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Short communication

BNIP3, a cell pro-apoptotic protein, involved in response to viral infection in orange spotted grouper, *Epinephelus coioides*

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ABSTRACT

BNIP3 is a kind of BH3-only protein that induces both cell death and autophagy. Here, a BNIP3 gene (EcBNIP3) was identified from orange spotted grouper, *Epinephelus coioides*. EcBNIP3 possessed 236 amino acids residues, contained a conservative BNIP3 domain and a transmembrane region. Besides, EcBNIP3 expressed at a relative high level in heart and spleen. EcBNIP3 transcript was up-regulated after SGIV infection *in vitro*. Subcellular localization analysis revealed that EcBNIP3 was predominantly localized in the cytoplasm and co-localized with mitochondria. In addition, overexpression EcBNIP3 accelerated SGIV infection induced cell death but inhibited viral genes transcription. Taken together, these results provided new evidence that fish BNIP3 might involved in response to virus infection.

1. Introduction

The B-cell lymphoma-2 (Bcl-2) family proteins act as crucial cell death regulators through controlling mitochondrial outer membrane permeabilization [1]. Bcl-2 family consisted of pro-apoptotic members and anti-apoptotic members. The pro-apoptotic Bcl-2 members are divided into multipledomain proteins (such as Bax and Bak) and BH3-only proteins (such as Bim and Bid). Bcl-2 E1B 19-KDa interacting protein 3 (BNIP3), an unique protein of BH3-only members that induces mitochondrial apoptosis as well as mitochondrial autophagy (mitophagy) [2]. Unlike other BH3-only proteins, BNIP3 can insert onto the mitochondrial outer membrane by their transmembrane domains independently of the formation of heterodimers with other BH1-4 multidomain Bcl2 proteins, and trigger mitochondrial permeablization and impair mitochondrial membrane potential [3,4]. To date, though the cellular function of BNIP3 during physiological or pathological processes have been well-documented in mammals, related information about fish BNIP3 remained largely unknown.

Groupers, *Epinephelus* spp. an important fish species widely farmed in China and Southeast Asian counties. But in recent years, the outbreak of infectious viral disease caused by Singapore grouper iridovirus (SGIV) and nervous necrosis virus (NNV) has led to significant economic losses in grouper culture [5–7]. Although different forms of cell death were explored during SGIV and NNV infection in different cell lines [8–11], the data concerned the roles of teleost Bcl-2 proteins on viral infection was still limit. In the current study, a BNIP3 homolog (EcBNIP3) was isolated from orange spotted grouper, *Epinephelus coioides*. The tissue distribution and expression pattern following SGIV challenge of EcBNIP3 were measured. Additionally, its intracellular localization and possible effects on viral infection were also investigated. These results will expand our knowledge on the roles of fish Bcl-2 family proteins in response to virus infection.

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2. Materials and methods

2.1. Fish, cell lines and virus

Orange-spotted groupers, *E. coioides* (50–60 g) were purchased and kept in a laboratory recirculating seawater system. Grouper spleen cells (GS) and fathead minnow (FHM) epithelial cells were cultured in Leibovitz's L15 medium and M199 medium containing 10% fetal bovine serum (Invitrogen, USA) at 25 °C, respectively [12]. Propagation of SGIV was performed as described previously [13].

2.2. Cloning of EcBNIP3 and bio-informatic analysis

The primers used for RACE PCR were design according to the EST sequence from grouper spleen transcriptome [14], the EcBNIP3 cDNA were amplified and assembly of EcBNIP3 cDNA were performed as described previously [15]. The nucleotide and predicted amino acid sequences of EcBNIP3 (Accession No. KY321170) were analyzed using Genetyx7.0 software. The similarities of EcBNIP3 with other BNIP3 proteins were analyzed using the BLASTP search program at the NCBI (http://www.ncbi.nlm.nih.gov/blast). Multiple-sequence alignment of the reported BNIP3 amino acid sequences was performed using Clustal X 2.0 and a phylogenetic tree was constructed using the MEGA 5.0 software.

Table 1
Sequences of primers used in this study.

Primers	Sequences (5′-3′)
EcBNIP3′GSP1	CAAATAAACGCAAATGCTGGCATGAGT
EcBNIP3′GSP2	AACTCTGCAGA CCTTGGAAGTGGTG
EcBNIP 5′NGSP1	GTCCTCCGATAAGGGTTTCATCACTCT
EcBNIP 5′NGSP2	AGAGTTTGACTCATGCCAGCATTTGCG
GFP-EcBNIP-F	CCGCTCGAGCT ATGTCCACCGCTGCTGCTCAACA
GFP-EcBNIP-R	CCCAAGCTT GTAAGAGCTGGTGGAGGCTGTGG
RT-EcBNIP-F	ATGAACAAAGAAGTAGATTGGGTCG
RT-EcBNIP-R	GTGAGATGAGTAAGGAAGGGATGA
RT-18S-F	ATTGACGGAAGGGCACCACCAG
RT-18S-R	TCGCTCCACCAACTAAGAACGG
RT-Actin-F	TACGAGCTGCCTGACGGACA
RT-Actin-R	GGCTGTGATCTCCTTCTGCA
RT-ORF115-F	CGGAAAGAACACAGATAACGG
RT-ORF115-R	AAAAAACACATGGCTTGCAAA
RT-ORF072-F	GCACGCTTCTCTCACCTTCA
RT-ORF072-R	AACGGCAACGGGAGCACTA
RT-ORF136-F	GATGCTGCCGTGTGAACTG
RT-ORF136-R	GCACATCCTTGGTGGTGTTG
RT-ORF086-F	ATCGGATCTACGTGGTTGG
RT-ORF086-R	CCGTCGTCGGTGTCTATTC

2.3. Tissue distribution of EcBNIP3

Total RNA was isolated from liver, spleen, head kidney, brain, intestine, heart, skin and muscle from healthy fish using TRIzol Reagent (Invitrogen, USA) according to manufacturer's instruction. Expression level of EcBNIP3 in various tissues was analyzed by qRT-PCR using RT primers listed in Table 1. The PCR conditions were applied as follow: 94 °C for 5 min, followed by 40 cycles of 5s at 94 °C, 10 s at 58 °C and 15 s at 72 °C. 18S was used as control with primers RT-18S-F and RT-18S -R (Table 1).

