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Transcriptome profiling combined with network analysis deepens the understanding of immune response mechanisms in blood of pacific oyster *Crassostrea gigas* infected by *Vibrio alginolyticus*

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Mass mortalities of oysters during the summer have been reported for years. Among the biological factors causing oyster death in summer, *Vibrio* infection is considered to be the most important factor. *Vibrio alginolyticus* is one of the main pathogens causing oyster diseases. *V. alginolyticus* can cause disease and death of fish, shrimp, shellfish, and other aquatic products. Human infection with *V. alginolyticus* can also cause skin infections, external ear canal infections, and other diseases. Hemocytes in the blood of *Crassostrea gigas* resemble vertebrate macrophages and play an important role in the immune regulation of organisms. Therefore, it is of great significance to analyze the blood transcriptome to study the anti-pathogen mechanism of the *C. gigas*. In our study, we performed a transcriptome profile of gene expression on *C. gigas* infected with *V. alginolyticus* at 12 and 48 hours, and identified 2,494 and 1,165 differentially expressed genes (DEGs) at two sampling time points. Then, the functions of DEGs were identified using GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) functional enrichment analysis, revealing that there were multiple significantly enriched GO terms and KEGG signaling pathways related to immunity. And a protein-protein interaction (PPI) network was constructed using DEGs enriched in significant enriched immune-related KEGG signaling pathways. At last, we screened and validated 12 key genes with multiple protein interaction numbers or involved in more KEGG signaling pathways, and verified their expression changes by quantitative RT-PCR (qRT-PCR). For the first time, we studied the immune defense mechanism of *C. gigas* blood based on protein-protein interaction network, which explains how *C. gigas* lacking specific immunity survive in a pathogen-rich environment. This research provides reference for solving the problem of high mortality of *C.*

gigas and other mollusks in summer, and provides reference for the future production of some disease-resistant *C. gigas*.

KEYWORDS

Crassostrea gigas, *Vibrio alginolyticus*, immune response, protein-protein interaction networks, transcriptome

Introduction

The innate or nonspecific responses exist in all living organisms, which is a quick response, efficient and ancient immune defense system (Macagno et al., 2010). Some mollusks, such as bivalves, live in estuaries rich in pathogenic microorganisms, and they concentrate these microorganisms when they eat (Riisgard and Larsen, 2010). They lack specific immunity and can only depend on innate immune system to resist foreign microorganisms (Gestal et al., 2008). In invertebrates, innate defense relies on two elements: cells (Hemocytes) and body fluids (antimicrobial peptides, lectins, and lysozymes) (Söderhäll, 2010; Bachère et al., 2015). Hemocytes are considered to be the main effective components in the immune response of invertebrates (Zhang et al., 2014). Hemocytes, similar to vertebrate macrophages, act as an important role in immune protection (Buchmann, 2014).

Pacific oyster (*Crassostrea gigas*) originated from East Asia, and is widely cultivated in the coastal sea area of Korea, Japan, and northern China at the present stage (Guo, 2009). As a filter-feeding and sessile invertebrate living in estuaries and intertidal areas, *C. gigas* must cope with and adapt to the dynamic and changeable environment. dynamic and changing environment (Wang et al., 2018). How *C. gigas* lacking specific immunity survive in this pathogen-rich environment is a question worth exploring. There is growing evidence that over a long process of evolution and adaptation, oysters have developed a very complicated immune system, which can respond to various pathogens and environmental pressures (Huang et al., 2017; Xing et al., 2018; Huang et al., 2019; Chen et al., 2021). Mass mortalities of oysters during the summer have been reported for years (De Decker et al., 2011). Among the biological factors causing oyster death in summer, *Vibrio* infection is considered to be the most important factor (Meng et al., 2015). In the process of breeding, many oyster diseases are caused by *Vibrio*. The reported pathogenic *Vibrio* including *Vibrio alginolyticus*, *Vibrio splendens*, *Vibrio giganteus*, etc (Javier et al., 2017). *V. alginolyticus* is a gram-negative bacterium that is widespread in oceans and estuaries, and is one of the main *Vibrio* species that cause oyster disease, and can be isolated from patients, seawater, and aquatic products in a variety of environments (Lajnef et al., 2012; Xie et al., 2020). *V. alginolyticus* can cause disease and

death of fish (Xie et al., 2020), shrimp (Xie et al., 2020), shellfish (Gómez-León et al., 2005; González-Escalona et al., 2006), and other aquatic products, and bring huge economic losses. If humans are infected with *V. alginolyticus*, they will suffer from skin infections, infections of the external ear canal and other diseases (Reilly et al., 2011; Zhou et al., 2021).

Recent research shows that transcriptome sequencing is a common method to study changes in biological immune regulation. For example, transcriptome analysis can be used to compare the changes of gene expression in different treatment conditions and tissues (Li et al., 2018). This technique is already widely used in bivalves such as *Crassostrea angulata* (Zeng et al., 2019), *Pinctada fucata* (Guan et al., 2017), *Crassostrea virginica* (Zhang et al., 2014). However, the transcriptome of blood of *C. gigas* infected with *V. alginolyticus* was not reported.

In our study, transcriptional profiles of oyster blood were examined at 12 and 48 h post-injection of *V. alginolyticus*. We then performed a bioinformatics analysis, including screening for differentially expressed genes (DEGs), Gene Ontology (GO) functional enrichment, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. We established protein interaction networks that is associated with immunity to analyze the role of hub genes in *C. gigas* immune process. Finally, we screened and verified the expression patterns of 12 key genes by quantitative RT-PCR (qRT-PCR). As a result of this research will provide an important basis for future studies on the immune mechanism of *C. gigas* infected with *V. alginolyticus*, and provide an important and valuable resource for the oyster aquaculture industry.

