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RESEARCH REPORT

Sequence characterization and expression analysis of a PDGF/VEGF related factor gene in swimming crab *Portunus trituberculatus*

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Abstract

PDGF/VEGF factors are closely associated with immune defense systems. In this study, a PDGF/VEGF related factor gene (designated as PtPVF) from swimming crab *Portunus trituberculatus* was identified and analyzed. Its full-length cDNA is 1320 bp, containing an open reading frame (ORF) of 582 bp, 5' untranslated regions (UTR) of 105 bp and 3' UTR of 633 bp, which encoded a polypeptide of 193 amino acid residues. The deduced amino acid sequence of PtPVF contained a signal peptide, a typical PDGF/VEGF domain, and a cysteine knot motif (CXCXC). Quantitative real-time PCR analysis demonstrated that the mRNA of PtPVF was detectable in gills, gonads, heart, hemocytes, hepatopancreas, intestines, muscles, optic stalk, stomach, and the highest level was found in hemocytes, followed by gill and hepatopancreas. After stimulation by *Vibrio alginolyticus*, pentachlorophenol (PCP) or cadmium chloride ($CdCl_2$), the relative mRNA expression levels of PtPVF in hemocytes reached their respective peaks at 3 h, 6 h and 6 h, and 36.61-fold, 3.06-fold and 21.26-fold compared with the original level, respectively, and the respond to *V. alginolyticus* and $CdCl_2$ is earlier than to PCP, while under the challenge of *V. alginolyticus* and $CdCl_2$, the mRNA expression level of PtPVF was significantly higher than that of PCP stimulation. Furthermore, the mRNA expression level of PtPVF first decreased at 3 h and then increased rapidly to a peak at 6 h after PCP challenge. All these results demonstrated that PtPVF might be involved in the response to invading pathogens and environmental pollution stimulants of *P. trituberculatus*.

Key Words: innate immunity; PDGF/VEGF related factor; *Portunus trituberculatus*; sequence features; expression profiles

Introduction

Platelet-derived growth factors (PDGFs) are disulphide-bonded dimers which assembled via homo- or heterodimerization, and thay are synthesized and released by various cells, including platelets, macrophages, and smooth muscle cells. Vascular endothelial growth factor (VEGF), also called vascular permeability factor (VPF), is a secreted mitogen specific for endothelial cells and mainly produced by macrophages and T-cell. PDGF/VEGF are cytokines widely present in vertebrates, characterized by highly conserved PDGF/VEGF homologous structural domains, conserved cysteine residues and characteristic cysteine knot motif, and play important roles in regulating the proliferation, migration, and differentiation of various types of immune cells in the immune system (Joukov *et al*., 1997). PDGF/VEGF related factors (PVFs) have been cloned and characterized in many vertebrates and invertebrates, for example, the human VEGF gene played a role in vascular disease and coronary artery recanalization (Inoue *et al*., 1998), while PDGF signaling was involved in epicardial function and coronary angiogenesis during heart regeneration in *Danio rerio* (Kim *et al*., 2010). Three PVFs (PVF1, PVF2, and PVF3) have been found in *Drosophila melanogaster*, which are related to oocyte migration (McDonald *et al*., 2003), induction of larval hemocytes proliferation (Munier *et al*., 2002), and maintenance of the monolayer epithelium of the wing disc (Duchek *et al*., 2001). *Caenorhabditis elegans*

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contained four possible PVF homologues, among which PVF-1 could bind mammalian VEGF receptors and participate in angiogenesis (Tarsitano *et al*., 2006). In recent years, PVF has been increasingly studied in aquatic invertebrates. EsPVF1, isolated and cloned from the Chinese mitten crab *Eriocheir sinensis*, was involved in regulation of the immune response and neuroendocrine system in crabs (Li *et al*., 2013). Moreover, PfPVF from the *Pinctada fucata* has also been shown to be an important player in the immune defense system and also contributes to wound healing during cell nuclear transplantation (Huang *et al*., 2017). All these studies suggest that PVF factors are not only involved in regulating immune responses, but also may have many other biological functions.

