Fish and Shellfish Immunology 94 (2019) 643–653

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Litopenaeus vannamei CK2 is involved in shrimp innate immunity by modulating hemocytes apoptosis

Qian Feng[a,b,1], Yueqian Huang[a,1], Defu Yao[a,b], Chunhua Zhu[c], Shengkang Li[a,b], Hongyu Ma[a,b], Jude Juventus Aweya[a,b,**], Yueling Zhang[a,b,*]

[a] Department of Biology and Guangdong Provincial Key Laboratory of Marine Biotechnology, Shantou University, Shantou, 515063, China
[b] STU-UMT Joint Shellfish Research Laboratory, Shantou University, Shantou, 515063, China
[c] College of Fisheries, Guangdong Ocean University, Zhanjiang, 524025, China

ARTICLE INFO

Keywords:
L. vannamei
*Lv*CK2
Shrimp immunity
Hemocytes apoptosis

ABSTRACT

Protein kinase CK2 (CK2) is a ubiquitous serine/threonine kinase with multiple cellular functions in vertebrates including apoptosis, differentiation, proliferation, survival, tumorigenesis, signal transduction, immune regulation and inflammation. In the current study, the catalytic and regulatory subunit homologs of *Litopenaeus vannamei* protein kinase CK2 (*Lv*CK2α and *Lv*CK2β) were cloned and characterized. *Lv*CK2α has a full-length cDNA sequence of 1764 bp with a 1053 bp open reading frame (ORF) encoding a putative protein of 351 amino acids, which contains a typical serine/threonine kinase domain. On the other hand, *Lv*CK2β has a 1394 bp full-length cDNA with an ORF of 663 bp encoding a protein with 221 amino acids, which contains a Casein kinase II regulatory subunit domain. Sequence and phylogenetic analysis revealed that *Lv*CK2 was evolutionary related with the CK2 of invertebrates. Quantitative reverse transcription PCR (RT-qPCR) analysis showed that *Lv*CK2α and *Lv*CK2β transcripts were widely expressed in all shrimp tissues tested, and were both induced in hemocytes and hepatopancreas upon challenge with *Vibrio parahaemolyticus*, *Streptoccocus iniae*, lipopolysaccharide (LPS), and white spot syndrome virus (WSSV), suggesting their involvement in shrimp immune response. Moreover, RNA interference (RNAi) of *Lv*CK2α resulted in increased hemocytes apoptosis, shown by high caspase 3/7 activity, increased number of apoptotic cells, coupled with an elevation in transcript levels of pro-apoptotic *Lv*Caspase3 and *Lv*Cytochrome C, and a reduction in mRNA levels of pro-survival *Lv*Bcl2, *Lv*IAP1, and *Lv*IAP2. In addition, *Lv*CK2α knockdown followed by *V. parahaemolyticus* challenge resulted in higher cumulative mortality of shrimp. Taken together, our current findings suggest that *Lv*CK2 modulates shrimp hemocytes apoptosis as part of the innate immune response to pathogens.

1. Introduction

Invertebrates are the most abundant animals on earth, although they lack specific or adaptive immune system and therefore only depend on their innate immunity [1]. Currently, the invertebrate innate immune system is best characterized in arthropods such as insects and crustaceans. The Pacific white shrimp, *Litopenaeus vannamei*, is one of the most important aquaculture shrimp species in the world, accounting for nearly 80% or two-thirds of the total annual produce [2,3]. Typical of other invertebrates, shrimp also lack a true adaptive immune system [4]. In the past two decades, several infections including those caused by *Vibiro*, *Pseudomonas* and *Aeromonas,* white spot syndrome virus (WSSV), etc. [5,6] have constrained *L. vannamei* culture, thereby reducing yield and impacting on trade [7]. To better understand the immune system of *L. vannamei* so as to curtail the impact of these pathogens on shrimp farming as well as prevent economic losses to farmers, research on shrimp immunity has intensified in recent years [8].

Protein kinase CK2 (hereafter CK2), a ubiquitous serine/threonine protein kinase that exists mainly as a holoenzyme composed of two catalytic (α and/or α′) and a dimer of regulatory (β) subunits, is believed to be among the most highly conserved proteins in nature [9]. The regulatory subunits of CK2 stimulate the catalytic subunits, thereby stabilizing the CK2 heterotetramer, a scaffold for specific kinase

* Corresponding author. Department of Biology School of Science Shantou University Shantou, Guangdong, 515063, China.
** Corresponding author. Department of Biology, School of Science, Shantou University, Shantou, Guangdong, 515063, China.
E-mail addresses: jjaweya@stu.edu.cn (J.J. Aweya), zhangyl@stu.edu.cn (Y. Zhang).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.fsi.2019.09.060
Received 7 June 2019; Received in revised form 23 September 2019; Accepted 25 September 2019
Available online 26 September 2019

partners [10]. In most mammalian studies, the expression and kinase activity of CK2 is reported to be higher in malignant tumor cells including stomach, kidney, prostate, heart, liver, lung, B-cells and hepatocytes [11–13] compared to normal cells. Apart from its ubiquitous distribution, CK2 is also a multifunctional protein kinase involved in the phosphorylation of more than 300 substrates and therefore plays a pivotal role in many biological and pathological processes including a dual function in DNA replication [14], gene transcription [15], cell proliferation [16], differentiation [17], apoptosis [18], signal transduction [19], immune regulation [20], inflammation [21], tumorigenesis [22], etc.

In spite of the fact that several studies have reported on the role of CK2 in physiological and pathophysiological functions in mammals, none is reported on CK2 homologs in arthropods. Moreover, limited studies have explored the role of CK2 in invertebrate immunity. In the current study, the role of CK2 in shrimp innate immunity was explored by first cloning and characterizing the subunit homologs of *L. vannamei* CK2 serine/threonine protein kinase (designated *Lv*CK2α and *Lv*CK2β). Tissue distribution analyses revealed that *Lv*CK2α and *Lv*CK2β were widely expressed in most shrimp tissues and were induced upon immune challenge. Knockdown of *Lv*CK2α increased hemocytes apoptosis in terms of high caspase3/7 activity and increase in the expression of *Lv*Caspase3 and *Lv*Cytochrome C, while a decrease in the expressions of *Lv*Bcl2, *Lv*IAP1, and *Lv*IAP2 were observed. Depletion of *Lv*CK2 followed by *V. parahaemolyticus* infection increased the cumulative mortality of shrimp. Thus, *Lv*CK2 plays an important role in shrimp immune response by modulating hemocytes apoptosis.

Table 1
Nucleotide sequences of primers and siRNA used in this article.

Primer	Sequence (5′-3′)	Amplicon size (bp)
PCR		
LvCK2α-F	ATGCCATTGGCAAGCCGTGC	1053
LvCK2α-R	CTATTCAGGAACTCCAGCTAGAGGGG	1053
LvCK2β-F	GCCTTCCTCCCTCGGCCTAGC	663
LvCK2β-R	CACAGAAGGGGACAGGAAGGAGAA	663
Real-Time qPCR		
LvCK2α-F	CGGAAGCGGTGGGAAAGGTT	188
LvCK2α-R	GGCGTGGGCGAGTTCATTT	188
LvCK2β-F	GTGGATTGCTTGGTTCTGTGGC	239
LvCK2β-R	AAATGTAGCGAGCGTGGATGAGG	239
LvBcl2-qF	GGTGGAATCACAAGAGAGCGA	83
LvBcl2-qR	TCTCCACGGTGTCTCACTTGG	83
LvCytochrome C-qF	AGGGAAAGAAGCTGTTCGTGC	148
LvCytochrome C-qR	GACTTGTTGGCGTCGGTGTA	148
LvCaspase3-qF	AGCGAGACTACCGCCTCCTAC	92
LvCaspase3-qR	CCAAATCTGAGCCACTCCTGTT	92
LvIAP1-qF	CCCGCACTGTCCATTTATCAG	144
LvIAP1-qR	CCACATTCATCTGTGCCACGT	144
LvIAP2-qF	CCAGTTGGGAACATACCTTTGG	189
LvIAP2-qR	GTCAATCGTGCTATCTGGCTCA	189
LvEF-1α-F	TATGCTCCTTTTGGACGTTTTGC	118
LvEF-1α-R	CCTTTTCTGCGGCCTTGGTAG	118
siRNA		
siLvCK2α-F	CCAAGACGAUUAUCAGCUUTT	21
siLvCK2α-R	AAGCUGAUAAUCGUCUUGGTT	21
Si-Non-F	UUCUCCGAACGUGUCACGUTT	21
Si-Non-R	ACGUGACACGUUCGGAGAATT	21

2. Materials and methods

2.1. Experimental animals and tissues collection

Healthy *L. vannamei* (approximate weight of 8–10 g each) were bought from a local shrimp farm, Shantou Huaxun Aquatic Product Corporation (Shantou, China), and cultured in tanks filled with seawater aerated at room temperature. Shrimp were acclimatized for 3–5 days prior to the experiments. Hemocytes were collected via the pericardial sinus with a sterile needle and syringe into an equal volume of precooled acid citrate dextrose (ACD) anti-coagulant buffer (19.65 g/L NaCl, 22.80 g/L glucose, 7.95 g/L sodium citrate and 3.35 g/L EDTA-Na2, pH 6.0). Hemocytes were harvested by centrifugation at 800 × *g* for 10 min at 4 °C. The other shrimp tissues such as hepatopancreas, intestine, heart, gill, muscle and eyestalk were collected and processed as previously described [23]. All animal experiments were carried out in accordance with the guidelines and approval of the Animal Research and Ethics Committees of Shantou University, China.

2.2. Total RNA extraction and cDNA synthesis

Total RNA was extracted from various shrimp tissues (gills, heart, hemocytes, hepatopancreas, intestine, muscle and stomach) using the RNAFAST 200 Kit (FeiJie, China) according to the manufacturer's instruction. The RNA was quantified using NanoDrop 2000 spectrophotometer (Nano-drop Technologies, Wilmington, DE) while RNA quality was ascertained using the A260/280 ratio (≥2) as well as checked on 1% agarose gel electrophoresis. The total RNA was either stored in aliquots at −80 °C or used immediately for cDNA synthesis. The cDNA samples were prepared with 1.0 μg of the total RNA using the PrimeScript™ RT reagent kit (TaKaRa, Japan) following the manufacturer's protocol. All cDNA samples were used immediately for downstream experiments or stored in aliquots at −20 °C.