Materials and methods

Oysters and bacteria

All the *C. gigas* used in this experiment came from Kongtong Island (Yantai, Shandong Province, China). These *C. gigas* were about half a year old, whose average shell length and average weight were 42.40 cm (range: 40.31 cm - 49.99 cm) and 26.59 g (range: 17.34 g - 39.08 g). These oysters were grown in filtered seawater at 25 ± 1°C and fed with spirulina and replaced with clean seawater daily. *V. alginolyticus* were presented by

researcher Bai Changming's team. After being retrieved, the bacteria were temporarily stored in at -20°C refrigerator for use. Before injection, *V. alginolyticus* was cultured in 2216E liquid medium at 28°C for 10 h to logarithmic proliferation phase.

Sample treatment and RNA preparation

We randomly divided healthy oysters into three groups: 10 oysters for blank control group (BCG), 70 oysters for PBS control group (PCG), 70 oysters for *V. alginolyticus* experiment group (VEG). The blank group was placed in normal aerated seawater, 50 μL *V. alginolyticus* (2×10^9 CFU) were injected into adductor muscle of oysters in VEG and oysters in PCG were injected with the same volume of PBS. In blank group, 6 oysters which blood was collected from the blood sinus were randomly selected before injection, then added with 1 mL TRIzol reagent and stored at -80°C for later extraction of blood RNA. At 12 h and 48 h post-injection, blood was collected in the PBS control group and *V. alginolyticus* experiment group in the same way. The blood of 6 oysters was randomly drawn at each sampling point for RNA extraction: BCG for 0 h (B-0h), PCG for 12 h (P-12h), VEG for 12 h (V-12h), PCG for 48 h (P-48h), and VEG for 48 h (V-48h). For each group, we randomly selected 6 oysters and divided them into 3 groups, and the RNA of the same mole ratio of oysters in each group was combined as templates for constructing RNA-Seq library. We used the remaining blood RNA after library construction for quantitative verification by quantitative real-time PCR.

Library construction and illumina sequencing

The extracted total RNA was used as the raw material for library construction. Total RNA was processed and library construction completed using the method of Li and Ge et al. (Li et al., 2018; Ge et al., 2018). Finally, the library preparation was sequenced on the Illumina NovaSeq platform to generate 150 bp paired reads.

Data processing and differential expression analysis

The raw data needs to be screened to ensure the quality and accuracy of subsequent analyses. It mainly includes removing reads with adapter, removing reads containing unknown base information, and removing inferior quality reads. Then calculate the cleaning data and GC content of Q20 and Q30. The clean reads were mapped to the *C. gigas* reference genome (Qi et al., 2021) by HISAT2 v2.0.5. Using Feature Counts v1.5.0-p3 count the number of reads mapped to each gene. The FPKM (fragments of transcribed sequence sequenced per million base

pairs) that can measure expression levels was then calculated for each gene based on the read counts and the gene length mapped to that gene. Using the DESeq2 R package (1.20.0) screen out differentially expressed genes between different treatment groups. Genes with $p\text{-value} \leq 0.05$ and $|\log_2\text{fold change}| \geq 1$ were designated as DEGs.

Functional annotation and enrichment analysis

We performed GO and KEGG enrichment analysis on the screened DEGs using DAVID v6.8 (Jiao et al., 2012), which enables us to better comprehend the high-level functions and roles of these DEGs in biological systems.

Functional protein association networks construction

The PPI interaction network was established by the default parameter of STRING v11.5 online tool to further study the connections and interactions between genes in immune regulatory pathways.

Gene expression validation by quantitative real-time PCR

We examined 12 DEGs using quantitative real-time RT-PCR to validate the accuracy of transcriptome sequencing. The experimental material consisted of 6 oysters from each sampling point. Specific primers for selected genes are designed by Primer Premier 5.0. *C. gigas* β -actin (ACTB) was used as the reference gene because its expression levels were relatively stable at various time points during the test. Quantitative RT-PCR was performed in a total volume of 20 μL containing 1 μL of template cDNA, 0.8 μL of forward and reverse primers, 10 μL of TB Green[®] Premix Ex Taq[™] II (Takara), and 7.4 μL of DEPC H₂O by using CFX connect Real-Time PCR Detection System at 95°C for 30 s predeformation, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. We used the $2^{-\Delta\Delta\text{CT}}$ method to determine the relative expression of selected genes at each sampling point. The primer information of the selected 12 genes is shown in Table 1.

Results

Transcriptome sequence and reads mapping

Transcriptome sequencing was performed at five sampling points, with three replicates per group. The RNA sequencing

TABLE 1 Information on primers used for validation of quantitative RT-PCR.

Gene name	Forward primer (5'-3')	TM(°C)	Reverse primer (5'-3')	TM(°C)	Amplicon length (bp)
BIRC2	GAATGACGCCTCCATCGCCATG	61	CGCAAGCCAAGTACGGACAGTC	61	127
BIRC3	CCTTGGACAGAACATGCCCGATG	61	GCTCCCTCTCCATTGCCTCTAG	61	131
CAV1	GCGGAAGTAGATGGCACTCATAGC	60	AGAGCAAAATCCAGTCCCCAGAAAC	60	146
CDC42	ATCCGCTCCTATCTCTCTCGTACAC	60	GACGCCATGTCCGACACTCTTG	61	143
COL6A5	CTGGCTCACGATGCTGGAATCTATG	60	CTGGCAATGGCACCGAGTTCTAC	61	83
COL9A1	AATTGGCGGTGGTATGTCAGATAGC	61	TCTCCTGGCATTCTGTGAGTCC	60	93
MYD88	CGACATTCGCCGAGGTTCTACGATTC	60	TCACCTGTTGATCCGCCTCTCTG	61	128
PIK3AP1	TGCTGTGATAGTCTGGTCTCTCTC	60	CATCCACGAGTCCCGCAAATC	61	149
PIK3CD	ACATCTCGGACACCCTCTACTG	60	TCCTGTGCTGCTGAGAATGGC	60	138
PIK3R1	CCAGAGGGAGCGAGAGACCAAC	61	GTCCACCACATTACGGCAGTCC	61	85
TEK	ACCATAGCAGCAAACGGCAGTG	61	CCTTGATTTCCGGACCCTAAACCAG	60	150
XIAP	GTAGTGCCAAGTCTCCACACCATC	60	AGTCTGTCTGGGTAGCGGTCTG	61	125

results are shown in Table 2. The screened clean reads were mapped to the reference genome (Qi et al., 2021), where the number of genes detected in each group was approximately 11,000. The resulting genes were then compared with the SwissProt database for further functional annotation.

