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Association analysis of polymorphisms of porcine LMP2 and LMP7 genes with haematological traits

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Abstract Low molecular weight polypeptides 2 (LMP2) and low molecular weight polypeptides 7 (LMP7) are located within the major histocompatibility complex and have been associated with autoimmune disease. In this study, polymorphisms of porcine LMP2 and LMP7 genes were analyzed by PCR-SSCP and DNA sequencing methods. Four SNPs (DQ659151:g.2115T>C; DQ659151:g.4343A>G; DQ872631:g.1232C>G; DQ872631:g.2847C>T) were identified. Four SNPs of genes were analyzed for association with 22 haematological traits in Large White ($n = 195$), Landrace ($n = 84$) and Songliao Black ($n = 86$) pig population. Of all the 22 traits, seven were significant associated with the SNPs of LMP2/LMP7 gene ($P < 0.05$). They included white blood cell count (WBC) ($P = 0.028$), neutrophilic granulocyte count (GRAN) ($P = 0.037$), monocytes percentage (MO%) ($P = 0.015$), red blood cell (RBC) ($P = 0.004$), red blood cell volume distribution width (RDW) ($P = 0.004$), mean platelet volume (MPV) ($P = 0.016$) and $CD4^+CD8^+$% ($P = 0.045$). These results suggest LMP2/LMP7 gene should be regarded as molecular marker to estimate animal's immune status for their effects on hematological traits.

Keywords Pig · LMP2 · LMP7 · SNP · SSCP · Haematological traits

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Introduction

Low molecular weight polypeptides 2 (LMP2) and Low molecular weight polypeptides 7 (LMP7) genes are two immune proteasome subunits that can alter the catalytic activities of 20s proteasome to facilitate the generation of endogenous peptide antigens presented by MHC class I molecules [1]. When cells are stimulated with the cytokines IFN-gamma or TNF-alpha, the syntheses of three 20s proteasome β-subunits, LMP2, LMP7 and multicatalytic endopeptidase complex subunit (MECL-1) are induced. LMP2 and LMP7 are located within the major histocompatibility complex (MHC) class II region. MHC class II genes control infectious disease responses and influence vaccine efficacy and specificity [2]. Considering their important restrictive role in antigen processing and presenting, LMP2 and LMP7 genes are attractive candidates as additional autoimmune disease susceptibility factors in human. Allele polymorphisms of LMP2/LMP7 gene are associated with an enhanced risk for arterial hypertension in adolescents [3]. LMP2 expression is deregulated in Sjogren's syndrome and its genotypes are involved in the genetic susceptibility to arthritis in Mexicans [4, 5]. Haematological traits are essential parameters for evaluating the health status of individual animals and herds. Differences in haematological traits among breeds and populations provide evidence of genetic control. The amount of additive genetic variation of some of the haematological traits argues for the existence of favorable and unfavorable gene variants that are involved in phenotypes. Identification of those variants might improve the diagnostic use of such traits and may be suitable for the genetic improvement through selection [6]. In our early study, we mapping seven significant QTL affecting haematological traits on porcine chromosome 7 in a pig

resource population consisting of 365 piglets of three breeds including Large White, Landrace and Songliao Black pig, and two QTL are mapped in the MHC region [7]. In this study, we chose LMP2 and LMP7 genes from the QTL region and further investigate the association of SNPs in the peptide-binding region of the LMP2/LMP7 gene with haematological traits in the same pig resource population.

Materials and methods

Genomic DNA isolation and traits detection

Genomic DNA was isolated from the ear tissue samples of a resource population consisting of 365 piglets of three breeds, the three breeds including Large White, Landrace and Songliao Black pig Blood samples were drawn twice from those piglets at 20- and 35-days-old, and the inoculation with the swine fever vaccine was at 21-days-old. Haematological traits which consist of mainly four components, including leukocyte traits, erythrocyte traits, platelet traits and T lymphocyte subsets, were measured. Twenty-two haematological traits include seven leukocyte traits [white blood cell count (WBC), neutrophilic granulocyte count (GRAN) and percentage (GR%), lymphocyte count (LYMF) and percentage (LY%), monocytes count (MONO), and percentage (MO%)], seven erythrocyte traits [red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and red blood cell volume distribution width (RDW)], four platelet traits (blood platelet counts (PLT), mean platelet volume (MPV), platelet distribution width (PDW), and plateletocrit (PCT)), and four T lymphocyte subset traits ($CD4^+CD8^+$%, $CD4^+\bar{CD8}$%, $\bar{CD4}CD8^+$%, and $\bar{CD4}\bar{CD8}$%) (Date of traits was presented in Table S1, online supplementary data).

