Research Article

Polymorphisms of exon 10 in the BPI gene and its association analysis with some reproductive traits and partial immune indexes in Meishan pigs

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Abstract: This study was conducted to detect polymorphisms of *BPI* gene exon 10 by PCR-RFLP in Meishan pigs and their associations with some important cytokine levels (IL-1β, IL-4, IL-6, IL-8, IL-10, TGF-1β, IFN-γ, TNF-α) and reproductive traits (total number born (TNB), number born alive (NBA), birth litter weight (BLW), and weaning litter weight (WLW)), with the aim of identifying effective genetic markers for molecular breeding. The results revealed three genotypes, including AA, AB, and BB, with the trend of AA > BB > AB in reproductive traits. Pigs with the AA genotype were significantly higher than the AB genotype in TNB and IFN-γ ($P < 0.05$), and extremely significantly higher in NBA and WLW ($P < 0.01$). Moreover, TGF-1β and TNF-α levels were significantly higher in the AA genotype than the BB genotype ($P < 0.05$). However, there were no significant differences among the three genotypes in the levels of other cytokines ($P > 0.05$). Comprehensive analysis indicated that the polymorphisms of exon 10 in the *BPI* gene have significant genetic effects on some reproductive traits and cytokines in Meishan pigs, for which the AA genotype was demonstrated to be the favorable genotype, which could be used as potential genetic markers for in-depth research and examination.

Key words: Pig, *BPI* gene, reproductive traits, immune indexes

1. Introduction

Bactericidal/permeability-increasing protein (BPI) is an endogenous antibacterial protein, which is mainly found in human and mammalian aniline blue particles of polymorphonuclear leukocytes (1). BPI is a member of the lipopolysaccharide binding protein family and an antimicrobial active protein. It plays an important role in the natural defense systems of animals (2). Studies have demonstrated that the BPI protein has multiple biological functions including promotion of complement activation, inhibition of inflammatory mediators' release, and infection by fungal and parasitic organisms. It also resists and kills bacteria (especially gram-negative bacteria) and neutralizes endotoxins or lipopolysaccharide (LPS) (3–5). In addition, the BPI protein has great potential in clinical applications such as in treating Crohn's disease, granulomatosis, and inflammatory diseases (6–9). Zhou et al. (10) successfully extracted and purified BPI protein from porcine neutrophils and proved that porcine BPI can also neutralize endotoxins and kill gram-negative bacteria through in vitro and in vivo biological activity experiments. Given the regulatory role of the *BPI* gene in killing gram-

negative bacteria, many studies were conducted on *BPI* polymorphisms. Shi et al. (11) found that polymorphisms in exon 4 of the *BPI* gene are associated with porcine *Salmonella* resistance. Moreover, Christopher et al. (12) detected the *Ava*II and *Hpa*II restriction polymorphisms in exons 4 and 10 of the *BPI* gene in multiple pig breeds, which are related to susceptibility to *Salmonella*.

Depending on different genetic bases, disease resistance can be divided into special disease resistance and general disease resistance. Previous studies have shown that the polymorphisms of exon 10 in the porcine *BPI* gene are mainly associated with specific disease resistance (such as *Escherichia coli* and *Salmonella* susceptibility), while there are few reports on the associations with general disease resistance. Our study analyzed the associations of this mutation site with several important cytokine levels for assessing the potential effects on general disease resistance. Moreover, it is also necessary to pay attention to the influence of genetic markers on important economic traits (such as reproduction traits, growth traits, and carcass traits) when identifying markers associated with disease resistance. Therefore, we detected the polymorphisms of

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exon 10 in the *BPI* gene of Meishan pigs and measured reproductive traits (total number born (TNB), number born alive (NBA), birth litter weight (BLW), and weaning litter weight (WLW)) and cytokine levels (IL-1β, IL-4, IL-6, IL-8, IL-10, TGF-1β, IFN-γ, and TNF-α). The correlations of the polymorphisms in exon 10 of the *BPI* gene with reproductive traits and immune indexes were further analyzed. The results could provide a theoretical basis for application of the polymorphisms as effective molecular markers in future disease-resistant breeding of Meishan pigs.

2. Materials and methods

2.1. Ethics statement
The animal study proposal was approved by the Institutional Animal Care and Use Committee (IACUC) of the Yangzhou University Animal Experiments Ethics Committee (permit number: SYXK (Su) IACUC 2012-0029). All experimental methods were conducted in accordance with the relevant guidelines and regulations.

