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A haplotype variant of porcine *IFIT2* increases poly(I:C)-induced activation of NF-κB and ISRE-binding factors

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Abstract

Interferon-induced protein with tetratricopeptide repeats (IFIT) 2 is associated with various viral infections and pathogenesis in humans and mice. However, there are few reports on IFIT2 in pigs and the polymorphic information remains unclear. Here, by using a direct PCR sequencing method, we identified four single nucleotide polymorphisms (SNPs), c.259G>A (p.Gly87Ser), c.520T>G (p.Phe174Val), c.571C>T (p.Pro191Ser), and c.879A>G (p.Glu293Glu), for the first time in the coding sequence of the porcine (p) *IFIT2* gene from a Chinese local breed (Hebao pig), Western commercial pig breeds (Yorkshire and Landrace), and a Chinese developed breed (Beijing Black pig). SNP c.520T>G (p.Phe174Val) leads to the addition of a tetratricopeptide repeat motif, characteristic structure of the IFIT family. SNPs c.259G>A and c.520T>G are medium polymorphic loci ($0.25 <$ polymorphic information content < 0.5) and distributed differently in Western pig breeds and the Chinese local pig, Hebao, which is well known for its strong resistance to disease. Additionally, they are completely linked. The four SNPs constituted five haplotypes with GTCA and AGCA as dominant. The haplotype variant AGCA, which is mainly present in Hebao pigs, significantly synergized the poly(I:C)-induced activation of transcription factors, including NF-κB and IFN-stimulated response element (ISRE)-binding factors, and the expression of interferon β, indicating that the variant contributes to the induction or magnitude of the immune response upon viral infection. The data showed that variant AGCA might be useful in improving the resistance of pigs to viruses through marker-assisted selection.

Keywords Pig · *IFIT2* · SNP · Haplotype · Role · Immune response

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Introduction

Upon infection with a virus, the host responds rapidly and effectively through activating the innate immune system, which comprises several molecules including pattern-recognition receptors (PRRs), interferons (IFNs), and numerous IFN-stimulated genes (ISGs). Upon recognizing viral pathogen-associated molecular patterns, such as double-stranded RNA (dsRNA) generated during virus replication in cells, PRRs first stimulate a signal cascade resulting in the activation and translocation of nuclear factor (NF)-κB and IFN regulatory factors (IRFs). Activated NF-κB and IRFs collaboratively induce the transcription of type I IFNs including IFNα and β [1], which leads to the formation and nuclear translocation of a transcription factor complex, IFN-stimulated gene factor 3 (ISGF3), through the Jak-STAT pathway. ISGF3 then triggers the transcription of ISGs by directly binding to an IFN-stimulated response element (ISRE), a *cis*-element present in the promoter of all ISGs [1–3].

ISGs, a big family encoding hundreds of antiviral effectors, play central roles in the antiviral innate immune response through the inhibition of viral replication, clearance of infected cells, and induction of an adaptive immune response [1, 4, 5]. Among the most prominent ISGs are IFN-induced proteins with tetratricopeptide repeats (IFITs). IFITs include *IFIT1, IFIT2, IFIT3, IFIT5*, several *IFIT*-like genes, and non-transcribed *IFIT*-related pseudogenes in mammals [6]. The encoded proteins are characterized by tetratricopeptide repeats (TPRs), degenerate helix-turn-helix motifs of 34 aa in length, which mediate a variety of protein–protein and protein–RNA interactions and thus TPR-containing proteins are involved in various cellular regulations [7].

Studies have shown that IFITs play critical roles in viral infection and pathogenesis in humans and mice. They are not transcribed under basal conditions but are induced immediately at high levels after virus infection in many cells [8]. IFITs recognize viral components directly [9–11] or through the formation of multiprotein complexes with the translation initiation factor, eIF3, to block viral infection [12]. IFIT members also enhance IFNα effector signaling and could be used as a biomarker predicting the response to IFNα therapy in hepatocellular carcinoma [13]. Additionally, there exists interaction among different family members [14, 15].