Primer design and polymerase chain reaction amplification

We firstly identified the 5,099 bp genomic structure of the LMP2 gene and the 2,995 bp genomic structure of the LMP7 gene. The LMP2 gene contains six exons and five introns (GenBank accession no. DQ659151) [8]. The LMP7 gene contains seven exons and six introns (GenBank accession no. DQ872631). Using the software Oligo6.01 [9], 13 pair of polymerase chain reaction (PCR) primers were designed for the coding region of the LMP2 and LMP7 genes. Each amplification reaction was carried out in a 25 μl reaction mixture containing 50 ng genomic DNA, 10 pmol of each primer, 1× buffer (including 1.5 mmol/l $MgCl_2$), 200 μmol dNTPs (dATP, dTTP, dCTP and dGTP), and 0.30 U TAQ DNA polymerase (TaKaRa Biotechnology, China). The cycling protocol was 5 min at 94°C, 34 cycles of denaturing at 94°C for 45 s, annealing at X°C for 1 min, extension at 72°C for 45 s, with a final extension at 72°C for 5 min (X was presented in Table S2, online supplementary data).

Single-stranded conformation polymorphism and sequencing

PCR-SSCP method was used to scan mutations within the amplified regions. Aliquots of 5 μl PCR products were mixed with 6 μl denaturing solution (95% formamide, 25 mM EDTA, 0.025% xylene-cyanogen and 0.025% bromophenol blue), heated for 10 min at 98°C and chilled in ice immediately. Denatured DNA was subjected to 10% polyacrylamide gel electrophoresis (PAGE) in 1× TBE buffer and constant voltage (140 V) for 16 h at a constant temperature of 4°C, then gels were stained with 0.1% silver nitrate and visualized with 2% NaOH solution (containing 0.1% formaldehyde). The PCR products which represented different PCR-SSCP genotype, including both homozygous and heterozygous genotypes were purified with the Gel Midi PCR DNA Purification Kit (Tiangen Biotechnology, China) and sequenced using the ABI377 sequencer from both directions. Sequences were aligned using the DNAMAN software (version 5.2.2).

Statistical analysis

Association analysis between the genotypes of the SNPs and the 22 haematological traits were examined separately for each population by fitting the following mixed models using in SAS9.13 software:

$$y = X\beta + Zb + e$$

where y is the vector of phenotype for immune traits analyzed, X is the design matrix for fixed effects, β is the vector of fixed effects parameter including breed, season, sex and genotype effect, Z is the design matrix of random animal effects, b is the mixed vector of random component including sires effect and dam effect within sires, e is the vector of residual effect.

Results and discussion

Polymorphisms of LMP2 genes

Based on PCR-SSCP detection, the number of bands and their positions in the gel clearly showed the occurrence of sequence variations (Fig. 1). In order to find which kind of genetic variation created this polymorphic, DNA amplification

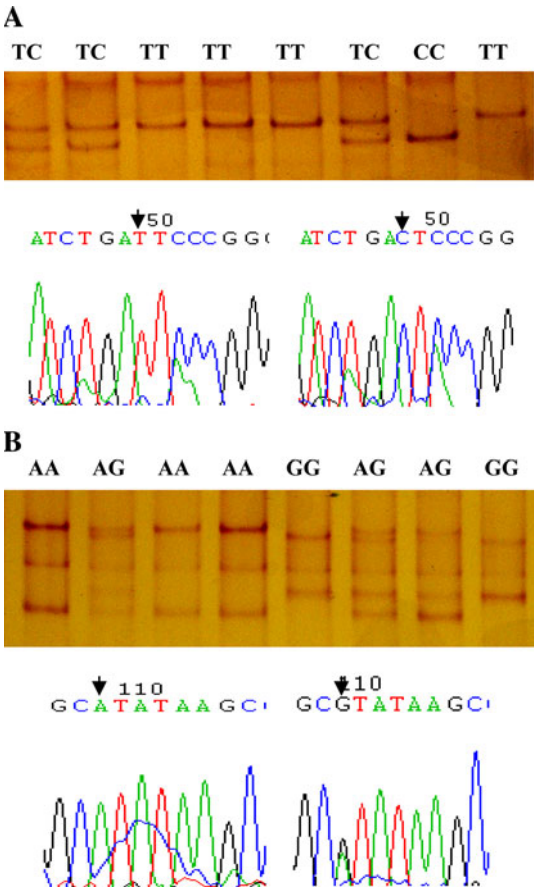

Fig. 1 The electrophoresis patterns of PCR-SSCP and sequencing maps for porcine LMP2 gene, SNPs are indicated by *black arrows*. **a** The electrophoresis patterns of PCR-SSCP for DQ659151:g.2115T>C. **b** The electrophoresis patterns of PCR-SSCP for DQ659151:g.4343A>G

