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A novel MKK gene (EcMKK6) in *Epinephelus coioides*: Identification, characterization and its response to *Vibrio alginolyticus* and SGIV infection

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ABSTRACT

Mitogen-activated protein kinase 6 (MKK6) is one of the major important central regulatory proteins response to environmental and physiological stimuli. In this study, a novel MKK6, EcMKK6, was isolated from *Epinephelus coioides*, an economically important cultured fish in China and Southeast Asian counties. The open reading frame (ORF) of EcMKK6 is 1077 bp encoding 358 amino acids. EcMKK6 contains a serine/threonine protein kinase (S_TKc) domain, a tyrosine kinase catalytic domain, a conserved dual phosphorylation site in the SVAKT motif and a conserved DVD domain. By in situ hybridization (ISH) with Digoxigenin-labeled probe, EcMKK6 mainly located at the cytoplasm of cells, and a little appears in the nucleus. EcMKK6 mRNA can be detected in all eleven tissues examined, but the expression level is different in these tissues. After challenge with *Vibrio alginolyticus* and Singapore grouper iridovirus (SGIV), the transcription level of EcMKK6 was apparently up-regulated in the tissues examined. The data demonstrated that the sequence and the characters of EcMKK6 were conserved, EcMKK6 showed tissue-specific expression profiles in healthy grouper, and the expression was significantly varied after pathogen infection, indicating that EcMKK6 may play important roles in *E. coioides* during pathogen-caused inflammation.

1. Introduction

MAPK kinase 6 (MKK6), known as MEK6 or MAP2K6, is of the MKK family members which are involved in the regulation of inflammation and apoptosis during an immune challenge [1–4]. MKK family members possess the same conserved domains: a serine/threonine protein kinase (S_TKc) domain, a conserved dual phosphorylation site (231 Ser and 235 Thr), and a conserved DVD domain [5–8]. MKK6 is activated upon phosphorylation of serine and threonine residues within subdomain VIII by upstream MAPKK kinases [3]. The interaction of the activating kinase with MKK6 is mediated by the DVD domain located in the C-terminal region of MKK6 [9].

In mammal, mouse knockout MKK3 or MKK6 will result in death during early development, indicating that MKK6 plays a role in thymocyte maturation [10,11]. MKK6-p38 signaling cascade regulates cyclooxygenase-2 expression which is catalyzes the rate-limiting step in delayed prostaglandin biosynthesis in cardiac myocytes of transgenic mice in vitro and in vivo [12]. MKK6 protein is up-regulated in esophagus, stomach, colon cancers and Alzheimer's disease which is also related to the granular structure of susceptible neurons in the hippocampus and cortex of patients with Alzheimer's disease [13,14]. The conditional induction of a dominant active form of MKK6, a direct upstream kinase of p38, in Langerhans-cells efficiently induces the up-regulation of costimulatory molecules and enhances their T-cell stimulatory capacity [15]. A constitutively active form of MKK6 which use adenoviral gene transfer in vitro was expressed in osteoclasts, phosphorylated p38 MAPK and enhanced the survival of osteoclasts [16].

In aquatic animals, several MKK6 has been identified, and its expression profile in carp *Cyprinus carpio*, Atlantic salmon *Salmo salar*, zebrafish *Danio rerio*, white shrimp *Litopenaeus vannamei*, Yesso scallop *Patinopecten yessoensis*, sea cucumber *Apostichopus japonicus* etc. was analyzed [5–7,17–21]. However, gene characters and expression pattern of MKK6 in *Epinephelus coioides* remained unknown.

Grouper *E. coioides* is an economically important cultured fish in

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Table 1
Primers used in this study.

MKK6-ORF-F	ATGGAGGGAGGGAGTGAGAAA
MKK6-ORF-R	TCAGTCCCCCAAGATGACTTTC
MKK6-RT-F	GGAGTGAGAAAGAAGGCAA
MKK6-RT-R	TCCGATGGTTACGCAAG
MKK6-pEGFP-F	tcgagctcaagcttcgaattcATGGAGGGAGGGAGTGAGAAA
MKK6-pEGFP-R	ggatcccgggcccgcggtaccTCAGTCCCCCAAGATGACTTTC
MKK6-pcDNA3.1 F	ggggtaccATGTCTCTGTCTAAAGGAGGGAAG
MKK6-pcDNA3.1 R	cggaattcctTCCCTGGTGCCCACTTACTGCC
β-actin-F	TGCTGTCCCTGTATGCCTCT
β-actin-R	CCTTGATGTCACGCACGAT

China and Southeast Asian counties [22–24]. With the development of high density culture, more and more diseases outbreak have caused huge economic losses in cultivated grouper. For example, *Vibrio alginolyticus* plays a pivotal role in causative agents of vibriosis which endangered the aquaculture of marine animals in the coastal provinces of South China [25–27]; Singapore grouper iridovirus, SGIV is one of the main epidemics of grouper virus disease, which is a highly pathogenic virus of grouper, and its mortality rate can reach more than 90% [28–31]. Based on this information, we cloned a MKK6 from *E. coioides*, studied its distribution in the tissues and expression patterns response to *V. alginolyticus* and SGIV infection, investigated the function of MKK6 in the activation of AP-1 using luciferase reporter assays, and explored the effects of EcMKK6 on the cell apoptosis using a flow cytometry system.