The swimming crab *Portunus trituberculatus* widely distribute along the coasts of East Asia countries, and is one of the important aquaculture and economic crabs in China (Li *et al*., 2011). The annual production of *P. trituberculatus* in China remains at approximately 105,000 tons (Gai *et al*., 2009; Liu *et al*., 2023). However, many factors are harmful to the growth of crabs, such as the infection of various pathogens and environmental pollution (Pan *et al*., 2016). Bacteria of the genus *Vibrio* and white spot syndrome virus (WSSV) are the main pathogenic agents causing crabs death (Chen *et al*., 2000; Marques *et al*., 2011; Nogami *et al*., 1992). As an invertebrate, *P. trituberculatus* does not possess an adaptive immune system and must rely on the innate immune defense system to defend itself against external aggression (Iwanaga *et al*., 2005; Lavine *et al*., 2002). In recent years, researchers have extensively studied *P. trituberculatus*'s immune genes, focusing on characterization and functional analysis, such as Toll-like receptors, NOD-like receptors, and immunoglobulins (Zhang *et al*., 2022; Zhou *et al*., 2015). In addition, six key immune-related genes, proPO, α2M, crustin, lysozyme, NOS, and NOX, in three-spot swimming crab were studied through pathogen infection experiments (Ren *et al*., 2017). These researches on immune genes provide important references for further exploring the immune mechanisms of this species.

In this study, we cloned a PDGF/VEGF-related factor gene (designated as PtPVF) from *P. trituberculatus* and analyzed its expression profiles in order to understand its immune roles in *P. trituberculatus*. The mRNA expression of PtPVF in different tissues was assessed using quantitative real-time PCR (qPCR). Additionally, we also investigated the mRNA expression of PtPVF in the hemocytes of crabs following the challenges with *Vibrio alginolyticus*, pentachlorophenol (PCP), and cadmium chloride ($CdCl_2$).

Materials and methods

Sample collection and processing

All the crabs used in this experiment were purchased from a breeding farm in Ningbo, Zhejiang, with an average weight of 50 ± 5 g. Before the experiment, the crabs were reared in aerated circulating seawater for two weeks, with a quarter of the water changed daily, during which time they were fed fresh small fish every day, to ensure that the crabs used in the experiments were healthy. Gill, gonad, heart, hemocytes, hepatopancreas, intestine, muscle, optic stalk, and stomach were collected to determine mRNA transcripts of PtPVF in different tissues, with five replications for every tissue and each replication was a mixture of three individuals. About 400 crabs were used in the challenge experiment and randomly divided into four groups. Three groups were immersed in *V. alginolyticus* (10^7 CFU/mL), PCP (0.01mg/L) and $CdCl_2$ (0.1mg/L), respectively, while untreated crabs served as the control group. At the time point of 0 h, 3 h, 6 h, 12 h, 24 h and 48 h post stimulation, hemolymph was extracted using a syringe from the last legs of the crabs, and mixed with anticoagulant solution (415 mM NaCl, 100 mM glucose, 30 mM sodium citrate, 26 mM citric acid, 30 mM-EDTA, pH 4.6). Then, the hemolymph was immediately centrifuged at 800 × *g* for 10 min at 4 °C to harvest the hemocytes. All the tissue samples were rapidly frozen in liquid nitrogen and used for RNA isolation.

Table 1 Primers used in this study

Primer	Sequence (5'-3')	Brief information
adaptor primer	GGCCACGCGTCGACTAGTAC	Anchor primer for 3' and 5' RACE
adaptor-oligo (dT)	GGCCACGCGTCGACTAGTACG_{10}HN	Olido (dT) for cDNA synthetize
adaptor-oligo (dG)	GGCCACGCGTCGACTAGTACT_{17}VN	Olido (dG) for cDNA synthetize
PtPVF- RACE-F1	CTGGAGGAGGACAAGGAGGAGGAA	Gene specific primer for 3` RACE
PtPVF- RACE-F2	CATCACACTCACAACTCAAGTTATCTC	Gene specific primer for 3` RACE
PtPVF- RACE-R1	GCAATGCGTGACAAGTGACTGCTTGTA	Gene specific primer for 5` RACE
PtPVF- RACE-R2	TACCACCAGCGTCAGTAACAGCAC	Gene specific primer for 5` RACE
PtPVF-qF	CAACAGGCCCACAGATTCC	Gene specific primer for qPCR
PtPVF-qR	GCACTTCATCGGGTATTCAGAG	Gene specific primer for qPCR
Pt-EF-1α-F	GAGACCTTCCAGAAGTATGCC	Internal control for qPCR
Pt-EF-1α-R	CACCTCCTTGATGACACCC	Internal control for qPCR

