Functional characterization of genetic variants in the porcine TLR3 gene

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ABSTRACT. Toll-like receptor 3 (TLR3) recognizes double-stranded RNA, which is a molecular signature of viruses, and plays a pivotal role in host defense against viral invasion. Polymorphisms in the human TLR3 gene have been shown to affect the receptor function and to be associated with a variety of diseases, suggesting correlations between TLR3 polymorphisms and the disease resistance/susceptibility in pigs. In this study, 5 known non-synonymous single nucleotide polymorphisms (SNPs) in the coding sequences of the porcine TLR3 gene - c.800C>T (p.T267M), c.933A>G (p.I311M), c.1116A>T (p.K372N), c.2129C>G (p.T710S), and c.2160T>G (p.I720M) - were analyzed for their effect on receptor function in transiently transfected PK-15 cells by using a luciferase reporter assay. In addition, the distribution of SNP c.933A>G was analyzed among pig populations. SNP c.933A>G significantly decreased the response to poly(I:C) (P < 0.05), as represented by the weaker induction of firefly luciferase relative to that achieved by wild-type TLR3. SNP c.933A>G results in the alteration of conserved amino acids in the highly conserved segment of the 12th leucine repeat region and is conserved among TLR3 orthologs from fishes to primates.

Moreover, together with the results of previous studies, the results of the present study revealed that SNP c.933A>G is found solely in local Chinese pig breeds. These results suggested that SNP c.933A>G plays a role in porcine disease resistance/susceptibility.

Key words: Porcine; TLR3; Polymorphism; Receptor function

INTRODUCTION

The immune system, which consists of the innate and acquired immune systems, fights against invading microbial pathogens and defends hosts from infection. Activation of the immune system can be elicited by the recognition of pathogen-associated molecular patterns (PAMPs), structures conserved among microbial species, by germline-encoded pattern-recognition receptors (PRRs) (Janeway and Medzhitov, 2002). The Toll-like receptor (TLR) family, comprising at least 12 functional members in mammals, is one of the most important PRRs and is essential for host defense against infection. Upon binding to PAMPs, TLRs trigger a series of signaling events, resulting in the induction of various cytokines and chemokines, which induce subsequent inflammation and immune responses (Akira et al., 2006; O'Neill and Bowie, 2007).

TLR3 recognizes double-stranded RNA (dsRNA), a product of viral multiplication. A synthetic dsRNA analog, poly(I:C), can activate TLR3 (Alexopoulou et al., 2001). After activation by dsRNA, TLR3 initiates the MyD88-independent signaling pathway either through the Toll/interleukin-1 receptor (TIR) domain-containing adaptor, which induces the interferon (IFN)-β adaptor protein, TRIF, or through the TIR domain-containing adaptor molecule, TICAM-1, which leads to the activation and nuclear translocation of the transcription factors NF-κB and IRF3, ultimately inducing the production of type I IFNs such as IFN-β and various proinflammatory cytokines (Yamamoto et al., 2002; Oshiumi et al., 2003).

Single nucleotide polymorphisms (SNPs) in TLR3 may affect receptor functions, including pathogen recognition and signaling and have been associated with host resistance/susceptibility to diseases. For example, a non-synonymous SNP (c.1234C>T; p.P412L) in the coding sequences of the TLR3 gene significantly decreased the capacity of the receptor for binding dsRNA, and these SNPs have been associated with geographic atrophy (Yang et al., 2008; Zhou et al., 2011), subacute sclerosing panencephalitis (Ishizaki et al., 2008), recurrent herpes labialis (Yang et al., 2012), hepatitis B virus infection (Al-Qahtani et al., 2012), etc. In addition, an SNP (-705A>G) at the promoter region may predispose individuals to hepatitis C virus infection (Medhi et al., 2011). Furthermore, SNP -764G>T at the promoter region of grass carp was significantly associated with resistance to the grass carp reovirus (Heng et al., 2011). Therefore, TLR3 is a strong candidate gene for a number of diseases, not only in humans, but also in domesticated animals.

