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Interaction of heat shock protein 60 (HSP60) with microRNA in Chinese mitten crab during *Spiroplasma eriocheiris* infection

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ABSTRACT: Heat shock protein 60 from the Chinese mitten crab *Eriocheir sinensis* (EsHSP60) was previously identified in relation to *Spiroplasma eriocheiris* infection by isobaric tags for relative and absolute quantitation labelling followed by liquid chromatography–tandem mass spectrometry. In the present study, to validate the immune function of this protein, the cDNA of the *EsHSP60* gene was cloned. Various crab tissues were assessed using real-time PCR, which showed that *EsHSP60* transcription occurred in all tissues examined. The expression profiles of *EsHSP60* in haemolymph at transcription and protein levels when infected with *S. eriocheiris* were investigated by real-time PCR and Western blot analysis, respectively. A significant increase of *EsHSP60* transcription and protein expression appeared post-injection in response to *S. eriocheiris* infection when compared to the control group. The double-luciferase reporter gene assay showed that the microRNA PC-533-3p interacted with the 3′-untranslated region of *EsHSP60* and inhibited the translation of EsHSP60. The expression profiles of PC-533-3p during *S. eriocheiris* infection were also investigated by real-time PCR. However, the change tendency of PC-533-3p was opposite to that of the *EsHSP60* after *S. eriocheiris* challenge. These data indicate that the EsHSP60 proteins may play an important role in mediating the immune responses of *E. sinensis* to an *S. eriocheiris* challenge.

KEY WORDS: *Eriocheir sinensis* · HSP60 · *Spiroplasma eriocheiris* stimulation

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INTRODUCTION

Heat shock proteins (HSPs) are highly conserved, occurring in all cells ranging from bacteria to humans, in a variety of intracellular locations and in various forms (Lindquist & Craig 1988). HSPs perform essential biological functions under both normal and stressed conditions (Sørensen et al. 2003), such as recognizing nascent polypeptide chains, preventing protein aggregation, and directly mediating protein folding (Hartl & Hayer-Hartl 2002). Based on their molecular weights, HSPs have been classified into 6 major families: HSP100, HSP90, HSP70, HSP60, HSP40 and small HSPs (Nover & Scharf 1997). In invertebrates, HSP60s appear to play important roles in responses to a variety of situations, such as salinity stress, thermal stress and bacterial challenge (Huang et al. 2011, Xu & Qin 2012). In vertebrates, the HSP60s play a crucial role as endogenous stress signal molecules in the immune defence against pathogens, and in autoimmune diseases (Vabulas et al. 2001, Tsan & Gao 2004). Other published sources have considered the capacity of HSP60s as self-antigens, carriers of other functional

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molecules and ligands for innate toll-like receptor signalling (e.g. Quintana & Cohen 2011). Similarly, research studies with the white shrimp *Litopenaeus vannamei* and the sea anemone *Anemonia viridis* indicated that HSP60 may play a critical role in mediating the immune responses to bacterial challenges (Zhou et al. 2010).

MicroRNAs (miRNAs) are small, non-coding RNAs that regulate gene expression at the post-transcriptional level (Bartel 2004) and play important roles in development, immunological defence and homeostasis functions (Wienholds & Plasterk 2005, Filipowicz et al. 2008, Ryan et al. 2010). Mature miRNAs, which are between 18 and 25 bp in length, contain characteristic stem–loop structures which are transcribed as primary-miRNA molecules (Inui et al. 2010). Recently, miRNAs have been proven to regulate gene function and expression by repressing specific target genes at post-transcriptional levels in various biological processes via complementary binding to the 3′-untranslated region (UTR) of target mRNAs, leading to mRNA cleavage or protein translation blockage (Li et al. 2013).

The Chinese mitten crab *Eriocheir sinensis* is an economically important freshwater species for aquaculture in China. Various diseases caused by bacteria, viruses and rickettsia-like organisms have severely impacted the sustainability of aquaculture populations of *E. sinensis.* (Wang et al. 2004a, Mu et al. 2009). Tremor disease is one of the most devastating diseases of this species (Wang et al. 2002), and a spiroplasma, named *Spiroplasma eriocheiris*, was previously identified as a novel causative pathogen of the disease (Wang et al. 2011). Our research group has been studying the role of *E. sinensis* HSP60 (EsHSP60) in *S. eriocheiris* infection by isobaric tags for relative and absolute quantitation labelling followed by liquid chromatography-tandem mass spectrometry. The results show that EsHSP60 belongs to a group of upregulated proteins in *E. sinensis* haemocytes appearing post-injection with *S. eriocheiris* (Meng et al. 2014).