Table 2 PDGF/VEGF related factors used in multiple sequence alignments and phylogenetic tree analysis

PDGF/VEGF related factors	Accession number
Drosophila melanogaster PDGF- and VEGF-related factor 1, isoform A	NP_523407.1
Drosophila melanogaster PDGF- and VEGF-related factor 2	AAF52484.2
Drosophila melanogaster PDGF- and VEGF-related factor 3, isoform E	NP_001097109.2
Eriocheir sinensis PDGF/VEGF-related factor 1	ADF87936.1
Mus musculus placenta growth factor isoform 1 precursor	NP_032853.1
Hydra vulgaris VEGF	ACN87994.1
Danio rerio VEGF A-like	XP_005157219.2
Danio rerio VEGF A-A isoform X1	XP_009290293.1
Danio rerio VEGF C isoform X1	XP_005160055.1
Danio rerio VEGF D precursor	NP_001035268.1
Homo sapiens VEGF A isoform a	NP_001020537.2
Homo sapiens VEGF B isoform VEGFB-186 precursor	NP_003368.1
Homo sapiens VEGF C preproprotein	NP_005420.1
Homo sapiens VEGF D preproprotein	NP_004460.1
Caenorhabditis elegans PDGF/VEGF-related factor 1	AAF60517.1
Tribolium castaneum PDGF/VEGF-related factor 3	EFA02909.1
PtPVF	KU361821

RNA isolation and cDNA synthesis

The collected tissue samples were used to extract total RNA with RNAiso Plus (9108, Takara, Japan). The concentration of RNA was determined using a measurement of the UV absorption at a wavelength of 260 nanometers, and its integrity was checked with 1.5% agarose gel. The first strand cDNA was then prepared using PrimeScript RT reagent Kit with gDNA Eraser (RR047A, Takara, Japan) with total RNA from different tissues as template and adaptor-oligo (dT) as primer. The reactions were performed at 42 °C for 1 h, terminated by heating at 95 °C for 5 min, then a homopolymeric tail was added using terminal deoxynucleotidyl transferase (2230, Takara, Japan) and dCTP (U1221, Promega, USA), and preserved at -80 °C.

Cloning of the full-length cDNA of PtPVF

An EST sequences (GW400075) homologues to previous identified PDGF/VEGF-related factors was selected to obtain the full-length cDNA sequence of PtPVF using rapid amplification of cDNA ends (RACE) method. Based on the obtained sequences, specific primers for RACE were designed using Primer Premier 5.00 (Table 1). The primers PtPVF-RACE-F1/F2 and adaptor primer-oligo (dT) were used to obtain 3' ends sequence, and the primers PtPVF-RACE-R1/R2 and adaptor-oligo (dG) were used to obtain 5' ends sequence. The other two gene-specific primers, PtPVF-ORF-F/R, were used to amplify and validate PtPVF ORF sequences. The PCR products were purified by gel and cloned into pMD18-T simple vector (D103A, Takara, Japan), and transformed into competent *Escherichia coli* strain DH5α (CB101, Tiangen, China). The positive recombinants were identified through anti-ampicillin selection, and three positive clones were picked for sequencing.

