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PfHMGB2 protects yellow catfish (*Pelteobagrus fulvidraco*) from bacterial infection by promoting phagocytosis and proliferation of PBL

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ABSTRACT

HMGB2, a member of the high mobility group box family, plays an important role in host immune responses. However, the mechanism of action of HMGB2 is not well understood. Herein, a homologue from yellow catfish (*Pelteobagrus fulvidraco*) was cloned and named PfHMGB2. The deduced amino acid sequence of PfHMGB2 possessed a typical tripartite structure (two DNA binding boxes and an acid tail) and shared 90% identity with the predicted HMGB2 from *I. punctatus*. The mRNA of PfHMGB2 was widely distributed in all 11 tested tissues in healthy fish bodies and was significantly induced in the liver and head kidney when yellow catfish were injected with inactivated *Aeromonas hydrophila*. Consistently, *PfHMGB2* mRNA could also be induced in yellow catfish peripheral blood leucocytes (PBL) by lipopolysaccharide. The recombinant PfHMGB2 protein was purified from *E. coli* BL21 (DE3):pET-28a/PfHMGB2 and showed DNA-binding affinity. Moreover, rPfHMGB2 improved the phagocytosis and proliferation activity and upregulated the mRNA expression of the pro-inflammatory cytokine TNFα in yellow catfish PBL. These results indicated that PfHMGB2 could protect yellow catfish from pathogen infection by activating PBL.

1. Introduction

The high-mobility group (HMG) family belong to chromatin-associated nonhistone proteins that organize and modify nucleosomes into high-order structures by binding chromosomal DNA [1]. According to their DNA binding domain and substrate binding specificity, HMG family proteins can be divided into the following three subfamilies: HMG-AT-hook family (HMGA), HMG-box (HMGB), and HMG-nucleosome binding family (HMGN) [2]. Mammalian HMGB proteins can be further classified into 4 subtypes, HMGB1, 2, 3, and 4. All HMGBs share the same tripartite structure, namely, an N-terminal A box, a central B box, and a C-terminal acidic tail, except for mammal HMGB4, which lacks the C-terminal acidic domain. The common acidic tails are negatively charged with Glu (D) and Asp (E) residues. In contrast, the two boxes are positively charged with Lys (P) and Arg (R) residues. Fish lack HMGB4, and some teleost even contain two co-orthologue HMGBs [1].

HMGB proteins bind DNA in a non-sequence-specific manner. However, they prefer to bind noncanonical and distorted DNA structures, such as bent DNA, single stranded DNA, supercoiled DNA, and damaged DNA. This affinity is modulated by its own C-terminal acid tail [2,3]. HMGB proteins can also interact with transcriptionally activated/inhabited factors, repair-related proteins, histones, virus proteins, and transcriptional factors to regulate gene expression [1].

HMGB1 is a well-known member of the HMGB family in mammals due to its necessity [4]. HMGB1 has diverse functions in DNA binding, transcriptional regulation, gene recombination, and DNA repair. It can also activate immune cells to evoke inflammation and maintain subsequent immune responses as a danger-associated molecular pattern (DAMP). Therefore, it is involved in many inflammatory diseases, such as hepatitis, neuronitis, and arthritis [5,6]. Recent studies revealed that fish HMGB1 also plays a vital role in the innate immune response, such as in inflammation and antibacterial and antiviral activities [7–11].

Given high identity (80%) between HMGB1 and HMGB2, HMGB2 may also play a role in immune system. However, little is known about HMGB2. Mice lacking Hmgb2 have a normal lifespan but show fertility and spermatogenesis defects [12]. The mouse *hmgb2* gene is broadly

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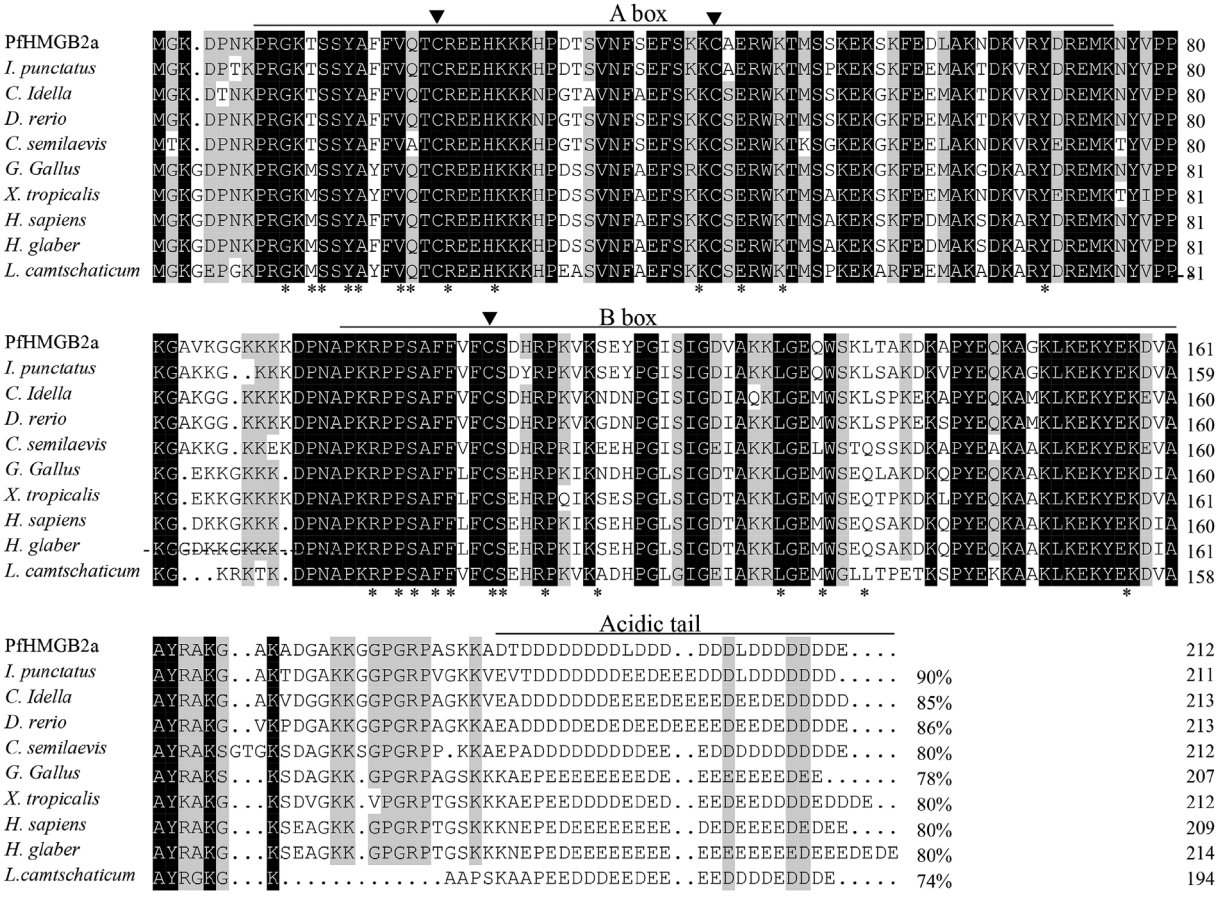

