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Genetic association of polymorphisms in porcine *RGS16* with porcine circovirus viral load in naturally infected Yorkshire pigs

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Abstract

Regulator of G protein signaling 16 (*RGS16*) is known to be associated with porcine circovirus type 2 (PCV2). PCV2 associated disease (PCVAD) is a serious problem in the swine industry. The representative symptoms of PCVAD are high viral titer proliferation and decreased average daily gain. In this study, we identified single nucleotide polymorphisms (SNPs) in the *RGS16* region, including the upstream region. Of the 22 identified SNPs, rs332913874, rs326071195, and rs318298586 were genotyped in 142 Yorkshire pigs. These SNPs were significantly associated with the PCV2 viral load. Moreover, the haplotype combination was also related to the PCV2 viral load. The haplotype and diplotype analysis also had a significant difference with the PCV2 viral load. Taken together, our results suggest that *RGS16* SNPs considerably affect the PCV2 viral load.

Keywords: PCV2, Viral load, *RGS16*, Single nucleotide polymorphism, Pig

INTRODUCTION

Porcine circovirus type 2 (PCV2), a small non-enveloped DNA virus, is perceived as an important pathogen in the swine industry worldwide [1]. PCV2 has four major open reading frames (ORFs). ORF1 encodes the replicase (Rep) and splicing replicase (Rep') proteins that participate in viral replication [2,3]. ORF2 encodes a viral capsid protein (Cap) [4,5]. The ORF3-encoded protein has been reported to be involved in the apoptosis of PCV2 infected cells [6]. The fourth ORF, named ORF4, has also been reported as a mitochondrial-targeting protein that interacts with adenine nucleotide translocase 3 via the mitochondrial pathways [7].

The proliferation of virus titers correlates with weight loss in post-weaning piglets [8,9]. The typical symptoms of PCV2 associated disease (PCVAD) are an increase in viral load and a decrease in average daily gain (ADG) [10–12]. Moreover, the appearance of PCVAD in swine herds leads to low productivity and economic losses for those farms [13–15]. In addition, PCVAD is known to be influenced by the host genetic background as to which genetically resistant or susceptible to PCV2 [16]. Therefore, the control of PCV2 viral load through host genetic effects is considered important.

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Availability of data and material
Upon reasonable request, the datasets of this study can be available from the corresponding author.

Authors' contributions
Conceptualization: Hong KC, Kim JM.
Data curation: Kim JM.
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Writing - review & editing: Kim JM.

Ethics approval and consent to participate
This study was conducted in accordance with the animal care guidelines of the Institutional Animal Care and Use Committee of the National Institute of Animal Science, Korea (2015-137).

Regulator of G protein signaling 16 (RGS16), a GTPase-activating protein, can regulate T-lymphocyte activation [17]. It has previously been reported that RGS16 is upregulated in inflammatory dendritic cells stimulated with lipopolysaccharide [18]. Furthermore, RGS16 is associated with the ORF3 protein of PCV2 and host response [19]. In pigs, the *RGS16* gene is located in the quantitative trait loci for ADG on *Sus scrofa* chromosome 9 [20,21]. Accordingly, RGS16 is thought to be associated with PCVAD. The aim of this study was to identify polymorphisms in the *RGS16* region and investigate the association between genotypes and the PCV2 viral load. Four-week-old unvaccinated piglets against PCV2 were used for measuring PCV2 viral load by blood collection. After 6 weeks, the viral load of PCV2 was measured again via re-blood collection.

MATERIALS AND METHODS

This study was conducted in accordance with the animal care guidelines of the Institutional Animal Care and Use Committee of the National Institute of Animal Science, Korea.

Animals

A total of 142 unvaccinated Yorkshire pigs (65 castrated males and 77 females) which were born from the 46 different sows selected from a single farm were chosen to have zero viral load at 4 weeks of age and were naturally infected with PCV2. Peripheral blood was collected from each pig at 4 and 10 weeks of age to assess PCV2 viral load and genomic DNA.

Measuring porcine circovirus type 2 viral load in serum

PCV2 viral DNA was extracted from serum using the QIAGEN™ virus mini elute kit (QIAGEN, MD, USA). PCV2 viral load was measured using TaqMan™ probe quantitative polymerase chain reaction (qPCR) with the primers listed in Table 1. These primers were designed from the complete PCV2 genome (Korea, GenBank: FR823451.1) using Primer3 [22]. The reaction mixture contained 500 nM of each primer, 250 nM probe, 25 μL of 2× TaqMan™ Universal Master Mix (Applied Biosystems, Waltham, MA, USA), and 2.5 μL template. Nuclease-free water was added to bring the final volume to 50 μL. Amplification was performed under universal cycling conditions (2 min at 50℃, 10 min at 95℃, and 45 cycles of 15 s at 95℃ and 1 min at 58℃).

