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ORIGINAL PAPER

A genomic variant in *IRF9* is associated with serum cytokine levels in pig

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Abstract The interferon regulatory factor 9 (*IRF9*) gene is a member of the IRF family and has been shown to play functionally diverse roles in the regulation of the immune system. Previous study revealed the *IRF9* gene resides within the reported quantitative trait locus (QTLs) for cytokine levels. The aims of this study were to identify genomic variants in *IRF9* and to test the association between the variants and cytokine levels in pig. A synonymous single-nucleotide polymorphism (*c.459A>G*) was identified in exon 4 of the *IRF9* gene. Association analysis in 300 piglets (Landrace, $n=68$; large white, $n=158$; and Songliao black, $n=74$) showed that this variant was significantly associated with the level of interferon (IFN)-γ and the ratio of IFN-γ to IL-10 in serum ($P<0.05$). Relative quantification of messenger RNA (mRNA) revealed that spleen had the highest expression level and individuals with genotype *AA* had higher expression than those with genotype *AG*. Transfection-based mRNA stability assay analysis further showed that the mutant allele *G* could reduce the RNA stability of *IRF9*. These findings suggest that the SNP (*c.459A>G*) could be a causative mutation for the association between *IRF9* and the serum cytokine levels in swine.

Keywords *IRF9* · Single-nucleotide polymorphism · Cytokine levels · Association analysis · Pig

Introduction

Infectious diseases caused by viral or bacterial pathogens severely limit production in current swine industry, and some of them belong to zoonoses, leading to potential risks to human health. Even though vaccination injection, antibiotic treatment, and isolation are commonly used in dealing with this issue, infectious diseases could not be solved completely (Visscher et al. 2002). In this context, including health traits in existing breeding schemes using indirect strategies is an emerging trend in pig breeding (Flori et al. 2011). Moreover, the swine is being increasingly exploited as an ideal animal model in human diseases based on the high similarity with human physiological characteristics (Swindle et al. 2012). Therefore, discovering new loci for disease resistance ability and revealing their genetic mechanisms in pig contribute to both productivity and human disease treatment.

Cytokines are important mediators of the adaptive immune response in various infections, inflammation, and even cancer development (Doster et al. 2010). The levels of a set of cytokines in serum, such as interferon (IFN) and interleukin (IL), vary with health and disease statuses. Among them, IFN-γ and IL-10 are known to play an important role in defense against virus (Chung and Chae 2003; Gomez-Laguna et al. 2010). IFN-γ, also called type II interferon, is critical for innate and adaptive immunity against viral and intracellular bacterial infections and for tumor control (Schoenborn and Wilson 2007). IL-10 has pleiotropic effects on immunoregulation and inflammation. IL-10 inhibits a broad spectrum of cellular responses, including suppressing the function of APCs and T cells by inhibiting co-stimulation, MHC class II

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expression, and chemokine secretion (Pestka et al. 2004). There is a positive feedback of IFN-γ and IL-10 on their own production and a negative control of each other's production (Mosmann and Moore 1991). The ratio of IFN-γ/IL-10 production reflects the capacity to activate or inhibit monocytic and T lymphocytic functions, and a higher ratio has also been shown to be associated with depressive disorders (Maes 1999).

Immunoglobulin G (IgG) is the most common immunoglobulin circulating in the blood. The presence of specific IgG corresponds to maturation of the antibody response, and IgG antibodies are involved predominantly in the secondary immune response. IgG protects the body against pathogens (viruses, bacteria, fungi, and so on) by agglutination and immobilization, complement activation, opsonization for phagocytosis, and neutralization of their toxins (Lu et al. 2013).

Differences in levels of cytokines and their ratios and IgG antibodies in serum among individuals under the same conditions provide evidence of genetic control on these traits. Aim to include immunocompetence in selection for improved health, a major challenge is to find the key genes controlling immune traits in animals with inter-individual variability in response to pathogens. Up to now, large amounts of quantitative trait locus (QTLs) for immune traits have been detected but were generally mapped in large confidence intervals and are usually inconsistent in different studies (Lu et al. 2013). Further, genome-wide association studies (GWAS) identified only the most significant single-nucleotide polymorphisms (SNPs) due to stringent statistical criteria necessary for minimizing false positive hit (Weng et al. 2011). With the burgeoning field of marker-assisted selection (MAS), exploring the quantitative trait nucleotides (QTNs) in functionally important gene could be a more practical way to improve the immune capacity in pork industry.

The interferon regulatory factor 9 (*IRF9*) gene belongs to the IRF family, which plays important roles in immune response to viral infection, cytokine signaling, cell growth regulation, and hematopoietic development (Honda and Taniguchi 2006). *IRF9* also regulates transcription of programmed cell death 1 (PD-1), which is an inhibitory receptor involved in T cell activation, tolerance, and exhaustion (Mathieu et al. 2013). In addition, the *IRF9* gene is located within the reported QTLs for IFN-γ levels (Animal QTLdb, http://www.animalgenome.org/cgi-bin/QTLdb/SS/traitmap?trait_ID=639). These findings strongly suggest that *IRF9* is a promising positional and functional candidate gene for immune traits in pig.

