

## SHORT COMMUNICATION

**A novel macrophage migration inhibitory factor gene from the Pacific white shrimp *Litopenaeus vannamei* (LvMIF2): comparative sequence and transcription analysis with LvMIF1****M Wang<sup>1</sup>, B Wang<sup>1</sup>, M Liu<sup>1</sup>, K Jiang<sup>1</sup>, L Wang<sup>1,2</sup>**<sup>1</sup>Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China<sup>2</sup>Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266237, China

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**Abstract**

Macrophage migration inhibitory factor (MIF) was an ancient cytokine and involved in innate immunity of vertebrates and invertebrates. In the present study, a novel MIF homologue (designated as LvMIF2) has been cloned from the Pacific white shrimp *Litopenaeus vannamei* via rapid amplification of cDNA ends (RACE) technique. The full-length cDNA sequence of LvMIF2 was 555 bp and contained a 97 bp 5' untranslated region (UTR) and a 3' UTR of 110 bp, and an open reading frame (ORF) of 348 bp which coded 115 amino acids. Quantitative realtime PCR (qRT-PCR) analysis indicated that LvMIF2 was constitutively expressed in all the tested tissues, including eyestalk, gill, gonad, heart, hemocytes, hepatopancreas, intestine, muscle, nerve and stomach, with the highest mRNA expression level in hemocytes and hepatopancreas. After *Vibrio parahaemolyticus* or white spot syndrome virus (WSSV) challenge, the mRNA expression levels of LvMIF2 in hemocytes and hepatopancreas were all sharply upregulated. Comparative sequence and transcription analysis between the previously identified LvMIF1 and LvMIF2 revealed that both LvMIF1 and LvMIF2 might play crucial and functionally differentiated roles in shrimp innate immune responses to bacterial and viral stimulation.

**Key Word:** *Litopenaeus vannamei*; macrophage migration inhibitory factor; innate immunity**Introduction**

Macrophage migration inhibitory factor (MIF) was first described as a cytokine 50 years ago, which was stemmed from activated T cells by its capacity to inhibit the migration of macrophages *in vitro*, and emerged in mammals as a mysterious cytokine with chemotactic, growth-promoting and pro-inflammatory activities (Calandra and Thierry, 2003). As an evolutionary ancient molecule, MIF and its homologues have been found from prokaryotes to eukaryotes (Sparkes *et al.*, 2017). In addition, MIF has recently gained substantial attention as a pivotal upstream regulator of both innate and adaptive immune responses (Sparkes *et al.*, 2017).

In vertebrates, MIF exhibits oxidoreductase activity and could utilize glutathione (GSH) as reductant to reduce insulin (Blocki *et al.*, 1993). Such

oxidoreductase activity plays a role in MIF-mediated monocyte and macrophage function (Mitchell *et al.*, 2002). In invertebrates, especially marine invertebrates, recent research achievements suggest that MIF is extensively involved in innate immune responses. For examples, in small abalone *Haliotis diversicolor supertexta*, the mRNA expression level of HdsMIF was significantly upregulated in hepatopancreas post *Vibrio parahaemolyticus* stimulation (Wang *et al.*, 2009). MIF of snail *Biomphalaria glabrata* (BgMIF) participated in hemocytes activation to resist parasitic infection *in vivo*, and could stimulate cell proliferation to suppress NO-induced apoptosis (Garcia *et al.*, 2010). In pearl oyster *Pinctada fucata*, the mRNA expression levels of PoMIF were significantly upregulated in digestive gland, gills, hemocytes and intestine, after pearl oyster was injected with *Vibrio alginolyticus*, and the recombinant PoMIF (rPoMIF) exhibited oxidoreductase activity and could utilize dithiothreitol (DTT) as reductant to reduce insulin (Cui *et al.*, 2011). MIF from Zhikong scallop *Chlamys farreri* (CMIF) was involved in innate immune responses

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by promoting fibroblast migration (Li *et al.*, 2011a). The mRNA transcripts of MIF in Chinese mitten crab *Eriocheir sinensis* (EsMIF) were responsive to *Vibrio anguillarum* challenge in hemocytes, hepatopancreas and gill (Li *et al.*, 2011b). Two previous reports identified three MIF in mud crabs *Scylla paramamosain* (SpMIF, SpMIF1 and SpMIF2), and the mRNA transcripts of SpMIF significantly increased after the crabs were challenge by *V. parahaemolyticus* (Fang *et al.*, 2013; Huang *et al.*, 2016). The mRNA expression level of MIF from the black tiger shrimp *Penaeus monodon* (PmMIF) was upregulated after bacterial infection, salinity challenge and heavy metals stress (Xie *et al.*, 2016). Two MIFs from starfish *Patiria (Asterina) pectinifera*, PpMIF1 and PpMIF2, could regulate larval immune cell chemotaxis (Furukawa *et al.*, 2016). The polymorphism of MIF in clam *Meretrix meretrix* (MmMIF) was associated with the resistance/susceptibility to *V. parahaemolyticus* (Zou and Liu, 2016). In contrast to other animals, the mRNA expression levels of MIF from mussel *Mytilus galloprovincialis* (MgMIF) were always down regulated following microbe stimulation, including *Vibrio splendidus*, *V. anguillarum*, *Micrococcus lysodeikticus*, *Fusarium oxysporum* and *Candida albicans* (Parisi *et al.*, 2012). All these research achievements indicated that MIF played a pivotal role in the innate immunity of marine invertebrates.

The Pacific white shrimp *Litopenaeus vannamei* is one of the most important and leading farm crustacean species and takes a large amount of the total shrimp production in the world (Li and Xiang, 2010, 2013a, b). However, during the past two decades, outbreaks of infectious disease such as acute hepatopancreatic necrosis disease (AHPND) or early mortality syndrome (EMS) caused by *V. parahaemolyticus* and white spot disease (WSD)

caused by white spot syndrome virus (WSSV), have become a major constraint, resulting in mass shrimp mortality and considerable economic losses (Lai *et al.*, 2015; Zhang *et al.*, 2016; Verma *et al.*, 2017). As invertebrates, shrimps lack an adaptive immune system and rely on the ancient innate immune defenses (Li and Xiang, 2013a, b). A better understanding of the innate immunity of shrimp is necessary for the health management of shrimp farming (Li and Xiang, 2010). A previous research identified a homolog of MIF in *L. vannamei* (designated as LvMIF1), and the mRNA expression level of LvMIF1 in hepatopancreas was significantly upregulated after WSSV injection, suggesting that LvMIF1 may be involved in the innate immune response to viral infection in shrimp (Zeng *et al.*, 2013). In the present study, a novel MIF gene (designated as LvMIF2) have been cloned and investigated in white shrimp, and the main objectives of the present study were (1) to characterize the molecular features of LvMIF2, (2) to detect the tissue distribution and temporal expression patterns after invading microbes stimulation of its mRNA transcripts and (3) to compare these features with the previous identified LvMIF1, in order to get a further understanding of the immunological roles of LvMIF1 and LvMIF2 in shrimp.