fragments were sequenced and two mutations were revealed. Compared with our reported sequence (GenBank accession no. DQ659151), one SNP showed a transversion T>C at position 2,115 bp (DQ659151:g.2115T>C), the other SNP showed a transversion A>G at position 4,343 bp (DQ659151:g.A4343G). After peptide-binding region predicted by DNAMAN software (version 5.2.2), the two SNPs in the peptide-binding region of LMP2 are synonymous substitution and not induce amino acid substitution. The genotypic and allelic frequencies of the two SNPs in three pig breeds are presented in Table 1. The two SNPs (DQ659151:g.2115T>C and DQ659151:g.4343A>G) showed segregation in three breeds. The frequency of T allele is greater than that of the C allele; The A allele is also obviously predominate in three breeds, especially in Landrace and Large White. Songliao Black pig is a Chinese indigenous breed, the frequency of genotype AG is higher than that of other genotype, which is different from two western introduced commercial breeds.

Polymorphisms of LMP7 gene

In the porcine LMP7 gene, polymorphisms were also detected through RCR-SSCP in each exon region and genotypes were identified in gel (Fig. 2). After sequenced amplification fragments, two mutations were revealed. Compared with our reported sequence (GenBank accession no. DQ872631), one SNP showed a transversion C>G at position 1,232 bp (DQ872631:g.1232C>G), the other SNP showed a transversion C>T at position 2,847 bp (DQ872631:g.2847C>T). The SNP (DQ872631:g.2847C>T) is not located in gene coding regions and the position is in intron 6 region. After peptide-binding region predicted by DNAMAN software (version 5.2.2), the DQ872631:g.1232C>G SNP induces amino acid substitution, i.e., alanine to serine. The genotypic and allelic frequencies of the two SNPs in three pig breeds are presented in Table 2. The two SNPs (DQ872631:g.1232C>G and DQ872631:g.2847C>T) also showed segregation in three breeds. The C allele of two SNPs is also obviously predominate in three breeds, but statistical genotyping results demonstrated that some difference among three pig breeds, the frequencies of heterozygosis genotype CG and CT were higher than that in the Songliao Black pig. It may be that the Chinese indigenous breed has not been under strong selection pressure and keep a high genetic diversity.

Association analyses

Base on statistical analysis, we found supporting relationship of the sequence variation found in four SNPs of

Table 1 Genotype and allele frequencies of the SNPs detected in the porcine LMP2 gene

Breeds	DQ659151:g.2115T>C					DQ659151:g.4343A>G				
	Genotype frequencies			Allele frequencies		Genotype frequencies			Allele frequencies	
	TT	TC	CC	T	C	AA	AG	GG	A	G
Landrace (84)	0.440	0.500	0.060	0.690	0.310	0.691	0.286	0.024	0.833	0.167
Large White (195)	0.457	0.343	0.201	0.627	0.373	0.539	0.432	0.030	0.754	0.246
Songliao black (86)	0.391	0.470	0.140	0.625	0.375	0.361	0.512	0.128	0.616	0.384

The number of animals in each breed is indicated between parentheses

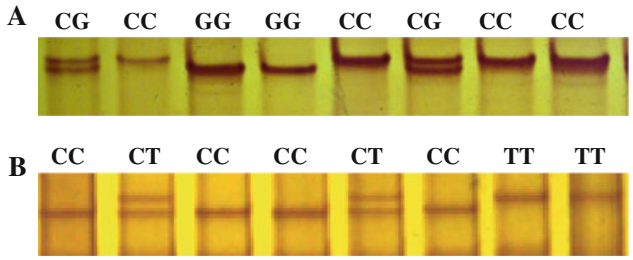

Fig. 2 The electrophoresis patterns of PCR-SSCP for porcine LMP7 gene. **a** The electrophoresis patterns of PCR-SSCP for DQ872631: g.1232C>G. **b** The electrophoresis patterns of PCR-SSCP for DQ872631:g.2847C>T

LMP2/LMP7 gene with haematological traits. Comparisons between the least squares means of the phenotypes evaluated and their respective standard errors for the genotypes of the LMP2/LMP7 polymorphisms are shown in Table 3.

