Gene 557 (2015) 172–181

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Molecular cloning, characterisation and expression analysis of melanoma differentiation associated gene 5 (MDA5) of green chromide, *Etroplus suratensis*

Aadil Bhat, Anutosh Paria, A. Deepika, K. Sreedharan, M. Makesh, Megha K. Bedekar, C.S. Purushothaman, K.V. Rajendran *

Central Institute of Fisheries Education, Off-Yari Road, Versova, Andheri (W), Mumbai 400061, India

ARTICLE INFO

Article history:
Received 25 October 2014
Received in revised form 11 December 2014
Accepted 13 December 2014
Available online 16 December 2014

Keywords:
MDA5
IPS-1
Etroplus suratensis
RLR
Gene expression
Poly I:C

ABSTRACT

Innate immune system recognises pathogen-associated molecular patterns (PAMPs) by limited number of germline encoded and non-clonally developed pathogen recognition receptors (PRRs). Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) are important cytosolic PRRs for sensing viral RNAs. The receptor encoded by melanoma differentiation associated gene 5 (MDA5), an RLR, recognises viral RNA and enhances antiviral response in host cells. The full-length MDA5 cDNA in *Etroplus suratensis* was cloned and found to have 3673 nucleotides encoding a polypeptide of 978 amino acids. The deduced amino acid sequence contains four main structural domains: two CARD domains in the N-terminal region, a DExDc (DEAH/DEAD box helicase domain), HELICc (C-terminal helicase) domain and a C-terminal regulatory domain (RD). Phylogenetic analysis revealed a close relationship of *E. suratensis* MDA5 (*Es*MDA5) with MDA5 of *Neolamprologus brichardi* and *Oreochromis niloticus*, both belonging to Cichlidae family. *Es*MDA5 transcripts were ubiquitously expressed in all the 12 tissues tested in healthy fish. Although, transcript level was found to be the highest in muscle, high expression was also detected in the spleen, head kidney and hindgut. In poly I:C-injected fish, *Es*MDA5 transcripts showed peak expression in the spleen, intestine and heart at 12 h post-injection (hpi). However, in gill and kidney tissues, maximum up-regulation of *Es*MDA5 was observed at 6 and 48 hpi, respectively. Further, liver tissue showed an increasing trend in expression profile from 6 to 48 hpi. Interferon promoter stimulator-1 (IPS-1) gene, an adaptor triggering RIG-I- and MDA5-mediated type I interferon induction, also showed up-regulated expression at initial time-points in poly I:C-injected *E. suratensis*. The constitutive expression and up-regulation of *Es*MDA5 and the IPS-1 genes in different tissues indicate that *Es*MDA5 may play an important role in sensing viral PAMPs in conjunction with IPS-1.

1. Introduction

Innate immune system is a fundamental defence mechanism in lower vertebrates because of less diversification of adaptive immune system in these groups (Hikima et al., 2011; Star et al., 2011; Zhu et al., 2013). In addition, it plays a crucial role in the activation of adaptive immune system through an array of germline encoded and nonclonal receptor proteins (Yoneyama et al., 2004; Kato et al., 2005, 2006; Holm et al., 2007). These are pattern recognition receptors (PRRs), which identify a vast number of highly conserved molecular components, specific to microbes, known as pathogen associated molecular patterns (PAMPs) (Pichlmair and Reis e Sousa, 2007; Kawai and Akira, 2009). PAMPs are crucial polysaccharides, glycans, bacterial DNA and viral RNA necessary for the survival of the microorganisms and, therefore, any alteration in these molecules will be detrimental to the microorganisms (Pichlmair and Reis e Sousa, 2007). This recognition results in the expression and up-regulation of many pro-inflammatory

Abbreviations: ALRs, absent in melanoma 2 (AIM2)-like receptors; ANOVA, analysis of variance; BLAST, basic local alignment search tool; CARD, caspase activation and recruitment domain; CCO, channel catfish ovarian cells; DDX58, DEAD (Asp-Glu-Ala-Asp) box polypeptide 58; DExH58, DExH (Asp-Glu-X-His) box polypeptide 58; GCRV, grass carp reovirus; HBSS, Hank's balanced salt solution; hpi, hour post-injection; IFIH1, interferon induced with helicase C domain 1; IPS-I, IFN-beta promoter stimulator-I; IRF, interferon regulatory factor; LGP2, laboratory of genetics and physiology 2; LYCK, large yellow croaker kidney cell line; MAVS, mitochondrial antiviral signalling protein; MDA5, melanoma differentiation associated gene 5; MEGA, molecular evolutionary genetics analysis; NCBI, National Center for Biotechnology Information; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NOD, nucleotide binding oligomerisation domain; ORF, open reading frame; PAMPs, pathogen associated molecular patterns; Poly I:C, polyinosinic:polycytidylic acid; PRRs, pathogen recognition receptors; RACE, rapid amplification of cDNA ends; RD, regulatory domain; ResIII, restriction enzyme III; RIG-I, retinoic acid-inducible gene I; RLR, retinoic acid inducible gene I like receptors; RTG-2, rainbow trout gonad 2; SMART, simple modular architecture research tool; TLRs, Toll-like receptors; UTR, un-translated region.

