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Transcriptome comparative analysis of immune tissues from asymptomatic and diseased *Epinephelus moara* naturally infected with nervous necrosis virus

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ABSTRACT

Epinephelus moara is an economically important fish in Southeast Asian countries but is suffering from nervous necrosis virus (NNV) infection. A deeper understanding of the host-NNV interaction mechanisms makes sense for disease control, however, at present, the pathogenesis of natural NNV infection and the resistance mechanism in host remains poorly understood. In this study, asymptomatic and diseased *E. moara* with clinical symptoms of viral nervous necrosis (VNN) from a grouper farm were both detected with a positive RT-PCR signal of NNV, then transcriptome sequencing of their immune tissues (liver, spleen and kidney) were performed for comparation analysis. The *de novo* assemblies yielded 53,789 unigenes which had a length varied from 201 to 19,675 bp and a N50 length of 2115 bp, and 29,451 unigenes were functionally annotated, with 83, 250 and 5632 unigenes being differentially expressed in liver, spleen and kidney respectively. KEGG pathway enrichment analysis of the DEGs showed many DEGs were enriched in immune related pathways. Although the expression of class I major histocompatibility complex (MHC) was significantly higher in three immune tissues of the diseased grouper, many immune related genes, including humoral immune molecules (such as antibodies), the cellular mediated cytotoxic molecules (such as perforin) and some adhesion related genes were down regulated in the diseased grouper. Our results provided many unigenes that might play important roles in NNV resistance for further research. Furthermore, a total of 8666 unigenes containing 11,623 simple sequence repeats (SSRs) were identified, which provided useful information for screening molecular markers associated with NNV resistance in *E. moara*.

1. Introduction

Nervous necrosis virus (NNV) is a pathogen lethal to more than one hundred fish species, NNV infection causes an acute infectious viral nervous necrosis (VNN) and mass mortality in larval and juvenile stages with vacuolation of the tissues in the central nervous system, while it cause persistent infection in adult stage which continues to spread the virus through spawning [1–3]. An infected fish often show abnormalities of movement, swim bladder control, sight and colour. NNV was a non-enveloped two single-stranded positive-sense RNA virus with sizes from 25 to 30 nm, which has been classified into the family *Nodaviridae*. The RNA1 sequence encodes the RNA-dependent RNA polymerase (RdRp) with a molecular weight (MW) of 110 kDa. RNA2 encodes the caspid protein of 42 kDa. According to International Committee on Taxonomy of Viruses, the genus Betanodavirus isolates were divided into four distinct clusters: TPNNV (Tiger Puffer Nervous Necrosis Virus), SJNNV (Striped Jack Nervous Necrosis Virus), BFNNV (Barfin Flounder Nervous Necrosis Virus) and RGNNV (Red-Spotted Grouper Nervous Necrosis Virus) [3,4].

Clinical signs of betanodavirus infection are characteristic and are the indication of disease onset. However, it is important to diagnose the preclinical state before disease occurs for disease control. Viral nervous

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necrosis can be diagnosed at least presumptively by clinical symptoms, histopathological identification, detection of virions, viral antigens or viral nucleotides, detection of specific antibodies in sera or body fluids and tissue culture of virus. Reverse-transcriptase polymerase chain reaction (RT-PCR) based on RNA2 segment is the most rapid and convenient method of diagnosing the infected fish. This method was widely used to detect the virus, and it could even be applied to screen the virus from asymptomatic fish [5,6]. The brood stock with NNV persistent infection caused the vertical transmission of NNV, and it can be controlled by selecting NNV-free spawners and disinfecting eggs using this detection method. However, there are still problems of horizontal transmission of NNV in fishes at grow-out stages obtained from the environment [3,7]. A deeper understanding of the host-NNV interaction mechanisms makes sense for VNN disease control, however, at present, the pathogenesis of natural NNV infection and the defense mechanism in host remains poorly understood.

The immune system plays an important role in fish resistance to viral infections [8–10]. It has been reported that the acute NNV infection in larvae and juveniles is largely due to their insufficient immune activity such as inactive interferon response while the adult fish with activated innate immune response show persistent infection [11]. Liver, spleen and kidney are the major lymphoid organs in teleost, therefore, they are eminently suitable organ for assessing immune defence ability and identification of immune-relevant genes in fish [12]. Although they were not the major lesion tissues of NNV, it has been reported that NNV RNA could be detected in the non-nervous tissues of NNV infectious fish by RT-PCR, such as spleen and kidney [13]. These immune organs showed immune respond to NNV infection in previous studies. The expression of a β-defensin gene of *Trachinotus ovatus* was most abundant in head kidney and spleen and was significantly up-regulated following NNV infection [14]. Intramuscularly vaccinating *E. coioides* by recombinant virus-like particles (VLPs) of OGNNV induced eleven genes associated with humoral, cellular and innate immunities in the liver, spleen and head kidney at 12 h post immunization [15]. Expression of type I interferon (IFN) and IFN-stimulated genes (ISGs) were significantly up-regulated after NNV-infection in the liver, spleen and kidney of *Oryzias latipes* [16]. However, little is known about the differences in immune response of these tissues between asymptomatic fish and diseased fish.

Grouper (*Epinephelus* sp.) is one of the most economically important aquaculture species in Southeast Asian countries, because of its delicious taste and high market price [17]. However, it is suffering from VNN disease which resulted in significant economic losses to grouper aquaculture [18,19]. *E. moara* is one of the main grouper species cultured in China, recently some diseased adults show the clinical signs of VNN in Laizhou Ming Bo Aquatic Co., Ltd., which is one of the biggest farms of grouper in north China. In this study, the pathogen of this disease was identified to be NNV by the use of a nested RT-PCR based on the NNV primers; in addition, the NNV RNA was also detected in asymptomatic fish. Whole transcriptome profiling analysis by rapidly developmented highthroughput sequencing technologies provides an efficient method for understanding the genetic response of host, especially of non-model organisms and those lacking reference genomes, to diseases and pathogens [20]. In order to understand the molecular mechanism in disease resistance, the differences in immune response of the immune organs were compared between asymptomatic and diseased groupers during NNV infection by transcriptomic analysis. Furthermore, putative simple SSRs were analyzed. These datasets provide a comprehensive understanding of the immune system in different clinical phenotype of *E. moara* naturally infected with NNV and will be useful for developing molecular markers associated with disease resistance.