Motivated by searching for potential genomic variants of *IRF9* associated with the serum cytokine levels in pig, we identified the variants in porcine *IRF9*; performed genotype-phenotype association analysis between the identified variant and cytokine levels (IFN-γ, IL-10, and IFN-γ/IL-10) and IgG blocking percentage to classical swine fever vaccine (CSFV) in serum; and explored the possible mechanisms of the association by expression analysis, alternative splicing analysis, and *in silico* analysis.

Methods

Ethics statement

The whole-study protocols for collection of the tissue samples of experimental individuals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of China Agricultural University.

The animals and collection of tissue samples

The animals used in this study were 300 piglets distributed in three different breeds (Landrace, n=68; large white, n=158; and Songliao black, n=74). All the animals were raised on the same farm under the same standard indoor conditions.

All piglets were vaccinated with four-dose live classical swine fever (CSF) vaccine (Rabbit origin, tissue virus≥ 0.01 mg/dose) (Qilu Animal Health Products Co. Ltd., Shandong, China) through intramuscular injection on day 21 after birth. CSF vaccine was a safe and efficacious modified live vaccine against CSF, which could lead to a high titer of neutralizing antibodies (Wang et al. 2013) and be used as a "stress" to detect the immune response for blood cytokine expression level. Blood samples were recruited from each piglet 1 day before the vaccination (day 20) and 2 weeks after the vaccination (day 35), respectively. All blood samples were directly injected into VACUETTEH® Serum Clot Activator tubes. Ear tissue samples of all pigs were collected for DNA extraction. In addition, on day 35, samples of other seven tissues, including liver, heart, lung, kidney, skeletal muscle, spleen, and stomach, and peripheral blood mononuclear cells (PBMCs) of eight large white piglets chosen randomly from the study population were collected within 30 min after slaughter and then immediately frozen in liquid nitrogen and stored at −80 °C for spatial expression analysis.

Measurement of phenotypes

IFN-γ and IL-10 concentrations in each serum sample were measured by a commercial enzyme-linked immunosorbent assay (ELISA) kit (Biosource, Carlsbad, CA) according to the standard manufacturer's instructions. All samples were arranged randomly in each plate, and the IFN-γ and IL-10 concentrations were calculated based on a standard curve.

IgG blocking percentage in serum was measured using the commercial CSF virus antibody test kit (IDEXX Laboratories, Liebefeld-Bern, Switzerland) in accordance with the manufacturer's instructions.

Genomic DNA extraction

Genomic DNA was extracted from ear tissues of all piglets with the method of standard phenol/chloroform and ethanol precipitation. The quality and quantity of all DNA samples were measured with 1 % agarose gel electrophoresis and NanoDrop™ 2000 Spectrophotometer (Thermo Scientific, USA).

SNP identification and genotyping

A total of eight pairs of PCR primers (Additional file 1: Table S1) were designed based on the porcine *IRF9* genomic sequence referring to Sscrofa 10.2 primary assembly (Ensembl Gene ID ENSSSCG00000002002) to amplify all exons and partial adjacent introns of *IRF9*. DNA samples of 30 piglets were selected randomly to construct a DNA pool with equal DNA concentration of 50 ng/μl for each individual. PCRs were performed in a 25 μl volume containing 50 ng pooled DNA, 2.5 μl of 10× PCR buffer, 5 mM of dNTPs, 10 pmol of forward and reverse primers, 0.625 U Taq DNA polymerase (Takara Biotechnology Co. Ltd.), and ddH$_2$O. The reaction conditions were as follows: an initial denaturation at 94 °C for 5 min, followed by 34 cycles at 94 °C for 30 s, annealing at 55–65 °C for 40 s and 72 °C for 40 s, and a final extension at 72 °C for 10 min. All PCR fragments were purified with a Gel Extraction Mini Kit (Beijing Tiangen Biotechnology, China) and then sequenced through using ABI 3730XL DNA analyzer (Applied Biosystems), and SNP discoveries were conducted by Chromas Software (version 2.3.1) and DNAMAN (version 6.0). Then, Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) (Squenom MassARRAY®; Bioyong Technologies Inc.) assay was applied for genotyping of the identified SNPs in 300 pigs.

Association analysis

To study the effect of *IRF9* variants on immune traits, we performed a single locus-based regression analysis based on the following linear mixed model:

$$\mathbf{y} = \mu + \mathbf{X\beta} + k\mathbf{f} + \mathbf{Zb} + \mathbf{e},$$

where **y** is the vector of trait observations (IFN-γ or IL-10 concentration or the ratio of IFN-γ/IL-10) of all piglets on day 35; μ is the overall mean; **β** is the vector of fixed effects including breed, ELISA plate, and genotype effects; **f** is the vector of trait observations on day 20; *k* is the regression coefficient of **y** on **f**; **b** is the vector of random litter effects; **X** and **Z** are the incidence matrices for **β** and **b**, respectively; and **e** is the vector of residual errors. Breed effect was included in the model to account for possible differences between breeds due to selective breeding in different breeds. The significance level was set as $\boldsymbol{\alpha}$=0.05.