With respect to pigs, some studies have been conducted on TLR3 polymorphisms (Morozumi and Uenishi, 2009; Yang et al., 2011), but to the best of our knowledge, the functional relevance of these SNPs remains unknown. Here, the physiological effect of five known missense SNPs in the porcine TLR3 gene, c.800C>T (p.T267M), c.933A>G (p.I311M), c.1116A>T (p.K372N), c.2129C>G (p.T710S), and c.2160T>G (p.I720M), on signal transduction was characterized in transiently transfected PK-15 cells. Additionally, the distribution

of one SNP, c.933A>G, was analyzed in pig populations. The results of this study will be useful for molecular marker characterization for breeding pigs with improved disease resistance.

MATERIAL AND METHODS

Primers

Primer pairs were designed based on the sequence deposited in GenBank (No. DQ266435) by using the Primer Premier 5.0 software. For cloning the complete coding sequence (CDS), a primer pair C1F/R, with enzyme restriction sites *Eco*RI and *Xho*I at the 5'-end, respectively, was designed. For site-directed mutagenesis, two primers with a mismatched base at the target position were designed for each SNP. Additionally, an extra primer, MR, containing the endonuclease recognition site, *Eco*RV, which is inherent to the CDS of the porcine TLR3 gene, was designed for mutant type (MT) eukaryotic expression construction. Detailed information of primer pairs is provided in Table 1.

Table 1. Primers used in this study.

Primers	Sequence (5'-3')	Position*
C1F	GAGAATTCATGAGCAGGAGTTTGCCTT	1-19
C1R	CGCTCGAGTTAATGTACTGAATTTCTGGA	2698-2718
M800F	ACCAGCAACAtGACTTTCTTTGG	790-812
M800R	CCAAAGAAAGTCaTGTTGCTGGTTTTGTA	784-813
M933F	ACAATATAgAGCGTTTGTCTTCT	926-948
M933R	ACGCTCcATATTGTTATACTCCA	917-939
M1116F	GCATAAAtAGGAATACTTTCACAGG	1109-1133
M1116R	GAAAGTATTCCTaTTTATGCCTGG	1105-1128
M2129F	TTCATGATAAgTGCCAGTATGCTACTGATTTT	2119-2150
M2129R	ATCAGTAGCATACTGGCAcTTATCATGAAAAAG	2110-2148
M2160F	TATgATACTGCTCATCCATTTCG	2156-2179
M2160R	ATGGATGAGCAGTATcATAAAGAT	2152-2175
MR	ATAAAAAGATATCCGCCAGCC	2182-2202

*Location of primers in the coding sequence of the porcine TLR3 gene (GenBank No. DQ266435). Enzyme recognition sites are underlined. Mismatched bases are presented with small letters.

Cloning

Total RNA was isolated from spleen tissues of Yorkshire pigs with the Trizol reagent according to manufacturer specifications (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized with 1 µg total RNA using Superscript II and Oligo(dT) primers (Invitrogen). Polymerase chain reaction (PCR) was performed with the C1F/R primer pair in a 25-µL final volume using Ex Taq DNA polymerase (TaKaRa, Dalian, China), cDNA was synthesized, and the product was inserted into a pMD-18T vector (TaKaRa) for confirmation by sequencing. The recombinant pMD-18T vector carrying the complete CDS, named pMD-18T-WT-TLR3, was used for site-directed mutagenesis and wild-type (WT) eukaryotic expression vector construction.

Site-directed mutagenesis

Site-specific mutation was introduced using a PCR-based method as reported previ-

ously (Li et al., 2011). Briefly, the mutation was introduced into the end of PCR products through two independent PCRs (Reactions A and B; Table 2) using primers containing mismatched bases, high-fidelity *Pfu* DNA polymerase (TransGen, Beijing, China), and pMD-18T-WT-TLR3 as a template. Next, the two obtained overlapping PCR fragments were spliced to introduce the mutation into the products (Reaction C; Table 2). The products of Reaction C were sub-cloned into the pMD-18T vector for sequencing confirmation.

Table 2. Primer pairs used in PCR-based site-directed mutagenesis.