2. Materials and methods

2.1. Ethics statement

This study was carried out in accordance with the animal care and use committee at the Yellow Sea Fisheries Research Institute. Efforts were taken to minimize fish suffering and included administering anesthesia. The study did not involve endangered or protected species.

2.2. Fish

The asymptomatic and sick *E. moara* were obtained from Laizhou Ming Bo Aquatic Co., Ltd. (Laizhou City, China). Clinical signs of three sick fish about 4–5 years old, with the weight of 2.9–10.5 kg were observed and collected. The swim bladder and abdominal cavities of the diseased fish were swelling severely and balance could not be kept. The eyeballs of the diseased fish protruded, the base of their pectoral fins festered, they swam abnormally and floated on the water surface with belly up and their feeding abilities were weak. Three asymptomatic fish about 4–5 years old, with the weight of 2.1–3.4 kg were also collected as control. The collected asymptomatic and diseased fish were from the same breeding group under the same breeding conditions.

2.3. Diagnosis of the disease in E. moara

Total RNA of liver, spleen and kidney from *E. moara* were extracted respectively using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. The RNA was then transcribed to cDNA using a PrimeScript™ RT reagent kit (TaKaRa, Dalian, China). With the spleen cDNA as the templates, the pathogen was identified by the use of a nested PCR based on the primers designed from consistent sequences of 4 reported NNV coat protein genes. PCR was performed as follows: 95 °C for 3 min, followed by 30 cycles of 95 °C for 5 s, 58 °C for 30 s, and 72 °C for 30 s. The DNA fragment was ligated into the pMD19-T vector and sequenced.

2.4. cDNA library construction and sequencing

Eighteen libraries of liver, spleen and kidney were constructed by the methods described by Ma et al. [21]. The amplified fragments were sequenced on the Illumina HiSeq2500 sequencing platform (Genedenovo Biotechnology Co., Ltd, Guangzhou, China) using 150 bp pair-end sequencing. All reads produced in this research have been deposited in the National Centre for Biotechnology Information (NCBI) and could be accessed in the short Read Archive (SRA) Database under accession number SRR8419168-8419173, SRR8987911-8987916, SRR8991368-8991369, SRR8991386-8991387, SRR8991356-8991357.

2.5. Assembly and functional annotation

Raw reads generated using the Illumina Hiseq 2500 platform were filtered to remove adaptors, sequences with unknown nucleotides larger than 5%, and low quality reads (more than 20% Q ≤ 10 bases). Following this, the clean reads were used for *de novo* assembly to produce unigenes using Trinity software (release-20130225) [22]. Functional assignment of unigenes was carried out with the protein databases NCBI nonredundant protein database (Nr, http://www.ncbi.nlm.nih.gov/), Swiss-Prot, KOG and Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.kegg.jp/) by Blastx with an E-value cutoff of $1e^{-5}$. The number of unigenes was annotated based on each database.

2.6. Analysis of differentially expressed genes

For analysis of gene expression, the number of unique-match reads was calculated and normalized to the FPKM (fragments per kilobase of

transcript per million mapped reads). Expression levels of each gene between the two groups were compared using the edgeR package to give an expression difference value [23]. K6 sample was removed in following analysis due to its poor repeatability. A threshold of false discovery rate (FDR) < 0.05 and an absolute value of log2Ratio > 1 were used to judge the significance of the differences in gene expression. The differentially expressed genes (DEGs) were used for GO classification and KEGG enrichment analyses to understand high-level functional information, according to a method similar to that described by Zhang et al. [24,25]. Both GO terms and KEGG pathways with a Q-value < 0.05 were considered significantly enriched in DEGs. All expression data statistics and visualizations were conducted using the R package software (http://www.r-project.org/).

2.7. Heatmap and gene co-expression network construction of some selected immune related genes

To exhibit the immune response differences, a heatmap of numerous immune related DEGs was constructed based on the Z-score normalized FPKM data and a cluster analysis was performed by using R program version. To reveal the relationship of these genes, expression correlation coefficients of these genes were calculated, and the top 200 relationships based on their absolute value were used to construct the gene networks using Cytoscape [26].

2.8. Identification of SSRs

The software MicroSAtellite (MISA) (http://pgrc.ipk-gatersleben.de/misa/) was used to detect putative SSRs from the assembled transcriptome. The parameters were adjusted to identify di-, tri, tetra-, penta- and hexanucleotide motifs with a minimum of 6, 5, 4, 4 and 4 repeats, respectively [27].

2.9. Quantitative real time PCR (qRT -PCR) analysis

The cDNA transcribed from liver, spleen and kidney RNA were used as the templates. Actin was used as reference gene. Primers used in this study were shown in Table S1. Quantification was performed using ABI 7500 Fast detection system (Applied Biosystems) with SYBR green Master Mix (TaKaRa). qRT-PCR was performed as follows: 94 °C for 30 s, followed by 40 cycles of 94 °C for 5 s, and 60 °C for 34 s. Transcription levels were analyzed by the comparative CT method and the data presented as mean ± SD of three samples with three independent replicates.

3. Results

3.1. Diagnosis of the disease

As the clinical symptoms of the sick *E. moara* obtained in this study were consistent with that of VNN disease (Fig. S1), we designed two pair of PCR primers for detection of NNV, and spleen cDNAs were used as the templates [13]. Using the nested RT-PCR with NNV primers, DNA fragments of length 245 bp were amplified from both asymptomatic and diseased spleen tissues respectively, and there were no corresponding bands in the control group. The DNA sequence cloned was shown in Fig. S2, and it showed high similarities of 99% with that of coat protein gene from RGNNV isolate MnNNV_12/06.

