An 192 bp ERV insertion in the first intron of TLR6 acts as an enhancer to increase *TLR6* and *TLR1* expression

xiaoyan wang

Yangzhou University <https://orcid.org/0000-0001-8521-2974>

Zixuan Chen

Yangzhou University

Eduard Murani

Leibniz Institute for Farm Animal Biology: Leibniz-Institut für Nutztierbiologie

Enrico D'alessandro

University of Messina

Yalong An

Yangzhou University

Cai Chen

Yangzhou University

Kui Li

Chinese Academy of Agricultural Sciences

Grazia Galeano

University of Messina

Klaus Wimmers

Leibniz Institute for Farm Animal Biology: Leibniz-Institut für Nutztierbiologie

Chengyi Song (✉ cysong@yzu.edu.cn)

Yangzhou University <https://orcid.org/0000-0002-0488-4718>

Research

Keywords: Pig, Retrotransposon, RIP, ERV, TLRs, TLR6, Enhancer, Expression, Polymorphism

Posted Date: November 5th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-101173/v1>

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Abstract

Background

Toll-like receptors (TLRs) play important roles in building innate immune and inducing adaptive immune responses. Associations of the *TLR* gene polymorphisms with diseases susceptibility, which are the basis of molecular breeding for disease resistant animals, have been reported extensively. Retrotransposon insertion polymorphisms (RIPs) were developed recently as a new type of molecular marker having great potential in population genetics and quantitative trait locus (QTL) mapping analysis. In this study, bioinformatics prediction combined with the PCR-based amplification was employed to screen for RIPs in porcine *TLR* genes. Their population distribution and impact on gene activity and phenotype of one RIP was further evaluated.

Results

Totally, five RIPs, located at the 3' flank of *TLR3*, 5' flank of *TLR5*, intron 1 of *TLR6*, intron 1 of *TLR7*, and 3' flank of *TLR8* respectively, were identified. These RIPs were detected in different breeds with an uneven distribution among them. By using the dual luciferase activity assay a 192 bp endogenous retrovirus (ERV) in the intron 1 of *TLR6* was proven to act as an enhancer increasing the activities of *TLR6* promoter and multiple mini-promoters. Furthermore, the real-time quantitative polymerase chain reaction (qPCR) analysis demonstrated that the ERV insertion significantly enhances the mRNA expressions of *TLR6*, the neighboring gene *TLR1*, and the downstream genes *MyD88* (Myeloid differentiation factor 88), *Rac1* (Rac family small GTPase 1), *TIRAP* (TIR domain containing adaptor protein), *Tollip* (Toll interacting protein) of TLR signaling pathway and the inflammatory factors *IL6* (Interleukin 6), *IL8* (Interleukin 8), and *TNFA* (Tumor necrosis factor alpha) in 30-day piglet tissues. In addition, the serum IL-6 and TNFα were also significantly upregulated by ERV insertion.

Conclusions

Overall, five RIPs were identified in several *TLRs*, and the 192 bp ERV insertion in the first intron of *TLR6* can improve the expressions of *TLR6*, *TLR1*, their downstream genes, and the inflammatory factors by acting as an enhancer affecting the regulation of TLR pathways, which may be applicable in the molecular breeding of disease resistant animals.

Introduction

Toll-like receptors (TLRs) play profound roles in motivating innate and adaptive immune responses. Protein(s) encoded by *TLRs* genes are important due to their ability of recognizing different types of pathogens and associated molecular patterns [1]. Polymorphisms in *TLR* loci and their influence on either susceptibility or resistance to major human infectious diseases, including tuberculosis, leishmaniasis,

malaria, filariasis, some autoimmune endocrine diseases, have been reported extensively [2–4]. It has been reported that C1174T of *TLR5* resulted in a common stop codon polymorphism and produced significantly lower levels of proinflammatory cytokines in comparison with individuals with the wild-type genotype which indicated that the *TLR5* stop codon polymorphism is associated with protection from the development of systemic lupus erythematosus [5]. The missense mutation of *TLR1* rs5743618, specific in Europeans can change the expression of 81 genes involved in the inflammatory response [6]. Chikungunya patients with rs179010-CC genotype of *TLR7* showed significantly high interferon alpha 1 (IFN- α) level, which might act as potential prognostic biomarkers for predicting Chikungunya susceptibility [7]. It is commonly accepted that *TLRs* are important candidate genes for some human diseases.

