Transcriptome Analysis of Immune-Related Gene in Hybrid Yellow Catfish in Reaction to Peptidoglycan Challenge

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Abstract: Hybrid yellow catfish (*Tachysurus vachelli* x *Tachysurus fulvidraco*) is one of the most important freshwater fish in China, intensive culturing has caused increased susceptibility to bacteria, viruses, and parasites. However, little is known about its adaptation mechanisms to pathogen infection. To better understand its immune system and immune related gene under pathogen infection, the transcriptome was analyzed by comparing the data of hybrid yellow catfish spleen stimulated by PGN and PBS. Fish in experimental group were injected with 100 μl baccilus subminis PGN (1mg/ml, 69,554, Sigma) intraperitoneally, and the fish in control group injected with 100 ul phosphate buffered solution (PBS). After 24 h injection, spleens were collected and immediately frozen in liquid nitrogen, then stored in -80 ℃ refrigerator. Total RNA from spleens of hybrid yellow catfish were extracted using Trizol reagent (Sangon, China) according to the manufacturer's instructions. The libraries were constructed using TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, USA) and sequenced on an Illumina HiSeq X Ten platform. There were 285 differentially expressed genes (DEGs) between PGN treated experimental groups and PBS control groups at 24 h, including 213 up regulated and 72 down regulated DEGs. The expression of qRT-PCR from five up regulated DEGs and five down regulated DEGs were consistent with RNA-Seq. In the analysis of GO enrichment, DEGs in the spleens of PGN treated hybrid yellow catfish were significantly enriched in GO items, including Biological Process, Cellular Component, and Molecular Function items. The identified DEGs and enriched GO terms and KEGG pathways were useful for understanding the physical and biochemical performances, and the transcriptome data greatly enriched the genetic information of hybrid yellow catfish.

Keywords: Differentially Expressed Genes, Genes and Genomes, Transcriptome, Ontology

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1 Introduction

Hybrid yellow catfish (*Tachysurus fulvidraco*♀×*Tachysurus vachellii*♂) is regarded as a good freshwater species in China for its delicious flesh and high nutrition. In recent years, intensive culturing of hybrid yellow catfish has caused increased susceptibility to bacteria, viruses, and parasites. Pathogen spread and disease outbreak resulted in huge economic losse to hybrid yellow catfish culturing. Knowing more about immune system in hybrid yellow catfish is essential to defense pathogen outbreak and establish effective measures to reduce financial losses.

Peptidoglycan (PGN) is a large molecular in cell wall of Gram-positive and Gram-negative bacteria [1], which has been studied in many fishes as immunostimulan [2-4]. Many

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defense related genes in fish innate immune system were reported, such as Cytokines, chemokines, Toll-like receptors, Immunoglobulin M, Vitellogenin, Cellular apoptosis susceptibility, Glutathione S-transferase [5]. Proteins of these defense related genes are important in protecing fish against invading viral, bacterial, and fungal. The spleen is an important immune organ, and regarded as an primordial secondary lymphoid organ [6], which has been reported to make great contribution to systemic immunity of fish and has the function to remove antigenic substances [7].

RNA-sequencing (RNA-Seq) is a mature technology, which uses high-through sequencing methods to determine all RNA transcripts of the specimin [8]. Transcriptome data can provide a basis to further analysize the immune system in various fishes, such as *Pelteobagrus fulvidraco* [9-12], *Sepia esculenta* [13], *Paralichthys olivaceus* [14], *Gymnodiptychus pachycheilus* [15], and *Salmo trutta* [16].

2 Materials and Methods

2.1 Experimental Samples

Healthy hybrid yellow catfish (24.60 ± 0.34 g) were maintained in 2000 L plastic tanks with recirculation system in Fisheries Institute, Anhui Academy of Agricultural Sciences, China. Two weeks later, fish were divided into experimental group and control group (twenty fish per group), and transferred into 200 L glass tanks. Fish in experimental group were injected with 100 μl baccilus subminis PGN (1mg/ml, 69,554, Sigma) intraperitoneally, and the fish in control group injected with 100 ul phosphate buffered solution (PBS). During the experiment, water temperature 26°C ~ 28°C, dissolved oxygen 5.0 mg/L ~ 6.0 mg/L, and pH 7.6 were maintained. Fish were fed with commercial formulated feed. After 24 h injection, spleens were collected and immediately frozen in liquid nitrogen, then stored in -80°C refrigerator. All procedures were carried out according to the Chinese legislation for animal experimentation guidelines.