3.2. De novo assembly and analysis of the E. moara liver, spleen and kidney transcriptome

A total of 18 libraries were constructed using total RNAs from tissues of asymptomatic and sick *E. moara* and sequenced. As a result, a total of approximately 506,433,120 raw reads were obtained. After filtering dirty reads from the raw reads, 468,390,766 clean reads were obtained, with Q20% (proportion of nucleotides with quality value larger than 20) of 97.29%, and GC percentage of 52.37%. These clean reads were further assembled using Trinity software into 53,789 unigenes. The length of assembled unigenes ranged from 201 bp to 19,675 bp, with a total length of 64,084,043 bp, average length of 1191 bp and N50 length of 2115 bp (Table 1). The length distribution of assembled unigenes revealed that most of the unigenes (63.9%) ranged from 200 bp to 999 bp, 18.5% of unigenes ranged from 1000 to 2000 bp, 8.5% of unigenes ranged from 2000 to 3000 bp, and 9.2% of unigenes were over 3000 bp in length (Fig. S3).

Table 1
Summary of the *E. moara* transcriptomes.

Item	Data stats
Clean reads	468,390,766
Average read length (bp)	151
GC percentage (%)	52.37
Q20 (%)	97.29
Unigenes	53789
Unigenes N50 (bp)	2115
Minimum unigene length (bp)	201
Maximum unigene length (bp)	19675
Average unigene length (bp)	1191

3.3. Annotation of assembled unigenes

53,789 unigenes were compared against the protein databases including the NR, Swiss-Prot, KOG and KEGG database by BLASTx. BLAST results revealed that 29,293 (54.46%), 23,149 (43.04%), 18,613 (34.60%) and 16,707 (31.06%) unigenes significantly matched with the annotated sequences in NR, Swiss-Prot, KOG and KEGG databases, respectively (Fig. 1). In total, 29,451 unigenes (54.75%) were annotated.

3.4. Identification of DEGs

The expression levels of unigenes in three tissues of asymptomatic and sick *E. moara* were compared. We obtained 83 (77 up-regulated and 6 down-regulated in diseased *E. moara*), 250 (89 up-regulated and 161 down-regulated in diseased *E. moara*) and 5632 (318 up-regulated and 5314 down-regulated in diseased *E. moara*) significant DEGs from liver, spleen and kidney respectively with a |log2Ratio| > 1 and FDR < 0.05 (Fig. 2). There were 14, 36 and 213 unigenes that could not be matched with any existing genes in liver, spleen and kidney respectively. Among them, there were 12, 26 and 213 DEGs with a |log2Ratio| > 3 and FDR < 0.05 (> 8 folds differences), indicating that these unknown genes played an important role in *E. moara*-pathogen interaction. There

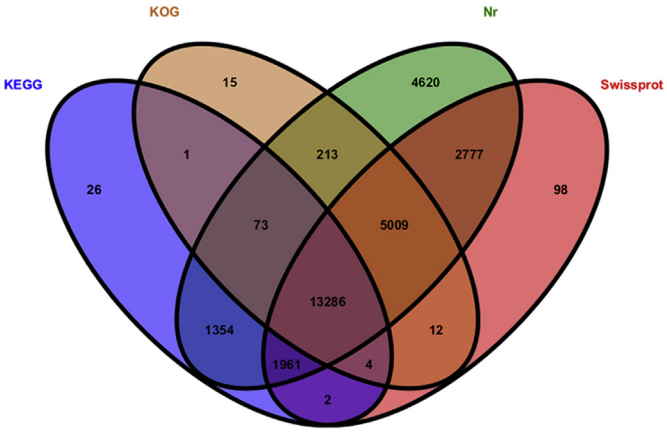

Fig. 1. Venn diagram showing the annotation of non-redundant consensus sequences.

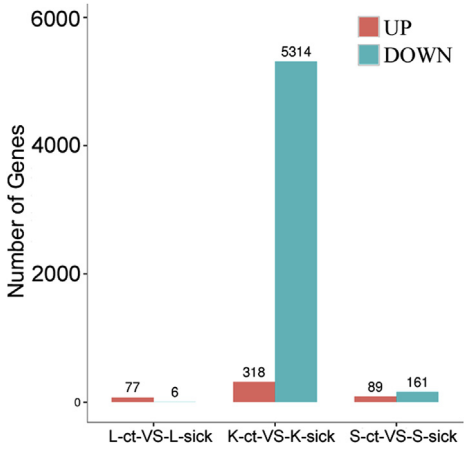

Fig. 2. DEGs statistics of liver, spleen and kidney transcriptome from asymptomatic (ct) and sick *E. moara*. L, liver; K, kidney; S, spleen; and the same below.

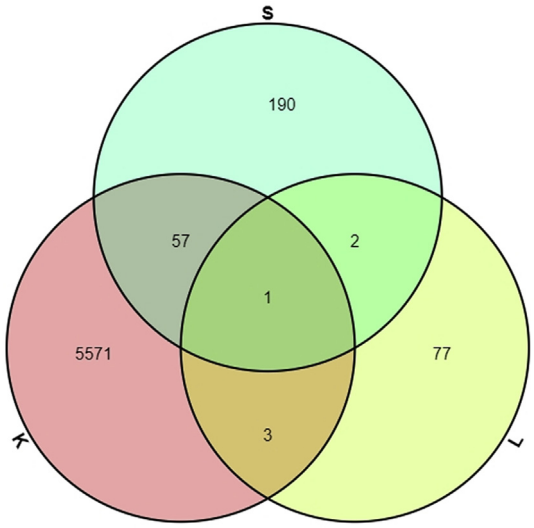

Fig. 3. Venn diagram of DEGs of liver, spleen and kidney transcriptome from asymptomatic and sick *E. moara*.

were 58 unigenes shared by kidney and spleen DEGs, 4 unigenes shared by kidney and liver DEGs, 3 unigenes shared in liver and spleen DEGs, while only 1 unigene showed significantly different expression in all three immune tissues (Fig. 3).