2. Materials and methods

2.1. Experimental fish and sampling

Healthy *E. coioides* (16.8 ± 4.6 g) from the Marine Fisheries Development Center of Guangdong Province, Guangdong, China, were maintained at 27 °C in a flow-through water system for two weeks before experimentation. 270 *E. coioides* were randomly separated in triplicate with 30 fish held in each tank for challenge with *V. alginolyticus* and SGIV, respectively. A group of untreated fish was used as the

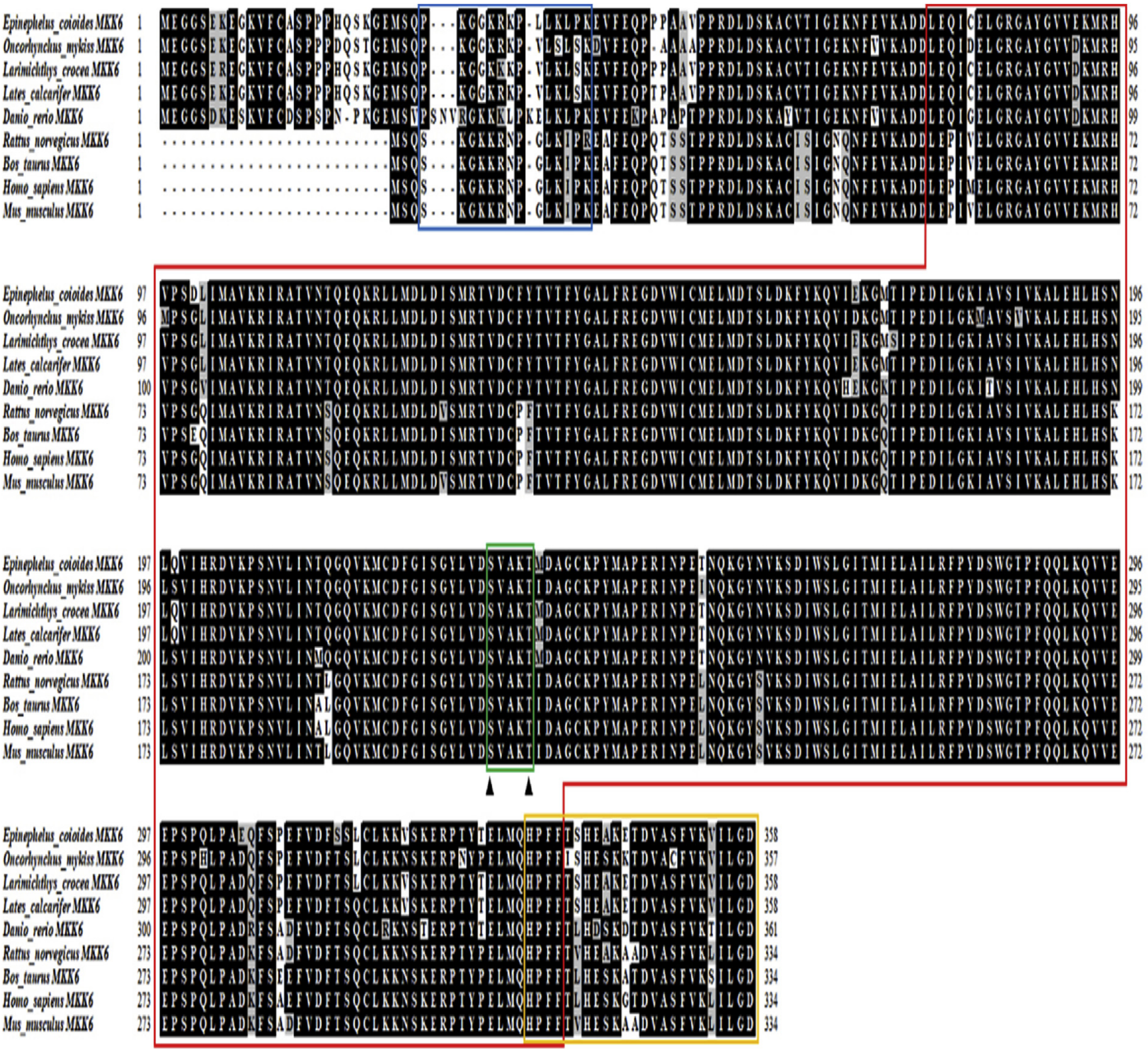

Fig. 1. The alignment of the amino acid sequence of EcMKK6 with other known sequences. Gaps, show as dashes (−), had been introduced to maximize the alignment to the sequences. Residues identical in all the sequences were shaded by black. The S-TKc domain and the tyrosine kinase catalytic domain were indicated was indicated by red box. The Low complexity region was indicated by blue box. a SVAKT motif was indicated by green box. The dual phosphorylation sites (Ser 231 and Thr 235) are showed with a black triangle below it. The conserved DVD domain was indicated by orange box. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