For domesticated farm animals, *TLRs* also have been suggested as the most promising candidate genes for immunity improvement or disease resistance by molecular breeding[8]. Genetic variants of *TLRs* associated with cattle mastitis, mycobacterial infection, and paratuberculosis have been identified [9–11]. Totally, ten *TLRs* in the pig genome were annotated and SNP screening in the pig *TLRs* and their expression patterns in immunity organs have been reported extensively[12]. It has been suggested that piglets with the T allele of *TLR5* (C1205T) exon exhibit impaired resistance to *Salmonella typhimurium* infection [13]. C506W of *TLR4* exon in Japanese wild boar populations caused loss of ability to induce nuclear factor- κ B activation after lipid A stimulation[14–16]. Association between SNPs in *TLR4* and *TLR5* with transcription levels of cytokines indicate that these SNPs are related to the modulation of the cytokine mediated immune response. Until now, all studies were focusing on the associations of SNPs in *TLRs* with diseases susceptibility, reports for retrotransposon insertion polymorphism in *TLRs* and their genetic effects are not available.

Retrotransposons are important components of plants and mammal genomes, which account for nearly half of the mammal genomes [17, 18], and can mobilize themselves to new genomic locations and generate polymorphic insertions. Retrotransposons can further be classified into LTR (Long Terminal Repeat elements, including endogenous retrovirus, ERV) and non-LTR (including LINE, Long Interspersed Nuclear Elements; and SINE, Short Interspersed Nuclear Elements) [19]. For a long time, transposable elements including retrotransposon has been considered as genomic parasites[20, 21]. However, recently, more and more evidences support that retrotransposons contribute to genome architecture and evolution even maintenance of three-dimensional chromatin organization in mammals. [18, 22–24]

Retrotransposon Insertion Polymorphism, RIPs, have been applied for genomic evolution and genetic diversity evaluations in plants as molecular markers[25, 26]. For human, RIPs have been identified as causative mutations for some diseases [27]. By genome-wide association studies, an intronic Alu insertion polymorphism inserted in the 97 bp downstream of a *CD58* exon is on a haplotype associated with multiple sclerosis risk due to the Alu insertion altering mRNA splicing [28, 29].

For domestic animals, RIPs also have been used for evolution and population genetic analysis of sheep [30], deer[31], cat[32], chicken [33], and rabbit [34]. Several RIPs have been defined as the causative

mutations for phenotype variations. An ERV insertion in the 5' flanking region of *SLCO1B3* causes blue eggshell by promoting the expression of *SLCO1B3* gene in the uterus (shell gland) of the oviduct in chicken[35, 36]. The henny feathering allele harbors an insertion of an intact avian ERV at the 5'end of *CYP19A1*[37]. The SINE insertion in follicle stimulating hormone beta (*FSHβ*) and protein disulfide isomerase associated 4 (*PDIA4*) genes were associated with litter size variation in pigs [38–39].

In the current study, the contribution of RIPs to the structural variations of TLR genes, the breed distribution of these RIPs and exemplarily the genetic effect of one RIP was investigated. We identified five RIPs for 10 TLR genes, and our data suggest that one RIP plays role in the regulation of TLR pathway by acting as an enhancer. These findings will contribute to the understanding the role of RIPs in the pig genomic and genetic variation, and one RIP may be useful for disease resistance selection in the pig breeding.

Material And Methods

RIP screen

Ten *TLR* genic and their flanking sequences (5 kb 5' flank and 3 kb 3' flank) were obtained from fifteen assembled non-reference genomes (Landrace, Yorkshire, Pietrain, Berkshire, Hampshire, Cross-breed of Yorkshire/Landrace/Duroc, Wuzhishan, Tibetan, Rongchang, Meishan, Bamei, Bama, and Jinhua, Goettingen, and Ellegaard Gottingen minipigs) and one reference genome (Duroc) deposited in NCBI database (<https://www.ncbi.nlm.nih.gov/>) to screen the structural variations by alignment with ClustalX program. Large structural variations (more than 50 bp) were remained for further analysis. Retrotransposon (SINE, LINE, and ERV) insertions were annotated by RepeatMasker (<http://www.repeatmasker.org/>) with a customer constructed library [40]. Promoters were predicted in <http://www.cbs.dtu.dk/services/Promoter/>. Transcription factor binding sites were searched at https://bip.weizmann.ac.il/toolbox/seq_analysis/promoters.html#databases and CPGs islands were recognized in https://www.novopro.cn/tools/cpg_islands.html?tdsourcetag=s_pctim_aiomsg. The predicted large structural variations (more than 50 bp) overlapping with retrotransposon (SINE, LINE, and ERV) insertions were designated as RIPs. These RIPs were further evaluated in seven Chinese native pig breeds (Diannan small-ear Pigs, Erhualian, Wuzhishan, Bama, Tibet, Meishan, Fengjing Pigs), three commercial pig breeds (Duroc, Landrace, Yorkshire), one cross breed (Sujiang) and wild boars (from Anhui province, Fujian province and Heilongjiang province) by PCR amplification (Vazyme, Nanjing, China). For each breed, two pooled DNA samples, each contained at least three animal samples, were used. Origins of pigs and primers used for RIP