S2, and Figure 1). Figure 2 shows all DEGs of *C. gigas* after infection with *V. alginolyticus*. Each of these DEGs may be involved in immunity of *C. gigas*, so a union of them (3,498) was selected for future analysis (Figure 2 and Table S4). Heatmap (Figure 3 and Table S4) visually presents the expression changes and clustering distribution of these DEGs.

Differential expression analysis

Differential expression analysis showed that two DEG lists were obtained in the comparison of V-12h versus P-12h and V-48h versus P-48h, generating 2,494 and 1,165 DEGs, respectively. Among these, 1,221 DEGs were up-regulated and 1,273 DEGs were down-regulated at 12h; 693 DEGs were up-regulated and 472 DEGs were down-regulated at 48h (Tables S1,

GO and KEGG pathway enrichment analysis of DEGs

In our research, we selected 3,498 DEGs and performed GO and KEGG functional enrichment analysis on them. GO functional enrichment analysis results were divided into three ontologies. There are 151 third-class subclasses in

TABLE 2 Summary of sequencing.

Time points	Read length (bp)	Raw reads	Clean reads	Clean Q20 (%)	Clean Q30 (%)	GC (%)	Mapped reads ratio (%)	Detected gene number
B-0h-1	150	48,546,432	47,633,394	98.11	94.24	40.39	55.37	11,015
B-0h-2	150	55,416,200	54,055,906	98.04	94.12	40.33	55.06	10,795
B-0h-3	150	60,373,410	58,630,392	97.94	93.90	39.25	52.05	10,776
P-12h-1	150	78,185,148	74,994,674	97.90	93.81	37.58	37.71	11,242
P-12h-2	150	47,445,178	46,215,588	98.21	94.57	40.27	57.22	10,947
P-12h-3	150	62,127,810	60,448,266	98.06	94.15	39.55	49.74	11,017
V-12h-1	150	45,405,838	44,297,618	98.20	94.57	40.84	58.96	11,120
V-12h-2	150	46,085,830	44,990,510	98.08	94.29	41.30	61.62	10,982
V-12h-3	150	43,103,976	41,924,102	98.20	94.58	40.78	58.84	11,158
P-48h-1	150	89,743,138	86,025,768	97.94	93.91	36.65	32.94	10,389
P-48h-2	150	56,864,038	55,358,874	97.30	92.21	41.28	64.47	10,965
P-48h-3	150	42,057,148	40,281,134	98.72	95.70	41.68	64.64	10,951
V-48h-1	150	44,446,260	43,227,842	98.18	94.55	41.04	56.62	10,982
V-48h-2	150	42,044,796	41,096,474	98.21	94.54	40.59	57.86	11,134
V-48h-3	150	45,414,700	43,809,598	98.18	94.52	41.93	64.39	10,849

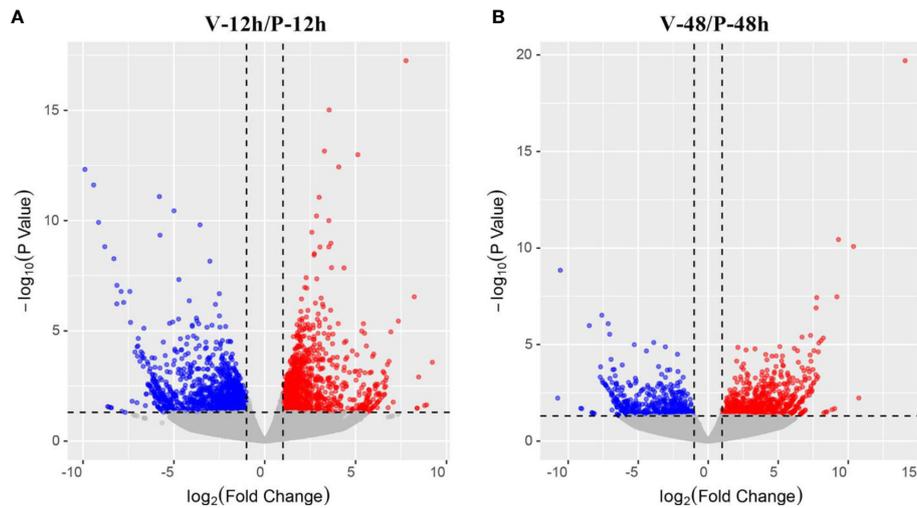


FIGURE 1

(A) Volcano plot of the distribution trend of DEGs between V-12h and P-12h groups. Each dot in this diagram represents a gene. Red dots represent up-regulated DEGs; blue dots represent down-regulated DEGs, and grey dots are not DEGs. (B) Volcano plot of the distribution trend of DEGs between V-48h and P-48h samples.

biological process, 46 third-class subclasses in cellular component and 57 third-class subclasses in molecular function (Table S5). Level-3 GO terms of the top 20 biological process or top 10 cellular component and molecular function are shown in Figure 4. Through KEGG pathway analysis, we can further understand the role of DEGs

in biological process, especially immune-related functions. Specifically, out of 3,498 DEGs, there are 339 annotated 156 KEGG signaling pathways (Table S6 and Figure 5). Among these pathways, there were 13 important and significantly enriched signaling pathways that are associated with immunity (Table 3).