2.2 RNA Extraction and Library Preparation

Total RNA from spleens of hybrid yellow catfish were extracted using Trizol reagent (Sangon, China) according to the manufacturer's instructions. RNA purity and quantification were evaluated using the NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Then the libraries were constructed using TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, USA) according to the manufacturer's instructions. The transcriptome sequencing was conducted by OE Biotech Co., Ltd. (Shanghai, China).

2.3 RNA Sequencing and Transcriptome Assembling

The libraries were sequenced on an Illumina HiSeq X Ten platform and 150 bp paired-end reads were generated. About 49 M raw reads were generated. Raw reads were firstly processed using Trimmomatic [17] and the low quality reads were removed to obtain the clean reads. Then about 48 M clean reads were retained for subsequent analyses. The clean reads were alligned to the *Tachysurus fulvidraco* reference genome (https://www.ncbi.nlm.nih.gov/genome/?term=yellow+catfish) using HISAT2 package to obtain annotation information [18].

2.4 Identification of Differentially Expressed Genes

Each gene was obtained by HTSeq-count [19]. Differentially expressed genes (DEGs) were analyzed using DESeq (2012) R package [20]. The threshold for DEGs was set to q value < 0.05 and $|\log_2 \text{foldchange}| \geq 1$. All DEGs were mapped to the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) [21] databases to determine their potential function and metabolic pathway.

2.5 Quantitative Real-Time PCR (qRT-PCR) Verification

To validate the results of RNA-Seq, ten DEGs were selected to analysis qRT-PCR by SYBR Premix Ex Taq kit (Invitrogen). RNA samples used for qRT-PCR were the same as the RNA used for transcriptome. Primers of qRT-PCR were listed in table 1. All reactions were performed using technical triplicates. β-Actin was

selected as the reference gene. Relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method [22].

3 Results

3.1 Transcriptome Sequencing and Annotation of the Unigene

Transcriptomes of the spleens from hybrid yellow fish treated by PBS or PGN were sequenced. After removing low quality sequences, number of clean reads in these six libraries ranged from 47424226 to 50845358, and the clean bases ranged from 7.04 G to 7.54 G. Q30 values of the sequenced libraries ranged from 93.05% to 94.98%, whereas the GC content of the libraries ranged from 47.22% to 47.40% (Table 2). These results confirmed that the sequenced data and transcriptome were reliable. In the transcriptome data for CS1, CS2, CS3 (PBS group), and ES1, ES2, ES3 (PGN group), the reads and percentage mapped to the yellow catfish genome is 36809302, 37225718, 39265044, 37309076, 37984493, 37472668, and 77.62%, 77.30%, 77.22%, 76.91%, 76.67%, 76.84%, respectively (Table 3). The raw reads of RNA-Seq were deposited in the Sequence Read Archive database of NCBI with the accession numbers of PRJNA833861.

Table 1 Sequences of primers used for qRT-PCR

name	primer sequence
ahsg2	F: TCTGTGCCTGATTGCGAG
	R: CTTGCACTTGAATGCTACG
serping1	F: GTCACACGAACGAAGAACTG
	R: CCTTCATCATTCCCTTGGCA
spon2a	F: TGTCCAATCATATCCTGCCT
	R: GACCACATGGAGACCTCG
SH2D1A	F: TCCTGATCCGAGACAGTG
	R: TTGCCTTCGAGCTGGTAGA
pglyrp2	F: ATCCACAGGTCTTCAGACT
	R: GTTTGTGAGGATTAGCAAGACG
NLRP3	F: GCAATGCCATTGATGTTGTAT
	R: GGTCTCCACTCCAAGTGA
FFAR3	F: AATGCCTGTTTGAATCCCT
	R: AATGAGTCTGCCTACGAATTG
BDKRB2	F: ATGCTGGGATTTGCGATAC
	R: GAACTCCAGGGATGAACCTC
Hdd11	F: AAACAACTCTCAAGGTGCC
	R: GATGGAATCAGGAGGAATCCAG
mmp25b	F: CCATCATGCGCCCATACTA
	R: TCTGAATGGCTTGCAGGT
β-Actin	F: GCACAGTAAAGGCGTTGTGA
	R: ACATCTGCTGGAAGGTGGAC

