INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
18–0231/201x/00–0–000–000
DOI: 10.17957/IJAB/15.0758
http://www.fspublishers.org

Full Length Article

The Regulatory Networks Conferred by IFN-γ in the Kidney of *Takifugu rubripes*

Derong Kong[1,*], Jun Cui[1,*], Zhicheng Wang[1], Lin Zang[1], He Sun[1], Ziwen Hu[1], Xin Li[1], Xuemei Qiu[1], Chen Jiang[1], Haiying Liu[2], Tao Zhang[3], Shengcong Liu[3], Zhiqiang Jiang[1], Xuesong Meng[3] and Xiuli Wang[1,]**

[1]*College of Fisheries and Life Science, Dalian Ocean University, Dalian, 116023, China*
[2]*College of Marine Science and Environment, Dalian Ocean University, Dalian, 116023 China*
[3]*Dalian Tianzheng Industrial Co. Ltd, Dalian, 116011, China*
[*]For **c**orrespondence: xiuliwang417@sina.com
[*]These authors contributed equally to this work

Abstract

Takifugu rubripies is one of the most important economic fishes. Recently, because of various infectious diseases, production of *T. rubripies* has been seriously hindered. Interferon gamma (IFN-γ) is critical for innate and adaptive immunity in fish. Here, a comparative RNA-Seq profiling study of the kidney of *T. rubripes* was performed to investigate the expression differences between the kidney samples with (Ts) and without (Cs) IFN-γ infection. A total of 109,140,188 raw reads were generated from the Ts and Cs samples, including 106,717,920 clean reads. Additionally, a total of 19,124 expressed genes were identified from the two samples. Among these expressed genes, 292 genes showed significantly differential expression including 171 up-regulated and 121 down-regulated genes in the Ts sample. The GO, KEGG pathway enrichment and hierarchical clustering analyses indicated that the G-protein coupled receptor, Cytokine-cytokine receptor interaction, Jak-STAT signaling pathways were likely to be affected by IFN-γ. In addition, the protein-protein interaction (PPI) result showed that the MAPK6 was located at the center of this regulatory network involving IFN-γ, suggesting that IFN-γ may activate the MAPK signaling pathway. These results demonstrate the IFN-γ-related molecular mechanisms in the kidney of *T. rubripes*, provide a basis for application of the recombinant IFN-γ protein as a vaccine candidate against pathogen infection in aquaculture, and provide candidates to enhance pathogen-resistance in *T. rubripes.* © 2018 Friends Science Publishers

Keywords: Differentially expressed genes; IFN-γ; Kidney; Regulatory network; RNA-Seq; *Takifugu rubripes*

Introduction

Takifugu rubripes is not only a model system for many scientific studies, including development and evolution, but also one of the most important economic fishes (Cui *et al*., 2014a). Its genome was finished in 2002 (Aparicio *et al*., 2002). As various infectious diseases, the production of *T. rubripes* was hindered to cause economic losses. So it is very important and significant to identify and study the function of immune related genes. Our previous work showed that a number of immune-related genes and pathways were identified in the gill of *T. rubripes* (Cui *et al*., 2014b), and it was also found that these immune-related genes contained a number of SNPs (Cui *et al*., 2014c). Kono *et al*. (2017) characterized the immunoregulatory function of neuromedin U (Nmu) in *T. rubripes*, and found that it played important roles in the activation of phagocytic cells and the elevation of the expression of cytokine mRNA in peripheral blood leukocytes (Kono *et al*., 2017). In addition, an immune-related gene, *Bax inhibitor-1* (*BI-1*), was identified and characterized in the pufferfish, which encodes an anti-apoptotic protein that protects cells from endoplasmic reticulum stress-induced apoptosis (Cheng *et al*., 2017).

Interferon gamma (IFN-γ) is a dimerized soluble cytokine and important for innate and adaptive immunity in animals (Gray and Goeddel, 1982). IFN-γ is a part of the innate immune response, which is produced dominantly by natural killer (NK) and natural killer T (NKT) cells. It can activate macrophages and induce the expression of MHC (Class II major histocompatibility complex) genes (Schoenborn and Wilson, 2007). In the studies on fish immunity, the *IFN-γ* gene was identified and characterized in a large yellow croaker *Larimichthys crocea*. After injection with *Vibrio harveyi*, a high expression of *IFN-γ* was found in 10 tissues during the first day (Chen *et al*., 2015). Likewise, the *IFN-γ* transcripts in head kidney and trunk kidney were also increased by bacterial peptidoglycan (PGN), lipopolysaccharide (LPS) and the interferon inducer polyI:C in the grass carp *Ctenopharyngodon idella* (Chen *et al*.,