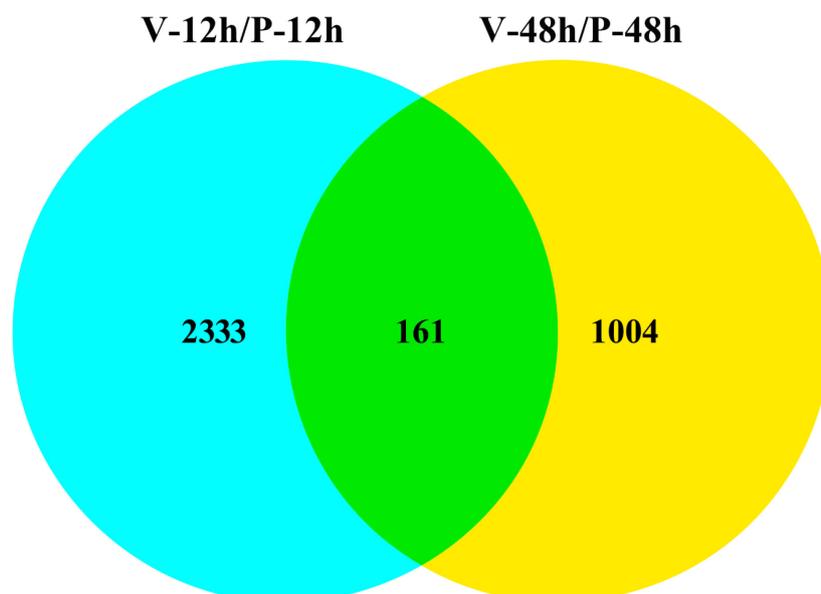
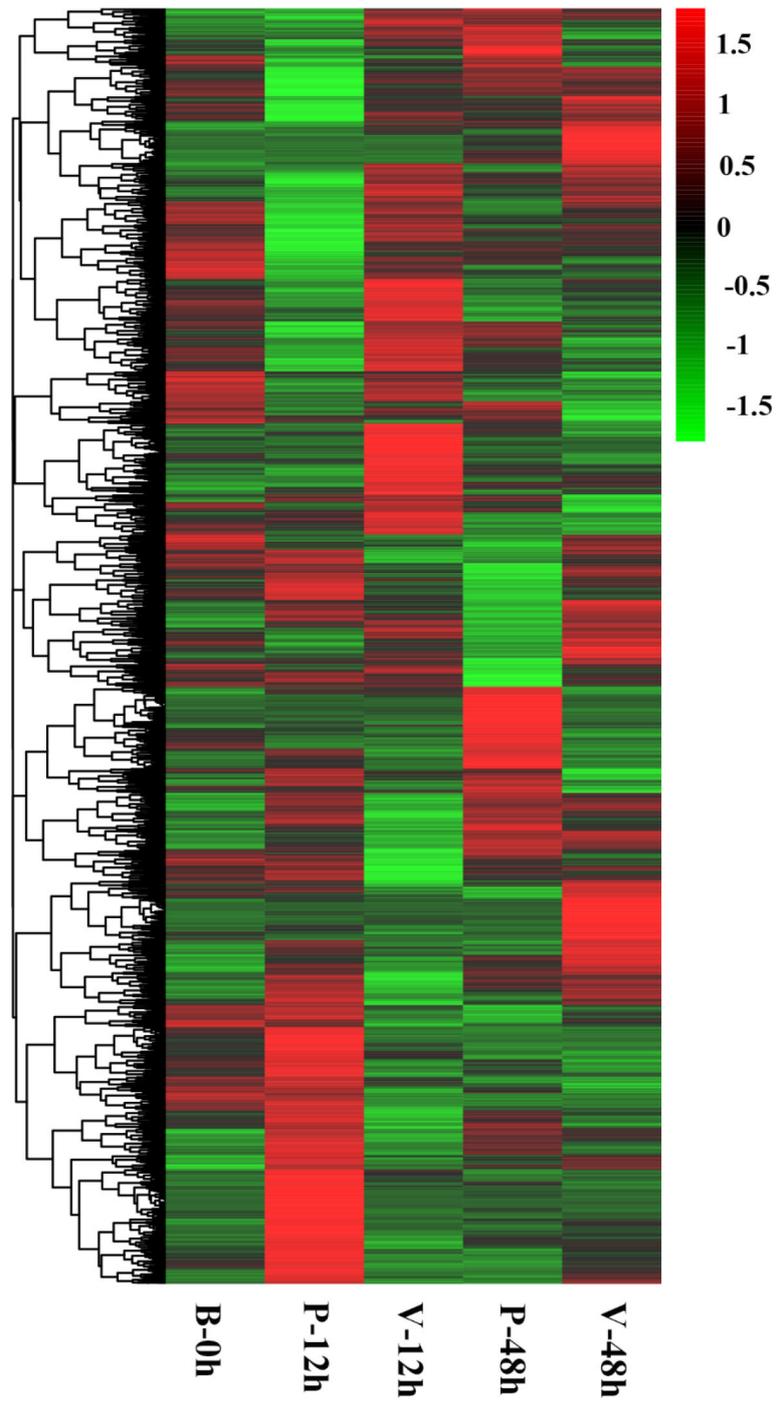
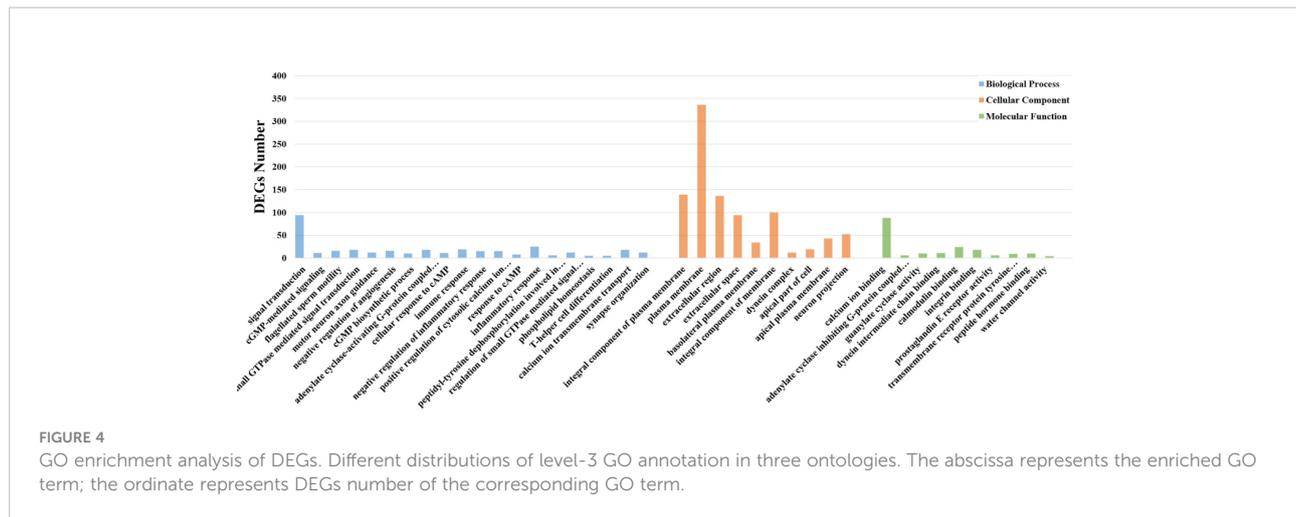