Sequence analysis

The sequence was identified by the BLAST program of the National Center for Biotechnology Information (NCBI) and multiple alignments were performed using ClustalW2. The PtPVF nucleotide sequence was translated into amino acid sequence using DANMAN 9.0. ExPASy server was used to predict the molecular mass and theoretical isoelectric point (pI). The simple modular architecture research tool (SMART) and the SignalP 6.0 Server were used to predict the functional domain and putative signal peptide. Phylogenetic tree was constructed using the neighbor joining (NJ) method in MEGA 11.0, and the reliability of the branches was tested with 1000 bootstrap trials. The sequences used in multiple sequence alignments and phylogenetic tree analysis were listed in Table 2.

Quantitative real-time PCR (qPCR) detection

The qPCR technique was utilized to measure the relative mRNA expression levels of PtPVF in different tissues of crabs, as well as the relative mRNA expression levels of PtPVF in hemocytes of crabs post various stimulation. We selected EF-1α as the reference gene for internal standardization to calibrate the cDNA template. PerlPrimer 1.1.21 was used to design primers PtPVF-qF and PtPVF-qR for PtPVF, and EF-1α-qR and EF-1α-qF for EF-1α. The efficiency of each primer pair was analyzed with

serial two-fold dilutions of cDNA (1, 1/2, 1/4, 1/8, 1/16 and 1/32) to determine that all pairs of primers have similar efficiency (Li *et al*., 2023; Sun *et al*., 2022), which were 99% for PtPVF-qF and PtPVF-qR, and 98% for EF-1α-qR and EF-1α-qF, respectively. The qPCR was carried out using Premix Ex Taq (Tli RNaseH Plus) with ROX (RR420A, Takara, Japan) in an ABI 7500 Real-time Thermal Cycler. The specific protocol settings for qPCR were one cycle at 94 °C for 30 s, 40 cycles at 94 °C for 5 s, and 60 °C for 31 s. The mRNA relative expression level of PtPVF and EF-1α were analyzed by comparative C_T ($2^{-\Delta\Delta Ct}$) method (Wang *et al*., 2011; Wang *et al*., 2014). All data were given in terms of the mRNA relative expression of PtPVF as the mean ± SD (n =5). The data were subjected to t-test to determine difference, and $P < 0.05$ was considered significant.

Result

Cloning and sequence analysis of PtPVF

The full-length cDNA of PtPVF was obtained by 5' and 3' RACE technique and had been submitted to the NCBI GenBank with the accession number KU36182. As is shown in Figure 1A, the full-length cDNA of PtPVF consisted of 1320 bp, including an open reading frame (ORF) of 588 bp, a 105 bp 5' untranslated region (UTR) and a 633 bp 3' UTR with a poly (A) tail. The ORF encoded a polypeptide of 193 amino acids, its calculated molecular mass was 58.5 kDa, and its pI was 6.9. In the deduced amino acid sequence, there were typical PDGF/VEGF domain and a signal peptide containing 16 amino acid residues. In addition, the typical cysteine junction motif (CXCXC) was also identified.

