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Association between the *MUC4* g.243A > G polymorphism and immune and production traits in Large White pigs

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Abstract: PCR-RFLP analysis of intron 17 of the *MUC4* gene was conducted in Large White pigs and associations with cytokine levels (IFN-γ, IL-1β, IL-4, IL-6, IL-8, IL-10, TGF-β, and TNF-α), production traits (100-kg-weight day age, back-fat depth, and eye muscle depth), and reproductive performance within different litters (total number born, number born alive, number in live weaning litter) were analyzed with the aim of identifying genetic markers of disease resistance. The results revealed AA, AG, and GG genotypes, with an A243G mutation identified in the GG genotype. Correlation analysis indicated that IL-8 and IL-10 levels and 100-kg-weight day age were significantly higher in the GG genotype compared with AA and AG genotypes ($P < 0.05$). No significant differences were detected in IL-8 and IL-10 levels or 100-kg-weight day age for AA and AG genotypes. The levels of other cytokine levels and reproductive performance from the first to fourth litter did not significantly differ among the 3 genotypes ($P > 0.05$). These data indicate that *MUC4* g.243A>G may enhance the antidisease capability and affect the growth rate of groups in the fattening period of Large White pigs without detrimental influences on resistance to disease, other production traits, and reproductive performance.

Key words: Swine, *MUC4* gene, genetic marker, immunity index, economic traits

1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) expressing the F4 (previously known as K88) fimbriae is a major cause of diarrhea in neonatal, preweaned, and postweaned piglets, which leads to considerable economical loss in the pig industry. The bacteria use fimbriae to adhere to specific receptors on brush borders of enterocytes of the small intestine. Colonizing bacteria secrete the deleterious enterotoxins that cause an increased secretion of electrolytes into the lumen. Subsequently, water flows into the lumen, resulting in diarrhea. Therefore, the pathogenicity of ETEC F4 in piglets is determined by the presence of its corresponding receptors in the brush border membrane of mucosal epithelial cells in their small intestines (1,2). It has been suggested that ETEC F4 resistance is inherited as an autosomal recessive trait (3,4). Three antigenic variants of F4 have been described: F4ab, F4ac, and F4ad, of which F4ac is the most prevalent (5).

Mucin 4 (MUC4) is a large membrane-bound O-glycoprotein that is abundant on the surface of gastrointestinal epithelial cells; it plays an important role in the lubrication and protection of mucosa, growth, fetal development, epithelial renewal and differentiation, epithelial integrity, carcinogenesis, and metastasis (6,7). The Fab/Fac receptor (F4bcR) locus has been mapped on porcine chromosome 13q41, in a region where the *mucin 4* (*MUC4*) gene is located (8,9). Studies suggested that the *MUC4* gene should be considered as one of the most promising candidate genes for F4abR/F4acR (10–13). Associations of genetic variation of the *MUC4* gene with susceptibility/resistance to ETEC F4 infection were reported. Joller et al. (14) reported that *XbaI* polymorphism in intron 7 of *MUC4* was shown to be in strong linkage disequilibrium with the ETEC F4ac receptor locus in pigs. Peng et al. (15) showed that the g.243A > G mutation in intron 17 of *MUC4* is significantly associated with susceptibility/resistance to ETEC F4ab/ac infection in pigs.

It is hoped that produced groups can resist infection while other forms of production performance remain stable or increase in the pigs, breeding resistance to disease. Based on important candidate genes and their molecular markers in molecular breeding to improve group special disease resistance, it is necessary to pay attention to the impact of marker-assisted selection of groups on general disease resistance and other important economic traits.