evaluation was listed in Table S1 and Table S2. All obtained RIPs were further confirmed by TA cloning(Tiagen, Beijing, China)following the manufacturer's instructions and sequencing.

RIP Genotyping

Totally, twelve breeds (Duroc, Landrace, Yorkshire, Erhualian, Meishan, Fengjing, Bama, Tibetan, Wuzhishan, Diannan small-ear, Sujiang, and Sicilian black) were used to genotype the RIP distribution. Among these breeds, Duroc, Landrace, and Yorkshire are three lean type breeds, Sicilian black, Erhualian, Meishan, and Fengjing are five fat type pigs and Bama, Diannan small-ear, Wuzhishan, and Tibet are four miniature pigs. Erhualian, Meishan, Fengjing, Bama, Tibetan Diannan small-ear, Wuzhishan, are Chinese native breeds, while Sujing is a middle-type pigs, which is a new cross breed with 62.5% Duroc, 18.75% Jiangquhia, and 18.75% Fengjing bloods. Sicilian black pigs are Italian native breeds. The genotype and the allele frequencies were calculated, and Hardy-Weinberg equilibrium were tested using χ^2 . Polymorphic information content (PIC) was calculated by the formula:

$$PIC = 1 - \sum_{i=1}^m P_i^2 - \sum_{i=1}^{m-1} \sum_{j=i+1}^m 2P_i^2 P_j^2$$

Dual luciferase reporter assay

The predicted core promoter regions (NC_010450.4, 30171883-30172870) of TLR6 with and without the ERV insertion was cloned from the Sujiang genomic DNA (primers were listed in Table S2), and verified by sequencing. Then the clones were inserted into the pGL3-Basic vectors (Ambion, Austin, American) to construct TLR6^{ERV+}-Luc(En) vector and TLR6^{ERV-}-Luc(En) Vector. In addition, β -globin and Oct4 mini-promoter were cloned from pEDV- β -globin-GFP and pTol2-Oct4-mCherry vector, respectively[41] and inserted into the pGL3-Basic vectors with or without the 192bp ERV for enhancer activity evaluation. A total of 2×10^4 PK15 and Hela cells were plated in a 24-well plates and transfected with the plasmids by using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, American). After 48 hours, cells were collected for luciferase activity evaluation by using the dual luciferase reporter system (Promega, Madison, American) according to the manufacturer's protocol.

Expression analysis

The Sujing piglets were genotyped and five piglets for each genotype ($ERV^{+/+}$, $ERV^{+/-}$, and $ERV^{-/-}$) were selected and slaughtered to collect tissue samples at 30 days. The mRNAs were extracted and cDNAs were prepared according to the manufacturer's protocol by using TAKARA Kit (Takara, Tokyo, Japan). Then, the mRNA expressions of *TLR6*, *TLR1*, *MyD88*, *RAC1*, *TOLLip*, *TIRAP*, *TNF α* , *IL-6*, and *IL-8* mRNA were evaluated by quantitative real-time PCR (qPCR) using the 7900HT Fast Real-Time PCR System (Applied Biosystems, New York, American) in a total volume of 20 μ l containing SYBR mix (10 μ l), primers (4 ng), and cDNA sample (50 ng) according to the manufacturer's instructions (Takara, Tokyo, Japan). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an endogenous control to normalize the target gene expression in four different tissues including liver, lung, kidney, and spleen. All gene expression was measured using the $2^{-\Delta\Delta C_t}$ method. PCR products were run on 1.5% ethidium bromide-stained agarose gels and confirmed using melting curve analyses to assess PCR product quality.

Measurement of serum TNF α , IL-6, and IL8 by enzyme linked immunosorbent assay (ELISA)

TNF α , IL-6, IL8 concentration in serum of 30-day Sujian piglets were measured using the Pig TNF α , IL-6, and IL8 ELISA Kit (Solarbio Science, Beijing, China) by following the manufacturer's protocol. All measurements were performed in 3 replicates, and mean values were used for statistical analysis.