3.5. GO and KEGG enrichment analysis of DEGs

The Go classification assigned all DEGs into three main categories, namely biological process, cellular component and molecular function. A total of 5, 6 and 30 GO terms with $q < 0.05$ were significantly enriched in liver, spleen and kidney tissues, respectively (Fig. 4a). Tetrapyrrole binding was enriched in both kidney and liver tissues. Liver DEGs were also enriched in transport and localization related pathways. In a biological process analysis, kidney DEGs were enriched in single-organism process, while in cellular component and molecular function analysis, numerous pathways were enriched. In spleen, the enriched GO terms focused on peptidase related pathways, potassium channel activity and integral component of membrane.

In order to identify possible biochemical pathways of function for predicted protein encoded by DEGs, KEGG pathway enrichment analysis was carried out (Fig. 4b). By KEGG analysis, 22.8% of spleen DEGs were annotated to 50 signaling pathways. KEGG pathway enrichment analysis of spleen DEGs (Corrected $p < 0.05$) showed significantly enriched cell adhesion molecules (CAMs), ECM-receptor interaction, phagosome, focal adhesion, neuroactive ligand-receptor interaction and regulation of actin cytoskeleton. In addition, DEGs appeared in the pathways of cytokine-cytokine receptor interaction, peroxisome, endocytosis, intestinal immune network for IgA production, Jak-STAT signaling pathway, p53 signaling pathway, apoptosis, Toll-like receptor signaling pathway and MAPK signaling pathway. By KEGG enrichment analysis, 22.9% of liver DEGs were annotated to 28 signaling pathways and were enriched in phagosome, antigen processing and presentation, PPAR signaling pathway and p53 signaling pathway ($p < 0.05$). In addition, DEGs appeared in the pathways CAMs and cytokine-cytokine receptor interaction. In kidney, 19.7% of DEGs were annotated to 165 signaling pathways and significantly enriched in metabolism related pathways and immune related pathways (CAMs, cytokine-cytokine receptor interaction, intestinal immune network for IgA production, ECM-receptor interaction, PPAR signaling pathway, focal adhesion, neuroactive ligand-receptor interaction and Fc gamma R-mediated phagocytosis) (Corrected $p < 0.05$). Kidney DEGs also appeared in the pathways NF-kappa B signaling pathway, Natural killer cell mediated cytotoxicity, B cell receptor signaling pathway, Fc epsilon RI signaling pathway, phagosome, TGF-beta signaling pathway, MAPK signaling pathway, Toll-like receptor signaling pathway, RIG-I-like receptor signaling pathway, endocytosis and NOD-like receptor signaling pathway. The pathways of CAMs, phagosome, lysosome and intestinal immune network for IgA production enriched by DEGs in kidney were shown in Figs. S4, S5, S6 and S7.

3.6. Immune related DEGs and the gene co-expressed network

To exhibit the immune response differences, numerous immune related DEGs were selected to build a heatmap (Fig. 5), the information of these genes were listed in Table S2. These genes included immune related DEGs shared by 2 or 3 immune tissues and DEGs annotated in immune related pathways. Many immune related genes showed lower expression in diseased grouper, such as genes of antibody, perforin, interleukin, complements, chemokines and chemokine receptors. Three kinds of immunoglobulin (Ig) antibodies were detected in *E. moara*, including *IgM, IgD, IgT/IgZ*, but their expression were significantly lower in diseased grouper; kidney showed down regulation of *IgD* by 5.1 folds, *IgM* was down regulated by 1.6 and 6.2 folds in spleen and kidney respectively, and the expression of *G4* was down regulated by 5.3 folds in kidney. Perforin 1 gene (*Prf1*) was down regulated by 1.9 and 1.5 folds in spleen and kidney respectively. In kidney, a significantly lower expression of complements (*C1, C3, C7, C8* and *C9*), chemokines (*CCL4, CXCL13, CXCL10, CCL3, CCL19, CXCL9, CXCL12, CCL20* and *CCL25*), chemokine receptors (*CCR9, CXCR4, CXCR3* and *CXR1*) and interleukin (*IL1, IL2, IL8, IL11, IL12* and *IL15*) were detected in diseased grouper; and partial of these unigenes were used for heatmap construction. Only few immune related genes showed higher expression in diseased grouper, among them MHC genes showed significantly higher expression in diseased fish. *HLA-A* were up regulated by 8.3 and 16.0 folds in spleen and kidney respectively, and *MR1* were up regulated by 4.5 and 5.8 folds in liver and spleen respectively. Higher expression of *C3, lysozyme, hepcidin* and *CCL19* were detected in liver or spleen of diseased grouper.

To reveal the relationship of the genes in the heatmap, gene co-expressed network was constructed based on the top 200 relationships calculated by absolute value of their expression correlation coefficients. As was shown in Fig. 6, these genes had strong expression correlations, and *ITGA2, CXCR1, Lag3, LAMA5, sox11-b, PTGER4, Rc3h2, IgD, Lamb2, IGM, ITGA6, LATS2, LATS2, Sema4b, GPR21* and *Prf1* were located in the center of the network, implying they might play important roles together in NNV infection control.