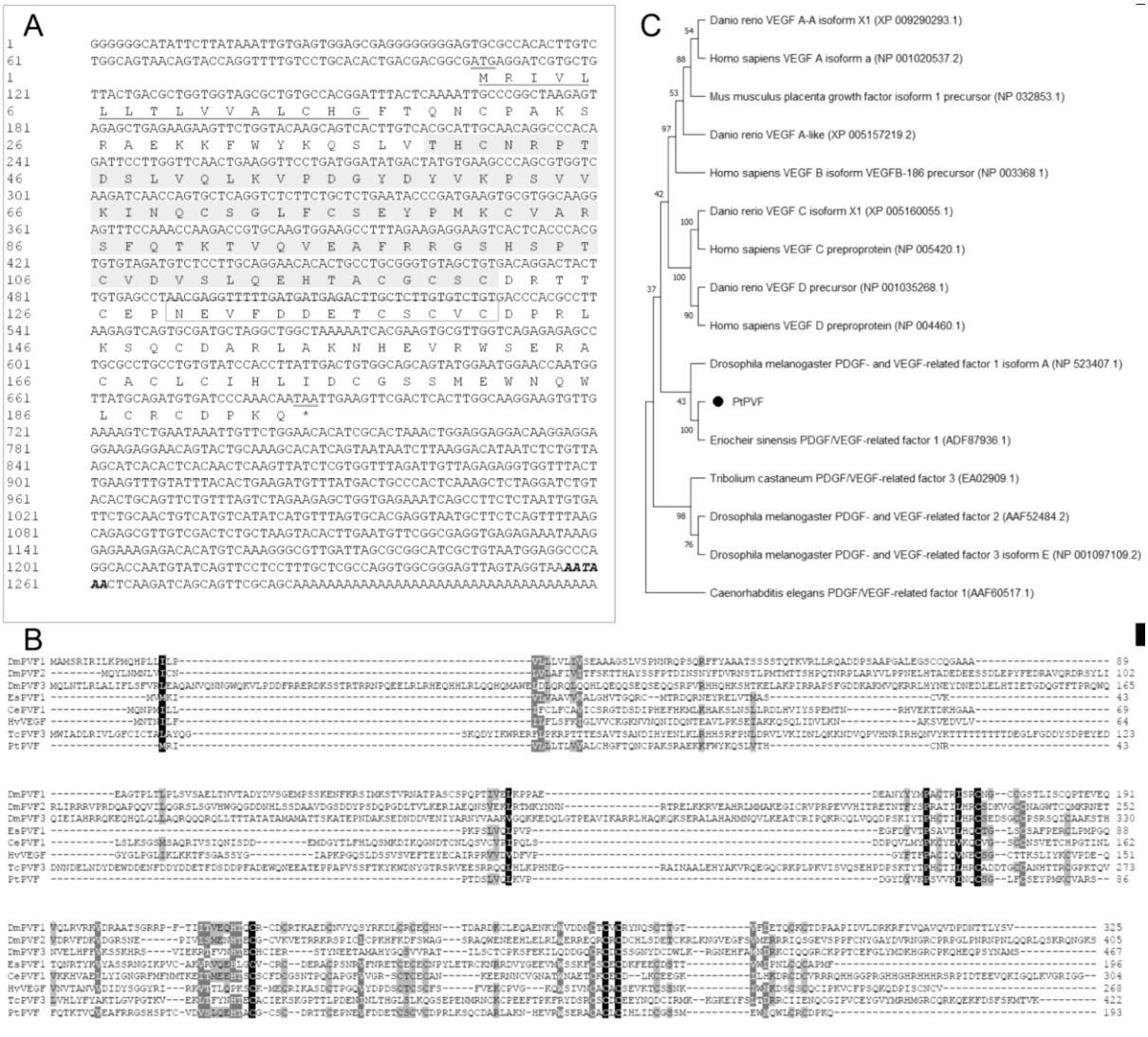

Fig. 1 Sequence features of PtPVF. A: Nucleotide and deduced amino acid sequences of PtPVF. The start codon (ATG) and the stop codon (TGA) are marked with double underlines. The signal peptide is indicated with underline and PDGF/VEGF homology domain is shadowed. The cysteine knot motif (CXCXC) with box. B: Neighbor-joining phylogenetic tree was constructed based on the PVF sequences from different organisms. The phylogenetic tree was constructed using MEGA software 11 and 10,000 bootstrap replications. C: Alignment of amino acid sequences of PtPVF with other selected PVFs. Amino acid residues that the same sequences are shaded in dark, and similar amino acids are shaded in gray. Dashes indicate gaps