To cite this paper: Kong, D., J. Cui, Z. Wang, L. Zang, H. Sun, Z. Hu, X. Li, X. Qiu, C. Jiang, H. Liu, T. Zhang, S. Liu, Z. Jiang, X. Meng and X. Wang, 201x. The regulatory networks conferred by IFN-γ in the kidney of *Takifugu rubripes*. *Int. J. Agric. Biol*., 00: 000–000

2010). In addition, the characterization, expression and function analyses of IFN-γ genes were also studied in other fishes, such as the Japanese flounder, zebrafish, and common carp (Wang *et al*., 2011; Chadzinska *et al*., 2014; Li *et al*., 2016; Hu *et al*., 2017; Ruan *et al*., 2017; Wu *et al*., 2017). In *T. rubripes*, an increased expression of *IFN-γ* gene was recorded in particulate silica stimulated *T. rubripes* head kidney cells (Morimoto *et al*., 2016*).* Though cytokines were tested in the *T. rubripes*, treated with recombinant IFN-γ, it was proved that *T. rubripes* IFN-γ might be an effective and important inducer (Biswas *et al*., 2016). In our previous study, we cloned a CDS of *IFN-γ* from the *T. rubripes* kidney, and its recombinant protein was produced in *E. coli BL21* (Ma *et al*., 2015).

The fish kidney is an important immune organ, which comprises cytokine-producing lymphoid cells (Geven and Klaren, 2017). In this study, we used IFN-γ recombinant protein to infect *T. rubripes* and the kidney samples were used to construct the RNA-Seq library. After comparative transcriptome analysis between the kidney samples with and without IFN-γ infection, the G-protein coupled receptor related GO terms and KEGG pathways affected by IFN-γ were identified. In addition, we found that MAPK6 was located at the center of the PPI regulatory network involving IFN-γ. Our results provide insight regarding the involvement of IFN-γ in *T. rubripes* immune responses, which demonstrates that the regulated networks involves IFN-γ in the kidney of *T. rubripes*, provides a basis for application of the recombinant IFN-γ protein as a vaccine and provides candidates for breeding to enhance pathogen-resistance in *T. rubripes.*

Materials and Methods

Sample Collection

A total of 50 *T. rubripes* (~18 cm) were sampled from Dalian Tianzheng Industrial Co. Ltd. (Dalian China). The *T. rubripes* were transferred to the experimental tanks. These individuals were kept in sea water at 20°C for 7 days to acclimate before the study. The treated group (Ts, 25 individuals) were injected with 200 μL of IFN-γ recombinant protein solution (25 μg/mL), which was dissolved in PBS (pH=7.4). 25 individuals, as control samples (Cs) were infected with 200 μL of PBS. After 24 h, the kidney samples were collected from the Ts and Cs and pooled. The tissues were placed into RNAlater (Ambion) and then moved to -80°C for storage until RNA isolation.

RNA Extraction, Library Construction and Sequencing

Isolation of total RNA from all samples was performed with using RNAiso plus (TaKaRa, Dalian China) (Wang *et al*., 2014). Agilent 2100 Bioanalyzer was used to measure the quantity and quality of the total RNA. The RNA-Seq library construction and next generation sequencing were performed by Biomarker Biotechnology Corporation (Beijing, China) using an Illumina HiSeq 2000 with 101 bp paired-end reads.

Reads Mapping

Based on the method of Cui *et al*. (2015), the clean reads were obtained. TopHat was used to map the clean reads to the *T. rubripes* genome (https://www.ncbi.nlm.nih.gov/genome/63?genome_assembly_id=22739).

Identification of New Genes

To supply and improve the annotation information of the *T. rubripes* genome, we used the RNA-Seq data to identify new genes. First, all the mapped reads were assembled by Cufflinks and mapped to the genome. Then, the sequences encoding shorter peptide chains (less than 50 amino acid residues) and containing single exons were removed. Last, the new genes identified above were used as query sequences to search against the Swiss-Prot, NR, COG, GO, Pfam, KOG, eggNOG and KEGG using BLASTx.