## Materials and Methods

### Shrimp and tissues sample collection

The white shrimps used in the present study were obtained from Ruizi Seafood Development Co. Ltd., Qingdao, China, and all the experiments were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH). The study protocol and all the experimental design

**Table 1** Oligonucleotide primers used in the present study

Primer	Sequence (5`-3`)	Tm (°C)	Brief information
adaptor primer	GGCCACGCGTCTGACTAGTAC	60	Anchor primer for 3' RACE
adaptor primer-oligo (dT)	GGCCACGCGTCTGACTAGTACT <sub>17</sub> VN	-	Olido (dT) for cDNA synthetize
LvEF-1α-qRT-F	GTATTGGAACAGTGCCCGT	60	Internal control for real-time PCR
LvEF-1α-qRT-R	CATCTCCACAGACTTTACCTCAG	60	Internal control for real-time PCR
LvMIF2-CDS-F	ATGCCTTTCGTAACCTGACTACC	64	Gene specific primer for CDS
LvMIF2-CDS-R	TCATCCCATTAGAGCCCCATTAC	64	Gene specific primer for CDS
LvMIF2-qRT-F	GCCAGCGGAAGAATTGACCA	60	Gene specific primer for real-time PCR
LvMIF2-qRT-R	GTTGAAGTCATCCCATTAGAGCCC	60	Gene specific primer for real-time PCR
LvMIF2-RACE-F1	GCTGGGACCCAGTGTGTGAGA	64	Gene specific primer for 3' RACE
LvMIF2-RACE-F2	CTGACCGAACTGGCATTCAACCTGCAA	64	Gene specific primer for 3' RACE
LvMIF1-qRT-F	TTAGTGAAACCATTGGGAAA	60	Gene specific primer for real-time PCR
LvMIF1-qRT-R	AACTGCATAAATCTTAGAAG	60	Gene specific primer for real-time PCR
M13-47	CGCCAGGGTTTTCCAGTCACGAC	56	Vector primer for sequencing
RV-M	GAGCGGATAACAATTTACACAGG	56	Vector primer for sequencing

**Table 2** Sequence feature of LvMIF1 and LvMIF2

Feature	LvMIF1	LvMIF2
Accession Number	KC513658	MF062461
cDNA length (bp)	689	555
5' UTR length (bp)	101	97
3' UTR length (bp)	225	110
ORF length (bp)	363	348
Polyadenylation signal sites	0	1
Polypeptide length (amino acid)	120	115
MIF domain information	from P <sup>2</sup> to H <sup>115</sup>	from P <sup>2</sup> to G <sup>115</sup>
Conserved tautomerase activity sites	P <sup>2</sup> and K <sup>33</sup>	P <sup>2</sup> and K <sup>33</sup>
Conserved oxidoreductase activity	C <sup>57</sup>	C <sup>58</sup>
Calculated molecular mass (Da)	13442.61	12534.52
Theoretical isoelectric point (pI)	7.034	7.116

were conducted with approval from Experimental Animal Ethics Committee of Institute of Oceanology, Chinese Academy of Sciences. White shrimps, with body weight 8 - 12 g, were cultured in 640 L cylindrical tanks with 500 L air-pumped circulating seawater at 20 ± 1 °C for two weeks before processing. Hemolymph was extracted from the ventral sinus of at least five untreated shrimps using a sterile syringe preloaded with equal volume of anticoagulant buffer (NaCl 510 mmol L<sup>-1</sup>, glucose 100 mmol L<sup>-1</sup>, citric acid 200 mmol L<sup>-1</sup>, tri-sodium citrate 30 mmol L<sup>-1</sup> and EDTA-2Na 10 mmol L<sup>-1</sup>, pH 7.4). Then the hemocytes were collected by centrifugation at 800 g for 10 min at 4 °C. Tissues including eyestalk, gill, gonad, heart, hepatopancreas, intestine (mid gut), muscle, nerve and stomach were collected from at least five untreated shrimps, kept in RNA<sup>later</sup> (AM7020, Thermo Fisher Scientific, USA) and stored at -80 °C until RNA isolation.

#### Immune stimulation and sample collection

Approximately 200 shrimps were employed for microbe stimulation assay. The *V. parahaemolyticus* suspension and WSSV stock were prepared according to previous reports (Yi *et al.*, 2014; Xia *et al.*, 2015; Sha *et al.*, 2016). The shrimps were randomly divided into three groups and each group contained about 60 - 70 individuals. The shrimps were received an injection at the abdominal segment with 100 µL phosphate buffered saline (PBS, pH 7.4, 10010023, Thermo Fisher Scientific, USA), *V. parahaemolyticus* suspension (1 × 10<sup>5</sup> CFUs µL<sup>-1</sup>, in PBS) and WSSV stock (1 × 10<sup>5</sup> copies µL<sup>-1</sup>, in PBS), respectively. The injected shrimps were returned to seawater tanks immediately and the hemocytes and hepatopancreas of at least five individuals were randomly sampled from each group at 3, 6, 12, 24 and 48 h post injection, kept in RNA<sup>later</sup> and stored at -80 °C until RNA isolation.

#### RNA isolation and cDNA synthesis

Total RNA was isolated from different tissues using TRIzol reagent (15596026, Thermo Fisher

Scientific, USA). The synthesis of first strand was carried out with Promega M-MLV (M1701, Promega, USA) using the DNase I (RQ1, M6101, Promega, USA) treated total RNA as template and adaptor primer-oligo (dT) as primer (Table 1). The reaction was performed at 42 °C for 1 h, terminated by heating to 95 °C for 5 min, and then stored at -80 °C.

#### Cloning the full-length cDNA of LvMIF2

The partial length sequence of LvMIF2 cDNA was obtained from the white shrimp transcriptome sequencing database (Qi *et al.*, 2017). Two gene-specific primers, LvMIF2-RACE-F1/2, were designed with Primer Premier 6.00 based on this partial length sequence to clone the 3' end of LvMIF2 cDNA by rapid amplification of cDNA ends (RACE) technique. And the coding sequence (CDS) of LvMIF2 was amplified and confirmed using another two gene-specific primers, LvMIF2-CDS-F/R, which was also designed with Primer Premier 6.00. All PCR amplification was performed in a MGL96G Peltier Thermal Cycler (LongGene, China). The PCR products were purified using Monarch DNA Gel Extraction Kit (T1020S, NEB, USA) and then cloned into the pMD18-T simple vector (D103A, Takara, Japan). After being transformed into the competent cells *Escherichia coli* strain DH5α (CB101, Tiangen, China), the positive recombinants were identified via anti-ampicillin selection and verified by PCR screening using M13-47 and RV-M primers (Table 1). Three of the positive clones were sequenced using a PRISM 3730XL automated sequencer (Thermo Fisher Scientific, USA).