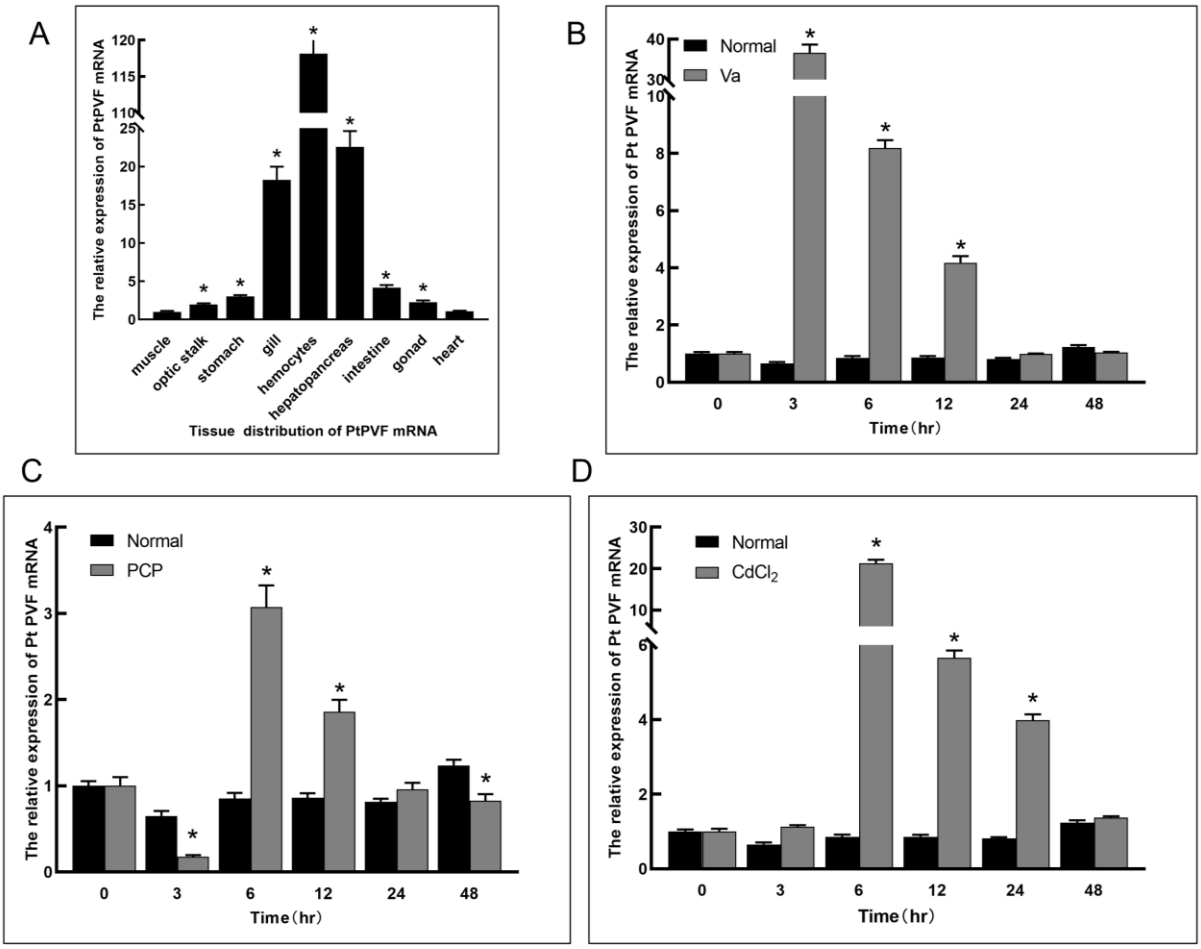

Fig. 2 Expression profiles of PtPVF. A: The mRNA expression level of PtPVF in different tissues. Significant differences ($P < 0.05$) of the mRNA expression level of PtPVF were indicated with asterisks. The EF-1α gene was used as an internal control to calibrate the cDNA template for each sample. B: The temporal mRNA expression of PtPVF in hemocytes of *P. trituberculatus* after Infection with *V. alginolyticus*. Significant differences ($P < 0.05$) of the mRNA expression level of PtPVF between the challenge and the control group were indicated with asterisks. EF-1α was used as an internal control to calibrate the cDNA template for all the samples. C: The temporal mRNA expression of PtPVF in hemocytes of *P. trituberculatus* after PCP contamination. Significant differences ($P < 0.05$) of the mRNA expression level of PtPVF between the challenge and the control group were indicated with asterisks. EF-1α was used as an internal control to calibrate the cDNA template for all the samples. D: The temporal mRNA expression of PtPVF in hemocytes of *P. trituberculatus* after $CdCl_2$ contamination. Significant differences ($P < 0.05$) of the mRNA expression level of PtPVF between the challenge and the control group were indicated with asterisks. EF-1α was used as an internal control to calibrate the cDNA template for all the samples

Multiple sequence alignment and phylogenetic analysis

According to the comparison of the amino acid sequences from some other known PDGF/VEGF related factors, the cystine residues were highly conserved and the typical structural characteristics of the PDGF/VEGF family were present in several species (Figure 1B). A phylogenetic tree of PVF gene was constructed using NJ method. As shown in Figure 1C, PtPVF was clustered with PVF1 of *E. sinensis*, indicating that most invertebrate PVFs were clustered together.