Table 2 Overview of spleen transcriptome sequencing data and quality filtering

	Raw Reads	Clean Reads	Clean Bases (G)	Q30 (%)	GC content (%)
CS1	48190336	47424226	7.04	94.98	47.22
CS2	48905310	48156158	7.14	94.34	47.36
CS3	51670678	50845358	7.54	94.06	47.37
ES1	50458942	49540920	7.34	93.89	47.4
ES2	49407308	48509062	7.19	93.05	47.47
ES3	49663718	48766782	7.22	93.34	47.25

Table 3 Transcriptome reads and percentage mapped to the yellow catfish genome

Sample	Clean reads	Mapped reads	mapped ratio
CS1	47424226	36809302	77.62%
CS2	48156158	37225718	77.30%
CS3	50845358	39265044	77.22%
ES1	49540920	37984493	76.67%
ES2	48509062	37309076	76.91%
ES3	48766782	37472668	76.84%

3.2 Identification and Analysis of DEGs

The DEGs between PGN treated experiment groups and PBS control groups were identified on the basis of q value < 0.05 and $|\log_2 \text{foldchange}| \geq 1$. There were 285 DEGs between PGN treated experimental groups and PBS control groups at 24 h, including 213 up regulated and 72 down regulated DEGs (Figure 1). In the volcano plot, significantly up regulated unigenes represented by red dots, significantly down regulated unigenes represented by green dots, and no significantly expressed unigenes represented by grey dots. The levels of magnitude changes in up regulated DEGs are lower than that of down regulated DEGs.

When yellow catfish challenged with lipopolysaccharide (LPS), 27 immune-related genes and 7 transcriptional genes were indentified [23]. 765 up regulated and 1757 down regulated genes were obtained from hepatopancreas, when *Procambarus clarkii* was challenged with PGN [24]. 466 up regulated genes and 834 down regulated genes were obtained from *Sesarmops*

sinensis hepatopancreas when challenged by PGN [25]. Transcription levels of G-CSFR, BPI/LBP and HSP70-4 were increased from the kidney of *Cyprinus carpio* by PGN stimulation [3].

3.3 Functional Annotation by GO and KEGG Analysis

In the analysis of GO enrichment, DEGs in the spleens of PGN treated hybrid yellow catfish were significantly enriched in 134 GO items, including 89 Biological Process (BP), 16 Cellular Component (CC), and 29 Molecular Function (MF) items. The significantly enriched five top BP items were "negative regulation of endopeptidase activity", "proteolysis", "complement activation, classical pathway", "complement activation, alternative pathway", and "digestion". The significantly enriched five top CC items were "extracellular space", "extracellular region", "blood microparticle", "membrane attack complex", and "cortical granule". The significantly enriched five top CC items were "serine-type endopeptidase activity", "serine-type endopeptidase inhibitor activity", "endopeptidase inhibitor activity", "cysteine-type endopeptidase inhibitor activity", and "complementcomponent C3b binding" (Figure 2). These results indicate that substantial changes emerged in energy metabolism and immune defenses in spleens under PGN stress. The KEGG database was used to investigate biochemical pathways of DEGs. The top 20 KEGG pathways included mineral absorption, vitamin digestion and absorption, fatty digestion and absorption, protein digestion and absorption, pancreatic secretion, insulin signaling pathway, complement and coagulation cascades (Figure 3).

