Expression, polymorphism of IRF5 gene and association with serum cytokine levels in pig

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ABSTRACT

Interferon regulatory factor 5 (IRF5) gene is a crucial transcription factor in immune system and has shown its functionally diverse roles in the regulation of antiviral responses. Previous study revealed the IRF5 gene resides in the reported quantitative trait locus (QTLs) for cytokine levels. In this report, the porcine IRF5 gene was selected to investigate its effect on cytokine level in serum. The mRNA expression of IRF5 was also detected by real time quantitative PCR and the results revealed that IRF5 mRNAs were widely expressed in all analysed tissues except muscle. A genomic variant (g.10764 T>C) in exon 7 of the IRF5 gene was identified by direct sequencing, and then the SNP was genotyped by MALDI-TOF MS assay method in three pig populations. The further association analysis indicated that the SNP was significantly associated with the level of interferon-γ (day 20), interleukin 10 (day 35) and ratio of interferon-γ/interleukin 10 (day 35) in serum (p < 0.05). An interferon known to be key in immune processes. These results suggested that IRF5 could serve as a promising candidate gene for pig disease resistance breeding.

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Introduction

The interferon regulatory factors (IRFs) consisting a family of transcription factors and emerging as critical regulators of innate and adaptive immune responses are involved in both immune cell differentiation and host defence against pathogens (Savitsky et al. 2010). The interferon regulatory factor 5 (IRF5) is one of the members of IRFs and play a crucial role in the control of inflammatory and the production of pro-inflammatory cytokines. IRF5 is also crucial for driving macrophages toward a proinflammatory phenotype by regulating cytokine and chemokine expression and modulating B-cell maturity and antibody production (Saliba et al. 2014). IRF5 may promote lymphocyte differentiation and apoptosis (Tamura et al. 2008). IRF5 was involved in several immune signal transduction pathways in leukocytes and epithelial cells. It could activate downstream of Toll like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I)-like RNA helicases, concerning interleukin-1 receptor-associated kinase and IkB kinase (IKK) (Honda and Taniguchi 2006).

In human, polymorphisms in IRF5 gene is associated with several inflammatory and autoimmune diseases, including systemic lupus erythematosus (SLE) and inflammatory bowel disease (Eames et al. 2016; Xu et al. 2016). In addition, the IRF5 gene is located within the reported QTL for IFN-γ level in pig chromosome 18 (Animal QTLdb, http://www.animalgenome.org/cgi-bin/QTLdb/SS/qdetails?QTL_ID =12345). These findings strongly suggest that IRF5 is a promising positional and functional candidate gene for immune traits in pig. In the previous study, we reported a member of porcine IRFs family (IRF1 gene) and revealed the polymorphisms was significantly associated with cytokine traits (Liu et al. 2011). However, so far, IRF5 gene has not been reported in pig.

In order to further provide more precise understanding of the porcine IRF5 gene in regulating IFN and also to be motivated by searching for potential genomic variants of IRF5 associated with the serum cytokine levels in pig, we identified the variant in porcine IRF5; performed genotype phenotype association analysis between the identified variant and cytokine levels (IFN-γ, IL10 and IFN-γ/IL10) in serum in three
pig populations; and we also explored the possible mRNA expression by RT-qPCR analysis.

Materials and methods

Animal population

The animals population consisted of 300 piglets distributed in three pig breeds, Landrace (68 piglets), Large White (158 piglets) and Songliao Black (74 piglets), which were raised under the same standard indoor conditions at the experimental farm of Chinese Academy of Agricultural Sciences, Beijing, China. All pigs were vaccinated with Classical Swine Fever (CSF) live vaccine at the 21-day age, as CSF vaccine was used as a ‘stress’ to detect the immune response for blood cytokine expression level.

Blood samples were recruited from each piglet one day before the vaccination (day 20) and two weeks after the vaccination (day 35), respectively, and then the serum was separated by ultracentrifugation. Ear tissue samples of all pigs were also collected for DNA extraction. Seven tissues including spleen, stomach, lung, liver, kidney, heart and muscle of three Large White pigs were collected at the 35th day after slaughter within 30 min, then immediately frozen in liquid nitrogen and stored at −80 °C for expression analysis.