IFIT2 is a heavily studied family member and extensively involved in the antiviral response. It protects mice from neuropathogenesis caused by lethal vesicular stomatitis virus [16, 17], rabies virus [18], neurotropic hepatitis virus [19], and West Nile virus [20]. Studies in vitro and in vivo have revealed that IFIT2 exerts a protective effect against virus mainly at the level of viral replication. IFIT2$^{-/-}$ mice have higher mortality and virus load than wild-type mice after infection of the respiratory tract with Sendai virus [21]. Knockdown of IFIT2 results in markedly increased virus replication in cultured human cells infected with hepatitis B virus [22]. Moreover, the mRNA level of IFIT2 could be used to predict virus infection in the nasopharynx and thus contribute to an early and accurate diagnosis of respiratory infection [23].

The extensive antiviral roles of IFIT2 indicate that it has potential in breeding pigs resistant to viral infection. However, little is known about porcine (p) IFIT2. We have characterized the cDNA sequence and transcriptional regulation mechanism of the *pIFIT2* gene [24]. Here, we have focused on the screening and functional characterization of single nucleotide polymorphisms (SNPs) in the coding sequence (CDS) of *pIFIT2* and found that a haplotype variant, mainly present in the Chinese local breed, Hebao pigs, affects pIFIT2 function in cells treated with the synthetic dsRNA, poly(I:C), a widely used viral analog in scientific studies [25, 26]. The results will contribute to breeding pigs with high disease-resistance through molecular anti-disease breeding programs such as marker-assisted selection.

Materials and methods

Animals and samples

All pigs, which included a Chinese local breed (Hebao pig), Western commercial pig breeds (Yorkshire and Landrace), and a Chinese developed breed (Beijing Black pig), were obtained from the Institute of Animal Husbandry, Heilongjiang Academy of Agricultural Sciences, Harbin, China. Ear tissues were collected from 34 individuals including ten Hebao pigs, nine Beijing Black pigs, seven Yorkshire pigs, and eight Landrace pigs. The protocol of animal treatment was in accordance with the guidelines of the Ministry of Science and Technology of China [27] and approved by the Experimental Animal Management Committee of Northeast Agricultural University. Spleen tissues were collected from one Yorkshire pig as described previously [25].

Polymorphisms

According to *pIFIT2* mRNA we cloned previously (GenBank No. JX070559) and its intron 1 sequence identified using the blat program in the Genome Browser Database (http://genome.ucsc.edu), a pair of primers, PF (5′-tcctatttgttatgctccctg-3′) and PR (5′-atgttttgaataccaactcgg-3′) (F, forward; R, reverse, the same as below), spanning intron 1 through a 3′ untranslated region (UTR) was designed using Primer Premier 5.0 software and synthesized by Beijing Genomic Institutes (BGI; Beijing, China).

Genomic DNA was isolated from ear tissue using the normal phenol/chloroform method. PCR was performed in a final volume of 25 μl containing $1 \times Ex$ Taq Buffer, 200 μM of each dNTPs, 200 nM of each primer, 50 ng genomic DNA, and 1 U *Ex* Taq DNA polymerase (Takara, Dalian, China). PCR conditions were as follows: 95 °C for 4 min, followed by 30 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 1.5 min, and 72 °C for 7 min.

SNPs were screened and genotyped by direct sequencing of the PCR products amplified from the ear tissue sample of Yorkshire, Landrace, Beijing Black, and Hebao pigs. The sequencing was conducted with an ABI 3730xl sequencer at the BGI (Beijing) with the same primer pair used in the PCR amplification. The reads obtained were assembled and aligned for detection of SNPs using the DNAMAN package (ver. 5.2.2). Manual inspection of sequence diagrams was performed to confirm SNPs and identify genotypes.