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Previous studies have shown that the *MUC4* g.243A > G mutation was strongly associated with ETEC F4ab/ac. Meanwhile, Balcells et al. (16) found that the g.243A > G mutation in intron 17 of *MUC4* was not associated with total number born (TNB) or number born alive (NBA). In this study, polymorphisms in intron 17 of the *MUC4* gene were identified by PCR-RFLP analysis and cytokine levels, growth rate, back-fat depth, eye muscle depth, and reproductive performance were determined from the first to fourth litter. The associations of polymorphisms in intron 17 of *MUC4* with general disease resistance index and economic traits were then analyzed based on these parameters in order to determine an experimental basis for marker-assisted selection in Large White pigs.

2. Materials and methods

2.1. Experiment material

Ear notches of 276 healthy Large White pigs were collected from Changzhou City Kangle Farming Co., Ltd. (Jiangsu Province, China). Approximately 1.0 g of each ear tissue sample was placed into a 1.5-mL Eppendorf tube in an ice box and transported to the laboratory for genomic DNA extraction according to a modified phenol and chloroform method (17).

2.2. PCR-RFLP analysis

Two primers (5′-TCTAAAGATGCTGGTGCTAC-3′ and 5′-CTGGCTGTATTTCTGTTGTG-3′) were designed according to the partial sequence of the *MUC4* gene in GenBank (Accession No. DQ124298.1) and synthesized by the Shanghai Biotechnology Co., Ltd., to produce a fragment of approximately 220 bp in length. The PCR system (total volume: 20 µL) consisted of 100 ng of genomic DNA, 5 pmol each primer, 10X PCR buffer, 2 mM dNTP mixture, and 1 U of Taq DNA polymerase (TaKaRa Biotechnology Dalian Co., Ltd., China). Thermal cycling was performed as follows: PCR at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 30 s; a final extension at 72 °C for 10 min; and preservation at 4 °C. PCR products (10 µL) were digested by *Hha*I (5 U/µL) overnight at 37 °C. The digested fragments were electrophoresed in 10% polyacrylamide gels in 1X TBE at a constant voltage of 120 V, silver-stained, and visualized under ultraviolet light. PCR products of homozygotic genotypes were purified with a Gel Extraction Kit (TaKaRa Biotechnology Dalian Co.) and sequenced using the ABI PRISM 377 DNA autosequencer by the Shanghai Biotechnology Co., Ltd.

2.3. Determination of cytokine levels, production traits and reproductive performance

Precaval venous blood from just-weaned piglets was collected in 50 mM EDTA at pH 8.0 to prevent coagulation. Fresh serum cytokine levels (IFN-γ, IL-1β, IL-4, IL-6, IL-8, IL-10, TGF-β, and TNF-α; pg/µL) were determined using the Procarta immunoassay kit according to the manufacturer's instruction from Affymetrix Inc. (Santa Clara, CA, USA). The 276 living pigs were scanned to measure their back-fat depth and eye muscle depth by B-mode ultrasound machine. Meanwhile, reproductive performance (TNB, NBA, and number alive in the weaning litter (NW)) was determined from the first to fourth litter by Changzhou City Kangle Farming Co., Ltd.

2.4. Statistical analysis

Allele and genotype frequencies were calculated according to the Hardy–Weinberg equilibrium principle, which is based on the difference between predicted and detected values ($p = P + H/2$, $q = Q + H/2$, where p and q represent the allele frequency at a certain position; $x^2 = \sum d^2/e$; $d = e - o$). A general linear model was established to analyze the genotype effects of the *MUC4* gene on immunity index and economic traits using $y_{ij} = \mu + G_i + e$, where y_{ij} represents immunity index or economic traits, µ represents the overall mean, G_i represents the genotypic effect of the *MUC4* gene, and e represents the residual error. These statistical analyses were carried out using SPSS 16.0.

3. Results

3.1. PCR amplification

PCR products were detected by 1% agarose electrophoresis. A clear specific DNA band was shown at position 220 bp, which was consistent with the predicted amplified fragment size.

