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One type of VEGFR is involved in WSSV infection to the Pacific whiteleg shrimp *Litopenaeus vannamei*

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ABSTRACT

VEGF signaling pathway plays vital roles in many physiological processes including cell proliferation, differentiation, migration, survival, cell–cell communication, vessel permeability and virus-host interaction in mammalian species. However, the VEGF signaling pathway and its biological function are still poorly understood in crustaceans. In the present study, an essential member of VEGF signaling pathway, VEGF receptor (*LvVEGFR*), was isolated from Penaeid shrimp *Litopenaeus vannamei* and its function during virus infection was analyzed. The deduced amino acid sequence of *LvVEGFR* possessed all common features of VEGFRs reported in other species, including a signal peptide, six IG-like domains, one immunoglobulin subtype 2 domain, a transmembrane domain, a juxtamembrane domain, a protein kinase domain separated by a kinase insert sequence, one ATP binding site and one tyrosine-protein kinase active site. *LvVEGFR* is mainly expressed in hemocytes and intestine. The transcriptional level of *LvVEGFR* could be obviously up-regulated in hemocytes and intestine after WSSV infection. Silencing of *LvVEGFR* gene by double-strand RNA (dsRNA) interference could not only lead to a decrease of virus copy number in WSSV infected shrimp, but also reduce the mortality of shrimp during WSSV infection. These data suggested that VEGF signaling pathway might play an important role during viral infection to shrimp.

1. Introduction

The vascular endothelial growth factor (VEGF) signaling pathway works essentially in many physiological processes such as cell proliferation, differentiation, migration, survival, cell–cell communication, vessel permeability, etc. (Cebe-Suarez et al., 2006; Nagy et al., 2008; Shibuya and Claesson-Welsh, 2006). VEGFs and VEGF receptors (VEGFRs) are main members of the VEGF signaling pathway. VEGF signaling pathway performs its biological function after activation of tyrosine kinase receptors (the VEGFRs) by combination with their ligand VEGFs (Neufeld et al., 1999; Tammela et al., 2005). In mammalian species, three kinds of VEGFRs, including VEGFR1, VEGFR2 and VEGFR3, have been identified interacting with five VEGF family members including VEGFA, VEGFB, VEGFC, VEGFD and placenta growth factor (PLGF). Activation of VEGFR-1 by VEGF-A, VEGF-B and PLGF, or activation of VEGFR-2 by VEGF-A, VEGF-C and VEGF-D, is required for normal development and angiogenesis (Rahimi, 2006), while VEGFR-3 is mainly involved in hematopoiesis and lymphogenesis by binding to VEGF-C and VEGF-D (Jussila and Alitalo, 2002). In *Drosophila*, the signaling pathway is designated as vascular endothelial growth factor/platelet-derived growth factor (VEGF/PDGF) signaling pathway, which is vital for regulating the proliferation (Munier et al., 2002), migration of hemocyte precursor cells in *Drosophila* embryos (Heino et al., 2001; Parsons and Foley, 2013).

VEGF signaling pathway also plays important roles in virus–host interaction. A new VEGF from parapoxvirus could activate host VEGFR-2 in bovine testis or ovine testis cells, further participates in promoting a distinctive pattern of epidermal proliferation and also contributes to virus replication (Savory et al., 2000). Once activated, the VEGF signaling pathway functions through phosphorylation and activation of downstream signaling pathways such as PI3K and MAPK/ERK signaling pathways (Takahashi et al., 2003), which are also important for virus infection and replication (Barber et al., 2002; Hsu et al., 2010).

Previously, we found that the transcription of genes in the VEGF signaling pathway was up-regulated in shrimp during acute infection of white spot syndrome virus (WSSV) based on shrimp transcriptome data (Li et al., 2013b). The expression of an identified VEGF member in *Eriocheir sinensis, EsPVF1*, was up-regulated by *Vibrio anguillarum, Pichia pastoris* GS115 injection or tissue injury (Li et al., 2013a). In *Marsupenaeus japonicas*, two types of VEGF, *MjVEGF* and *MjVEGF2*, were also identified and considered playing

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function in innate immunity (Inada et al., 2013). These data indicated that the VEGF signaling pathway might play important roles in the immune system in crustacean. However, there is no report on VEGFRs in crustacean and the biological function of VEGF signaling pathway in immunity is still not clear.

In order to understand the function of VEGF signaling pathway in shrimp immunity, we identified a VEGFR gene from the whiteleg shrimp *Litopenaeus vannamei* and characterized its function during WSSV infection in the present study. To our knowledge, this is the first time to report a VEGFR gene in crustacean and describe its biological function during WSSV infection.

2. Materials and methods

2.1. Experimental animals

Indoor cultured whiteleg shrimp, *L. vannamei*, with a body length of 12.5 ± 0.4 cm and a body weight of 25.1 ± 2.5 g, were used for tissue dissection and WSSV infection experiments. Shrimp with a body length of 6.8 ± 0.5 cm and a body weight of 4.4 ± 1.0 g were used for RNA interference to analyze the function of *LvVEGFR* during WSSV infection. Before experiments, shrimp were acclimated in the aerated seawater at 25 ± 1 °C and fed with shrimp food pellet.

2.2. Tissue collection

Hemolymph from 10 healthy shrimp was collected from the ventral sinus located at the first abdominal segment using a syringe with equal volume of anticoagulant-modified Alsever solution (Rodriguez et al., 1995). Hemocytes were isolated by centrifugation at 800 g, 4 °C, for 10 min and then preserved in liquid nitrogen. Tissues including muscle, gill, hepatopancreas, nerve cord, intestine, stomach, heart, skin, lymphoid organ, eyestalk, ovary and testis were dissected from these shrimp and preserved in liquid nitrogen for total RNA extraction.

