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Sequence and expression analyses of porcine *ISG15* and *ISG43* genes

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ABSTRACT

The coding sequences of porcine interferon-stimulated gene 15 (*ISG15*) and the interferon-stimulated gene (*ISG43*) were cloned from swine spleen mRNA. The amino acid sequences deduced from porcine *ISG15* and *ISG43* genes coding sequence shared 24–75% and 29–83% similarity with ISG15s and ISG43s from other vertebrates, respectively. Structural analyses revealed that porcine ISG15 comprises two ubiquitin homologues motifs (UBQ) domain and a conserved C-terminal LRLRGG conjugating motif. Porcine ISG43 contains an ubiquitin-processing proteases-like domain. Phylogenetic analyses showed that porcine ISG15 and ISG43 were mostly related to rat ISG15 and cattle ISG43, respectively. Using quantitative real-time PCR assay, significant increased expression levels of porcine *ISG15* and *ISG43* genes were detected in porcine kidney endothelial cells (PK15) cells treated with poly I:C. We also observed the enhanced mRNA expression of three members of dsRNA pattern-recognition receptors (PRR), TLR3, DDX58 and IFIH1, which have been reported to act as critical receptors in inducing the mRNA expression of *ISG15* and *ISG43* genes. However, we did not detect any induced mRNA expression of *IFNα* and *IFNβ*, suggesting that transcriptional activations of *ISG15* and *ISG43* were mediated through IFN-independent signaling pathway in the poly I:C treated PK15 cells. Association analyses in a Landrace pig population revealed that *ISG15* c.347T>C (BstUI) polymorphism and the *ISG43* c.953T>G (BccI) polymorphism were significantly associated with hematological parameters and immune-related traits.

1. Introduction

Interferon-stimulated genes 15 (ISG15) encodes a 15 kDa ubiquitin-like protein which is transcriptionally activated by IFN treatment or virus infection (Korant et al., 1984; Martensen and Justesen, 2004). The protein can be conjugated to both IFN-induced and constitutively expressed proteins through a system similar to ubiquitylation, referred to as ISG15 conjugation (ISG15ylation). The process of ISG15ylation is involved in amplification of interferon signaling (Lu et al., 2006; Arimoto et al., 2008). The ISGylation process of ISG15 is a reversible enzymatic cascade that is controlled by a 43 kDa protease ISG43 (Malakhov et al., 2002), the only deconjugating protease with specificity for ISG15. Previous studies revealed that *ISG43* is strongly up-regulated by LPS stimulation, IFN treatment and virus infection (Malakhova et al., 2002; Afonso et al., 2004). Further, it was shown that ISG43$^{-/-}$ mice exhibit an enhancement in the level of protein ISGylation which is associated with the prolonged type I IFN signaling (Malakhova et al., 2003). Thus, the *ISG15* and *ISG43* genes have been suggested to play an important role in innate immunity.

The double-strand RNA (dsRNA) is a genetic material or an intermediate during the multiplication of most viruses (Huang et al., 2006). It has been considered to be an important trigger for host antiviral defenses (Long and Burke, 1971; Vilcek et al., 1968). In mammals, synthesis of IFN is through the recognition of the invading virus by pattern-recognition receptors (PRRs) (Seth et al., 2006). Soon after, the IFN and PRRs mediated signaling pathway dependently or independently activates the transcription of a subset of genes which are so-called interferon-stimulated genes (ISGs) (Andrejeva et al., 2004). Increasing studies confirmed that specific PRRs including Toll-like receptor 3 (TLR3), DExD/H RNA Helicases (including DEAD (Asp-Glu-Ala-Asp) box polypeptide 58 (DDX58) and interferon induced with helicase C domain 1 (IFIH1)) are implicated in the induction of *ISG15* and *ISG43* genes in the dsRNA treatment in a cell type-specific manner (Kato et al., 2006; Matsukura et al., 2007; Matsumoto and Seya, 2008). Despite utilizing the different adaptors, the two classes of PRRs converge at the same nodes, two kinases TANK binding kinase-1 (TBK1) and IκB kinase (IKK)-related kinases (IKKi) which are essential for transcriptional activation of *ISG15* and *ISG43* genes in a temporal and spatial manners (Ritchie and Zhang, 2004; Solis et al., 2007). Subsequently, the up-regulated *ISG15* and *ISG43* exert their antiviral activity through altering the function of host cells or viral proteins and ultimately facilitate virus clearance from host cells (Giannakopoulos et al., 2005; Zhao et al., 2005).