3.2. PCR-RFLP analysis

The g.243A > G mutation in intron 17 of *MUC4* in the GG genotype came into being at the *Hha*I recognition site, rendering digestion possible and yielding 2 fragments of 166 bp and 54 bp. The AA genotype without an *Hha*I restriction recognition site, rendering digestion impossible, yielded a 220-bp fragment. Incomplete *Hha*I digestion of these alleles in AG heterozygotes yielded a mixture of these fragments (220 bp/166 bp/54 bp) (Figure 1).

3.3. Sequence analysis

PCR products of AA and GG homozygotes were purified with a gel extraction kit (TaKaRa Biotechnology Dalian Co.) and sequenced by the Shanghai Biotechnology Co., Ltd. Results indicated that the sequence in the AA genotype (defined as the wild type) was in accordance with that of the GenBank sequence, while that of the GG genotype (defined as the mutant type) exhibited an A > G mutation in intron 17 of the *MUC4* gene at position 243 (Figure 2).

3.4. Genotype and allele frequency analyses of *MUC4* g.243A > G in Large White pigs

Allele and genotype frequencies were calculated according to the Hardy–Weinberg equilibrium principle. Three

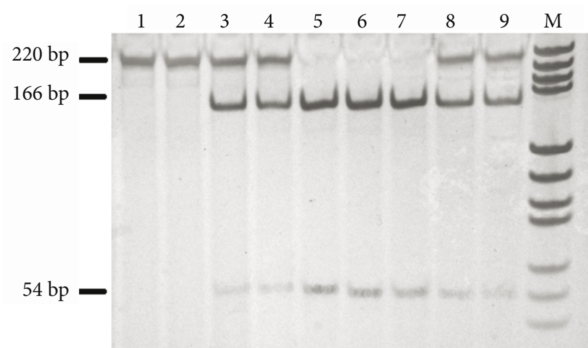

Figure 1. Polyacrylamide (10%) gel electrophoresis band patterns following complete *Hha*I digestion of *MUC4* gene intron 17. Lanes 3, 4, 8, 9: AG; lanes 5, 6, 7: GG; lanes 1, 2: AA; M: pBR322 DNA/*Bsu*RI marker.

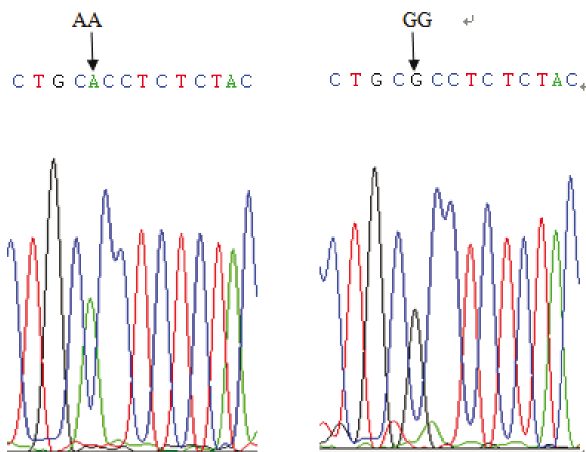

Figure 2. Sequence analysis of AA and GG genotypes. Arrows indicate the A > G mutation in the *Hha*I restriction site of *MUC4* gene intron 17.

genotypes and 2 alleles were detected, with A being the more frequent allele. Chi-square fitness analysis indicated that *Hha*I polymorphic sites in the *MUC4* gene were in Hardy–Weinberg equilibrium in Large White pigs ($P > 0.05$) (Table 1).

3.5. Change of cytokine levels in Large White pigs according to *MUC4* g.243A > G polymorphisms

Correlation analysis indicated that IL-8 and IL-10 levels were significantly higher in the GG genotype compared with the AA and AG genotypes ($P < 0.05$). No significant differences were detected in IL-8 and IL-10 levels for the AA and AG genotypes or the levels of other cytokine levels among the 3 genotypes ($P > 0.05$) (Table 2).