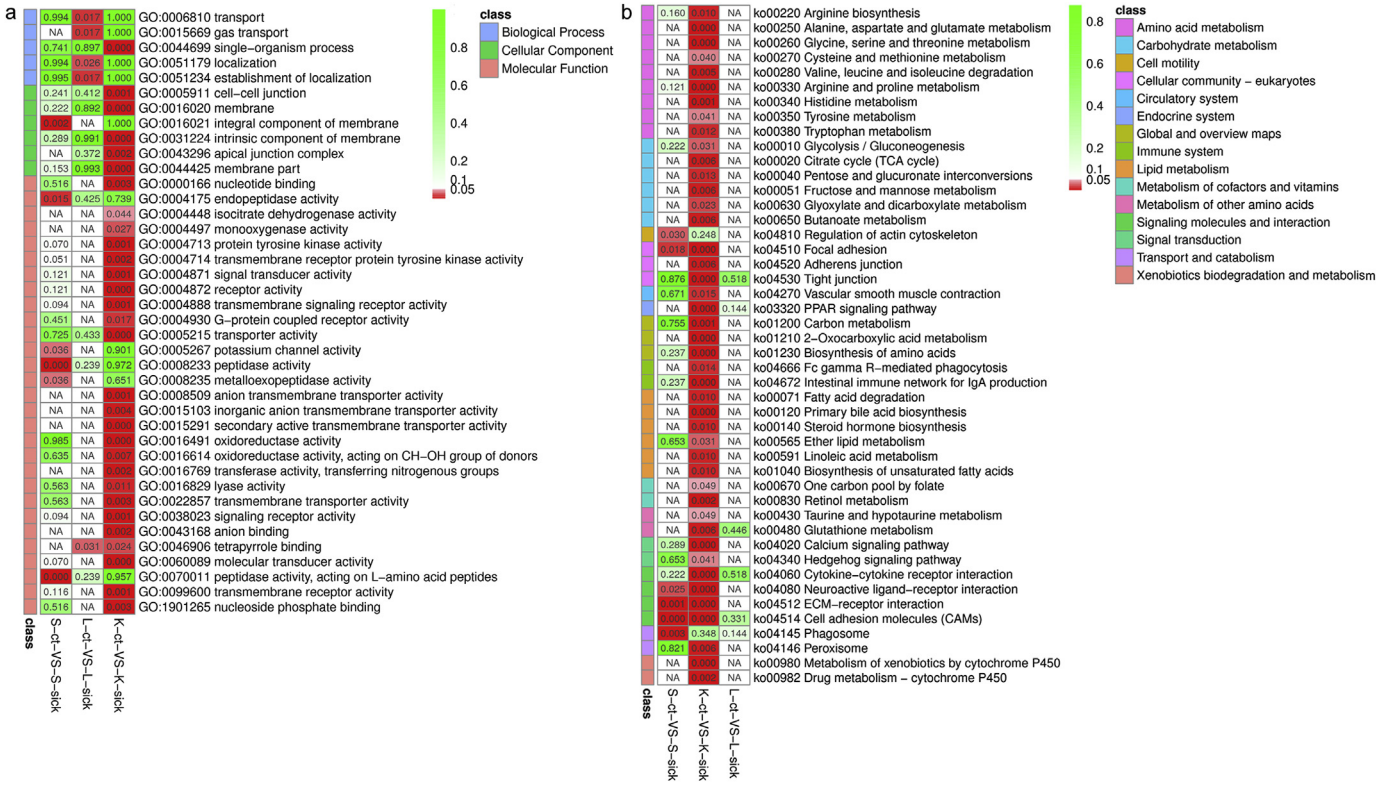

Fig. 4. GO term and KEGG pathway enrichment of DEGs in three groups of RNA-seq. a Enriched GO terms in the spleen, liver and kidney ($q < 0.05$). b Enriched KEGG pathways in the spleen, liver and kidney ($q < 0.05$). The color bar indicates q value from low (red) to high (green). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.7. Analysis of gene expression by quantitative real-time PCR (qRT-PCR)

In order to validate the expression changes, we detected the relative expression of partial unigenes in the heatmap using qRT-PCR, including 8 unigenes in spleen (*SAA1, Lyg2, CCL19, CD28, IGHV3-23, IGHM, FCGR3* and *MR1*), 6 unigenes in kidney (*HLA-A, CCL4, LAMA5, Mylk, IL8* and *Vtcn1)* and 1 unigenes *(HIPK3)* in liver. As shown in Fig. 7, the relative expression of *HLA-A, SAA1, Lyg2, CCL19, CD28* and *MR1* increased significantly in diseased grouper, compared with the expression of these genes in the control fish. In contrast, the expression of *IGHV3-23, IL8, FCGR3, IGHM, CCL4, LAMA5, Mylk, HIPK3* and *Vtcn1* decreased significantly in diseased grouper. These qRT-PCR results are basically consistent with RNA-seq results.

3.8. Identification of SSRs

SSRs are repeated sequences of 1–6 base pairs of DNA with conservative flanking sequence, which can be developed for genomic mapping, DNA fingerprinting, and marker-assisted selection in many species [26]. To avoid overestimation of SSRs given the fact that mono-nucleotides may be the result of sequencing errors and assembly mistakes, mono-nucleotide repeats were excluded in further analysis. Using MISA software, a total of 8666 unigenes containing 11,623 SSRs were identified from 53,789 unigenes, with 2074 unigenes containing more than one SSR (Table 2). On average, one SSR existed for about every 5.5 kb in the *E. moara* transcriptome. Within these SSRs, the most abundant type of repeat motif was di-nucleotide repeats (5969, 51.4%), followed by tri- (3995, 34.4%), tetra- (1161, 10%), penta- (289, 2.5%) and hexa- (209, 1.8%) (Table 3). The most abundant nucleotide repeats was AC/GT (40.9%), followed by AGG/CCT (10.5%), AGC/CTG (8.3%) and AG/CT (7.0%) (Fig. S8). By KEGG enrichment analysis, 66.7% (7747) of SSRs were annotated to 116 signaling pathways and were significantly enriched in pathways of focal adhesion, ECM-receptor interaction, Vascular smooth muscle contraction, CAMs and Glycosaminoglycan biosynthesis - heparan sulfate/heparin (Fig. 8).

4. Discussion

The pathogenesis of intracellular infection entails a continuous battle between the defence mechanisms of the host and specific mechanisms utilized by the pathogens to evade the immune response and to promote intracellular replication and survival; and this interaction process directly affects the course of infection and clinical manifestations. In this study, we obtained two phenotypes of adult *E. moara* naturally infected with RGNNV from a grouper farm, asymptomatic fish and diseased fish showing clinical symptoms. In fact, there has been several reports of nodaviruses infecting fish without evidence of disease [1,4,5]. However, the mechanism of viral persistent carrier state without clinical symptoms in adult is not yet clear, and we wonder which genes are important in controlling the infection process of RGNNV. To increase our knowledge of the immune system of adult *E. moara* and the differences in defence mechanism between asymptomatic and diseased fish naturally infected with RGNNV, we compared the transcriptome profiles of their immune tissues. In this study, 53,789 genes with a total length of 64,084,043 bp, average length of 1191 bp and N50 length of 2115 bp were obtained. The length distribution pattern, mean length and GC content of the unigenes suggested that the data from *E. moara* immune tissues was effectively assembled. The distribution of significant BLASTX hits over different organisms was analyzed. Due to the lack of *E. moara* genomic information, the majority of sequences in the libraries matched genes or fragments from *Lates calcarifer* (Fig. S9). BLAST results revealed that 29,451 (54.75%) unigenes of *E. moara* significantly matched with the annotated sequences in NR. This annotation ratio is relatively higher than those reported in previous *de novo* transcriptome sequencing studies for other fishes, such as *E. fuscoguttatus* (48.8%), *Scophthalmus maximus* (44.84%),