Haplotype analysis was performed with the PROC HAPLOTYPE procedure of SAS9.1 by using the Expectation–Maximization algorithm. Confidence intervals

and standard errors were estimated with default binomial assumption. Individual haplotypes estimated with probabilities < 99% were verified through sequencing the T–A clone, that is, the fragments were sub-cloned into the pMD18-T vector (Takara) and then sequenced. In each case, at least three sub-clones randomly selected were sequenced to assure both alleles of an individual were sequenced. Linkage disequilibrium analysis was conducted using the SHEsis program (http://analysis.bio-x.cn/myAnalysis.php).

Eukaryotic expression plasmid

Total RNA was extracted from spleen tissue with Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized from 1 μg of total RNA using a PrimeScript® RT reagent kit (Perfect Real Time) (Takara) and oligo(dT) primer. Complete CDS of *pIFIT2* was amplified as described above with 1 μl cDNA template and a primer pair (CF: 5′-ggatccatgagtgagaccacaaagaac-3′; CR: 5′-gctctagattattcctcagctaaagatact-3′. Underlined are *Bam*HI and *Xba*I recognition site, respectively.) designed according to the mRNA sequence of *pIFIT2* (GenBank No. JX070559). The PCR products were first cloned into a pMD18-T vector and sequenced for verification, and then inserted into pcDNA3.1+ (Invitrogen) using *Bam*HI and *Xba*I recognition sites to construct a eukaryotic plasmid expressing one dominant haplotype of *pIFIT2*.

Additionally, a complete CDS containing mutations at positions c.259 and c.520 simultaneously was obtained using overlap extension PCR as described previously [25]. The double mutations were introduced by two continuous steps. Briefly, we first obtained the fragment with one mutation at position c.259 using a *pIFIT2* cloning vector template, and then the resultant PCR products were used as a template to create the other mutation at position c.520. The primers used for site-directed mutation are as follows, M259F: 5′-cacgc**t**agccaggcagaaat-3′, M259R: 5′-tgcctggct**a**gcgtgttctc-3′, M520F: 5′-acccagaa**g**tcacctctggt-3′, M520R: 5′-ggtga**c**ttctgggtccttag-3′ (mismatched bases are bolded). The primer pairing and experiment scheme is shown in Table 1. After verification by sequencing the fragment containing the double mutations were ligated into pcDNA3.1+ (Invitrogen) to construct a eukaryotic plasmid expressing the other dominant haplotype of *pIFIT2*.

Table 1 Primer pairing scheme of overlap extension PCR

Steps	Reaction I	Reaction II	Splicing reaction*
First	CF/M259R	M259F/CR	CF/CR
Second	CF/M520R	M520F/CR	CF/CR

*Products of reactions I and II are spliced into a bigger one, which introduces the mutation into the inner of fragment

Luciferase activity analysis

PK-15 cells were cultured as described previously [28]. Each eukaryotic expression plasmid was cotransfected into PK-15 cells using Lipofectamine 2000 (Invitrogen) with a firefly luciferase reporter, pNF-κB-Luc (Stratagene, La Jolla, Canada) or pISRE-Luc (Stratagene, La Jolla, CA, USA). A *Renilla* luciferase reporter (pRL-TK) (Promega, Madison, WI), constitutively expressed in cells, was used as an inner control. At 24 h after transfection, the cells were treated with 5 μg/ml poly(I:C) (Invivogen, San Diego, CA) for another 12 h and then collected for luciferase activity analysis using a Dual-Glo Luciferase Assay System (Promega). Relative luciferase activity was calculated as a ratio of firefly to *Renilla* luciferase activity. A T test was used to compare the effects of the two haplotypes on activation of NF-κB or ISRE-binding factors induced by poly(I:C).