Loci	Reaction A	Reaction B	Reaction C
c.800C>T	C1F/800R	800F/C1R	C1F/C1R
c.933A>G	C1F/M933R	M933F/MR	C1F/MR
c.1116A>T	C1F/M1116R	M1116F/MR	C1F/MR
c.2129C>G	C1F/2129R	2129F/C1R	C1F/C1R
c.2160T>G	C1F/2160R	2160F/C1R	C1F/C1R

Eukaryotic expression vector construction

For construction of the WT eukaryotic expression vector, pMD-18T-WT-TLR3 and pcDNA3.1+ (Invitrogen) were double-digested with *Eco*RI and *Xho*I (TaKaRa), respectively; the target fragments were purified using the DNA Gel Extraction Kit (Watson, Shanghai, China), and were ligated using T4 DNA ligase (TaKaRa). After transformation into *Escherichia coli* JM109, the recombinant plasmids were extracted from bacterial colonies and identified by double-digestion using *Eco*RI and *Xho*I. Sequencing was performed by the Beijing Genomics Institute (China) for confirmation. The positive recombinant plasmids were named pcDNA3.1-WT-TLR3. For construction of the MT expression vector, the pcDNA3.1-WT-TLR3 and the pMD-18T vector carrying mutant TLR3 were double-digested with *Eco*RI and *Xho*I (for 800, 2129, and 2160 loci) or *Eco*RI and *Eco*RV (for 933 and 1116 loci), respectively, and the target fragments were purified, ligated, and sequenced as described above.

Transfection and dual-luciferase assay

PK-15 cells were cultured as described previously (Li et al., 2011). The cells were transiently co-transfected with one of the TLR3 expression vectors (WT or MT), the IRF3-dependent firefly luciferase construct (pISRE-Luc) (Stratagene, La Jolla, CA, USA), and the *Renilla* luciferase reporter plasmid (pRL-TK) (Promega, Madison, WI, USA) using the Lipofectamine 2000 reagent (Invitrogen). At 24 h post-transfection, cells were stimulated with poly(I:C). Firefly and *Renilla* luciferase activities were measured using the Dual-Glo Luciferase Assay System (Promega). The firefly luciferase activity was normalized by dividing it by the *Renilla* luciferase activity. To determine whether the difference between the WT and MT allele was significant, the Student *t*-test was performed using the SAS 9.1 software (SAS, Cary, NC, USA).

Genotyping

The distribution of SNP c.933A>G in 42 Landrace pigs and in 220 individuals

from a three-way crossbreed [Piétrain x (German Landrace x German Large White)] was analyzed. Genomic DNA was isolated from whole blood using the phenol-chloroform method. Genotyping was performed using the created restriction site PCR-restricted fragment length polymorphism method. Specifically, a mutant base was introduced into the amplification fragment by the primer to create a recognition site of the endonuclease *Xba*I with one kind of base, A, at the polymorphic site. The primer pair was as follows: 5'-tatttctttctggagtataacaat<u>c</u>t-3' (forward; the underlined base represents the mismatched base) and 5'-tgcctggaaagttgttatct-3' (reverse). The PCR and digestion reactions were performed normally, and the subsequent fragment separation was conducted on 4% agarose gel for 1 h at 100 V.

RESULTS

SNPs of porcine TLR3

Morozumi and Uenishi (2009) and Yang et al. (2011) previously searched for SNPs in CDS of the porcine TLR3 gene among different groups and identified 7 non-synonymous SNPs that led to amino acid substitutions (Table 3). SNPs c.4A>G and c.95G>A were present with very low frequencies, and in only one study among the 96 individuals from 11 breeds analyzed (Morozumi and Uenishi, 2009). Furthermore, SNPs c.800C>T, c.933A>G, and c.1116A>T were present in the leucine rich repeat (LRR) domain. SNPs c.1116A>T and c.2129C>G were prevalent alleles, being represented in >10% of populations sampled by Morozumi and Uenishi (2009) and Yang et al. (2011), respectively. Additionally, SNP c.2160T>G was identified at a high frequency (22.3%) in populations consisting of 87 individuals of Chinese indigenous breeds and Western commercial pig breeds, and 10 Chinese wild boars (Yang et al., 2011); however, its frequency was lower (4.8%) in a sample panel investigated by Morozumi and Uenishi (2009). LRR domains are involved in PAMPs recognition (Chang, 2010). In addition, the prevalence of SNPs suggests that they have potential to be used as molecular markers in pig breeding. Therefore, SNPs c.800C>T (p.T267M), c.933A>G (p.I311M), c.1116A>T (p.K372N), c.2129C>G (p.T710S), and c.2160T>G (p.I720M) were selected for functional analysis in cultured cells.

