EFFECT OF C7 COMPLEMENT COMPONENT GENOTYPES ON HAEMOLYTIC COMPLEMENT ACTIVITY IN PIG

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SUMMARY

C7 is one of the important components of the membrane attack complex (MAC) of the complement system, which can cause lysis of the target cells. Herein we described cDNA sequence variation of the porcine C7 gene (pC7) obtained in different breeds (Hampshire, German Landrace, Duroc, Pietrain, Berlin Miniature), assigned the pC7 to chromosome as well as evaluated the effect of its genetic variation on the haemolytic complement activity (CH50) and C3c serum concentration (C3c) in a F_2 DUMI resource population. In this study, six SNPs, in which three of them had amino acid exchange 724A→G (52Ile→Val, *Bsr*DI), 320C→T (67Thr→Met, *Mae*II) and 1001A→G (294Lys→Arg, *Mbo*II) according to GenBank accession number NM_214282, were detected by using PCR-RFLP. Some of them belonged to the rich-cysteine immuno protein domains as thrombospondin type 1 repeats (TSP1), membrane-attack complex/ perforin (MACPF) and complement control protein (CCP). Genotypic frequency at SNPs 990C→T and 1001A→G was in a strong agreement with Hardy-Weinberg equilibrium for populations. In addition, the pC7 was assigned to chromosome 16q14 (Retention fraction 38 %, linked marker S0077, 21 cR in distance, LOD =17,94) by using IMpRH mapping. Statistically significant difference was found for 1001A→G genetic association with CH50 (p=0.008) and C3c (p=0.0405). The results promote the porcine C7 gene as a candidate gene for improvement of disease resistance and porcine health, one of the main targets of animal breeding program.

Keywords: *serum C3c concentration, porcine C7 gene, genetic variation, and haemolytic complement activity*

INTRODUCTION

The complement system is a highly regulated and complex set of interacting proteins in blood plasma and on the cell surfaces that can recognize, bind to, and kill, or remove invading microbes. There are three pathways of complement activation, the classical, the alternative and the lectin pathway that lead to the formation of the MAC. Assembly of the MAC containing C5b, C6, C7, C8 and C9 plays an important role in formation of transmembrane pores causing bacterial cell death (Wimmers *et al.*, 2003; Do 2010). The seventh complement component (C7) has been characterized in human (DiScipio *et al.*, 1988) and in pig (Agah *et al.*, 2000). Recent reports indicated that the human C7 deficiency due to point mutations can cause recurrent infections, meningococcal meningitis, for example (Ki *et al.*, 2005; Barroso *et al.* 2006). Agah *et al.* (2000) demonstrated that porcine and human C7 are highly conserved, sharing structural and functional characteristics. Therefore, in this study, we aimed at analyzing genetic variation of the porcine C7, mapping the gene, and associating with its haemolytic complement activity.

MATERIALS AND METHODS

Animals, DNA extraction and cDNA synthesis

Animals of the breeds Hampshire (HA, n=1), German Landrace (DL, n=30), Duroc (DU, n=1), Pietrain (PI, n=30), Berlin Miniature (MI, n=1) (originating from Vietnamese potbelly pig x Saddle Back Pigs x German Landrace) and Muong Khuong (MK, a Vietnamese miniature pig kept in Lao Cai province of Vietnam, n=25) were used to detect polymorphism in pC7. Genomic DNA from tail or ear sample of experimental animals was obtained by ProteinaseK digestion followed by phenol-chloroform extraction and ethanol precipitation. The genomic DNA samples were resuspended in TE buffer and stored at 4°C. Total RNA was isolated from fresh liver tissue with TRIzol reagent procedure (Sigma, Steinheim, Germany) and treated with Dnase1 (Roche, Mannheim, Germany). The cDNA synthesis was performed using oligo (dT)13 VN primer (Promega Co., Germany) and Super Script™III. The cDNA solution was diluted 1:5 and stored at −20°C for the subsequent PCR reaction.

