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# Identification and characterization of two novel vascular endothelial growth factor genes in *Litopenaeus vannamei*

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ABSTRACT

Vascular endothelial growth factor (VEGF) signaling pathway induces endothelial cell proliferation, promotes cell migration, and inhibits apoptosis. Although three VEGF and two VEGF receptor genes have been identified in *Litopenaeus vannamei* and demonstrated their roles in WSSV infection, another two novel VEGF genes (*LvVEGF4*, *LvVEGF5*) were isolated and their involvements in the WSSV infection of shrimp were studied in the present study. The deduced amino acid sequences of both *LvVEGF4* and *LvVEGF5* contained a signal peptide, a typical PDGF/VEGF domain and a cysteine knot motif (CXCXCX). Tissue distribution analysis showed that *LvVEGF4* was predominantly expressed in gill and hemocytes, while *LvVEGF5* was mainly detected in hemocytes and intestine. WSSV infection could cause up-regulation of the transcriptional levels of *LvVEGF4* and *LvVEGF5*. Their functions were studied by double-strand RNA interference. The results showed that knock-down of *LvVEGF4* and *LvVEGF5* led to a decrease of the viral copy number in WSSV infected shrimp. Yeast two-hybrid analysis showed that both LvVEGF4 and LvVEGF5 could interact with LvVEGFR1 rather than LvVEGFR2. In addition, knock-down of *LvVEGF4* and *LvVEGF5* could reduce the expressional levels of downstream genes *FAK* and *PI3K*. The present study provides new clues in demonstrating that the VEGF signaling pathway is involved in the process of WSSV infection in shrimp.

## 1. Introduction

White spot syndrome virus (WSSV), which usually causes 100% mortality of the shrimp in three to five days, is a great threat in shrimp aquaculture [1,2]. As a kind of invertebrates, shrimp generates immune responses to pathogens mainly through innate immune system [3]. When infected by microorganism, they mainly rely on the pattern recognition receptor (PRR) to identify pathogens and then activate cell signaling pathways. Signal transduction induces specific antimicrobial peptides (AMPs) to kill the pathogens [4,5]. Toll, IMD and JAK/STAT pathways are regarded as important pathways regulating the innate immune responses of invertebrates including shrimp [6–8].

VEGF signaling pathway is involved in a variety of biological processes including vascular proliferation, cell differentiation, cell migration and so on [9]. As reviewed by Neufeld et al. there are five VEGFs and three VEGFRs in mammals [10]. Different VEGF binds to specific receptors and exerts different biological functions. VEGF (VEGF-A) binds to VEGFR1 and VEGFR2 to perform as a highly specific mitogen for vascular endothelial cells [11]. VEGF-B binds to VEGFR-1 to regulate the activity of plasminogen activator in endothelial cells [12]. VEGF-C is proved to bind with VEGFR-2 or VEGFR-3 to regulate vascular development and maintenance of the lymphatic vessels [13]. VEGF-D is a ligand for VEGF receptor 2 (Flk-1) and VEGF receptor 3 (Flt-4) and it could promote tumor angiogenesis and growth [14]. VEGFRs belong to tyrosine-kinase receptor family by the presence of seven immunoglobulin-like loops in their extracellular part and a split tyrosine-kinase domain in their intracellular part. When performing functions, VEGF dimer might bind and link two VEGF receptors together to form homo- or heterodimers of receptors [10]. Moreover, VEGF could regulate other innate immune signaling pathways such as MAPK/ERK, PI3K, JAK/STAT etc. [15–17], which are involved in pathogen infection [18–20].

Recently, the VEGF signaling pathway has also been reported directly participating in pathogen infection. When the human pancreatic

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**Table 1**
Primer sequences and corresponding annealing temperature.

| Primer name | Primer sequence(5'-3') | Expected size (bp) | Annealing temperature (°C) |
|---|---|---|---|
| LvVEGF4-F | ATTAGAATTCTTACGCTCGTG | 594 | 55 |
| LvVEGF4-R | TTAAGATTCCATCACGTAACG | | |
| LvVEGF5-F | TCGGCGACGCTCATTATC | 1029 | 55 |
| LvVEGF5-R | CAGCAAGGAACAGTTGT | | |
| LvVEGF4-qF | TGGAGGCTCACAGATTGA | 137 | 57 |
| LvVEGF4-qR | GAAGTTGCGGTTGAACAC | | |
| LvVEGF5-qF | AATATGAGCCAGACGACAG | 134 | 57 |
| LvVEGF5-qR | CACCACAAGTGAGCAGAA | | |
| 18S-F | TATACGCTAGTGGAGCTGGAA | 136 | 56 |
| 18S-R | GGGGAGGTAGTGACGAAAAAT | | |
| LvVEGF4-dsF | TAATACGACTCACTATAGGGAGCTGTGGCATAAACAAGGTG | 545 | 59 |
| LvVEGF4-dsR | TAATACGACTCACTATAGGGTTGTGAGGAGTCGTGGTGTCT | | |
| LvVEGF5-dsF | TAATACGACTCACTATAGGGCGGCGAATAATTGAGTATAAGAGTA | 556 | 59 |
| LvVEGF5-dsR | TAATACGACTCACTATAGGGTGCAAATGCCAGTGTAAGGA | | |
| EGFP-dsF | TAATACGACTCACTATAGGGCAGTGCTTCAGCCGCTACCC | 289 | 59 |
| EGFP-dsR | TAATACGACTCACTATAGGGAGTTCACCTTGATGCCGTTCTT | | |
| VP28-qF | AAACCTCCGCATTCCTGTGA | 141 | 55 |
| VP28-qR | TCCGCATCTTCTTCCTTCAT | | |
| LvFAK-qF | ATTACTCAACACCAGCAACC | 172 | 57 |
| LvFAK-qR | GTTCCCTCGGACTCCACCTT | | |
| LvPI3K-qF | TATGAAGTAACCCGTAGTGCCA | 187 | 57 |
| LvPI3K-qR | TGCCCACATCTCCTGACTGA | | |
| LvVEGF4-YF | GGATCCATTAGAATTCTTACGCTCGTG | 591 | 55 |
| LvVEGF4-YR | GAATTCTTAAGATTCCATCACGTAACG | | |
| LvVEGF5-YF | GGATCCTCGGCGACGCTCATTATC | 1029 | 56 |
| LvVEGF5-YR | GAATTCCAGCAAGGAACAGTTGTAGC | | |
| LvVR1(2–5)-YF | ACTTTGACCACATACAAGTA | 1127 | 57 |
| LvVR1(2–5)-YR | TCCATGTTCATTGGAGGTTT | | |
| LvVR2(1–4)-YF | TTCAACCCAGTGCATGTTGAGGAG | 1473 | 58 |
| LvVR2(1–4)-YR | CACAACTACTAGCCTCAGTTTGCG | | |

