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Molecular characterization and function of a PTEN gene from *Litopenaeus vannamei* after *Vibrio alginolyticus* challenge

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ABSTRACT

PTEN, a tumor suppressor gene, suppresses cell survival, growth, apoptosis, cell migration and DNA damage repair by inhibiting the PI3K/AKT signaling pathway. In this study, the full-length *Litopenaeus vannamei* PTEN (LvPTEN) cDNA was obtained, containing a 5′UTR of 59bp, an ORF of 1269bp and a 3′UTR of 146bp besides the poly (A) tail. The PTEN gene encoded a protein of 422 amino acids with an estimated molecular mass of 48.3 KDa and a predicted isoelectric point (pI) of 7.6. Subcellular localization analysis revealed that LvPTEN was distributed in both cytoplasm and nucleus, and the tissue distribution patterns showed that LvPTEN was ubiquitously expressed in all the examined tissues. *Vibrio alginolyticus* challenge induced upregulation of LvPTEN expression. Moreover, RNAi knock-down of LvPTEN in vivo significantly increased the expression of LvAKT mRNA, while reducing that of the downstream apoptosis genes LvP53 and LvCaspase3. LvPTEN knock-down also caused a sharp increase in cumulative mortality, bacterial numbers, and DNA damage in the hemolymph of *L. vannamei* following *V. alginolyticus* challenge, together with a sharp decrease in the total hemocyte count (THC). These results suggested that LvPTEN may participate in apoptosis via the PI3K/AKT signaling pathway in *L. vannamei*, and play an important role in shrimp innate immunity.

1. Introduction

The Pacific white shrimp, *Litopenaeus vannamei*, is one of the most economically important shrimp species in the global aquaculture industry. Pathogenic invasions cause a number of problems in shrimp aquaculture that result in high shrimp mortality (Briggs et al., 2005). There is a growing body of evidence showing that invertebrates such as shrimp rely exclusively on their cellular and humoral innate immune responses for defense against pathogens (Bachere et al., 2004; Magnadottir, 2006). Tissues and cell types associated with the innate immune system, such as hemocytes and the hepatopancreas, thus play vital roles in pathogen defense. *Vibrio alginolyticus*, a gram-negative marine bacterium, is one of the most serious threats to aquacultured shrimp (Reboucas et al., 2011). Shrimp infected with *V. alginolyticus* exhibit reduced total hemocyte counts and increased levels of reactive oxygen species (ROS) (Cha et al., 2015; Huang et al., 2015), which damage important biomolecules such as DNA and induce apoptosis (Halliwell, 1999). Because the innate immune system is the primary means by which shrimp resist such infections, it is important to understand its workings in detail in order to facilitate the development of shrimp aquaculture.

Phosphatase and tensin homologue deleted on chromosome ten (PTEN) was originally identified as a tumor suppressor gene that is frequently lost from a region of chromosome 10q23 in various human tumors (Li et al., 1997; Steck et al., 1997). It is expressed in both the cytoplasm and the nucleus of normal cells (Gimm et al., 2000), and functions as a tumor suppressor by negatively regulating the PI3K/AKT intracellular signaling pathway (Stambolic et al., 1998), which is regulates several cellular events (Cantrell, 2001). It achieves this negative regulation by catalyzing the dephosphorylation of 3, 4, 5-triphosphorylated phosphoinositide (PIP3), low PIP3 concentrations lead to the down-regulation of AKT, which itself regulates cellular processes relating to survival, growth, migration and DNA repair (Hopkins et al., 2014; Waite and Eng, 2002). The PI3K/AKT pathway has been reported to play an important role in immune responses to pathogenic invaders in

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several organisms, including shrimp (Ruan et al., 2014a).

Many reports have suggested that PTEN is a key regulator of apoptosis (Torres et al., 2003). Its overexpression inhibits the growth of Jurkat cells, and renders cells more susceptible to apoptosis induced by various stimuli (Xu et al., 2002). Similarly, PTEN-silencing reduced the occurrence of apoptosis by inhibiting a Bcl-2 related apoptotic pathway in oxygen and glucose deprived BMSC cells (Chen et al., 2011). There is also a growing body of evidence indicating that PTEN plays a complex regulatory role in controlling the expression of P53, a well-known tumor suppressor protein that plays a pivotal role in apoptosis and maintaining cellular homeostasis (Vogelstein et al., 2000). Specifically, PTEN up-regulation can abrogate the activity of AKT and MDM2, leading to P53 up-regulation and the induction of apoptosis (Selvendiran et al., 2007). Another piece of evidence linking PTEN to apoptosis regulation is that the expression of Caspase3, a major apoptotic executioner protease, was shown to correlate significantly and positively with that of PTEN (Sun et al., 2004; Yang et al., 2008). Recent studies have also shown that PTEN contributes to immune responses to pathogen infection. For example, PTEN deficiency rendered several cells types hyper-susceptible to mycobacterial infection (Huang et al., 2012). In murine pneumococcal pneumonia, myeloid PTEN expression can promote inflammation via the PI3K/AKT pathway but it also impairs bactericidal activity by reducing neutrophil influxes and the phagocytic capacity of macrophages (Schabbauer et al., 2010).