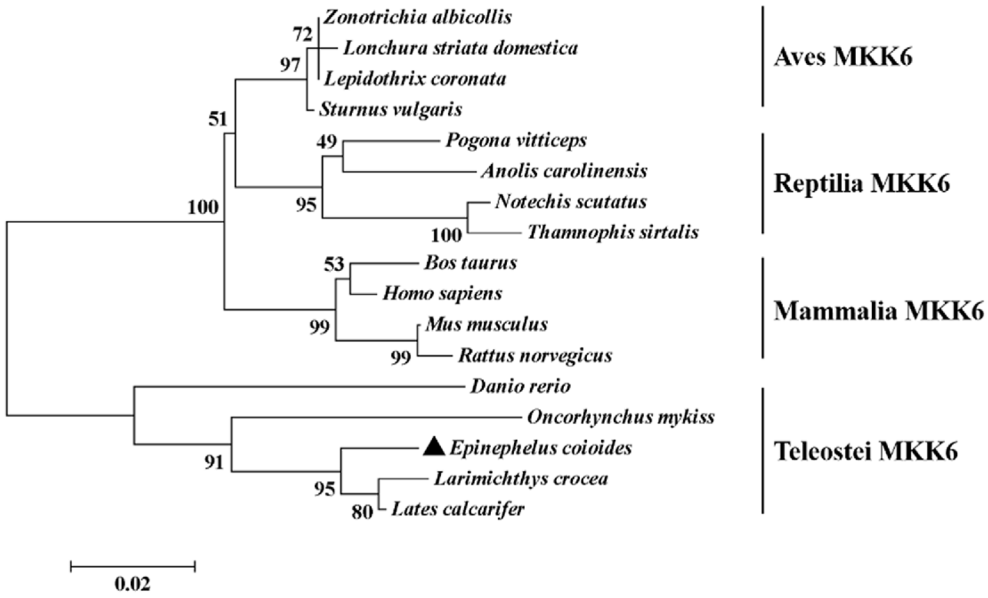

Fig. 2. A phylogenetic tree using the deduced amino acid sequences of MKK6 from the present study and those available in GenBank by the neighbor-joining method in the MEGA program. Node values represent percent bootstrap confidence derived from 1000 replicates. The scale bar is 0.02.

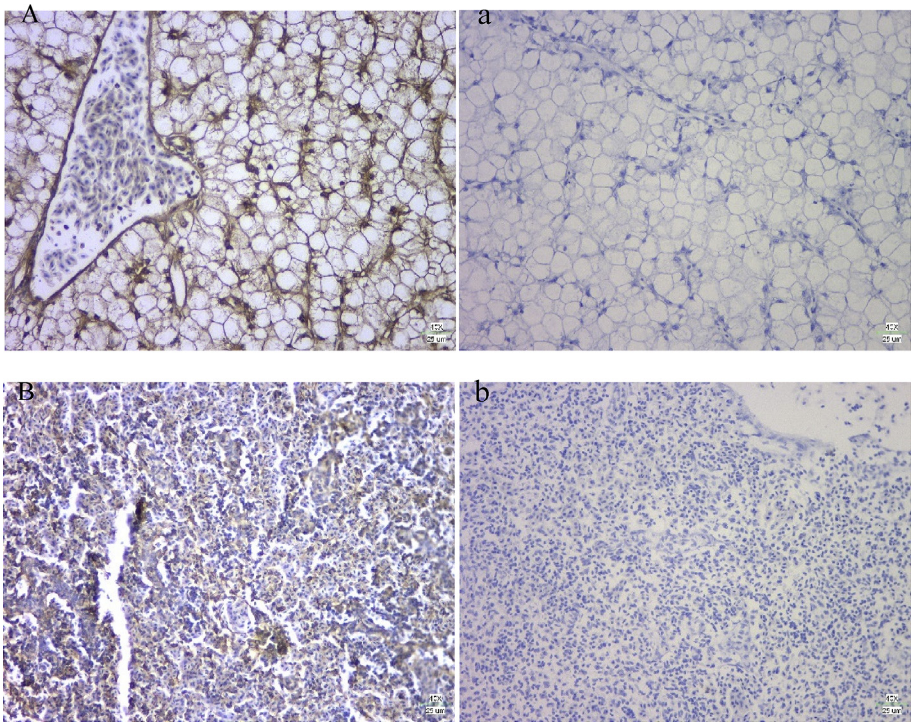

Fig. 3. In situ hybridization for MKK6 in grouper tissues using DIG-labeled DNA probe. DNA probe without DIG-label was used as negative control of hybridization. Positive ISH signals were shown in A (liver) and B (spleen). Negative controls were shown in a (liver) and b (spleen). Scale bars: 25 μm.

control. 3 fish per each tank were randomly selected at 0, 3, 6, 12, 24, 48 and 72 h after challenge. Subsequently, the tissues of the blood, skin, muscle, liver, intestine, spleen, trunk kidney, gill, head kidney, heart and brain were collected and stored at −80 °C for further analysis.