2.3. Cloning and sequence analysis of full-length LvCK2 cDNA

The full-length cDNA sequence of the unigenes encoding putative *Lv*CK2α and *Lv*CK2β proteins were retrieved from our in-house *L. vannamei* transcriptome data [24]. Next, gene specific primers (Table 1) were designed with Primer Premier 5 software for PCR analysis of the open reading frames (ORF) of *Lv*CK2α and *Lv*CK2β so as to further confirm the sequence. The PCR reaction, which contained 10 μL 2x PCR Mix (GenStar, Beijing, China), 1 μL cDNA (10 ng/uL) template (from hemocytes), 1 μL each of forward and reverse primers (10 μmol), plus ddH_2O to a total volume of 20 μL, was carried out in a thermal cycler (Biometra T Gradient, Germany). The PCR conditions were: 94 °C for 3 min initially, then 35 cycles of 94 °C for 30 s, 59 °C for 30 s and 72 °C for 1 min, and finally 72 °C for 10 min. The PCR products were cloned into the pMD-19T vector (Takara, Japan) and sequenced using the Sanger sequencing technology at Beijing Genomics Institute (GBI, Shenzhen, China).

2.4. Bioinformatics analysis

The BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) was used to search and analyze the nucleotide sequences of *Lv*CK2 against other species. The simple modular architecture research tool (SMART) online program (http://smart.emblheidelberg.de) was used to predict the protein functional domains of *Lv*CK2 and then compare to CK2 of other species. Analysis of *Lv*CK2 sequence and multiple sequence alignment was performed using the DNAMAN program (version 6.0.3.99). The MEGA 7.0 software [25] was used to construct the phylogenic tree and the evolutionary history of *Lv*CK2 inferred using the Neighbor-Joining method based on the full-length amino acid sequences of *Lv*CK2α (351 amino acids) and *Lv*CK2β (221 amino acids) [26]. Briefly, the phylogenetic tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used for the tree. The evolutionary distances were computed using the Poisson correction method, and are in units of the number of amino acid substitutions per site. The analysis involved 20 amino acid sequences. Bootstrap values were determined based on 1000 replications. The position of *Lv*CK2 on the tree is marked with a triangle.

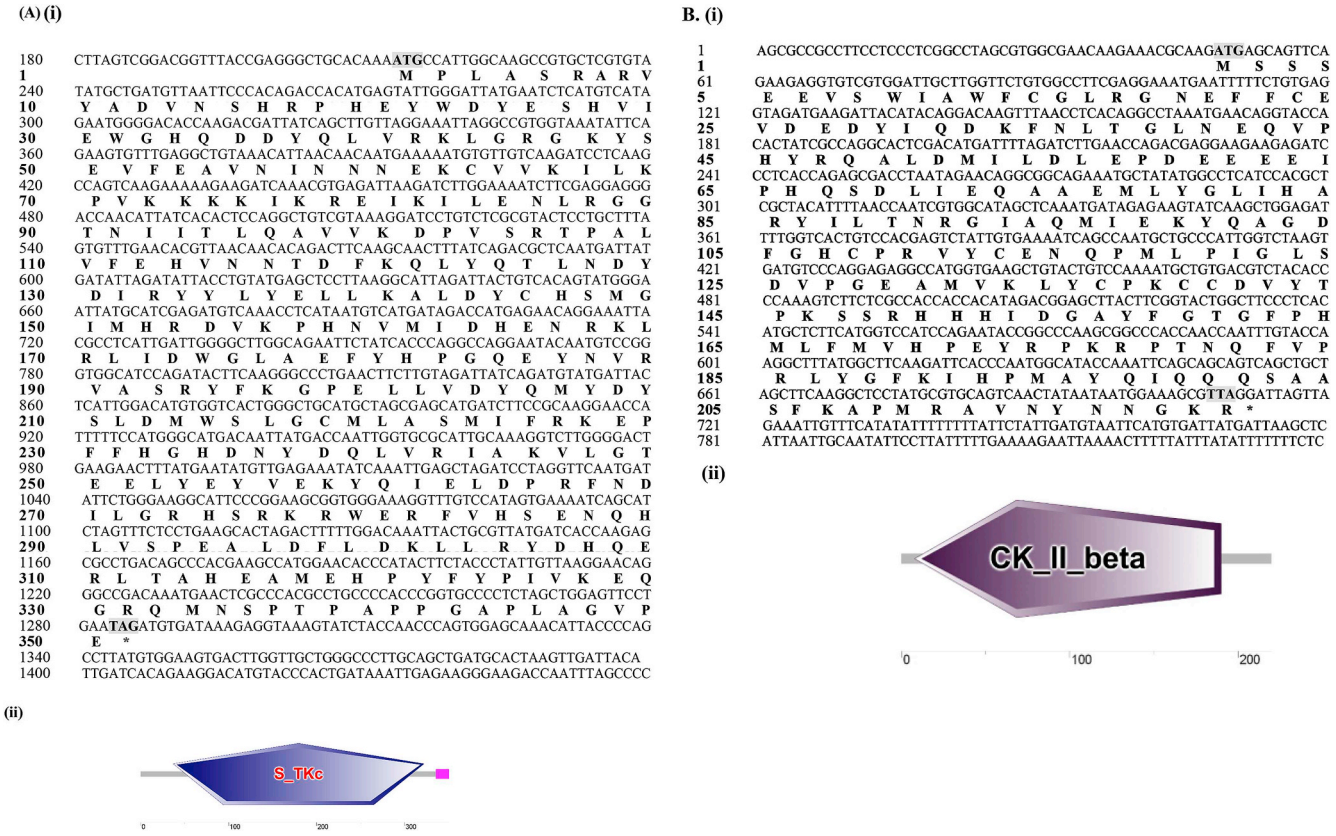


Fig. 1. The nucleotide sequence of *L. vannamei* CK2α (Genbank accession number KY038483.1) and *L. vannamei* CK2β (Genbank accession number KY038484.1) with deduced amino acid sequence and structural domain. (A) (i) The ORF of the amino acid sequences of *Lv*CK2α are shown with one-letter codes. Nucleotides and amino acids are numbered on the left of the sequences. The initiation codon (ATG) and the stop codon (TGA) are shaded. (ii) The S/TKc structural domain of *Lv*CK2α. (B) (i) The ORF of the amino acid sequences of *Lv*CK2β are shown with one-letter codes. Nucleotides and amino acids are numbered on the left of the sequences. The initiation codon (ATG) and the stop codon (TGA) are shaded. (ii) The CK_II_beta (Casein kinase 2 regulatory subunit) domain of *Lv*CK2β.

2.5. Tissue distribution of *LvCK2* transcript

To analyze the expression of *Lv*CK2 in different tissues, total RNA from fifty (50) healthy shrimp was extracted, as outlined in section 2.2 above, from the hemocytes, hepatopancreas, muscle, gill, nerve, intestine, heart, stomach and eyestalk for cDNA synthesis. Gene specific primers (Table 1) designed based on the full-length sequence of *Lv*CK2 and the Elongation factor 1 alpha gene of *L. vannamei* (*Lv*EF1α), were then used for RT-qPCR analysis with the Master SYBR Green I mix (GenStar, Beijing, China) on qTOWER 2.0 (Analytikjena, Germany). The qPCR reaction mixture contained 10 μL of 2 × RealStar Green power mixture, 1 μL each of forward and reverse primers, 1 μL of cDNA and 7 μL of Milli-Q water. The cycling conditions of the qPCR were as follows: one cycle at 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Samples were analyzed in triplicates, with *Lv*EF1α used as the internal control. Gene expressions were computed using the relative quantification method and normalized to *Lv*EF1α. All samples were analyzed in triplicate, with the *Lv*EF1α gene used as the internal control gene, and relative mRNA expression calculated using the $2^{-\Delta\Delta CT}$ method [27].

2.6. Immune challenge

Three hundred (300) healthy shrimp were divided into five groups, with 60 shrimp per group. For the challenge groups, 100 μL of *V. parahaemolyticus* (1×10^7 CFU/ml), *Streptococcus iniae* (1×10^7 CFU/ml), or WSSV (1×10^4 virus copies/ml), Lipopolysaccharides (LPS) (0.5 mg/ml), was injected at the third abdominal segment of each shrimp. As control, 100 μl of 0.01 M PBS (1 L ddH_2O containing 2.90 g Na_2HPO_4..12H_2 O, 0.296 g NaH_2PO_4·2H_2O, 8.5 g NaCl, pH 7.4) was used. Hemocytes were collected from five shrimp per group at 0, 6, 12, 24, 48, and 72 h post injection (hpi). Total RNA was extracted and cDNA synthesized as described in subsection 2.2 above, while the *Lv*CK2 transcript level was determined by RT-qPCR as outlined above in subsection 2.5.

2.7. *LvCK2α* knockdown and expression of apoptosis-related genes

The *Lv*CK2α siRNA (si*Lv*CK2α) and scrambled control siRNA (siNon) were designed and chemically synthesized by GenePharma (Suzhou, China). For the knockdown experiments, shrimp were divided into two groups (20 shrimp per group). Experimental group shrimp were each intramuscularly injected with 2.5 μg si*Lv*CK2α, while the control group shrimp were injected with equivalent amounts of siNon. Knockdown efficiency of *Lv*CK2α was ascertained by both Western blot analysis as previously described [28] and RT-qPCR as outlined in subsection 2.5. Briefly, hemocytes were collected from six shrimp per group and pooled together. After being washed three times with PBS (Section 2.2), hemocytes were lysed at 4 °C for 30 min in lysis buffer (25 mM HEPES, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA - Na_2·2H_2O, PH 7.4) containing a mixture of protease inhibitors (Roche, Switzerland) and phenylmethylsulfonyl fluoride (PMSF) (Beyotime, Beijing, China). Supernatants were collected by centrifugation at 20,000 *g*, 4 °C for 10 min. The protein concentration of the samples were determined using a modified Bradford assay (Bio-Rad, USA) with BSA as standard, and used immediately for the next experiment or stored at −20 °C for later use. Next, 20 μL loading buffer (42 mmol/LTris-HCl, containing 100 mL/L glycerol, 23 g/L SDS, 50 g/L 2-