Cloning of the porcine *IRF9* gene coding region and identification of splice variant

Total RNA was extracted from the samples of each of seven tissues with TRIzol Reagent (Life Technology, Carlsbad, CA, USA) following the manufacturer's protocols. The quality of extracted RNA was detected by 1 % agarose gel electrophoresis and quantified with the NanoDrop™ 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RNA was then purified and reversely transcribed into complementary DNA (cDNA) by PrimerScript® RT Reagent Kit with gDNA Eraser (Takara Biotechnology Co. Ltd.). The specific primer pair *IRF9*F and *IRF9*R (Fig. 1 and Additional file 1: Table S2) was designed to amplify the entire coding region of the *IRF9* gene according to the *IRF9* messenger RNA (mRNA) reference sequence (Ensembl Gene ID ENSSSCG00000002002). Conditions for PCR were 4 min at 94 °C, followed by 35 cycles at 94 °C for 30 s, annealing at 59.5 °C for 30 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR products from the seven pooled cDNA of different tissues of eight piglets were purified with a Gel Extraction Mini Kit (Beijing Tiangen Biotechnology, China) and cloned into the pMD18-T vector (Takara Biotechnology, Tokyo, Japan). Ten colonies per sample were selected randomly for sequencing.

To confirm the putative porcine *IRF9* splice variant, *IRF9-TV*-specific primers *IRF9*F1 and *IRF9*R1 (Fig. 1 and Additional file 1: Table S2) locating at the skipped region were designed to amplify the region including part of exon 7 from the bacteria solution. PCR products then were sent to BGI (Beijing, China) for direct sequencing using an ABI 3730XL instrument.

Relative quantitative analysis of *IRF9* expression

The mRNA expression levels of *IRF9* in seven different tissues and PBMCs of eight randomly selected 35-day-old large white pigs were investigated by real-time quantitative PCR using LightCycler® 480 II instrument (Roche Diagnostics GmbH, Germany). The reaction system contained 10 μl of 2× SYBR Green I mixture, 10 pM of each of the forward and reverse primers, and 20 ng of cDNA in a final volume of 20 μl. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was applied as an internal reference gene to normalized gene expression. Primers for the amplification of *IRF9* (IF and IR), *IRF9-001* (IF-001 and IR-001), and *GAPDH* (GF and GR) are presented in Additional file 1: Table S2. The reaction conditions were as follows: 95 °C for 10 min, 45 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s. Each quantitative PCR (qPCR) assay was carried out in triplicate. The relative gene expression was calculated by using the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen 2001).

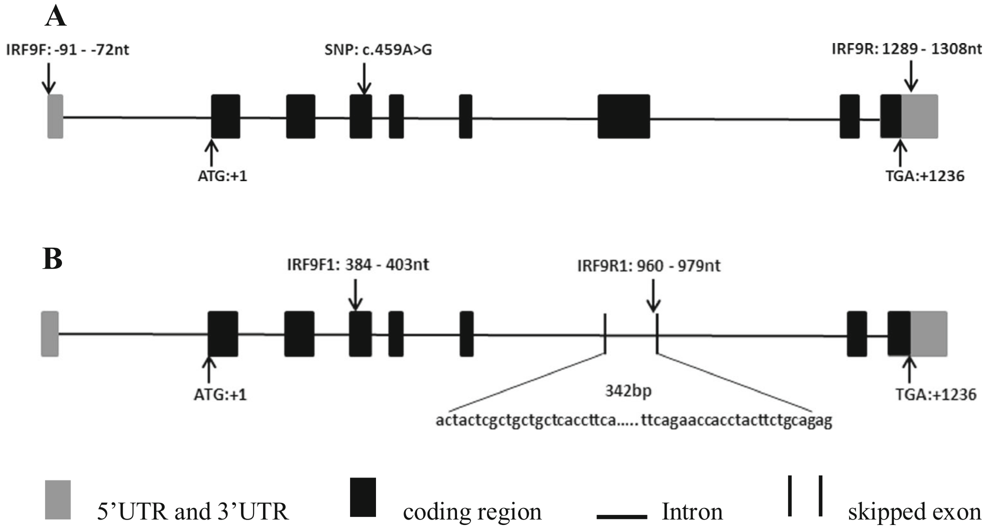


Fig. 1 Schematic illustration of the genomic structure of the porcine *IRF9* gene (**a**) and the splicing pattern of the novel splice variant (named *IRF9-TV*) (**b**). The porcine *IRF9* gene consists of nine exons with 1236 bp of coding sequence. The position of the *A* nucleotide in the start codon (ATG) is defined as +1. A SNP (*c.459A>G*) was identified in exon 4. The *IRF9-TV* transcript skipped a 342-bp sequence in exon 7

Bioinformatics analysis of identified SNP

The potential impacts of the variants in *IRF9* on the secondary structures of its transcripts were predicted using RNAstructure (version 5.4) (Reuter and Mathews 2010) under the default conditions of the software, and the full lengths of the mRNA sequences were modeled to ensure that the truncation did not affect the predicted secondary structure.

RNA stability assay

Generation of constructs The constructs used for in vitro transcription encompassed the full length of *IRF9* coding region. Primers *IRF9*-CDF and *IRF9*-CDR (Additional file 1: Table S2) were used to amplify coding sequence (CDS) of *IRF9* from the cDNA of a heterozygous individual. The resulted wild-type and mutant PCR products were then cloned into a pEGFP-C1 mammalian expression vector (Clontech, USA). The accuracy of cloning was verified by direct sequencing of the constructs.