2.2. RNA isolation and cDNA synthesis

Total RNA was isolated from the tissues using TRIzol reagent (Invitrogen, Canada), and digested with DNase I (Promega, USA). The concentration was measured spectrophotometrically, and RNA integrity was examined by 1% agarose gel electrophoresis. The first-strand cDNA was synthesized using ReverTra Ace-a reverse transcriptase according to the manufacturer's instruction (Toyobo, Japan) and was used as a template in following polymerase chain reaction (PCR) (Takara, Japan) and real-time PCR (Thermo, USA).

2.3. Identification of grouper EcMKK6 sequences

The transcriptome data of grouper was obtained in our lab. According to the transcriptome data and the BLAST information, the primers, MKK6-ORF-F/MKK6-ORF-R (Table 1), were designed to amplify the longest open reading frames (ORF) of grouper MKK6. Each

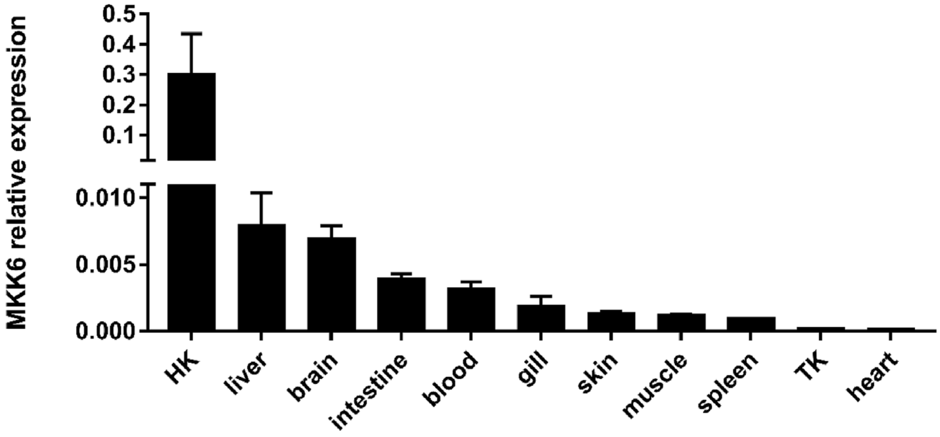

Fig. 4. The expression pattern of MKK6 from healthy grouper tissues. The histogram showed the relative expression level of MKK6 in different tissues of grouper normalized by β-actin transcript, calibrated using the expression of the epithelia in the control group as 1. TK: trunk kidney; HK: head kidney. All data are presented as Mean ± SD, N = 4. Different letters represent the significant difference of the gene in an individual tissue. Duncan test (*P < 0.05*).

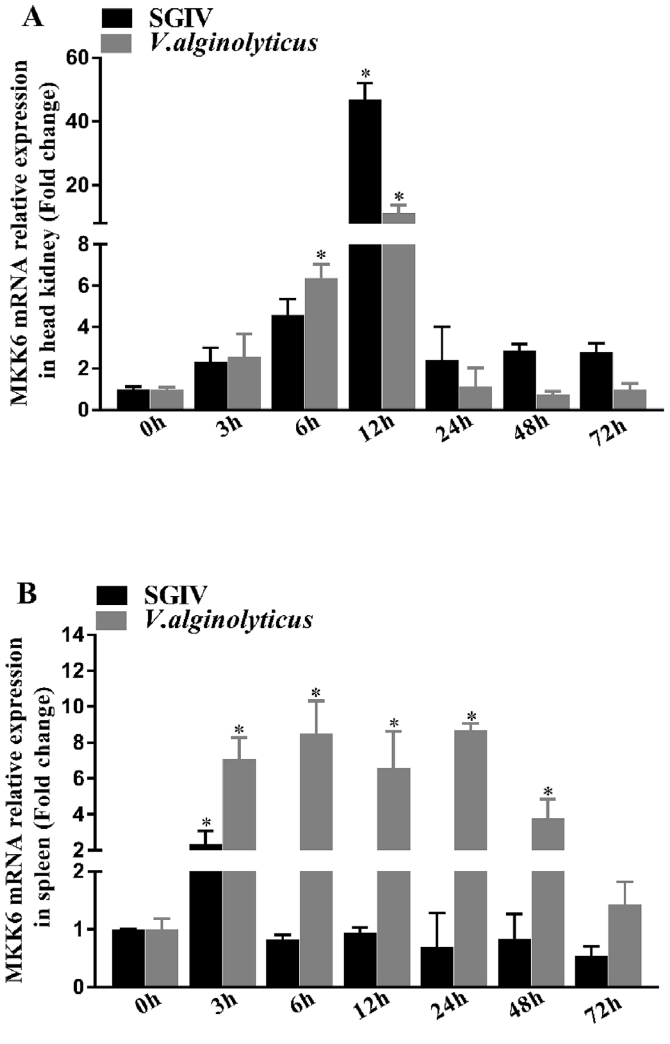

Fig. 5. Expression of EcMKK6 in the *E. coioides* head kidney (A) and spleen (B) challenge with *V. alginolyticus* and SGIV. Significant differences of the genes expression between control and pathogen infection group at each time points is indicated with * (significant increase, *P < 0.05*). All data are presented as Mean ± SD, N = 4.

