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Association of *Mx1* gene polymorphism with some economic traits in Meishan pigs

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Abstract: This study was conducted to determine the association of polymorphisms in exon 14 of the *Mx1* gene with significant economic traits in Meishan pigs, with the aim of identifying genetic markers of disease resistance. PCR-RFLP analysis of exon 14 of the *Mx1* gene was conducted and associations with some cytokine levels (IFN-α, IFN-γ, IL-2, IL-6, IL-10, and IL-12), early body weight (birth weight, 20-day weight, and 40-day weight), and reproductive performance within different parities (total number born, number born alive, number in live weaning litter, birth weight, and weaning weight) were analyzed. The results showed that there were AA, AB, and BB genotypes defined, while a G36T mutation was identified in BB genotype individuals of Meishan pigs. Correlation analysis indicated that the IFN-γ levels were significantly higher in the AA genotype than the AB genotype ($P < 0.05$). However, there were no significant differences in the levels of other cytokines, early body weight, and reproductive performance among the three genotypes. These data indicated that selective molecular breeding practices may enhance the antiviral capability of Meishan pigs without detrimental influences on early body weight, development, and reproductive performance.

Key words: *Mx1* gene, pig, genetic marker, cytokine level

1. Introduction

The myxovirus resistance protein (Mx) is the key component of the antiviral state induced by type I (α/β) interferon (IFN). Resistance or susceptibility to myxovirus is inherited as a single autosomal characteristic controlled by the $Mx1^+$ or $Mx1^-$ allele, respectively (1). Experiments in vivo and in vitro have indicated that *Mx1* gene expression is normally silent but rapidly induced by infection with a variety of viruses through the action of virus-induced type I (α/β) IFN. This effect has been shown to protect infected animals from severe influenza and death (2,3).

The porcine *Mx1* gene is located on SSC13, containing an open reading frame of 663 amino acids and exhibiting high homology to the human *MxA* (myxovirus resistance protein A) gene (4). Horisberger (5) reported that Mx proteins in porcine cells inhibited the growth of influenza virus, vesicular stomatitis virus (VSV), and mengovirus. Furthermore, Zhang et al. (6) indicated that porcine *Mx1* expression was induced by infection with the porcine reproductive and respiratory syndrome virus. Morozumi et al. (7) identified two novel polymorphisms in exon 14 of porcine *Mx1* by DNA sequencing and confirmed their presence in different breeds. Wu et al. (8) identified six genotypes and three alleles based on polymorphisms in exon 14 of the *Mx1* gene in wild boar and 16 native and exotic pig breeds. To date, associations between polymorphisms in exon 14 of porcine *Mx1* and economic traits have been extensively reported (9,10).

Pigs are important vectors in transmission of the influenza virus between animals and humans. The *Mx* gene has antiviral properties. Consequently, investigation of genetic variation in the pig *Mx1* gene is likely to yield important information regarding strategies for controlling the spread of the influenza virus. Meishan pigs, known for high fertility and strong disease resistance, are not only outstanding local varieties in China but have also played an important role in cultivation of varieties in the world. Kunshan City Meishan Pigs Breeding Conservation Co., Ltd. is an important national conservation base in China. In this study, polymorphisms in exon 14 of the *Mx1* gene were identified by PCR-RFLP analysis and cytokine levels, early body weight, and reproductive performance were determined for the third and fourth parities. The association between polymorphisms in exon 14 of *Mx1* and economic traits were analyzed for further marker-assisted selection of Meishan pigs in the future.

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2. Materials and methods

2.1. Experiment material

The ear notches of 136 healthy Meishan pigs were collected from Kunshan City Meishan Pigs Breeding Conservation Co., Ltd. (Lujia, Kunshan, Jiangsu Province, China). Approximately 1.0 g of fresh ear tissue sample was placed into a 1.5-mL Eppendorf tube in ice box and transported to the laboratory for genomic DNA extraction according to a modified phenol and chloroform method (11). The 1% agarose gel electrophoresis and NanoDrop-1000 spectrophotometer detected the purity and concentration of the DNA, respectively. The genomic DNA was diluted to 100 ng/μL and then stored at –20 °C.