Table 3. Non-synonymous SNPs in the porcine TLR3 gene.

SNPs	Amino acid	Domain	Domain position	MAF (%)[1]	MAF (%)[2]
c.4A>G	p.S2G	SP	1-24	1.2	-
c.95G>A	p.R32K	LRRNT	25-32	0.6	-
c.800C>T	p.T267M	LRR10	251-276	1.8	-
c.933A>G	p.I311M	LRR12	301-324	0	4.6
c.1116A>T	p.K372N	LRR14	358-381	16.7	41.38
c.2129C>G	p.T710S	TM	705-724	21.4	12.64
c.2160T>G	p.I720M	TM	705-724	4.8	22.3

MAF = minor allele frequency. [1]Data from Morozumi and Uenishi (2009). [2]Data from Yang et al. (2011). (-) = not investigated by Yang et al. (2011). Domains were predicted according to the description of Matsushima et al. (2007).

Effect of SNPs on TLR3 function

The complete CDS of the TLR3 gene in Yorkshire pigs was successfully obtained us-

ing the reverse transcriptase PCR method. Compared with the sequence deposited in GenBank (No. DQ266435), 3 synonymous SNPs, c.36C>T, c.2643T>C, and c.2649A>G, were identified. No missense mutation was found. The nucleotides at positions 800, 933, 1116, 2129, and 2160 were C, A, A, C, and T, respectively, which are the most common nucleotide sequences among pig populations (Morozumi and Uenishi, 2009; Yang et al., 2011). Therefore, the sequence obtained was used as the WT TLR3 allele.

To determine whether the five SNPs resulting in amino acid substitutions affected TLR3 function, a cell-based assay was used to analyze TLR3-dependent activation of a firefly luciferase reporter, pISRE-Luc. pISRE-Luc contains direct repeats of the IFN-stimulated response element (ISRE) in the promoter region, which drives the expression of the downstream luciferase gene through binding activated IRF3. Therefore, the assay measures TLR3 function by the amount of firefly luciferase activity produced through activation of the IRF3 transcription factor. pRL-TK, constitutively expressing the *Renilla* luciferase, was used as a transfection control.

As shown in Figure 1, TLR3 responded to poly(I:C) in a dose-dependent manner with the best stimulation observed at 5 μg/mL for 12 h. Furthermore, transiently transfected cells can produce weak luminescence without stimulation. Thus, in subsequent experiments, results were measured as fold-change in induction of firefly luciferase activity in the presence of poly(I:C) relative to the unstimulated control.

Upon stimulation with 5 μg/mL poly(I:C) for 12 h, transfectants with c.800C>T, c.1116A>T, c.2129C>G, and c.2160T>G mutants showed firefly luciferase activity comparable to that of the WT allele with no significant difference ($P > 0.05$). Whereas, the transfectant with the c.933A>G mutant showed a significant decrease in activity ($P < 0.05$), which indicates weaker ability of the mutant protein to induce activation of IRF3 (Figure 2).
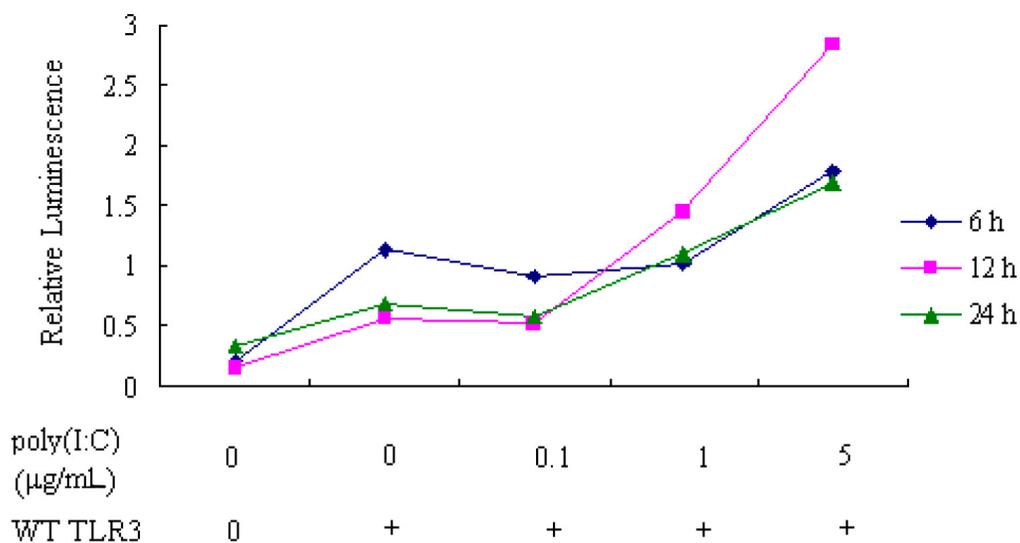

