic analysis

Phylogenetic analysis of the amino acid sequences of *SpHsp90* was performed to study the evolutionary relationship with the reported HSP90 of vertebrates from the GenBank database (Fig. 3). The results showed that HSP90 α and HSP90 β were clustered, and *SpHsp90* was grouped in the HSP90 β of Teleostei and different from the HSP90 β of Mammalia, Aves, and Amphibia.

Tissue distribution of *SpHsp90* expression in *S. prenanti*

Expression of *SpHsp90* mRNA was examined by qPCR in different tissues from five fish, with *18s rRNA* and β -actin as the internal controls. As shown in Fig. 4, *SpHsp90* transcripts were ubiquitously expressed in all tissues examined, including the gills, brains, hearts, muscles, skin, blood, kidneys, hepatopancreas, intestines, and spleens. The relative expression of *SpHsp90* was significantly higher in the kidney, hepatopancreas, blood, spleen, intestine, and gill than in other tissues ($P < 0.05$). Indeed, the level of expression of *SpHsp90* in the kidney was nearly three times than that in the spleen and

blood. Moreover, the lowest relative mRNA expression of *SpHsp90* appeared in the muscle (Fig. 4).

Expression patterns of *SpHsp90* after challenge with *S. agalactiae*

To determine the change in *SpHsp90* after bacterial infection, the level of transcription was determined in the kidney, hepatopancreas, blood, and spleen by qPCR. After *S. agalactiae* infection, *SpHsp90* expression was rapidly upregulated in the blood and hepatopancreas and reached the maximum point at 6 h (Fig. 5a, b). The transcription of *SpHsp90* was gradually upregulated in the kidney and in the spleen up to 24 h after *S. agalactiae* challenge (Fig. 5c, d). The level of mRNA expression of *SpHsp90* in the blood remained equal with the respective level in the control sample at day 5, but the levels of expression of *SpHsp90* in the spleen and kidney remained significantly different.

Discussion

HSP90, one of the HSPs, plays an important role in protecting cell resistance to stress damage. In this study, we identified and analyzed *SpHsp90* from *S. prenanti* for the first time. *Hsp90* is highly conserved at the amino acid sequence level. Analysis alignment of the amino acid sequence of *Hsp90* from other species, such as *C. carpio*, showed a high structural similarity. *SpHsp90* contains five conserved HSP90 family

Fig. 3 Phylogenetic tree of Hsp90 from vertebrates. The phylogenetic tree was constructed using p-distance model and 95 % partial deletion method in MEGA 6.0. The statistical robustness of the tree was estimated by bootstrapping with 1000 replicates

Fig. 4 Tissue relative mRNA expression of *SpHsp90*. Quantitative data for *SpHsp90* mRNA levels were obtained by qPCR. The results were expressed as relative expression levels after standardization by β -actin and *18S-rRNA*, which served as controls to verify the quality and amount of samples. *Mu* muscle, *Sk* skin, *Br* brain, *He* heart, *Gi* gill, *In* intestine, *Bl* blood, *Sp* spleen, *Hep* hepatopancreas, *TK* kidney. Different letters represent significant differences ($P < 0.05$). Error bars represent standard error of the mean ($n = 5$ fish/group)

signatures and one ATP binding domain, which are essential for the basic functions of Hsp90 protein (Hartl and Hayer-Hartl 2002). A conserved motif (G-X-G) in the ATP binding domain is necessary for ATP binding to the ATP binding domain (Prodromou et al. 1997). The C-terminal-conserved

MEEVD motif was determined in the amino acid sequence of *SpHsp90*; thus, *SpHsp90* belongs to the cytosolic HSP90 family because it has the characteristics of cytosolic HSP members. The function of the MEEVD motif mediates inter-domain communication and peptide-binding capacity (Gupta 1995). The results suggest that *SpHsp90* may have the same functions as the *Hsp90* from other organisms.