All experiments were conducted in the Animal Hospital of Yangzhou University according to the regulations for the administration of affairs concerning experimental animals (Ministry of Science and Technology, China, revised in June 2012) and approved for an experimental animal usage permit, No. SYXK (Su) 2012-0029.

2.2. Determination of early body weight and reproductive performance

The important parameters determined in this study were as follows: early body weight (birth weight, 20-day weight and 40-day weight) (kg) and reproductive performance for the third and fourth parities (total number born (TNB), number born alive (NBA), number alive in the weaning litter (NW), birth weight, and weaning weight).

2.3. PCR-RFLP analysis

Two primers (5'-TGAAGGAGCGGCTGATGC-3' and 5'-GGCGGGGCTCATTCAAGT-3') were designed according to the partial sequence of the *Mx1* gene in GenBank (Accession No. AB164037) (12) and synthesized by the Shanghai Biotechnology Co., Ltd. (Shanghai, China), to produce a fragment of approximately 290 bp in length. The PCR reaction system consisted of 2.5 μL of 10X PCR buffer, 2.2 μL of $MgCl_2$, 1.5 μL of 2.5×10^3 μM/L dNTPs, 1 μL of 10 μM/L primers (upstream), 1 μL of 10 μM/L primer (downstream), 0.2 μL of Taq enzyme (5 U/μL) (TaKaRa Biotechnology Dalian Co., Ltd., Liaoning Province, China), 1 μL of 100 ng/μL DNA template, and 10.6 μL of ddH_2O; the total volume was 20 μL. Thermal cycling was performed as follows: PCR was carried out at 95 °C for 5 min, with 30 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 45 s, and extension at 72 °C for 45 s, and a final extension at 72 °C for 10 min and preservation at 4 °C. The PCR-products were detected by 1% agarose gel. The PCR products (10 μL) were digested by *Hin*6I enzyme (5 U/μL) 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. The PCR products of homozygotic genotypes were purified with a Gel Extraction Kit (TaKaRa Biotechnology Dalian Co., Ltd.) and sequenced using the ABI PRISM 377 DNA autosequencer of the Shanghai Biotechnology Co., Ltd. (Shanghai, China).

2.4. Determination of cytokine levels

Precaval venous blood from just-weaned piglets (35 days) was collected in 50 mM/L EDTA at pH 8.0 to prevent coagulation. Blood was held for 30 min at 4 °C, then centrifuged at 4 °C followed by centrifugation at 1000 rpm to separate serum. Fresh serum cytokine levels (IFN-α, IFN-γ, IL-2, IL-6, IL-10, and IL-12) (pg/μL) were determined using the Procarta immunoassay kit (Affymetrix Inc., Santa Clara, CA, USA).

2.5. Statistical analysis

Chi-square fitness tests were used to compare the observed numbers of each genotype with those expected for the population using Hardy–Weinberg equilibrium. An analysis of the genotypic effects of the *Mx1* gene was carried out using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). The fixed model was $Y_{ijk} = \mu + B_i + S_j + G_k + e_{ijk}$, where Y_{ijk} represents the observed value of individual from the breed i, μ represents the least square means of the observed values, B_i represents the effective value of breed I, S_j represents the effective value of the sex j, G_k represents the genotypic effect of the *Mx1* gene, and e_{ijk} represents the residual effect corresponding to the observed value.