Identification of genomic sequence variations

To identify polymorphisms, between or within four different pig breeds (Yorkshire, Landrace, Duroc, and Berkshire; n = 20 per breed), direct sequencing analysis was performed using porcine-specific primers containing the upstream and genetic regions of porcine *RGS16*. The primer sets were designed using the Primer3 software [22] (Table 2). PCR was performed using a Mastercycler™ gradient (Eppendorf, Hamburg, Germany) in a total volume of 20 μL containing 100 ng porcine genomic DNA template, 10 pmol of each primer, 0.25 mM of each dNTP, 1× PCR buffer, and

Table 1. Primers and probe used for analyzing PCV2 viral load in serum

Oligo	Length (nt)	Primer sequence	Location in PCV2 genome[1)]
Forward	22	5′-TCG ATC TCA AGG ACA ACG GAG T-3′	380–401
Reverse	24	5′-TTG GTC TTC CAA TCA CGC TTC TGC-3′	530–553
Probe	27	5′-CAG AGC AGC ACC CTG TAA CGT TTG TCA-3′	457–483

[1)]GenBank: FR823451.1.
PCV2, porcine circovirus type 2.

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Table 2. Primers for polymorphism identification in porcine *RGS16* gene

Primer number	Primer sequence	Binding region	Size (bp)
P1	5′-CTT GGC AGC TGC ATA ATG A-3′	Upstream	760
P2	5′-GGA GCG TGT TTT TGT GAC TC-3′	Upstream	760
P3	5′-GGT GGA ACC TCT TCT CTG CT-3′	Upstream	828
P4	5′-TCG GAA CTA AGA GGG TGT GA-3′	Upstream	828
P5	5′-TCC AGT GAA CAG CTG ATG TG-3′	Upstream	791
P6	5′-CGT GCC CAA GAT CAG AGT TA-3′	Upstream	791
P7	5′-GTT TTA GCG AAA GGG ATT GG-3′	Exon 1	881
P8	5′-CAG CTG GAC AAG CTC CTC TT-3′	Exon 1	881
P9	5′-GAC AAC GTG CGA CTT CTC TG-3′	Exon 1	870
P10	5′-AAA AAC ACT TCC CTG CCT TC-3′	Exon 1	870
P11	5′-CTG CCT GGA GAA AGA AGT CC-3′	Intron1	704
P12	5′-AGC TGT GCC TCC ATA GGT CT-3′	Intron1	704
P13	5′-CAA TCT TTG CTG AAG CTG GA-3′	Exon 2	710
P14	5′-GCC CTG GGT AGA TTT GTC TG-3′	Exon 2	710
P15	5′-TTA GGA CAT GTC AGG GTG GA-3′	Exon 3	749
P16	5′-ACA ACA GGG TCC CAC TCA CT-3′	Exon 3	749
P17	5′-CAT TTG AGG AGT CAG CTC CA-3′	Exon 4	824
P18	5′-TTT TCA GTC TCC AGC CTC CT-3′	Exon 4	824

1.25 U DNA polymerase (i-Max II, Intron biotechnology, Seongnam, Korea). Sequencing analysis was performed using an ABI PRISM 3730 Genetic Analyzer (Applied BiosystemsUSA). Sequence assembly and polymorphism identification were executed using SeqMan [23].

Genotyping

Genotyping was performed on the rs332913874, rs326071195, and rs318298586 loci. Genomic DNA was extracted from peripheral blood using a DNA isolation kit (G-Dex™ IIb, Intron Biotechnology). These single nucleotide polymorphism (SNP) genotypes were identified via direct sequencing analysis. PCR was performed using the aforementioned conditions. The amplification conditions were as follows: 94℃ for 10 min; 30 cycles of 94℃ for 1 min, 61℃ for 1 min, and 72℃ for 1 min; and a final extension at 72℃ for 10 min.