2.2. Experimental animals
A total of 137 Meishan pigs were obtained from the Meishan Pig National Breeding Conservation Center in Taicang City (Jiangsu Province, China). About 1.0 g of ear tissue sample from each individual was collected, placed into a 1.5-mL Eppendorf tube in an ice box, and transported to the laboratory for genomic DNA extraction.

2.3. Genomic DNA extraction
Genomic DNA was extracted using the conventional phenol-chloroform method. The concentration and purity of DNA were checked using the NanoDrop ND-1000 nucleic acid concentration analyzer (Thermo Scientific, Waltham, MA, USA). The A_{260}/A_{280} ratio of samples was 1.8–1.9, indicating no significant protein contamination or degradation. The final concentration was diluted to 100 ng/μL.

2.4. Primer design and sequence synthesis
According to the *BPI* gene sequence in GenBank (Accession No. FP339579.2), primers were designed by Primer Premier 5.0 software with product length of 142 bp. The primer sequences were as follows: forward primer 5'-CCCAACATGGAGATGCAGTTC-3' and reverse primer 5'-CAATGAATCAATGAGCACACC-3'. All primers were synthesized by Shanghai Sangon Biological Engineering Technology (Shanghai, China).

2.5. PCR amplification
The PCR amplification system (total volume: 20 μL) was prepared as follows: 1 μL of template DNA (100 ng/μL), 10 μL of PCR Master Mix (2X), 1 μL of PCR forward primer (10 μM), 1 μL of PCR reverse primer (10 μM), and double-distilled (ddH$_2$O) to make up the final volume of 20 μL. The reaction conditions were 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s with an extension at 72 °C for 8 min and final preservation at 4 °C. PCR products were assayed in 1% agarose gel electrophoresis.

2.6. PCR-RFLP analysis
The RFLP-PCR reaction system (total volume: 10 μL) consisted of 2.5 μL of PCR products, 0.3 μL of *Hpa*II (5 U/μL), 1 μL of 10X buffer, and 6.2 μL of ddH$_2$O. PCR products were digested by *Hpa*II restriction enzyme at 37 °C for 3 h. The digested fragments were electrophoresed on 10% polyacrylamide gel in TBE (1X), then stained with silver and photographed under UV light. According to the result of PCR-RFLP analysis, individuals with different genotypes were selected for sequencing.

2.7. Determination of reproductive traits and immune indexes
As the reproductive traits of primiparous sows are unstable, the reproductive records of 2–4 parities of multiparous sows were collected and analyzed in this study. The reproductive traits included total litter size (TNB), number born alive (NBA), birth litter weight (BLW), and weaning litter weight (WLW).

The blood samples were collected from the anterior vena cava and serum was routinely separated. Fresh serum samples were processed using an ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA), and concentrations of cytokines (ng/L) IL-1β, IL-4, IL-6, IL-8, IL-10, TGF-1β, IFN-γ, and TNF-α in the serum were calculated by the standard curve.

2.8. Statistical analysis
The frequencies of gene and genotype were calculated by the formulas p = P+H/2, q = Q+H/2, and $\times^2$ = Σd2/e, in which d = e-o, and p and q represent the allele frequency. The chi-square fitness test was performed to analyze whether the allele frequency matches the Hardy–Weinberg equilibrium principle. The heterogeneity (He), homozygosity (Ho), effective number of alleles (Ne), and polymorphism information content (PIC) values were calculated using PopGene 32 software and PIC software. The relationships between genotypes of the *BPI* gene and reproductive traits and immune indexes were analyzed by least squares using the general linear model in SPSS 21.0. The results are shown as the least squares mean ± standard deviation. Statistical models are used to analyze gene effects including fixed effects (parity effects), *BPI* gene genotype effects, and random effects (random residual effects).

The following statistical model was used: yijk = μ+ Gi +Pj + eijk, where Y represents reproductive performance or immune indexes, μ represents the overall mean, Gi represents genotypic effect of the *BPI* gene, Pj represents parity effect, and eijk represents random residual error. These statistical analyses were carried out using SPSS 21.0 (IBM Corp., Armonk, NY, USA).

3. Results

3.1. Results of PCR amplification
In this study, the product length of the amplified fragment should be 445 bp. The PCR product was detected on 1.0% agarose gel electrophoresis, which confirmed the size of the predicted amplified fragment.