Polymorphism and genotyping

Primer pairs were designed from the porcine C7 cDNA sequence (GenBank accession number NM_214282) to produce overlapping PCR fragments for comparative sequencing (Do 2010). PCR reactions were performed in 20 µl total volume containing 50 ng of liver cDNA or 100 ng of genomic DNA, 0.2 mM of each primer (forward or reverse primer), 50 µM of each dNTP, 0.5 U of Taq polymerase (Promega, Mannheim, Germany), in 1xTaq buffer, 1.5 mM of $MgCl_2$ (Sigma, Taufkirchen, Germany). The PCR thermal cycling program was set up with an initial denaturation step of 94°C for 4 min, followed by 40 amplification cycles (94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min) and terminated by an extension at 72°C for 5 min. PCR products obtained from individuals of the breeds HA, DU, DL, PI, MI, and MK were comparatively sequenced. SNP sites were confirmed using resequencing and PCR-RFLP thereafter (Figure 3). All samples of DL, PI and MK had been used for genotyping. The restricted digestion reaction was performed in a total volume of 20 µl containing 15µl aliquot of PCR product and 10 U of appropriate enzyme (Table 1).

IMpRH mapping

In order to perform physical mapping of the candidate gene, the INRA-University of Minnesota porcine 7000Rad Radiation Hybrid panel (IMpRH) containing 118 pig/ hamster DNA hybrid clones (Hawken *et al*, 1999; Yerle *et al*, 1998) was employed. The IMpRH typing experiment was performed twice, and data were scored independently. PCR products were scored as present (1), absent (0), or ambiguous (2). Multi-point and two-point linkage analysis were done using the IMpRH mapping tool available at the IMpRH Web Server (http://imprh.toulouse.inra.fr).

Phenotyping

Parameters of *in vivo* C3c serum concentration and *in vitro* complement activity (haemolytic complement activity in the classical pathway, CH50) were obtained from 473 animals of the F_2 DUMI resource population that is based on reciprocal crossing of Duroc and Berlin Miniature Pig (Hardge *et al.*, 1999). Animals were vaccinated with Mycoplasma (Mh), Aujeszky (ADV) and PRRSV (Porcine reproductive and respiratory syndrome) vaccines at 6, 14 and 20 weeks of age. EDTA blood samples were taken before (day 0) and after Mh and ADV vaccination (day 4 and 10) but only on day 10 after PRRSV vaccination. For estimation of haemolytic complement activity (CH50) dilution series of sera were mixed with antibody sensitized sheep erythrocytes and the amount of serum caused a 50% haemolysis of in the reaction mixture were determined (Wimmers *et al.*, 2003). C3c serum concentration was determined immunonephelometrically (Wimmers *et al.*, 1999).

Statistical analysis

The SAS procedure PROC MIXED (The SAS software package, release 9.1) with the repeated measured statement was applied for analyses of variance. The mixed linear model was given as follows:

$$y_{ijklmno} = \mu + sire_i + dam_j + parity_k + treatment_l + genotype_m + time_n + sex_o + animal_{ijklmno} + (genotype \times time)_{mn} + \varepsilon_{ijklmno}$$

where:

$y_{ijklmno}$: CH50, C3c;

μ: overall mean;

$Sire_i$: fixed-effect of sire; i = 1 – 3;

$Genotype_m$: fixed-effect of C7 genotype; m = 1 - 3;

Dam_j: fixed-effect of dam; j = 1 - 11;

$Parity_k$: fixed-effect of parity; k = 1 - 5;

Sex_o: fixed-effected of sex, 0 = 1 - 2;

$Animal_{ijklmno}$: random effect of animal;

$\varepsilon_{ijklmno}$: residual error;

$Treatment_l$: fixed-effect of treatment-vaccinated trail/unvaccinated control; l = 1 - 2;

$Time_n$: fixed-effect of time point of measurement prior to and after vaccinations; m = 1 - 8;

$(Genotype \times time)_{mn}$: interaction between C7 genotype and time point.

RESULTS AND DISCUSSION

Genetic variation

Six polymorphic sites within the candidate gene were identified by sequencing and confirmed using PCR-RFLP in six different breeds of pig HA, DU, DL, PI, MI, MK. The figure 1 showed the

representative pattern of PCR-RFLP using *Mbo*II to detect SNP at position 1001A→G. Among six SNPs, three led to amino acid substitutions (Table 1). Most of them belonged to the rich-cysteine immunoprotein domains as TSP1, MACPF and CCP. These may make differentiation for function and/or role of the domains as well as of gene during the formation of the MAC and/or baterial killing probability of complement system.