Quantitative real-time PCR

PK-15 cells were transiently transfected with one of the eukaryotic expression plasmids using Lipofectamine 2000 (Invitrogen) for 24 h, and treated with 5 μg/ml poly(I:C) (Invivogen) for another 12 h. Then the cells were collected for quantitative real-time PCR (qPCR) to measure the induction of endogenous IFNβ mRNA. qPCR was performed with a primer pair (F: 5′-cgataccaacaaaggagcag-3′; R: 5′-ggtttcattccagccagt-3′) as described previously [29]. Each sample was run in a total volume of 10 μl in triplicate with SYBR Green I detection (Takara). The $2^{-\Delta\Delta C_t}$ method [30] was used to calculate the relative expression level of IFNβ with β-actin (F: 5′-catcaccatcggcaacga-3′; R: 5′-gcgtagaggtccttcctgatgt-3′) as a reference. A T test was used to compare the effects of the two haplotypes on the induction of endogenous IFNβ.

Results

SNP identification

The *pIFIT2* gene is composed of two exons with the first one having a 5′ UTR and five nucleotides of CDS the same as its counterpart in humans. There are few reports on *IFIT2* in pigs and the polymorphic information remains unclear. Using primer pair PF/R, Specific products were obtained and can be directly sequenced for SNP identification as observed on 1.5% agarose gel electrophoresis. The fragment amplified is 1566 bp long containing 1402 bp of CDS and 154 bp of 3′ UTR. SNPs were identified among a total of 34 individuals including seven Yorkshire pigs, eight Landrace pigs, nine Beijing Black pigs, and ten Hebao pigs. Four SNPs, c.259G>A (p.Gly87Ser), c.520T>G (p.Phe174Val),

c.571C>T (p.Pro191Ser), and c.879A>G (p.Glu293Glu), were found in the CDS, among which three are non-synonymous SNPs resulting in amino acid (aa) substitution. SNP c.520T>G (p.Phe174Val) leads to the addition of a TPR motif, a characteristic structure of the IFIT family (Table 2). SNP c.571C>T (p.Pro191Ser) leads to the deletion of an HMG motif and alters the aa polarity. No SNPs were found in the amplified 3′ UTR.

TPR motifs have been involved in various physiological and pathological process through mediating protein–protein interactions and play important roles in maintaining protein function [7]. The number of TPR motifs is distinctive among IFIT family members but constant among species. There are six, three, four, and five TPRs in human IFIT1, two, three, and five, respectively [31]. We have found that both porcine IFIT2 and IFIT5, having three and five TPRs, respectively, contain the same TPR structures as their counterparts in humans [24, 29]. The addition of the TPR motif might affect the role of pIFIT2.

Genotyping and haplotype analysis

SNPs c.259G>A and c.520T>G showed a medium level of polymorphism (polymorphism information content (PIC) = 0.37) in all populations studied. Both of the SNPs have three genotypes in each of the populations studied. Additionally, dominant alleles at these two polymorphic sites are different among species. Alleles G and T at sites c.259 and c.520, respectively, are dominant in Yorkshire, Landrace, and Beijing Black individuals, while alleles A and C are dominant in Hebao pigs. The remaining SNPs, c.571C>T and c.879A>G, are present at a relatively low frequency ($PIC < 0.25$) and not detected in all breeds studied. SNP c.879A>G is not detected in Landrace and Hebao pigs; SNP c.571C>T is not detected in Beijing Black pigs, and no homozygote is found in Yorkshire pigs (Table 3).