FIGURE 2

Venn diagram of overlapping DEGs at 12 h post-injection (blue) and 48 h post-injection (yellow). 2,333 DEGs are differentially expressed only at 12 h of infection; 1,004 DEGs are differentially expressed only at 48 h of infection; and 161 DEGs are differentially expressed at both two time points.

**FIGURE 3**

Heatmap analysis of expression patterns of all DEGs at the three time points. In this figure, each row represents a gene, and each column represents a sample group. Green to red indicates the increase of gene expression level.



Construction of immune-related protein interaction networks

Protein is the material basis for the immune defense function of organisms, and are involved in tissue formation and organ development of the immune system. Constructing protein-protein interaction (PPI) network is helpful to discover important genes in immune regulation. In this study, we used 71 DEGs which were significantly enriched in 13 important immune-related signaling pathways to set up the PPI network (Figure 6). Information about specific network parameters is shown in (Table 4). From this table, we can see that the number of protein interaction edges corresponding to the 43 immune-related DEGs we selected is higher than the expected number of edges, indicating that there is a significant interaction relationship between these genes.

Analysis of important DEGs associated with immune responses

In our study, we chiefly analyzed the interaction between immune-related DEGs. In total, we validated 12 key DEGs with multiple interactions or involvement in various signaling pathways (Table 5). These 12 key genes are divided into 5 categories: Inhibitor of apoptosis family, Collagen family, PI3K-Akt signaling pathway, NOD-like receptor signaling pathway and other important genes that are involved in various immune processes in oysters. Through these immune-related gene families and pathways, the immune defense mechanism of *C. gigas* was studied.

Quantitative RT-PCR validation

In order to verify the results of transcriptome analysis, we detected the relative expression changes of 12 immune-related

DEGs at 3 time points by using quantitative RT-PCR. The fold change detected by qPCR of selected DEGs was compared with fold change detected by RNA-Seq. As shown in Figure 7, the expression trends of the selected genes at each time point were verified by qRT-PCR to be the same as the sequencing results, indicating that the RNA-Seq sequencing results in this study were accurate.