As the first tumor suppressor with phosphatase activity to be discovered, there is considerable interest in PTEN and it is being studied intensively. However, little is known about its role in the immune responses of invertebrates, and nothing has been reported about its role in crustaceans' immune systems. To address this knowledge gap, we investigated its role in the immune response of *L. vannamei* to *V. alginolyticus* challenge. To this end, we cloned and characterized the full-length LvPTEN cDNA of *L. vannamei*. In addition, the subcellular localization of LvPTEN and its expression after *V. alginolyticus* challenge were determined. Finally, an RNAi experiment was performed to investigate how down regulating LvPTEN expression in vivo affects the total hemocyte count, bacterial numbers, DNA damage, cumulative mortality, and AKT/PI3K related genes of shrimp following *V. alginolyticus* challenge.

2. Materials and methods

2.1. Animals

Healthy *L. vannamei* (10–15 g) obtained from an aquaculture farm in Panyu in the Guangdong Province of China were used for cDNA cloning and tissue samples for expression analysis. In addition, healthy *L. vannamei* (3–4 g in weight and 5.5–6.5 cm in length) from the same farm were selected for *V. alginolyticus* challenge and dsRNA injection experiments. Before the experiments, the shrimp were acclimated in a circulating water tank containing continuously aerated seawater (10‰ salinity, pH 7.9–8.1, 24 ± 2 °C) for two weeks, during which they were fed three times daily with commercial feed.

2.2. Gene cloning and sequence analyses

The full-length LvPTEN cDNA was obtained by the polymerase chain reaction (PCR) and rapid amplification of cDNA ends (RACE). A pair of degenerate primers (Table 1) was designed based on the highly conserved nucleotide sequence of PTEN from other species in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) to obtain the LvPTEN fragment. Gene-specific primers and nested primers (Table 1) for 5′ and 3′ RACE were then designed. The RACE template was obtained using the BD SMART RACE cDNA reaction kit (BD Bioscience Clontech, USA).

Table 1
The primer sequences used in this study.

Name	Sequence(5′–3′)
For cDNA cloning	
LvPTEN-F	ATWGCYATGGGMTWTCCAGC
LvPTEN-R	CCCTTTCCAGCTTTACARTG
LvPTEN-3′RACE1	TCCCGCTTCCTAGAAGAGAGGCACA
LvPTEN-3′RACE2	CCATAACCCACCGCCTCTGATTGACA
LvPTEN-5′RACE1	TGTGCCTCTCTTCTAGGAAGCGGGA
LvPTEN-5′RACE2	CGACACCAAGTTCCTTATGCCTTTGCTC
For subcellular localization	
LvPTEN-pEGFP-F	CCC**AAGCTT**CGATGAGCAAAGGCATAAGGAACT
LvPTEN-pEGFP-R	CGC**GGATCC**CAGCAGCACCTCCGAYCCTTCC
For RNAi	
dsRNA-LvPTEN-F	GGCACAAGGACCACTAT
dsRNA-LvPTEN-R	GCCACTTCGTCTTTTACTA
dsRNA-LvPTEN-T7-F	TAATACGACTCACTATAGGGGCACAAGGACCACTAT
dsRNA-LvPTEN-T7-R	TAATACGACTCACTATAGGGCCACTTCGTCTTTTACTA
dsRNA-LvGFP-F	GTGCCCATCCTGGTCGAGCT
dsRNA-LvGFP-R	TGCACGCTGCCGTCCTCGAT
dsRNA-LvGFP-T7-F	TAATACGACTCACTATAGGGTGCCCATCCTGGTCGAGCT
dsRNA-LvGFP-T7-R	TAATACGACTCACTATAGGTGCACGCTGCCGTCCTCGAT
For real-time RT-PCR	
RT-LvPTEN-F	CTCAGAAGTTGGAGGGTGTGTAC
RT-LvPTEN-R	CAATCAGAGGCGGTGGGTTA
RT-LvAKT-F	TCAGAATGTCCAAATCCAGCC
RT-LvAKT-R	CCAAATGTCCCTTTCCCAAGT
RT-LvP53-F	CGAATCCCCACATCCACG
RT-LvP53-R	GGCGGCTGATACACCACC
RT-LvCaspase3-F	ACGAGAAGTCGCCAGGAGGT
RT-LvCaspase3-R	CGGTCGCATTGTGATGATAAAA
RT-LvEF-1α-F	GTATTGGAACAGTGCCCGTG
RT-LvEF-1α-R	ACCAGGGACAGCCTCAGTAAG

The deduced amino acid sequence of LvPTEN was used as the query in a Blast search of the NCBI protein sequence database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and an additional search was performed to identify conserved domains in the sequence (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Multiple sequence alignment was performed using the ClustalW2 program, and a phylogenetic tree was constructed on the basis of the sequence alignment using the neighbor-joining (NJ) algorithm as implemented in the MEGA 5.1 software package, with 1000 bootstrap replications.

2.3. Plasmid construction, cell transfection and subcellular localization

For the subcellular localization assay, the open reading frame (ORF) of LvPTEN was cloned into the pEGFP-N3 vector using the primers listed in Table 1. The successful formation of the desired recombinant plasmid was confirmed by DNA sequencing.

Transfection of the recombinant plasmid was performed using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Briefly, HeLa cells were grown in 6-well plate. Separately, Lipofectamine 2000 and the plasmids were mixed for 25 min before the cell transfection. Then, HeLa cells were incubated with the mixture for 6 h at 25 °C, after which they were cultured in fresh medium. The cultured cells were washed with PBS, fixed with 4% paraformaldehyde, stained with DAPI (1 mg/ml) (Huang et al., 2008), and examined by the fluorescence microscopy.

2.4. Immune challenge experiment with V. alginolyticus

Samples of *V. alginolyticus* (ATCC33787) culture maintained in