Both multiple alignment and phylogenetic analyses revealed that the *SpHsp90* shares a high amino acid identity with the *Hsp90* of other vertebrates (87.0–95.5 %). In vertebrates, *Hsp90* has two groups (*Hsp90 α* and *Hsp90 β*) which have evolved from the same ancestral gene (Moore et al. 1989). Alignment indicated that *SpHsp90* is highly similar to *Hsp90 β* of vertebrates and amino acid sequence analysis indicated a lack of the glutamine-rich sequence (QTQDQ) in the N-terminal domain, which characterizes all α -isoforms (Fu et al. 2011; Theodoraki and Mintzas 2006). In the phylogenetic tree, *SpHsp90* and the *Hsp90 β* of other vertebrates cluster, and *SpHsp90* is located on the branch of teleosts. This fact suggests that the *SpHsp90* gene is more closely related to vertebrate β -isoforms.

SpHsp90 was extensively expressed in all tissues of *S. prenanti*, which was measured by qPCR. The findings herein were similar to the findings in *M. miiuy* (Wei et al. 2013), *M. amblycephala*, and *I. punctatus* (Xie et al. 2015). The intestines, hepatopancreas, kidneys, and gills are important metabolic organs in fish. *SpHsp90*, with a high level of

Fig. 5 Transcript level of *SpHsp90* in tissues of *S. prenanti* by *S. agalactiae* infection at different time points. **a** Blood, **b** hepatopancreas, **c** spleen, **d** kidney. Quantitative data for *SpHsp90* mRNA levels was obtained by qPCR. The results were expressed as relative expression levels after standardization by β -actin and *18S-rRNA*, which served as a control to verify the quality and amount of samples. Asterisks represent significant differences between groups at the same time point ($P < 0.05$). Error bars represent standard error of the mean ($n = 8$ fish/group)

expression, was observed in these organs as molecular chaperones, especially in the kidney and hepatopancreas. In addition, the stable level of expression of *SpHsp90* gene in the spleen and blood showed that *SpHsp90* may have an immunity-related function; however, intestines and gills, as open tissue samplings, are vulnerable to interference or pollution. We finally chose the hepatopancreas, kidney, spleen, and blood for qPCR analysis of the level of expression of *Hsp90* after bacterial infection.

The level of expression of *SpHsp90* mRNA in the hepatopancreas, spleen, kidney, and blood was significantly increased ($P < 0.05$) after challenge. A similar *Hsp90* pattern of expression was observed in *M. amblycephala* after *A. hydrophila* infection (Ding et al. 2013). In the current study, the blood, hepatopancreas, and kidney rapidly responded initially (after 0 h), but the spleen reaction was late (after 6 h), and related to organ function and route of bacterial infection. After intraperitoneal injection, bacteria invade the host tissues and organs through the blood. The blood functions to maintain homeostasis, immunologic and defensive functions, and transportation (Ferencz et al. 2012). Thus, *SpHsp90* in the blood reached a maximum at 6 h, and the relative level of expression in the infection group was five times the control group. Although followed by a rapid decline in the blood, *SpHsp90* was still significantly higher than the control group in the first 24 h. As previously shown, the hepatopancreas and kidney are the main infection sites for *S. agalactiae* infections (Suwannasang et al. 2014). We observed that the level of expression of *SpHsp90* gene increased during the first 24 h in the hepatopancreas and kidney. In addition to bacterial infection, the level of expression of *Hsp90* changed significantly in the liver after viral infection in Atlantic salmon (Gadan et al. 2012). There was no change in *SpHsp90* in the spleen the first 6 h after infection, but the response persisted longer than the blood and hepatopancreas after 24 h. Thus, *Hsp90* may be involved in immune regulation against bacterial infections in fish. Considering the development of fish disease after bacterial infection by intraperitoneal injection, the course of disease was divided into four stages according to the time: acute stage (6 h), early stage (24 h), intermediate stage (72 h), and late stage (120 h) (Cha et al. 2013). The level of expression of *SpHsp90* was significantly higher than that of the control group in the blood, hepatopancreas, and kidney in the acute (6 h) and early stages (24 h) after infection. Therefore, we speculated that *Hsp90* may be a potential early warning marker of *S. agalactiae* infection in the blood, hepatopancreas, and kidney. The *AiHsp90* mRNA transcripts in hemocytes of *A. irradians* were measured after challenge by *V. anguillarum* or *M. luteus*, and the expression of *AiHsp90* mRNA increased and reached a maximum at 9 h (Gao et al. 2008). Other studies also indicated that bacterial infection induces significant changes in the expression of *Hsp90* during acute and early stages. Previous studies showed that