Statistical analysis

Experimental results were processed by statistical SPSS17.0 software package (SPSS, Chicago, USA) using one-way analysis of variance with Tukey's post hoc test, and the data were expressed as mean $\pm$ S.D.

Animal welfare

All treatments and protocols involving animals in this study were strictly done in accordance with the guidelines of the Animal Experiment Ethics Committee of Yangzhou University (approval number: YZUDWSY2018-12).

Results

Five RIPs generated by retrotransposon insertions in the pig TLR gene cluster

Ten *TLR* genes and their flanking sequences from sixteen assembled pig genomes, representing lean type pigs (Cross-breed of Yorkshire/Landrace/Duroc, Duroc, Landrace, Yorkshire, Pietrain, Berkshire, and Hampshire), fat type pigs (Rongchang, Meishan, Bamei, and Jinhua), and miniature pigs (Bama, Wuzhishan, Tibetan, Goettingen, and Ellegaard Gottingen) were used for screening of structural variations by alignment with ClustalX program (version 2.0) [42]. Totally, we identified 53 large SVs (more than 50 bp and less than 1000bp) or large frameshift variants (more than 1000bp), and 35 of them were

predicted as retrotransposon insertion polymorphisms, including 15 SINE, 11 ERV and 6 LINE insertions, which were summarized in additional file 1 (Table S3). Then all these predicted insertions were further evaluated by wet PCR via the specific primer pairs spanning the insertions in pooled samples. Finally, 5 RIPs, including two SINE insertions, two ERV insertions and one L1 insertion, were obtained by using 11 domesticated pig breeds and wild boar samples (Fig. 1a). All these insertions were confirmed again by TA cloning and sequencing. One 288 bp and one 294 bp SINE insertions in the 3' flanks of *TLR3* and *TLR8* were detected, respectively. Moreover a single 357 bp L1 insertion in the 5' flank of *TLR5* was found, and in addition two ERV insertions were detected, one 192 bp and one 413 bp in first intron of *TLR6* and *TLR7* were identified, respectively. We named those insertion as *TLR3*-SINE-RIP, *TLR5*-LINE-RIP, *TLR6*-ERV-RIP, *TLR7*-ERV-RIP, and *TLR8*-SINE-RIP, respectively (Fig.1b and 1c).

RIP distribution in different pig breeds

A total of eleven breeds (Duroc, Landrace, Yorkshire, Erhualian, Meishan, Fengjing, Bama, Tibetan, Wuzhishan, Diannan small-ear and Sujiang) were used for all RIPs evaluation, while extra breeds, the Sicilian black from Italy and the Landrace and the Yorkshire from Germany, were used for *TLR6*-ERV-RIP evaluation. For breeds displaying polymorphic RIPs in Fig.1 an additional individual samples were tested (Table 1). The PCR genotyping revealed that *TLR3*-SINE-RIP in six breeds (Duroc, Erhualian, Bama, Tibetan, Meishan, and Fengjing), *TLR5*-LINE-RIP in three breeds (Duroc, Yorkshire, and Wuzhishan), *TLR6*-ERV-RIP in three breeds (Sujiang, Bama, and Fengjing), *TLR7*-ERV-RIP in two breeds (Landrace and Wuzhishan), and *TLR8*-SINE-RIP in four breeds (Landrace, Yorkshire, Sujiang, and Wuzhishan) display polymorphisms. Most RIPs in most breeds were in Hardy-Weinberg equilibrium ($P>0.05$), while *TLR3*-SINE-RIP in Meishan, *TLR6*-ERV-RIP in Sujiang and Fengjing, *TLR7*-ERV-RIP in Landrace, *TLR8*-SINE-RIP in Wuzhishan deviated from the Hardy-Weinberg equilibrium ($P<0.05$). The SINE^{+/+} genotype of *TLR3*-SINE-RIP in Duroc and Tibetan, the ERV^{-/-} genotype of *TLR6*-ERV-RIP in Fengjing, and the ERV^{+/+} genotype of *TLR7*-ERV-RIP in Landrace and Wuzhishan were not detectable. As for polymorphic information content (PIC), RIPs in most breeds display moderate polymorphism (ranging from 0.239 to 0.375), except for the Duroc and the Tibetan, where *TLR3*-SINE-RIP shows low polymorphism (low than 0.150).