To validate the immune function of this gene, we cloned *EsHSP60* from the Chinese mitten crab and analysed the *EsHSP60* gene expression pattern and tissue profiles using real-time PCR. The *EsHSP60* expression pattern at the protein level was also examined by Western blotting. In addition, the double-luciferase reporter gene assay was used to identify the miRNA (PC-533-3p) that interacts with the 3′-UTRs of the *EsHSP60* gene. Real-time PCR was used to verify the expression profiles of the miRNA. Our results provide valuable evidence that EsHSP60 plays an important function in the immune system.

MATERIALS AND METHODS

Haemolymph collection

Eriocheir sinensis (mean ± SD weight = 50 ± 3 g) were purchased from a market in Nanjing, China, and cultivated in aquaculture tanks containing aerated freshwater at 28°C. Healthy *E. sinensis* were verified by *Spiroplasma eriocheiris*-negative results using haemolymph transmission electron microscope (TEM) negative staining methods, and no evidence of *S. eriocheiris* was found using PCR 16S rRNA sequence analysis. Crab haemolymph was drawn using a 1 ml syringe, mixed and quickly added into an anticoagulant solution (glucose, 0.08 M; trisodium citrate dehydrate, 0.045 M; citric acid, 0.025 M). Samples were immediately centrifuged at 2500 × *g* (4°C, 10 min) to collect the haemocytes.

Rapid amplification of cDNA ends (RACE) of *EsHSP60*

Total RNA was extracted using TRIzol Reagent (TaKaRa) following the manufacturer's protocol. The absorbance at OD_{260} was used to determine the total RNA concentration, and RNA integrity was checked by electrophoresis. The *EsHSP60* partial cDNA sequence was acquired from unpublished *E. sinensis* transcriptome data of our research group. The full length of the *EsHSP60* was extended using 5′ and 3′ RACE (SMART^{TM} cDNA kit). A total of 8 gene-specific primers (Table 1) were designed based on the partial cDNA sequences for the gene. The 3′ RACE PCR reaction was carried out in a total volume of 50 µl. The reaction constituents were as follows: 2.5 µl (800 ng µl^{-1}) of the first-strand cDNA used as a template, 5 µl of 10× Advantage 2 PCR buffer, 1 µl of 10 mM dNTPs, 5 µl (10 mM) gene-specific primer (*EsHSP60*-3F1, *EsHSP60*-3F2), 1 ml of Universal Primer A Mix (UPM; Clonetech), 34.5 µl of sterile deionized water and 1 U 50× Advantage 2 polymerase mix (Clonetech). For the 5′ RACE, UPM was used as the forward primer in the PCR reactions in conjunction with the reverse gene-specific primers (*EsHSP60*-5R1, *EsHSP60*-5R2). PCR amplification conditions for both the 3′ and 5′ RACE were as follows: 5 cycles at 94°C for 30 s, 72°C for 3 min; 5 cycles at 94°C for 30 s, 70°C for 30 s and 72°C for 3 min; 20 cycles at 94°C for 30 s, 68°C for 30 s and 72°C for 3 min. After linking into the vector, the samples were sequenced at Invitrogen.

Table 1. Primers used for cloning and real-time quantitative analyses of *EsHSP60* and PC-533-3p

Name	Purpose	Sequence (5′–3′)
EsHSP60-3F1	3′ RACE PCR	TCA TCA CAG AGA TCC CGA AGG AG
EsHSP60-3F2	3′ RACE PCR	TTG TGG AGG CGG GAA TCA TTG AC
EsHSP60-5R1	5′ RACE PCR	TCC ACA GCC TTT GCC ACC GTC ACT C
EsHSP60-5R2	5′ RACE PCR	TAA GTG TCT TGC CAT CCT TGA CCG T
EsHSP60-ORF-F	Gene fragment cloning	ATG TAC CGT GCT GCC TCC
EsHSP60-ORF-R	Gene fragment cloning	TCA CAT CAT GCC ACC CAT C
EsHSP60-3UTR-F	PCR amplification	CTG CTC GAG CTC ACC CCA GCC AAT GAA GA
EsHSP60-3UTR-R	PCR amplification	ATG GCG GCC GCC ACC AGC ACA GCA GCA CTT T
EsHSP60-qPCR-F	Real-time PCR	CCA GCC AAT GAA GAC CAG AA
EsHSP60-qPCR-R	Real-time PCR	CTC CAC CTT GTT GAC GAT GA
EsGAPDH-qF:	Real-time PCR	GTC TCT TGC GAT TTC ACT GG
EsGAPDH-qR:	Real-time PCR	ATG ACA CGG TTG GAG TAG CC
EsPC-533-3p-RT:	Reverse transcription PCR amplification	CTC AAC TGG TGT CGT GGA GTC GG CAA TTC AGT TGA GTG TTC GGA
EsPC-533-3p-F:	Real-time PCR	ACA CTC CAG CTG GGG CAT ACT GTC ACG CTC
EsU6-RT	Reverse transcription PCR amplification	AAC GCT TCA CGA TTT TGC GT
EsU6-F	Real-time PCR	CTT GCT TCG GCA GAA CAT ATA CT
miRNA-R	Real-time PCR	AAC TGG TGT CGT GGA G