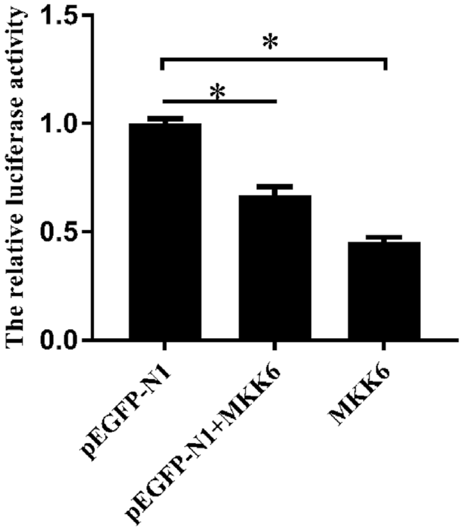

Fig. 6. Detection of EcMKK6 in AP-1 activity. Relative luciferase activity is measured as a ratio of firefly luciferase activity to Renilla luciferase activity. The transfection amount: pEGFP-N1 (800 ng), pEGFP-N1 (400 ng) + MKK6 (400 ng), MKK6 (800 ng). 48 h after transfection, cells were harvested and lysed according to the manual for dual luciferase reporter assay analysis. Results are expressed as means ± standard errors (n = 4).

amplification reaction was performed in a final volume of 50 µl containing template DNA, 10 mM Tris–HCl, 50 mM KCl, 3 mM MgCl$_2$, 200 µM deoxyribonucleotide triphosphates, 0.5 pmol of each primer, and 2.5 U Taq polymerase (Takara) in a thermocycler (Bio-Rad) under the following conditions: 35 cycles of 94 °C, 15 s; 55 °C, 15 s; and 72 °C, 90 s, followed by 72 °C for 5 min. The PCR products were purified and cloned into the pEASY®-Blunt Simple Cloning vector (Trans-Gen Biotech, China), and sequenced by the Life Technologies Corporation, Guangdong, China.

2.4. Bioinformatic analysis

The ORF of EcMKK6 was found by NCBI ORF finder tool (https://www.ncbi.nlm.nih.gov/orffinder/). Conserved domain was searched in (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The isoelectric point (pI) and molecular weight (Mw) of MKK6 were calculated with pI/Mw tool (http://web.expasy.org/compute_pi/). Amino acid sequence of MKK6 was compared with other species by CLUSTALW server (http://www.ebi.ac.uk/clustalw). The domain and location were predicted by the SMART tool (http://smart.embl-heidelberg.de/). A neighbor-joining phylogenic tree based on the sequences of the amino acids was performed by the MEGA 5.04, and bootstraps was set as 1000.

2.5. In situ hybridization for MKK6 in grouper tissues

The brain, heart, liver and spleen were obtained from grouper, washed and placed in a fixing solution (DEPC water preparation) for