Figure 1. TLR3 responds to poly(I:C) in a dose-dependent manner in PK-15 cells. The values were measured as a ratio of firefly luciferase to that of *Renilla* luciferase.
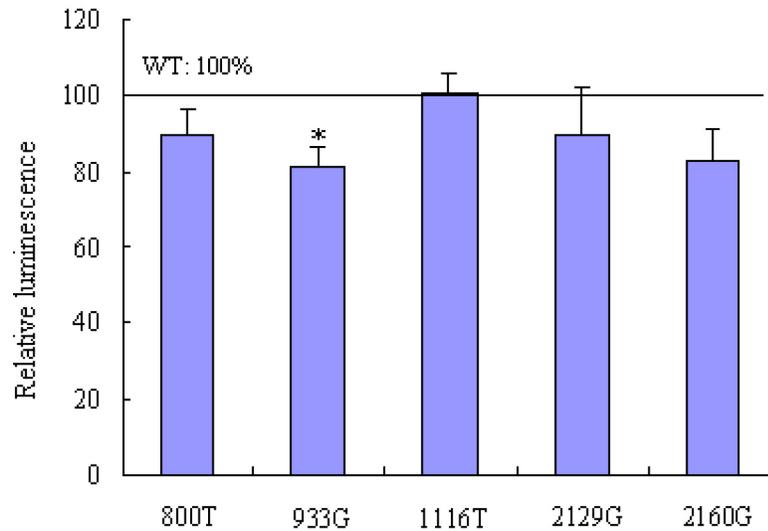
Figure 2. Effect of SNP in porcine TLR3 on the recognition of poly(I:C). The firefly luciferase activities of MT allele are displayed relative to that of the WT TLR3, which was normalized as 100%. Data are reported as means ± SE from three independent experiments, each with three replicates. Significant difference from the WT TLR3 was indicated ($*P < 0.05$).

Genotyping

Morozumi and Uenishi (2009) and Yang et al. (2011) analyzed SNPs of the porcine TLR3 gene and their distribution among pig populations, and found that SNP c.933A>G was only present in local Chinese pig breeds, including Min and Beijing black pigs (Tables 3 and 4). To obtain further information of SNP c.933A>G in Western commercial pig breeds, we genotyped Deutsche Landrace animals and individuals of a three-way crossbreed [Piétrain x (German Landrace x German Large White)]. Our results showed that SNP c.933A>G is rare in the two populations selected, because neither homozygote nor heterozygote carriers were found (Table 4).

Table 4. SNP c.933A>G distribution among pig populations.

Allele	Frequency (%)			
	M (30)*	B (12)*	L (42)	C (220)
Allele A	98.3	91.7	100	100
Allele G	1.7	8.3	0	0

M = Min pig; B = Beijing black pig; L = Landrace; C = Crossbreed pig. *Data from Yang et al. (2011).

DISCUSSION

TLRs are type I transmembrane glycoproteins. Its ectodomain contains tandem LRRs, which have been suggested to be involved in ligand recognition (Chang, 2010). All LRRs