3.2. PCR-RFLP analysis for *BPI* gene
Exon 10 of the *BPI* gene has a *Hpa*II restriction site, which is located at chr5: 41378475 loci. After digestion of the PCR product by *Hpa*II, a total of three phenotypes were generated, which were wild AA-type (445 bp),

heterozygous AB-type (445 bp/304 bp/142 bp), and homozygous BB-type (304 bp/142 bp) (Figure 1).

3.3. Results of sequence analysis
The amplified PCR product fragments of AA, AB, and BB genotypes were sequenced and compared with the reference sequence using BioEdit software. The results showed that the AA type was consistent with the published sequence in GenBank (Accession No. FP339579.2) and was defined as the wild type (Figure 2). Comparing the BB genotype with the AA genotype, a missense mutation (Leu → Arg) occurred at chr5: 41378475 loci, which resulted
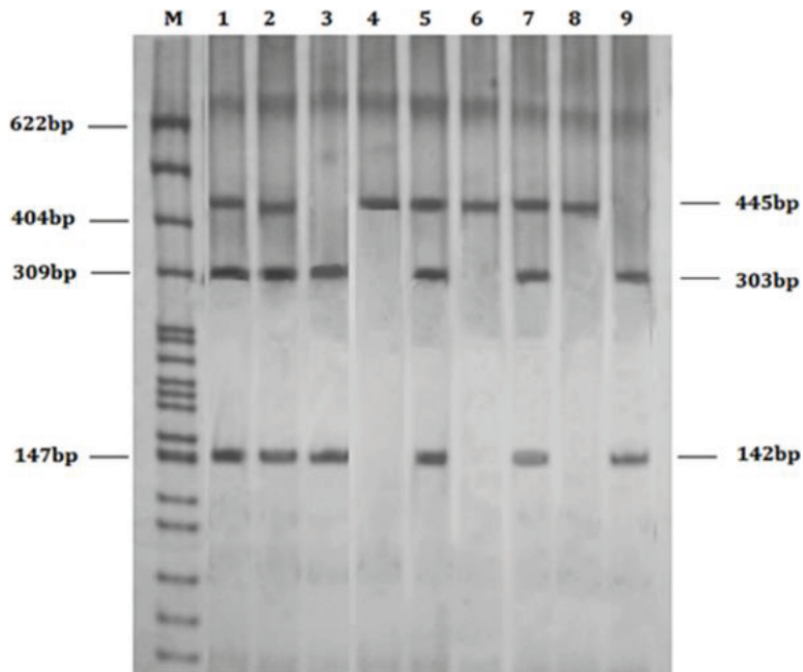

Figure 1. The genotyping results of *Hpa*II digestion in *BPI* gene exon 10 for Meishan pigs. Lanes 4, 6, and 8 = AA genotype; Lanes 1, 2, 5, and 7 = AB genotype; Lanes 3 and 9 = BB genotypes; Lane M = pBR322 DNA/*MSPI* (*Hap*II) marker.
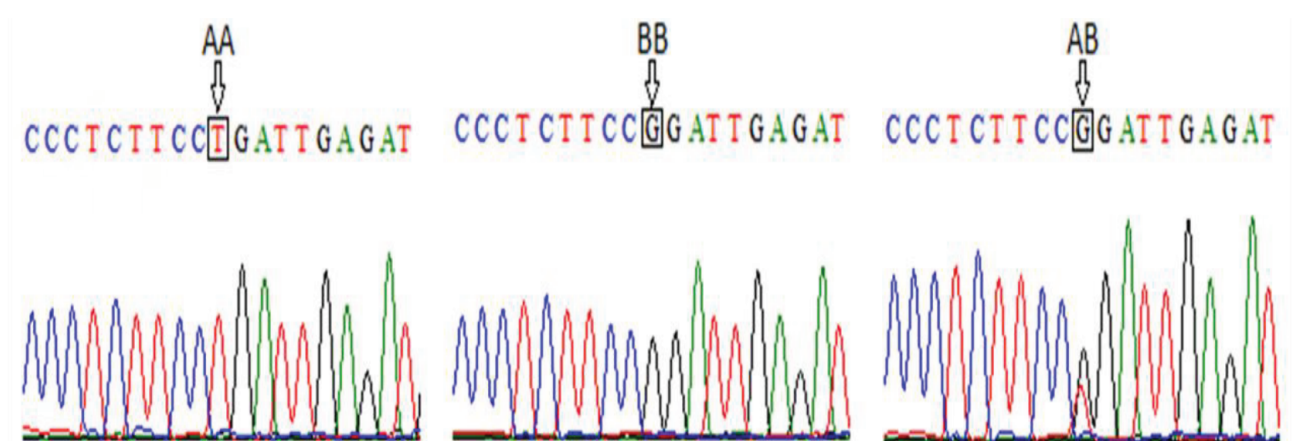

Figure 2. Sequencing plot of G/T mutation in AA, AB, and BB genotypes at chr5: 41378475 loci of *BPI* gene exon 10.

in the disappearance of the restriction recognition site. Therefore, the BB genotype was defined as the mutant type.