Fig. 1. Alignment of yellow catfish PfHMGB2 with its homologues constructed by ClustalX 2.1. The dashes are introduced for maximum matching. Identities of 100% are shaded dark, while similarities ≥75% are shaded in grey. The HMG A box, B box, and acidic tail are overlined. The DNA-binding sites within the A and B box are asterisked. The cysteines are indicated with inverted triangles. The GenBank numbers are as follows: *Ictalurus punctatus*, XP_017318880.1; *Ctenopharyngodon Idella*, AFR33805.1; *D anio rerio*, NP_001032501.1; *Cynoglossus semilaevis*, NP_001284515.1; *Gallus gallus*, NP_990817.1; *X enopus tropicalis*, NP_988904.1; *Homo sapiens*, NP_002120.1; *Heterocephalus glaber*, EHB04011.1; and *Lethenteron camtschaticum*, AEH59760.1.

expressed during embryogenesis but becomes restricted mainly in the thymus and testis in adult mice. In teleosts, the zebrafish *hmgb2a* gene is most highly expressed during embryonic development [13]. In contrast, HMGB2 is broadly expressed in examined tissues from *Cynoglossus semilaevis* [14], *Ctenopharyngodon Idella* [15], and *Sebastes schlegelii* [16]. In host immunity, *HMGB2* not only responds to bacterial/viral PAMP (pathogen-associated molecular pattern) challenge as a sensor [15,17] but also exhibits antibacterial and antiviral functions [14,16]. The antibacterial and antiviral properties of HMGB2 protein are largely independent on its C-terminal acidic domain. Moreover, purified HMGB2 from the human intestinal tract can directly inhibit pathogens, such as *E. coli K-12, B.* adolescentis, and *S. salivarius* subsp. *thermophilus*, in a concentration-dependent manner, and the two DNA-binding domains (HMG boxes A and B) are crucial for its antibiotic functions [18].

In this study, a full-length cDNA sequence of PfHMGB2 from yellow catfish, a valuable cultured fish in southern China, was cloned and characterized [19,20]. Its mRNA expression profiles were investigated under normal and stimulated conditions. Moreover, recombinant protein rPfHMGB2 was overexpressed and purified from *E. coli* and its effects in a host immune system were examined.

2. Materials and methods

2.1. cDNA cloning and analysis

Part of the PfHMGB2 cDNA sequence was obtained by sequencing a normalized yellow catfish kidney cDNA library constructed in our laboratory [21]. It shared high identity with HMGB2 from other species based on BLASTn. To obtain the full length cDNA sequence, its 5′-end and 3′-end were separately amplified using specific primers via 5′-RACE (HMGB2-5′, 5′-ATGCGGCAACATCCTTCTCATA-3′, and HMGB2-5′-1, 5′-GCTCGTATGGAGCTTTATCCTTG-3′) and 3′-RACE (HMGB2-3′, 5′-GCCAAGGATAAAGCTCCATACG-3′, and HMGB2-3′-1, 5′-TGAGAAG GATGTTGCCGCATAC-3′) as previously described [20]. The full-length cDNA sequence was analysed via BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi/).

2.2. Phylogenetic analysis

The deduced amino acid sequence of PfHMGB2 was aligned with HMGBs from other fishes, amphibians, mammals, and invertebrates. Based on this alignment, a phylogenetic tree was constructed with HMGB1 and HMGB3 used as the extra clusters.