We next performed haplotype analysis and found that the four SNPs constitute five haplotypes with GTCA and AGCA as dominant (Table 4). The proportion of these two haplotypes is 80.9% among all breeds studied. Haplotype AGCA mainly distributes in Hebao pigs with a frequency of 85%, while GTCA is dominant in other pig breeds studied with a frequency of 50%. Among ten Hebao pigs, there are 17 haplotypes composed of AGCA and eight individuals are homozygotes of this haplotype. Among Yorkshire, Landrace, and Chinese developed Beijing Black pigs, most individuals possess (14 out of 24) haplotype GTCA out of which ten homozygotes were found. The remaining three haplotypes, GTCG, AGCG, and GTTG, are not present in all breeds studied and the frequencies are very low. In particular, haplotype GTCG only exists in one individual of Beijing Black pigs in a homozygous manner, which might be indicative of the breeding origin. In all haplotypes, G and T (or A and G) at sites c.259 and c.520, respectively, are always present together, meaning that

Table 2 Missense SNPs identified

SNP	Amino acid	Motif	Amino acid polarity
c.259G>A	p.Gly87Ser		Polar/polar
c.520T>G	p.Phe174Val	Additon of a TPR motif	Nonpopular/nonpopular
c.571C>T	p.Pro191Ser	Deletion of a HMG motif	Nonpolar/polar

Table 3 Allele distribution among populations studied

Sites	Genotype	Genotype frequency %				Allele	Allele frequency %				Total	PIC
		Y (7)	L (8)	B (9)	H (10)		Y (7)	L (8)	B (9)	H (10)		
c.259	GG	42.9	50.0	55.6	10.0	G	57.2	62.5	61.2	15.0	47.06	0.37
	GA	28.6	25.0	11.1	10.0							
	AA	28.6	25.0	33.3	80.0	A	42.8	37.5	38.8	85.0	52.94	
c.520	TT	42.9	50.0	55.6	10.0	T	57.2	62.5	61.2	15.0	47.06	0.37
	TG	28.6	25.0	11.1	10.0							
	GG	28.6	25.0	33.3	80.0	G	42.8	37.5	38.8	85.0	52.94	
c.571	CC	85.7	87.5	100	90.0	C	85.7	93.8	100	95.0	94.1	0.10
	CT	0	12.5	0	10.0							
	TT	14.3	0	0	0	T	14.3	6.2	0	5.0	5.9	
c.879	AA	71.4	100	55.6	100	A	85.7	100	66.7	100	88.2	0.19
	AG	28.6	0	22.2	0							
	GG	0	0	22.2	0	G	14.3	0	33.3	0	11.8	

Breed abbreviations as follows: *Y* Yorkshire pig, *L* Landrace pig, *B* Beijing Black pig, *H* Hebao pig

Table 4 Haplotype distribution among populations

Haplotype	Distribution				Total	Percent (%)
	Y (7)	L (8)	B (9)	H (10)		
GTCA	6/2*	9/4	9/4	1/0	25/10	36.8/29.4
AGCA	4/1	6/2	3/1	17/8	30/12	44.1/35.3
GTCG	0	0	2/1	0	2/1	2.9/2.9
GTTG	2/1	1/0	0	2/1	5/2	7.4/5.9
AGCG	2/0	0	4/1	0	6/1	8.8/2.9

*Indicates haplotype number/homozygote number of the haplotype

they are completely linked. The complete linkage is further confirmed by the online program SHEsis.

Hebao pigs, breed in northeast China, are well known for their strong resistance to disease, whereas, through the long-term intensive breeding for growth rate and lean meat percentage, disease-resistance in Yorkshire and Landrace pigs has decreased. The results suggested that SNPs c.259G>A and c.520T>G might be related to porcine anti-disease ability. At the same time, they occurred with a high frequency and distributed in all breeds studied, which makes it a suitable candidate as a marker in molecular breeding of pigs. Although missense SNP c.571C>T (p.Pro191Ser), leading to deletion of a functional domain, might affect the protein function and has the potential for the disease-resistant breeding of pigs, it occurs rarely and belongs to a locus with low polymorphism ($PIC < 0.25$). We thus focused on revealing the role of SNPs c.259G>A and c.520T>G in the following experiments.

Effect of haplotype variant on pIFIT2 function

Missense SNPs leading to an aa substitution might be associated with a particular trait and thus valuable for marker-assisted selection. There are many reports on association analysis of SNPs with production, reproduction, or growth traits in farm animals, which could reveal the possibility of SNPs for use in production and breeding. However, association analysis of SNPs with disease-related traits is not common because of many obstacles such as difficulty in trait collection and high cost.