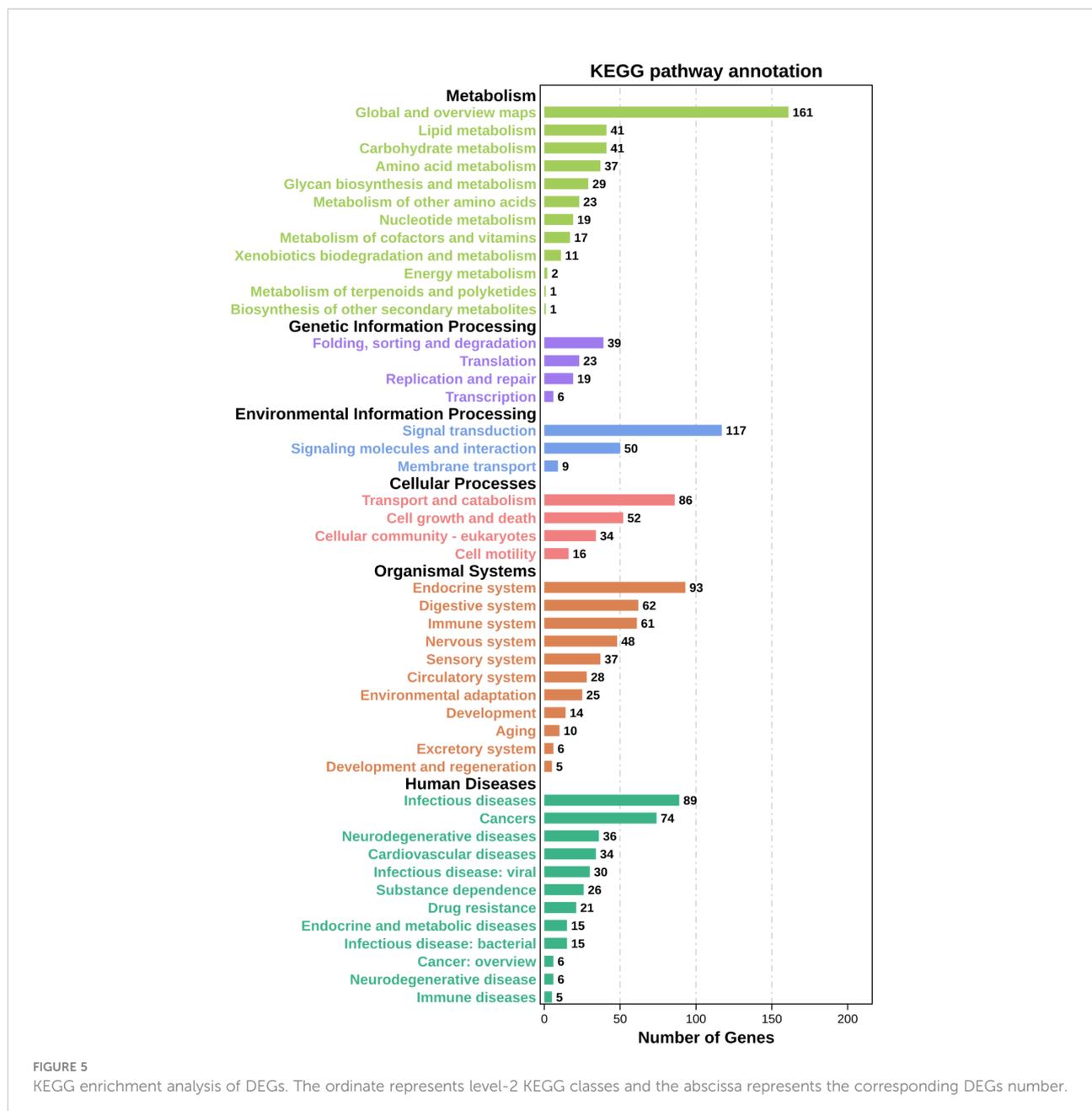
Discussion

The purpose and significance of transcriptome study

The high mortality of oysters in summer seems to be caused by the complex physiological interaction between host, environment and invasive pathogens (Samain, 2011). It was found that *Vibrio* infection was an important factor for serious reduction of oyster production in summer (Saulnier et al., 2010). Hemocytes of *C. gigas*, similar to vertebrate macrophages, play vital function in immune protection. Sequencing the transcriptome of the blood of *C. gigas* can help us understand preferably how *C. gigas* adapts to the environment affluent in pathogens. In order to better expound the changes in immune regulation of oyster infected with *V. alginolyticus*, we identified 2,494 and 1,165 DEGs at 12 h and 48 h post-injection, respectively. Every DEG may be involved in the oyster immune process, so we choose to use their union (3,498 DEGs) for the following analysis.

Enrichment of immune-related GO terms and KEGG pathways

GO enrichment analysis of DEGs after *V. alginolyticus* stimulation showed that some immune-related terms including



immune response, negative regulation of immune response, and immune response were significantly enriched in biological processes (Figure 4). Some pathways significantly enriched in KEGG pathway, such as PI3K-Akt signaling pathway and NOD-like receptor signaling pathway are related to immunity. These results fully indicate that there are affluent immune-related genes in the blood of *C. gigas*, and various pathways in immune regulation are activated to eliminate the threat posed by *V. alginolyticus*. In-depth discussion of these enriched GO terms and KEGG Pathways provides us with ideas to explore the immune regulation mechanism of *C. gigas* infected with *V.*

alginolyticus, find out the reasons for the high mortality of *C. gigas* in summer and ways to improve them.

Speculation of hub genes

Protein is the material basis of the immune defense function of the organism. Systematic analysis of the interaction between a large number of proteins and genes in the biological system is of great significance to explore the biological signals and immune regulation of *C. gigas* under special physiological conditions.

TABLE 3 Summary of 13 significant immune-related signaling pathways.

Pathways	Number of DEGs
Rap1 signaling pathway	22
PI3K-Akt signaling pathway	20
Proteoglycans in cancer	12
Inflammatory mediator regulation of TRP channels	10
NOD-like receptor signaling pathway	8
Transcriptional misregulation in cancer	8
Endocytosis	7
Pathways in cancer	6
Leukocyte transendothelial migration	4
Fc epsilon RI signaling pathway	4
p53 signaling pathway	3
Apoptosis	3
Bacterial invasion of epithelial cells	2

TABLE 4 Network Statistics of immune-related proteins.

Network Stats	
Number of nodes	63
Number of edges	474
Average node degree	15
Clustering coefficient	0.541
Expected number of edges	263
PPI enrichment p-value	1.0E-16

Therefore, we constructed an immune-related PPI using the 71 DEGs obtained by KEGG enrichment analysis. As can be seen

from Table 4, the number of interactions between the selected proteins is greater than expected. These results suggest that proteins interact as a group to regulate biological function. Therefore, proteins with many interactions are defined as immune-related hub proteins, and the genes corresponding to these hub proteins are hub genes that require attention and validation.

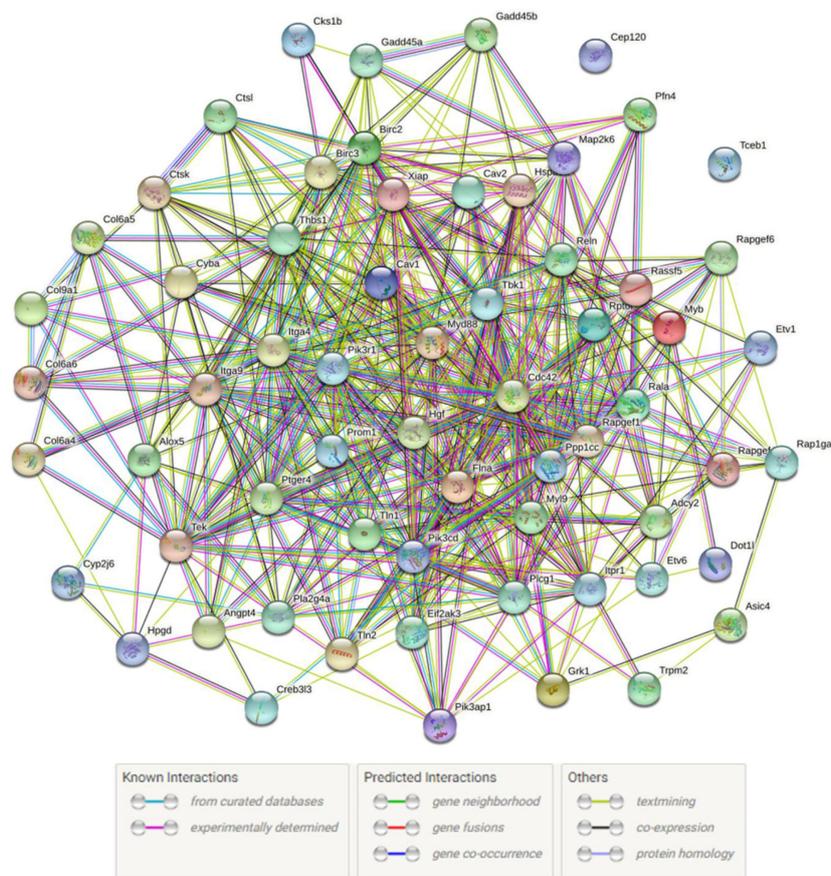


FIGURE 6 Immune-related PPI networks. Network nodes represents proteins. The legend represents the interaction relationship between the individual proteins.