2.3. PfHMGB2 RNA distribution in different tissues

To analyse *PfHMGB2* RNA expression in different tissues of healthy yellow catfish, wild yellow catfish obtained from Pingxi Lake in Pingdingshan city, Henan province were cultured in the laboratory for at least 2 weeks as described previously [20]. Then, the healthy fishes were sacrificed to dissect 11 tissues (n = 3), including the trunk kidney, intestine, blood, spleen, muscle, skin, liver, head kidney, fin, gill, and heart. Total RNA was extracted using the Trizol Reagent (Invitrogen, USA). Residual DNA was removed, and the RNA was reverse

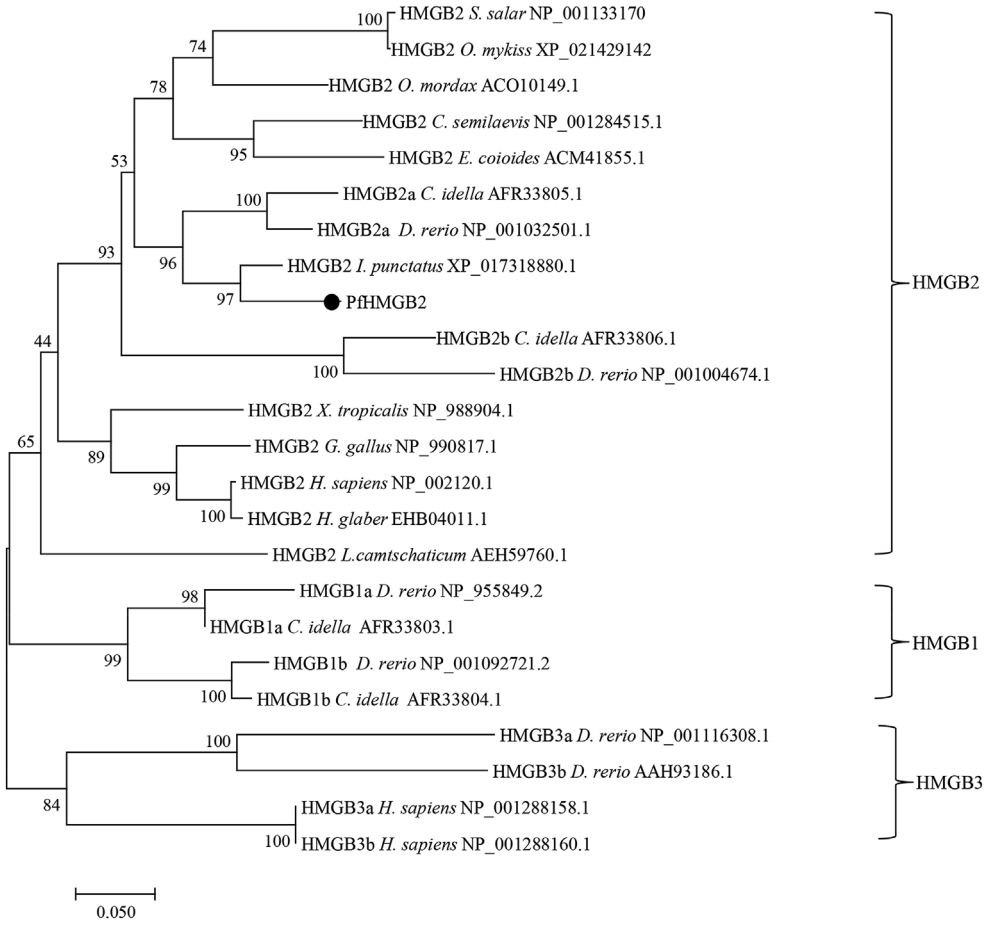

Fig. 2. Phylogenetic analysis of PfHMGB2 protein with its homologues. The tree was constructed by the Neighbour-joining algorithm with 1,000 bootstraps in MEGA 7.0 based on the multiple sequence alignment performed by ClustalX 2.1. The bar (0.050) indicates the genetic distance.

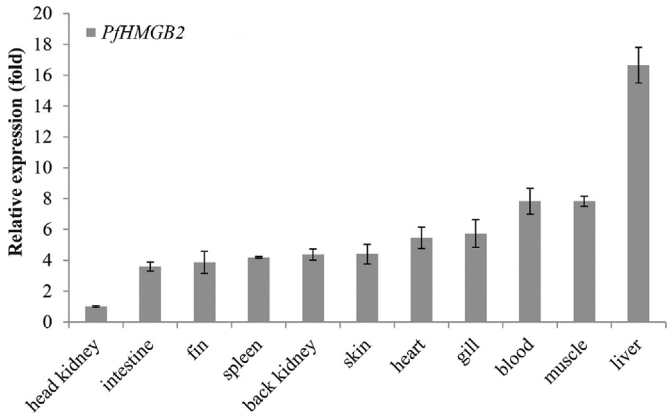

Fig. 3. *PfHMGB2* mRNA in 11 tissues from healthy yellow catfish detected by qPCR, with 18S rRNA as a reference gene. The relative expression was calibrated against that of head kidney. The data were expressed as the mean ± SD (n = 3).

transcribed with a Prime Script RT reagent Kit using gDNA Eraser (TaKaRa, Japan) [20,22]. Using a SYBR Green Premix Ex-Taq (TaKaRa, Japan), cDNA copies were quantified with 0.2 μM gene-specific primers (HMGB2-RTF, 5′-GACCAAAGGTGAAGAGCGAGTA-3′, and HMGB2-RTR, 5′-CTCGTATGGAGCTTTATCCTTGG-3′). 18S rRNA served as the reference gene [20]. The relative fold expression was calculated with the $2^{-\triangle\triangle CT}$ method [21,23].

2.4. PfHMGB2 RNA expression in tissues post-challenge

To explore the temporal expression of *PfHMGB2* in the immune tissues post-challenge *in vivo*, healthy yellow catfish were injected with 200 μL PBS or inactivated *A. hydrophila* (1×10^7 cfu). Then, the liver and head kidney were collected at 0, 6, 12, 24, 72, 120, or 168 h from each group (n = 3). *PfHMGB2* mRNA expression was detected using qPCR as described above.