Understanding and regulating C5b-9 formation may lead to potential therapeutics that inhibit C5b-9-mediated tissue injury where C7 may be a potential therapeutic target for inhibition of C5b-9 formation and inflammation (Agah *et al.*, 200). Additionally, the SNPs at nt990 and nt1001 were genotyped in three pig breeds (Table 2). Frequency of genotypes and alleles revealed no deviation from Hardy-Weinberg equilibrium. For both SNPs, two alleles were detected segregating in all three breeds and appeared with different frequencies.

Table 1. Characterization of single mutation points.

Variation (nt)	Variation (aa)	Codon	Exon	Enzyme	Module
724A→G	Ile→Val	52ATT→GTT	4	*Bsr*DI	TSP1
320C→T	Thr→Met	67ACG→ATG	4	*Mae*II	TSP1
666C→T		182AAC→AAT	6	*Mae*II	
990C→T		290AG**C**→AG**T**	8	*Hin*6I	MACPF
1001A→G	Lys→Arg	294AAA→AGA	8	*Mbo*II	MACPF
1884A→G	-	588ACA→ACG	17	*Mae*II	CCP

Table 2. Genotypic and allelic frequencies at two point mutations in three commercial pig breeds.

		German Landrace n (%)	Pietrain n (%)	Muong Khuong n (%)	F2 DUMI n (%)
Genotype frequency	nt 990C→T	30	30	25	
	CC	0 (0.00)	0 (0 00)	7 (0.28)	
	CT	13 (0.43)	12 (0 40)	16 (0 64)	
	TT	17 (0.57)	18 (0.60)	2 (0.08)	
	nt 1001A→G	30	30	25	
	AA	0 (0.00)	0 (0 00)	7 (0.28)	252 (0 53)
	AG	13 (0.43)	12 (0 40)	16 (0.64)	207 (0 44)
	GG	17 (0.57)	18 (0.60)	2 (0.08)	14 (0 03)
Allele frequency	nt 990C→T				
	C	13 (0.22)	12 (0 20)	30 (0.60)	
	T	47 (0.78)	48 (0.80)	20 (0 40)	
	nt 1001A→G				
	A	13 (0.22)	12 (0.20)	30 (0 60)	711 (0,75)
	G	47 (0 78)	48 (0 80)	20 (0 40)	235 (0 25)

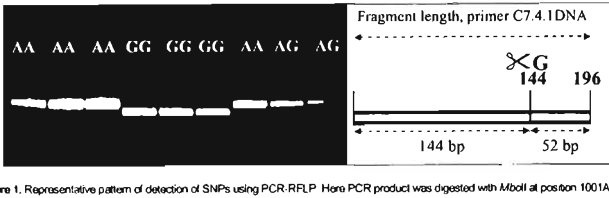

Figure 1. Representative pattern of detection of SNPs using PCR-RFLP. Here PCR product was digested with *Mbo*II at position 1001A→G.

Chromosomal location

By RH mapping using the IMpRH panel, the pC7 was assigned to chromosome 16q14 (IMpRH: Retention fraction (38 %); linked marker (2pt analysis) S0077; 21 cR in distance; LOD =17.94) (Do, 2010). Genetic mapping in 21 full sib families of the DUMI resource population correspondingly revealed linkage of the pC7 to pC9 and AGXT2 that have previously been assigned to the q-arm of SSC16 (Wintero *et al.*, 1998, Thomsen *et al.*, 1998; Ponsuksili *et al*., 2001). Mapping of the pC7 is in line with the current status of the human-porcine comparative map (Meyers *et al.*, 2005).

Association study

The genotypes of the SNP nt1001A→G were used to analyze association with *in vitro* haemolytic complement activity in the classical pathway (CH50) in the DUMI resource population. It showed the highest lysis activity on day 4 after ADV vaccination. There is highly significant difference between genotypes and CH50 with the allele "A" being favourable in terms of complement activity (Figure 2).