3. Results

3.1. PCR-RFLP and sequence analysis

Complete digestion of the *Hin*6I restriction recognition site in exon 14 of the *Mx1* gene in the AA genotype yielded two fragments of 255 bp and 35 bp. The G →T mutation at this site in the BB genotype destroyed the *Hin*6I recognition site, rendering digestion impossible and yielding a 290-bp fragment. Complete *Hin*6I digestion of these alleles in AB heterozygotes yielded a mixture of these fragments (255 bp/35 bp/290 bp) (Figure 1). Sequencing results indicated

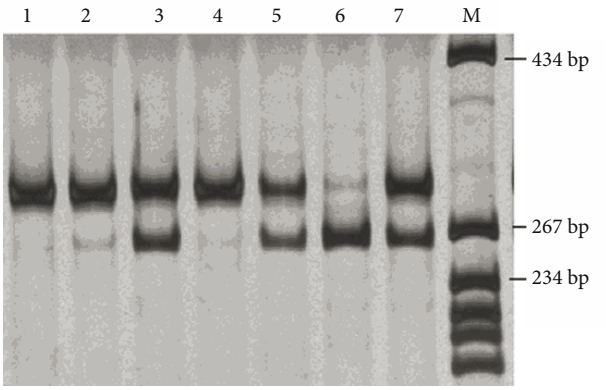

Figure 1. Polyacrylamide (10%) gel electrophoresis band patterns following complete *Hin*6I digestion of *Mx1* gene in exon 14. Lanes 1, 2, 4 – BB; lanes 3, 5, 7 – AB; lane 6 – AA; M – pBR322DNA/*Bsu*RI marker.

that the sequence of the restriction enzyme recognition site in the AA genotype (defined as the wild type) was in accordance with that of the GenBank sequence, while that of the BB genotype (defined as the mutant type) exhibited a G > T mutation in exon 14 of the *Mx1* gene at position 36 (Figure 2).

3.2. Genotype and allele frequency analyses of exon 14 of *Mx1* gene

The genotype (allele) frequency and chi-square fitness test were calculated according to Hardy–Weinberg equilibrium (Table 1). Among the 136 Meishan pigs, 48 showed the AA genotype, 52 showed the AB genotype, and 36 showed the BB genotype at *Mx1* exon 14 site. The frequencies of the AA, AB, and BB genotypes were 0.35, 0.38, and 0.27, respectively, and allele A was the dominant allele. Chi-square fitness analysis indicated that *Hin*6I polymorphic sites in the *Mx1* gene were in Hardy–Weinberg equilibrium in Meishan pigs.

3.3. Association analysis of *Mx1* gene with cytokine level, early body weight, and reproductive performance

There were no significant differences in the performance of 136 Meishan pigs from 10 lineages, so these pigs were analyzed without partition. For some cytokines and early body weight, the association analysis indicated that IFN-γ levels were significantly higher in the AA genotype compared with AB ($P < 0.05$). No significant differences were detected in the levels of other cytokines and early body weight among the three genotypes (Table 2). For reproductive performance, the association analysis indicated there were no significant differences in reproductive performance of the third and fourth parities among these three genotypes (Tables 3 and 4).

4. Discussion

In this study, we identified three genotypes and two alleles of *Mx1* gene, with A being the dominant allele in Meishan pigs. These results were consistent with those of Wu et al. (8) and Zhao et al. (13). Moreover, the result of chi-square analysis indicated that long-term artificial selection had no influence on the frequency of the *Mx1* gene in Meishan pigs. Additionally, the effects of *Mx1* gene variation on virus infection have been reported. Native antiviral specificity based on the Mx protein is affected by amino acid variations at position 631 in chickens. The genotype with Asn at position 631 corresponds to the positive antiviral phenotype, while the genotype with Ser at position 631 corresponds to the negative phenotype (14). A Glu-to-Arg substitution mutation near the carboxyl terminal of the human *MxA* gene inhibits influenza virus multiplication but has no effect on vesicular stomatitis virus infection (15). Porcine Mx proteins inhibited the growth of influenza virus, VSV, and mengovirus (12). Porcine *Mx1* gene polymorphisms have been analyzed to explore effects on viral infections (7,8,12).