Nucleotide and amino acid sequence analyses

The full-length cDNA sequence of *EsHSP60* was analysed for similarity with BLAST gene library sequences at the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/). The deduced amino acid sequence was analysed with the Expert Protein Analysis System (http://us.expasy.org/tools/). The signal peptide was predicted by the SignalP 3.0 program (www.cbs.dtu.dk/services/SignalP/).

Protein expression, purification and antibody preparation

EsHSP-ORF-F and *EsHSP*-ORF-R (Table 1) were designed to amplify the open reading frame (ORF) of the *EsHSP60* gene (including a potential mitochondrial signal sequence). The product was ligated into the p*EASY*-E1 expression vector (TransGen Biotech). *Escherichia coli* BL21 (DE3) (TransGen Biotech) was transformed with the resulting recombinant plasmid pEASY-EsHSP60 using isopropyl β-D-1-thiogalactopyranoside (IPTG)-induced recombinant expression (final IPTG concentration of 0.5 mM). After being induced at 37°C for 4 h, the bacterial cells were harvested. The recombinant proteins were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). High-affinity nickel-nitrilotriacetic acid resin was used to purify the recombinant proteins according to the manufacturer's instructions (Jinsite). New Zealand white rabbits were immunized with 150 µg of purified protein that was homogenized in complete Freund's adjuvant. Rabbits were injected 3 times at 2 wk intervals. A booster injection in incomplete Freund's adjuvant was given for another week. Rabbit serum was collected 7 d after the last immunization.

Tissue expression of *EsHSP60*

Samples of total RNA from nerve, muscle, gill, intestine, heart, hepatopancreas and haemocytes were extracted as described above. Total RNA was reverse-transcribed into cDNA with a PrimeScript RT reagent kit (TaKaRa). For quantification of *EsHSP60* expression, a pair of gene-specific primers (*EsHSP60*-qPCR-F, *EsHSP60*-qPCR-R) was used, and the primers *EsGAPDH*-qF and *EsGAPDH*-qR were used to amplify the *EsGAPDH* fragment as a housekeeping gene. The PCR reaction was performed in a 20 µl volume using a SYBR Premix ExTaq™ Kit (TaKaRa), 2 µM of each specific primer and 1 µl of cDNA in a Mastercycler ep realplex. The procedure was as follows: initial denaturation at 95°C for 2 min; followed by 40 cycles of amplification (95°C for 10 s, 55°C for 30 s and 72°C for 30 s). The relative expression levels of *EsHSP60* in different tissues were calculated according to the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001).

EsHSP60 haemolymph mRNA expression profile against a pathogen challenge

S. eriocheiris was isolated from *E. sinensis* with tremor disease using the methods described by Wang

et al. (2004b) and cultured in R2 medium at 30°C (Moulder et al. 2002). *E. sinensis*, shown to be pathogen-negative by PCR and TEM negative staining methods, were cultivated for 1 wk before treatment. The *E. sinensis* employed in this experiment were randomly divided into 2 groups. Crabs in the experimental group (50 individuals) each received an injection of *S. eriocheiris* (10^6 spiroplasmas in 100 µl). Crabs used as a control group (50 individuals) each received an injection of 100 µl R2 medium. Three individuals from each group (treatment or control) were randomly collected at 0, 2, 4, 6, 8 and 10 d post-injection. The total RNA from haemolymph was extracted from different groups by the Trizol technique. After reverse-transcribing into cDNA, real-time PCR was carried out as described above to measure the transcription levels of the *EsHSP60* gene.

Western blot analysis

The expression of EsHSP60 relative to varying protein levels in the haemolymph, when analysed at different exposure times during the pathogenic challenge, was examined using Western blot analysis. Crab haemolymph samples were collected as described above. Total protein was extracted with lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris-HCl, pH 8.5) containing 1 mM phenylmethanesulphonyl fluoride and 2 mM EDTA on ice. After 5 min, 10 mM dithiothreitol was added. After sonication and centrifugation (13 000 × *g*, 15 min, 4°C), the resulting supernatants were collected for protein concentration measurement. Protein concentrations were determined by the bicinchoninine acid assay. Fifty µg of protein were loaded in separate lanes of a 12% SDS-PAGE gel and electrophoretically separated. The proteins in the gels were blotted onto polyvinylidene fluoride membranes. Blotted membranes were incubated in phosphate-buffered saline containing 0.05% Tween-20 (PBST) with 5% BSA at room temperature for 2 h, and then in anti-EsHSP60 antibody diluted 1:2500 with PBST at 4°C for 24 h. After washing in PBST 3 times, the membranes were incubated overnight while treated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Trans) diluted 1:5000 at 4°C; after which the bands were visualized using an enhanced chemiluminescence kit (ECL, Vazyme), and the luminance of bands was represented by the gray ratio. The expression of β-actin was used as a control.