The mRNA expression level of PtPVF in different tissues

The PtPVF mRNA transcripts were all detectable in gill, gonad, heart, hemocytes, hepatopancreas, intestine, muscle, optic stalk, and stomach (Figure 2A). The highest mRNA expression level of PtPVF was found in hemocytes (117.41-fold compared with muscle, $P < 0.05$), followed by hepatopancreas (22.44-fold compared with muscle, $P < 0.05$) and gill (18.15-fold compared with muscle, $P < 0.05$).

Temporal mRNA expression profiles of PtPVF after different stimulation

The mRNA expression profiles of PtPVF in hemocytes after the stimulation of *V. alginolyticus*, PCP or $CdCl_2$ were analyzed by qPCR. As shown in Figure 2B, after stimulation with *V. alginolyticus*, the relative mRNA expression levels of PtPVF significantly increased, peaked at 3 h (36.61-fold compared with the original, $P < 0.05$), and maintained a high level at 6 h (8.17-fold compared

with the original, $P < 0.05$) and 12 h (4.18-fold compared with the original, $P < 0.05$). Then, the relative mRNA expression levels of PtPVF in the challenge group was down-regulated continuously and had no significant difference with the control group at 24 h. Figure 2C illustrates the changes in PtPVF relative mRNA expression levels in the hemocytes post PCP challenge. First, the relative mRNA expression levels of PtPVF decreased to the lowest point at 3 h (0.18-fold compared with the original, $P < 0.05$), and then increased rapidly with the peak at 6 h (3.06-fold compared with the original, $P < 0.05$). Subsequently, the relative mRNA expression levels of PtPVF slowly decreased and reached 1.85-fold of the original level at 12 h, then returned to normal levels at 24 h. While under $CdCl_2$ stimulation, the relative mRNA expression levels of PtPVF in hemocytes was up-regulated. As shown in Figure 2D, the relative mRNA expression levels of PtPVF in hemocytes after $CdCl_2$ challenges were significantly up-regulated at 6 h (21.26-fold compared with the original, $P < 0.05$), 12 h (5.65-fold compared with the original, $P < 0.05$) and 24 h (3.97-fold compared with the original, $P < 0.05$) with the peak at 6 h. Then, the relative mRNA expression levels of PtPVF gradually declined from the peak at 6 h and returned to normal levels at 48 h. In addition, the relative mRNA expression levels of PtPVF did not exhibit any significant changes in the control group.

Discussion

PDGF/VEGF factors are closely associated with biological immune defense systems. According to previous studies, all known PDGF/VEGFs have characteristic PDGF/VEGF domains (Owji *et al*., 2018). The typical cystine knot motif CXCXC involved in inter- and intra-disulphide bonding of dimeric growth factor (Pötgens *et al*., 1994). The full-length cDNA of PtPVF in *P. trituberculatus* was successfully cloned and characterized in this study. The deduced amino acid sequence of PtPVF was conformity to the conserved domains of those identified PVFs, which included a signal peptide of 16 amino acids, a typical PDGF/VEGF homology growth factor domain of 83 amino acids, and the typical cystine knot motif CXCXC. In the phylogenetic tree, PtPVF is the closest relative to PVF1 of *E. sinensis*. And it is clustered together with most invertebrate aggregates. Therefore, based on these characteristics, PtPVF was considered as a novel member of the PDGF/VEGF family, which indicated that the PtPVF could have similar functions with those from other invertebrates.