2.5. Recombinant expression of PfHMGB2

To overexpress PfHMGB2, specific primers were designed (HMGB2-28a-F, 5′- TTGAGAATTCATGGGGAAAGATC-3′, and HMGB2-28a-R, 5′-CAAAATTCTCGAGAAATTTATTCGT-3′) with the *Eco*R I and *Xho* I (underlined) restriction enzyme sites to amplify the *PfHMGB2* ORF. The amplified products were ligated to the pMD18-T vector, sequenced, and dissected with *Eco*R I and *Xho* I. Then, the collected *PfHMGB2* ORF fragment was ligated to the pET-28a vector, resulting in the pET-28a/PfHMGB2 expression plasmid. The recombinant plasmid was transformed into *E. coli* BL21 (DE3) to induce overexpression of rPfHMGB2 with 1 mM IPTG at 28 °C for 4 h. Protein was purified with a Ni column according to the manufacturer's protocols (Novagen, USA), dialyzed with PBS, and its concentration was determined with a Bradford Protein Assay Kit (CWBIO, China). Last, the dialyzed rPfHMGB2 was mixed with high-efficiency endotoxin affinity chromatography resin FF (Beijing Wechsler Bohui Chromatography, China) to remove residual endotoxin and checked with the limulus lysate agent (Zhangjiang A&C, China).

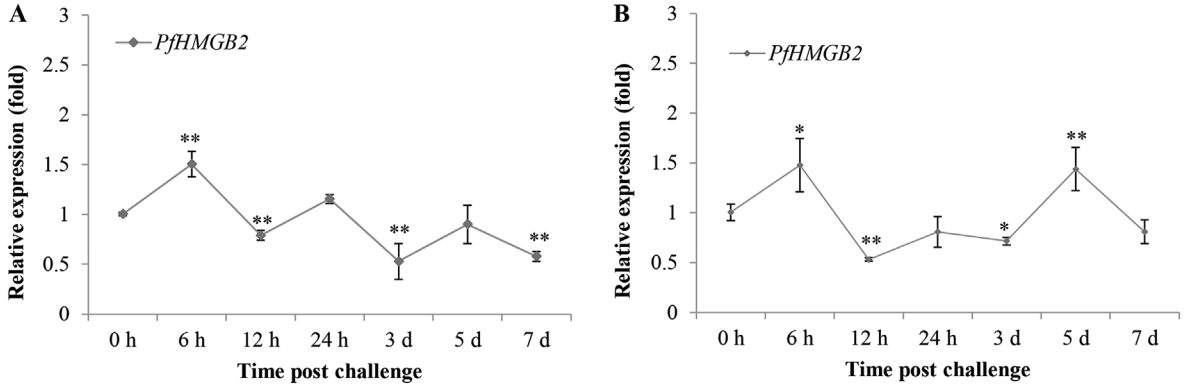

Fig. 4. Temporal expression of *PfHMGB2* mRNA in liver (A) and head kidney (B) of yellow catfish post-challenge. The healthy fish were injected with PBS or inactivated *A. hydrophila*. The relative expression levels were analysed by qPCR. The 18S rRNA served as a control. Values were expressed as the mean ± SD (n = 3); *P < 0.05 and **P < 0.01.

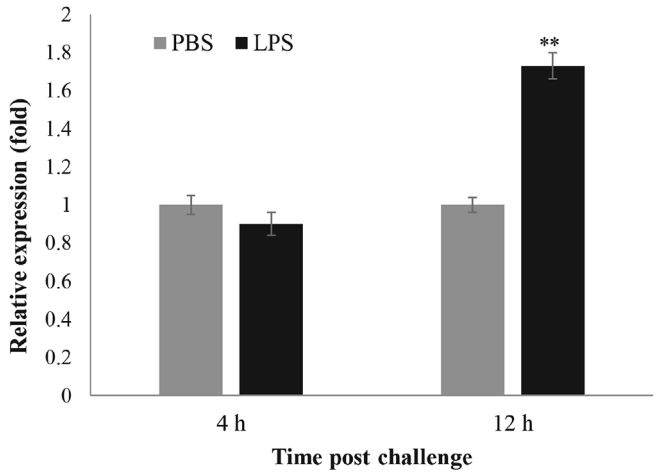

Fig. 5. *PfHMGB2* mRNA expression in peripheral blood leukocytes (PBL) from yellow catfish post-LPS challenge. The relative expression levels were analysed by qPCR, with 18S rRNA as a housekeeping gene. Values were expressed as the mean ± SD (n = 3); *P < 0.05 and **P < 0.01.

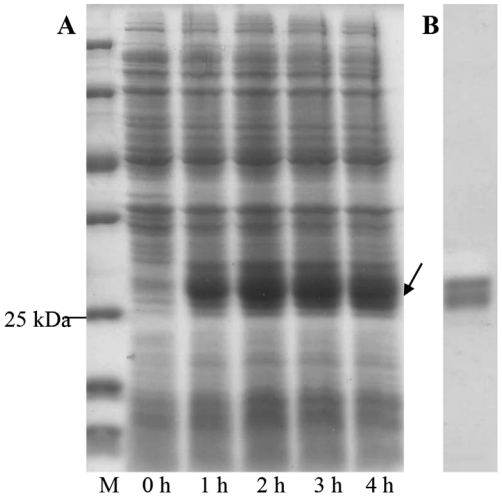

Fig. 6. The induced expression (A) and purification (B) of rPfHMGB2 protein. (A) *E. coli* BL21 (DE3) transformed with pET-28a/PfHMGB2 plasmid was induced by 1 mM IPTG at 0, 1, 2, 3, or 4 h and analysed by SDS-PAGE. The target band is arrow marked. (B) rPfHMGB2 was purified with a Ni-bond column.