Transfection experiments The 293T cells were transfected with wild-type and mutant *IRF9* constructs, respectively, using the Lipofectamine® 2000 Transfection Reagent (Invitrogen, USA) at a reagent to DNA ratio of 3:1. Forty-eight hours after transfection, 5 μg/ml actinomycin D (Sigma, USA) was added and cells were grown for further 12 and 24 h before being harvested. All transfection experiments were carried out in triplicate.

Quantification of transcripts The TRIzol Reagent (Life Technology, Carlsbad, CA, USA) was used to extract total RNA from transfected cells, before and after incubation with actinomycin D. Quantitative real-time PCR (RT-PCR) was used to analyze the RNAs that were obtained from each transfected construct using LightCycler® 480 II instrument (Roche Diagnostics GmbH, Germany). After reverse transcription, cDNAs were amplified using IF and IR primers. *Neo*-F and *Neo*-R primers (Additional file 1: Table S2) were used to amplify the neomycin cDNA, which was utilized as an internal control. The reaction system and reaction conditions were the same as that used for measurement of mRNA expression levels in tissues.

Results

Alterations of cytokine levels in peripheral blood after challenge

The descriptive statistics of the measured traits in the study population on day 35 (the day 2 weeks after vaccination) and day 20 (the day before vaccination) are shown in Table 1. Compared with the measurements on day 20, the IL-10 and the IgG levels on day 35 decreased obviously, while the IFN-γ concentration and the ratio of IFN-γ to IL-10 changed only slightly and tended to be retained after challenge. Large inter-individual variations (standard deviations) were observed for all of the three traits on the two measurement days.

Polymorphisms detected and their functional prediction

Only one SNP (*c.459A>G*; Fig. 1a) in exon 4 was discovered in this study. This SNP is a synonymous mutation, which does not induce a substitution of amino acid. The predicted secondary structures of the transcripts with respect to this mutation are shown in Fig. 2. *In silico* analysis revealed that this point mutation disrupted the way of base-pairing (Fig. 2) and thus might have an effect on the mRNA structures and may carry a splicing code (Pervouchine et al. 2012).

Table 1 Descriptive statistics of the traits in the study population (n=300)

Trait	Test day	Mean	Standard deviation
IFN-γ (pg/ml)	20	28.43	60.77
	35	31.14	68.24
IL-10 (pg/ml)	20	111.95	191.42
	35	84.58	141.82
IFN-γ/IL-10	20	1.21	2.23
	35	1.18	2.08
IgG (%)	20	33.40	17.36
	35	22.96	11.96

Table 2 Genotype and allele frequencies of the SNP (*c.459A>G*) in the study population

Breed	Number	Genotype frequencies			MAF	*P* value[a]
		AA	*AG*	*GG*		
Landrace	68	0.618	0.338	0.044	0.213	0.947
Large white	158	0.709	0.259	0.032	0.162	0.603
Songliao black	74	0.608	0.338	0.054	0.365	0.829

MAF minor allele frequency

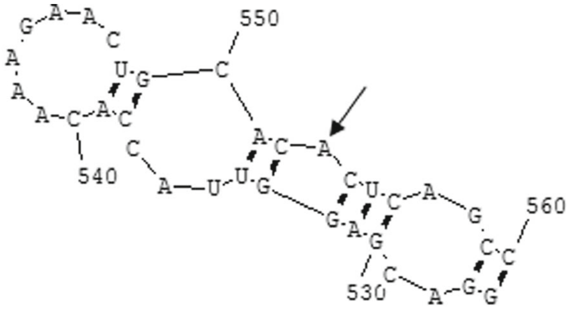
[a] *P* value for Hardy-Weinberg equilibrium test

Association analysis

The SNP (*c.459A>G*) was genotyped in 300 piglets by applying MALDI-TOF MS (Squenom MassARRAY®; Bioyong Technologies Inc.) assay. The genotype and allele frequencies and the significance of deviations from HWE of this SNP are shown in Table 2. In all of the three breeds, allele *A* is the dominant allele. Association analyses revealed that this variant had little effect on the levels of IL-10 and IgG to CSFV in swine (P>0.05) (Table 3), while it is significantly associated with the IFN-γ level and the ratio of IFN-γ to IL10 (P<0.05) (Table 3). Multiple comparisons further revealed that piglets with genotype *AA* or *AG* had significantly higher (P<0.05) IFN-γ levels but lower ratio of IFN-γ to IL-10 than those with genotype *GG*, while no significant differences (P>0.05) in the two traits were found between piglets with genotypes *AA* and *AG*.