3.6. Change of production traits and reproductive performance of different litters in Large White pigs according to *MUC4* g.243A > G polymorphisms

Analysis results indicated that 100-kg-weight day ages were significantly higher in the GG genotype compared with the AA and AG genotypes ($P < 0.05$). No significant differences were detected in 100-kg-weight day ages of AA and AG genotypes or reproductive performance from the first to fourth litter among the 3 genotypes ($P > 0.05$) (Tables 3–5).

4. Discussion

Mucins are a group of epithelial cells, composed of membrane-bound and secretory glycoprotein of the macromolecular protein family. They play an important role in lubrication, protection of mucosa, and regulation of cell signal transduction. As a member of the sprawling transmembrane mucin family, *MUC4* also plays an extremely important role in lubrication, protection of surface epithelium, cell proliferation and differentiation of epithelial cells, and immune responses (7). It has been suggested that the *MUC4* gene has been mapped on porcine chromosome 13q41; meanwhile, the g.243A > G mutation in intron 17 of *MUC4* is significantly associated with susceptibility/resistance to ETEC F4ab/ac infection in pigs, and allele g.243G is predominant in those resistant animals, indicating that this polymorphism is in a significant linkage disequilibrium with the ETEC F4ab/ac receptor locus (15). Studies suggested that the *MUC4* gene should be considered as one of the most promising candidate genes for F4abR/F4acR (10–13).

In this study, we identified 3 genotypes and 2 alleles, with A being the dominant allele in Large White pigs. These results were consistent with those of Peng et al. (15).

Table 1. Genotype and allele frequency analysis of *MUC4* g.243A > G in Large White pigs.

Samples	Genotype frequency			Allele frequency		χ^2 value
	AA	AG	GG	A	G	
276	0.42(116)	0.46(128)	0.12(32)	0.65	0.35	0.07

Notes: χ^2 test value indicates that the different genotypes are in Hardy–Weinberg equilibrium: $\chi^2 0.05(1) = 3.84$, $\chi^2 0.01(1) = 6.63$.

Table 2. Change of cytokine levels in Large White pigs according to *MUC4* g.243A > G polymorphisms.

Cytokine levels (pg/μL)	Genotype		
	AA (n = 116)	AG (n = 128)	GG (n = 32)
IFN-γ	7.475 ± 0.159	7.203 ± 0.117	7.286 ± 0.269
IL-1β	17.375 ± 0.477	16.878 ± 0.351	16.286 ± 0.806
IL-4	9.375 ± 0.334	9.054 ± 0.246	8.714 ± 0.565
IL-6	25.250 ± 2.027	23.297 ± 1.490	23.643 ± 3.426
IL-8	36.550 ± 4.126[a]	39.365 ± 3.034[a]	58.571 ± 6.974[b]
IL-10	7.025 ± 0.388[a]	7.311 ± 0.285[a]	8.857 ± 0.655[b]
TGF-β	10.075 ± 0.843	10.527 ± 0.620	10.571 ± 1.425
TNF-α	9.675 ± 0.349	9.649 ± 0.257	9.571 ± 0.590

Note: Means with different superscripts within the same column differ significantly ($P < 0.05$).

Table 3. Change of production traits in Large White pigs according to *MUC4* g.243A > G polymorphisms.

Genotype	Production traits		
	100-kg-weight day age	Back-fat depth	Eye muscle depth
AA (n = 116)	190.38 ± 11.92[a]	9.80 ± 2.02	5.97 ± 0.65
AG (n = 128)	190.80 ± 12.94[a]	9.32 ± 1.97	6.01 ± 0.58
GG (n = 32)	202.37 ± 12.42[b]	8.74 ± 2.04	6.10 ± 0.80

Note: Means with different superscripts within the same column differ significantly ($P < 0.05$).

Table 4. Change of reproductive performance for the first and second litters in Large White pigs according to *MUC4* g.243A > G polymorphisms.