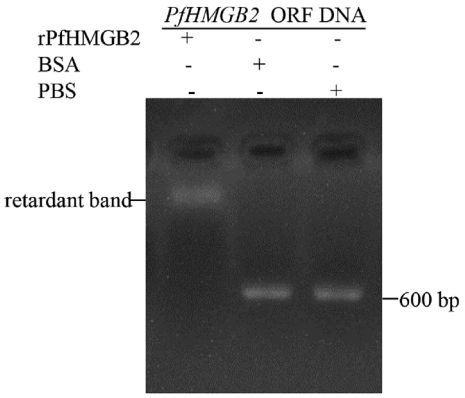

Fig. 7. DNA-affinity activity of rPfHMGB2 analysed by simple electrophoretic mobility shift assay (EMSA). *PfHMGB2* DNA fragments were incubated with rPfHMGB2 protein, BSA or PBS.

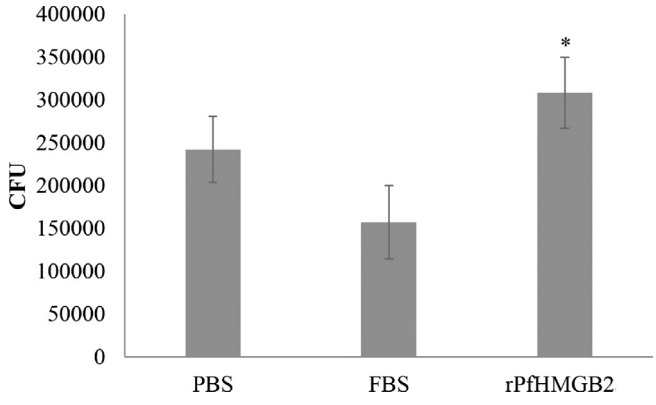

Fig. 8. The phagocytosis ability of PBL stimulated by rPfHMGB2. PBL (1×10^7 cells/mL) were incubated with PBS, foetal bovine serum (FBS), or rPfHMGB2 for 4 h and then incubated with *A. hydrophila* (1×10^8 cfu) that had been serum-opsonized. Bacterial recovery (shown as colony forming unit, CFUs) from the PBL was determined by plate counting. Values are expressed as the mean ± SD (n = 6); *P < 0.05 and **P < 0.01.

2.6. rPfHMGB2 DNA affinity assay

To validate the DNA binding ability of rPfHMGB2, simplified electrophoretic mobility shift assay (EMSA) were performed. A 600-bp dsDNA fragment of the *PfHMGB2* ORF was amplified using the HMGB2-28a-F/R primers. One hundred nanograms of recovered DNA was incubated with 2 μg rPfHMGB2 in 50 μL PBS at 37 °C for 1 h with BSA or

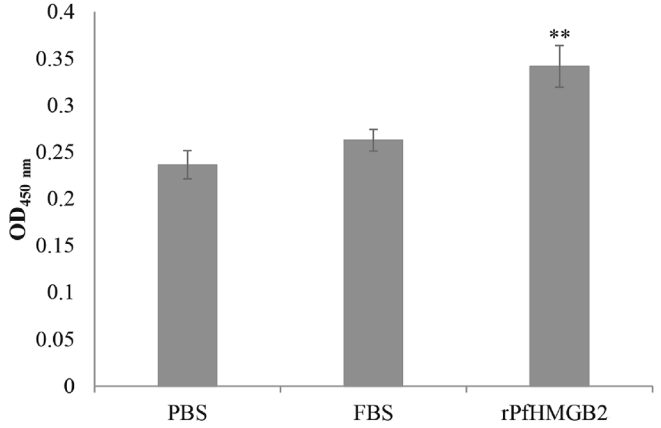

Fig. 9. The proliferation ability of PBL stimulated by rPfHMGB2. PBL (4×10^4 cells/mL) were incubated with PBS, FBS, or rPfHMGB2 for 4 h and then incubated with CCK-8 for 3 h. The $OD_{450\ nm}$ absorption values are shown as the mean ± SD (n = 4); *P < 0.05 and **P < 0.01.

PBS as controls. Then, the protein-dsDNA complexes were electrophoresed on 2% agarose gels for 10 min and visualized with GelRed (Biotum, USA) using a gel documentation system (Syngene, UK).

2.7. Peripheral blood leucocyte isolation

Peripheral blood leukocytes (PBL) were isolated from yellow catfish using a leucocyte separation kit for fish peripheral blood (TBD, China). Briefly, blood was drawn from the caudal vein of fish using a 1 ml syringe containing 0.2 ml heparin sodium (Amresco, USA), diluted with sample diluent, and centrifuged with separation liquid for 25 min at 500 g. After centrifugation, the PBL, namely, the middle milk-white cell layer, was collected and cultured in M199 medium containing 100 U ampicillin and streptomycin and 5% foetal bovine serum (FBS). Isolated PBL were 99% alive using a 0.4% trypan blue exclusion test for cell viability.

2.8. PfHMGB2 RNA expression in PBL post-challenge

To detect *PfHMGB2* RNA expression in PBL modulated by lipopolysaccharide (LPS), isolated PBL were seeded in a 6-well plate (1×10^7 cells/ml) for 2 h. Then, they were incubated with 100 μL PBS or LPS (100 ng/μL, Sigma) for 4 or 12 h. Last, total RNA was extracted for qPCR analysis of *PfHMGB2* RNA expression as previously described.