Fig. 2 Predicted secondary structures surrounding the *c.459A>G* polymorphism site and their full-length mRNA free energies. The positions of the polymorphic sites are indicated by *arrows*. The *numbers* are the nucleotide positions

a) Allele A, free energy = **- 646.3kal/mol**



b) Allele G, free energy = **- 646.4kal/mol**

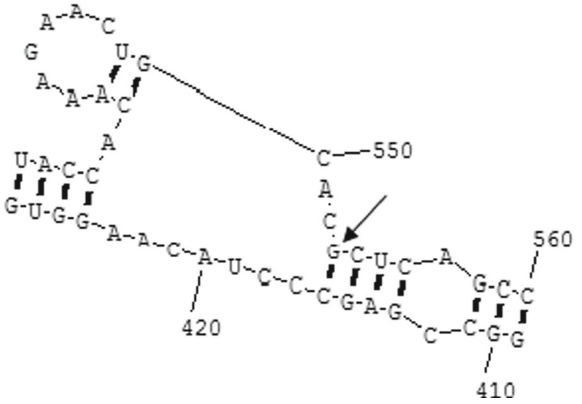


Table 3 Association of the SNP (*c.459A>G*) in *IRF9* with cytokine concentrations (LSM±SE)

Trait	Genotypes			*P* value
	AA (*n*=199)	*AG* (*n*=89)	*GG* (*n*=12)	
IFN-γ	36.541 ± 27.392^{a}	32.058 ± 24.386^{a}	28.365 ± 24.965^{b}	0.0353
IL-10	84.763±17.008	83.909±28.361	80.740±30.597	0.8391
IFN-γ/IL-10	1.026 ± 0.156^{a}	1.159 ± 0.261^{a}	1.298 ± 0.541^{b}	0.0217
IgG	24.47±1.74	22.70±2.73	19.06±2.88	0.2800

Note that means in a row with different superscript letters differ significantly ($P<0.05$)

Identification of alternative splicing variants

The porcine *IRF9* gene is located on chromosome 7 and consists of nine exons and eight introns (Fig. 1a). These exons may express to form a number of transcripts corresponding to different alternative splice variants, which may be structurally and functionally different. Five transcripts have been annotated in the Vega database (ID OTTSUSG00000002857). Specific *IRF*9F and *IRF*9R primers were used to amplify the 5′-untranslated region (5′UTR) to the 3′UTR region of the porcine *IRF9* gene (Fig. 1) from the seven pooled cDNAs of different tissues of eight piglets. It turned out that, in addition to the expected major 1587-bp PCR product of *IRF9-001* (ID OTTSUST00000006796), a smaller band, not same as any of the five annotated transcripts, was also detected in all of the seven tissues by electrophoresis on 2 % agarose gel (Fig. 3a), indicating that a novel splice variant might exist. We purified and cloned the PCR products into the pMD18-T vector. A total of 70 clones of each band were sequenced individually. Again, we obtained a major transcript, which is the annotated alternative splicing isoform *IRF9-001*, and a minor transcript, which is different from all of the five annotated transcripts. We name the novel splice variant *IRF9-TV*. Introns of both transcripts conform to the GU-AG rule. BLAST analysis indicated that, compared to the *IRF9-001* transcript, the novel *IRF9-TV* skipped all 342 nucleotides of exon 7 and missed 114 amino acids (Fig. 1b).

To further demonstrate the evidence of *IRF9-TV* in pig, sequences including the skipped region were amplified using the *IRF9-TV*-specific primers *IRF*9F1 and *IRF*9R1 (Fig. 1 and Additional file 1: Table S2). As expected, a 596-bp PCR product was detected when using the major band as templates, while nothing was obtained when using the smaller band as templates for PCR (Fig. 3b). By sequencing the PCR amplification products, we identified the sequence retaining the 342 nucleotides in exon 7 of *IRF9-001*. Failing to obtain PCR product from the smaller band proved the existence of *IRF9-TV*. These results give strong evidence of the novel splicing variant *IRF9-TV*. The comparisons of the mRNA sequences and peptide sequences of the two transcripts are shown in Additional file 2: Figs. S1 and S2.

To determine whether the *c.459A>G* nucleotide substitution would lead to the exclusion of exon 7 and cause aberrant splicing, we analyzed the mRNA expressions of *IRF9-001* and *IRF9-TV* with different genotypes in four immune-related tissues and PBMCs. Through RT-PCR experiments, the *IRF9-TV* transcripts were found to be expressed in all immune-related tissues and PBMCs of pigs possessing allele *A* or allele *G*. Further, the percentage of occurrence of *IRF9-TV* transcript with *AA* and *AG* genotypes had little difference with *IRF-001* (Additional file 3: Figs. S3 and S4).

Expression analysis of the porcine *IRF9* gene

To study the function of the *IRF9* gene and to resolve in vivo their expression patterns in different tissues, relative quantification of the total mRNA in seven different tissues and PBMCs were performed by RT-qPCR from eight randomly selected 35-old-day large white piglets. The result showed an extensive expression pattern of *IRF9*, with the highest expression level in spleen, followed by that in stomach, lung, liver, PBMCs, kidney, and heart (Fig. 4a). Almost no expression of *IRF9* was observed in skeletal muscle, which is not an immune-related tissue (Fig. 4a). To explore the possible effect of the *c.459A>G* mutation on the expression of *IRF9*, we analyzed the expression of *IRF9* in four tissues with higher *IRF9* expression (liver, spleen, lung, and stomach) and in PBMCs of animals with different genotypes. The results showed that in all tissues, the total mRNA expressions of *IRF9* of piglets with genotype *AA* (*n*=5) had higher mRNA levels than that of genotype *AG* (*n*=3); in particular, in spleen, the difference was statistically significant ($P<0.05$) (Figs. 4b). The same expression tendencies were also observed for *IRF9-001* and *IRF9-TV* (Additional file 3: Figures S3 and S4), although the differences did not reach statistical significance. The results indicated that allele *G* might lead to a reduced expression of *IRF9* in immune-related tissues, although we did not have samples with genotype *GG* because of its low frequency in the study population.