Evidence of enhancer activity of the 192 bp ERV insertion near the core promoter of *TLR6*

Both of *TLR6* and *TLR7* genes contain an ERV insertion in the first intron, and further analysis revealed that the 192 bp ERV insertion was near the core promoter region of *TLR6*, and diverse regulatory elements such as CpG islands and transcription factor binding sites (Fig. 2a). To further evaluate the potential roles of ERV insertions in the regulation of *TLR6* activity, the core promoter regions with or without the 192 ERV insertion allele were cloned into a luciferase reporter vector (pGL3-Basic), respectively (Fig.2a), and then submitted for luciferase activity evaluation. The dual luciferase activity assay revealed that the ERV insertion significantly improve the promoter activity of *TLR6*, the luciferase activity in cells transfected with the vector with ERV insertion allele (*TLR6*^{ERV+}-Luc(En)) were almost two times ($P<0.01$) than that without ERV insertion allele (*TLR6*^{ERV-}-Luc(En)) in both porcine PK15 and human Hela cells (Fig. 2b),

which suggested that the 192 bp ERV may act as an enhancer in the regulation of *TLR6* activity. To further confirm this deduction, we evaluated the enhancer activity of the ERV insertion by cloning it into the luciferase reporter vector containing mini-promoter, but absent SV40 enhancer, which is generally used for enhancer activity evaluation. Two type mini-promoters (β -globin, and OCT4) were evaluated, and the schematic diagram of vectors were shown in Fig.3a. Again, the luciferase activity assay revealed that the ERV insertion allele significantly improve all these mini-promoter activities in both PK15 (Fig.3b) and Hela cell lines (Fig.3c). These data strongly support that the 192 bp ERV insertion acts as an enhancer and may be involved in the regulation of TLRs.

ERV insertion alter the expression of pig *TLR6* and *TLR1* and their downstream genes in multiple tissues

To further illustrate the biological roles of the 192 bp ERV insertion in the Toll like receptor signaling pathway, we investigated the mRNA expressions of *TLR6* and *TLR1*, which are neighboring genes located on chromosome 8, and their downstream genes (*MyD88*, *Rac1*, *TOLLip*, *TIRAP*, *IL-6*, *IL-8*, and *TNF α*) in the same pathway by qPCR in multiple tissues (liver, spleen, lung and kidney) for different genotypes in 30-day piglets. The qPCR results revealed that, generally, the ERV insertion improve the expression of *TLR6* and *TLR1* in these tissues. In detail, in the spleen, kidney and liver tissues, the expression of *TLR6* and *TLR1* in the pigs with ERV^{+/+} genotype were significantly higher ($P<0.05$) than that in those animals with ERV^{+/-} and ERV^{-/-} genotypes. In lung, there were significant expression differences ($P<0.01$) of *TLR6* between the ERV^{+/+} animals and the ERV^{-/-} animals (Fig.4a and 4b). *MyD88*, *Rac1*, *TOLLip* and *TIRAP* are the downstream genes of *TLR6* and *TLR1* in the Toll like receptor signaling pathway and play key roles in innate immune mechanisms as central molecules. The qPCR results revealed that ERV insertion enhanced the expressions of most downstream genes of TLRs. In detail, in the spleen, lung and kidney, the expression levels of *MyD88* and *Rac1* were significantly higher ($P<0.05$) in the animals with ERV^{+/+} than in the animals with ERV^{+/-} and ERV^{-/-} genotypes (Fig.4c and 4d). Expression of *TIRAP* and *Rac1* in liver, lung and kidney of ERV^{+/+} homozygous animals were significantly ($P<0.05$) higher than those of heterozygote (ERV^{+/-}) and homozygote (ERV^{-/-}) animals. In spleen, significantly ($P<0.05$) difference was only observed for the expression of *TIRAP* gene between homozygotes ERV^{+/+} and ERV^{-/-} animals (Fig.4e and 4f). Inflammatory factors *IL-6*, *IL-8*, *TNF α* are important genes in the end of Toll like receptor signaling pathway. The expression of *TNF α* , *IL-6*, *IL-8* increased significantly ($P<0.05$) in the spleen, lung and kidney of homozygous of ERV^{+/+} compared with that in ERV^{-/-} genotype piglets (Fig.4g, 4h, 4i). These results indicated that the 192 bp ERV insertion allele near the core promoter of *TLR6* increases not only the expressions of *TLR6* and *TLR1*, but also the expressions of the downstream genes of the TLR signaling pathway.