* Corresponding author.
E-mail address: rajendrankv@hotmail.com (K.V. Rajendran).

http://dx.doi.org/10.1016/j.gene.2014.12.028
0378-1119/

Table 1
List of primers used in the study.

Primers	Sequence (5′–3′)
Gene cloning	
MDA5-F1	AAGCGGTAAAACCAGAGTTGCA
MDA5-R1	TGAAGCAGTCAGGCCAAGTATC
MDA5-F2	GCCAGGGATCGTTTTCAC
MDA5-R2	GCAGCTTCGCCAGCTAAACT
MDA5-F3	AAGAACAGAAGGCTGATGAAGGA
MDA5-R3	CTGCGACGTGTTTTGGTGAA
MDA5-F4	CCGAAGTTGAGGCAGAGAATG
MDA5-R4	CCTGCTGTCCAGATGTTTTCTG
IPS-F1	ACGGCGGAATATGGCAATC
IPS-R1	CAGATGACCGGCAGATGGT
RACE PCR	
MDA5-5′R1	AAGAAGCTCACGGCTCCATTCCTG
MDA5-5′NR1	TTGGGAGACAGCACCTCAATAAGCC
MDA5-3′F1	ACTCTGGGCTGGCTGAAAAGGAGT
MDA5-3′NF1	TCCGTCTAATCTGAAGTTTAGCTGCC
UPM-long	CTAATACGACTCACTATAGGGCAAGCAG TGGTATCAACGCAGAGTCTAAT
UPM-short	ACGACTCACTATAGGGC
NUP	AAGCAGTGGTATCAACGCAGAGT
Real-time PCR	
*Es*EF1α-F	CTGTACCCGTCGGTCGTGTT
*Es*EF1α-R	GGTGCATCTCCACGGACTTC
*Es*MDA5-qF1	TGAGGTGCTGTCTCCCAATCT
*Es*MDA5-qR1	CGGCTTCAATCCTGTCTTTGTC
*Es*IPS-qF1	GCTCACGACAGGGATGTTATTG
*Es*IPS-qR1	CTGCTCACACGCCTCAAGTG

cytokines and antiviral molecules in the early phase of infection (Kawai and Akira, 2006; Wilkins and Gale, 2010).

Based on the function and localisation, PRRs are categorised in three groups; soluble PRRs, endocytic PRRs and signalling PRRs (Jeannin et al., 2008; Faure and Rabourdin-Combe, 2011). Among these three types, signalling PRRs are one of the main components of the innate immune system in vertebrates. Signalling PRRs including membrane-bound Toll-like receptors (TLRs) and the cytosolic nucleotide oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and absent in melanoma 2 (AIM2)-like receptors (ALRs), are involved in cell activation (Akira et al., 2006; Meylan et al., 2006; Chen et al., 2009; Franchi et al., 2010; Jones et al., 2010; Hansen et al., 2011).

RLRs, initially identified by transcriptional profiling, belongs to superfamily 2 (SF2) helicases/ATPases (Lu et al., 2009). RLRs are key cytoplasmic sensors that specifically recognise intracellular viral nucleic acid and trigger the signalling cascades that result in the expression of type I interferons (Takeuchi and Akira, 2008; Zou et al., 2009). Three genes encode RIG-I-like receptors: retinoic acid-inducible gene I (RIG-I) also known as DDX58, melanoma differentiation associated gene 5 (MDA5) also known as helicard or IFIH1, and laboratory of genetics physiology 2 (LGP2), also known as DExH58 (Sun, 1997; Cui et al., 2001; Kang et al., 2002). RLR family shares some uniform structural domains, including a C-terminal regulatory domain (RD), a central DExD/H box helicase, and two N-terminal caspase activation and recruitment domains (CARDs), but LGP2 lacks CARD (Yoneyama et al., 2004, 2005; Holm et al., 2007; Venkataraman et al., 2007; Yoneyama and Fujita, 2007, 2008; Zou et al., 2009). RD is involved in signalling repression, nucleic acid recognition and protein dimerization (Saito et al., 2007; Cui et al., 2008). The central helicase domain is used in translocation, ATP hydrolysis and RNA binding (Yoneyama et al., 2005). The binding of CARD domains of RLRs and mitochondrial antiviral signalling protein (MAVS) triggers interferon response through the phosphorylation of interferon regulatory factors (IRF) 3 and 7 (Kawai et al., 2005; Xu et al., 2005; Satoh et al., 2010). The absence of RIG-1 receptor due to gene loss in some fish genome or divergence into a non-recognisable form (Zou et al., 2009) suggests that MDA5 plays a pivotal role in sensing of cytosolic viral RNA PAMPs in these fish species.