Identification of miRNAs targeting *EsHSP60* and luciferase activity assay

To identify the miRNAs targeting *EsHSP60*, the 3′-UTR of *EsHSP60* and miRNA data were analysed by 3 software programs: TargetScan (www.targetscan.org/mamm_31/), Miranda (www.microrna.org/microrna/getMirnaForm.do) and PITA (www.pita.ps/). Two miRNAs, i.e. PC-533-3p and esi-mir-217, interacted with the 3′-UTR of *EsHSP60* and were selected to be examined by a luciferase activity assay to confirm the interaction. To construct the luciferase reporter vector, the 3′-UTR of *EsHSP60* was PCR-amplified using the primers *EsHSP60*-3UTR-F and *EsHSP60*-3UTR-R. The sense and antisense primers were supplemented with *Xho*I and *Not*I sites, respectively. After being digested and purified, the amplified fragment was cloned to psiCHECK2.0 plasmid (Promega). All constructs were confirmed by sequencing.

The cell line HEK 293 T was obtained from the Chinese Academy of Science cell bank (Shanghai), and maintained in Dulbecco's modified Eagle's medium with 10% inactivated foetal bovine serum (Biowest), supplemented with glutamine and penicillin/streptomycin (100 U ml^{-1}). Cells were seeded in a 24-well plate in antibiotic-free media 1 d prior to transfection. When the cells reached about 80% confluence (2×10^5 cells $well^{-1}$), transfections were done using Lipofectamine 2000 reagent according to the manufacturer's protocol (Invitrogen). The transfection mixtures contained 0.2 µg of psiCHECK2.0 recombinant plasmid and 0.03 µg of synthetic miRNA mimics (Bio Shanghai) in a final volume of 0.5 ml. Each sample was transfected in triplicate. The negative predictive miRNA PC-19-5p was selected as a control.

Cell lysates were harvested 24 h after transfection, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Beyotime). The knockdown levels of luciferase were normalized and shown by the ratio of relative luciferase activity.

Quantitative real-time PCR assay of PC-533-3p

MiRNA expression levels were assayed by real-time PCR using a SYBR premix kit (TaKaRa) according to the manufacturer's protocol. Total RNAs were extracted from haemolymph samples that were randomly collected at 0, 2, 4, 6, 8 and 10 d during *S. eriocheiris* treatment and were reverse transcribed using Moloney-murine leukemia virus reverse transcriptase (TaKaRa) and a stem-looped antisense primer

(*EsPC-533-3p*-RT). The resultant cDNA was submitted for amplification of mature miRNAs using a miRNA-specific primer (*EsPC-533-3p*-F) and a universal primer (miRNA-R). The U6 snRNA gene was employed as an endogenous control. The relative expression levels of miRNAs were calculated according to the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001). Statistical analysis of data was performed by 1-way ANOVA using the software SPSS.

RESULTS

cDNA cloning, sequence characterization of *EsHSP60* and homology analysis

The full-length *EsHSP60* cDNA comprised 2248 bp, containing 117 bp in the 5′-UTR, 1734 bp in the ORF and 397 bp in the 3′-UTR with a poly (A) tail. The ORF encoded a polypeptide of 577 amino acids, including a 25 amino acid extension at the N-terminus, which may serve as a mitochondrial targeting signal. The conserved domain of *EsHSP60* was identified (see Fig. S1 in the Supplement at www.int-res.com/articles/suppl/d125p207_supp.pdf). A conserved domain search of the NCBI database, and application of the program patmatmotifs in EMBOSS, identified a conserved ATP-binding site, hinge regions and stacking interaction sites.