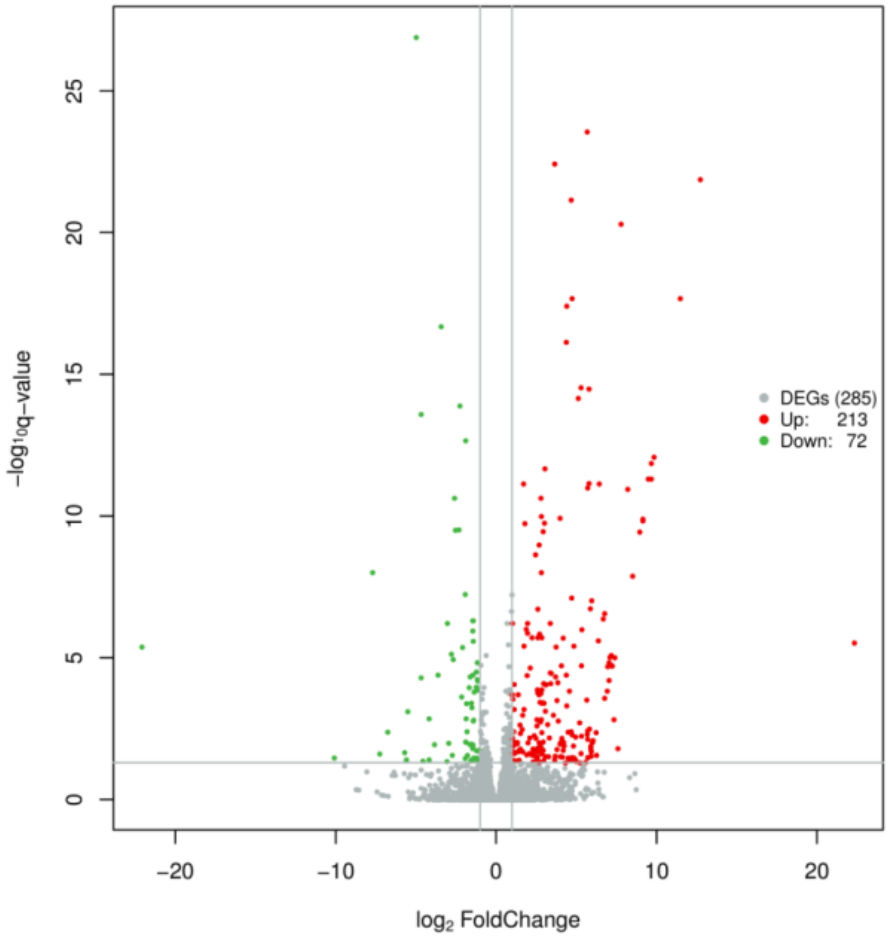

Figure 1 Volcano plot of differentially expressed genes (DEGs) from PGN-treated and PBS control groups in hybrid yellow catfish. The x-axis represents the fold change between the PGN and PBS groups; the y-axis indicates the significance of differential expression. Grey dots represented no significantly expressed unigenes, while red and green dots represent up- and down- regulated unigenes, respectively (q value < 0.05 and |log2foldchange| ≥ 1)