To reveal the functional role of the SNPs c.259G>A and c.520T>G, we constructed eukaryotic expression plasmid containing two dominant haplotypes, that is, GTCA and AGCA. The fragment amplified from cDNA has the same sequence as that deposited in GenBank (No. JX070559), and the nucleotides at positions c.259, c.520, c.571, and c.879 are G, T, C and A, respectively. The haplotype variant, AGCA, containing A and G at positions c.259 and c.520, respectively, was created with a PCR-based method using haplotype GTCA as a template. There is no other difference except for the two target sites between haplotypes GTCA and AGCA.

There is little effect of ectopic *pIFIT2* containing the haplotype GTCA on the expression of each luciferase reporter stimulated by poly(I:C), whereas ectopic expression of the allele possessing the haplotype AGCA markedly synergized the induction of the pNF-κB-Luc reporter, and displayed moderate potentiating effect on the pISRE-Luc reporter under the same condition (Fig. 1). The difference in the induction of the pNF-κB-Luc and pISRE-Luc reporter was highly significant ($p < 0.01$) and significant ($p < 0.05$) between haplotypes GTCA and AGCA, respectively. The pNF-κB-Luc and pISRE-Luc reporter has NF-κB binding sites and ISRE sequences, respectively, in the promoter region, and is, therefore, driven by activated NF-κB and ISRE-binding factors. The results have indicated that haplotype variant AGCA could change the pIFIT2 ability for mediating activation of NF-κB and ISRE-binding factors induced by poly(I:C).

Compared with NF-κB, the activation of ISRE-binding factors was much lower in cells transfected with haplotype AGCA. In addition to IRF9 in the ISGF3 complex, other members of the IRF family including IRF3 and IRF7 could also recognize the ISRE structure [32, 33], that is, ISRE-binding factors comprise a series of transcription factors. These factors are induced and function distinctively, and interaction might exist as well, which complicates the induction of pISRE-Luc expression. Additionally, we used cells cotransfected with the pNF-κB-Luc and *pIFIT2* expression vector to find the optimal time for poly(I:C) induction in preliminary experiments, and the time selected might not be the best for pISRE-Luc expression. To further corroborate the function, effects of haplotype variant on the induction of endogenous IFNβ, containing both NF-κB binding sites and ISRE sequences in the promoter, was measured by qPCR. Consistently, ectopic expression of haplotype GTCA hardly affected the mRNA level of poly(I:C)-induced IFNβ, whereas haplotype AGCA could markedly potentiate the induction of IFNβ, and the difference is highly significant ($p < 0.01$) between haplotypes GTCA and AGCA (Fig. 2). These data suggest that haplotype AGCA of *pIFIT2*, not GTCA, synergizes the activation of the NF-κB and ISRE-binding factors during virus infection.
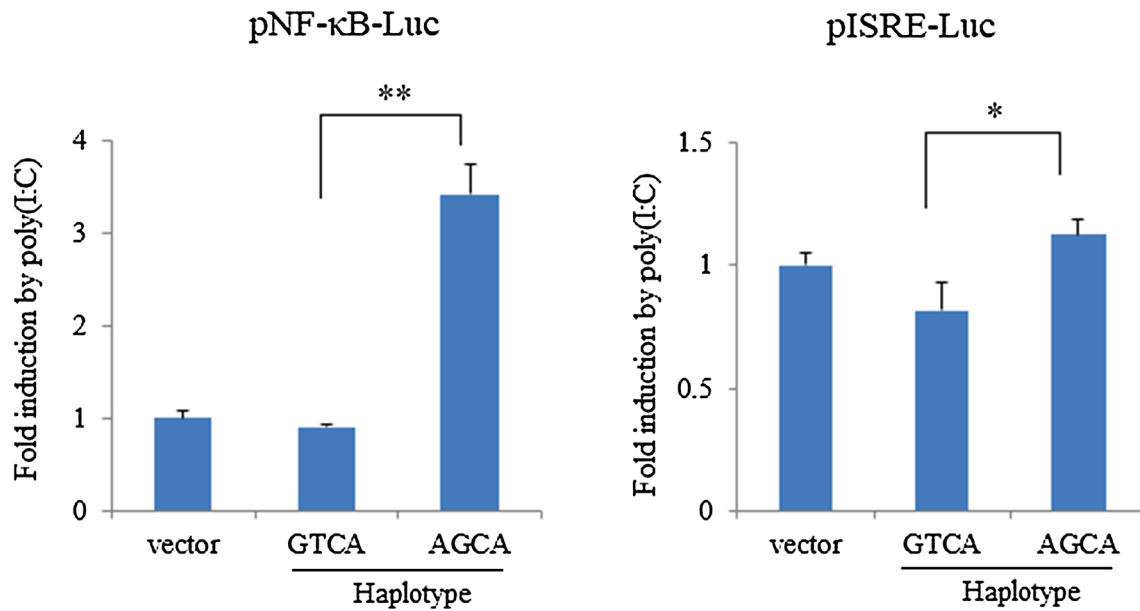
pNF-κB-Luc