		Genotype		
		AA (n = 116)	AG (n = 128)	GG (n = 32)
First litter	TNB	10.48 ± 2.15	10.23 ± 1.66	10.94 ± 1.84
	NBA	10.16 ± 2.17	10.02 ± 1.70	10.75 ± 1.98
	NW	9.24 ± 1.53	9.20 ± 1.64	9.56 ± 1.41
Second litter	TNB	11.14 ± 1.76	10.91 ± 1.79	11.87 ± 1.75
	NBA	10.97 ± 1.78	10.77 ± 1.70	11.69 ± 1.45
	NW	9.86 ± 1.59	9.69 ± 1.37	10.00 ± 1.27

TNB: Total number born; NBA: number born alive; NW: number alive in the weaning litter.

Table 5. Change of reproductive performance for the third and fourth litters in Large White pigs according to *MUC4* g.243A > G polymorphisms.

		Genotype		
		AA (n = 116)	AG (n = 128)	GG (n = 32)
Third litter	TNB	11.45 ± 2.05	11.08 ± 1.88	10.94 ± 1.91
	NBA	11.21 ± 2.06	10.89 ± 1.90	10.81 ± 1.80
	NW	9.86 ± 1.28	9.78 ± 1.20	9.75 ± 0.93
Fourth litter	TNB	10.76 ± 1.87	11.03 ± 1.83	11.12 ± 1.71
	NBA	10.40 ± 1.79	10.78 ± 1.90	11.00 ± 1.63
	NW	9.31 ± 1.48	9.47 ± 1.17	9.31 ± 0.95

TNB: Total number born; NBA: number born alive; NW: number alive in the weaning litter.

Chi-square fitness analysis showed that polymorphisms in the *Hha*I restriction site of the *MUC4* gene were in Hardy–Weinberg equilibrium in Large White pigs (P > 0.05), indicating that long-term artificial selection had no influence on the frequency of the *MUC4* gene in Large White pigs.

Based on important candidate genes and their molecular markers in molecular breeding to improve groups' special disease resistance, it is necessary to pay attention to the impact of marker-assisted selection of groups on general disease resistance and other important economic traits. Fontanesi et al. (18) suggested that the g.8227C > G mutation was associated with average daily gain and back-fat depth. Balcells et al. (16) reported that the g.243A > G mutation in intron 17 of *MUC4* was not associated with TNB or NBA in an Iberian × Meishan F2 population. To date, associations of the g.243A > G mutation in intron 17 of *MUC4* with the body's immune response ability and important economic traits have not been reported. Cytokines, used as signal protein composed of a set of proteins and peptides, play an important role in innate and adaptive immune response, indirectly reflected in the body's immune response ability and general disease resistance. In this study, analysis results indicated that IL-8 and IL-10 levels were significantly higher in the GG genotype compared with the AA and AG genotypes (P < 0.05), suggesting strong antidisease capability in the GG genotype individuals. However, no significant differences were detected in IL-8 and IL-10 levels for AA and AG genotypes or the levels of other cytokine levels among the 3 genotypes (P > 0.05). These observations indicated that selective molecular breeding based on the genotype will have no detrimental effects on resistance to disease, but might enhance the antidisease capability of Large White pigs. The study also indicated that 100-kg-weight day ages were significantly higher in the GG genotype compared with the AA and AG genotypes (P < 0.05), suggesting that GG genotype individuals grow slower in the fattening period. No significant differences were detected in 100-kg-weight day ages of AA and AG genotypes or reproductive performance from the first to fourth litter among the 3 genotypes (P > 0.05). These observations also indicated that this type of selective molecular breeding will not have a negative influence on other production traits and reproductive performance, but might affect growth rate of the groups in a way.

The results of this study suggest that the genetic effects of g.243A > G polymorphisms in intron 17 of the *MUC4* gene should be further studied, focusing on investigation of associations with important immune indexes, production traits, reproduction performance, and *MUC4* gene expression. This information will provide a reliable basis for molecular breeding strategies for the generation of disease resistance in Large White pigs.

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