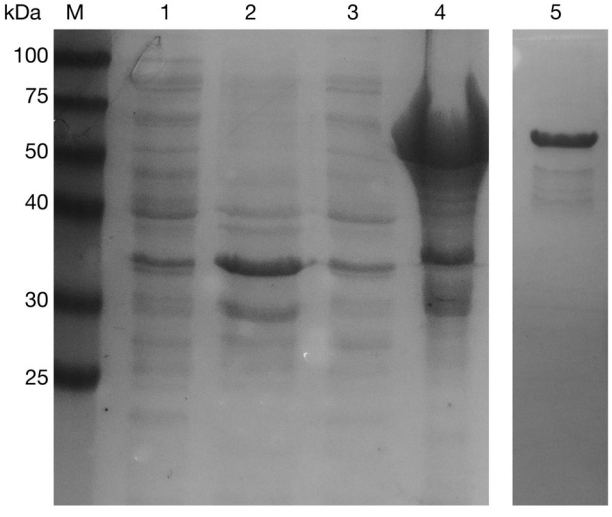

Fig. 1. Analysis of recombinant EsHSP60 by SDS-PAGE. Lane M: molecular weight standards; Lane 1: soluble protein of *E. coli* BL21 (DE3); Lane 2: insoluble protein of *E. coli* BL21 (DE3); Lane 3: soluble protein of *E. coli* BL21 (DE3) with pEASY-EsHSP60 after protein expression; Lane 4: insoluble protein of *E. coli* BL21 (DE3) with pEASY-EsHSP60 after protein expression; Lane 5: EsHSP60 purified by affinity chromatography

The deduced amino acid sequence of EsHSP60 was homologous to that of *Scylla paramamosain* HSP60 (identity = 95%), *Portunus trituberculatus* HSP60 (93%), *Macrophthalmus japonicus* HSP60 (88%) and *Litopenaeus vannamei* HSP60 (88%) (Fig. S2).

Protein expression, purification and antibody preparation

After being expressed, recombinant EsHSP60 formed the inclusion body and had an apparent molecular weight of around 63 kDa. After denaturation and renaturation, the recombinant EsHSP60 was purified by affinity chromatography (Fig. 1). The purified recombinant EsHSP60 with the His-tag yielded a single band of 63 kDa on SDS-PAGE gels after Coomassie blue staining. Rabbit antiserum was also reactive with a constituent that had an apparent molecular weight 63 kDa, corresponding to the molecular weight predicted for *EsHSP60* cDNA of the crab (see Fig. 4A).

Tissue distribution of *EsHSP60* mRNA

Real-time PCR analyses were employed to quantify the *EsHSP60* mRNA expression in nerve, intestine, muscle, gill, heart, haemolymph and hepatopancreas. *EsHSP60* expression was detected in all tissues of *Eriocheir sinensis* (Fig. 2), with the highest *EsHSP60* expression level found in gill tissues.

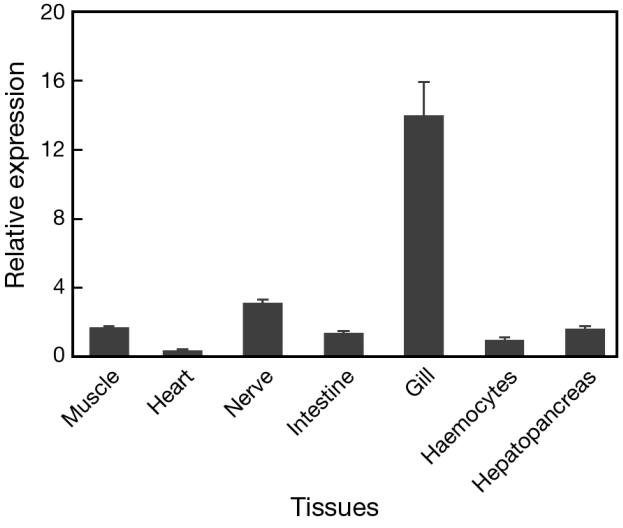

Fig. 2. Expression levels of the *EsHSP60* mRNA in different tissues, collected from 5 individual Chinese mitten crabs *Eriocheir sinensis*. Each bar represents the mean + SE based on 3 replicates

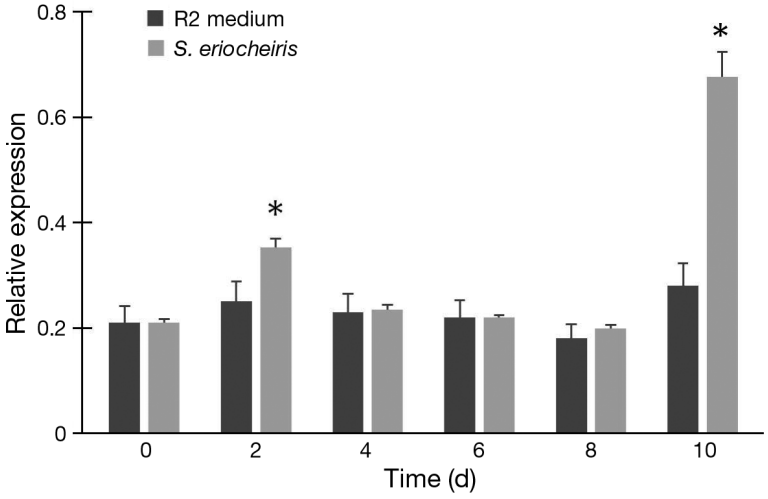

Fig. 3. mRNA expression levels of *EsHSP60* in haemocytes of Chinese mitten crabs *Eriocheir sinensis* using real-time PCR analysis of the responses to *Spiroplasma eriocheiris* challenge. Significant differences from samples at 0 h post injection are indicated with an asterisk at $p < 0.05$. Each bar represents the mean + SE based on 3 replicates