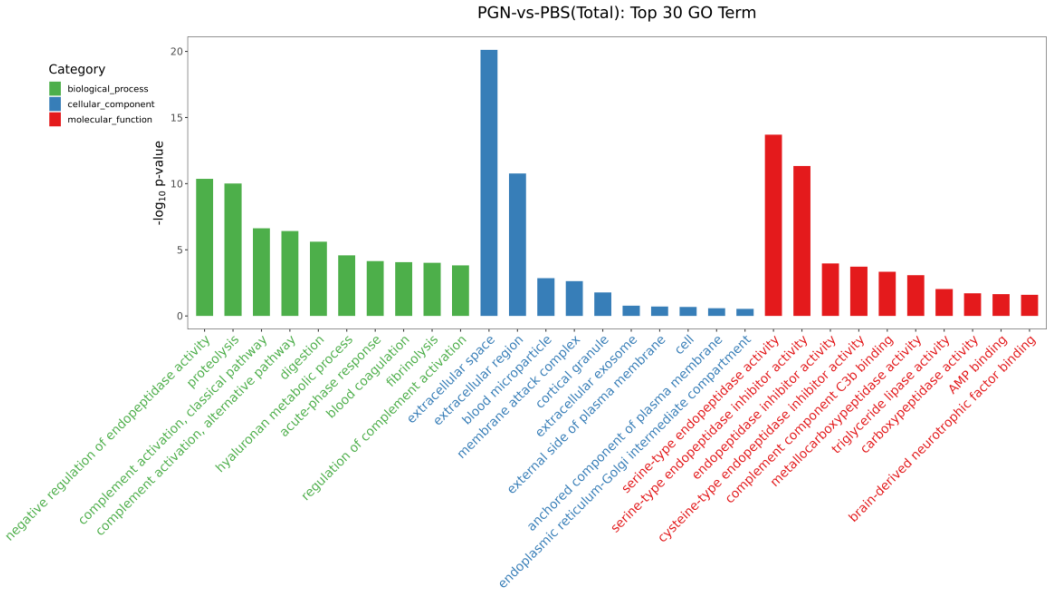

Figure 2 Gene ontology (GO) TOP 30 terms enriched by differentially expressed genes (DEGs). The results are summarized in three main categories: biological process, cellular component, and molecular function. The x-axis indicates the second term of GO and the y-axis indicates gene percentage

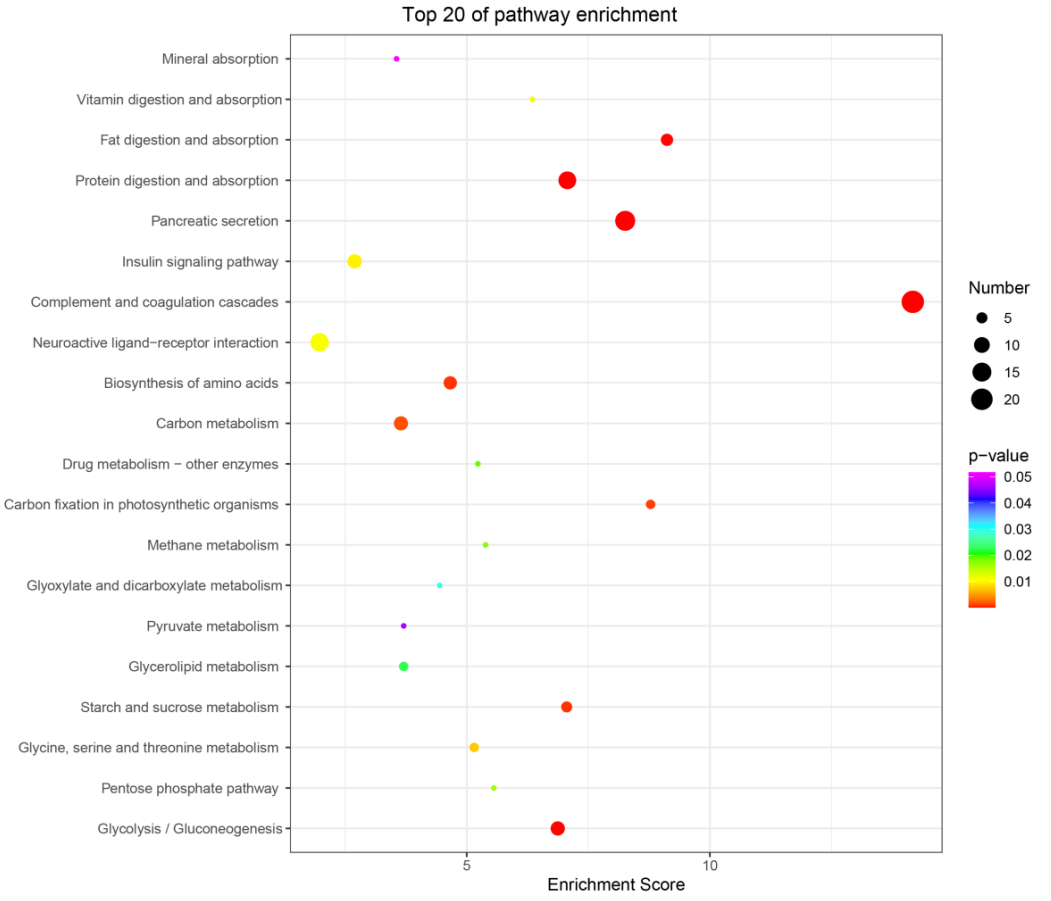

Figure 3 Top 20 significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of DEGs. Color and gene ratio indicate the P-value and the ratio of genes with each pathway, respectively. The x-axis indicates the enrichment score of KEGG and the y-axis indicates the name of the top 20 pathways