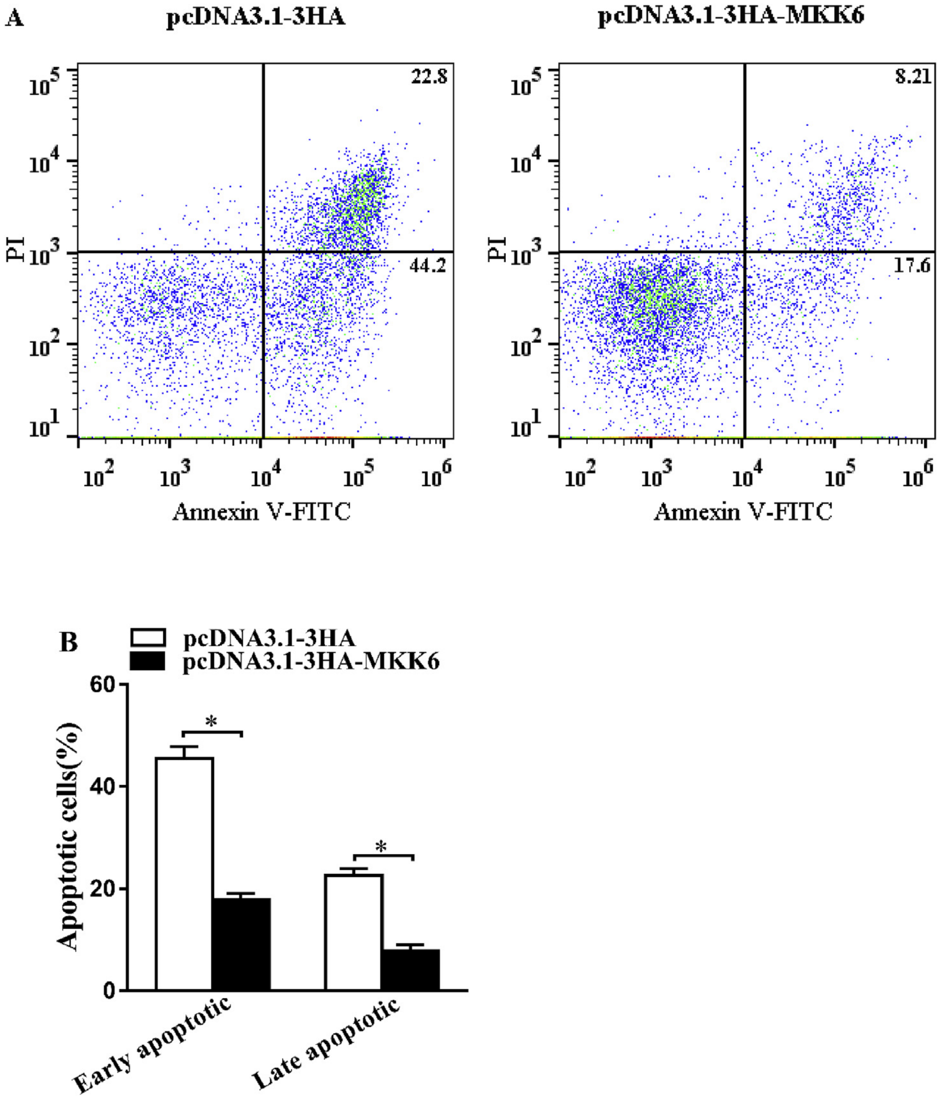

Fig. 7. The effect of EcMKK6 on apoptosis of FHM cells. Cells were treated with the pcDNA3.1-3HA/pcDNA3.1-3HA- EcMKK6 plasmid transfections, infected by SGIV for 24 h, and then analyzed using flow cytometry. A, Annexin-V + /PI-cells and annexin-V + /PI + cells are early apoptotic cells and late apoptotic cells, respectively. B, This graph is based on the average of three parallel experimental data.

2–12 h. It is dehydrated by gradient alcohol dehydration, embedded in wax and sliced for 2 h at 62 °C. The slides were dewaxed to water with sequentially placed in xylene I, 15 min; xylene II, 15 min; anhydrous ethanol I, 5 min; anhydrous ethanol II, 5 min; 85% alcohol, 5 min; 75% alcohol, 5 min and DEPC water. The slices are boiled in the repair solution for 10 min, digested by the proteinase K (20 μg/ml) at 37 °C for 25 min and washed with PBS. The slices added 3% methanol-H_2O_2 were incubate at room temperature for 15 min in the dark, and placed the slide in PBS (pH 7.4) for 3 times. Pre-hybridization solution was added dropwise and incubated at 37 °C for 1 h. The pre-hybridization solution was decanted, and the hybridization solution containing the MKK6 probe was added dropwise (8 ng/μl), then slides were incubated in a humidity chamber and hybridized overnight at 37 °C. In day two, the hybridization solution was washed in 2 × SSC for 10 min; (1 × SSC for 5 min) × 2; 0.5 × SSC for 10 min, BSA for 30 min. The mouse anti-digoxigenin-labeled peroxidase (anti-DIG-HRP) was added at 37 °C for 40 min. After the slice is slightly dried, the freshly prepared DAB coloring solution is added dropwise in the circle. Harris hematoxylin, 1% hydrochloric acid alcohol, ammonia, different concentrations of alcohol, xylene were used and prepared.

2.6. Expression analysis

Quantitative real-time PCR was used to determine the tissue expression profile of grouper EcMKK6 in both healthy fish and fish with *V. alginolyticus* and SGIV infection as described previously [23]. As shown in Table 1, the primers, MKK6-RT-F/MKK6-RT-R were used for quantification of MKK6, and β-actin-F/β-actin-R was used to amplify the housekeeping gene β-actin as the reference genes. Real time PCR was performed with SYBR Green Real-Time PCR Master Mix (Toyobo) on a Thermo Scientific Varioskan LUX (Thermo,USA),The conditions were one cycle of 95 °C for 1min, followed by 40 cycles of 95 °C, 15 s; 60 °C, 15 s; 72 °C, 45 s. Melting-curve analysis and sequencing were used to confirm the specificity of PCR products. All simples were verified in triplicate. The expression of the target gene was normalized to reference gene and calculated with the $2^{-\Delta\Delta Ct}$ method [24].

2.7. Dual luciferase reporter assays

To clarify whether MKK6 plays a role in AP-1 transcriptional regulation, plasmid (pEGFP-N1-EcMKK6) was used for luciferase reporter assay. Cells of grouper spleen (College of Marine Sciences, South China Agricultural University), GS cells, which were propagated with

Leibovitz's L15 medium and the 10% fetal bovine serum (FBS, Gibco, USA) at 28 °C [32], were seeded in 24 well plates overnight. pEGFP-N1 (800 ng, control), pEGFP-N1-EcMKK6 (800 ng), both pEGFP-N1 (400 ng) and pEGFP-N1-EcMKK6 (400 ng) were co-transfated with 150 ng of AP-1 dependent firefly luciferase reporter plasmid and 40 ng of renilla luciferase vectors. 48 h after transfection, cells were harvested and lysed according to the manual for dual luciferase reporter assay analysis. Relative luciferase activity is measured as a ratio of firefly luciferase activity to Renilla luciferase activity.