Among the seven leukocyte traits, the DQ659151: g.2115T>C SNP was found to be significantly associated with WBC ($P = 0.028$), GRAN ($P = 0.037$) and MO% ($P = 0.015$), the three traits with CC genotype of DQ659151:g.2115T>C SNP was significantly higher than other genotype ($P < 0.05$); Among the four platelet and four T lymphocyte subsets traits, the DQ659151: g.4343A>G SNP was found to have significant association with MPV ($P = 0.016$), PCT ($P = 0.050$) and $CD4^+CD8^+$% ($P = 0.045$), PCT and $CD4^+CD8^+$% with GG genotype were significantly higher, but MPV with GG genotype was significantly lower than other genotype. Among the seven erythrocyte traits, the DQ872631: g.1232C>G and DQ872631:g.2847C>T SNPs were found to have significant association with RBC ($P = 0.004$) and RDW ($P = 0.004$), respectively, RBC with CC genotype and RDW with TT genotype were significantly higher than other genotypes.

Although the DQ872631:g.2847C>T SNP are not located in gene coding regions, the association between the markers and RDW shown in this study suggests that they are in linkage or are in strong linkage disequilibrium with the polymorphisms responsible for the phenotypic alterations. Furthermore, it is known that non-coding RNAs transcribed from intronic regions are involved in different biological processes such as transcriptional and post-transcriptional control of gene expression [10]. Synonymous SNPs also can affect protein expression by alteration or increase in the stability of the mRNA [11, 12]. Additionally, Kimchi-Sarfaty et al. [13] has also revealed that a "silent" polymorphism changes substrate specificity. Therefore, the association result indicated that the SNP also may simply be used as a genetic marker linking to quantitative trait loci with effects on red blood cell volume distribution width (RDW).

Haematological traits are controlled by multiple genes and can vary with health and disease status of animal [14]. In order to identify the gene(s) responsible for effects on haematological traits, Gong et al. [7] identified 22 significant QTL affecting 18 haematological traits by a partial genome scan in the same resource population as we used in this study, among which seven QTL on chromosome 7 were found to have effect on WBC, GRAN, MCHC, HGB, PLT, MCV and RDW, respectively. Reiner et al. [6] also mapped a QTL in the interval between tumour necrosis factor beta (TNF-β) and SW2428 on SSC7 with pleiotropic effects on erythroid traits including HB, RBC, HCT and MCHC in a Pietrain/Meishan family. The porcine LMP7 gene was reported to be located in the SLA class II region on the q-arm of SSC7 (7q1.1) [15, 16]; we also mapped the porcine LMP2 gene to the same region using the IMpRH panel and is closely linked to microsatellite SW472, which is near TNF beta (Fig. 3). LMP2 and LMP7 genes are key antigen presenting participant molecules in cell immunity, they efficiently generate peptide fragments to form complexes with MHC class I molecules for presentation to CD8 T lymphocytes [17]. Polymorphisms of LMP2/LMP7 gene are one of the important host factors which independently affect on the infection of hepatitis B virus [18]. Mice lacking LMP7 gene are highly susceptible to infection with *T. gondii* and showed a reduced number of functional $CD8^+$ T cells [19]. The location of the LMP2/LMP7 gene from the MHC region on SSC7 and their function should make them strong candidates for the observed effects. Our association results indicated that LMP2/LMP7 may be a genetic marker linking to quantitative trait loci with effects

Table 2 Genotype and allele frequencies of the SNPs detected in the porcine LMP7 gene

Breeds	DQ872631:g.1232C>G					DQ872631:g.2847C>T				
	Genotype frequencies			Allele frequencies		Genotype frequencies			Allele frequencies	
	CC	CG	GG	C	G	CC	CT	TT	C	T
Landrace (84)	0.750	0.107	0.143	0.807	0.193	0.643	0.298	0.060	0.791	0.209
Large White (195)	0.608	0.131	0.261	0.674	0.327	0.568	0.408	0.024	0.772	0.228
Songliao Black (86)	0.400	0.314	0.291	0.552	0.448	0.489	0.419	0.093	0.697	0.303

The number of animals in each breed is indicated between parentheses

Table 3 Least square means of haematological traits of the LMP2/LMP7 SNPs in three pig populations