3.4. Genotype distribution and allele frequency of *BPI* gene in Meishan pigs

The PCR-RFLP method was used to detect the genotypes of Meishan pigs. The genotypic and allelic frequencies at the position of chr5: 41378475 loci (that is, the *Hpa*II restriction site in exon 10 of the *BPI* gene) were calculated according to Hardy–Weinberg equilibrium (Table 1). The results demonstrated that there were two alleles (A and B), and three genotypes (AA, AB, and BB) were detected after digestion with *Hpa*II in the *BPI* gene, of which A was the dominant allele (0.58). The frequencies of AA, AB, and BB genotypes were 0.23, 0.70, and 0.07, respectively. Chi-square (x^2) goodness-of-fit test results showed that the *Hpa*II polymorphism sites significantly deviated from Hardy–Weinberg equilibrium (P < 0.01). Genetic analysis (Table 2) showed that the PIC value of Meishan pigs at the mutation site was 0.367 (0.25 < PIC < 0.50), indicating moderate polymorphism.

3.5. Relationship between *BPI* gene polymorphisms and reproductive traits

The reproductive traits and cytokine levels of 3 multiparous sows were analyzed and the results were not significantly different, so they were combined for genotypic effect analysis. The correlation analysis indicated that the traits of total litter size, number born alive, birth litter weight, and weaning litter weight in Meishan pigs displayed the trend of AA > BB > AB at the mutation site in the *BPI* gene. The AA genotype was significantly higher than the AB genotype in total litter size (P < 0.05) and extremely significantly higher in number born alive and weaning litter weight (P < 0.01). The birth litter weight showed no significant differences among the three genotypes (P> 0.05). Moreover, allele A showed a positive effect on all reproductive traits, and allele B had the opposite effect in multiparous sows (Table 3).

3.6. Relationship between *BPI* gene polymorphism and immune indexes

The relationship between *BPI* gene polymorphisms and immune indexes is shown in Table 4. Individuals with the AA genotype had significantly higher TGF-1β and TNF-α than those individuals with the BB genotype (P < 0.05). In addition, IFN-γ was significantly higher in individuals with the AA genotype than the AB genotype (P < 0.05). The other immune indexes showed no significant differences among individuals with different genotypes (P > 0.05).

4. Discussion

Recent studies have reported the associations of the porcine *BPI* gene and disease resistance. *BPI* is an integral part of the natural defense system, and its functional amino acid fragment has nonspecific inhibitory and killing effects against gram-negative bacteria, especially *Escherichia coli*, *Salmonella*, *Proteus*, *Shigella dysenteriae*, and *Neisseria gonorrhoeae*. Therefore, the *BPI* gene is an important molecular marker for disease resistance. *BPI* is the most powerful substance in neutralizing endotoxin. It not only binds to free LPS, but also has high affinity to LPS on the cell membrane. The binding of BPI with LPS can promote bacterial killing inside and outside the cell and competitively inhibits the binding of LPS to lipopolysaccharide binding protein, which ultimately reduces LPS-induced local and systemic inflammatory responses (13). BPI protein represents the core component of innate immunity, directly fights against microbial infection, and regulates the subsequent adaptive responses (14). Considering the important biological functions of the porcine *BPI* gene, it has attracted extensive attention as an important candidate gene for disease-resistant breeding.

In this study, the polymorphism distribution of exon 10 in the *BPI* gene was detected in Meishan pigs. There were 3 genotypes, of which A was the predominant allele, which is consistent with the results of Wu et al. (15). The suitability test results showed that *Hpa*II polymorphisms of exon 10 in the *BPI* gene deviated from Hardy–Weinberg equilibrium (P < 0.01). This indicated that the Meishan pigs retained a genotype after long-term artificial selection, which has a significant effect on the distribution of polymorphisms in the genetic variation of the population. The PIC value showed that the *BPI* gene carried moderate polymorphism in Meishan pigs. Meanwhile, the population was rich in genetic diversity and had strong adaptability to the external environment, which was beneficial to the development of genetic breeding.