biomarkers should be quick, easy, and inexpensive to detect in a wide variety of organisms; the *Hsp90* is very conservative protein from prokaryotes to eukaryotes; and the mRNA expression level use the qPCR detected are faster than protein detected. So the results suggested that *Hsp90* may be a potential biomarker, not only for environmental stress but also for bacterial infection to monitor the health status of fish.

The advantage of blood as a test sample is that it is not necessary to sacrifice animals; however, one must be cognizant of rapid increase 6 h after infection. Other studies have reported similar results (Liu et al. 2014). The level of expression of *Hsp90* mRNA in hemocytes of *S. paramamosain* was reported to be upregulated after challenge, and the level of *Hsp90* mRNA peaked at 6 h in the live bacteria group, then decreased 12 and 24 h post-infection (Zhang et al. 2014). It is worth noting that the level of *Hsp90* in kidneys of the infection group was significantly higher than the control group at every time point, suggesting that kidney is the most suitable for detecting *SpHsp90* after bacterial infection. Furthermore, the regulation of *Hsp90* gene expression is very complex, and the level of gene expression usually occurs at both the transcription and translation levels. It has been reported that the level of expression of *Hsp90* protein is consistent with the mRNA levels after infection. Thus, the next step will be to focus on determining the basis for the level of expression of protein in infected *S. prenanti*.

In conclusion, we have cloned *SpHsp90* cDNA from *S. prenanti*. The *SpHsp90* gene is highly similar to *Hsp90* β of teleosts. *SpHsp90* mRNA is extensively expressed in all tissues (from high to low): kidney, hepatopancreas, blood, spleen, intestine, gill, heart, brain, skin, and muscle. *Hsp90* may be a potential early warning marker for *S. agalactiae* infection in blood, hepatopancreas, and kidney. Indeed, kidney is the most suitable tissue for detecting *SpHsp90* expression after *S. agalactiae* infection.

Acknowledgments This work was supported by the double branch plan of Sichuan Agricultural University.

References

- Basu N, Todgham A, Ackerman P, Bibeau M, Nakano K, Schulte P, Iwama GK (2002) Heat shock protein genes and their functional significance in fish. *Gene* 295:173–183
- Cha IS, Kwon J, Park SB, Jang HB, Nho SW, Kim YK, J-i H, Aoki T, Jung TS (2013) Heat shock protein profiles on the protein and gene expression levels in olive flounder kidney infected with *Streptococcus parauberis*. *Fish & shellfish immunology* 34:1455–1462
- Choi YK, Jo PG, Choi CY (2008) Cadmium affects the expression of heat shock protein 90 and metallothionein mRNA in the Pacific oyster, *Crassostrea gigas*. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 147:286–292
- Ding Z, Wu J, Su L, Zhou F, Zhao X, Deng W, Zhang J, Liu S, Wang W, Liu H (2013) Expression of heat shock protein 90 genes during early