#### Bioinformatical analysis of cDNA and protein sequences

The search for protein sequence similarity was conducted with blastp 2.6.0. The deduced protein sequences were analyzed by the EditSeq module in Lasergene program suite 14.0.0.88. The function domains were predicted with Simple Modular Architecture Research Tool (SMART) 7.0. Multiple sequence alignments were performed with Clustal Omega 1.2.4 and visualized using multiple alignment

**A**

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1 GAACGCCACGCCAACACTGAGCGGACCTGACGTTTCGAGAAAAGAGTCTTGCAATTCAAA
1 M P Y L E I A
61 ATTATCTAGCAGGAAGTAGAGCATTGAGGAAGGTATAAAAATGCCTTATTTGGAGATTG
8 T N I P R D R V T P E I V S T F S K I L
121 CAACAAATATTCTAGAGACAGAGTGACTCCTGAGATTGTTTCAACCTTTAGTAAAATTC
28 S E T I G K P E Q Y C V V R V I P D Q L
181 TTAGTGAAACCATTGGGAAACCAGAACAGTATTGTGTTGTTAGAGTAATTCCTGATCAGC
48 M S F G G T F D P C G S A I L M S L G K
241 TGATGAGTTTTGGAGGCACATTTGATCCTTGTGGTTCTGCAATTTTGTGAGCCTTGGCA
68 L G V E E N K T Y A S K I Y A V V E K Y
301 AGTTAGGGGTTGAAGAAAACAAAACGTATGCTTCTAAGATTTATGCAGTTGTGGAGAAAT
88 L G I P N D R M Y I Q F I D K Q T S E V
361 ACCTGGGATTCCTAATGATCGAATGTACATCCAATTCATAGATAAACAGACATCAGAAG
108 G F K G T T F H Q I L G R *
421 TTGGCTCAAAGGAACAACCTCCACCAGATATTGGGACGTTAAAAAGTCAGAAAGTTCGC
481 AAGATCCTCAAGTGAATGAAGATATTGCAATATGCTTACTGTGCACACTTCTGCATCA
541 GCACCATACATTTACATGCAGGATAATATGAGCAGATATCGTTTATTGCTTGTATGCTT
601 GTGATTTCATGGTATCTTTAGATGCTAAGGAATGATACTCATATCATAATCTAGATAGCT
661 TATTATTCATCCTTGACAACCTGTCAGTAG

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**B**

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1 GCTCATTCTCCCTTTTAGTCAGCTGATAAATCCCGTCCACGCAGCAGTAGTTGTAGTAAC
1 M P F V T L T T
61 ATCAAGTGATAGTATTTTTTAAAAGGATCTTCTAATATGCCTTTCGTAACCCTGACTAC
9 N L S S E K V D K A F S E K F S E K L A
121 CAATCTGTCTTCTGAAAAAGTGGACAAAGCCTTTTCGGAGAAGTTTTCGGAAAAGTTAGC
29 E T L G K P I E R I S V T V V A G Q Q M
181 AGAACTTTAGGGAACCCATAGAACGTATTTCACTGACTGTAGTGGCTGGGCAGCAGAT
49 C R G G S W D P V C E I H V L A I G L D
241 GTGCCGAGGTGGAAGCTGGGACCCAGTGTGTGAGATTCACGTGTTAGCCATTGGACTAGA
69 S S E K T Q K P A E E L T K F V S D R T
301 TTCCTCAGAGAAAACCCAAAAGCCAGCGGAAGAATTGACCAAATTCGTGTCTGACCGAAC
89 G I Q P A R I C C T F R P L L P H Q V S
361 TGGCATTCAACCTGCAAGAATCTGCTGCACCTCCGACCACTACTACCTCATCAGGTTTC
109 V N G A L M G *
421 CGTGAATGGGGCTCTAATGGGATGACTTCAACGACCATCGAGTGTGACTTCAGCCATAAT
481 AGATGATTTGGAATGAGCTAGCAATAAACTTTTTATAGGGCAAAAAAAAAAAAAAAAAA
541 AAAAAAAAAAAAAAAAAA

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**Fig. 1** Nucleotide and deduced amino acid sequences of *LvMIF1* and *LvMIF2* (A: *LvMIF1*, B: *LvMIF2*). The nucleotides and amino acids were numbered along the left margin. The function domain was in shade. The conserved tautomerase activity sites (P<sup>2</sup> and K<sup>33</sup>) are boxed. The conserved oxidoreductase activity site (C<sup>57</sup>/C<sup>58</sup>) is marked by circle. The asterisks indicated the stop codon. The single typical polyadenylation signal (AATAAA) was double underlined.

show module in Sequence Manipulation Suite 2.0. A Neighbor-Joining (NJ) phylogenetic tree was constructed with MEGA 7.0.21. To derive confidence value for the phylogeny analysis, bootstrap trials were replicated 1,000 times.

#### Expression pattern analysis via real-time quantitative RT-PCR

The mRNA transcripts of *LvMIF2* and the previously identified *LvMIF1* in different tissues or their temporal expression patterns in hemocytes and hepatopancreas of shrimps stimulated with *V. parahaemolyticus* or WSSV were investigated by quantitative real-time PCR (qRT-PCR) technique. All qRT-PCR reactions were performed with the SYBR