Differentially Expressed Gene Analysis

Expression levels of the genes were measured with normalized read counts by their respective lengths using Cufflinks. FPKM were applied to represent the expression levels of each gene. DEGseq was used to identify the differentially expressed genes (DEGs). The rules were| fold change | > 2 and False Discovery Rate (FDR) < 0.01. The fold change was calculated with the following equation:

$$\text{Fold Change} = log_2 \frac{FPKM_{Ts}}{FPKM_{Cs}}$$

GO, KEGG Enrichment and Protein-Protein Interaction (PPI) Analyses

The GOseq was used to perform Gene Ontology (GO) enrichment analysis and KEGG pathway enrichment analysis was performed using KOBAS (Mao *et al*., 2005).

The PPI of DEGs was predicted using the STRING database (http://string-db.org/), and the Cytoscape was used to construct the interaction network (Shannon *et al*., 2003).

Results

RNA-Seq and Mapping

Next-generation sequencing was performed to generate more than 100,000,000 raw sequence reads from the two samples. As shown in Table 1, clean reads accounted for more than 97% of the raw reads. TopHat was used to map the clean reads to the *T. rubripes* genome. Of these clean reads, more than approximately 70.89% and 69.64% were mapped reads

Table 1: Statistical data of the RNA-Seq reads for two samples

Samples	Raw reads	Clean reads	Q30%	Mapped reads	Unique mapped reads
Cs	59,879,012	58,831,130	85.02	41,703,857	40,434,259
Ts	49,261,176	47,886,790	85.04	33,349,030	32,329,272

Table 2: The statistics of new genes annotation

Annotated databased	New gene number
COG	47
GO	201
KEGG	251
KOG	236
Pfam	353
Swiss-Prot	221
eggNOG	616
NR	719
All	733

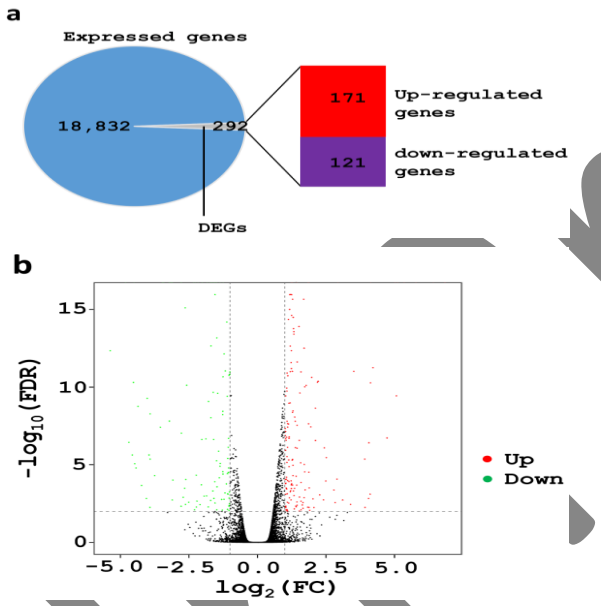

Fig. 1: The DEGs between the Ts and Cs samples. **a**, The number of expressed genes, DEGs, up-regulated genes and down-regulated genes. **b**, Volcano plot of the significant DEGs between Ts and Cs samples. The y-axis represents the logarithm of FDR, and the x-axis represents the logarithm of the Fold change

in Cs and Ts, and more than 68.73% and 67.51% were unique mapped reads in Cs and Ts. Unique mapped reads were assigned to intergenic regions (6.98%/6.39%, Cs/Ts), introns (4.48%/4.43%, Cs/Ts) and exons (88.54%/89.18%, Cs/Ts). The RNA-Seq datasets have been submitted to NCBI. The accession number are SRR6675807 and SRR5625907.

In addition, after analysis of the assembly and mapping, we identified 994 new genes. By BLASTx search against NR, Swiss-Prot, GO, COG, KOG, Pfam, eggNOG and KEGG, respectively, 733 new genes were annotated (Table 2).

DEGs Analysis

As shown in Fig. 1a, a total of 19,124 genes were identified as expressed genes in these two samples. A total of 292 genes showed significantly differential expression in the kidneys of the Ts samples compared with the Cs samples, including 171 up-regulated genes in the Ts samples, and 121 up-regulated genes in the Cs samples (Fig. 1b). Of these 292 DEGs, 277 were annotated to one or more database. Hierarchical clustering based on expression patterns indicated the DEGs were classified in four major groups (Fig. 2). In the third and fourth groups, the expression of the DEGs was the lowest in the Ts and Cs samples, respectively.