2.9. Phagocytic test in vitro

A phagocytic test was performed to detect the effects of rPfHMGB2 on the phagocytosis activity of PBL [24]. Briefly, 100 μL detached PBL (1×10^7 cells/mL) were seeded in a 96-well plate and incubated with 10 μL PBS, FBS, or rPfHMGB2 (0.1 μg/μL) for 4 h. Meanwhile, overnight-cultured *A. hydrophila* were washed with PBS twice, suspended in PBS containing 10% FBS, and spinning at 6 rpm/min for 30 min. Then, 10 μL serum-opsonized bacteria (1×10^8 cfu) were incubated with the treated PBL for 30 min. After that, PBL were collected by centrifugation at 150*g* at 4 °C for 5 min and washed with ice-cold PBS twice to remove the attached bacteria (n = 6). Last, the washed cells were lysed via suspension in sterile water for 1 h. The released bacteria were counted by plate counting.

2.10. Cell proliferation assay

To explore the effects of rPfHMGB2 on the PBL, a cell proliferation assay was performed using a CCK-8 kit (Beyotime, China). The detached PBL were seeded in a 96-well plate at a density of 4×10^4 cells/well for 2 h. Then, the cells were treated with PBS, FBS, or rPfHMGB2 (0.1 μg/μL) in quadruplicate for 4 h. Last, the treated cells were incubated with CCK-8 for 3 h, and the absorption at 450 nm was measured by using a microplate reader (PE, USA).

2.11. Immune-related gene expression

To explore the effects of rPfHMGB2 on immune-related gene expression in PBL, isolated PBL were seeded in a 6-well plate (1×10^7 cells/well) for 2 h. Then, they were stimulated with 100 μL purified rPfHMGB2 (0.1 μg/μL) for 4 h. FBS served as a control. Then, the cells were collected, and their total RNA was extracted and reverse-transcribed into cDNA. The cDNA was used as template of qPCR to detect the expression of the inflammatory genes TNFα, IL-1β [9] and MAPK [21] using gene-specific primers for TNF-α (TNF-α-F-80, 5′-CAGTGGGTGAGTGGAGTG-3′, and TNF-α-R-80, 5′-TCGTGGGGAATGAGGATA-3′), IL-1β (IL-1β-F-112, 5′-TAGGCATAGAGGGTAA-3′, and IL-1β-R-112), 5′-AAGGTGTTCAGGGAGTCA-3′), and MAPK (MAPK-RTF, 5′-TCAAGCTGTGTGACTTTGG

C-3′, and MAPK-RTR, CTCTACCAGTGAAAGCCCCA-3′).

2.12. Statistical analysis

Significant differences in the data from three, four or six repeats were examined by ANOVA using SPSS 19.0 and defined as significant as follows: *P < 0.05 and **P < 0.01.

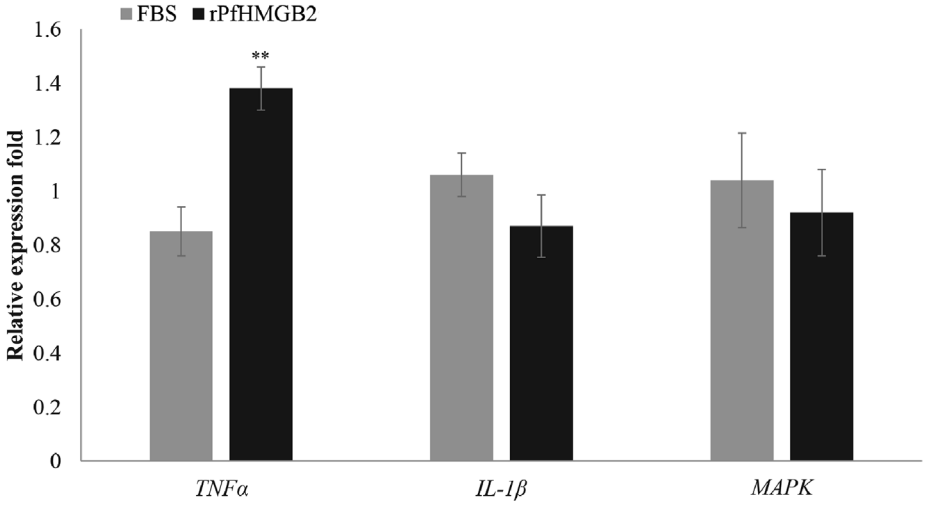

Fig. 10. Immune-related gene expression in PBL stimulated by rPfHMGB2. The isolated PBL (1×10^7 cells/mL) were treated with FBS or rPfHMGB2 for 4 h. Immune-related gene expression was detected by qPCR, with 18S rRNA serving as a housekeeping gene. Values were expressed as the mean ± SD (n = 3); *P < 0.05 and **P < 0.01.

3. Results

3.1. Cloning and identification of full-length PfHMGB2 cDNA

The full-length cDNA sequence of PfHMGB2 (GenBank accession number MK381266) was obtained by sequencing a yellow catfish cDNA library constructed in our laboratory, as well as by 5′- and 3′-RACE. It consists of 1090 bp, including an 84-bp 5′-untranslated region (UTR), a 639-bp open reading frame (ORF), and a 367-bp 3′-UTR with a polyadenylation signal AATAAA and two instability motifs (ATTTA). The identified ORF encodes a protein with theoretical pI/Mw of 8.47/23.85 kDa. The deduced PfHMGB2 protein has a typical tripartite structure, an N-terminal A box (8–75), a middle B box (96–161), and a C-terminal acid tail (187–212). There is also a nuclear localization sequence (NLS) located at residues 79–100 (Fig. 1).

3.2. Phylogenetic analysis of PfHMGB2 protein

The deduced amino acid sequence of PfHMGB2 shares 74–90% identities with that of other species from bony fish to tetrapods (Fig. 1). It displays the highest identity (90%) with the predicted HMGB2 from *I. punctatus*. To further analyse their phylogenetic relationships, a phylogenetic tree was constructed in MEGA 7.0 based on the multiple sequence alignment (Fig. 2). The tree is subdivided into the HMGB1, 2 and 3 subfamilies. HMGB2s are more closely related to HMGB1. The HMGB2 subfamily expands from the jawless lamprey *L. camtschaticum*. Among HMGB2s, PfHMGB2 is most closely related to HMGB2 from *I. punctatus*.