Note: T7 promoter sequences were underlined. *BamH* I and *EcoR* I sites were wave-underlined.

carcinoma cell lines were infected by *Vaccinia virus* (VV), increased expression of VEGF-A could enhance VV gene expression, replication, and cytotoxicity [21]. *Bovine papular stomatitis virus* (BPSV) could encode a novel VEGF homologue which could bind to VEGFR-2 with high affinity and contribute to the proliferation and highly vascularized nature of BPSV lesions [22]. In the genome of *Orf virus*, a gene encoding a homology to mammalian VEGF was discovered [23], which was proved to activate VEGFR-2 and participated in virus infection [24]. In *Drosophila*, the expression of one VEGF, *pvf2*, was up-regulated after infected by *E. coli* [25]. In the crustacean *Eriocheir sinensis*, a VEGF homologous gene, *EsPVF1* could respond to the infection of *Vibrio anguillarum* and *Pichia pastoris* [26]. Two VEGF homologous genes, *MjVEGF-1* and *MjVEGF-2* were proved to participate in the process of immune response in *Marsupenaeus japonicas* [27].

Previously, three types of VEGF genes and two types of VEGFR genes were identified in *L. vannamei* and they all participated into WSSV infection [28–31]. In the present study, another two members of VEGF family (*LvVEGF4*, *LvVEGF5*) was identified from *L. vannamei* and their functions during WSSV infection were explored. Furthermore, the interaction between LvVEGF4, LvVEGF5 and LvVEGFRs was also studied by the yeast two-hybrid system. The current study will provide new evidence for understanding the role of the VEGF signaling pathway during WSSV infection in shrimp.

## 2. Materials and methods

### 2.1. Experimental animals

Healthy adult Pacific whiteleg shrimp cultured in our lab, with a body length of 12.5 ± 0.4 cm and a body weight of 25.1 ± 2.5 g, were used for tissue distribution analysis and WSSV challenge experiments. Shrimp with a body length of 8.80 ± 2.30 cm and a body weight of 6.55 ± 3.19 g were used for dsRNA injection and WSSV replication detection after gene silencing. All shrimp were acclimated in air-pumped circulating sea water at 25 °C before experiments.

### 2.2. Tissues collection and WSSV challenge experiments

In order to detect the tissue distribution of *LvVEGF4* and *LvVEGF5*, different tissues from 12 individuals were dissected. Firstly, hemolymph was obtained by using a sterile syringe preloaded with equal volume of modified anticoagulant Alsever solution and then centrifuged immediately at 800 g at 4 °C for 10 min [32]. The supernatant was discarded and the hemocytes were collected and preserved in liquid nitrogen. Then the hepatopancreas, heart, eyestalk, muscle, lymphoid organ, intestine, stomach, gill, epidermis, testis and ovary were collected and kept in liquid nitrogen.

For virus challenge experiment, WSSV was purified from the cephalothoraxes of infected *Exopalamon carincauda* and quantified according to the method previously described [33]. Healthy adult shrimp were divided into two groups including WSSV group and PBS group, with 80 individuals in each group. In WSSV group, each shrimp was injected with 20 μl WSSV solution containing $10^4$ copies of WSSV, while in PBS group each was injected with 20 μl PBS as the negative control. At 0.5, 3, 6, 12, 24 and 48 hours (h) post WSSV infection, gill, intestine and hemocytes of 12 shrimp in each group were collected separately and frozen in liquid nitrogen.

### 2.3. Total RNA extraction and cDNA synthesis

To detect the transcriptional level of *LvVEGF4* and *LvVEGF5*, total RNA of each sample was extracted using RNAiso Plus reagent (TaKaRa, Japan) following the manufacturer's protocol. The concentration of extracted RNA was detected by Nanodrop 2000 (Thermo Fisher Scientific, USA) and the RNA quality was assessed by electrophoresis on 1% agarose gel. The cDNA samples were synthesized using PrimeScript RT Reagent Kit (TaKaRa, Japan) with 1 μg total RNA. Genomic DNA was firstly removed by gDNA Eraser. And then the first-strand cDNA