The Annotation of DEGs and Enrichment Analysis

As shown in Fig. 3, a total of 166 DEGs were assigned with one or more GO terms for the Biological Process (BP), Molecular Function (MF) and Cellular Component (CC). The highly represented GO terms were the following: catalytic activity(GO:0003824) and binding(GO:0005488) for the MF; cell (GO:0005623), membrane (GO:0016020) and cell part (GO:0044464) for CC; metabolic process (GO:0008152), cellular process (GO:0009987) and single-organism process (GO:0044699) for BP.

The results of the hierarchical clustering and GO enrichment showed that the GO terms, apicultural plasma membrane, ammonium transmembrane transport, ammonium transmembrane transporter activity, cytoplasmic vesicle membrane and carbonate dehydratase activity, belonged to the third group. The G-protein coupled receptor signaling pathway (GO:0007186), G-protein coupled receptor activity (GO:0004930) and protein dimerization activity (GO:0046983), belonged to the fourth group (Table 3).

In addition to the GO analysis, we also performed the KEGG pathway analysis. After the enrichment analysis, the top 20 KEGG enrichment pathways are shown in Fig. 4. The DEGs mainly were enriched in cytokine-cytokine receptor interaction (ko04060), intestinal immune network for IgA production (ko04672), Jak-STAT signaling pathway (ko04630) and others.

PPI Network Analysis

A total of 11 modules and 59 protein pairs were obtained based on the STRING database (Fig. 5). Of these 11 modules, one module contained the most genes (33 genes), and the interaction network was also the most complex. In this module, MAPK6 was located at the center of the regulatory network, suggesting that MAPK6 may affect in the pathway involving IFN-γ in the kidney of *T. rubripes*.

Discussion

T. rubripes is one of the most important economic fishes. Various infectious diseases occur during the feeding period,

Table 3: The top enriched GO terms in the four groups classified by hierarchical clustering

Group	GO term	*P*-value
Group I	hydrolase activity, acting on acid anhydrides	1.058962e-07
	V(D)J recombination	4.754109e-05
	chemokine activity	1.140420e-04
	actomyosin, actin portion	6.911452e-04
	endocardial cushion formation	7.003809e-04
Group II	neuron differentiation	0.0001896626
	extracellular region	0.0006024408
	structural molecule activity	0.0008683244
	intermediate filament	0.0008993523
	motor neuron axon guidance	0.0031272068
Group III	apicolateral plasma membrane	0.001023869
	ammonium transmembrane transport	0.001388475
	ammonium transmembrane transporter activity	0.002654530
	cytoplasmic vesicle membrane	0.004601219
	carbonate dehydratase activity	0.006437544
Group IV	G-protein coupled receptor signaling pathway	0.001626457
	G-protein coupled receptor activity	0.002058420
	protein dimerization activity	0.035998731

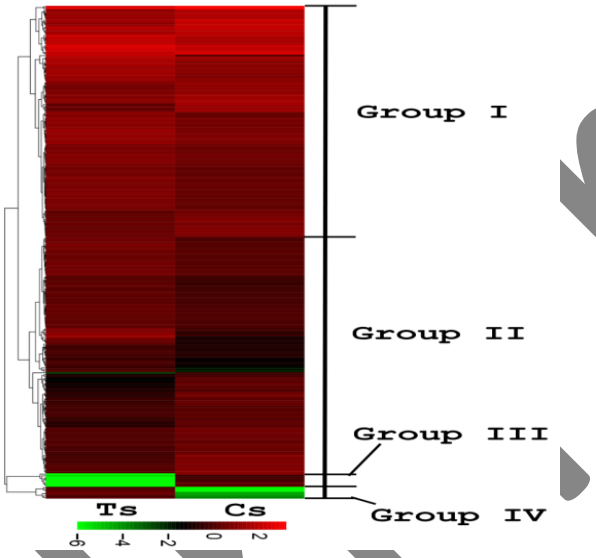

Fig. 2: Expression profiling of the DEGs in the Ts and Cs samples. Hierarchical clustering shows that the genes can be classified into four groups

and these are regarded as a major threat to *T. rubripes* production, causing serious economic loss for *T. rubripes.* Although the studies on the immune response to pathogens in *T. rubripes* have made progresses, the molecular mechanisms of the key immune genes are unknown. It is necessary to study the regulatory networks conferring the key immune genes. The recombinant IFN-γ protein from *T. rubripes* was produced in our previous study (Ma *et al*., 2015). However, the reports on IFN-γ-related molecular mechanisms in *T. rubripes* are few. This limits application of recombinant IFN-γ protein as a vaccine candidate. In this study, a comparative RNA-Seq analysis between the kidney samples with and without IFN-γ infection was performed to identify the genes, GO terms and KEGG pathways which were likely to be affected by IFN-γ. This not only provide a base for further study into the diagnosis and application of vaccines against pathogen infection in aquaculture, but also help reducing antibiotic use and environmental pollution. In addition, this information on IFN-γ-related molecular mechanisms will provide candidates for breeding to enhance pathogen-resistance in *T. rubripes.*