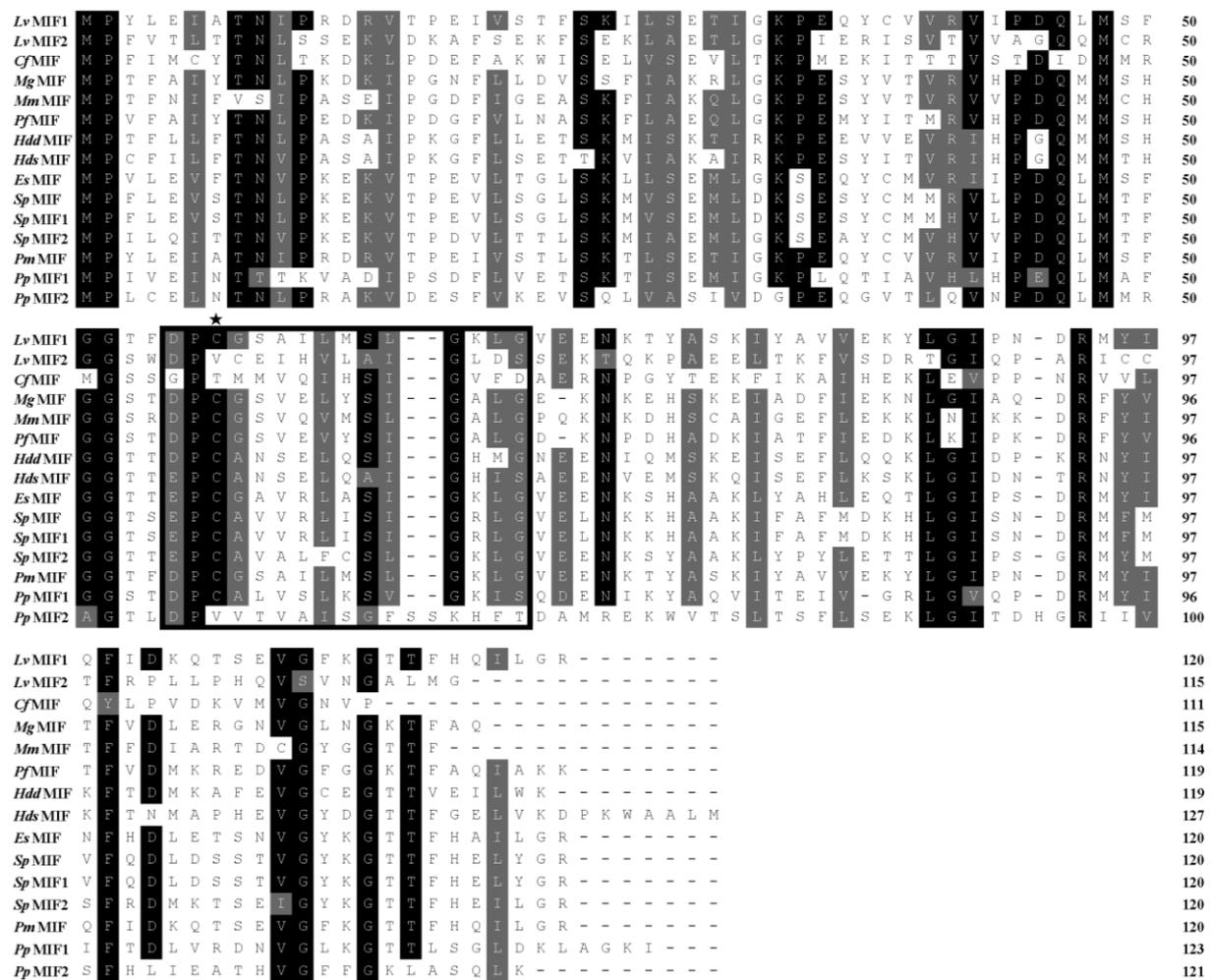
premix ExTaq (Tli RNaseH plus) (RR420, Takara, Japan) using 100 ng cDNA template in a LineGene K FQD-48A (A4) Fluorescence Quantitative PCR Detection System (Bioer, China). All the primers for qRT-PCR were designed with PerlPrimer 1.1.21 and listed in Table 1. The mRNA expression levels of *LvMIF2* and *LvMIF1* were normalized to those of elongation factor 1  $\alpha$  (EF-1 $\alpha$ ) for each sample. The relative mRNA expression levels of *LvMIF2* and *LvMIF1* were generated using comparative C<sub>T</sub> method (2<sup>- $\Delta\Delta C_t$</sup>  method) (Schmittgen and Livak, 2008). The data were subjected to one-way analysis of variance (ANOVA) followed by a multiple comparison using IBM SPSS Statistics 23.0.0.0, and the *p* values less than 0.05 were considered statistically significant.

## Results

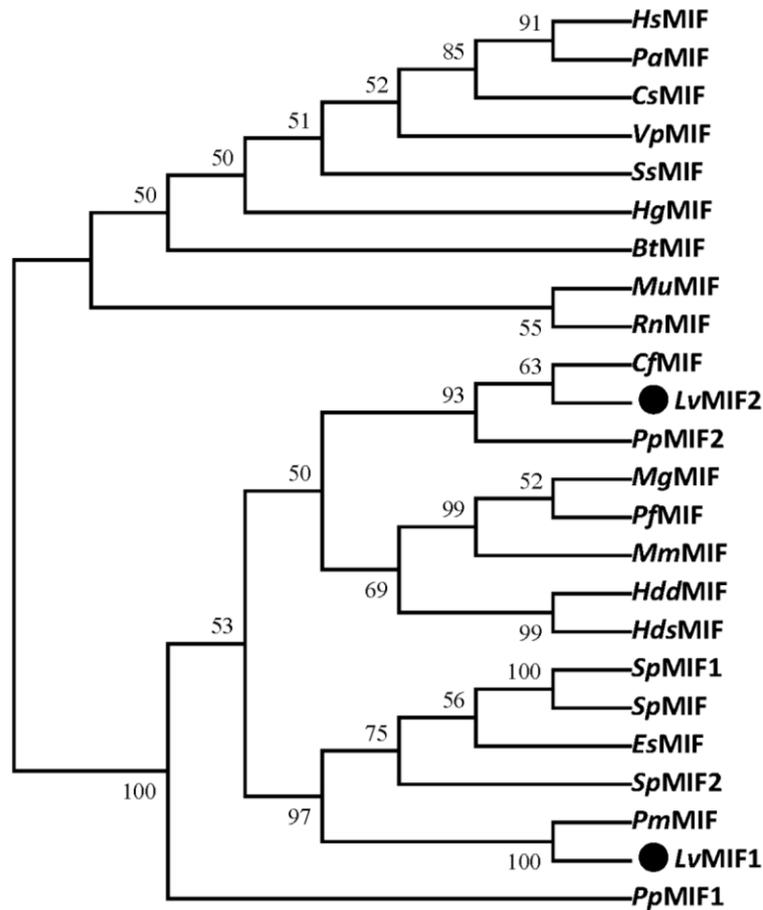
### Sequence features of *LvMIF2*

The full-length cDNA sequence of *LvMIF2* was obtained by 3' RACE technique, and deposited in GenBank under the accession number MF062461. It comprised 555 bp, containing a 5' untranslated regions (UTR) of 97 bp, a 3' UTR of 110 bp with a poly A tail and an open reading frame (ORF) of 348 bp. The ORF encoded a polypeptide of 115 amino acid residues with a calculated molecular mass of approximately 12.53 kDa and a theoretical isoelectric point (pI) of 7.12 (Table 2). The deduced protein sequence of *LvMIF2* contained a MIF domain (from P<sup>2</sup> to G<sup>115</sup>), two conserved tautomerase activity sites (P<sup>2</sup> and K<sup>33</sup>) and one conserved oxidoreductase activity (C<sup>58</sup>) (Fig. 1). An alignment