Also, significant effect of the interaction of pC7 genotypes and time point of measurement along the vaccination program on *in vivo* complement activity, i.e. C3c serum concentration, was found ($p<0.05$) (Figure 3). The time course of haemolytic complement activity depends on the pC7 genotypes. In the F_2 DUMI resource population, probably the allele "A" originates from the Berlin Miniature Pig grandparents. Berlin Miniature Pigs were bred from Vietnamese potbelly pig, Saddle Back Pig and German Landrace with the first being likely the source of the "A" allele. Interestingly, the higher "A" allele frequency in the MK was observed in comparison to DL and PI. The MK breed may be a promising genetic resource for disease resistance and responsiveness to microorganisms. According to Agah *et al.* (2000) porcine and human C7 genes are highly conserved, sharing structural and functional characteristics. Their haemolytic activity concentrated at 40 µg/ml. Thus, addition of purified pC7 restored the hemolytic activity of C7-depleted human sera in a dose-dependent manner. The hemolytically inactive C5b-7 complex may induce cellular activation *in vitro* and inflammation *in vivo* (Wang *et al.*, 1996; Wang *et al.*, 1999). These results as well as the position of the gene in a previously detected QTL region (Quantitave trait loci) for complement activity promote pC7 as a candidate gene that may be suitable to be addressed with regards to genetically enriching animal health.

CONCLUSION

The pC7 is a single glycoprotein with an approximate molecular mass of 90 kDa and 100 kDa (Agah *et al*., 2000). The study had succeeded in detection of SNPs in the pC7 gene as well as assigment of the gene to chromosome. Also, it indicated the valuable genetic association of SNP 1001A→G with CH50 and C3c. Interestingly, this SNP located in MACPF domain, one of the rich cysteine immuno functional domains. Therefore, they had really provided further evidences for important role of the pC7 in innate immune system of host against viral (ADV, PRRSV) and/or bacterial (Mh) pathogens. It is meaningful in breeding programe aiming at porcine disease resistance and health.

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ẢNH HƯỞNG CỦA NHỮNG KIỂU GEN BỊ THIẾU C7 LÊN HOẠT ĐỘNG TIÊU MÁU Ở LỢN

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TÓM TẮT

C7 là một trong những thành phần quan trọng của phức hợp tấn công màng tế bào (Membrane Attack

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Complex, MAC) của hệ thống bổ thể, có chức năng trong sự dung giải tế bào mục tiêu. Nghiên cứu này (i) mô tả sự khác biệt trong trình tự chuỗi cDNA của gen pC7 giữa các giống lợn khác nhau (Hampshire, German Landrace, Duroc, Pietrain, Berlin Miniature), (ii) định vị gen pC7 trên nhiễm sắc thể và (iii) đánh giá ảnh hưởng của sự khác biệt di truyền của gen pC7 với hoạt động tiêu máu (CH50) và nồng độ C3c trong huyết thanh ở quần thể F_2 DUMI. Vì vậy, kỹ thuật PCR-RFLP được sử dụng để phát hiện sai khác di truyền tại 6 vị trí đột biến điểm (single nucleotide polymorphism - SNP). Trong đó, có 3 điểm đột biến có sự trao đổi amino acid 724A→G (52Ile→Val, *Bst*DI), 320C→T (67Thr→Met, *Mae*II) và 1001A→G (294Lys→Arg, *Mbo*II) theo GenBank số NM_214282. Một vài SNPs nằm trong miền protein có chức năng miễn dịch giàu cysteine như TSP1, MACPF và CCP. Tần số kiểu gen tại các đột biến điểm 990C→T and 1001A→G tuân theo định luật Hardy-Weinberg đối với các quần thể lợn được nghiên cứu. Thêm vào đó, gen pC7 được xác định nằm trên nhiễm sắc thể 16q14 (tỷ lệ giữ lại 38 %, gần marker S0077, khoảng cách 21 cR, LOD =17,94) bằng kỹ thuật IMpRH. Sự liên kết di truyền tại điểm SNP 1001A→G với CH50 (p=0.008) và C3c (p=0.0405) có sự khác biệt thống kê có ý nghĩa. Nghiên cứu gợi ý rằng pC7 là gen ứng viên tiềm năng cho sự cải thiện khả năng kháng bệnh và nâng cao sức khỏe lợn, một trong những mục tiêu chính của chương trình gây giống vật nuôi.

Từ khoá: *Đa hình, gen pC7, hoạt động tiêu máu, lợn, nồng độ C3c huyết tương*