Statistical analysis

The significance of differences in genotype and allele frequencies within the population was determined using the Hardy–Weinberg equilibrium (HWE) calculation (chi-square test). The association of SNPs with viral load was evaluated using the MIXED model procedure in the SAS software (ver. 9.4, SAS Institute, Cary, NC, USA). The model was used as follows: $y_{ijkl} = \mu + S_i + B_j + D_k + e_{ijkl}$, where y_{ijklm} is the observation of traits, μ is the general mean, S_i is the fixed effect of SNP genotypes i, B_j is the fixed effect of batch j, D_k is the random effect of dam l, and e_{ijkl} is the random error. Haplotype combinations of three SNPs were reconstructed by PHASE software [24] and analyzed with the same MIXED procedure using SAS. Results are presented as the mean of the least squares with standard error. Additive and dominance effects were calculated using the method previously described by Hasenstein et al. [25]. A value of $p < 0.05$ was considered statistically significant.

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RESULTS

Identification of SNPs in *RGS16* in pig populations

We identified 22 SNPs in the upstream and genetic regions of *RGS16* in Yorkshire pigs (Fig. 1). Of these, 10 SNPs, including rs332913874 and rs326071195, were located in the upstream region, whereas 12 others, including rs318298586, spread over the intron region. These 22 SNPs were registered on dbSNP and are presented in Supplementary Table S1.

The allelic and genotypic frequencies of rs332913874, rs326071195, and rs318298586 are shown in Table 3. In rs332913874, the G and A allele frequencies were 0.64 and 0.36, respectively, in the Yorkshire population. Genotype frequencies for the homozygote (GG and AA) and heterozygote (GA) genotypes were 0.38, 0.10, and 0.52, respectively. The genotype and allele frequencies of rs326071195 were found to be as follows: T allele, 0.52; C allele, 0.48, TT genotype, 0.19; TC genotype, 0.67; and CC genotype, 0.14. Furthermore, the genotype and allele frequencies of another SNP (rs318298586) were as follows: A allele, 0.51; T allele, 0.49; AA genotype, 0.19; AT genotype, 0.65; and TT genotype, 0.16. Chi-square tests were used to check for deviation from the HWE. We found that the rs332913874 genotypes conformed to HWE; however, the genotypes of the other two SNPs did not follow HWE in our population. Based on these frequency data, we conducted an association analysis between genotypes and the PCV2 viral load in our population.

Haplotype analysis

Haplotype analysis of the three SNPs is presented in Table 4. We observed three major haplotypes

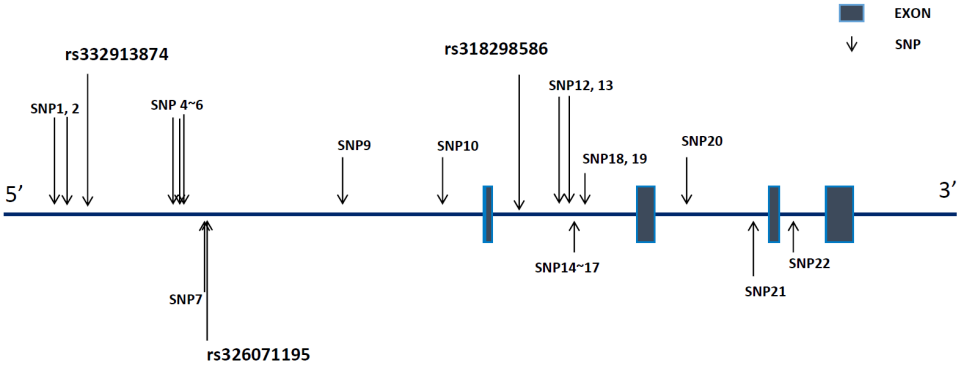

Fig. 1. Diagrammatic representation of the *RGS16* gene and identified polymorphisms. Arrows indicate polymorphisms from upstream and genomic regions of *RGS16*; text represents SNP IDs that were identified by us and used in this study. SNP, single nucleotide polymorphism.

Table 3. Allele and genotype frequencies of polymorphisms in porcine *RGS16* gene

SNP ID	N	Genotype			Allele		HWE[1] *p*-value
rs332913874	142	GG	AG	AA	G	A	0.12
		0.38 (54)[2]	0.52 (74)	0.10 (14)	0.64	0.36	
rs326071195	142	TT	TC	CC	T	C	< 0.05
		0.19 (27)	0.67 (95)	0.14 (20)	0.52	0.48	
rs318298586	142	AA	AT	TT	A	T	< 0.05
		0.19 (27)	0.65 (92)	0.16 (23)	0.51	0.49	

[1]Hardy–Weinberg equilibrium (HWE) by the χ^2-test (df = 1, $\chi^2_{0.05}$ = 3.841).