of the protein sequence of *LvMIF2* with those of previously identified MIFs was shown in Figure 2, and a MIF family signature (from D<sup>55</sup> to G<sup>69</sup>) was revealed. The deduced protein sequence of *LvMIF2* exhibited high similarity with other previously identified MIFs, such as 52 % identity with that of *SpMIF1* (AKT09427) (Huang *et al.*, 2016). The NJ phylogenetic tree based on protein sequences of multiple MIFs was positioned separately into two main branches, the vertebrate one and the invertebrate one, and *LvMIF1* was clustered with its homologue from the black tiger shrimp *P. monodon*, *PmMIF*, and located in the Crustacean sub-branch, while *LvMIF2* were clustered with its homologue from Zhikong scallop *C. farreri*, *CfMIF*, and located in the Mollusca sub-branch (Fig. 3).



**Fig. 2** Multiple alignments of *LvMIF1* and *LvMIF2* with previous known MIFs. The MIF family signatures were boxed. The sites of MIF tautomerase activity were indicated with ▼. The conserved oxidoreductase activity site was indicated with ★. The sequences and their accession numbers are as follows: *CfMIF*, *Chlamys farreri*, ADF87941; *MgMIF*, *Mytilus galloprovincialis*, AEN25591; *MmMIF*, *Meretrix meretrix*, AKN56918; *PfMIF*, *Pinctada fucata*, ADU19847; *HddMIF*, *Haliotis discus discus*, ACJ65690; *HdsMIF*, *Haliotis diversicolor supertexta*, ABX76741; *EsMIF*, *Eriocheir sinensis*, ADM86239; *SpMIF*, *Scylla paramamosain*, AGB07600; *SpMIF1*, *Scylla paramamosain*, AKT09426; *SpMIF2*, *Scylla paramamosain*, AKT09428; *PmMIF*, *Penaeus monodon*, ANZ80588; *PpMIF1*, *Patiria pectinifera*, BAR92638 and *PpMIF2*, *Patiria pectinifera*, BAR92639.



**Fig. 3** Consensus neighbor-joining phylogenetic based on the amino acid sequences of MIFs from different organisms. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1,000 replicates was taken to represent the evolutionary history of the taxa analyzed. All positions containing gaps and missing data were eliminated. The numbers at the forks indicated the bootstrap value. The sequences and their accession numbers are as follows: CfMIF, *Chlamys farreri*, ADF87941; MgMIF, *Mytilus galloprovincialis*, AEN25591; MmMIF, *Meretrix meretrix*, AKN56918; PmMIF, *Pinctada fucata*, ADU19847; HddMIF, *Haliotis discus discus*, ACJ65690; HdsMIF, *Haliotis diversicolor supertexta*, ABX76741; EsMIF, *Eriocheir sinensis*, ADM86239; SpMIF, *Scylla paramamosain*, AGB07600; SpMIF1, *Scylla paramamosain*, AKT09426; SpMIF2, *Scylla paramamosain*, AKT09428; PmMIF, *Penaeus monodon*, ANZ80588; PpMIF1, *Patiria pectinifera*, BAR92638; PpMIF2, *Patiria pectinifera*, BAR92639; HsMIF, *Homo sapiens*, NP\_002406; PaMIF, *Pongo abelii*, XP\_009232451; CsMIF, *Carlito syrichta*, XP\_008064641; VpMIF, *Vicugna pacos*, NP\_001274126; SsMIF, *Sus scrofa*, NP\_001070681; HgMIF, *Heterocephalus glaber*, NP\_001266756; BtMIF, *Bos taurus*, NP\_001028780; MuMIF, *Meriones unguiculatus*, XP\_021509432 and RnMIF, *Rattus norvegicus*, NP\_112313.

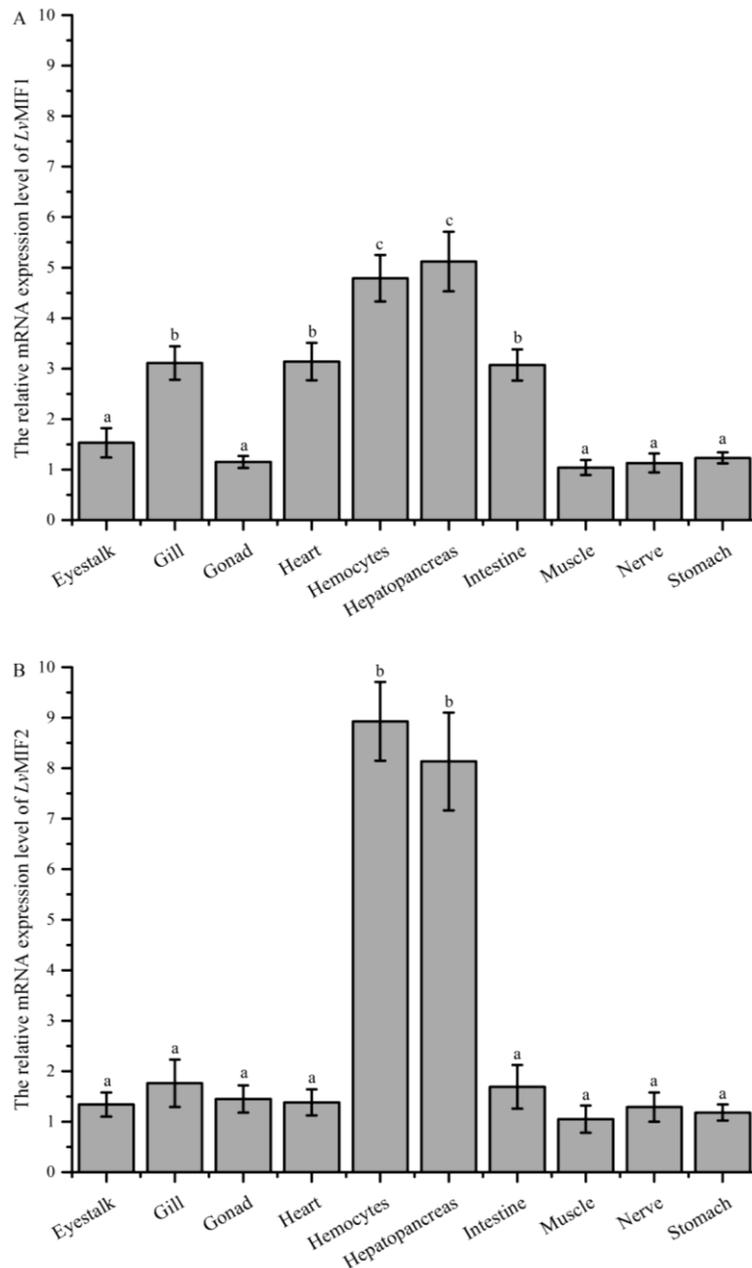
#### Tissue distribution of LvMIF1 and LvMIF2