Measurement of IFN-γ and IL10 concentrations in serum

IFN-γ and IL10 concentrations in each serum sample (day 20; day 35) were measured using a commercial ELISA kit (Biosource, Carlsbad, CA) based on the standard instructions from manufacturer. The kit contains an antibody coated 96-well test plate, standards of known IFN-γ/IL10 concentrations, standard diluent buffer, wash buffers and a biotin monoclonal antibody specific to IFN-γ/IL10, a streptavidin-HRP diluent solution and a stop solution. All samples were randomly arranged on each plate and were used to calculate IFN-γ and IL10 concentrations based on a standard curve.

Genomic DNA isolation and total RNA extraction

Genomic DNA was extracted from ear tissues of all pigs with the method of standard phenol/chloroform and ethanol precipitation (Sambrook et al. 1989). The qualities of all DNA samples were measured with 1% agarose gel electrophoresis and NanoDrop™ 2000 Spectrophotometer (Thermo Scientific, Waltham, MA). The total RNA was isolated from different tissues using Trizol reagent (Invitrogen, Carlsbad, CA) and quantified using the NanoDrop™ 2000 spectrophotometer (Thermo Scientific, Waltham, MA). The integrity of the RNA samples was detected by 1% agarose gel electrophoresis before the first-strand cDNA was synthesised. RNA was purified and reversely transcribed into cDNA using PrimerScript® RT reagent kit with gDNA eraser (TaKara Biotechnology Co., Ltd, Dalian, China) following the manufacturer’s instructions.

Tissues expression analysis of the porcine IRF5 gene

cDNA sequence of IRF5 gene was obtained using ENSEMBL pig database (Sscrofa 10.2, Gene ID:ENSSSCG00000016573). Expression levels of mRNA 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 each of the forward and reverse primers, 20 ng of cDNA in a final volume of 20 μL. A gene-specific primer set for the mRNA expression pattern of IRF5 gene was also designed to eliminate potential confounding results from genomic DNA contamination: F: 5’-GCCAAGGAGACAGGAAGTAC-3’, R: 5’-TCTCCCTCCCTCCCTCCCT-3’. The GAPDH (glyceraldehyde-3-dehydrogenase) gene was applied as an internal reference gene for normalisation and the primers were: F: 5’-GTCCACTGTTGCTTACGA-3’, R: 5’-GCTGACGATCTTGAAGGGAGT-3’. All measurements were carried out in triplicate and normalised to GAPDH by the 2^−ΔΔCt method (Livak and Schmittgen 2001).

SNP identification and genotyping

A total of nine pairs of PCR primers were designed based on the porcine IRF5 genomic sequence referring to Sscrofa 10.2 primary assembly (Ensembl Gene ID: ENSSSCG00000016573) to amplify all exons and partial adjacent introns (Table 1). DNA samples of 30 piglets from three breeds 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 primer, 0.625 U Taq DNA polymerase (Takara Biotechnology Co., Ltd.) and ddH2O. The reaction conditions were as follows: an initial denaturation at 94 °C for 5 min, followed by 34 cycles of 94 °C for 30 s, annealing at 55–65 °C for 40 s, 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 ABI3730xl DNA analyser (Applied Biosystems, Foster, CA) and SNPs detection were conducted by Chromas 2.3.1 (Technelysium, South Brisbane, Australia) and DNAMAN 6.0 software (Lynnon, San Ramon, CA) software. Then MALDI-TOF MS (Squenom MassARRAY™, Bioyong Technologies Inc., Beijing, China) assay was applied for genotyping of the identified SNP in 300 pigs.