Traits	*P*-value				Polymorphism genotypes (least square mean and SE)		
	DQ65915 12115T>C	DQ659151 4343A>G	DQ872631 1232C>G	DQ87263 12847C>T			
WBC	0.028*	0.380	0.917	0.296	DQ659151:g.2115T>C		
					TT	TC	CC
					19.84 ± 1.56^{A}	17.08 ± 1.26^{B}	20.58 ± 1.49^{A}
GRAN	0.037*	0.892	0.050	0.374	DQ659151:g.2115T>C		
					TT	TC	CC
					6.41 ± 1.05^{A}	4.52 ± 0.90^{B}	5.69 ± 1.01^{A}
MO%	0.015*	0.700	0.989	0.129	DQ659151:g.2115T>C		
					TT	TC	CC
					5.26 ± 0.61^{A}	5.57 ± 0.50^{A}	6.68 ± 0.58^{B}
PCT	0.677	0.050*	0.289	0.114	DQ659151:g.4343A>G		
					AA	AG	GG
					3.90 ± 0.58^{A}	4.51 ± 0.60^{A}	4.49 ± 0.66^{B}
MPV	0.685	0.016*	0.828	0.313	DQ659151:g.4343A>G		
					AA	AG	GG
					4.40 ± 0.99^{A}	3.73 ± 1.03^{A}	2.82 ± 1.09^{B}
$CD4^{+}CD8^{+}$	0.205	0.045*	0.489	0.845	DQ659151:g.4343A>G		
					AA	AG	GG
					9.99 ± 0.95^{A}	9.63 ± 0.96^{A}	12.36 ± 1.39^{B}
RBC	0.466	0.441	0.004**	0.403	DQ872631:g.1232C>G		
					CC	CG	GG
					6.21 ± 0.15^{A}	6.41 ± 0.22^{A}	5.69 ± 0.23^{B}
RDW	0.148	0.108	0.329	0.004**	DQ872631:g.2847C>T		
					CC	TC	TT
					17.68 ± 0.48^{A}	16.88 ± 0.50^{B}	18.62 ± 0.90^{A}

WBC white blood cell count, g/l; *GRAN* neutrophilic granulocyte count, g/l; MO%, monocytes percentage, %; *RBC* red blood cell, g/l; *RDW* red blood cell volume distribution width, %; *MPV* mean platelet volume, fL; *PCT* plateletocrit, %; $CD4^{+}CD8^{+}$ T lymphocyte subsets, %

* $P < 0.05$; ** $P < 0.01$; ^{A,B}Statistically different of least square means ($P < 0.05$)

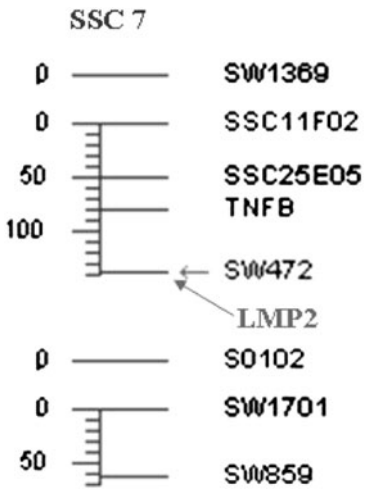

Fig. 3 Mapping LMP2 gene to microsatellite SW472 of chromosome 7 by IMpRH panel

on porcine haematological traits. In addition, we also consider a possible explanation that it should be in linkage disequilibrium with other immune genes in the MHC region for a bigger QTL effect to impact haematological traits. Further studies on the relationship between SNPs and immune traits are currently in progress by genome-wide association method in our resource population.

In summary, we firstly identified the genomic structure of the porcine LMP2 and LMP7 genes. Base on SSCP analysis, polymorphisms in the LMP2 and LMP7 genes were detected. The PCR products of different SSCP variants were sequenced. The SNPs (DQ659151:g.2115T>C; g.2115T>C and DQ872631:g.2847C>T; g.1232C>G) of porcine LMP2 and LMP7 genes were identified. Four SNPs were analyzed for association with 22 haematological traits in three pig breed population. Seven haematological traits, including white blood cell count (WBC), neutrophilic granulocyte count (GRAN), monocytes percentage (MO%), red blood

cell (RBC), red blood cell volume distribution width (RDW), mean platelet volume (MPV) and $CD4^+CD8^+$%, were significant associated with the SNPs of LMP2/LMP7 gene ($P < 0.05$). The association results in our study indicated that LMP2/LMP7 gene should be regarded as molecular marker and may supply us an initial exploration to estimate animal's hematological traits.

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