In order to further investigate the potential biological roles of PtPVF, we used qPCR technique to analyze the mRNA expression profiles of PtPVF in multiple tissues of *P. trituberculatus*. According to previous studies, hemocytes have been proved to play a key role in the innate immune defense of crustaceans (Wang *et al*., 2015; Wang *et al*., 2018). Hepatopancreas was an important immune and detoxification organ in crustaceans (Song *et al*., 2015; Wang *et al*., 2016), which was closely related to the function of disease defense. In addition, gill, as an organ for water exchange with the outside world, was the first defense line against pathogens (Wang *et al*., 2019; Wang *et al*., 2022). The results of tissue specific tests in this study showed that the mRNA transcripts of PtPVF were detectable in all examined tissues, including gills, gonads, heart, hemocytes, hepatopancreas, intestines, muscles, optic stalk, stomach, with different expression levels. The highest mRNA expression levels of PtPVF mRNA were found in hemocytes, followed by hepatopancreas and gill. It is speculated that PtPVF may be involved in the innate immune regulation of *P. trituberculatus*.

In order to further explore the role of PtPVF in immune responses and environment pollution of crabs, hemocytes were selected as the target tissues to detect the temporal expression profiles of PtPVF in response to *V. alginolyticus*, PCP or $CdCl_2$ challenge. In decapod crustaceans, the characterization of PDGF/VEGF factors and its role in immunomodulation has been already reported. A PDGF/VEGF-related factor (EsPVF1) in Chinese mitten crab *E. sinensis* not only participated in immune response, but also regulated the neuroendocrine system through the induced release of norepinephrine and dopamine (Li *et al*., 2013). Similar results have also been reported in other invertebrates. Bacterial infection can induce differentiation of *D. melanogaster* mbn-2 cells and slightly increase PVF2 transcription levels (Cho *et al*., 2002). After LPS stimulation, the mRNA expression of PfPVF in the pearl oyster *P. fucata* was significantly down-regulated, indicating that PfPVF participates in the immune response to stimulation (Huang *et al*., 2017). In this study, after *V. alginolyticus* stimulation, the relative mRNA expression levels of PtPVF sharply increased at 3 h, indicating that PtPVF may protect organisms against pathogens challenges. Cadmium is a highly toxic and cumulative heavy metal pollutant, it can inhibit the phagocytosis function of macrophages and destroy the immune system (Wang *et al*., 2009). After $CdCl_2$ challenged, the relative mRNA expression levels of PtPVF also increased rapidly to a peak at 6 h after the experiment, and did not return to the initial level until 48 h. Unlike *V. alginolyticus* stimulation, PtPVF had different time points to reach the highest relative mRNA expression levels after $CdCl_2$ stimulation. The reaction to *V. alginolyticus* stimulation was earlier than to $CdCl_2$ stimulation. PCP was a kind of organic pollutants which can produce compound toxicity, it can greatly affect the development of zebrafish blood and immune cells (Namit *et al*., 2022). However, the mRNA expression profiles of PtPVF after PCP challenge were different. Initially, the stimulation of PCP caused a decrease in PtPVF which then increased slightly to its highest point at 6 h. At the peak, the relative mRNA expression levels of PtPVF were 3.06-fold compared with the original, much lower than 36.61-fold and 21.26-fold of the *V. alginolyticus* challenge group and the $CdCl_2$ challenge group. We concluded that PCP may inhibit the mRNA expression of PtPVF. These results suggested that the expression profiles of PtPVF would be influenced by multiple stimulation types. Different stimuli will affect the PtPVF response degree and speed. What' s more, PtPVF mainly responds to bacterial challenge and heavy metal stimulate.

In summary, we cloned and identified a PDGF/VEGF related factor gene (PtPVF) from *P. trituberculatus*. PtPVF was widely expressed in all the tested tissues and highly expressed in hemocytes, hepatopancreas and gills. The PtPVF was significantly increased in hemocytes after *V. alginolyticus*, PCP and $CdCl_2$ stimulation. It was proved that PtPVF may be involved in immune defense and stress response of environmental pollution of *P. trituberculatus*.

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