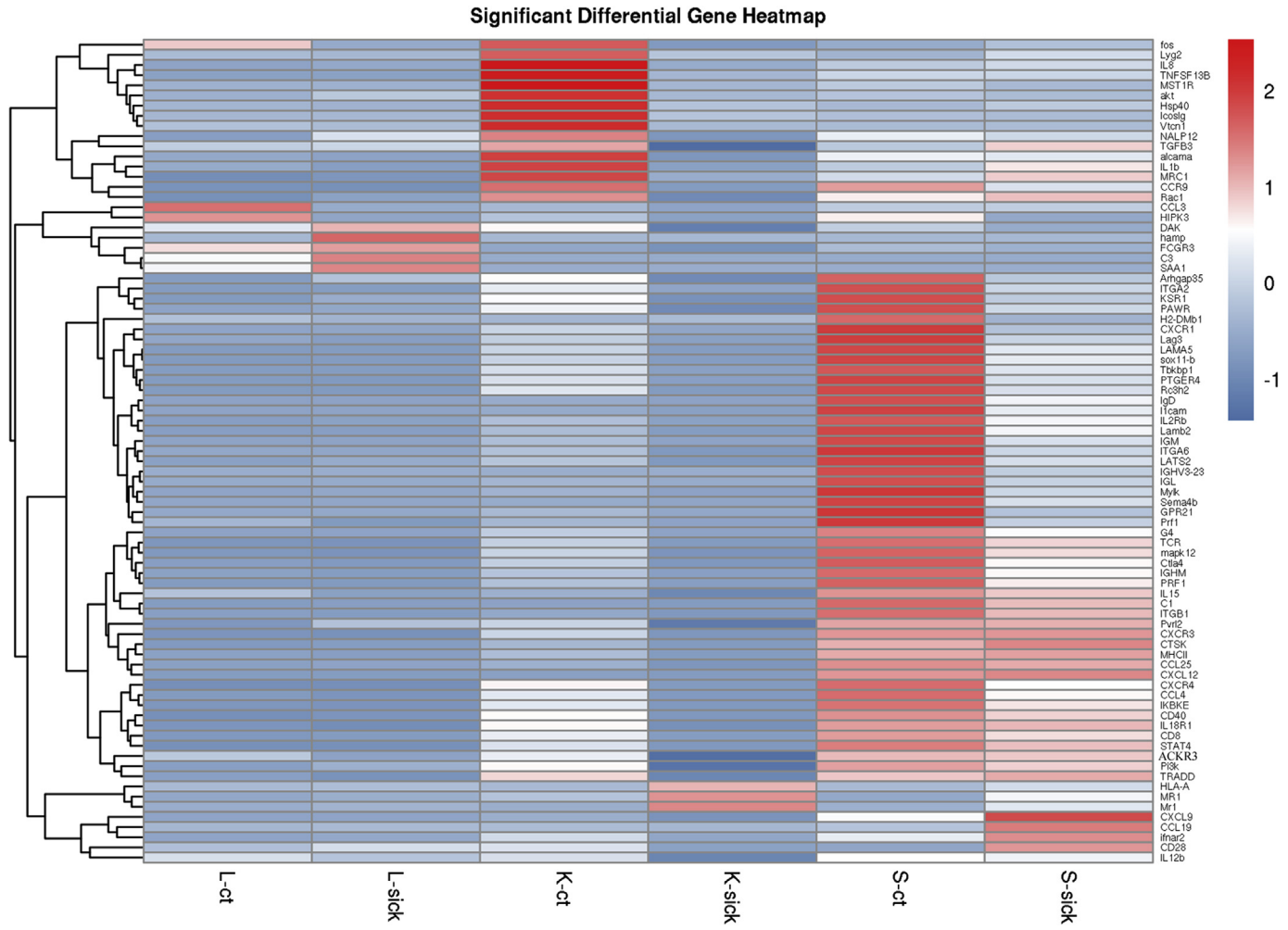

Fig. 5. The heatmap of numerous immune related DEGs including 30 shared DEGs by 2 or 3 immune tissues and 51 tissue specific DEGs. The gene cluster dendrogram were constructed by genes' correlation coefficients. The color bar indicates expression levels from low (blue) to high (red). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

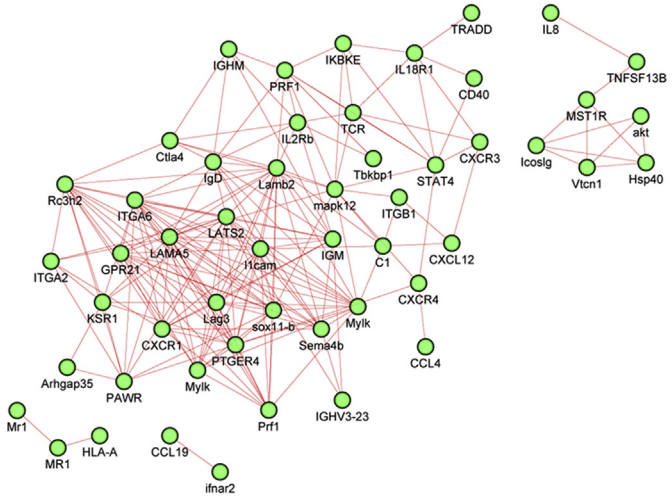

Fig. 6. The network relationship of numerous immune related DEGs.