Table 1. Genotype and allele frequency of *BPI* gene at chr5: 41378475 loci in Meishan pigs.

Gene	Sample size	Genotype frequency			Allele frequency		x^2 values
		AA	AB	BB	A	B	
BPI	137	0.23 (31)	0.70 (96)	0.07 (10)	0.58	0.42	25.95**

** Significant (*P* < 0.01); x^2 test value indicates that the distribution of different genotypes is in Hardy–Weinberg equilibrium; $x^2_{0.05}$ (df = 1) = 3.841; $x^2_{0.01}$ (df = 1) = 6.635.

Table 2. Hereditary character of *BPI* gene at chr5: 41378475 loci in Meishan pigs.

Gene	Ho	He	Ne	PIC
BPI	0.513	0.487	2.052	0.367

Ho: Observed homozygosity; He: expected heterozygosity; Ne: number of effective alleles; PIC: polymorphism information content.

Until now, there have been few reports on the effects of *BPI* polymorphisms on reproductive traits. As the reproductive traits of primiparous sows are not stable, the reproduction traits of multiparous sows are more valuable. Our results showed that polymorphisms of exon 10 in the *BPI* gene have a significant genetic effect on the reproductive traits of multiparous sows, and the detected individuals were significantly affected by the additive effect on the traits of total number born and number born alive and weaning litter weight. AA was a favorable genotype, individuals of which were significantly higher in number than those with the AB genotype ($P < 0.05$), and also higher than those with the BB genotype ($P > 0.05$). Therefore, the results further support that polymorphisms of exon 10 of the *BPI* gene can be used as potential genetic markers for reproductive traits in Meishan pigs.

Many studies have proven that the polymorphisms of the porcine *BPI* gene are associated with specific disease resistance. Liu et al. (16) found that the *Hpa*II restriction site of exon 10 in the *BPI* gene was closely related to resistance to *E. coli* F18, wherein the AA genotype had

Table 3. Effects of *BPI* gene polymorphisms on the reproductive traits in Meishan pigs.

Parities	Genotypes	TNB	NBA	BLW	WLW
2–4 parities	AA (31)	13.95 ± 2.14[a]	12.43 ± 3.02[A]	13.24 ± 2.40	89.04 ± 30.82[A]
	AB (96)	12.99 ± 2.68[b]	10.39 ± 3.55[B]	12.56 ± 2.49	72.51 ± 29.96[B]
	BB (10)	13.54 ± 2.96[ab]	11.93 ± 3.94[AB]	13.20 ± 1.83	80.00 ± 39.34[AB]
	a	−0.205	−0.250	0.020	−4.520
	d	−0.755	−1.790	−0.660	−12.01
	A	0.196	0.327	0.055	3.859
	B	−0.139	−0.231	−0.039	−2.726

[a,b] Different superscripts within the same column represent significant differences ($P < 0.05$). [A,B] Different superscripts within the same column represent extremely significant differences ($P < 0.01$).
a represents addictive effect; d represents dominant effect; A represents A allele effect; B represents B allele effect.

Table 4. Relationship between polymorphism of exon 10 of *BPI* gene and partial immune indexes in Meishan pigs.

Immune indexes (ng/L)	Genotypes		
	AA (31)	AB (96)	BB (10)
IL-1β	188.67 ± 47.39	171.88 ± 43.81	127.52 ± 8.55
IL-4	189.79 ± 39.26	182.24 ± 45.52	144.56 ± 10.20
IL-6	178.53 ± 49.18	182.26 ± 60.61	218.87 ± 130.93
IL-8	44.86 ± 11.21	48.94 ± 13.07	31.33 ± 3.00
IL-10	178.51 ± 38.26	182.39 ± 36.99	136.21 ± 27.07
TGF-1β	580.32 ± 180.42[a]	497.21 ± 156.82[ab]	297.79 ± 138.01[b]
IFN-γ	318.38 ± 54.36[a]	274.41 ± 59.34[b]	318.34 ± 83.37[ab]
TNF-α	149.14 ± 36.09[a]	123.09 ± 39.20[ab]	88.16 ± 14.53[b]

[a,b] Different superscripts within the same row represent significant differences ($P < 0.05$).