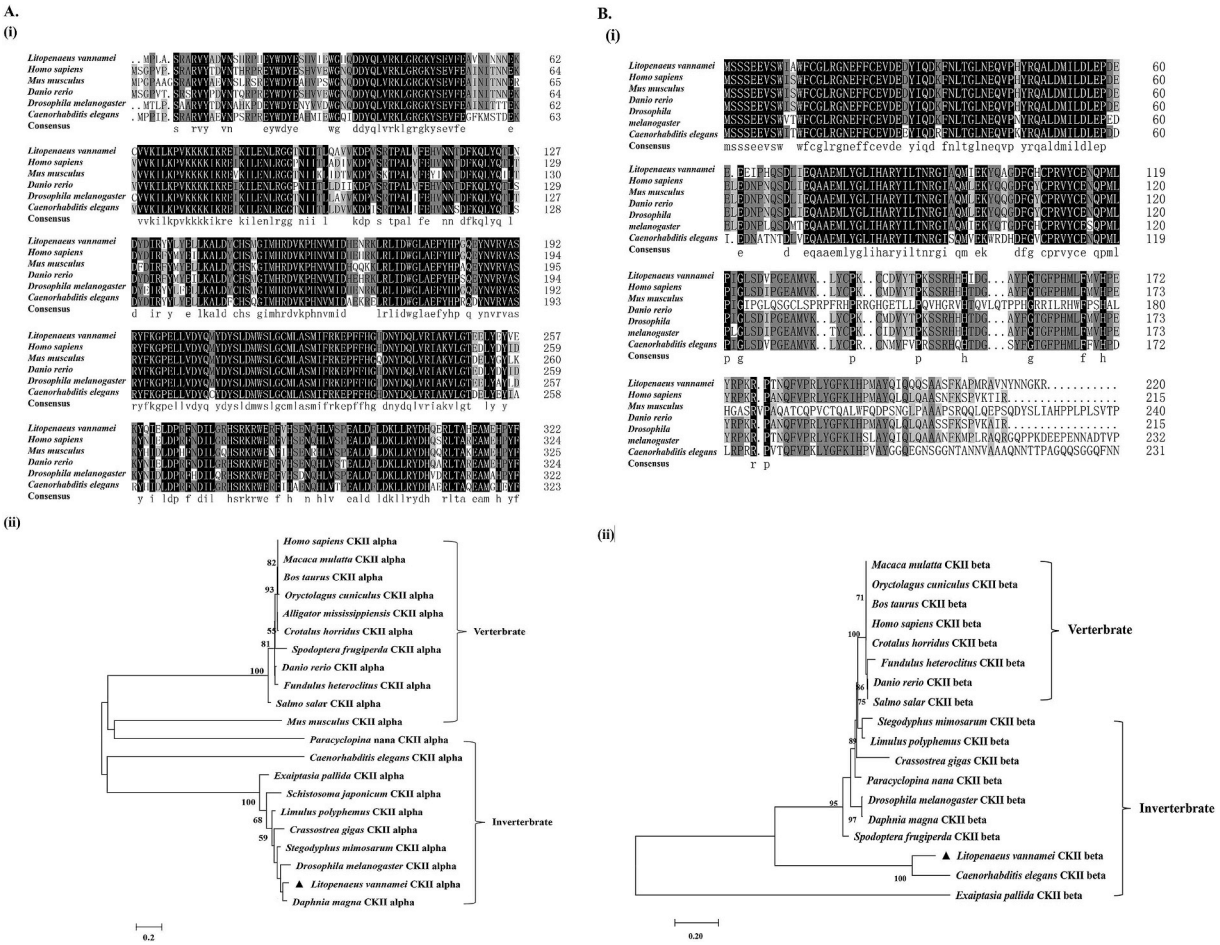


Fig. 2. Multiple sequence alignment and phylogenetic analysis of the CK2 proteins. (A) (i) Multiple sequence alignment between *Lv*CK2α and other CK2α proteins. CK2α proteins used for the analysis include: *Mus musculus* CK2α (NP_034104.1), *Drosophila melanogaster* CK2α (NP_001287153.1), *Caenorhabditis elegans* CK2α (CCD61302.1), *Homo sapiens* CK2α (NP_808227.1), *Danio rerio* CK2α (NP_001002164.1). Identical amino acid residues are shaded in black and similar residues in gray. (ii) The neighbor-joining phylogenetic tree based on the sequences of *Lv*CK2α and other known vertebrates and invertebrates CK2 proteins. The numbers marked on the tree branches represent the bootstrap values. The location of *Lv*CK2α is indicated by a black filled triangle. Species included in construction of phylogenetic tree were all retrieved from NCBI database and accession numbers of the CK2α are : *Homo sapiens* CK2α (NP_808227.1), *Macaca mulatta* CK2α (NP_001247806.1), *Bos taurus* CK2α (NP_777060.2), *Alligator mississippiensis* CK2α (KYO41035.1), *Crotalus horridus* CK2α (JAG45466.1), *Oryctolagus cuniculus* CK2α (NP_001153756.1), *Fundulus heteroclitus* CK2α (JAR36498.1), *Danio rerio* CK2α (NP_001002164.1), *Salmo salar* CK2α (NP_001134812.1), *Spodoptera frugiperda* CK2α (AAC24041.1), *Drosophila melanogaster* CK2α (NP_001287153.1), *Daphnia magna* CK2α (KZS14530.1), *Stegodyphus mimosarum* CK2α (KFM81306.1), *Limulus polyphemus* CK2α (ANO53976.1), *Crassostrea gigas* CK2α (EKC28117.1), *Caenorhabditis elegans* CK2α (CCD61302.1), *Schistosoma japonicum* CK2α (CAX75618.1), *Exaiptasia pallida* CK2α (KXJ22091.1), *Mus musculus* CK2α (NP_034104.1). (B) (i) Multiple sequence alignment between *Lv*CK2β and other CK2β proteins. CK2β proteins used for the analysis include: *Danio rerio* CK2β (NP_571262.1) , *Homo sapiens* CK2β (NP_001311.3) , *Drosophila melanogaster* CK2β (NP_996415.1) , *Caenorhabditis elegans* CK2β (AAA27983.1), *Mus musculus* CK2β (NP_001290405.1). Identical amino acid residues are shaded in black and similar residues in gray. (ii) A neighbor-joining phylogenetic tree based on the full-length amino acid sequences of *Lv*CK2β and some vertebrates and invertebrates CK2 proteins. The numbers marked on the tree branches represent the bootstrap values. The location of *Lv*CK2β is indicated by a black filled triangle. Species included in construction of phylogenetic tree were all retrieved from NCBI database and accession numbers of the CK2β are : *Macaca mulatta* CK2β (NP_001244617.1), *Oryctolagus cuniculus* CK2β (NP_001153755.1), *Bos taurus* CK2β (NP_001039919.1), *Homo sapiens* CK2β (NP_001311.3), *Crotalus horridus* CK2β (JAG47227.1), *Fundulus heteroclitus* CK2β (JAR36519.1), *Danio rerio* CK2β (NP_571262.1), *Salmo salar* CK2β (ACN11473.1), *Crassostrea gigas* CK2β (EKC23043.1), *Stegodyphus mimosarum* CK2β (KFM82016.1), *Limulus polyphemus* CK2β (ANO53990.1), *Spodoptera frugiperda* CK2β (AAC24042.1), *Drosophila melanogaster* CK2β (NP_996415.1), *Daphnia magna* CK2β (NP_001259462.1), *Caenorhabditis elegans* CK2β (AAA27983.1), *Exaiptasia pallida* CK2β (KXJ19257.1).

mercaptoethanol and 0.02 g/L bromophenol blue) was added to 80 μL of each lysate, before being boiled for 5 min. Samples were then separated on 10% SDS–PAGE gels and transferred onto PVDF membranes (Millipore, USA) using the Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad, USA). The membranes were blocked for 2 h at room temperature in 5% skimmed milk dissolved in TBST buffer (20 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.6), and subsequently incubated with primary antibodies (rabbit anti-CK2α antibody 1:1000 dilution, Thermo Fisher, USA) for 2 h or (mouse anti-tubulin antibody 1:3000 dilution, Sigma-Aldrich, USA) for 1 h at room temperature. Next, membranes were washed three times with TBST buffer (15 min each time), followed by incubation for 1 h at room temperature with secondary antibodies (goat anti-rabbit IgG-HRP 1:3000 dilution, or goat anti-mouse IgG-HRP 1:3000 dilution, Thermo Scientific, USA). After being washed three times with TBST buffer, signals were visualized using Millipore Immobilon™ Western Chemiluminescent HRP Substrate 280 (Millipore, USA) detection system. For the RT-qPCR analysis, total RNA was extracted from pooled hemocytes and used for cDNA synthesis as described above (subsection 2.2). The knockdown efficiency of *Lv*CK2α and the relative expression of *Lv*CK2β, *Lv*Cytochrome C, *Lv*Caspase 3, *Lv*Bcl2, *Lv*IAP1 and *Lv*IAP2 were determined using gene specific primers (Table 1).

2.8. Analysis of hemocytes apoptosis after LvCK2α knockdown

The caspase activity of shrimp hemocytes after *Lv*CK2α knockdown was measured by using the Caspase-Glo® 3/7 assay kit (Promega, USA). Shrimp in the experimental group were intramuscularly injected with 2.5 μg si*Lv*CK2α. One of the two control groups shrimp were injected with 2.5 μg siNon, while the other control group shrimp were left untreated. At 120 h post-siRNA injection, hemocytes were collected from six shrimp per group and pooled together for caspase 3/7 activity assay and flow cytometry analysis. For the caspase 3/7 activity assay, the Caspase-Glo® 3/7 buffer and lyophilized Caspase-Glo® 3/7 substrate were equilibrated to room temperature before being mixed to obtain the reaction buffer. Next, 6000 cells were seeded in a volume of 30 μL per well into an opaque 96-well plate and then mixed with 30 μL of reaction buffer. Samples were mixed gently on a rocker and incubated for 3 h at room temperature [29]. The enzyme activity was measured as the luminescence of each sample with a Multimode microplate reader (Infinite™ F200, Tecan, Switzerland). Samples were analyzed in triplicates each time for three independent samples. For the flow cytometry analysis of hemocytes apoptosis, pooled hemocytes were collected as described above and stained with the YO-PRO™-1 dye/Propidium Iodide (PI) double staining kit (Thermo Fisher, USA) following the manufacturers' instructions. Briefly, a minimum of 3×10^5 hemocytes were washed and re-suspended in 0.5 ml of anti-coagulant buffer. Next the YO-PRO-1 dye was added at a final concentration of 20 μg/ml, while PI was added to a final concentration of 50 μg/ml. Hemocytes were stained for 15 min in the dark at room temperature. At least 10000 events were collected, recorded on a dot plot, The fluorescence signal was determined and analyzed on an Accuri C6 flow cytometer (BD Bioscience, SanDiego, USA). The number of necrotic, living, early apoptotic, and late apoptotic or dead cells were characterized using dot plots, where the number of dots counted in the four different quadrants, i.e., upper left (UL), upper right (UR), lower left (LL), and lower right (LR), represent primary necrotic cells, late apoptotic or secondary necrotic cells, viable or live cells, and cells undergoing early apoptosis, respectively.

2.9. Analysis of cumulative mortality rate

Four groups (30 shrimp per group) were used for the cumulative mortality rate determination after knockdown of *Lv*CK2α followed by challenge with *V. parahaemolyticus*. Two groups (80 shrimp in each group) were first injected with si*Lv*CK2α or siNon. At 120 h post injection, shrimp from each group were further divided into two subgroups, with one subgroup injected with 50 μL V. *parahaemolyticus* (2.0×10^6 CFU/ml) while the other was injected with 50 μL sterile PBS. Shrimp mortality was recorded every 6 h and the cumulative mortality calculated for 5 d. One-way analysis of variance (one-way ANOVA) was used to determine statistical significance. Significance was considered at $p < 0.05$.