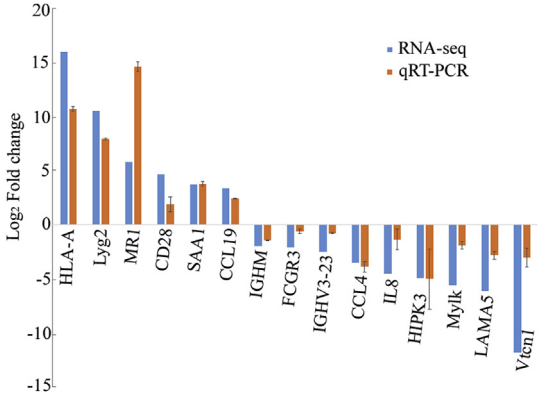

Fig. 7. Quantitative real-time RT-PCR for validation of RNA-seq. Expression of target genes was normalised to b-actin as a reference gene. The fold changes of asymptomatic grouper vs sick grouper detected by RNA-Seq and qRT-PCR were calculated by $2^{-\Delta\Delta Ct}$ and FPKM, respectively. qRT-PCR data were shown as means ± SD (n = 3).

Gymnocypris przewalskii (48.9%) and *Paralichthys olivaceus* (22.14%) [28–31]. This is the first report of comprehensive analysis of immune response towards NNV infection in *E. moara* immune tissues with different clinical signs, and the large amount of reliable transcriptome information obtained in this study could be a valuable resource for investigating specific processes, functions, and pathways in *E. moara*.

The difference in individual weight within the same grouper breeding population was relatively large, so the weights of the diseased fish used in this study showed huge difference. In the grouper farm where we obtained the diseased and asymptomatic *E. moara*, about

Table 2
Simple sequence repeats (SSRs) item in *E. moara*.

Item	Number
Total number of sequences examined	53789
Total size of examined sequences (bp)	64,084,043
Total number of identified SSRs	11623
Number of SSR containing sequences	8666
Number of sequences containing more than 1 SSR	2074
Number of SSRs present in compound formation	1123
Di-nucleotide	5969
Tri-nucleotide	3995
Tetra-nucleotide	1161
Penta-nucleotide	289
Hexa-nucleotide	209

Table 3
Length distribution of SSRs based on the number of repeat units obtained from immune tissues of *E. moara*.

Repeat Numbers	Motif length					Total
	Di-	Tri-	Tetra-	Penta-	Hexa-	
4	0	0	677	180	128	985
5	0	1989	231	43	61	2324
6	2081	902	65	30	2	3080
7	941	495	83	4	1	1524
8	644	96	39	4	7	790
9	387	47	12	9	7	462
10	297	239	14	2	2	554
11	180	45	12	1	0	238
12	59	24	8	5	1	97
13	9	35	5	3	0	52
14	26	24	2	1	0	53
> =15	1345	99	13	7	0	1464

3%–5% of farmed *E. moara* were diseased ranging from 1-year age to 5-year age, and the weights of the diseased *E. moara* were from 300 g to 11 kg. The disease showed no direct correlation with the weights of the fish, thus the difference in the fish body weights would not lead to huge differences in the expression of disease related genes. In addition, a threshold of false discovery rate (FDR) < 0.05 and an absolute value of log2Ratio > 1 were used to judge the significance of the differences in gene expression, which could filter out many DEGs due to the weight differences. In this study, through comparative transcriptome analysis, 83, 250 and 5632 DEGs were obtained from liver, spleen and kidney of *E. moara*, which implied kidney was the primary immune response organ. By KEGG pathway enrichment analysis, many DEGs were significantly enriched in immune related pathways, such as phagosome, CAMs, ECM-receptor interaction, focal adhesion, intestinal immune network for IgA production, Toll-like receptor signaling pathway, RIG-I-like receptor signaling pathway, NOD-like receptor signaling pathway, Cytokine-cytokine receptor interaction, Jak-STAT signaling pathway, p53 signaling pathway, apoptosis, TGF-beta signaling pathway and MAPK signaling pathway. Particularly, the DEGs annotated in most of immune related pathways showed consistently decreased expression in diseased grouper. In addition, many DEGs were unannotated by any database but expressed with significant differences; these unknown genes might play an important role in *E. moara*-RGNNV interaction and need to be studied in further research. In previous study, endoplasmic reticulum (ER) stress response was shown to be prominently affected in NNV-infected grouper kidney cells; while high immune cell active signaling, surface receptor and interferon expression were triggered in brain tissue of *E. malabaricus* during persistent infection [32,33]. In this study, although ER stress response was not a significantly enriched pathway, 18 DEGs in kidney and 1 DEG in liver and spleen were annotated in this pathway respectively.

Because NNV is an intracellular pathogen, it is likely that both B cell and cytotoxic T-cells (CTLs) activities are needed to provide an effective adaptive response against the virus. The type and route of antigen delivered is known to influence this response. Presentation of antigens