The healthy shrimp were also used for infection experiment. Each shrimp was injected with 8000 copies WSSV suspended in 20 μl PBS. Shrimp injected with equal volume PBS were set as control. At 1, 6, 12 and 24 hour (h) post injection, hemocytes and intestine from 10 treated individuals were collected and preserved following the above-mentioned procedures, respectively. Hemocytes and intestine from 10 untreated individuals were also collected and taken as samples at 0h.

2.3. Total RNA extraction, cDNA synthesis

Total RNA from each tissue was extracted with Unizol reagent (UnionGene, China) following the manufacturer's protocol. RNA quality was assessed by electrophoresis on 1% agarose gel.

Total RNA was treated with RQ1 RNase-free DNase (Promega, USA) to remove the contaminated DNA. cDNA was synthesized from 1 μg total RNA by Moloney-Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega, USA) following the manufacturer's protocol with Hexamer primer (NNN NNN) (Sangon, China).

2.4. cDNA cloning and sequence analysis

An assembled nucleotide sequence encoding VEGFR was obtained from an Illumina-based transcriptome sequencing database of *L. vannamei* (Wei et al., 2014). Four pairs of primers, LvVEGFR-cF1/LvVEGFR-cR1, LvVEGFR-cF2/LvVEGFR-cR2, LvVEGFR-cF3/LvVEGFR-cR3 and LvVEGFR-cF4/LvVEGFR-cR4, were designed to amplify and validate the assembled sequence. The nucleotide sequences and annealing temperature of primers were shown in Table 1.

Table 1
Nucleotide sequences of primers used for *LvVEGFR* cloning and expression analysis.

Name	Nucleotide sequence (5′-3′)	Annealing temperature (C)	Product size (bp)
LvVEGFR-cF1	GCCAGGGCAAGGCAAGGTT	58	962
LvVEGFR-cR1	AATGTTGATTCGCAGCCCAC		
LvVEGFR-cF2	CCCAAGCCTGCTATTGAGTGG	60	1757
LvVEGFR-cR2	GGCAATAACTCAGCTTGTTCG		
LvVEGFR-cF3	CGGACGAACAAGCTGAGTTA	59	831
LvVEGFR-cR3	CTCGGTAGTATCCAACAGATC		
LvVEGFR-cF4	TCTGTTGGATACTACCGAGAC	58	1590
LvVEGFR-cR4	CTGGAAAGCAAAGCACCGC		
LvVEGFR-qF	GTTTGGCAGCAATCCTCCTC	57	128
LvVEGFR-qR	GAAGATCAGTTGTCCAGTTCC		
LvVEGFR-dsF	TAATACGACTCACTATAGGG CAAGTGTAGATGTCGGTGAG	55	479
LvVEGFR-dsR	TAATACGACTCACTATAGGG GACCTGGGTGTACTGTGAG		
EGFP-dsF	TAATACGACTCACTATAGGG CAGTGCTTCAGCCGCTACCC	55	289
EGFP-dsR	TAATACGACTCACTATAGGG AGTTCACCTTGATGCCGTTCTT		
18S-F	TATACGCTAGTGGAGCTGGAA	56	150
18S-R	GGGGAGGTAGTGACGAAAAAT		
VP28-F	TGTGACCAAGACCATCGAA	55	281
VP28-R	CCACACCTTGAATGTTCCC		
VP28-qF	AAACCTCCGCATTCCTGTGA	55	141
VP28-qR	TCCGCATCTTCTTCCTTCAT		
LvFAK-qF	ATTACTCAACACCAGCAACC	57	172
LvFAK-qR	GTTCCCTCGGACTCCACCTT		
LvPI3K-qF	TATGAAGTAACCCGTAGTGCCA	57	187
LvPI3K-qR	TGCCCACATCTCCTGACTGA		

The underlined nucleotide sequences are T7 promoters.

Protein domains of LvVEGFR were predicted with BLAST algorithm (NCBI, http://www.ncbi.nlm.nih.gov/BLAST/) and InterProScan software (http://www.ebi.ac.uk/interpro/). Signal peptide and transmembrane domain were predicted by CBS prediction servers (http://www.cbs.dtu.dk/services). Phylogenic analysis was constructed on VEGFR from different organisms (Table 2) using MEGA software version 4.0 (Tamura et al., 2007).

2.5. Preparation of dsRNA

A pair of primers with T7 promoter sequences, LvVEGFR-dsF and LvVEGFR-dsR (Table 1), were designed to amplify a 479 bp cDNA fragment of *LvVEGFR* gene. The PCR program was performed as follows: one cycle of 94 °C for 4 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s; followed by one cycle of 72 °C for 10 min. The PCR product was purified by QIAquick PCR Purification Kit (Qiagen, Germany) and used as the template for dsRNA synthesis with TranscriptAid T7 High Yield Transcription Kit (Thermo Fisher Scientific, USA). Primers of EGFP-dsF and EGFP-dsR with the T7 promoter sequences (Table 1) were used to clone a 289 bp DNA fragment of enhanced green fluorescent protein (EGFP) gene based on pEGFP-N1 plasmid for dsRNA synthesis. Redundant single-strand RNA was digested by RNaseA (TaKaRa, China). Synthesized dsRNA (dsLvVEGFR or dsEGFP) was assessed on 1.2% agarose gel, and the concentration was measured by Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, USA) and stored at –70 °C until use.

2.6. RNA interference and accumulated death rate experiment

After optimization to the dsRNA dosage for interference (data not shown), 4 μg dsRNA per shrimp was used in the RNAi experiment. Cephalothoraxes of four individuals were sampled at 48 h after injection with dsEGFP or dsLvVEGFR, respectively. Total RNA extraction and cDNA synthesis followed the procedure described in 2.3.