2.8. Cell apoptosis analysis

To explore the function of MKK6 in cell apoptosis during SGIV infection, primers MKK6-pcDNA3.1-F/MKK6-pcDNA3.1-R were designed for construction of pcDNA3.1-EcMKK6. The plasmid, pcDNA3.1 or pcDNA3.1-EcMKK6 was transfected into FHM cells for 24 well plates with each well of 800 ng and 2 μl Lipofectamine 2000 (Invitrogen) by three replicates. After 24 h of SGIV infection, the cells were harvested,and apoptosis was detected by flow cytometry using the annexin V-FITC apoptosis detection kit (Beyotime, China) according to the manufacturer's instructions. Each sample was analyzed in triplicate. Data acquisition and analysis were performed using a flow cytometry system (Beckman Coulter, USA) and FlowJo VX software.

2.9. Statistical analysis

All of the data that expressed as mean ± standard error of the mean (SD) were analyzed with SPSS using one-way analysis of ANOVA followed by Duncan's test. significance was set at $P < 0.05$.

3. Results

3.1. Sequence characterization and phylogenetic analysis

As expected, the longest ORF of *E. coioides* MKK6 was obtained, and submitted to GenBank under accession no. MK423963. The ORF of EcMKK6 is 1077 bp, encoding sequences of 358 amino acids with a molecular mass of 40.25 kDa and a theoretical PI of 5.90. As shown in Fig. 1, there is no signal peptides in the deduced amino acids sequence of EcMKK6 suggesting it was not the secretory protein. There are a region of low compositional complexity starts at position 28 and ends at position 41, the conservative S_Tkc domain and tyrosine kinase catalytic domain at sites 77–338, a conserved dual phosphorylation site (231 Ser and 235 Thr) in the SVAKT motif at sites 231–235 and a conserved DVD domain at sites 335–358.

To analyze the evolutionary relationships, a phylogenetic tree was constructed according to the amino acid sequences. As shown in Fig. 2, it was clustered into four branches, and EcMKK6 was divided into the teleostei group. Homology analysis showed that EcMKK6 has 87%–98% identity with other teleost fish, 88%–89% identity with aves,87% identity with mammalian and 86%–87% identity with reptilia. EcMKK6 shares the closest homology with teleostei, and has the highest amino acid identity (98%) with *Laters calcarifer* MKK6.

3.2. In situ hybridization for MKK6 in grouper tissues

As shown in Fig. 3, EcMKK6 DIG-labeled DNA probe reacted positively in all samples examined. The nucleus of hematoxylin was stained blue, and the positive signal of DAB was brown-yellow. The positive signal of EcMKK6 in the liver (Fig. 3A) and spleen (Fig. 3B) were detected, and no signals were obtained in the liver (Fig. 3a) and spleen (Fig. 3b) with DNA probe without DIG-label (negative control). The results showed that MKK6 mainly located at the cytoplasm of cells, and a little appears in the nucleus.

3.3. Tissue distribution in healthy E. coioides

To examine the tissue distribution, the total RNA of eleven tissues including blood, liver, spleen, head kidney, trunk kidney, gill, heart, skin, muscle, brain and intestine from healthy *E. coioides* was extracted, and real-time quantitative PCR was used to quantify the expression. As shown in Fig. 4, EcMKK6 mRNA were detected in all of the tissues examined, but the expression level is different: the expression level of EcMKK6 was higher in the head kidney, followed by the liver, brain, intestine, blood, gill, spleen, skin, muscle, trunk kidney and heart.

3.4. Genes expression pattern response to V. alginolyticus and SGIV infection

To explore the expression profile of EcMKK6 in response to pathogen, *V. alginolyticus* and SGIV were used to infect healthy *E. coioides*, and the target gene EcMKK6 was detected using real-time quantitative PCR. At each time point, the expression and fold change of the gene EcMKK6 were compared to the control fish as shown in Fig. 5.

As shown in Fig. 5A, after challenge with *V. alginolyticus* and SGIV, the relative expression of EcMKK6 in the head kidney was up-regulated at 3 h, and reached the peak at 12 h (11.20 fold with *V. alginolyticus* infection; 46.92 fold with SGIV infection), then decreased since 24 h.

In the spleen (Fig. 5B), the expression of EcMKK6 was up-regulated since 3 h post infection. The peaks occurred at 3 h (2.38 fold) after SGIV infection, and 24 h (8.69 fold) after *V. alginolyticus*, respectively. Subsequently, the expression was decreased.

3.5. Luciferase reporter assays of MKK6

As shown in Fig. 6, dual-luciferase reporter assays were performed to research the function of EcMKK6 in the activation of AP-1. The ORF of EcMKK6 was ligated to pEGFP-N1 and over-expressed in GS cells. The relative luciferase activity showed that EcMKK6 down-regulates the activation of AP-1 compared to the mock ($P < 0.05$).