TABLE 5 Summary of 12 key DEGs.

Gene name (abbreviation)	Gene name(official full name)	Number of protein-protein interaction	Number of KEGG signaling pathway
CDC42	cell division cycle 42	41	3
CAV1	caveolin 1	31	3
PIK3R1	phosphoinositide-3-kinase regulatory subunit 1	31	5
XIAP	X-linked inhibitor of apoptosis	30	3
MYD88	myeloid differentiation primary response gene 88	27	3
TEK	TEK receptor tyrosine kinase	26	1
BIRC2	baculoviral IAP repeat containing 2	23	1
BIRC3	baculoviral IAP repeat containing 3	23	1
PIK3CD	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta	23	1
PIK3AP1	phosphoinositide-3-kinase adaptor protein 1	12	1
COL6A5	collagen type VI alpha 5 chain	7	1
COL9A1	collagen type IX alpha 1 chain	7	1

Functional analysis of hub genes and KEGG signaling pathways

In this study, the transcriptome analysis of the blood of the *C. gigas* compared the changes of the immune response at different time points after *C. gigas* infection with *V. alginolyticus*, and revealed the special molecular response of the *C. gigas* to the stimulation of *V. alginolyticus*. At last, twelve hub genes were identified, which have various protein-protein interactions and participate in multiple immune-related KEGG signaling pathways.

Inhibitor of apoptosis family

Apoptosis is a basic biological phenomenon by which many organisms can clear damaged and infected cells (Gervais et al., 2016). Inhibitor of apoptosis (IAP) protein maintains the stability between cell expansion and cell death and promote the immune responses (Qu et al., 2015). In contrast to necrosis, apoptosis is actively performed by the cell. Typical features of apoptosis have been described in oysters, such as DNA fragmentation, cell shrinks, and rapid blebbing and chromatin condenses (Sunila and Labanca, 2003; Gervais et al., 2016). In this study, XIAP, BIRC2, and BIRC3 were selected as hub genes for functional research. In this study, the expression levels of XIAP, BIRC2 and BIRC3 in oysters infected with *V. alginolyticus* at 12 h and 48 h increased first and then decreased compared with the control group. XIAP can effectively inhibit endogenous and exogenous pathway stimuli to regulate caspase activation and apoptosis (Kamada, 2013). Previous studies have demonstrated that transcripts encoding IAP proteins are significantly up-regulated during the initial phase of oyster exposure to *Vibrio* (Tanguy et al., 2018), which consistent with our study. Overexpression of IAP in the blood of *C. gigas* indicates that apoptosis is activated during the stress period of *V. alginolyticus* to maintain homeostasis.

NOD-like receptor signaling pathway

NOD-like receptor (NLR) protein is a common pattern recognition receptor that regulate cellular pathways that control growth and immune responses to stimuli, and is pivotal in cellular infection, pathogen recognition and innate immune responses (Shaw et al., 2010; Platnich and Muruve, 2019). Some recent studies have suggested that NLRs also play a rather important role in different immune processes including inflammatory responses (Zhen and Zhang, 2019) and cell death (Heim et al., 2019). Many hub genes are enriched into NOD-like receptor signaling pathway in the blood transcriptome of *C. gigas*. Genes enriched in pathways, MYD88 and CYBA, were up-regulated at 12 h post-injection and down-regulated at 48 h post-injection. MYD88 is an essential component of the innate immune, and is also pivotal in priming and activating the immune defense system (Du et al., 2013). Many MYD88 isoforms have been found in different mollusks, and the expression of MYD88 isoforms tended to be up-regulated under pathogen attack (Qiu et al., 2007; Guo et al., 2018; Priyathilaka et al., 2018). The CYBA gene encodes P22^{phox} protein that is capable of destroying foreign bacteria and fungi as well as other pathogens (San Jose et al., 2008; Stasia, 2016). The results showed that many genes were enriched in the NOD-like receptor signaling pathway when *C. gigas* was infected with *V. alginolyticus*, which indicate that NLR plays a very important role in innate immune response of *C. gigas* and helps protect it from pathogens.

PI3K-Akt signaling pathway

PI3K-Akt signaling pathway can be activated by a variety of different types of bacteria and toxins, and takes part in the regulation of many basic cellular activities, such as cell growth, proliferation, survival, migration, invasion, apoptosis and metabolism (Cao et al., 2019; Liu et al., 2020). In this study, many hub genes such as phosphoinositide 3-kinases (PI3K)