3.3. PfHMGB2 mRNA expression in different tissues

qPCR was performed to detect the relative expression of *PfHMGB2* in 11 tissues from healthy fish bodies (Fig. 3). Different expression levels in different tissues were observed, with the lowest level being observed in the head kidney, which was calibrated as 1. The highest expression was observed in the liver, followed by the muscle. The expression level in the liver is about 17-fold more than in the head kidney and 2-fold more than in the muscle.

3.4. PfHMGB2 mRNA expression post-challenge

qPCR was performed to detect the temporal expression of *PfHMGB2* in the immune tissues post-*A. hydrophila* challenge (Fig. 4). In the liver, the relative expression of *PfHMGB2* mRNA significantly increased at 6 h, but decreased at 12 h, 3 d and 7 d. In the head kidney, it remarkably increased at 6 h and 5 d but declined at 12 h and 3 d. *PfHMGB2* expression was remarkably modulated by bacteria, but in a dynamic manner.

qPCR was also performed to detect *PfHMGB2* expression in immune cells after LPS challenge (Fig. 5). *PfHMGB2* mRNA in PBL was not affected by LPS at 4 h, but remarkably induced at 12 h.

3.5. DNA affinity of rPfHMGB2

Recombinant PfHMGB2 was purified and analysed by SDS-PAGE (Fig. 6A). Unexpectedly, the purified protein showed double bands close to one another and appeared higher than the 25 kDa marker, which is somewhat bigger than the expected molecular mass of 23.85 kDa (Fig. 6B). To identify the two bands, they were recovered from the gel for mass spectrum analysis (Sangon Biotech, China). The identified peptides aligned with the predicted amino acid sequence of PfHMGB2 with high scores. Moreover, the identified peptides showed no identity with the *E. coli* protein. Mass spectrometry analysis also indicated that there are acetyl (protein N-term) and oxidation (Met) modifications in rPfHMGB2. Therefore, both bands are rPfHMGB2 and may have different posttranslational modifications. This phenomenon has been observed in some preparations of recombinant HMGB1 [25].

Purified rPfHMGB2 was incubated with dsDNA fragments to detect its DNA-affinity activity with BSA and PBS as controls. The protein-DNA complexes were subjected to electrophoresis. An obvious retardant band was observed near the comb hole (Fig. 7). rPfHMGB2 retarded the migration of *PfHMGB2* DNA. In contrast, no retardation was detected by BSA or PBS.

3.6. Effects on the phagocytic abilities of PBL

The effects of rPfHMGB2 on the phagocytosis of PBL was analysed by plate counting. PBL were first incubated with PBS, FBS, or rPfHMGB2, and then infected with serum-opsonized *A. hydrophila*. Subsequent analysis showed that the number of bacteria engulfed by rPfHMGB2-treated PBL was the highest (Fig. 8). The phagocytic activity of PBL was enhanced by rPfHMGB2.

3.7. Effects on the proliferation of PBL

The effects of rPfHMGB2 on the cell proliferation/cytotoxicity of PBL were determined using a CCK-8 kit. The results showed that rPfHMGB2 obviously enhanced the proliferation of PBL compared with PBS and FBS (Fig. 9).

3.8. Effects on expression of immune-related genes in PBL

qPCR was used to study the effects of rPfHMGB2 on the mRNA expression of immune-related genes, including TNFα, IL-1β and MAPK. TNFα mRNA in PBL was significantly increased after stimulation with rPfHMGB2 (Fig. 10). However, the IL-1β and MAPK mRNAs were not affected.

4. Discussion

As a chromatin-associated non-structure protein, HMGB2 not only regulates gene expression by interacting with transcriptional activators/repressors but also the innate immune response by binding to immunogenic nucleic acid [26]. In this study, a PfHMGB2 was identified from yellow catfish, which protects the yellow catfish from bacterial infection.

4.1. PfHMGB2 was a member of the HMGB2 subfamily

The cloned *PfHMGB2* cDNA sequence was an unstable sequence, as it contained two “AUUUA” motifs in its 3′-UTR; A/U-rich elements (adenylate uridylate-rich element, ARE) in the 3′-UTR are destabilizing sequences that interact with *trans*-acting actors, such as RNA binding proteins, to modulate its own mRNA instability for its precise regulation and transient expression [27]. mRNA instability is an important post-transcriptional modulation of inflammatory and immune-associated genes. In fact, most inflammatory and immune-associated gene mRNAs are unstable in eukaryotes [27,28].

The PfHMGB2 amino acid sequence shared high identity with that from teleosts. It even showed 80% identity with that from *H. sapiens*, indicating the extreme conservation in the evolution of HMGB2 protein. The PfHMGB2 amino acid sequence showed the highest identity (90%) with HMGB2 from *I. punctatus*, suggesting their evolutionary relationship [19,20]. Moreover, HMGB2 and HMGB1 were grouped together in the phylogenetic tree versus HMGB3 (Fig. 2). Sharma et al. (1997) indicated that HMGB1 and HMGB2 split from the HMGB protein [29].

4.2. Expression of PfHMGB2 mRNA was induced by bacteria and LPS

Different from the restricted expression pattern of HMGB2 in mice [12], *PfHMGB2* mRNA is constitutively distributed in all of the tested tissues in yellow catfish, as is also observed in *C. idella* [15], *C.*

semilaevis [14], and *S. schlegelii* [16]. The ubiquitous expression of HMGB2 hinted to its diverse functions in fish physiological processes.