The fish kidney is one of the important immune organs. For example, the *Viperin* gene was rapidly and significantly up-regulated in vivo after poly (I:C) challenge in the head kidney of *Larimichthys crocea* (Zhang *et al*., 2017). Meanwhile, interferon regulatory factor (Li *et al*., 2017a), interleukin (Wang *et al*., 2017), major histocompatibility complex class II (Li *et al*., 2017b) and others were found to be involved in immune response in the head kidney of fish. Previous study showed that the *IFN-γ* gene was mainly expressed in the head kidney of Nile tilapia, with limited expression observed in other tissues (Velázquez *et al*., 2017). The same results were also found in other fish. For example, black seabream *IFN-γ* transcript was examined in all tissues, and its expression level was the highest in the kidney (Xiang *et al*., 2017). IFN-γ also activates other immune genes, such as the members of IL family in common carp and goldfish (Arts *et al*., 2010; Grayfer *et al*., 2010). The IFN-γ recombinant protein of Nile tilapia produced in *E. coli* induced the *Mx* gene transcription in head kidney primary culture cells (Velázquez *et al*., 2017). Meanwhile, the expression of *STAT1*, *STAT2* and *IRF 9* genes was rapidly and transiently stimulated though the overexpression of *AsIFN-γ* in cultured black seabream (Xiang *et al*., 2017). In this study, the IFN-γ recombinant protein was infected in *T. rubripes* and after comparative transcriptome analysis, we found that 171 genes showed higher expression in kidneys with IFN-γ infection and that 121 genes showed lower expression (Fig. 1).

In this study, hierarchical clustering based on the expression patterns indicated that the DEGs were classified in four major groups. In the fourth group, the expression of the DEGs was the lowest in the Cs samples and highest in the Ts samples (Fig. 2). GO enrichment and hierarchical clustering analysis showed that the DEGs from the fourth group were assigned to the GO terms associated with, G-protein coupled receptor (GPCR) (Table 3). Previous study showed that the GPCRs affected the function of sensory receptors and were involved in the defense responses for survival against pathogens (Gupta and Singh, 2017). After KEGG enrichment analysis of the DEGs, we observed significant KEGG pathway enrichment in the kidney with and without IFN-γ infection. The pathways, cytokine-cytokine receptor interaction, intestinal immune network for IgA production, Jak-STAT signaling pathway and others in the top enriched pathways had been reported to affect the essential function in the immune and stress responses (Fig. 4). IFNγ is involved in the pathways such as cytokine-cytokine receptor interaction. It was found that IFN-γ also

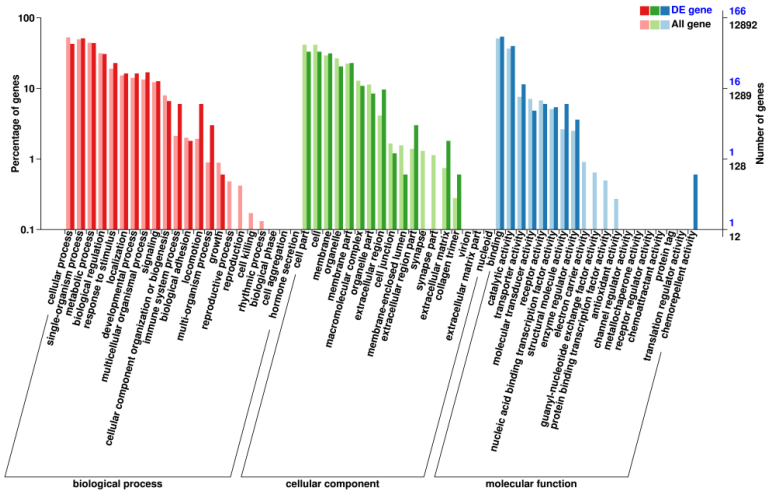

Fig. 3: Gene ontology (level 2) for all the expressed genes and DEGs under molecular functions, cellular components, and biological processes