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Table 1
Primer pairs designed for amplifications of porcine *ISG15* and *ISG43* genes.

Primer	Primer sequences (5′–3′) (forward/reverse)	Primer location	Tm (°C)	Size (bp)
ISG15F1	GCTGGTGAAGGGAGGTG	5′-UTR		
ISG15R1	CAGACGCTGCTGGAAGG	Exon1	63	343
ISG15F2	ATGGGTAGGGAACTGAAGGTG	Exon1		
ISG15R2	AATTTTGCCACAGCTTTATTACTAG	3′-UTR	64	525
ISG43F1	GGAAGGCAGGTTGTGTGG	5′-UTR		
ISG43R1	CATCGTGCTGGACAAACAT	Exon3	54	333
ISG43F2	CTGAGGAGCAGAGGAGAAATGT	Exon2.3		
ISG43R2	GGAGAGTCGGTGACCAATAC	3′-UTR	58	1352

In this study, we report the characterization of cDNA sequences of porcine *ISG15* and *ISG43* genes. To better understand the role of *ISG15* and *ISG43* in swine antiviral system, we investigated the induced expression levels of the *ISG15* and *ISG43* mRNA by the synthetic dsRNA analog polyinosinic acid–polycytidylic acid (poly I:C) in PK15 cells. The results indicated that the transcriptional activation of *ISG15* and *ISG43* genes were mediated through of IFN-independent signaling pathway in the poly I:C treated PK15 cells. We also performed association analyses between the polymorphisms identified in the two genes and the immune-related traits in a Landrace pig (*Sus scrofa*) population.

2. Materials and methods

2.1. Isolating of the coding sequence of the porcine ISG15 and ISG43 genes

The cDNAs of human *ISG15* and *ISG43* genes (GenBank accession nos. NM_005101 and NM_017414) were used as queries to search homologous pig (*S. scrofa*) expressed sequence tags (ESTs) available in the TIGR porcine EST database (http://www.tigr.org/tdb/tgi/), respectively. The entire coding regions of *ISG15* and *ISG43* were obtained respectively by PCR with four pairs of primers (ISG15F1–R1; ISG15F2–R2; ISG43F1–R1; ISG43F2–R2) (listed in Table 1) designed based on the pig EST contigs retrieved. Total RNA was extracted from spleen tissue of an adult Chinese indigenous Tongcheng pig (Hubei Province, China) with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RT-PCR was performed using Taq polymerase (Fermentas, Vilnius, Lithuania), M-MLV reverse transcriptase (Promega, Madison, WI, USA). PCR-amplified fragments were cloned into the pGEM-T vector (TaKaRa Bio Inc., Otsu, Japan) and sequenced commercially.

The molecular weight (MW) determination was carried out using the program Protparam (http://ca.expasy.org/tools/protparam.html). Prediction of potential biologically significant sites was performed by PROSITE (http://www.expasy.org/prosite) in the Expert Protein Analysis System (ExPASy) proteomics server of the Swiss Institute of Bioinformatics (SIB). The alignment of the amino acid sequences of porcine *ISG15* and *ISG43* genes were performed based on the amino acid sequences of the prepro-forms using the program of ClustalW (http://www.ebi.ac.uk/clustalw/).