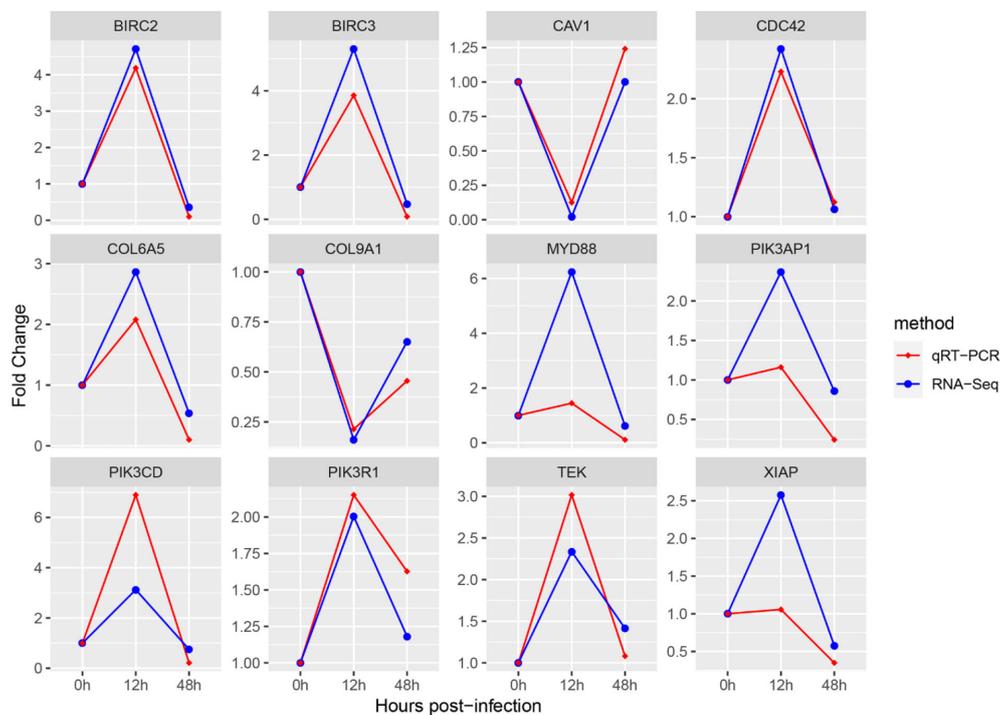


FIGURE 7

Comparison of 12 hub genes quantitative RT-PCR and RNA-Seq expression changes. The transcriptional expression levels of the selected DEGs were all normalized by the expression levels of the β -actin gene. The abscissa represents the change of sampling time, and the ordinate represents the fold change of experimental group compared with control group.

family and collagen family are enriched in this pathway. The genes of the PI3K family were all up-regulated after *V. alginolyticus* stress compared with the control group. Activation of PI3K can improve the immune defense ability of organism against pathogens and environmental pollutants and coordinate the phagocytosis of mollusk (Sun et al., 2016). The previous researches have shown that collagen and integrin can participate in the migration of oyster hemocytes, allowing hemocytes to penetrate into infected tissues and can also enhance the phagocytosis of hemocytes (Li et al., 2019; Lv et al., 2020). These results show that PI3K-Akt signaling pathway was activated by *V. alginolyticus* infection of *C. gigas*, and the up-regulated expression of hub immune related genes improved the immune ability of the oyster, so as to cope with the harm caused by *V. alginolyticus*. The up-regulated expression of many immune-related genes enriched in the pathway enhanced the resistance of oyster to pathogens.

The top three hub genes in the number of interactions

In this research, we focused on the interaction between different immune genes, so as to analyze the changes of immune defense mechanism of *C. gigas* after infection with *V. alginolyticus*. We screened and analyzed the top three genes for

protein interaction: CDC42, CAV1 and PIK3R1, which are also involved in multiple immune signaling pathways. CDC42, a small GTP-binding protein of the Rho family, is a core protein involved in cell cycle regulation and plays key roles in various signaling and biological processes in many organisms (Gerasimcik et al., 2017). CDC42 as a regulator controls the generation of functional granulocytes in *C. gigas*, and also regulates phagocytosis of granulocytes (Mao et al., 2020). CAV1 is an important functional protein in the caveolin family (Udayantha et al., 2018), and involve in many common physiological processes such as lipid transport, membrane transport and signal transduction (Liu et al., 2002). CAV1 can regard as the core of immune-related signaling pathways, such as NF-kappa B signaling pathway (Garrean et al., 2006) or Jak-Stat signaling pathway (Guo et al., 2011). PIK3R1 actively regulates the PI3K-Akt signaling pathway, promoting apoptosis and metabolic activity (Fan et al., 2020). Among them, CDC42, PIK3R1 were up-regulated at 12 h post-injection and slightly up-regulated at 48 h post-injection. These results suggest that these two genes may take part in the immune regulation of *C. gigas*. However, CAV1 was down-regulated at 12 h post-infection, and this result needs further research and verification. These three genes are involved in various immune processes, and have the largest number of protein interactions in

our research. Therefore, it is necessary to explore the functions of these three genes in the blood immunity of *C. gigas*.

Other KEGG signaling pathways and hub genes

In addition to the signaling pathways and hub genes discussed above, we also identified other genes and pathways involved in the *C. gigas* immune response to *V. alginolyticus*. For example, inflammatory mediator regulation of TRP, Fc epsilon RI signaling pathway, TEK. These pathways and hub genes are involved in the immune defense of many organisms and have been validated in previous studies (Yano et al., 1997; Klemm and Ruland, 2006; Duitama et al., 2021). The details of the signaling pathways and genes involved in the immune process in the blood of *C. gigas* infected with *V. alginolyticus* require us to further explore in future studies.

Conclusion

In this research, we performed the transcriptome analysis of *C. gigas* blood after infection with *V. alginolyticus* and constructed an immune-related PPI network. A total of 12 hub genes were identified, which participate in various KEGG signaling pathways and having various protein interactions with each other. For the first time, we studied the immune defense mechanism of *C. gigas* blood based on protein-protein interaction network. This research provides abundant reference data for future research on the specific response of *C. gigas* to *V. alginolyticus* infection. At the same time, it also provides reference for solving the high mortality of *C. gigas* and other mollusks in summer and help breed new varieties with stronger antibacterial properties.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA861156&acc_s%3Aa

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Author contributions

JY, ZL and WW designed and supervised the study. EZ, LD, XB, XY, YL and YF prepared the samples. EZ, XB and ZL analyzed all sequencing data. EZ, ZL and WW wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2022.1017445/full#supplementary-material>

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