**Association analysis**

The association between the genotypes of the SNP and immune traits was examined by fitting the following mixed models using in SAS software (Version 9.2):

\[ y = \mu + X\beta + Tv + bm + Za + e \]

where \( y \) is the vector of phenotypic values of immune traits analysed; \( \mu \) is the overall mean; \( \beta \) is the vector of fixed effects including breed with three levels, sex with two levels and ELISA plate effect with five levels; \( v \) is the vector of random litter effects; \( m \) is the vector of SNP genotype with three levels; \( a \) is the vector of the residual polygenetic effects with \( a \sim N(0, A\sigma_a^2) \), where \( A \) is the numerator relationship matrix; \( X, T \) and \( Z \) are the incidence matrices for \( \beta, v \) and \( a \), respectively; \( b \) is the regression coefficient of phenotypes on SNP genotypes; \( e \) is the vector of residual errors with \( e \sim N(0, I\sigma_e^2) \).

**Results and discussion**

**Tissues expression analysis of the porcine IRF5 gene**

The relative expression results of porcine IRF5 showed that total mRNA of IRF5 was expressed in all analysed tissues except skeletal muscle, with the highest expression level in spleen, followed by lung, kidney, liver and lower expression level in heart and stomach (Figure 1). Our results were consistent with the research by Zhu et al. (2016), in which tissues...
expression of the common carp IRF5 mRNA by RT-PCR was found.

**Association analysis of porcine IRF5 gene**

Porcine IRF5 gene is located on chromosome 18 and full length of genomic sequence is 12,430 bp. It is consisted of eight exons. One SNP (g.10764 T > C) in exon 7 was detected and genotyped in 300 pigs. The Hardy–Weinberg equilibrium for genotypic distributions tested by employing the Chi-square ($\chi^2$) test revealed that the alleles meet the requirements of Hardy–Weinberg equilibrium in our experimental population ($p > .05$). Genetic variation analysis also demonstrated that allele frequencies are not significantly different among three pig breeds ($p > .05$) (Table 2). The allele T is obviously predominant in three detected pig breeds. In contrast, the allele C has lower frequencies and the CC genotype is detected in three detected pig breeds. In contrast, the allele C has lower frequencies and the CC genotype is detected lower in Landrace, Large White and Songliao Black.

A preliminary association study was also performed herein to determine whether the SNP influenced the level of some cytokines in pigs. The results showed that the SNP (g.10764 T > C) had significant difference in level of IFN-γ (day 20), IL10 (day 35) and the ratio of IFN-γ to IL10 (day 35) ($p < .05$) (Table 3). The IFN-γ and IL10 level in serum of pigs with genotype TT were significantly higher than those of pigs with genotype CC ($p < .05$), but the ratio of IFN-γ to IL10 in serum of pigs with genotype TT was significantly lower than those of pigs with genotype CC ($p < .05$) (Table 3). On the other hand, the pig breed is also a significant factor for these serum cytokine traits, the level of IL10 (day 20; day 35), IFN-γ (day 20; day 35) and the ratio of IFN-γ to IL10 (day 20; day 35) among the pig breeds were significantly different ($p < .05$) (Table 4). Songliao Black had higher level of IL10 (day 20; day 35), IFN-γ (day 35) than other two pig breeds, but the lower level in ratio of IFN-γ to IL10 (day 20).

Serum cytokine are valuable biomarkers for health and disease status. IFN-γ is pivotal in the wave of innate and adaptive immunity against viral and intracellular bacterial infections (Schoenborn and Wilson 2007). IL10 is an anti-inflammatory cytokine mainly produced by regulatory T cells and helper T cells, which functions through suppressing macrophage/T cell cytokine expression and it plays a critical role in down-regulating the expression of Th1 cytokines and MHC class II antigens (Redpath et al. 1999; Mocellin et al. 2003). Besides the active role of IFN-γ and IL10, the ratio of them also affects the capacity to activate or inhibit monocyctic and T lymphocytic functions, and a high ratio has also been reported to have relevance.