2.2. Phylogenetic analysis

Amino acid sequences of ISG15s and ISG43s in vertebrate were downloaded from the Genbank database and aligned using the multiple sequence alignment feature of the Clustal X program (Shin et al., 2007). The exported alignment file was imported into the MEGA 4.1 program (Tamura et al., 2007) to reconstruct molecular phylogenetic trees with the maximum-parsimony method (Kim et al., 2009). A phylogenetic tree containing all available ISG15 sequences was constructed with human FAT10 as an outgroup, and phylogenetic tree of ISG43 sequences was constructed with goldfish ISG43 protein as an evolutionary outgroup. Maximum-parsimony (MP) analysis was carried out with tree search options of close-neighbor interchange (CNI) (search level = 1) with an initial tree by random addition (101 replicates). For MP analyses, gap and missing data were completely deleted. Robustness of tree topology was estimated by bootstrap analyses with 1000 replicates.

2.3. In vitro treatment of pig kidney cells with poly I:C

PK15 cells, a cell line derived from pig kidney epithelial cells, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum. Cells were incubated at 37 °C with 5% humidified CO_2.

Cells were seeded in 6-well cell culture plates at a density of 5×10^6 cells per well. The stock solution of poly I:C (Sigma, St. Louis, MO, USA) was made up to 2 mg/mL in sterilized phosphate buffered saline (PBS). The cells were treated with poly I:C to a final concentration of 20 μg/mL and harvested at 0, 6, 12, 24, and 48 h post-stimulation. All time points were carried out in three independent experiments, each performed in duplicates. Non-adherent cells were removed by a single wash in PBS.

Total cellular RNA was extracted using the TRIzol reagent kit (Invitrogen, Groningen, The Netherlands) according to the manufacturer's instructions and purified with TURBO DNA-freeTM Kit (Ambion, Austin, TX, USA). The cDNA was synthesized using M-MLV Reverse Transcriptase (Promega) and oligo-$(dT)_{12-18}$ (TaKaRa) and the RT-PCR were carried out as described by Yu et al. (2004).

2.4. Quantitative real-time PCR analysis for gene expression

For the evaluation of mRNA expression of *IFNα*, *IFNβ*, *TLR3*, *DDX58*, *IFIH1*, *IKKi*, *ISG15* and *ISG43* in cultured porcine kidney cells induced with poly I:C (20 μg/mL), semi-quantitative RT-PCR was firstly performed to detect the basal expression of each gene at 0, 6, 12, 24, and 48 h post-stimulation. The PCR reaction was performed in triplicate. The primers designed for RT-PCR are given in Table 2. The results showed that neither *IFNα* nor *IFNβ* gene was expressed even at a basal level. Therefore, expression levels of the rest of the six genes (*TLR3*, *DDX58 IFIH1*, *IKKi*, *ISG15* and *ISG43*) in response to the poly I:C stimulation were further detected by quantitative real-time PCR method. Quantitative real-time PCR was performed using ICycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA, USA) and using Core kit for SYBR (Toyobo) in a total volume of 25 μL. The ribosomal protein L32 gene (RPL32) was used for the internal control to normalize gene expression profiles. Relative gene expression was analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). RNA of the cells harvested at 0 h post-stimulation was used as a calibrator for relative quantification.

Table 2
Primer pairs designed for quantitative real-time PCR in this study.

Primer	Primer sequences (5′–3′)	Tm (°C)	Size (bp)
ISG15	GATGCTGGGAGGCAAGGA		
	CAGGATGCTCAGTGGGTCTCT	62	229
ISG43	CCAATGTTTGTCCAGCACGA		
	GGGCATCCTCCAGGGTTTT	63	241
TLR3	CATTGCCTGGTTTGTTAGTT		
	ATCAAAAAGAATCACTGGGAG	66	118
DDX58	GCCACAACACCAGCAAAC		
	CGAGGCAGTCAGTCCAAT	66	113
IFIH1	ACAGGCAACTCCTTAGC		
	CTCCTCCACACATTTATCC	66	168
IKKi	TCACCACCCCTTATGGCTACT		
	GGACCCTATTCAACCCTTC	66	142
IFNα[a]	AGAATCTCTCCCTTCTCCTG		
	GAGTCTGTCTTGCAGGTTTC	64	420
IFNβ	CGATACCAACAAAGGAGCAGC		
	ACGGTTTCATTCCAGCCAGT	64	230

[a] Primer sequences come from Jung and Chae (2006).