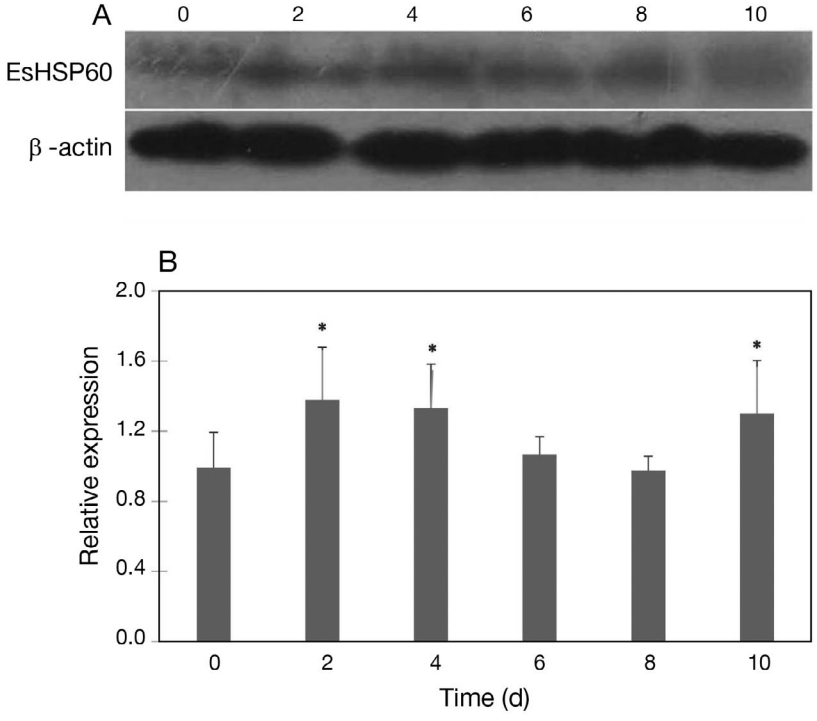

Fig. 4. (A) Protein expression levels of EsHSP60 in haemocytes of Chinese mitten crabs *Eriocheir sinensis* using Western blotting analysis of responses to *Spiroplasma eriocheiris* challenge and (B) the relative gray ratio analysis of EsHSP60 by Photoshop CS5. EsHSP60 levels were analysed and standardized according to the β-actin levels. Each bar represents the mean + SE based on 3 replicates. Significant differences relative to the control are indicated with an asterisk ($p < 0.05$)

EsHSP60 level changes in haemocytes under challenge

To study the function of EsHSP60 in the immune response of *E. sinensis*, the transcription of *EsHSP60* in haemolymph after pathogen challenge was measured through real-time PCR. During *S. eriocheiris* stimulation, the *EsHSP60* mRNA levels increased markedly at 2 d ($p < 0.05$), then recovered to control levels from 4 to 8 d. However, at 10 d after stimulation, the *EsHSP60* transcription level was significantly upregulated ($p < 0.05$) compared with the control group (Fig. 3). Similar to the real-time PCR results from the haemolymph, Western blot analysis showed that the EsHSP60 protein was upregulated ($p < 0.05$) at the early stages (2 and 4 d), then recovered to control levels from 6 to 8 d, and increased again at 10 d compared with the controls (Fig. 4).

Identification of the miRNAs targeting the *EsHSP60* gene

Based on analysis of the prediction software, 2 miRNAs (PC-533-3p and esi-mir-217) may interact with the 3′-UTR of *EsHSP60* (Fig. 5B). To determine whether there are direct interactions between PC-533-3p, esi-mir-217 and their target sites of the *EsHSP60* gene, we used a luciferase 3′-UTR reporter assay to measure the inhibitory effects of these miRNAs. Using co-transfected HEK 293T cells, PC-533-3p mimics significantly reduced the luciferase activity of the reporter plasmid containing the *EsHSP60* 3′-UTR, whereas esi-mir-217 mimics had no effect on the luciferase activity (Fig. 5C). These results indicated that PC-533-3p can downregulate target gene expression by binding the 3′-UTR of *EsHSP60*.