2.4. Expression profile of EcBNIP3 after SGIV infection in vitro

GS cells were grown in L15 medium containing 10% fetal bovine serum (Invitrogen, USA) at 25 °C. For virus challenge *in vitro*, GS cells were infected with SGIV at a multiplicity of infection (MOI) of 1. The cells were harvested at 0, 4, 6, 12, 24 and 48 h post infection for further analysis. The qRT-PCR analysis was performed as section 2.3.

2.5. Subcellular localization of EcBNIP3

To elucidate the potential function of EcBNIP3 *in vitro*, the full length of EcBNIP3 was cloned into pEGFP-C1 (the used primers were shown in Table 1) and confirmed by DNA sequencing. To demonstrate the intracellular localization of EcBNIP3 *in vitro*, pEGFP-C1 or pEGFP-EcBNIP3 and pDsRED2-Mito (Takara, JAPAN) were co-transfected into GS cells using Lipofectamine 2000

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ACGCGGGATGTTGATGAATGCTAAAACTGGGTAGTCAACATGTCCACCGCTGCTGCTCAACAAAACAATAACGAAGAGCCCGGGCTTCACGGCTCCTGGG
                                        M  S  T  A  A  A  Q  Q  N  N  N  E  E  P  G  L  H  G  S  W  V

TAGAGCTGGAGCTGAATGGGAACACAGGGGCTCAGGGCGTGCAGACCAACCAGCCAATGACACCCGCTGCAGACCAAATAAACGCAAATGCTGGCATGAG
  E  L  E  L  N  G  N  T  G  A  Q  G  V  Q  T  N  Q  P  M  T  P  A  A  D  Q  I  N  A  N  A  G  M  S

TCAAACTCTGCAGACCTTGGAAGTGGTGCCTGAGAGTGATGAAACCCTTATCGGAGGACTAGAGCATGTGCCGTCGTCCTCCTCTATTCACAATGGAGAC
  Q  T  L  Q  T  L  E  V  V  P  E  S  D  E  T  L  I  G  G  L  E  H  V  P  S  S  S  S  I  H  N  G  D

ATGGAAAAGATCCTCTTGGATGCACAGCATGAATCCAGCCAGAGTAGCTCCTCTTGTGATAGTCCTCACAGGCCACCAAGTCCAGATCAGGACGAGGGTC
  M  E  K  I  L  L  D  A  Q  H  E  S  S  Q  S  S  S  S  C  D  S  P  H  R  P  P  S  P  D  Q  D  E  G  Q

AGATAACTTTTGATGTGGAGATGTCCAGCCCAAGAGATAGTCAGTCAGAGGAAGATGGTCCAGAGAAGGACAGAGAGGAGGACATCCTGATGAACAAAGA
  I  T  F  D  V  E  M  S  S  P  R  D  S  Q  S  E  E  D  G  P  E  K  D  R  E  E  D  I  L  M  N  K  E

AGTAGATTGGGTCGcTGATTGGTCCAGCAGACCGGAAAACGTTCCACCAAAGGAGTTCCACTTCAGGCACCCCCGACGTTCAGTATCTCTCAGTATGAGA
  V  D  W  V  A  D  W  S  S  R  P  E  N  V  P  P  K  E  F  H  F  R  H  P  R  R  S  V  S  L  S  M  R

AAGAGTGGAGTCATGAAGAAAGGCGGCATCTTCTCTGCTGAATTCCTCAAAGTGTTCATCCCTTCCTTACTCATCTCACACATCCTTGCTCTTGGCCTCG
  K  S  G  V  M  K  K  G  G  I  F  S  A  E  F  L  K  V  F  I  P  S  L  L  I  S  H  I  L  A  L  G  L  G

GGGTGTACATTGGGAAGAGAATTGCCACAGCCTCCACCAGCTCTTACTGAATAGAAGACGAAGCAGTGCCTGGATGTTCTCCTGTCTCCAAGTCTCTCAA
  V  Y  I  G  K  R  I  A  T  A  S  T  S  S  Y  *

CCATCCTCCTGTAGTCAAGCTGTCTCTCTGCATCTATCGTACGCCCACATTTCCCCTCCAATCAGCATTAAATTCTGTCACATTGTAGATGTAGTTATAG

AGTTATACACATGACGTAATGTGGTTGCATTCCATCACATTTTGGCACAGGGGCAACGTTGTACCCAGCAGCTGCCACCGACTTGAATCCAGTATATGAC

TGACCCTGTTAGAGCTGCAAAATATTAGTCATCCCGTTCAACTCAAAAAAAAAAAAAAAAAAAAAA
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Fig. 1. The nucleotide and deduced amino acid sequences of EcBNIP3. The predicted BNIP3 domains was underlined and marked as respective domain. The putative transmembrane (TM) region indicated in shade.