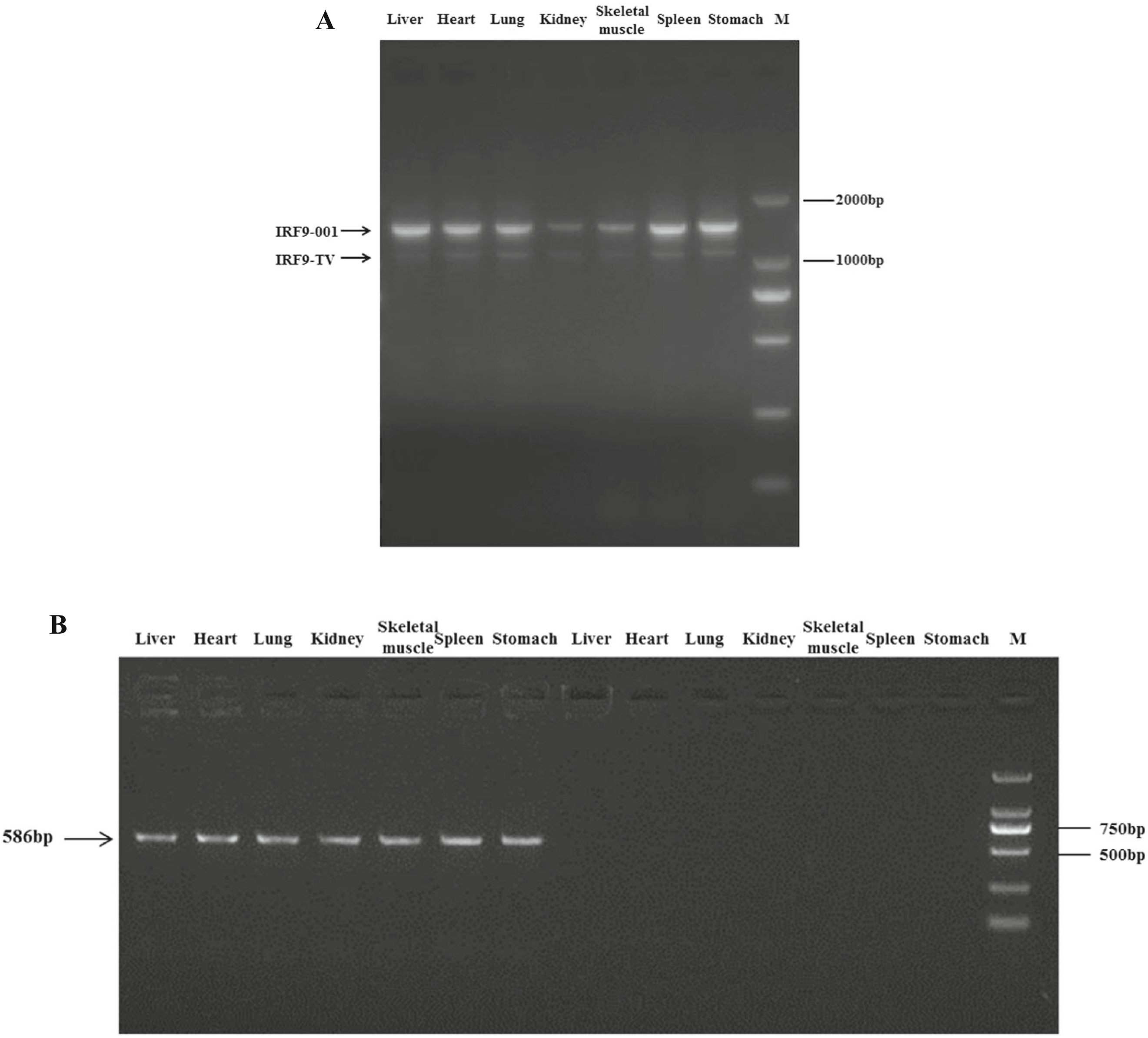


Fig. 3 RT-PCR products of full-length coding sequence of *IRF9* (**a**) and sequence including part of the skipped region (**b**) in seven different tissues. The larger band in **a** is the expected 1399-bp PCR product of *IRF9-001*, and the smaller one is an aberrant splice variant (named *IRF9-TV*). Line 1 to line 7 in **b** are the expected 596-bp PCR products using the major band in **a** as templates, and line 8 to line 14 are the PCR products when the smaller band in **a** was used as templates. M: DNA Marker 2000 (Takara, China)

Effect of SNP (*c.459A>G*) on mRNA stability of *IRF9*

To assess transcript stability directly, we transfected 293 T cell lines with the wild-type and mutant constructs encompassing the full coding region of *IRF9*, respectively, and quantified *IRF9* mRNAs before and after incubation with an inhibitor of transcription (i.e., actinomycin D) by using quantitative RT-PCR and the *Neo* mRNA (which is also transcribed from the transfection vector) as an internal control. It turned out that, at 12 h, the relative mRNA levels of the wild-type and mutant transcripts were 1.10 and 0.95 of that of *Neo* mRNAs. At 24 h, the relative mRNA levels of the wild-type and mutant transcripts were 1.29 and 0.87 of that of *Neo* mRNAs (Fig. 5). These results indicated that the mutant transcript decayed much faster than the wide-type transcript and that the mutation (*c.459A>G*) would reduce the mRNA stability of *IRF9*.