a higher immune response capability, which may be a potential molecular marker for piglets against *E. coli* F18. Meanwhile, Ye et al. (17) further reported that high expression of the AA genotype is in connection with special disease resistance by detecting *BPI* gene expression in Sutai pigs with different genotypes, consistent with the results of Liu et al. (16). With respect to genetic variation analysis of the exon 10 polymorphisms of the *BPI* gene among different pig breeds, the results suggested that individuals with the AA genotype may have high immune response ability against gram-negative bacteria. Moreover, no significant differences were observed between varieties and genetic backgrounds in Chinese and foreign pig breeds for this mutation site (18). The polymorphic analysis at 103 loci of exon 4 in the *BPI* gene in multiple pig breeds revealed that GG was the favorable genotype and individuals with allele G had higher disease resistance to *Salmonella choleraesuis* than those with allele A at this site (19). Cytokines are not only widely involved in antiinflammatory reactions but also regulate homeostasis mechanisms and can effectively resist pathogenic infections. Their levels can reflect the general disease resistance of the body.

Transforming growth factor-1β (TGF-1β) is an antiinflammatory cytokine, which is secreted by mononuclear macrophages induced by LPS, and regulates most immune functions. Many studies have confirmed that TGF-1β is the strongest endogenous inhibitor of lymphocyte proliferation. It can also inhibit killer cells' formation, prevents the body's immune system's excessive proliferation and activation, and plays an important role in immune system regulation and tolerance (20). The reduction of TGF-1β release can spontaneously induce the activity of the porcine TGF-1β-damaged part and promote granulation tissue formation, and ultimately heal the wound effectively (21). Meanwhile, TGF-1β is a key factor in maintaining intestinal homeostasis and regulating the intestinal immune system (22). In our study, the TGF-1β of individuals with the AA genotype was significantly higher than that of BB (P < 0.05), which further demonstrated that the AA genotype may be more favorable in coping with trauma repair and intestinal immune system regulation. Tumor necrosis factor alpha (TNF-α), an important cytokine that maintains homeostasis and resists pathogenic bacteria invasion, is indispensable for inflammatory response and immune response. TNF-α produces an antitumor immune response by regulating B-cell-mediated tumor suppression activity (23). TNF-α may also modulate type I immune activation by suppressing the proliferation of CD4 and CD8 T cells, and inhibit the occurrence of harmful immune responses (24). In this study, the TNF-α levels in individuals with the AA genotype were significantly higher than those with the BB genotype (P < 0.05), indicating that the AA genotype had stronger immune response and antiinflammatory activity than the AB and BB genotypes. Interferon gamma (IFN-γ)

is a cytokine with biological functions of antivirus and antitumor, which plays an important role in increasing the expression of MHC-class I and MHC-class II, promoting the differentiation of T and B cells, and activating mononuclear macrophages during immune responses (25,26). Additionally, porcine IFN-γ is an important component to exert its immune function, which can effectively inhibit infectious viruses (e.g., FMDV, PRRSV, ASFV) that cause huge economic losses in the pig industry (27,28). In this study, IFN-γ levels were significantly higher in individuals with the AA genotype compared with the AB genotype (P < 0.05) and BB genotype (P > 0.05). The results indicated that the AA genotype was more favorable for immune regulation and antiviral capability than the AB and BB genotypes. Interleukins (ILs) are an indispensable part of the immune system. Under normal physiological conditions, they reflect the comprehensive immune response capability of body, and are also characteristic of general disease resistance. In this study, no significant difference in IL levels was found between individuals with the AA genotype and those with AB and BB genotypes (P > 0.05). To some extent, the results suggested that molecular breeding for this mutation site will increase the body's disease resistance and will not have a significant impact on the general disease resistance regulated by the body's interleukins.

In the processes of pig breeding, it is necessary to pay attention to the potential negative effects on important economic traits when enhancing the group's disease resistance via molecular marker-assisted selection. Based on the effects of the polymorphisms of exon 10 in the *BPI* gene on reproduction traits and immune indexes, the findings suggest that increasing the frequency of allele A may improve the body's specific disease resistance to some extent and not impact general disease resistance regulated by other cytokines. The influence of this mutation site will also contribute to the improvement of important economic traits. Therefore, it is feasible to use the polymorphisms of exon 10 in the *BPI* gene as genetic markers for molecular breeding of Meishan pigs. Thus, in-depth systemic studies for this mutation site should be carried out with a larger sample size. As the important economic traits may be regulated by multiple genes or pathways, more genetic studies on reproductive traits are needed to provide reliable guidance for disease-resistant breeding of Meishan pigs.

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