MDA5 is a double stranded RNA dependent helicase initially identified by Kang et al. (2002) through subtraction hybridisation. Due to the presence of helicase and CARD, it plays a vital role in growth, differentiation and apoptosis. MDA5 recognises long dsRNA (>1 kb) and single stranded molecules with 5′ triphosphate, and induces the secretion of type I IFN. In fishes, MDA5 was first cloned and characterised in grass carp (Su et al., 2010). Later, it has been characterised in Atlantic salmon (Sun et al., 2009), rainbow trout (Chang et al., 2011), Japanese flounder (Ohtani et al., 2011), goldfish (Sun et al., 2011), channel catfish (Rajendran et al., 2012), and orange spotted grouper (Accession number; HQ880665).

Etroplus suratensis, an important food and aquarium fish, and closely related to Nile tilapia, has not been a subject of study to identify any of the pathogen recognition receptors. In the present study, we have cloned and sequenced a full-length cDNA sequence of MDA5 gene from *E. suratensis* (*Es*MDA5). We have also studied the normal expression profile in the different tissues of healthy animals and estimated the expression in fishes induced with poly I:C. Further, we have investigated the expression of an adaptor triggering RIG-I- and MDA5-mediated type I interferon induction, interferon promoter stimulator-1 (IPS-1) after poly I:C-induction to get an idea about the functional role played by this gene in the non-specific immune system of cichlids.

2. Materials and methods

2.1. Fish and experimental conditions

Fish (average body weight — 20 g) were procured from a fish farm in Kerala, India. The fishes were reared in tanks with water salinity 5‰ maintained at ambient temperature of 25–30 °C and fed with commercial feed. After acclimatisation, fishes were intramuscularly injected with 100 μL (100 μg/fish) poly I:C (Sigma, USA), suspended in Hank's Balanced Salt Solution (HBSS) and the control animals were injected with 100 μL HBSS. Sampling was done at 6, 12, 24, 48 and 72 h post-injection (hpi) from both control and experimental groups. Six fishes were sampled at each time-point and tissues such as the gill, heart, spleen, kidney, liver and intestine were collected. Tissues pooled from two animals (3 pools at each time-point) were used in the expression analysis. To study the normal expression of MDA5 and IPS-1 in different tissues of healthy *E. suratensis*, nine animals were sampled and 12 different tissues such as the brain, gill, heart, intestine, muscle, spleen, liver, stomach, trunk kidney, head kidney, midgut and hindgut were collected. Tissues from nine fish were pooled and three such pools were used for RNA extraction and subsequently for PCR amplification.

2.2. RNA extraction and cDNA synthesis

Total RNA was isolated from the different tissues of healthy and experimental animals using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. Two micrograms of total RNA was treated with DNase I (Thermo Scientific, USA) to remove the genomic DNA. One microgram of DNase-treated RNA was reverse-transcribed using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA), primed with OligodT primer.

2.3. Amplification and full-length cloning of E. suratensis MDA5 cDNA

For the amplification of *Es*MDA5 cDNA, a set of primers was designed based on the conserved regions of MDA5 gene sequences from *Maylandia zebra* (accession no. XP_004557958), *Haplochromis burtoni* (accession no. XP_005918275), *Oreochromis niloticus* (accession no. XP_003447455) and *Paralichthys olivaceous* (accession no. ADW78349), using the Primer Express software version 3.0. For PCR amplification, cDNA prepared from kidney tissue was used. Amplified PCR products were cloned into pTZ57R/T vector (Fermentas, USA) and sequenced using ABI Big DYE terminator method. From the obtained sequence, gene-specific primers for 5′ and 3′ RACE were designed and used for the amplification of 5′ and