**Table 2.** Genotype frequencies and allelic frequencies of IRF5 gene determined by MALDI-TOF-MS method in three pig populations.

| Breed            | Number | TT  | TC  | CC  | T   | C   |
|------------------|--------|-----|-----|-----|-----|-----|
| Landrace         | 68     | 31  | 26  | 11  | 0.65| 0.35|
| Large White      | 158    | 96  | 43  | 19  | 0.74| 0.26|
| Songliao Black   | 74     | 36  | 25  | 13  | 0.66| 0.34|

**Table 3.** Association analysis and multiple tests of the SNP (g.10764 T > C) of IRF5 gene with immune traits in three pig populations.

| Traits          | Genotypes (means ± standard error of means) |
|-----------------|---------------------------------------------|
|                 | TT ($n = 163$) | TC ($n = 94$) | CC ($n = 43$) | p value |
| IFN-γ (day 20)  | 46.541 ± 27.392$^a$ | 42.058 ± 24.386$^a$ | 35.365 ± 24.965$^b$ | .0248$^b$ |
| IFN-γ/IL10 (day 20) | 163.475 ± 42.573 | 158.702 ± 40.952 | 156.846 ± 41.854 | .8325 |
| IFN-γ (day 35)  | 0.997 ± 0.365 | 1.019 ± 0.384 | 1.007 ± 0.375 | .7547 |
| IFN-γ/IL10 (day 35) | 63.917 ± 29.307 | 61.382 ± 29.124 | 63.324 ± 28.413 | .6382 |
| IL-10 (day 20)  | 119.912 ± 34.971$^a$ | 121.479 ± 33.413$^a$ | 110.740 ± 30.597$^b$ | .0442$^b$ |
| IL-10 (day 35)  | 46.541 ± 27.392$^a$ | 42.058 ± 24.386$^a$ | 35.365 ± 24.965$^b$ | .0248$^b$ |

$^a,b$Statistically different of least square means ($p < .05$).

$^a$Statistically different of least square means (< .05).

**Table 4.** Association analysis of immune traits in three pig breeds.

| Traits          | Breeds (Means ± standard error of means) |
|-----------------|------------------------------------------|
|                 | Landrace ($n = 68$) | Large White ($n = 158$) | Songliao Black ($n = 74$) |
| IFN-γ (day 20)  | 46.269 ± 27.393$^a$ | 45.777 ± 26.713$^a$ | 32.409 ± 28.572$^b$ |
| IL-10 (day 20)  | 127.400 ± 43.484$^a$ | 100.610 ± 40.699$^a$ | 212.070 ± 43.032$^b$ |
| IFN-γ/IL10 (day 20) | 1.459 ± 0.518$^a$ | 1.299 ± 0.390$^a$ | 1.409 ± 0.609$^b$ |
| IFN-γ (day 35)  | 24.459 ± 31.910$^a$ | 22.378 ± 29.354$^a$ | 36.380 ± 35.845$^b$ |
| IL-10 (day 35)  | 122.020 ± 19.202$^a$ | 86.743 ± 34.820$^a$ | 147.620 ± 36.251$^b$ |
| IFN-γ/IL10 (day 35) | 1.125 ± 0.483$^a$ | 1.036 ± 0.479$^a$ | 1.324 ± 0.535$^b$ |

$^a,b$Statistically different of least square means ($p < .05$).

$^a$Statistically different of least square means (< .01).
with depressive disorders (Maes 1999). Our results showed that the ratio of IFN-γ/IL10 in Songliao Black pig was the lowest, which could be explained as this breed may have better T lymphocytic balance and immune capacity than other two western commercial pig breeds.

As the immune system plays an essential role in disease resistance of animals, genes which involved in the immune response could be regarded as the important candidate genes for selection. Our association analysis results provided a straightforward insight that the IRF5 gene which has effects on serum IFN-γ, IL10 level and ratio of IFN-γ to IL10 in serum in three pig populations, and it could serve as a promising candidate gene for cytokines level in pig breeding. However, the number of pigs analysed here is limited, further investigation will be required among other pig populations to confirm the association between the SNP and immune traits.

Conclusions

In summary, tissue expression results revealed that the IRF5 mRNA was expressed widely in all analysed tissues except skeletal muscle. One missense SNP in the peptide-binding region of the IRF5 gene was identified and it was significantly associated with the level of IL10 (day 20), IFN-γ (day 35) and ratio of IFN-γ/IL10 (day 35) in serum in three pig populations. All these results indicated that IRF5 could be a promising candidate gene for serum cytokine level in pig disease resistance breeding program.

Disclosure statement

The authors have declared that no competing interests exist.

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