Quantification of miRNA expression

To identify differentially expressed miRNA profiles during *S. eriocheiris* treatment, real-time PCR was used. After normalization against U6 snRNA, the relative ex-

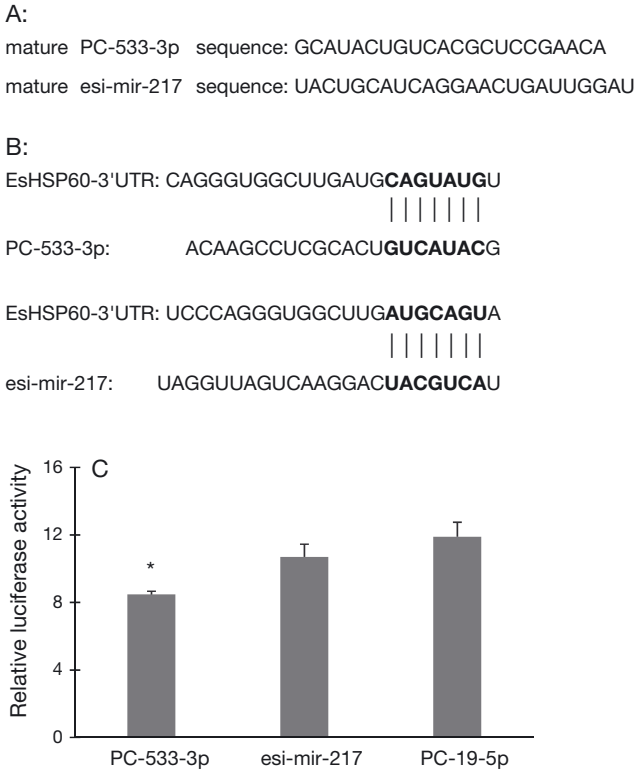

Fig. 5. (A) Sequence and (B) potential miRNA target sites of PC-533-3p and esi-mir-217 in the 3′-untranslated region (UTR) of *EsHSP60* in Chinese mitten crabs *Eriocheir sinensis* when analysed by TargetScan. (C) Luciferase 3′-UTR reporter assays of the miRNAs silencing effects using psiCHECK2.0/*EsHSP60* 3′-UTR reporter vectors. Firefly luciferase activity was evaluated 24 h after cotransfection. Each bar represents the mean + SE based on 3 replicates. Asterisks indicate a significant difference ($p < 0.05$) compared with the negative control group of *PC-19-5p*

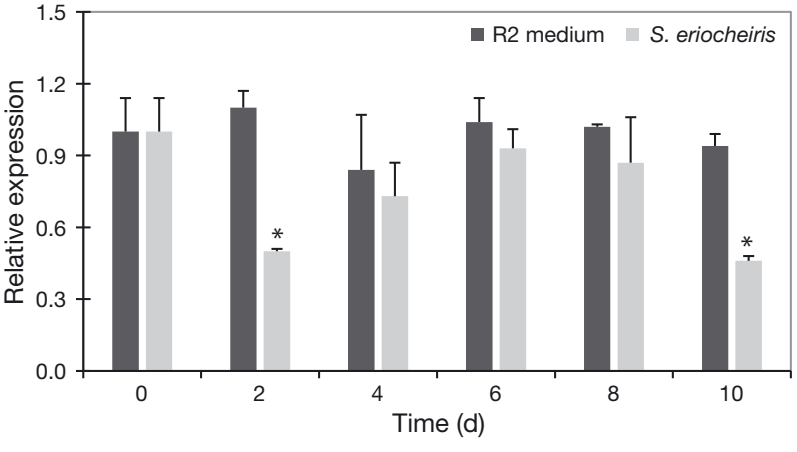

Fig. 6. Expression levels of microRNA EsPC-533-3p in haemocytes of Chinese mitten crabs *Eriocheir sinensis* using real-time PCR analysis of responses to a *Spiroplasma eriocheiris* challenge. Expression of EsPC-533-3p was analysed for significance using a post hoc Duncan multiple range test. Significant differences from samples at 0 h post injection are indicated with an asterisk ($p < 0.05$). Each bar represents the mean + SE based on 3 replicates

pression level of EsPC-533-3p significantly decreased ($p < 0.05$) at 2 and 10 d during *S. eriocheiris* treatment (Fig. 6). These results are consistent with the expression profiles of *EsHSP60* in haemolymph at transcription, and protein levels during *S. eriocheiris* treatment. This experiment further confirms that EsPC-533-3p plays an important role in regulating EsHSP60 expression.