[2]Number of animals.

SNP, single nucleotide polymorphism.

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Table 4. Haplotype combination from the three SNPs in the upstream region and intron 1 of the *RGS16* gene

Haplotype	Allele combination	Count	Estimated frequency	Cumulated frequency	SE
Haplo1	GTA	144	0.507	0.507	0.001
Haplo2	GTT	5	0.018	0.525	0.001
Haplo3	GCA	1	0.004	0.529	0.001
Haplo4	GCT	32	0.113	0.642	0.001
Haplo5	ACA	1	0.004	0.646	0.001
Haplo6	ACT	101	0.355	1.001	0.001

SNP, single nucleotide polymorphism.

that accounted for 97.5% of all possible haplotype combinations in the population of animals. Based on these three haplotype combinations (Haplo1, Haplo4, and Haplo6), we performed association analysis with haplotype combinations and the PCV2 viral load. Results of the analyses are presented in Table 5. The Haplo1 (GTA) and Haplo6 (ACT) combinations showed significant differences in PCV2 viral loads. Animals containing one copy of the Haplo1 combination had a significantly higher PCV2 viral load than those containing two copies ($p = 0.04$); However, compared to that of a single copy of Haplo1, the absence of Haplo1 combination did not significantly affect PCV2 viral load. The Haplo4 (GCT) combination did not significance. The diplotype frequencies of haplotype combinations were showed in Supplementary Table S2. The frequency of the Haplo1/Haplo6 (GTA/ACT) combination which had the most count was 0.465. The second and the third count of diplotype combinations were Haplo1/Haplo1 (GTA/GTA) and Haplo1/Haplo4 (GTA/GCT). The frequencies of these two combination were 0.176 and 0.169 each. The forth frequency of diplotype combinations (Haplo6/Haplo6, ACT/ACT) was 0.099. The associations among the top 4 diplotype combinations against viral load were presented in Supplementary Table S3. The viral load of Haplo1/Haplo6 (GTA/ACT) combination was significantly higher than Haplo1/Haplo1 (GTA/GTA) combination ($p = 0.01$).

Associations between porcine circovirus type 2 viral load and *RGS16* genotypes

The animal frequency against viral load at 10-week old is showed using a histogram in Supplementary Fig. S1. The interval of the viral load was set by positive integer values. The results of the analyzed associations between the genotypes of SNPs in *RGS16* and PCV2 viral loads are presented in Table 6. The rs332913874 SNP was significantly associated with PCV2 viral load ($p = 0.03$). Animals with the heterozygous genotype had a higher viral load than those with the homozygous GG genotype. However, the heterozygous GA genotype did not show any statistical significance with another homozygous AA genotype. The association analysis of rs326071195 and PCV2 viral load revealed that the viral load in animals with the TC genotype was higher than that in animals

Table 5. Significant haplotype effect of polymorphisms in *RGS16* on the PCV2 viral load in 142 Yorkshire pigs

Combination	Significant trait	Haplotype						*p*-value
		Absent		1 copy		2 copies		
		No.	LSM ± SE	No.	LSM ± SE	No.	LSM ± SE	
Haplo1 (GTA)	Viral load	23	2.99 ± 0.39^{ab}	94	3.43 ± 0.19^{a}	25	2.36 ± 0.39^{b}	0.04
Haplo4 (GCT)	Viral load	110	3.20 ± 0.20	32	3.34 ± 0.35	0	N/A	0.71
Haplo6 (ACT)	Viral load	55	2.75 ± 0.26^{b}	73	3.64 ± 0.22^{a}	14	2.57 ± 0.49^{b}	0.01

^{a,b}Means with different superscript letters are significantly different ($p < 0.05$).

PCV2, porcine circovirus type 2; LSM, least squares mean.

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Table 6. Significant genotype effect of polymorphisms in *RGS16* on the PCV2 viral load in 142 Yorkshire pigs

Locus (Allele1/Allele2)	Significant trait	Genotype						*p*-value	Additive	Dominance
		Allele1/Allele1		Allele1/Allele2		Allele2/Allele2				
		No.	LSM ± SE	No.	LSM ± SE	No.	LSM ± SE			
rs332913874 (G/A)	Viral load[1)]	54	2.80 ± 0.27[b]	74	3.59 ± 0.22[a]	14	2.58 ± 0.49[ab]	0.03	−0.11	1.81*
rs326071195 (T/C)	Viral load	27	2.33 ± 0.37[b]	95	3.45 ± 0.19[a]	20	2.96 ± 0.42[ab]	0.02	0.31	1.61*
rs318298586 (A/T)	Viral load	27	2.31 ± 0.37[b]	92	3.47 ± 0.19[a]	23	2.99 ± 0.39[ab]	0.02	−0.34	1.63*

[1)]The unit of viral load: Log PCV2 copies/mL.