- development and infection in *Megalobrama amblycephala* and evidence for adaptive evolution in teleost. *Developmental & Comparative Immunology* 41:683–693. doi:10.1016/j.dci.2013.08.007
- Ferencz Á, Juhász R, Butnariu M, Deér A, Varga I, Nemcsók J (2012) Expression analysis of heat shock genes in the skin, spleen and blood of common carp (*Cyprinus carpio*) after cadmium exposure and hypothermia. *Acta Biol Hung* 63:15–25
- Fu D, Chen J, Zhang Y, Yu Z (2011) Cloning and expression of a heat shock protein (HSP) 90 gene in the haemocytes of *Crassostrea hongkongensis* under osmotic stress and bacterial challenge. *Fish & shellfish immunology* 31:118–125. doi:10.1016/j.fsi.2011.04
- Gadan K, Marjara IS, Sundh H, Sundell K, Evensen Ø (2012) Slow release cortisol implants result in impaired innate immune responses and higher infection prevalence following experimental challenge with infectious pancreatic necrosis virus in Atlantic salmon (*Salmo salar*) parr. *Fish & shellfish immunology* 32:637–644
- Gao Q, Song L, Ni D, Wu L, Zhang H, Chang Y (2007) cDNA cloning and mRNA expression of heat shock protein 90 gene in the haemocytes of Zhikong scallop *Chlamys farreri*. *Comp Biochem Physiol Part B: Biochem Mol Biol* 147:704–715
- Gao Q, Zhao J, Song L, Qiu L, Yu Y, Zhang H, Ni D (2008) Molecular cloning, characterization and expression of heat shock protein 90 gene in the haemocytes of bay scallop *Argopecten irradians*. *Fish & shellfish immunology* 24:379–385
- Geng Y, Wang K, Huang X, Chen D, Li C, Ren S, Liao Y, Zhou Z, Liu Q, Du Z (2012) *Streptococcus agalactiae*, an emerging pathogen for cultured Ya-fish, *Schizothorax prenanti*, in China. *Transbound Emerg Dis* 59:369–375
- Gupta RS (1995) Phylogenetic analysis of the 90 kD heat shock family of protein sequences and an examination of the relationship among animals, plants, and fungi species. *Mol Biol Evol* 12:1063–1073
- Hartl FU, Hayer-Hartl M (2002) Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* 295:1852–1858
- Hermesz E, Ábrahám M, Nemcsók J (2001) Identification of two hsp90 genes in carp. *Comp Biochem Physiol Part C: Toxicol Pharmacol* 129:397–407
- Jiravanichpaisal P, Söderhäll K, Söderhäll I (2004) Effect of water temperature on the immune response and infectivity pattern of white spot syndrome virus (WSSV) in freshwater crayfish. *Fish & shellfish immunology* 17:265–275
- Lin F, Zhou C, Chen H, Wu H, Xin Z, Liu J, Gao Y, Yuan D, Wang T, Wei R (2014) Molecular characterization, tissue distribution and feeding related changes of NUCB2A/nesfatin-1 in Ya-fish (*Schizothorax prenanti*). *Gene* 536:238–246
- Liu T, Pan L, Cai Y, Miao J (2015) Molecular cloning and sequence analysis of heat shock proteins 70 (HSP70) and 90 (HSP90) and their expression analysis when exposed to benzo (a) pyrene in the clam *Ruditapes philippinarum*. *Gene* 555:108–118
- Liu X, Ji C, Zhao J, Wang Q, Li F, Wu H (2014) Metabolic profiling of the tissue-specific responses in mussel *Mytilus galloprovincialis* towards *Vibrio Harveyi* challenge. *Fish & shellfish immunology* 39:372–377
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻ΔΔCT method. *Methods* 25:402–408
- Mohindra V, Tripathi RK, Yadav P, Singh RK, Lal KK (2015) Hypoxia induced altered expression of heat shock protein genes (Hsc71, Hsp90α and Hsp10) in Indian Catfish, *Clarias batrachus* (Linnaeus, 1758) under oxidative stress. *Mol Biol Rep* 1–13