The qRT-PCR was employed to detect the tissue distribution of LvMIF1 and LvMIF2 mRNA transcripts with EF-1 $\alpha$  as internal control. The LvMIF1 mRNA transcripts could be detected in all the tested tissues, including eyestalk, gill, gonad, heart, hemocytes, hepatopancreas, intestine, muscle, nerve and stomach. The highest mRNA expression level of LvMIF1 was found in hepatopancreas and hemocytes, which was 5.12-fold and 4.79-fold of that in muscle ( $p < 0.05$ ), respectively, followed by heart, gill and intestine, which were 3.14-fold, 3.11-fold and 3.07-fold of that in muscle ( $p < 0.05$ ), respectively (Fig. 4A). The mRNA transcripts of LvMIF2 were also constitutively

expressed in all the selected tissues of untreated shrimps, with the specific highest expression level in hemocytes and hepatopancreas, which was 8.93-fold and 8.13-fold of that in muscle ( $p < 0.05$ ), respectively (Fig. 4B).

#### The mRNA expression profiles of LvMIF1 and LvMIF2 post bacterial stimulation

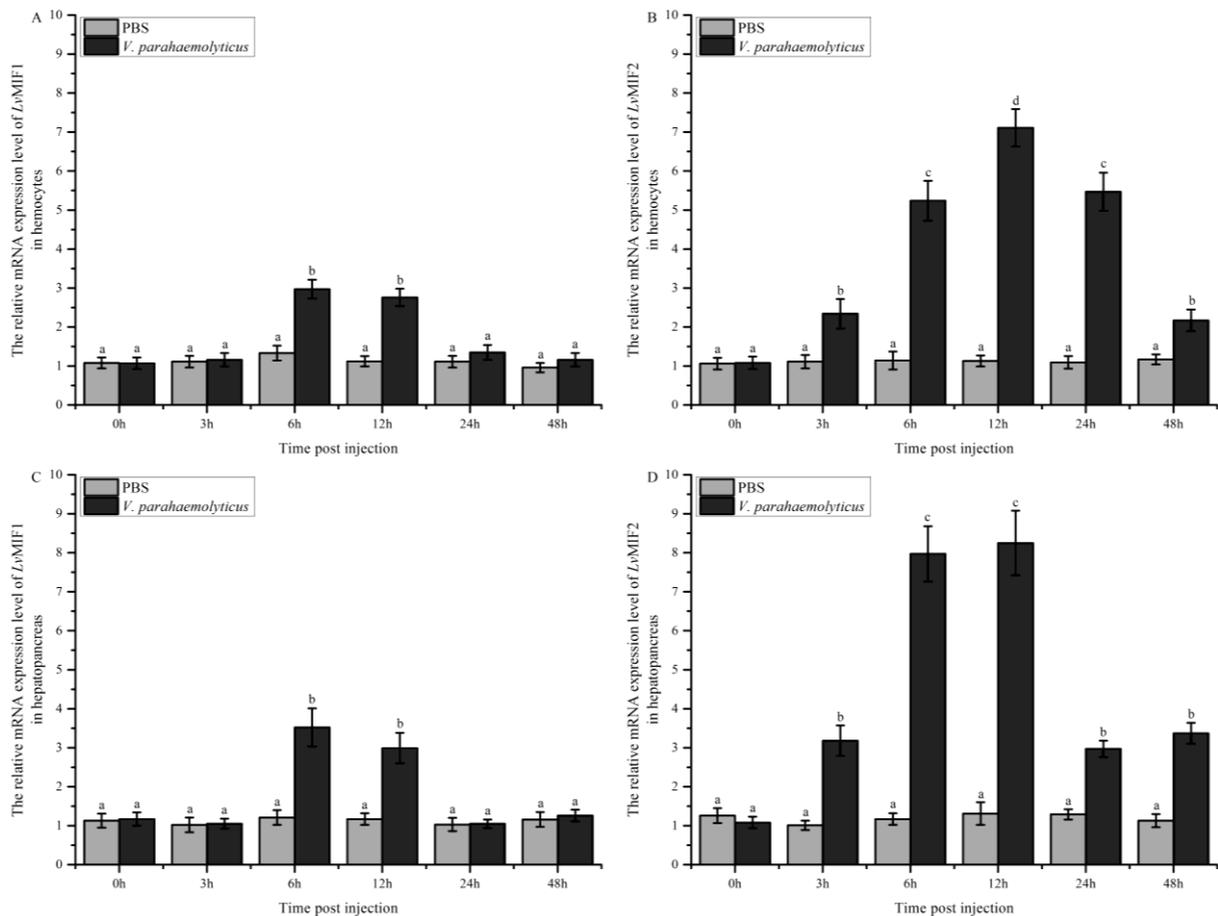
The qRT-PCR was performed to analyze the mRNA expression patterns of LvMIF1 and LvMIF2 in hemocytes and hepatopancreas of shrimp challenged by *V. parahaemolyticus*. The mRNA transcripts of LvMIF1 in hemocytes significantly increased and reached the peak at 6 h post *V. parahaemolyticus* stimulation (2.97-fold compared



**Fig. 4** Tissue distribution of LvMIF1 and LvMIF2 mRNA transcripts detected by qRT-PCR technique (A: LvMIF1, B: LvMIF2). The mRNA expression levels in eyestalk, gill, gonad, heart, hemocytes, hepatopancreas, intestine, muscle, nerve and stomach of five untreated shrimps were normalized to that of muscle. The EF-1 $\alpha$  gene was used as an internal control to calibrate the cDNA template for each sample. Vertical bars represent mean  $\pm$  SD (n = 5), and bars with different characters were significantly different ( $p < 0.05$ ).

with the origin level,  $p < 0.05$ ), kept at a high level at 12 h (2.76-fold,  $p < 0.05$ ), and then decreased to the origin level at 24 h (Fig. 5A). After stimulated with *V. parahaemolyticus*, LvMIF2 mRNA transcripts in hemocytes significantly increased at 3 h post stimulation (2.33-fold,  $p < 0.05$ ) and reached the peak level at 12 h (7.11-fold,  $p < 0.05$ ), kept at a high level at 24 h (5.47-fold,  $p < 0.05$ ) and then decreased but still higher than the origin level at 48 h (2.17-fold,  $p < 0.05$ , Fig. 5B). The mRNA expression profile of LvMIF1 in hepatopancreas was similar with that in hemocytes, it also significantly increased and

reached the peak at 6 h post *V. parahaemolyticus* stimulation (3.52-fold,  $p < 0.05$ ), kept at a high level at 12 h (2.99-fold,  $p < 0.05$ ), and then decreased to the origin level at 24 h (Fig. 5C). While LvMIF2 mRNA transcripts in hepatopancreas significantly increased at 3 h post bacterial stimulation (3.18-fold,  $p < 0.05$ ) and reached the peak level at 6 h and 12 h post stimulation (7.97-fold and 8.25-fold, respectively,  $p < 0.05$ ), and then decreased but still higher than the origin level at 24 h and 48 h (2.97-fold and 3.37-fold, respectively,  $p < 0.05$ , Fig. 5D).