3.6. Effects of EcMKK6 on the cell apoptosis

To investigate the involvement of EcMKK6 in the procedures of apoptosis, pcDNA3.1/EcMKK6 was transfected into FHM cells. As is shown in Fig. 7A, and the percentage of early SGIV-induced apoptosis in FHM/pcDNA3.1 cells was 44.2%, while that of FHM/pcDNA3.1-EcMKK6 cells was 17.6%, indicating that over-expression of exogenous EcMKK6 significantly inhibit the apoptosis in FHM cells.

4. Discussion

Mitogen-activated protein kinase 6 (MKK6) is one of the major important central regulatory proteins response to environmental and physiological stimuli, and it can be activated in cytokine producing cells or in cells receiving those inflammatory signals in an inflammatory environment [33–36]. In this study, a novel fish MKK6 was cloned for the first time from *E. coioides* and characterized their function response to *V. alginolyticus* and SGIV infection.

As expected, five conserved fields including a low compositional complexity, a S_TKc domain, a tyrosine kinase catalytic domain, a SVAKT motif with dual phosphorylation site (231 Ser and 235 Thr) and a conserved DVD domain were found on the sequence of EcMKK6. Analysis of phylogenetic relationships and BLAST illustrated that the sequence EcMKK6 was highly conserved, and shared 83–88% identity with mammals, and 83–98% identity with teleostei, indicating that functions of MKK6 might be similar in fishes than other species.

The subcellular localization of EcMKK6 was analyzed by in situ hybridization, and the result showed that EcMKK6 is mainly distributed in the cytoplasm, and a little appears in the nucleus suggesting that EcMKK6 may not only play a role in the cytoplasm but also participate in some physiological processes in the nucleus. Similar results were found in the grass crap *Ctenopharyngodon idella* and white shimp *L. vannamei* [6,20]. MKK6 receives the upstream kinase MAPKKK signal and transmits a signal to the downstream kinase MAPK in the cytoplasm, inducing expression of kinds specific target genes, confirms that the distribution of MKK6 in the cytoplasm is reasonable [19].

MKK6 is widely distributed in various tissues and organs of vertebrates and invertebrates [6,7,15,19,20,37]. In L. *vannamei*, MKK6 has also been widely expressed in the stomach, intestines, blood, heart, eyestalk, nerve, gill, epithelium, scape, pyloric cecum, muscle and hepatopancreas [20]. The transcript levels of CiMKK6 in eight tissues, intestine, liver, blood, heart, kidney, gill, muscle and spleen of adult grass carp were analyzed by qRT-PCR, and results showed that MKK6 is ubiquitous in all organs, expression patterns can emphasize different functions [6]. In this study, EcMKK6 mRNA was detected in all of the tissues examined in healthy *E. coioides*, and the tissue distributions were different due to the tissue types, and similar results were found in grass carp *C. idella* and yesso asclop *P. yessoensis* [6,7].

Previous studies demonstrated that MKK6 may be activated in mice, *L. vannamei* etc. response to inflammatory cytokines, environmental stress, bacterial and virus [1,3,20,38–40]. However, whether the MKK6 can be activated in *E. coioides* remained unknown. In this study, the differential expressions of the EcMKK6 in head kidney and spleen were explored response to bacterial and virus infection. After challenge with bacteria or virus, the expressions in both spleen and head kidney were up-regulated, indicating that EcMKK6 might play vital roles in response to pathogen infection.

In the MAPK signaling pathway, MKK6 receives upstream MKKK signaling which can phosphorylate the TGY motif of the threonine and tyrosine residues of downstream p38 MAPKs, stimulated activation of downstream transcription factors and induce specific target genes expression, and p38 MAPKs/ERKs functioned as the upstream protein kinases involving in AP-1 transactivation [5–8,13,41–44]. In this study, dual-luciferase reporter assays were performed to research the function of EcMKK6 in the activation of AP-1.

SGIV infection induced typical apoptosis in FHM cells [38], and over-expression of p38 MAPK inhibited SGIV induced typical apoptosis in fish cells [31]. In mammals, MKK6 is essential for the activation of p38 MAPK induced by environmental stress [4]. In this study, we found over-expression of *E. coioides* MKK6 can inhibit the apoptosis induced by SGIV in fish cells, indicating that MKK6 might be involved in the cell apoptosis via p38 MAPK.

In summary, a new EcMKK6 was successfully identified from *E. coioides*. Ec-MKK6 mainly located at the cytoplasm of cells, and a little appears in the nucleus. The EcMKK6 can be detected in all of the eleven tissues examined of healthy *E. coioides,* and the expressions significantly varies response to *V. alginolyticus* and SGIV infection. EcMKK6 has an inhibitory effect on SGIV-induced apoptosis. These findings provide new insights into how the gene's expression profile changes with pathogen invasion of *E. coioides* and lay a foundation for further functional studies on MKK6s in teleosts.

Acknowledgments

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