The highest and lowest *PfHMGB2* mRNA expression was observed in the liver and head kidney, respectively. Its expression increased significantly in both tissues at early stages post-bacterial challenge. This rapid response suggests its important antibacterial effects in immune tissues [14–17]. However, the heightened levels of *PfHMGB2* mRNA were not very high, probably due to its high abundance under normal conditions. It is almost comparable to the housekeeping gene 18S rRNA in qPCR analysis (data not shown). The zebrafish *hmgb2a/hmgb2b* genes are also highly expressed [13]. Additionally, the *PfHMGB2* mRNA responded in a dynamic manner post-challenge, which may be related to its own mRNA instability and post-transcriptional regulation.

Inactivated *A. hydrophila* can induce *the* expression of *PfHMGB2* in the liver and head kidney; moreover, LPS (the PAMPs of G negative bacteria) can also upregulate *PfHMGB2* expression in PBL (Fig. 5). PBL are the important blood immune cells that consist of lymphocytes, monocytes, neutrophils, eosinophils, and basophile granulocytes. HMGB2 and HMGB1 are abundant in the immune cells, especially in monocytes [30,31]. Thus, the upregulation of *PfHMGB2* mRNA in PBL may be necessary for defending the body from infection. In *Ctenopharyngodon idella* kidney cells, *CiHMGB2a* and *CiHMGB2b* mRNA levels are also significantly induced by LPS [15]. In *Lampetra japonica*, *Lj-HMGB2* mRNA was particularly upregulated in the intestines after challenge with LPS [17].

4.3. rPfHMGB2 protein regulated PBL to participate in host immune response

HMGB2, like HMGB1, is located in the nucleus under normal conditions. When released into the extracellular milieu, HMGB2 can trigger inflammatory responses as an endogenous danger signal [32,33]. However, little is known about its mechanism of action. Thus, rPfHMGB2 protein was overexpressed and purified. Purified protein can retard the migration of DNA, which confirms its DNA-binding affinity. In fact, the two HMG boxes of HMGB2 protein can bend DNA and prefer to bind distorted, single-strand, and supercoiled DNA. They interact with the small groove, unwinding and binding to the small groove in DNA [34]. The C-terminal acidic tail in the HMGB2 molecule is also necessary for inducing higher affinity to the peculiar distorted structures [35].

Although teleost HMGB2 plays an important role in defending the body from bacterial infection [14,16], and purified HMGB2 from the human intestinal tract can directly inhibit *E. coli* [18], no direct inhibitory activity was found in rPfHMGB2 (data not shown). However, rPfHMGB2 may play a role via the regulation of immune cells from yellow catfish.

When yellow catfish PBL were treated with endotoxin-free rPfHMGB2, their phagocytic abilities were obviously enhanced. Neutrophil granulocytes and monocytes in PBL are the main phagocytic cells that eliminate bacteria [36]; thus, they were potentially activated by rPfHMGB2. Gu et al. (2018) indicated that knockdown of HMGB1 can affect phagocytosis in recombinant *Clostridium difficile* TcdB-treated CT26 cells [37].

rPfHMGB2 not only enhanced the phagocytosis of PBL but also the proliferation. Therefore, we speculated that activated phagocytosis in PBL may be associated with enhanced cell proliferation. Suzuki et al. (2009) showed that HMGB2 is associated with cell proliferation in hepatocellular carcinogenesis in rats and humans. Additionally, knockdown of HMGB2 expression resulted in inhibition of rat hepatocellular carcinoma cell growth [38]. Pusterla et al. (2009) revealed that HMGB2 secreted by THP-1 cells can promote the proliferation and migration of endothelial cells [39]. The TLR4-dependent JNK and PI3K/Akt signal pathways are involved in HMGB1-induced proliferation of hepatic stellate cells [40]. Thus, rPfHMGB2 may have immunostimulatory properties.

qPCR showed that rPfHMGB2 also induced TNFα mRNA expression in PBL. TNFα is an important pro-inflammatory cytokine in immune cells. Thus, HMGB2, similar to HMGB1, may trigger inflammatory immune responses as a cytokine. For example, both HMGB1 and HMGB2 from *Plasmodium falciparum* can induce the pro-inflammatory cytokine TNFα in mouse peritoneal macrophages [41]. Disruption of the parasite *hmgb2* gene can attenuate inflammatory responses by *plasmodium berghei* [32]. In addition, rLj-HMGB2 can induce the generation of TNFα in an activated human acute monocytic leukaemia cell line [17]. However, the TNFα released from PBL after rPfHMGB2 challenge could not be detected due to the lack of specific antibodies. In addition to its role in the regulation of inflammation, TNFα can also regulate cell growth or apoptosis as a cell signalling protein. It binds to its receptor, TNFR, to accelerate cell growth or induce apoptosis by forming different complexes. Complex I formation will activate NF-κB to inhibit cell apoptosis and promote cell survival. Complex II formation will activate caspase family proteases to induce cell apoptosis [42]. Further studies are required to confirm whether the effects of rPfHMGB2 on cell proliferation result from TNFα regulation.

5. Conclusion

A HMGB2 homologue, PfHMGB2, was cloned and characterized from yellow catfish. The expression of PfHMGB2 could be induced by bacteria and LPS. The purified rPfHMGB2 protein possessed DNA-binding abilities but no direct antibacterial activities. However, rPfHMGB2 could activate phagocytosis, enhance cell proliferation, and induce TNFα expression in yellow catfish PBL. These results suggest that PfHMGB2, as a cytokine, regulates immune responses in PBL and protects yellow catfish from bacterial infection.

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