**Fig. 5** Temporal mRNA expression profiles of LvMIF1 and LvMIF2 detected via qRT-PCR technique in white shrimp hemocytes and hepatopancreas post bacterial stimulation (A: LvMIF1 in hemocytes, B: LvMIF2 in hemocytes, C: LvMIF1 in hepatopancreas, D: LvMIF2 in hepatopancreas). The EF-1 $\alpha$  gene was used as an internal control to calibrate the cDNA template for each sample. Vertical bars represent mean  $\pm$  SD ( $n = 5$ ), and bars with different characters were significantly different ( $p < 0.05$ ).

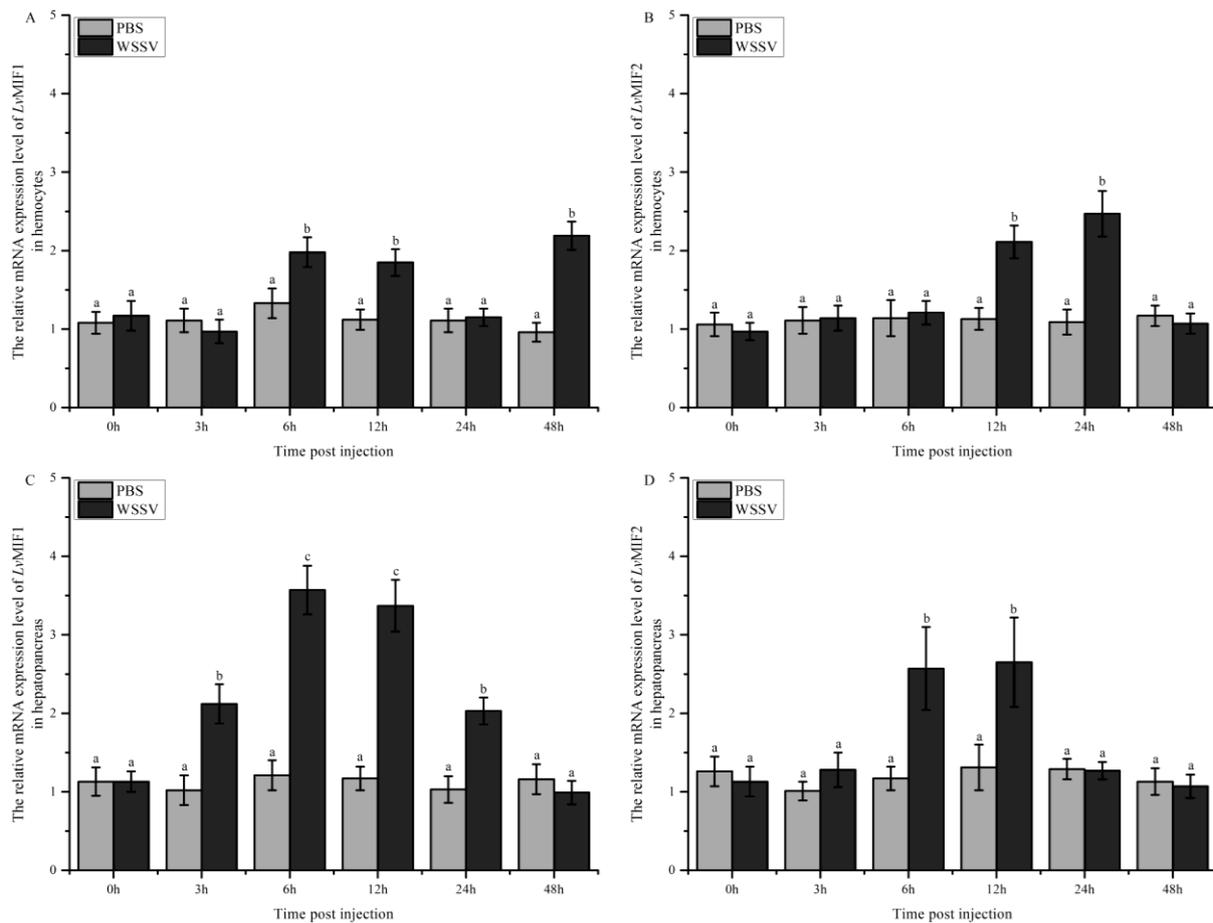
#### The mRNA expression profiles of LvMIF1 and LvMIF2 post viral stimulation

The qRT-PCR was performed to analyze the mRNA expression patterns of LvMIF1 and LvMIF2 in hemocytes and hepatopancreas of shrimp challenged by WSSV. After challenged by WSSV, the mRNA expression level of LvMIF1 in hemocytes was significantly upregulated at 6 h and 12 h (1.98-fold and 1.85-fold, respectively,  $p < 0.05$ ), downregulated to the origin level at 24 h, and then significantly upregulated again at 48 h (2.19-fold,  $p < 0.05$ , Fig. 6A). During the WSSV challenge, the mRNA transcripts of LvMIF2 in hemocytes significantly increased at 12 h and 24 h (2.11-fold and 2.47-fold, respectively,  $p < 0.05$ ) and then decreased to the origin level at 48 h (Fig. 6B). In hepatopancreas, LvMIF1 mRNA transcripts significantly increased at 3 h post stimulation (2.12-fold,  $p < 0.05$ ) and reached the peak level at 6 h and 12 h (3.57-fold and 3.37-fold, respectively,  $p < 0.05$ ), kept at a high level at 24 h (2.03-fold,  $p < 0.05$ ) and then decreased to the origin level at 48 h (Fig. 6C). While the mRNA expression level of LvMIF2 in

hepatopancreas was significantly upregulated at 6 h and 12 h (2.57-fold and 2.65-fold, respectively,  $p < 0.05$ ) and then decreased to the origin level at 24 h (Fig. 6D).