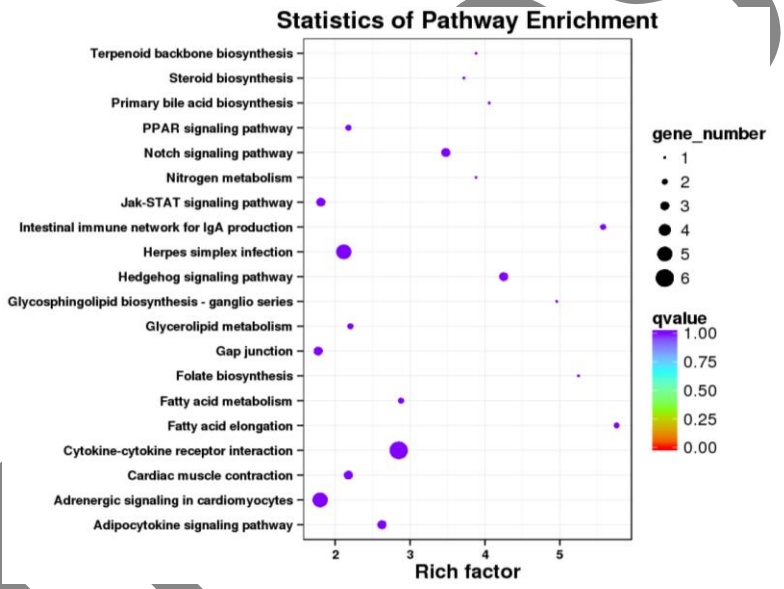

Fig. 4: The scatter diagram of the top 20 enriched KEGG pathways of the DEGs

regulates other members of cytokine-cytokine receptor interaction. For example, common carp and goldfish IFN-γ could induce other cytokines, such as the members of IL family and TNF-α (Arts *et al*., 2010; Grayfer *et al*., 2010). The Jak-STAT signaling pathway also has an important effect in the immune responses. It was found that recombinant IFN-γ activated the STAT signaling pathway in hepatocytes (Pirault *et al*., 2017). *AsIFN-γ*-overexpression transgenic black seabream brain cells exhibited significantly enhanced expression levels of *STAT1*, *STAT2 and IRF9* in JAK-STAT signaling pathway (Xiang *et al*., 2017).

Besides the Jak-STAT signaling pathway, previous study showed that IFN-γ also regulated downstream engagement of the MAPK signaling pathway through the activation of interferon-dependent PKC-theta (Srivastava *et al*., 2004). The MAPK signaling pathway affects the function of signal transmission after treatment of multiple extracellular signals or stimuli, such as hormones, growth factors, and cytokines (Yao *et al*., 2017). In this study, MAPK6 was found to locate at the center of the PPI regulatory network conferring IFN-γ (Fig. 7). The expression level of *MAPK6* was up-regulated by RNA-Seq analysis. These findings suggest that IFN-γ may induce the expression of *MAPK6* to regulate other pathways in the *T. rubripes* immune response.

Conclusion

A comparative RNA-Seq study between the kidney with and without IFN-γ infection in *T. rubripes* were performed.

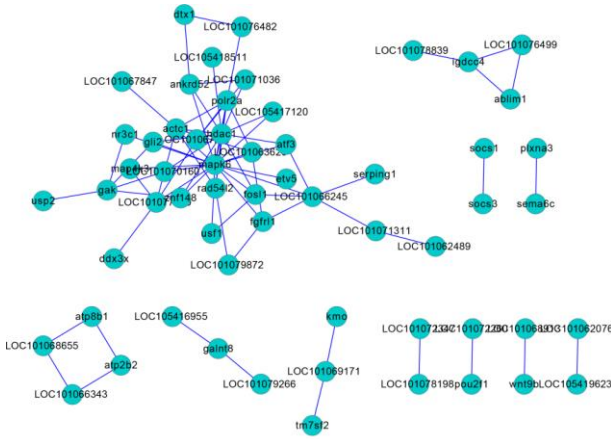

Fig. 5: Protein-protein interaction (PPI) network conferred by IFN-γ in the kidney of *Takifugu rubripes*

A number of differentially expressed genes in the kidney were identified. The GPCR-related, cytokine-cytokine receptor interaction, Jak-STAT, and MAPK signaling pathways are highly likely to be affected by IFN-γ. These results will benefit not only understanding the molecular mechanisms underlying IFN-γ in the immune response, but also application of the recombinant IFN-γ protein as a vaccine and future molecular breeding of *T. rubripes*.

Acknowledgments

This study was supported by the National Marine Public Benefit Research Foundation, China (No. 201405003-2) and the China Agriculture Research System (CARS-47).

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(Received 12 February 2018; Accepted 14 April 2018)