3. Results

3.1. Identification and sequence analysis of LvCK2

The full-length cDNA sequences of *Lv*CK2α and *Lv*CK2β were analyzed by first using PCR cloning. The full-length cDNA of LvCK2α was 1764 bp (deposited at GenBank with the accession number: KY038483.1) with an ORF of 1053 bp encoding a protein with 351 amino acids (Fig. 1A(i)). On the other hand, *Lv*CK2β had a full-length cDNA of 1394 bp (deposited at GenBank with the accession number: KY038484.1) with an ORF of 663 bp encoding a protein with 221 amino acids (Fig. 1B(i)). Protein functional domain analysis using the SMART program revealed that *Lv*CK2α contains a Serine/Threonine protein kinase catalytic domain (S/TKc) located at 37–322 aa (Fig. 1 A (ii)), while *Lv*CK2β has a Casein kinase II regulatory subunit domain located at 8–190 aa (Fig. 1 B(ii)). Multiple sequence alignments shows that *Lv*CK2α shares 84.3% homology with *Drosophila melanogaster* CK2α (NP_034104.1), 80.4% with *Mus musculus* CK2α (NP_001287153.1), 78.3% with *Caenorhabditis elegans* CK2α (CCD61302.1), 79.3% with *Homo sapiens* CK2α (NP_808227.1), and 77.1% with *Danio rerio* CK2α (NP_001002164.1) (Fig. 2A(i)). Similarly, full-length *Lv*CK2β shares 87.8% homology with *Danio rerio* CK2β (NP_571262.1), 86.9% with *Homo sapiens* CK2β (NP_001311.3), 80.9% with *Drosophila melanogaster* CK2β (NP_996415.1), 70.5% with *Caenorhabditis elegans* CK2β (AAA27983.1), and 50.1% with *Mus musculus* CK2β (NP_001290405.1) (Fig. 2B(i)). Neighbor-joining (NJ) phylogenetic trees (Fig. 2) of *L. vannamei* CK2 (*Lv*CK2α and *Lv*CK2β) with CK2 of other species revealed that *Lv*CK2α and *Daphnia magna* CK2α were clustered together, while *Lv*CK2β and *Drosophila melanogaster* CK2β were clustered together. This is an interesting observation as *Lv*CK2α and LvCK2β share 14.5% identity.

3.2. Tissue distribution of LvCK2 transcripts in shrimp

The distribution of *Lv*CK2α and *Lv*CK2β transcripts in various tissues of healthy shrimp were determined using RT-qPCR including levels in hemocytes, hepatopancreas, muscle, gill, nerve, intestine, heart, stomach and eyestalk. The results showed that *Lv*CK2α and *Lv*CK2β were expressed in all tissues examined, with high levels mainly found in gill, intestine, nerve and hemocytes, while lower levels were generally observed in eyestalk, muscle, stomach and hepatopancreas (Fig. 3). Notably, the two subunits of *Lv*CK2 displayed different tissue distribution, with higher levels of *Lv*CK2α found in hemocytes (Fig. 3A) while the highest expression of *Lv*CK2β were in intestine (Fig. 3B).

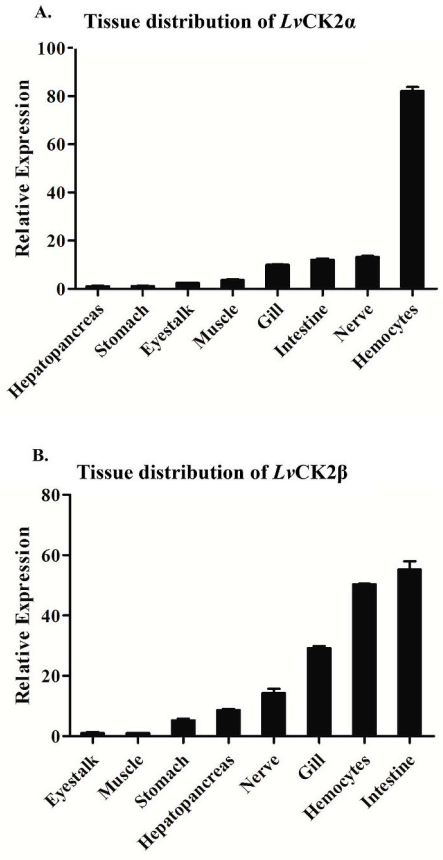
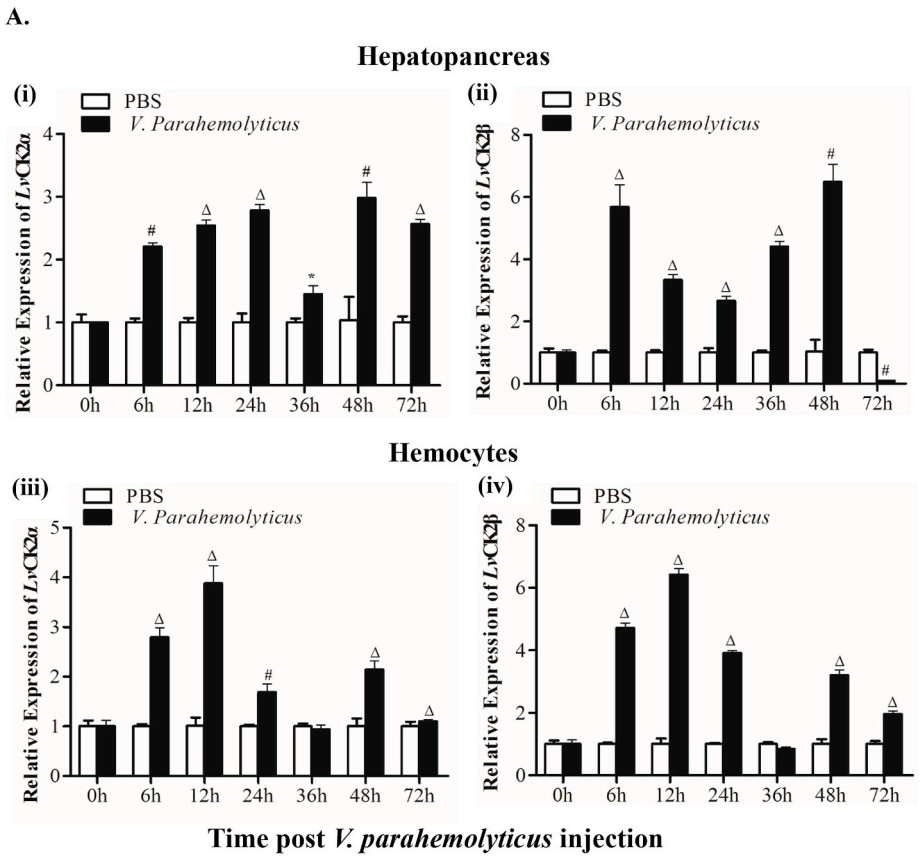
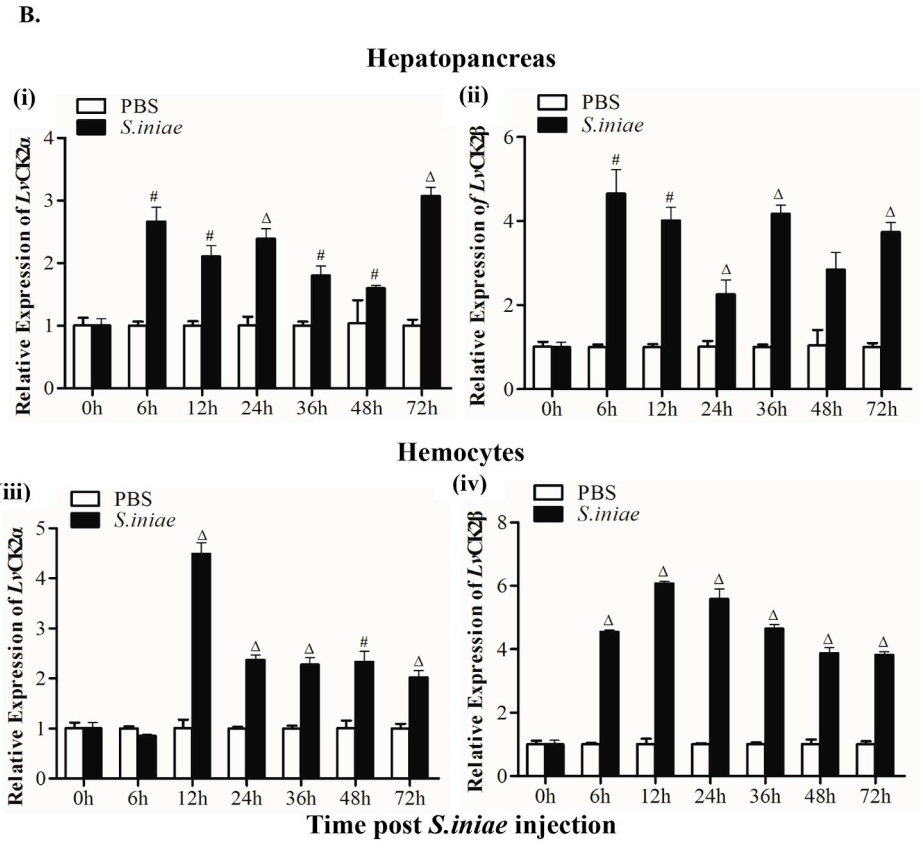


Fig. 3. Tissue distribution of *Lv*CK2α and *Lv*CK2β in healthy *L. vannamei.* The expression of (A) *Lv*CK2α and (B) *Lv*CK2β were determined relative to LvEF-1α expression using RT-qPCR analysis. Data represent mean ± SD (n = 50) of three independent experiments.





(caption on next page)

Fig. 4. Expression patterns of *Lv*CK2α and *Lv*CK2β in hepatopancreas and hemocytes upon immune challenge. Transcript levels of *Lv*CK2α and *Lv*CK2β (A–D) in hepatopancreas and hemocytes at 0, 6, 12, 24, 48, and 72 h post-injection with *V. parahemolyticus*, *S. iniae*, LPS, and WSSV, determined by RT-qPCR analysis relative to *Lv*EF1α expression. The expression level of *Lv*CK2α and *Lv*CK2β at 0 h of each group was set to 1.0. Data represent mean ± SD (n = 5) of three independent experiments. Statistical difference between experimental groups and control groups at same time points were determined by Student's t-test ($^{*}p < 0.05$, $^{\#}p < 0.01$ and $^{\Delta}p < 0.001$).