contain a highly conserved segment (HCS) and a variable segment. The HCS is composed of an 11-residue stretch, LxxLxLxxNxL, in which "L" is L, I, V, or F, "N" is N, T, S, or C, and "x" is any amino acid (Kobe and Kajava, 2001). According to their cellular localization, TLRs can be divided into the cell-surface group (TLR1, 2, 4, 5, 6, and 10) and the cytoplasmic vesicle group (TLR3, 7, 8, and 9) (Chang, 2010). Compared with TLRs expressed on the cell surface, intracellular TLRs contain fewer non-synonymous SNPs in the protein-coding region (Shinkai et al., 2006; Morozumi and Uenishi, 2009). Only 4 and 7 non-synonymous SNPs were found in the human and porcine TLR3 gene, respectively (Ranjith-Kumar et al., 2007; Morozumi and Uenishi, 2009; Yang et al., 2011). Additionally, LRR domains of TLR3 and TLR7 show the lowest evolutionary rate from fishes to primates among all 15 TLRs identified thus far, except for TLR10 (Mikami et al., 2012). Together, this indicates that TLR3 has been subjected to severe selective pressures during evolution and any polymorphisms might therefore influence the receptor function. Genetic differences in the human TLR3 gene have been shown to influence the innate immune response through changing the receptor expression and/or responsiveness to ligands, and have been associated with various diseases (Al-Qahtani et al., 2012; Clifford et al., 2012; Nuolivirta et al., 2012; Yang et al., 2012). These results suggest correlations between TLR3 polymorphisms and the disease resisitance/susceptibility in pigs.

In this study, we investigated 5 known non-synonymous SNPs, c.800C>T, c.933A>G, c.1116A>T, c.2129C>G, and c.2160T>G, in the CDS of the porcine TLR3 gene for their functional relevance using a luciferase reporter assay in cultured PK-15 cells. One of the SNPs, c.933A>G, was found to significantly impair the responsiveness of the receptor to poly(I:C) ($P < 0.05$), as represented by decreased production of IRF3. SNP c.933A>G (p.I311M) results in the alteration of a conserved amino acid, the last "L", in the HCS of the 12th LRR (Matsushima et al., 2007). Moreover, I311 is conserved among TLR3 orthologs from fishes to primates (Figure 3). These observations suggest that I311 is crucial for the receptor function, which is consistent with our results.

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           LxxLxLxxNxL
                 *
    Sus    LKYFFLEYMNIERLSSRSLYGLSN    324
   Homo    LEYFFLEYMNIQHLFSHSLHGLFN    323
    Mus    LRYLSLEYMNIQRLSPRSFYGLSN    324
 Rattus    LKYLSLEYMNIQRLTPHSFRGLSN    324
    Bos    LEYLSLEYMNIEHLSSRSFYGLSN    324
  Equus    LEYFFLEYMNIEHLYVHSFYGLFN    323
  Felis    LEYFFLGYMNIEHLFSHSLYGLLS    323
   Ovis    LEYLSLEYMNIEHLSSRSFYGLSS    324
    Pan    LEYFFLDYMNIQHLFSHSLHGLFN    323
 Macaca    LEYFFLEYMNIQHLLSHSLHGLFN    323
 Gallus    LEYLNLEDMNIINVSSHLFYGLSS    323
  Danio    LEFLSLEHNTIRHLTNDSFSGLGN    324
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Figure 3. Alignment of the 11th LRR domain of TLR3 among species. p.311I in porcine was marked with asterisk. Arabic numerals indicate the position of the last base in the corresponding coding sequence. Homologous sequences were downloaded from GenBank with No. DQ266435 for *Sus scrofa*, NM003265 for *Homo sapiens*, NM198791 for *Rattus norvegicus*, NM001135928 for *Ovis aries*, NM126166 *for Mus musculus*, NM001079829 for *Felis catus*, NM001008664 for *Bos taurus*, NM001081798 for *Equus caballus*, EU204934 for *Macaca mulatta*, NM001130470 for *Pan paniscus*, NM001011691 for *Gallus gallus*, NM001013269 for *Danio rerio*.

SNP c.933A>G was found solely in local Chinese pig breeds (Tables 3 and 4). Local Chinese and Western commercial pig breeds have different disease resistance owing to variations in their breeding environments. Local Chinese breeds, especially the Min pig, are famous for their high general resistance to disease. Therefore, this SNP might provide certain advantages to local Chinese pig breeds. However, the attenuating TLR signaling plays dual roles in host defense responses. For example, SNP c.1234C>T in the human TLR3, which results in a reduced response to poly(I:C), is associated with susceptibility to recurrent herpes labialis on the one hand (Yang et al., 2012), whereas it confers natural resistance to HIV-1 infection and protects against geographic atrophy, on the other hand (Zhou et al., 2011; Sironi et al., 2012). Therefore, in order to determine to which disease SNP c.933A>G originally provided the carrier resistance, further studies of specific virus infection are needed.

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