- Moore SK, Kozak C, Robinson EA, Ullrich SJ, Appella E (1989) Murine 86- and 84-kDa heat shock proteins, cDNA sequences, chromosome assignments, and evolutionary origins. *J Biol Chem* 264:5343–5351
- Ni M, Wen H, Li J, Chi M, Ren Y, Song Z, Ding H (2014) Two HSPs gene from juvenile Amur sturgeon (*Acipenser schrenckii*): cloning, characterization and expression pattern to crowding and hypoxia stress. *Fish Physiol Biochem* 40:1801–1816
- Peng G, Zhao W, Shi Z, Chen H, Liu Y, Wei J, Gao F (2015) Cloning HSP70 and HSP90 genes of kaluga (*Huso dauricus*) and the effects of temperature and salinity stress on their gene expression. *Cell Stress and Chaperones* 21(2):349–359
- Prodromou C, Roe SM, O'Brien R, Ladbury JE, Piper PW, Pearl LH (1997) Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. *Cell* 90:65–75
- Roberts R, Agius C, Saliba C, Bossier P, Sung Y (2010) Heat shock proteins (chaperones) in fish and shellfish and their potential role in relation to fish health: a review. *J Fish Dis* 33:789–801
- Ryan J, Hightower L (1996) Stress proteins as molecular biomarkers for environmental toxicology. In: *Stress-inducible cellular responses*. Springer 411–424
- Ryan JA, Hightower LE (1994) Evaluation of heavy-metal ion toxicity in fish cells using a combined stress protein and cytotoxicity assay. *Environ Toxicol Chem* 13:1231–1240
- Suwannasang A, Dangwetngam M, Issaro A, Phromkunthong W, Suanyuk N (2014) Pathological manifestations and immune responses of serotypes Ia and III *Streptococcus agalactiae* infections in Nile tilapia (*Oreochromis niloticus*) Songklanakarin. *J Sci Technol* 36(5):499–506
- Tedeschi J, Kennington W, Berry O, Whiting S, Meekan M, Mitchell N (2015) Increased expression of Hsp70 and Hsp90 mRNA as biomarkers of thermal stress in loggerhead turtle embryos (*Caretta caretta*). *J Therm Biol* 47:42–50
- Theodoraki M, Mintzas A (2006) cDNA cloning, heat shock regulation and developmental expression of the hsp83 gene in the Mediterranean fruit fly *Ceratitis capitata*. *Insect Mol Biol* 15:839–852
- Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22:4673–4680
- Wang E, Wang K, Chen D, Wang J, He Y, Long B, Yang L, Yang Q, Geng Y, Huang X (2015) Evaluation and selection of appropriate reference genes for real-time quantitative PCR analysis of gene expression in Nile tilapia (*Oreochromis niloticus*) during vaccination and infection. *Int J Mol Sci* 16:9998–10015
- Wei T, Gao Y, Wang R, Xu T (2013) A heat shock protein 90 β isoform involved in immune response to bacteria challenge and heat shock from *Müchthys müy*. *Fish & shellfish immunology* 35:429–437
- Wu C-X, Zhao F-Y, Zhang Y, Zhu Y-J, Ma M-S, Mao H-L, Hu C-Y (2012) Overexpression of Hsp90 from grass carp (*Ctenopharyngodon idella*) increases thermal protection against heat stress. *Fish Shellfish Immunol* 33:42–47
- Xie Y, Song L, Weng Z, Liu S, Liu Z (2015) Hsp90, Hsp60 and sHsp families of heat shock protein genes in channel catfish and their expression after bacterial infections. *Fish & shellfish immunology* 44:642–651
- Zhang F, Jiang K, Zhang D, Ma C, Ma H, Ma L (2014) Molecular cloning, characterization and expression analysis of heat shock protein 90 (HSP90) from the mud crab *Scylla paramamosain*. *Afr J Biotechnol* 11:6296–6304
- Zhou C, Lin H, Huang Z, Wang J, Wang Y, Yu W (2015) Effects of dietary soybean isoflavones on non-specific immune responses and hepatic antioxidant abilities and mRNA expression of two heat shock proteins (HSPs) in juvenile golden pompano *Trachinotus ovatus* under pH stress. *Fish & shellfish immunology* 47:1043–1053