3.3. Transcript levels of LvCK2 after pathogens challenge

The expression profile of *Lv*CK2 in the hepatopancreas and hemocytes of shrimp after challenge with *V. parahaemolyticus*, *S. iniae*, LPS, WSSV, and PBS (as negative control) at different time points was determined using RT-qPCR. There were different expression patterns in the transcript levels of *Lv*CK2 in the two tissues (Fig. 4). Compared with control (PBS), transcript levels of *Lv*CK2α and *Lv*CK2β in the hepatopancreas were significantly increased at all infection time points post *V. parahaemolyticus* challenge, except at 36 h for *Lv*CK2α (Fig. 4A (i)) and 72 h for *Lv*CK2β (Fig. 4A(ii)), where their expression levels was not significant. In hemocytes, levels of *Lv*CK2α and *Lv*CK2β post *V. parahaemolyticus* challenge were also significantly elevated at all time points, with a decrease to baseline at 36 h and 72 h for *Lv*CK2α (Fig. 4 A (iii)) and 36 h for *Lv*CK2β (Fig. 4 A (iv)). In response to *S. iniae* challenge, the mRNA levels of *Lv*CK2α (Fig. 4B(i)) and *Lv*CK2β (Fig. 4B(ii)) in the hepatopancreas increased significantly throughout the time points. While in hemocytes, transcript levels of *Lv*CK2α post *S. iniae* infection was at baseline at 6 h but increased significantly from 12 h to 72 h post challenge (Fig. 4B(iii)), with the levels of *Lv*CK2β elevated significantly at all challenged time points (Fig. 4B(iv)). For LPS treatment, mRNA levels of both *Lv*CK2α and *Lv*CK2β in the hepatopancreas increased significantly between 6 h and 72 h, except at 36 h for *Lv*CK2α (Fig. 4C(i)) and 48 h for *Lv*CK2β (Fig. 4C(ii)), where their expression levels was not significantly different from control. Similarly, in hemocytes, transcript levels of *Lv*CK2α (Fig. 4C(iii)) and *Lv*CK2β (Fig. 4C(iv)) were significantly elevated between 6 h and 72 h post LPS treatment, except at 6 h where the expression of *Lv*CK2α was same as baseline. When shrimp were challenged with WSSV, transcript levels of *Lv*CK2α in the hepatopancreas increased significantly at only 24 h, 48 h and 72 h, with no significant change at 6 h, 12 h and 36 h (Fig. 4D(i)), while the levels of *Lv*CK2β in the hepatopancreas increased significantly throughout the challenged period (Fig. 4D(ii)). In hemocytes, mRNA levels of *Lv*CK2α (Fig. 4D(iii)) and *Lv*CK2β (Fig. 4D(iv)) were both significantly increased between 6 h and 72 h post WSSV infection, except at 6 h and 36 h for LvCK2α where transcript levels were not significantly different from control samples.

3.4. LvCK2 is involved in shrimp immune response to pathogens

To further explore whether *Lv*CK2 was involved in shrimp immune response to pathogens, we employed the technique of RNAi. First, the knockdown efficiency of *Lv*CK2α in shrimp hemocytes using siLvCK2α was determined by RT-qPCR and Western blot analysis. As shown in (Fig. 5A(i) and 5A(ii)), at 120 h post siRNA injection, a significant ($p < 0.01$) decrease in the mRNA and protein expression of *Lv*CK2α was observed compared to control. At the same time, we went on to detect the expression of *Lv*CK2β after depletion of *Lv*CK2α. The results showed that the mRNA level of *Lv*CK2β in hemocytes was also significantly ($p < 0.01$) reduced after *Lv*CK2α knockdown (Fig. 5A(iii)), suggesting a successful knockdown of both subunits of *Lv*CK2. Next, the effect of *Lv*CK2 knockdown on the immune response of shrimp to *V. parahaemolyticus* infection was determined in terms of cumulative mortality. Shrimp successfully depleted of *Lv*CK2α were injected with *V. parahaemolyticus* and the mortality recorded at 6 h interval post injection. As shown in Fig. 5B, the cumulative mortality of siLvCK2α depleted shrimp challenged with *V. parahaemolyticus* was relatively higher than siNon treated shrimp injected with *V. parahaemolyticus*. For instance, the cumulative mortality following *Lv*CK2α knockdown increased sharply from 6 h to 120 h, reaching 93.3% at 120 h post *V. parahaemolyticus* injection, while the highest shrimp cumulative mortality in the siNon control group was 66.6% at the same time point. Some mortality was recorded in the *Lv*CK2α depleted shrimp injected with sterile PBS at 120 h (53.3%), compared with control siNon shrimp injected with PBS injection at 120 h (30.0%), suggesting that *Lv*CK2α plays an important antibacterial role in shrimp.

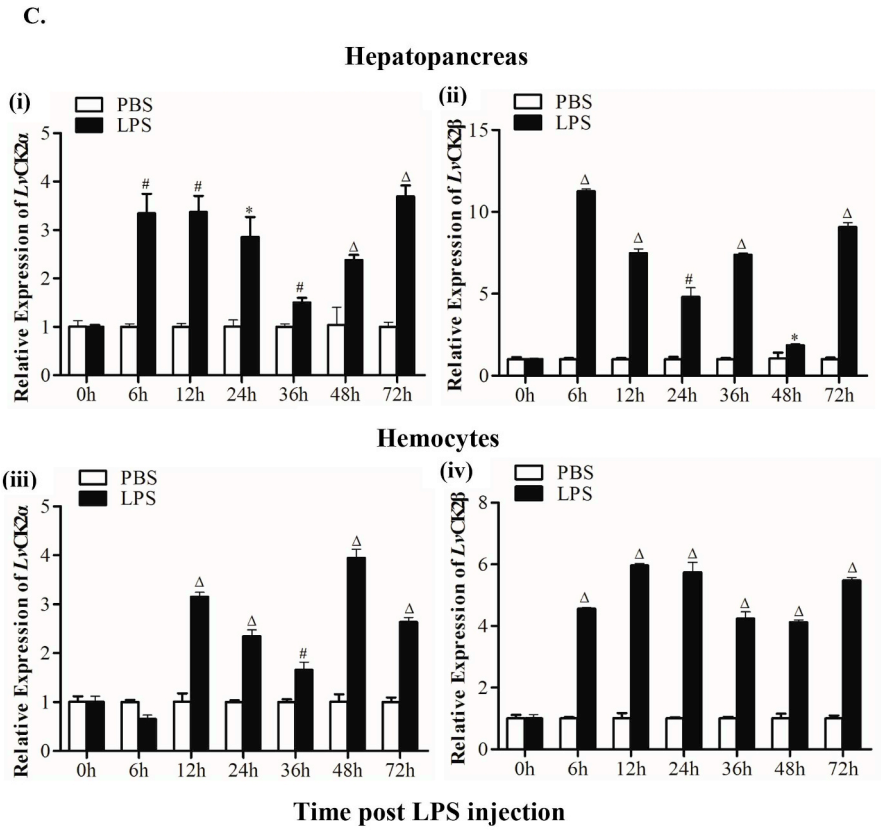
3.5. LvCK2 affects hemocytes apoptosis

The effect of *Lv*CK2α on shrimp hemocytes apoptosis was determined using RT-qPCR and flow cytometry. In terms of the expression of apoptosis-related genes, significant increase in the levels of pro-apoptotic *Lv*Cytochrome C ($p = 0.036$, Fig. 6A(i)) and *Lv*Caspase 3 ($p = 0.033$, Fig. 6A(ii)) were observed after *Lv*CK2α silencing, while significant decrease in expression of the anti-apoptotic genes *Lv*IAP1 ($p < 0.01$, Fig. 6A (iii)), *Lv*IAP2 ($p = 0.046$, Fig. 6A (iv)) and *Lv*Bcl2 ($p = 0.048$, Fig. 6A (v)) were observed after *Lv*CK2α knockdown. Similarly, the there was a significant increase ($p < 0.01$) in *Lv*Caspase3/7 activity in shrimp hemocytes after *Lv*CK2α knockdown (Fig. 6B(i)) compared with control (siNon). In addition, after successful *Lv*CK2α knockdown, flow cytometry analysis of hemocytes apoptosis using YO-PRO-1/PI double staining revealed that the ratio of viable cells (7000 events) decreased, while the percentage of late apoptotic cells and PI/YO-PRO1 positive cells increased from 5% (350 events) to 13% (910 events) Fig. 6B(ii).

4. Discussion

The CK2 serine/threonine protein kinase plays a role in mammalian cell survival, proliferation and suppression of apoptosis [11,30]. Emerging evidence also implicates CK2 protein kinases in mammalian immunity and inflammation [31,32]. For instance, CK2 is involved in antiviral interferon (IFN) response and temporal induction of IFN response when CK2 is blocked [20]. Gibson et al., revealed that CK2 plays an important role in adaptive immunity as a critical regulator of the Th17/Treg cell axis [33]. Similarly, CK2 was identified as a new molecular target required for the suppression of Th2 immune responses by T cells [34]. Protein kinase CK2 has also been shown to be a critical regulator of epithelial homeostasis in chronic intestinal inflammation [21]. However, in shrimp the role of CK2 in immune regulation and inflammatory response remains unclear.

In this study, the CK2 homolog in *L. vannamei* was identified (designated *Lv*CK2) and characterized. The identified *Lv*CK2 had two subunits composed of a catalytic subunit (*Lv*CK2α) and a regulatory subunit (*Lv*CK2β), which is synonymous with mammalian protein kinase CK2 [35]. *Lv*CK2α has an ORF of 1053 bp, encoding a putative protein of 351 amino acids and contains a typical serine/threonine protein kinase catalytic domain (S/TKc) at 37–322 aa. On the other hand, *Lv*CK2β has an ORF of 663 bp encoding a putative protein of 221 amino acids with a Casein kinase II regulatory subunit domain located at 8–190 aa. The putative *Lv*CK2 kinases share high sequence homology with other known CK2 protein kinases, with *Lv*CK2α sharing 77%–84% homology with the CK2 protein kinases of *Mus musculus, Drosophila melanogaster, Caenorhabditis elegans, Homo sapiens* and *Danio rerio*, while *Lv*CK2β shares 50%–88% sequence homology with the CK2α of *Mus musculus, Drosophila melanogaster, Caenorhabditis elegans, Homo sapiens* and *Danio rerio*. Phylogenetic analysis based on amino acid sequences revealed that *Lv*CK2α clustered with CK2α of *Daphnia magna*, while