DISCUSSION

In eukaryotes, one of the widely investigated and most abundant higher-order taxonomic groups, the HSP60s form a group of proteins with characteristic motifs and conserved sequences (Feder & Hofmann 1999, Kregel 2002). In this study, conserved sequences and characteristic motifs were found in the deduced EsHSP60 amino acid sequence, which included HSP60 family signatures, ATP-binding site (Marchler-Bauer et al. 2007), conserved Gly–Gly–Met repeats at the C-terminal end (Sanchez et al. 1999), as well as the major structural and functional domains typically found in HSP60s (Choresh et al. 2004).

Under normal, non-infected conditions, the *EsHSP60* gene was ubiquitously expressed in all examined tissues of *Eriocheir sinensis*, indicating that *EsHSP60* may be required to maintain cell homeostasis. The observation that *EsHSP60* mRNA is expressed most notably in the gills is in agreement with *Scylla paramamosain HSP60* and *Portunus trituberculatus HSP60* (Xu & Qin 2012, Yang et al. 2013). In crustaceans, gills are most directly in contact with the external environment compared to other organs. In addition, gills are known to play key roles in osmoregulation, detoxification and defence mechanisms (Clavero-Salas et al. 2007).

In invertebrates, haemolymph plays an important role in fighting infection (Plows et al. 2006, Chang et al. 2007, Hong et al. 2007). Moreover, evidence of *S. eriocheiris* was mainly seen in the haemolymph during infection (Wang et al. 2004a). Therefore, haemolymph was used as our target tissue to examine the expression profiles of *EsHSP60* after an *S. eriocheiris* challenge. After the crabs were challenged with *S. eriocheiris*, the transcription of *EsHSP60* in haemolymph was significantly upregu-

lated at 2 d and reached a peak at 10 d. The detection results based on the mRNA levels of *EsHSP60* had the same tendency as the results obtained based on the protein level. The upregulation of EsHSP60 was observed early during the onset of infection, which suggested that EsHSP60 was induced rapidly to protect the denatured proteins in the haemolymph when infected with *S. eriocheiris.* Similarly, other studies have demonstrated that a similar pattern of rapid expression levels of the *HSP60* gene was observed in *Litopenaeus vannamei* after a *Vibrio alginolyticus* challenge (Zhou et al. 2010). In addition, the proliferation of *S. eriocheiris* in *E. sinensis* haemolymph was similar to that of *Procambarus clarkii* (Ding et al. 2014); and furthermore, copies increased and reached the maximum at 10 d. The crabs developed typical signs of tremor disease and died after 10 d post challenge (Wang et al. 2004b), which suggested that EsHSP60 was induced to repair damaged tissue from infection and take part in a defensive response in reaction to newly expressed proteins. In our study, EsHSP60 may possess different response patterns at different times after *S. eriocheiris* challenge in crabs.

The miRNA can regulate gene expression patterns *in vivo*, thereby changing the amount of mRNA targets (Krol et al. 2010, Leung & Sharp 2010). In a previous study, many miRNAs such as PC-533-3p may have significantly contributed to *S. eriocheiris* disease progression (Ou et al. 2012). Our study showed that there are direct interactions between PC-533-3p and its target sites in the *EsHSP60* gene. PC-533-3p was found differentially expressed in *S. eriocheiris*-infected *E. sinensis*, indicating that it might play an important role in the innate immunity of the crabs. Increasing lines of evidence have shown that miRNAs can serve important roles in the innate immunity of shrimp (Lu & Liston 2009, Yang et al. 2012). Both miRNA-146 and miRNA-125 play important roles in the immune response of *Vibrio parahaemolyticus*-infected *Scylla serrata* (Kim et al. 2012). Measured at the same time point, the trend in the relative expression of PC-533-3p is opposite to that of *EsHSP60* after an *S. eriocheiris* challenge. These results also indicated that PC-533-3p negative regulation of the expression of EsHSP60 is responsible for *S. eriocheiris* stimulation. HSP60 is a critical regulator in response to stress both *in vitro* and *in vivo* (Yan et al. 2012). Thus, it is not surprising that HSP60 is tightly regulated by multiple mechanisms, such as miRNAs. To sum up, PC-533-3p regulates the expression of HSP60s and plays an important role in the immune responses in *E. sinensis*.

In conclusion, to validate the immune function of this gene, we described differential expression patterns of EsHSP60 in response to *S. eriocheiris* stimulation. The transcription level and protein level of EsHSP60 was upregulated when crabs received an injection of *S. eriocheiris*. Double-luciferase reporter gene assays showed that PC-533-3p interacted with the 3′-UTRs of the *EsHSP60* gene. The expression profiles of PC-533-3p during *S. eriocheiris* treatment were investigated by real-time PCR. Our results support that EsHSP60 may participate in the crab immune response. Moreover, this finding provides insight into the post-transcriptional regulation mechanism of innate immunity in crabs.

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