To date, associations between porcine *Mx1* gene polymorphisms and economic traits have been extensively studied. Li et al. (9) reported a significant association between intron 9 polymorphisms in the porcine *Mx1* gene and some immunity traits (nitroblue tetrazolium, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin concentration, red cell distribution width). Zhao et al. (13) showed a relationship between polymorphisms in exon 14 of the *Mx1* gene and cytokine levels in Hebao and Large White pigs, indicating significantly higher IL-4 and SlgA levels in the BB genotype

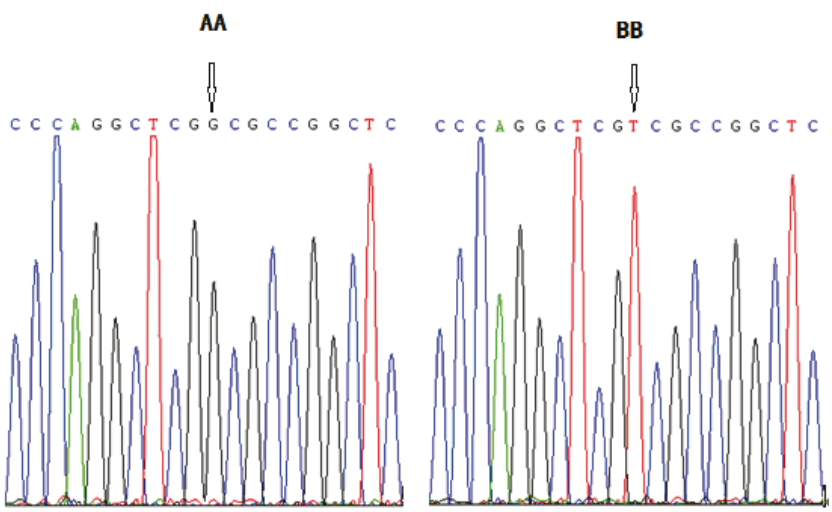

Figure 2. Sequence analysis of AA and BB genotypes. Arrows indicate the G>T mutation in the *Hin*6I restriction site of *Mx1* gene in exon 14.

Table 1. Genotype and allele frequency analysis of *Mx1* gene exon 14 in Meishan pigs.

Sample	Genotype frequency			Allele frequency		χ^2 value
	AA	AB	BB	A	B	
136	0.35 (48)	0.38 (52)	0.27 (36)	0.54	0.46	3.58

Note: 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. Association of *Mx1* gene in Meishan pig with partial cytokine levels and early growth performance.

Index	Genotype		
	AA (48)	AB (52)	BB (36)
IFN-α (pg/μL)	107.40 ± 32.55	113.73 ± 31.39	114.42 ± 30.15
IFN-γ (pg/μL)	26.10 ± 9.73 [a]	19.31 ± 8.49 [b]	23.01 ±8.97 [ab]
IL-2 (pg/μL)	219.07 ± 80.95	216.31 ± 79.03	233.20 ± 87.50
IL-6 (pg/μL)	29.42 ± 11.03	33.76 ± 11.85	37.27 ± 15.65
IL-10 (pg/μL)	78.23 ± 33.79	61.37 ± 31.12	79.88 ± 38.18
IL-12 (pg/μL)	47.27 ± 29.49	49.72 ± 23.76	59.70 ± 18.19
Birth weight (kg)	0.97 ± 0.29	0.87 ± 0.21	0.82 ± 0.21
20-day weight (kg)	3.38 ± 0.65	3.10 ± 0.52	3.06 ± 0.64
40-day weight (kg)	7.00 ± 2.25	7.12 ± 1.52	6.43 ± 1.32

Note: Different superscripts within the same row indicate significant differences ($P < 0.05$).

Table 3. Association of *Mx1* gene in Meishan pig with reproductive performance in the third litter.