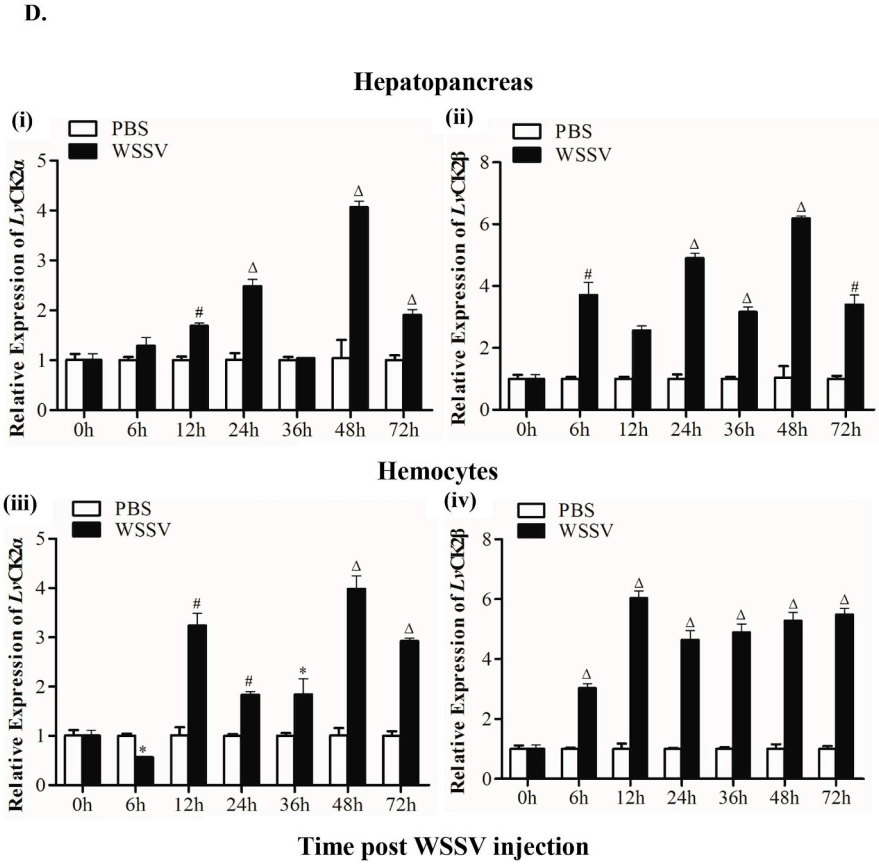


Fig. 4. (*continued*)

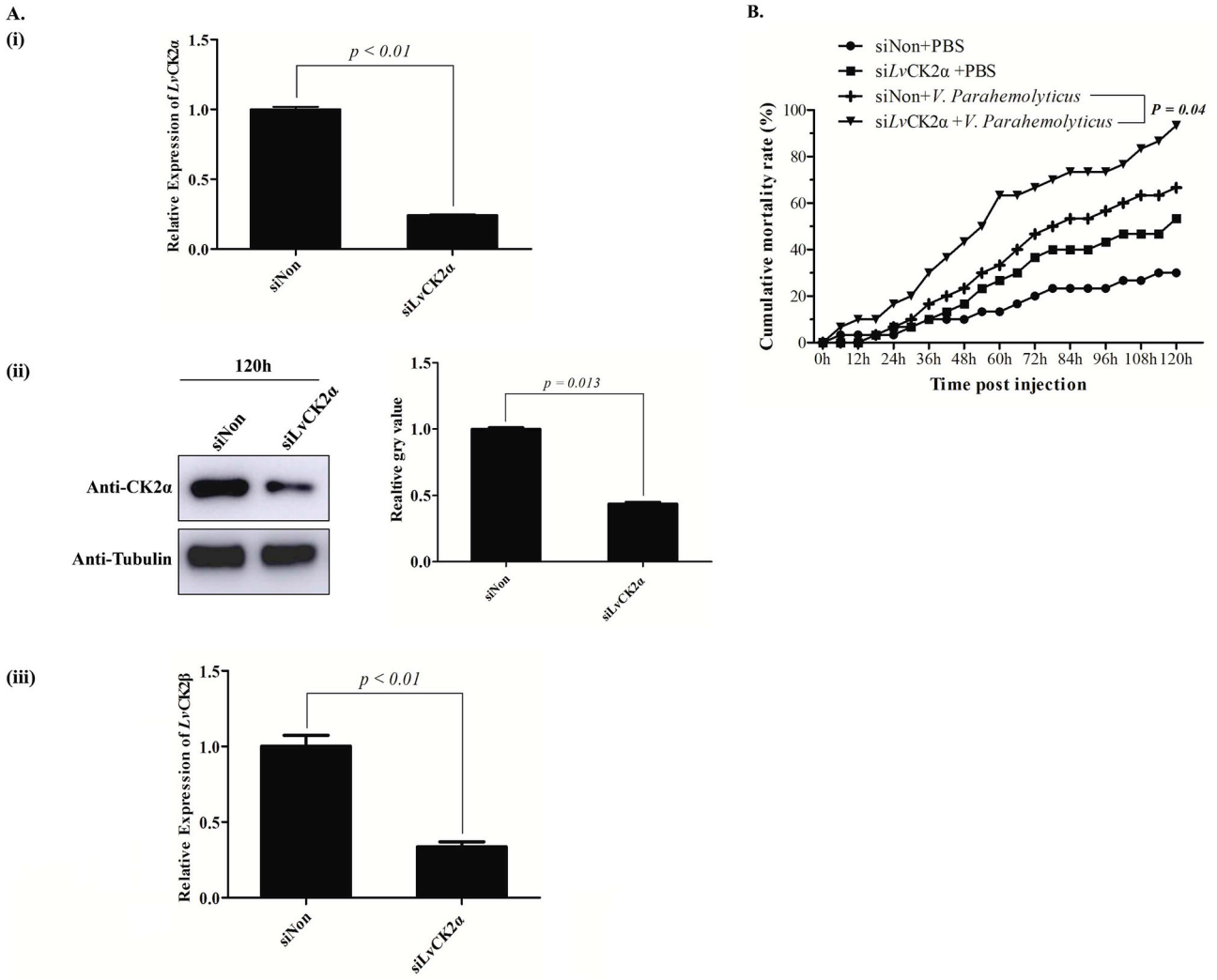
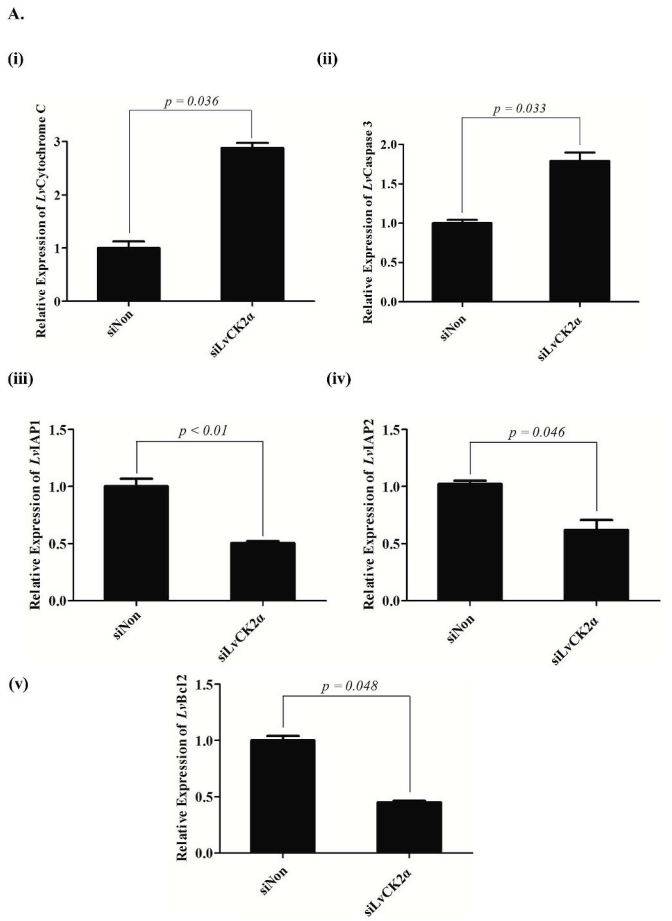


Fig. 5. *Lv*CK2 RNA interference (RNAi) and shrimp mortality. (A) Transcript and protein levels after *Lv*CK2α RNA interference (RNAi). At 120 h post-injection with siNon and si*Lv*CK2α, expression levels of *Lv*CK2α in shrimp hemocytes was determined by (i) RT-qPCR and (ii) Western blot analysis, as well as (iii) Transcript levels of *Lv*CK2β. The mRNA expression of *Lv*CK2α and *Lv*CK2β were determined relative to *Lv*EF-1α expression, with the relative expression for siNon in each group set to 1.0. All experiments were repeated at least three times. Statistical difference between the experimental groups and control groups at the same time points were determined by Student's t-test. Asterisks indicate statistical significance. (B) Shrimp (n = 30 per group) initially intramuscularly injected with siNon or si*Lv*CK2α, were further injected 120 h later with *Vibrio parahemolyticus* or PBS as negative control. Cumulative mortality was recorded at 6 h interval.

*Lv*CK2β clustered with CK2β of *Crassostrea gigas* in the invertebrate clade. These data strongly suggest that *Lv*CK2α and *Lv*CK2β are members of the CK2 serine/threonine protein kinase family.

Transcript levels of *Lv*CK2 were detected in all shrimp tissues examined, with highest level of *Lv*CK2α mRNA found in hemocytes, while that of *Lv*CK2β was in intestines. This observation is consistent with the reported ubiquitous distribution of mammalian CK2 in tissues such as kidney, heart, liver, and head [36], and also indicates the importance of CK2 in most tissues. Notably, the observed high constitutive expression of *Lv*CK2 in hemocytes, intestine and gill, suggest a key role played by *Lv*CK2 in immune-related functions in shrimp. On the other hand, the differences in the levels of *Lv*CK2 in different tissues also suggest the diverse functions of CK2 either as an effector or mediator in various cellular and pathophysiological responses [37,38]. It was therefore not surprising that transcript levels of *Lv*CK2 were significantly induced in shrimp hemocytes and hepatopancreas upon challenge with *V. parahaemolyticus*, *S. iniae*, LPS and WSSV, albeit at different levels. This observation is synonymous with previous studies where CK2 was implicated in immune and inflammatory response [31]. For instance, in RAW 264.7 macrophage cells, CK2 was shown to respond to LPS stimulation [39]. Similarly, under steady state conditions and in herpes simplex virus (HSV) infection, CK2 was reported to be involved in the antiviral immune response, by serving as a negative regulator of IFN responses [20]. It is believed that the role played by CK2 in immunity could be via several signaling pathways, as CK2 is able to promote the activation of the NF-κB, PI3K–Akt–mTOR, and JAK–STAT pathways in mammals, which are used by immune cells to respond to inflammatory insults [40].