Discussion

Based on the knowledge of biological functions as well as the significant QTL signals of *IRF9*, we speculate that *IRF9* may be of importance in immune response in swine. We explored the genomic variants of the porcine *IRF9* gene and tested their

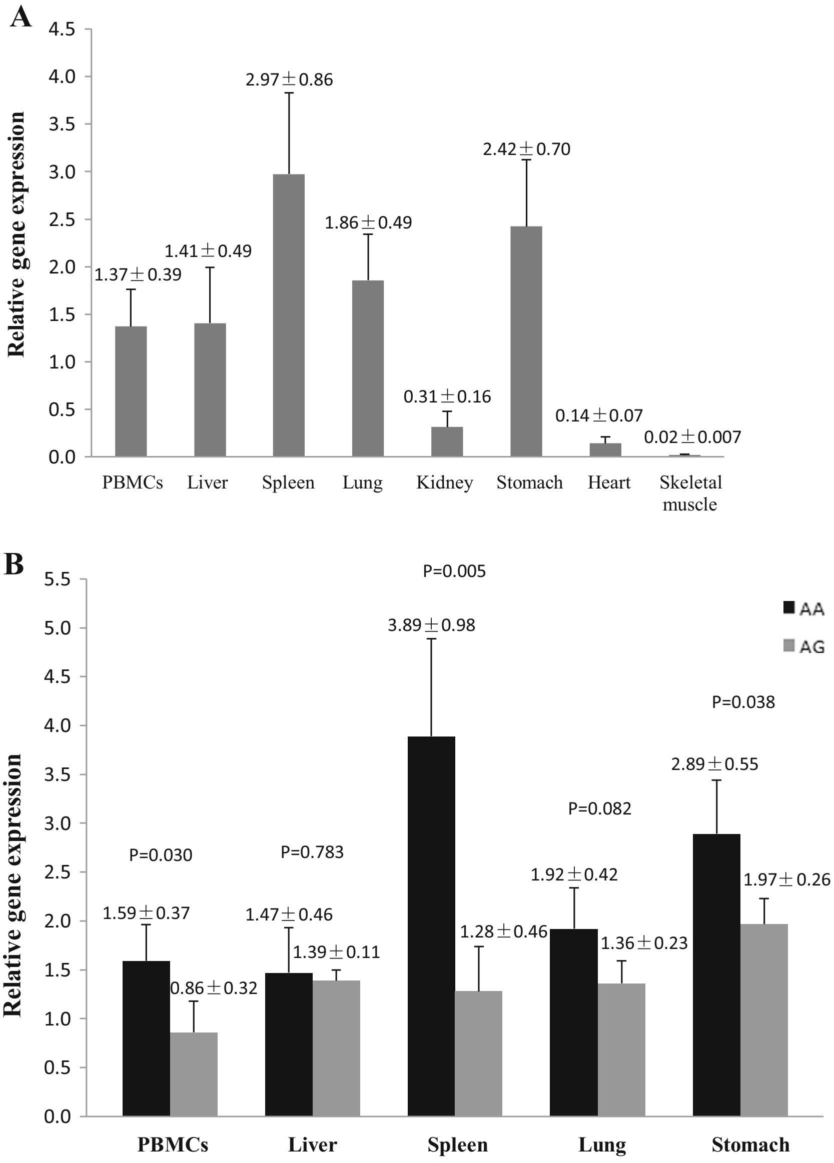


Fig. 4 Relative quantification of mRNA expression levels of the porcine *IRF9* gene in eight different tissues of eight piglets (**a**) and in tissues with genotypes *AA* (*n*=5) and *AG* (*n*=3) of the SNP (*c.459A>G*) (**b**). The values were normalized to the internal reference gene *GAPDH*

association with cytokine levels (IFN-γ, IL-10, and IFN-γ/IL-10) in serum. Our association analysis results indicated that a synonymous SNP (*c.459A>G*) in exon 4 of *IRF9* is associated significantly with the IFN-γ concentration and the ratio of IFN-γ/IL-10 after challenged with CSF vaccine (Table 3). In addition, the highest mRNA expression level of *IRF9* was detected in spleen (Fig. 4a), an important peripheral lymphoid organ, while almost no expression was observed in skeletal muscle, a non-immune-related tissue. These results confirmed our speculation.