#### Discussion

For approximately half a century, MIF has been considered as a mysterious cytokine (Calandra and Thierry, 2003). Recently, MIF has assumed an important role as a pivotal modulator of innate immunity (Sparkes *et al.*, 2017). In the present study, the full-length cDNA of a novel MIF homologue, LvMIF2, was obtained from white shrimp *L. vannamei*. The deduced polypeptide of LvMIF2 consisted of 115 amino acids, and its calculated molecular weight was 12.53 kDa, which was close to the previously identified LvMIF1. The protein sequence of LvMIF2 shared 52 % similarities with the previously identified SpMIF1. Moreover, a MIF domain and a MIF family signature were revealed from the amino acid sequence of LvMIF2. The amino terminal proline residue (P<sup>2</sup>), invariant lysine residue



**Fig. 6** Temporal mRNA expression profiles of LvMIF1 and LvMIF2 detected via qRT-PCR technique in white shrimp hemocytes and hepatopancreas post viral stimulation (A: LvMIF1 in hemocytes, B: LvMIF2 in hemocytes, C: LvMIF1 in hepatopancreas, D: LvMIF2 in hepatopancreas). The EF-1 $\alpha$  gene was used as an internal control to calibrate the cDNA template for each sample. Vertical bars represent mean  $\pm$  SD (n = 5), and bars with different characters were significantly different ( $p < 0.05$ ).

(K<sup>33</sup>) and conserved oxidoreductase activity (C<sup>58</sup>), which are critical active sites of tautomerase activity in mammalian MIF, were also revealed (Xie *et al.*, 2016). The conserved function domain and signature sequence of LvMIF2 and high similarity with other previously identified MIFs collectively suggested that LvMIF2 was a novel member of invertebrate MIF family, and it could have similar functions to those from vertebrates and other invertebrates. While in the NJ phylogenetic tree, LvMIF1 were clustered with its homologues from crustacean, and LvMIF2 were clustered with its homologues from Mollusca, indicating LvMIF2 might be more ancient than LvMIF1.

Previous researches showed that MIF was ubiquitously expressed in various tissues (Lue *et al.*, 2002). In the present study, qRT-PCR analysis indicated that both LvMIF1 and LvMIF2 mRNA transcripts were also constitutively expression in all the investigated tissues from healthy white shrimp, which were consistent with the previously observation in other crustacean (Li *et al.*, 2011b; Fang *et al.*, 2013; Xie *et al.*, 2016), and the ubiquity

of MIF mRNA in all tissues in crustacean might be attributed to the open circulatory system (McMahon and Burnett, 1990). The highest mRNA expression level of LvMIF1 was found in hepatopancreas and hemocytes, followed by heart, gill and intestine, which was consistent with the previous results (Zeng *et al.*, 2013), and also confirmed that the qRT-PCR analysis in the present study were credible. While the highest expression level of LvMIF2 mRNA was also detected in hemocytes and hepatopancreas. Similarly, in *E. sinensis*, EsMIF was highest expressed in hepatopancreas (Li *et al.*, 2011b). In *S. paramamosain*, SpMIF was highly expressed in hepatopancreas and hemocytes (Fang *et al.*, 2013). And in *P. monodon*, the highest level of PmMIF mRNA was detected in hepatopancreas, followed by gills and heart (Xie *et al.*, 2016). All the results collectively confirmed the hypothesis that hemocytes, hepatopancreas and gills might play pivotal roles in the innate immune system of crustacean (Cerenius *et al.*, 2010).

MIF could be released in response to bacterial stimulation (Calandra and Thierry, 2003). In order to

clarify the potential role of LvMIF1 and LvMIF2 in regulating shrimp innate immunity, their temporal expression profiles in both hemocytes and hepatopancreas post *V. parahaemolyticus* stimulation was detected and compared by qRT-PCR technique. The mRNA expression levels of LvMIF1 in both hemocytes and hepatopancreas were significantly upregulated at 6 h and 12 h post injection. Compared with LvMIF1, LvMIF2 exhibited a fast and intense mRNA expression pattern, and the LvMIF2 mRNA transcripts in both hemocytes and hepatopancreas sharply increased at 3 h post injection and reached the maximum at 12 h. Likewise, in *E. sinensis*, the mRNA expression level of EsMIF in hepatopancreas was sharply upregulated from 6 h post *V. anguillarum* challenge and reached the peak at 12 h (Li *et al.*, 2011b). The mRNA expression level of SpMIF from *S. paramamosain* in hemocytes increased significantly after a 6 h challenge by *V. parahaemolyticus* and peaked at 8 h (Fang *et al.*, 2013). And in *P. monodon*, after the infection of *V. harveyi*, PmMIF mRNA expression level in hepatopancreas was sharply upregulated at 6 h post infection and reached the maximum at 12 h (Xie *et al.*, 2016). These results indicate that both LvMIF1 and LvMIF2 were involved in the innate immune system of shrimp. While as a fast releasing and intensive responsible defense molecule, LvMIF2 might play a more important role in the defense to bacterial stimulation in the innate immunity of shrimp.

To further understand the immunological roles of LvMIF1 and LvMIF2 in shrimp, their temporal expression profiles in both hemocytes and hepatopancreas post WSSV stimulation was also detected and compared by qRT-PCR technique. After challenged by WSSV, the mRNA expression level of LvMIF1 in hemocytes was significantly upregulated at 6 h, downregulated to the origin level at 24 h, and then significantly upregulated again at 48 h, while the mRNA transcripts of LvMIF2 in hemocytes significantly increased at 12 h and 24 h and then decreased to the origin level at 48 h. In hepatopancreas, LvMIF1 significantly increased at 3 h post stimulation, reached the maximum at 6 h, kept at a high level at 24 h and then decreased to the origin level at 48 h, which was consistent with the previous results (Zeng *et al.*, 2013), while the mRNA expression level of LvMIF2 was significantly upregulated at 6 h and 12 h and then decreased to the origin level at 24 h. All the results collectively indicated that both LvMIF1 and LvMIF2 might participate in the shrimp immune response to viral infection, and LvMIF1 might play a more important role than LvMIF2.

In conclusion, the full-length cDNAs of a novel MIF, LvMIF2, were cloned from white shrimp, and its mRNA expression profiles were detected and compared with the previously identified LvMIF1, when the shrimps were exposed to bacterial and viral stimulation. These results of comparative sequence and transcription analysis suggested that both LvMIF1 and LvMIF2 may play crucial and functionally differentiated roles in innate immune responses to bacterial and viral stimulation. The results of this study would enrich the understanding of the shrimp immune immunity.

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