To further delineate the involvement of *Lv*CK2 in shrimp immune defense, the technique of RNAi followed by immune challenge was employed. Interestingly, knockdown of *Lv*CK2α resulted in concomitant decrease in *Lv*CK2β transcript levels, which is believed to be due to the rapid degradation of *Lv*CK2β [41], as it could not assemble with the depleted *Lv*CK2α. This observation is in accordance with previous studies in mammals [42]. Most importantly, knockdown of *Lv*CK2 made shrimp more susceptible to *V. parahaemolyticus* infection, as there was an increase in shrimp mortality compared with control group shrimp. This suggest an important role of *Lv*CK2 in shrimp immune response to bacterial infection. The results thus far indicate a significant role of *Lv*CK2 in shrimp immunity. *Lv*CK2 thus seems to play a role in shrimp



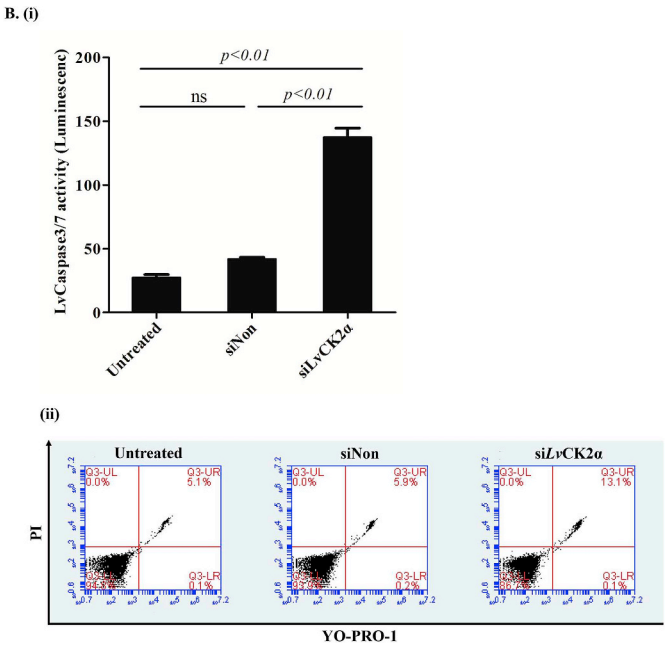


Fig. 6. (*continued*)

Fig. 6. (A) *Lv*CK2 knockdown and hemocytes apoptosis. After successful *Lv*CK2α knockdown, RT-qPCR analysis was used to determine the transcript levels of the apoptosis-related genes (i) *Lv*Cytochrome C, (ii) *Lv*Caspase 3, (iii) *Lv*IAP1, (iv) *Lv*IAP2, and (v) *Lv*Bcl2, in shrimp hemocytes at 96 h post siNon and si*Lv*CK2α injection. The transcript levels were determined relative to *Lv*EF-1α expression, with the relative expression for siNon in each group set to 1.0. All experiments were repeated at least three times and the statistical difference between the siNon and si*Lv*CK2α groups at the same time point determined by Student's t-test. Asterisks indicate statistical significance. (B) *Lv*CK2 knockdown affects hemocytes apoptosis. (i) *Lv*Caspase3/7 activity in shrimp hemocytes. Experiments were repeated at least three times and the statistical difference between the control (untreated or siNon) and si*Lv*CK2α groups determined by Student's t-test. Asterisks indicate statistical significance. (ii) Number of apoptotic cells. The number of apoptotic cells was determined by Flow cytometry using the YO-PRO™-1 dye/Propidium Iodide double staining kit. Figure represents one of three independent experiments.

immune response to various pathogens, synonymous with other shrimp serine/threonine kinases such as *Lv*Pim1 [29], *Lv*-p38 [43], *Lv*MST4 [44], *Lv*GSK3β [45] and *Lv*ERK [46].

The CK2 protein kinase is reported to exhibit anti-apoptotic activity and functions downstream of Wnt, NF-κB, JAK/STAT and AKT signaling pathways [47,48]. Similarly, CK2α suppresses prosurvival signaling pathways such as the PI3K/AKT pathway, with CK2α knockdown reported to attenuate AKT activation [49]. In mammalian cells, inhibition of CK2 expression leads to apoptosis induction [50,51]. In the current study, knockdown of *Lv*CK2 resulted in an increase in hemocytes apoptosis, in terms of high caspase 3/7 activity, increased number of apoptotic cells, upregulated expression of proapoptotic genes (i.e., *Lv*Caspase 3 and *Lv*Cytochrome C), and attenuation in the expression of prosurvival genes (i.e., *Lv*Bcl2, *Lv*IAP1, and *Lv*IAP2). These results are consistent with previous studies, where knockdown of CK2α reduced the expression of prosurvival PCNA, Bcl-xl, and Bcl-2 proteins, while increasing the levels of proapoptotic Bax, cleaved caspase-3, cleaved caspase-9 and cleaved caspase-8 [52]. In fact, it has been shown that CK2α is able to decrease the level of Bcl-2/Bax in cells [50,53], with CK2α knockdown reported to decrease bladder cancer cells survival [54], while inhibition or knockdown of CK2α in human lung cancer cells reduces Notch1 signaling, thereby attenuating cell growth and decreasing cancer stem-like cell population [55]. Collectively, the current data strongly suggest an essential role of *Lv*CK2 in shrimp immunity.

In conclusion, *Lv*CK2 (*Lv*CK2α and *Lv*CK2β), the *L. vannamei* CK2 protein kinase homologs, were cloned and characterized. The findings here revealed the involvement of *Lv*CK2 in shrimp immunity via modulation of hemocytes apoptosis, and therefore indicates the importance of *Lv*CK2 in shrimp immune and inflammatory responses. There have been no previous findings on the involvement of CK2 in the immunity of shrimp or other arthropods. Future work would further explore the molecular mechanisms behind *Lv*CK2 involvement in shrimp immune response.

Acknowledgments

This work was sponsored by National Natural Science Foundation of China (No.31872596), Natural Science Foundation of Guangdong Province (No. 2017A030311032) and Department of Education of Guangdong Province (No. 2017KZDXM033).

References

[1] A.F. Rowley, A. Powell, Invertebrate immune systems–specific, quasi-specific, or nonspecific? J. Immunol. 179 (11) (2007) 7209–7214.
[2] M. Briggs, S. Fungesmith, R.P. Subasinghe, M. Phillips, Introductions and Movement of Two Penaeid Shrimp Species in Asia and the Pacific, (2005), pp. 1–57 Fao Fisheries Technical Paper 476.
[3] D. Sookying, D.A. Davis, Pond production of Pacific white shrimp (*Litopenaeus vannamei*) fed high levels of soybean meal in various combinations, Aquaculture 319 (1) (2011) 141–149.
[4] A. Tassanakajon, K. Somboonwiwat, P. Supungul, S. Tang, Discovery of immune molecules and their crucial functions in shrimp immunity, Fish Shellfish Immunol. 34 (4) (2013) 954–967.
[5] G.D. Stentiford, D.M. Neil, E.J. Peeler, J.D. Shields, H.J. Small, T.W. Flegel, J.M. Vlak, B. Jones, F. Morado, S. Moss, J. Lotz, L. Bartholomay, D.C. Behringer, C. Hauton, D.V. Lightner, Disease will limit future food supply from the global crustacean fishery and aquaculture sectors, J. Invertebr. Pathol. 110 (2) (2012)