Genotype	Third litter				
	TNB	NBA	NW	Birth weight (kg)	Weaning weight (kg)
AA (48)	14.17 ± 3.19	14.00 ± 2.97	13.17 ± 1.84	0.99 ± 0.09	10.14 ± 0.91
AB (52)	14.53 ± 3.01	13.68 ± 2.52	12.63 ± 1.54	1.03 ± 0.12	10.48 ± 1.59
BB (36)	14.00 ± 1.73	12.60 ± 1.14	12.60 ± 1.14	0.99 ± 0.02	10.48 ± 0.67

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

Table 4. Association of *Mx1* gene in Meishan pig reproductive performance in the fourth litter.

Genotype	Fourth litter				
	TNB	NBA	NW	Birth weight (kg)	Weaning weight (kg)
AA (48)	15.50 ± 5.13	14.83 ± 3.71	13.17 ± 2.04	1.01 ± 0.06	9.36 ± 0.51
AB (52)	15.37 ± 2.91	14.00 ± 1.67	13.63 ± 1.50	0.99 ± 0.12	9.57 ± 1.19
BB (36)	17.60 ± 2.41	15.80 ± 2.49	14.20 ± 2.28	0.96 ± 0.06	9.47 ± 1.71

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

compared with the AA and AB genotypes, while significant differences were detected in the levels of IL-4 and SlgA in the AA and AB genotypes. Furthermore, there were no significant differences in the IFN-α level among the three genotypes. Wu et al. (16) reported that polymorphisms in intron 8 of porcine *Mx1* were significantly associated with dressing percentage, lean meat percentage, and loin eye depth. Furthermore, Wu et al. (10) showed a relationship between polymorphisms in exon 14 of *Mx1* and reproductive performance in Sutai pigs in which the BC genotype was associated with significantly enhanced reproductive performance ($P < 0.05$) compared with the BB, AA, AB, AC, and CC genotypes. As a potential disease resistance gene marker in pig genetic selection, it is interesting and promising to know whether the Mx1 *Hin*6I restriction polymorphism associates with economic traits in Chinese local pig breeds. In this study, there were no significant differences in reproductive performance of birth weight, 20-day weight, 40-day weight, and third litter, indicating polymorphism without detrimental influences on early body weight, development, and reproductive performance.

Mx1 is present in all vertebrate species; it plays an important role in the immune response, induction of apoptosis, and signal transduction. The presence of Mx1 protein in peripheral blood leukocytes of patients with acute viral infections first indicated the possibility of using it as a biomarker to discriminate between viral and bacterial infections (17). Thereby we enabled further study of the functions of the *Mx1* gene in immune-competent situations, including considering it as candidate gene for immune capacity. In this study, the AA genotype was associated with significantly higher IFN-γ levels than AB ($P < 0.05$). IFN-γ is a critical immune factor involved in antiviral and antitumor responses as well as in immunoregulation. Haller et al. (2) reported that Mx1 was the key component of the antiviral state induced by interferons in many species. The IFN-induced Mx1 protein is one of the best-studied determinants of innate immunity to viral infection (18,19). This result of association with IFN-γ suggested strong antiviral capability in AA genotype individuals. In addition, no significant differences were detected in the levels of other cytokines among the three genotypes. These observations indicated that selective molecular breeding based on the genotype will have a certain effect on resistance to disease.

In conclusion, the *Hin*6I polymorphisms of the *Mx1* gene had a certain effect on immune capacity, such as the level of IFN-γ, which indicated that it may be a potential marker for resistance to disease. Most economic traits in this study showed no significance for genotyping in a small population, which also indicated that the *Mx1* gene as a potential antiviral marker has no detrimental effects on early body weight, reproductive performance, and general immune performance. Further investigations in more populations are needed to confirm whether this marker can be used for molecular breeding of disease resistance in Meishan pigs. Meanwhile, the more valuable antiviral marker of the *Mx1* gene should be detected and further studied to serve as a reliable basis for molecular breeding strategies for the generation of disease resistance in Meishan pigs.

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