[a,b]Means with different superscript letters are significantly different ($p < 0.05$).

Significant levels of additive and dominance effects: *$p < 0.05$.

PCV2, porcine circovirus type 2; LSM, least squares mean.

with the TT genotype; however, no significance was observed between the viral loads in animals with the CC and heterozygous genotypes. The results of residual normality test are showed in Supplementary Fig. S2. All of the residual was satisfied normality. In the case of rs318298586, the viral load of pigs with the AT genotype was higher than that of pigs with the AA genotype. Conversely, no significant difference against viral load was found between the heterozygous and TT genotypes.

DISCUSSION

In this study, we investigated the association between PCV2 viral load and SNP genotypes in porcine *RGS16*. PCV2, a small non-enveloped, circular ssDNA virus, leads to PCVAD with clinical signs of wasting disease, enlarged lymph nodes, and diarrhea [9,11,16,26]. PCVAD is an endemic disease that causes reduced productivity in the swine industry [1,10,27]. PCV2 is commonly known to have 11 ORFs [16,28]; however, the functions of only four of these ORF proteins have been identified. ORF1 codes for nonstructural replicase proteins known as Rep and Rep' [5,29]. ORF2 encodes the Cap protein [30,31]. ORF3 proteins have been previously reported to induce apoptosis in PK-15 cells [32]. ORF4 is known to be a mitochondrial-targeting protein that triggers apoptosis in the host cell [7].

Various types of research on PCV2 have revealed that the viral load of experimental or naturally infected pigs is associated with PCVAD clinical signs, including postweaning multisystemic wasting syndrome (PMWS) [33–36]. To prevent PCVAD, vaccinations against PCV2 are done in the swine farms. Nevertheless, the swine industry has been consistently damaged by PCV2. Therefore, researchers have experimented on host genetic susceptibility to PCV2 using infection and genome-wide association studies [37,38]. Walker et al. reported that porcine *SYNGR2* polymorphism is related to the PCV2 viral load. Zhang et al. also reported that some miRNAs are associated with PCVAD susceptibility to PCV2 infection in lung tissues. In this study, we investigated the differences in viral load based on genotype in the *RGS16* polymorphism in naturally infected Yorkshire pigs. We identified SNPs in porcine *RGS16* gene. A total of 22 SNPs were identified, including those in the 5′UTR and 3′UTR regions. In the statistical analysis model, we did not include sex as a fixed effect. There was no significant effect between sex in the statistical model including sex as a fixed effect. The previous study reported that the difference of sex effect against PCV2 was not founded [39]. Therefore, it is estimated that there was no difference in infection with gender in our study. Three SNPs located in the intron region were associated with the PCV2 viral load. In particular, the heterozygous genotypes of these three SNPs were related to high PCV2 viral loads.

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Furthermore, the reference homozygous genotypes (Allele1/Allele1) were associated with a low viral load. In addition, the association between SNP and viral load did not follow the additive effects and the complete dominance effect. This phenomenon is presumed to be the effect of hybrid weakness, but more follow-up studies seem to be needed. As previously described, PCV2 viral load is a triggering factor that causes PCVAD, including PMWS. Moreover, *RGS16* has been previously reported to be involved in the activation of immune cells, with the important exception of macrophages in rats [40,41]. Another study has reported that RGS16 activated by inflammatory stimulation and the PCV2 ORF3 protein colocalize in porcine peripheral blood mononuclear cells [19]. In the present study, we did not identify differences in the expression levels of RGS16 in porcine whole blood among the genotypes. In addition, the PCV2 viral load was not measured every week in the study animals; hence, it was difficult to trace the change of aspect against viral load from the initial point to the end point.

CONCLUSION

Although the measurement of viral load in this study showed two points, our findings confirm that the polymorphisms in the *RGS16* gene have considerable effects on the PCV2 viral load in naturally infected pigs. Therefore, these polymorphisms can be regarded as an effective marker for estimating the proliferation of PCV2 viral load.

SUPPLEMENTARY MATERIALS

Supplementary materials are only available online from: https://doi.org/10.5187/jast.2021.e105.

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