141–157.
[6] S.A. Soto-Rodriguez, B. Gomez-Gil, R. Lozano-Olvera, M. Betancourt-Lozano, M.S. Morales-Covarrubias, Field and experimental evidence of *Vibrio parahaemolyticus* as the causative agent of acute hepatopancreatic necrosis disease of cultured shrimp (*Litopenaeus vannamei*) in northwestern Mexico, Appl. Environ. Microbiol. 81 (5) (2015) 1689–1699.
[7] X.W. Wang, J.X. Wang, Pattern recognition receptors acting in innate immune system of shrimp against pathogen infections, Fish Shellfish Immunol. 34 (4) (2013) 981–989.
[8] F. Li, J. Xiang, Recent advances in researches on the innate immunity of shrimp in China, Dev. Comp. Immunol. 39 (1–2) (2013) 11–26.
[9] K. Niefind, B. Guerra, I. Ermakowa, O.G. Issinger, Crystal structure of human protein kinase CK2: insights into basic properties of the CK2 holoenzyme, EMBO J. 20 (19) (2014) 5320–5331.
[10] A.C. French, B. Luscher, D.W. Litchfield, Development of a stabilized form of the regulatory CK2beta subunit that inhibits cell proliferation, J. Biol. Chem. 282 (40) (2007) 29667–29677.
[11] H. Lian, M. Su, Y. Zhu, Y. Zhou, S.H. Soomro, H. Fu, Protein kinase CK2, a potential therapeutic target in carcinoma management, Asian Pac. J. Cancer Prev. APJCP 20 (1) (2019) 23–32.
[12] G.H. Eom, Y.K. Cho, J.H. Ko, S. Shin, N. Choe, Y. Kim, H. Joung, H.S. Kim, K.I. Nam, H.J. Kee, H. Kook, Casein kinase-2alpha1 induces hypertrophic response by phosphorylation of histone deacetylase 2 S394 and its activation in the heart, Circulation 123 (21) (2011) 2392–2403.
[13] M. Pizzi, F. Piazza, C. Agostinelli, F. Fuligni, P. Benvenuti, E. Mandato, A. Casellato, M. Rugge, G. Semenzato, S.A. Pileri, Protein kinase CK2 is widely expressed in follicular, Burkitt and diffuse large B-cell lymphomas and propels malignant B-cell growth, Oncotarget 6 (9) (2015) 6544–6552.
[14] S. Schuck, C. Ruse, A. Stenlund, CK2 phosphorylation inactivates DNA binding by the papillomavirus E1 and E2 proteins, J. Virol. 87 (13) (2013) 7668–7679.
[15] H. Basnet, X.B. Su, Y. Tan, J. Meisenhelder, D. Merkurjev, K.A. Ohgi, T. Hunter, L. Pillus, M.G. Rosenfeld, Tyrosine phosphorylation of histone H2A by CK2 regulates transcriptional elongation, Nature 516 (7530) (2014) 267–271.
[16] Z.M. Jia, X. Ai, J.F. Teng, Y.P. Wang, B.J. Wang, X. Zhang, p21 and CK2 interaction-mediated HDAC2 phosphorylation modulates KLF4 acetylation to regulate bladder cancer cell proliferation, Tumor Biol. 37 (6) (2016) 8293–8304.
[17] B. Guerra, O.G. Issinger, Protein kinase CK2 and its role in cellular proliferation, development and pathology, Electrophoresis 20 (2) (2015) 391–408.
[18] K.A. Ahmad, G. Wang, G. Unger, J. Slaton, K. Ahmed, Protein kinase CK2–a key suppressor of apoptosis, Adv. Enzym. Regul. 48 (1) (2008) 179.
[19] C.S. Gowda, C. Song, Y. Ding, M. Kapadia, S. Dovat, Protein signaling and regulation of gene transcription in leukemia: role of the Casein Kinase II-Ikaros axis, J. Investig. Med. 64 (3) (2016) 735–739.
[20] M. Du, J. Liu, X. Chen, Y. Xie, C. Yuan, Y. Xiang, B. Sun, K. Lan, M. Chen, S.J. James, Y. Zhang, J. Zhong, H. Xiao, Casein kinase II controls TBK1/IRF3 activation in IFN response against viral infection, J. Immunol. 194 (9) (2015) 4477–4488.
[21] S. Koch, C.T. Capaldo, R.S. Hilgarth, B. Fournier, C.A. Parkos, A. Nusrat, Protein kinase CK2 is a critical regulator of epithelial homeostasis in chronic intestinal inflammation, Mucosal Immunol. 6 (1) (2013) 136–145.
[22] J.S. Duncan, D.W. Litchfield, Too much of a good thing: the role of protein kinase CK2 in tumorigenesis and prospects for therapeutic inhibition of CK2, Biochim. Biophys. Acta 1784 (1) (2008) 33–47.
[23] P. Ning, Z. Zheng, J.J. Aweya, D. Yao, S. Li, H. Ma, F. Wang, Y. Zhang, *Litopenaeus vannamei* notch affects lipopolysaccharides induced reactive oxygen species, Dev. Comp. Immunol. 81 (2017) 74–82.
[24] Z. Zheng, F. Wang, J.J. Aweya, R. Li, D. Yao, M. Zhong, S. Li, Y. Zhang, Comparative transcriptomic analysis of shrimp hemocyte in response to acute hepatopancreas necrosis disease (AHPND) causing Vibrio parahemolyticus infection, Fish Shellfish Immunol. 74 (2018) 10–18.
[25] S. Kumar, G. Stecher, K. Tamura, MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets, Mol. Biol. Evol. 33 (7) (2016) 1870–1874.
[26] N. Saitou, M. Nei, The neighbor-joining method: a new method for reconstructing phylogenetic trees, Mol. Biol. Evol. 4 (4) (1987) 406–425.
[27] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods 25 (4) (2001) 402–408.
[28] W. Zhao, Z. Yu, J.J. Aweya, F. Wang, D. Yao, H. Ma, J. Lun, Y. Zhang, Molecular cloning and functional characterization of a homolog of the transcriptional regulator CSL in *Litopenaeus vannamei*, Dev. Comp. Immunol. 88 (2018) 152–160.
[29] J.J. Aweya, W. Wang, Y. Zhang, D. Yao, S. Li, F. Wang, Identification and molecular characterization of the Pim1 serine/threonine kinase homolog in *Litopenaeus vannamei*, Fish Shellfish Immunol. 74 (2018) 491–500.
[30] N.A. Stdenis, D.W. Litchfield, Protein kinase CK2 in health and disease: from birth to death: the role of protein kinase CK2 in the regulation of cell proliferation and survival, Cell. Mol. Life Sci. 66 (2009) 11–12.
[31] S.A. Gibson, E.N. Benveniste, Protein Kinase CK2: an emerging regulator of immunity, Trends Immunol. 39 (2) (2018) 82–85.
[32] J. Huang, Z. Chen, J. Li, Q. Chen, W. Gong, P. Liu, H. Huang, Protein kinase CK2α catalytic subunit ameliorates diabetic renal inflammatory fibrosis via NF-κB signaling pathway, Biochem. Pharmacol. 132 (2017) 102.
[33] S.A. Gibson, W. Yang, Z. Yan, Y. Liu, A.L. Rowse, A.S. Weinmann, H. Qin, E.N. Benveniste, Protein kinase CK2 controls the fate between Th17 cell and regulatory T cell differentiation, J. Immunol. 198 (11) (2017) 4244–4254.
[34] A. Ulges, M. Klein, S. Reuter, B. Gerlitzki, M. Hoffmann, N. Grebe, V. Staudt, N. Stergiou, T. Bohn, T.J. Bruhl, S. Muth, H. Yurugi, K. Rajalingam, I. Bellinghausen, A. Tuettenberg, S. Hahn, S. Reissig, I. Haben, F. Zipp, A. Waisman, H.C. Probst, A. Beilhack, T. Buchou, O. Filhol-Cochet, B. Boldyreff, M. Breloer, H. Jonuleit, H. Schild, E. Schmitt, T. Bopp, Protein kinase CK2 enables regulatory T cells to suppress excessive TH2 responses in vivo, Nat. Immunol. 16 (3) (2015) 267–275.
[35] C. Franchin, C. Borgo, L. Cesaro, S. Zaramella, J. Vilardell, M. Salvi, G. Arrigoni, L.A. Pinna, Re-evaluation of protein kinase CK2 pleiotropy: new insights provided by a phosphoproteomics analysis of CK2 knockout cells, Cell. Mol. Life Sci. 75 (11) (2018) 2011–2026.
[36] L. Volodina Iu, A.A. Shtil, Casein kinase 2, the versatile regulator of cell survival, Mol. Biol. 46 (3) (2012) 423–433.
[37] J.S. Bae, S.H. Park, K.M. Kim, K.S. Kwon, C.Y. Kim, H.K. Lee, et al., CK2α phosphorylates DBC1 and is involved in the progression of gastric carcinoma and predicts poor survival of gastric carcinoma patients, Int. J. Cancer 136 (4) (2015) 797–809.
[38] H.S. Kim, Y.G. Chang, H.J. Bae, J.W. Eun, Q. Shen, S.J. Park, et al., Oncogenic potential of CK2α and its regulatory role in EGF-induced HDAC2 expression in human liver cancer, FEBS J. 281 (3) (2014) 851–861.
[39] O.V. Glushkova, S.B. Parfenyuk, T.V. Novoselova, M.O. Khrenov, S.M. Lunin, E.G. Novoselova, The role of p38 and CK2 protein kinases in the response of RAW 264.7 Macrophages to lipopolysaccharide, Biochemistry (Mosc.) 83 (6) (2018) 746–754.
[40] M.M. Chua, C.E. Ortega, A. Sheikh, M. Lee, H. Abdul-Rassoul, K.L. Hartshorn, I. Dominguez, CK2 in cancer: cellular and biochemical mechanisms and potential therapeutic target, Pharmaceuticals 10 (1) (2017).
[41] N.N. Singh, D.P. Ramji, Protein kinase CK2, an important regulator of the inflammatory response? J. Mol. Med. (Berl.) 86 (8) (2008) 887–897.
[42] J.N. Kreutzer, R. Maria, G. Barbara, Enhancing chemosensitivity to gemcitabine via RNA interference targeting the catalytic subunits of protein kinase CK2 in human pancreatic cancer cells, BMC Canc. 10 (1) (2010) 440-440.
[43] S. He, Z. Qian, J. Yang, X. Wang, X. Mi, Y. Liu, F. Hou, Q. Liu, X. Liu, Molecular characterization of a p38 MAPK from *Litopenaeus vannamei* and its expression during the molt cycle and following pathogen infection, Dev. Comp. Immunol. 41 (2) (2013) 217–221.
[44] C.S. Zhao, D. Huang, T. Peng, M.Z. Huang, C.Y. Xie, J. Chen, J.R. Kong, R.C. Xie, Y. Liu, W.N. Wang, Molecular cloning, characterization and function of a germinal center kinase MST4 gene from *Litopenaeus vannamei* in response to Vibrio alginolyticus challenge in TLR-TRAF6 signaling pathway, Dev. Comp. Immunol. 73 (2017) 206.
[45] L. Ruan, H. Liu, H. Shi, Characterization and function of GSK3beta from *Litopenaeus vannamei* in WSSV infection, Fish Shellfish Immunol. 82 (2018) 220–228.
[46] H. Shi, X. Yan, X. Xu, L. Ruan, Molecular cloning and characterization of a cDNA encoding extracellular signal-regulated kinase from *Litopenaeus vannamei*, Fish Shellfish Immunol. 33 (4) (2012) 813–820.
[47] Y. Zheng, H. Qin, S.J. Frank, L. Deng, D.W. Litchfield, A. Tefferi, A. Pardanani, F.T. Lin, J. Li, B. Sha, A CK2-dependent mechanism for activation of the JAK-STAT signaling pathway, Blood 118 (1) (2011) 156–166.
[48] T.L. Quotti, N.S. Canovas, A. Brancalion, B.E. Doriguzzi, S. Manni, E. Mandato, F. Zaffino, P. Macaccaro, M. Carrino, K. Gianesin, Protein kinase CK2 regulates AKT, NF-kB and STAT3 activation, stem cell viability and proliferation in acute myeloid leukemia, Leukemia 31 (2) (2016) 292–300.
[49] Y. Zheng, B.C. Mcfarland, D. Drygin, H. Yu, S.L. Bellis, H. Kim, M. Bredel, E.N. Benveniste, Targeting protein kinase CK2 suppresses prosurvival signaling pathways and growth of glioblastoma, Clin. Cancer Res. 19 (23) (2013) 6484–6494.
[50] L. Jiang, J.H. Zhang, N.F. Hu, A.C. Liu, H.L. Zhu, L.Q. Li, Y.Y. Tian, X. Chen, L.N. Quan, Lentivirus-mediated down-regulation of CK2 alpha inhibits proliferation and induces apoptosis of malignant lymphoma and leukemia cells, Biochem. Cell Biol. 96 (6) (2018) 786–796.
[51] Q. Li, K. Li, T. Yang, S. Zhang, Y. Zhou, Z. Li, J. Xiong, F. Zhou, X. Zhou, L. Liu, R. Meng, G. Wu, Association of protein kinase CK2 inhibition with cellular radiosensitivity of non-small cell lung cancer, Sci. Rep. UK 7 (2017) 16134.
[52] A. Wu, Q. Zhang, Z. Mai, S. Li, Z. Yang, Down-regulation of CK2α expression inhibits the proliferation and apoptosis of A549 cell line in lung adenocarcinoma, J. Mod. Oncol. 25 (16) (2017) 2541–2545.
[53] J.T. Wang, S.S. Gong, [Effects of protein kinase CK2α on apoptosis and ultrastructure of laryngeal carcinoma cells], Zhonghua Yixue Zazhi 90 (90) (2010) 2869–2872.
[54] X. Zhang, X. Yang, C. Yang, P. Li, W. Yuan, X. Deng, Y. Cheng, H. Yang, J. Tao, Targeting protein kinase CK2 suppresses bladder cancer cell survival via the glucose metabolic pathway, Oncotarget 7 (52) (2016) 87361.
[55] S. Zhang, H. Long, Y.L. Yang, Y. Wang, D. Hsieh, W. Li, A. Au, H.J. Stoppler, Z. Xu, D.M. Jablons, Inhibition of CK2α down-regulates Notch1 signalling in lung cancer cells, J. Cell Mol. Med. 17 (7) (2013) 854–862.