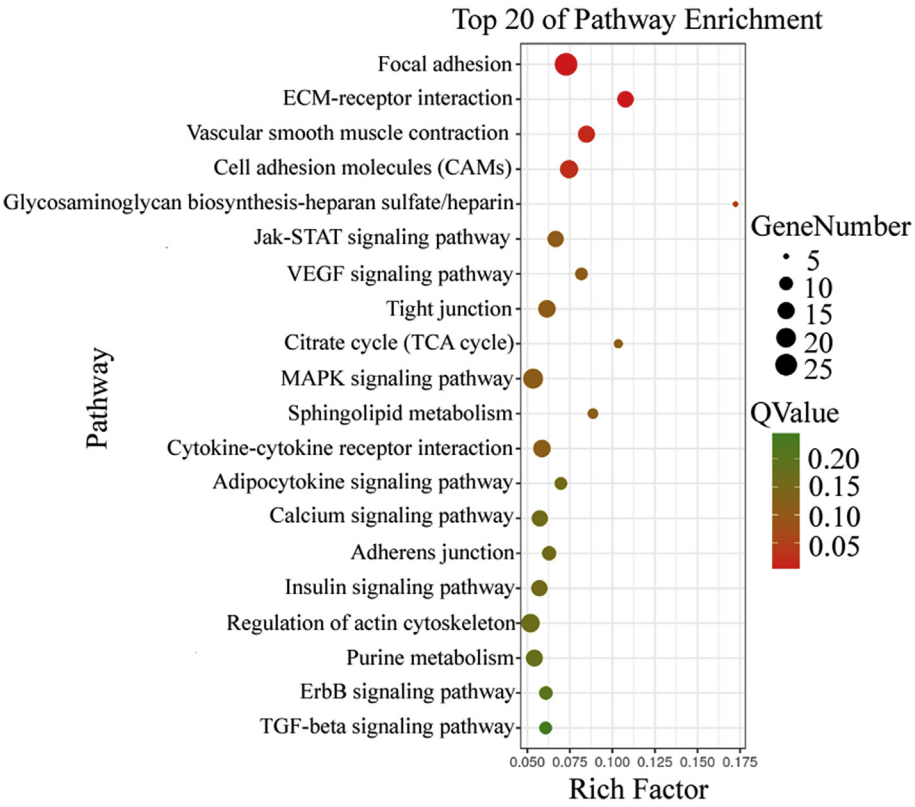

Fig. 8. KEGG pathway enrichment of differentially expressed SSRs. Rich factor stands for the ratio of the number of target SSRs belonging to a pathway to the number of all the annotated SSRs located in the pathway. The higher rich factor represents the higher level of enrichment. The size of the dot indicates the number of target SSRs in the pathway, and the color of the dot reflects different Q value range. Q value < 0.05 was considered to have enrichment significance. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

through an intracellular route induces MHC-I restricted $CD8^+$ responses (i.e. cytotoxic T lymphocytes, CTLs) e.g. live, attenuated or DNA vaccines, while extracellular delivery induces MHC-II restricted $CD4^+$ responses (i.e. antibody responses) e.g. inactivated virus and recombinant proteins [34,35]. In this study, many immune related genes showed significant different expression in asymptomatic and diseased fish, including antibody, complement, MHC, chemokines, chemokines receptors, antimicrobial peptides and interleukin. Among them, the expressions of Class I MHC were all significantly higher in three immune tissues of diseased grouper, implying a higher activity or replication capacity of the virus antigen in cells of sick grouper. Perforin is an important immune molecules involved in CTL cytotoxicity, which directly contributes to T-cell mediated death via apoptosis or necrosis by permeabilizing target cell membrane and ensuing translocation of pro-apoptotic granzymes into the cytoplasm, and it has been suggested as a potential biomarker against some diseases [36,37]. In addition, lots of DEGs in phagosome and lysosome pathways were down regulated in diseased fish (Fig. S6 and Fig. S7). In this study, although more class I MHC mRNA were expressed in the immune tissues of the diseased grouper, a significantly lower expression of antigen process related genes was shown, which suggested the cytotoxicity of CTLs was weaker in them.

It has been shown that the activation of antibodies is an important host immune response during NNV infections in teleosts, because the antibodies produced can neutralise the virus, preventing it from causing damage, and the detection of *IgM* levels has been used to evaluate the effectiveness of the vaccine for VNN disease control [38,39]. Class II MHC generally presents exogenous antigens to T helper lymphocytes to induce antibody response. In this study, the expression of Class II MHC was significantly lower in spleen and kidney of diseased *E. moara*, and three kinds of antibodies were detected in *E. moara*, including *IgM, IgD, IgT/IgZ*, but their expression were also significantly lower in diseased grouper. The same significantly reduced expression of Class II MHC was also shown in spleen of *E. coioides* injecting with NNV-like particles (VLPs), but the mechanism was not known [38]. The DEGs analysis indicated many immune related genes were differently expressed, although more Class I MHC mRNA was expressed for antigen presentation in diseased grouper, the insufficient expression of some immune molecules (such as antibodies and perforin) to neutralize and eliminate antigens might exacerbate the infection to show clinical signs. Thus, this study provided many unigenes that might play important roles in NNV resistance for further research.

The innate immune system clearly provides a front-line defense, and interferons (IFN) and IFN inducible protein (Mx) are the anti-viral immune molecules most studied in fish [11,40], however in this study there were no differences in the expression of *IFN* and *Mx* between asymptomatic and diseased fish. The antimicrobial peptides (AMP), component and chemokines were important innate immune molecules [8,9], in this study we detected higher expression of *c3, lysozyme, hepcidin* and *CCL19* in liver and spleen of diseased grouper, which was consistent with the previously reported phenomenon that these genes were more activated in the susceptible fishes to NNV infection [41]. The acute phase protein serum amyloid A (SAA) has been shown to possess many functional properties including chemoattraction and opsonization of Gram-negative bacteria which facilitated phagocytosis by macrophages [42]; CD28 plays a crucial role in the maintenance of regulatory T cell (Treg) pool size through promoting the development and proliferation of these cells [43]. In this study, high expression of *SAA* and *CD28* were seen in the spleen of sick *E. moara,* however, in kidney lower expression of *CD28* were seen in sick *E. moara.* In kidney, a significantly lower expression of complements (*C1, C3, C7, C8* and *C9*), chemokines (*CCL4, CXCL13, CXCL10, CCL3, CCL19, CXCL9, CXCL12, CCL20* and *CCL25*), chemokine receptors (*CCR9, CXCR4, CXCR3* and *CXR1*) and interleukin (*IL1, IL2, IL8, IL11, IL12* and *IL15*) were detected in diseased grouper. Thus, the immune response to NNV infection was different in three immune tissues, and the diseased fish showed significantly weaker immune response in kidney.

SSRs have been developed for genomic mapping, DNA fingerprinting, and marker-assisted selection in many species. However, little information of SSRs were known in groupers. In this study, lots of SSRs were provided and the differentially expressed SSRs were significantly enriched in several immune related pathways, such as CAMs and focal adhesion. The large amount of reliable transcriptome and SSRs information obtained in this study could be a valuable resource for investigating specific processes, functions, pathways and marker-assisted anti-VNN selection in *E. moara*. Also, the transcriptome study revealed a comprehensive view of the immune system in different clinical phenotype of *E. moara* naturally infected with NNV and provided many genes associated with RGNNV resistance for future functional research.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.07.020.

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