Accumulating evidence have indicated that synonymous SNPs could have functional consequences (Sauna and Kimchi-Sarfaty 2011), although they do not induce amino acid substitutions of the encoded proteins. A number of studies have demonstrated the significant contribution of synonymous SNPs to human disease (Ho et al. 2011; Macaya et al. 2009; Ramser et al. 2008). Synonymous mutations could influence the mRNA expression by modifying mRNA stability (Capon et al. 2004; Nackley et al. 2006). Our quantitative RT-PCR analysis with respect to the synonymous mutation (*c.459A>G*) in *IRF9* revealed that genotype *AA* (*n*=5) had higher expression levels than genotype *AG* (*n*=3) in four immune-related tissues and in PBMC (Fig. 4b), indicating that mutant allele *G* might lead to a reduced expression of *IRF9*. By using transfection-based mRNA stability assays, we were able to show that allele *G* caused a reduction of the mRNA stability of *IRF9* (Fig. 5).

A total of five transcripts of *IRF9* (*IRF9-001* to *IRF9-005*) have been annotated in the Vega database (ID OTTSUSG00000002857). The fragments of *IRF9-002* and *IRF9-003* are CDS 3′ incomplete, and *IRF9-004* and *IRF9-005* are CDS 5′ and CDS 3′ incomplete. These four

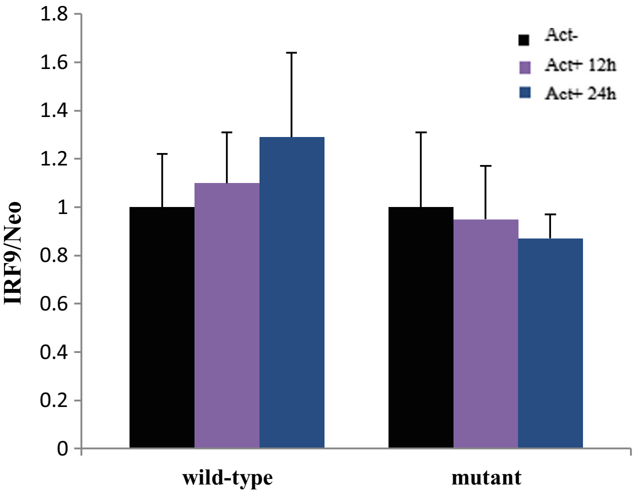


Fig. 5 Relative mRNA levels of the wild-type and mutant transcripts of *IRF9*, as determined by quantitative RT-PCR before (Act−) and after (Act + 12 h and Act + 24 h) actinomycin treatment. The values were normalized to the internal reference gene *Neo* and the relative value before actinomycin treatment was set to be 1

types of transcripts are just putative and have not yet been proved by experiments. In this study, only *IRF9-001* and a novel transcript splice variant of *IRF9*, named *IRF9-TV*, were detected. Sequencing and BLAST revealed that this novel splice variant skipped 342 nucleotides of exon 7 and missed 114 amino acids (Fig. 1) in comparison with the dominant transcript *IRF9-001*. Alternative splicing (AS) is a process by which exons can be either excluded or included in generating multiple mRNA isoforms (Markovic 2013). Consequences of AS are that either the mRNA never gets translated into a protein (McGlincy and Smith 2008) or the translated protein isoforms are different in their sequences and functions (Marshall and Geballe 2009). Recent survey of various mammalian tissues, developmental stages, and cell lines indicated that at least 90 % of the protein-coding genes employ alternative splicing events (Sun et al. 2011). Recent evidence suggest that SNPs are major contributors to the generation of alternative splice variants (Menoud et al. 2012; Drogemuller et al. 2011). RNA structures could be involved in the regulation of splicing in mammals through structure-mediated changes in spatial positioning of *cis*-acting elements with respect to each other (Pervouchine et al. 2012). *In silico* analysis revealed that the SNP (*c.459A>G*) in exon 4 of *IRF9* disrupts the ways of base-pairing (Fig. 2) and thus might have an effect on the mRNA structures. To test whether this SNP plays a role in the aberrant splicing, we analyzed the mRNA expression of *IRF9-TV* with different genotypes in four immune-related tissues and PBMCs by RT-PCR. It turned out that both *AA* and *AG* genotypes expressed the transcript of *IRF9-TV*. And the percentage of occurrence of *IRF9-TV* in both genotypes had little difference with *IRF9-001* (Additional file 3: Figs. S3 and S4). So, it seems that the aberrant splicing is not due to the *c.459A>G* nucleotide substitution. There may be other reason for the aberrant splicing of *IRF9-TV* transcript.

Conclusions

We identified a synonymous SNP (*c.459A>G*) in the porcine *IRF9* gene and found that the substitution of *A* to *G* significantly decreased the IFN-γ concentration and increased the ratio of IFN-γ/IL-10 in serum of piglets after challenged with CSF vaccine. Meanwhile, the individuals with genotype *AA* showed a higher expression level of *IRF9* in immune-related tissues and in PBMCs than those with genotype *AG* because of the reduced mRNA stability caused by the mutant allele *G*. We found a novel splice variant of *IRF9*, which is not due to *c.459A>G* nucleotide substitution. Results of this study provide useful information about the function of the porcine *IRF9* gene in disease resistance in pig. Further functional studies are needed to validate the effects of this SNP with a larger number of samples and more critical assay conditionals.

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Compliance with ethical standards

Competing interests The authors have declared that no competing interests exist.

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