3.4 qRT-PCR Analysis

Five upregulated DEGs (ahsg2, serping1, spon2a, SH2D1A, pglyrp2) and five downregulated DEGs (NLRP3, FFAR3, BDKRB2, Hdd11, mmp25b) were chosen for qRT-PCR analysis. The results showed the expression of qRT-PCR were consistent with that of RNA-Seq (Figure 4). The results showed that serping1, SH2D1A, pglyrp2, were upregulated at least two times, and all the downregulated DEGs were downregulated at least two times of the control. Pglyrp2, BDKRB2, and Hdd11 were particularly responsive to the PGN stimulation. In this study, pglyrp2 was upregulated eight times in hybrid yellow catfish in response to PGN, Whereas BDKRB2 and Hdd11 were downregulated four times when compared to the control. These immune related genes maybe involved in innate immune defense against PGN in *T. fulvidraco*.

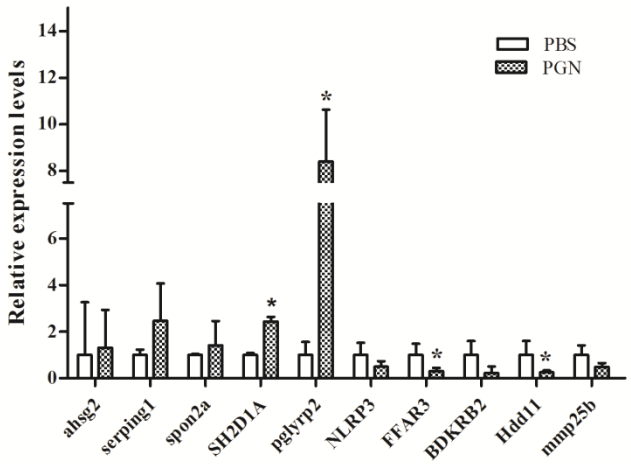

Figure 4 Five upregulated and five downregulated immune related genes treated by PGN stimulation relative to PBS were selected to validate RNA-seq by qRT-PCR.

4 Discussion

In this study, transcriptome profiles of hybrid yellow catfish was used to invest immune mechanism when the fish challenged with PGN. Tanscriptome sequencing results showed that immune related DEGs occupied large portion of all the DEGs.

Pglyrp2 was a inducible and constitutive acute-phase protein [26], and had antimicrobial and peptidoglycan-lytic amidase activity in fish [27, 28]. In our study, the expression of pglyrp2 was up-regulated when the hybrid yellow catfish was challenged with PGN, suggesting that pglyrp2 has important role in recogniting bacteria and initiating down stream antimicrobial pathway.

BDKRB2 is a component of kallikrein-kinin system, which participates in physiological and pathological processes [29]. Current study showed that BDKRB2 was downregulated when challenged by PGN. Previously study showed that BDKRB2 were expressed relatively higher at the time of pre-fertilization and pre-hatching in the ovarian of rockfish [29]. Bdkrb2 was also related to cerebrovascular dysfunction with the development of rat's Alzheimer's disease, and expressed lower at 20 day and 18 month [30].

HDD11 is an innate immune protein, which showed transcriptional suppression in our study. HDD11 transcripts significantly lower exrpessed (~25-fold) in sea slice parasitezed group compared with the non infected group, suggesting that sea slice can exert immunosuppressive in fins of salmon [31].

5 Conclusion

In conclusion, the transcriptome of hybrid yellow catfish immune response upon PGN injection were investigated. A total of 285 DEGs between PGN treated experimental groups and PBS control groups at 24 h, including 213 up regulated and 72 down regulated DEGs. These DEGs contribute to anti-bacterial responses after yellow catfish challenged with PGN. Results of this study could be benefit for better understanding the defense mechanisms and innate immune system of hybrid yellow catfish by the challenge of PGN, and valuable information for further studying on fish innate immune system.

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