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Wnt5b regulates apoptosis in *Litopenaeus vannamei* against white spot syndrome virus

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ABSTRACT

The Wnt signaling mediated by Wnt proteins that orchestrate and influence a myriad of cellular processes, such as cell proliferation, differentiation, tumorigenesis, apoptosis, and participation in immune defense during microbe infection. Wnt5b is one of the Wnt signaling molecules that initiate the cascade. In this study, we cloned and characterized a Wnt5b homolog from *Litopenaeus vannamei* designed as *LvWnt5b*. The full length of *LvWnt5b* transcript was 1726 bp with an 1107 bp open reading frame that encoded a 368 aa protein, which contained 24 discontinuous and highly conserved cysteine. Real-time quantitative PCR showed that the transcriptional level of *LvWnt5b* was down-regulated when infected with white spot syndrome virus (WSSV). Knock-down of LvWnt5b resulted in inhibition of the transcriptional level of WSSV gene *ie1*, indicating that LvWnt5b mediated signaling pathway may play an important role in defense against WSSV infection. When LvWnt5b was silenced, caspase3/7 activity in hemocytes was increased significantly, and the transcription of viral gene was decreased as well. Moreover, overexpression of LvWnt5b in HEK293T cells led to inhibition of caspase3/7 activity, which further proved the role of LvWnt5b in restraining apoptosis. The study showed that the shrimp may decrease the expression of LvWnt5b initiatively to act as an immune defense mechanism against WSSV infection via promoting apoptosis. It will be helpful for understanding the function of Wnt signaling pathway in virus invasion and host defense.

1. Introduction

Viral infection has been the principal factor resulting in high mortality of shrimp, and causes enormous economic losses in shrimp culture industry [1]. White spot sydrome virus (WSSV), which has a wide range of host, is both lethal and virulent to crustaceans. A lot of researches have been taken to realize the immune responses of shrimps to WSSV. It was found that signaling transduction pathways could play important roles in the interaction between the host and WSSV. For example, Toll/IMD−NF−κB and JAK/STAT pathways were involved in regulating the immune response of shrimp to microbes [2–5]. On the other hand, WSSV could also regulate the signaling pathway to facilitate their proliferation. Previous researches had revealed that the shrimp NF-κB and STAT could activate viral gene transcription [6,7].

Wnt signaling cascade is highly conserved among different species that is integrally involved in growth, development, metabolism and cell maintenance [8,9]. The pathway that has been well recognized is the so-called canonical Wnt signaling pathway, which regulates the expression of specific target genes by β-catenin [10]. When Wnt is inactive, β-catenin forms a complex with Axin, adenomatosis polyposis coli (APC) and glycogen synthase kinase 3-β (GSK3-β), and gets phosphorylated and targeted for degradation. While Wnt exists, β-catenin is uncoupled from the degradation complex and translocates into the nucleus, where it binds to Tcf/Lef transcription factors and activates the expression of target genes [11]. The other two branches termed as noncanonical Wnt signaling pathways include Wnt/planar cell polarity (Wnt/PCP) pathway [12] and Wnt/calcium (Wnt/Ca^{2+}) pathway [13]. All of these pathways need Wnt to bind to its receptors Frizzled (Fzd) [14] and low-density-lipoprotein receptor related protein5/6 (LRP5/6) [15] to initiate the signaling.

Wnt genes encode a family of secreted glyco-protein ligands that contain a transmembrane region and a Wnt1 domain with 24 discontinuous and highly conserved cysteine residues [16]. Wnt members are distinguished by sequence rather than functional properties. They

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Table 1
Primers used in this work.

Primers	Sequence (5′-3′)
Primers used for 5′-Rapid Amplification of cDNA Ends	
LvWnt5b-5RACE1	CACGGTGGAGTCGTCAACAGTGGAG
LvWnt5b-5RACE2	GGGTCCGAACACGGTGGAGTCGTCAAC
LvWnt5bF	ATGGGGGTCGTGGGCCTCC
LvWnt5bR	TTATTTGCATGTGTGAAGGTC
Primers used for RNAi	
dsRNA-LvWnt5b-F	CCTCCTCAACCTGCTCCTG
dsRNA-T7-LvWnt5b-R	[a]GGATCCTAATACGACTCACTATAGGGCCCCGCTTGTAGTTCTTC
dsRNA-LvWnt5b-R	GCCCCGCTTGTAGTTCTTC
dsRNA-T7-LvWnt5b-F	[a]GGATCCTAATACGACTCACTATAGGCCTCCTCAACCTGCTCCTG
dsRNA-eGFP-F	GTGCCCATCCTGGTCGAGCT
dsRNA-T7-eGFP-R	[a]GGATCCTAATACGACTCACTATAGGTGCACGCTGCCGTCCTCGAT
dsRNA-eGFP-R	TGCACGCTGCCGTCCTCGAT
dsRNA-T7-eGFP-F	[a]GGATCCTAATACGACTCACTATAGGGTGCCCATCCTGGTCGAGCT
Primers used for Reai-time quantitative PCR	
LvWnt5brtF	CTACTTGGACGAATCTCCCGACTAC
LvWnt5brtR	CAGCATAAGAGTCCACAGCCATCC
LvactinrtF	AGGCTAACCGCGAGAAGATGAC
LvactinrtR	GTAGCACAGTTTCTCCTTGATG
LvtubulinrtF	GCCTCGTGCCATCCTTGTTG
LvtubulinrtR	CCCTTAGCCCAGTTGTTTCCAG
wsv069rtF	GCACAACAACAGACCCTACCC
wsv069rtR	GAAATACGACATAGCACCTCCAC