pISRE-Luc

Fig. 1 Effects of haplotype variant on activation of NF-κB or ISRE-binding factors induced by poly(I:C). **indicates highly significant difference ($p < 0.01$); *indicates significant difference ($p < 0.05$); The data are mean fold induction ± SEM, and the value of cells transfected with empty vector was used as 1. The data was representative of three independent experiments, each with three replicates
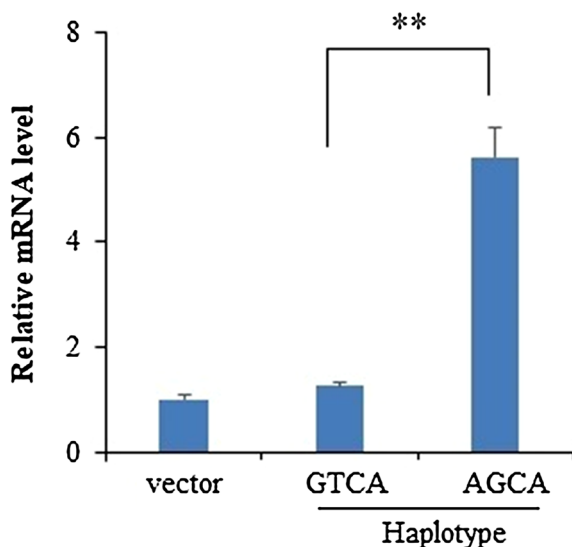

Fig. 2 Effect of haplotype variant on endogenous IFN-β expression induced by poly(I:C). **indicates highly significant difference ($p < 0.01$); relative level of cells transfected with empty vector was used as 1. The data was representative of three independent experiments, each with three replicates

Taken together, on the basis of the *pIFIT2* mRNA sequence cloned previously [24], we identified four SNPs in the CDS with a direct PCR sequencing method. In addition, we verified that the haplotype variant AGCA, mainly present in the Chinese local breed, Hebao pig, has a distinct role compared with the wild-type allele in that it markedly potentiates poly(I:C)-induced activation of NF-κB and ISRE-binding factors and subsequent expression of their regulated gene, IFNβ, and therefore helps the induction or magnitude of the immune response upon viral infection. The results showed that the *pIFIT2* gene is a candidate for anti-disease breeding in pigs and the haplotype variant AGCA might be a useful marker. Next, we will focus on revealing its effect on the immune response and health in vivo using pigs treated with poly(I:C) or virus.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All animal handling conformed to the requirements of Guidelines on Humane Treatment of Laboratory Animals of China.

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