[a] The sequences of T7 promoter were underlined.

are evolutionarily highly conserved from fruit flies to humans [17] and play important roles in development, disease, immunity and so on. In Dorsophila, Wnt5/PCP pathway could regulate axonal development, which is fundamental for circuit formation in the nervous system [18]. In human, Wnt5b is required for adipogenesis that occurs in Type 2 diabetes mellitus, and could also inhibit the stimulation of β-catenin [19]. Moreover, Samudra K also found that Wnt5a can facilitate melanoma metastasis via the i-nduction of EMT (Epithelial-Mesenchymal Transition) [20].

Wnt signaling pathway could also play a role in innate immune of *Litopenaeus vannamei*. In previous report, LvWnt5 and other five Wnt genes were cloned and characterized. It was found that the transcrptional level of Wnts could respond to WSSV infection, suggesting that Wnt genes may play a role in WSSV infection [21]. Also the downstream molecule β-catenin, a positive regulator in antiviral process, could respond to WSSV infection and activate the expression of several antimicrobial peptides (AMPs) [22]. In this study, a wnt gene, designed as *LvWnt5b*, was cloned, and it had a down-regulated expression profile after WSSV infection. Further study revealed that LvWnt5b could inhibit the viral gene transcription by activating the apoptosis of the shrimp hemocytes. The results would helpful for understanding the role of Wnt signaling pathway in innate immune.

2. Materials and methods

2.1. Shrimp culture and WSSV extraction

Healthy live shrimp, *L. vannamei*, length in 12–13 cm and weight in 12–15 g, were bought from local farmer's market. Several shrimps were selected randomly to detect WSSV before experiments.

WSSV particles were extracted from hemocytes of infected crayfish *Proca-mbarus clarkii* and quantified according to Yang's description [23,24].

2.2. Total RNA isolation and cDNA synthesis

Total RNAs were isolated from different tissues of three individual shrimps using TRIzol reagent (Molecular Research Center, Inc) according to manufacturer's instruction. After treatment with DNaseI(Takara) at 37 °C for 0.5h, the first-strand cDNA was synthesized by reverse transcriptase M-MLV (Takara) with Oligo(dT)$_{18}$.

2.3. Cloning of full-length cDNA

Partial sequence (~1150 bp) of *LvWnt5b* was obtained from *L. vannamei* transcriptome analyzed in our lab. The missing 5′-terminal sequence of *LvWnt5b* was acquired by 5′-RACE (5′-Rapid Amplification of cDNA Ends) using the specific primer, LvWnt5b-5RACE1 and LvWnt5b-5RACE2 (Table 1). Based on the full-length sequence of *LvWnt5b,* the primers LvWnt5bF, LvWnt5bR (Table 1) was designed for PCR amplification of open reading frame (ORF) of *LvWnt5b*.

2.4. Sequence and phylogenetic analysis

Characteristic domains of LvWnt5b were predicted using a research tool called SMART (http://smart.embl.de/) [25]. The sequence of LvWnt5b and its homologues from other species were singled out from the National Center For Biotechnology Information (NCBI) databases. The phylogenetic tree was constructed utilizing the MEGA6 software [26]with the Neighbor-Joining (NJ) Method based on the full-length amino-acid sequence of Wnt5b proteins.

2.5. Tissue distribution analysis

Following the protocol referred above, total RNAs were extracted from different tissues including the hemocyte, gill, hepatopancreas, heart, intestine and muscle. Then the cDNAs were synthesized. The first-strand cDNA was diluted to 1:10 for the Real-time quantitative PCR with the primer pairs LvWnt5brtF/LvWnt5brtR (Table 1). The shrimp *β-Actin* gene was used as the internal standardization. Relative mRNA levels of *LvWnt5b* were analyzed using the $2^{-\Delta\Delta Ct}$ method [27].