Impact of ERV insertion on the serum immune cytokine

To investigate the impact of the ERV insertion allele near the core promoter of *TLR6* on the immune response, several serum immune cytokines were measured by ELISA for different genotypes of 30-day piglets. The ELISA analysis revealed that, consistent with the higher expression of *IL-6* and *TNF α* in the

important immune tissues (spleen and kidney) of ERV^{+/+} piglets compared that in other genotype piglets (ERV^{+/-} and ERV^{-/-}), the serum concentrations of IL-6 and TNF α in the animals with ERV^{+/+} genotype were also significantly higher than that in the ERV^{-/-} genotype animals ($P<0.05$) (Figure 8B). But there is no significant difference of serum IL8 among different genotype.

Discussion

Retrotransposons are dominant components in most land plant genomes and mammals [18, 26, 43] and regarded as important drivers of species diversity and putative actors in evolution and adaptation [26, 44–46]. Various methods, such as inter-retrotransposon amplified polymorphism (IRAP) and retrotransposon-microsatellite amplified polymorphism (REMAP), have been developed to exploit RIPs [47] (Kalendar and Schulman, 2006). They have been successfully applied in the studies of plant genetic diversity and QTL mapping [48]. Furthermore, some RIPs have been proved as a causal mutation to change the plant phenotype [49–51]. It is believed that SINE of RIPs are “nearly ideal” genetic markers to facilitate plant breeding [52]. In domestic animals, RIPs also have been applied to genetic diversity, evolution and variety identification [18], and display great potentials in animal molecular breeding. Our previous study revealed that LINE, LTR, and SINE are highly enriched in the pig genome, and totally account for about 37.13% of the genome sequence [40]. According to the insertion age analysis, differential evolution profiles were observed for different families and subfamilies of retrotransposons. Most retrotransposons in the pig genome are ancient and no longer jumping, and cannot generate polymorphic insertions in populations, whereas some of them were thought to be younger retroelements, such as SINEA, L1D, ERV6 subfamilies [40]. These retrotransposons still play roles in shaping genomes and gene evolution and contribute to the genomic variations and their insertions tend to generate polymorphisms, which can be used as genetic markers. In addition, it has been suggested that transposable elements affect the genome in both destructive and constructive ways [43]. Natural selection and genetic drift could shape the distribution and accumulation of TEs and insertions with destructive effects are rapidly removed from the population [53]. In the current study, we identified five RIPs in *TLRs* genic and flanking regions by alignment and combining the PCR validation. They located in different genic positions including introns, 5' and 3' flank of *TLRs*. Two ERV insertions, One of 192 bp and the other of 413 bp in length were identified in the first intron of both *TLR6* and *TLR7*, respectively, and based on the bioinformatics analysis, the 192 bp ERV insertion in the intron 1 of *TLR6* was predicted to be in the core promoter region, which may influence the gene expression. Since the ERV insertion fragment contained the U3-R-U5 sequences, which were thought to act as transcriptional regulators because of U3 region [54], it may act as enhancer or promoter that could be involved in the regulation of expression pattern of target genes [55–57]. Here, the luciferase assay analysis strongly supported that the 192 bp ERV insertion serves as an enhancer, which not only can increase the *TLR6* promoter activity, but also can improve the activities of diverse mini-promoters.

Population distribution analysis of these RIPs revealed that most loci are in genetic equilibrium, while some polymorphic insertion loci (*TLR5*-LINE-RIP) are in genetic disequilibrium, indicating that these loci

may have experienced strong selection and play biological roles in gene regulation and phenotype variation. *TLR6*-ERV-RIP in Sujiang and Fenjing deviated from the Hardy-Weinberg equilibrium ($P < 0.05$), indicating that this locus may have experienced strong purification selection considering the ERV insertion playing positive roles in the immune response. The deletion allele of *TLR6*-ERV-RIP were only found in China native pig breeds (Bama, Fenjing) or cross breeds of Sujiang (Duroc × Jiangquhai × Fengjing), while all analysed western pigs including Landrace, Yorkshire, Duroc, and Sicilian Black from Italy are monomorphic and only contain the ERV insertion allele (ERV⁺), suggesting that the deletion allele (ERV⁻) seems to originate from Asian pig breeds. Base on the positive effect of this allele on the disease resistance, it can be used to improve the disease resistance performance (such as Sujiang) or to be introduced into other China native breeds with marker assisted selection.

An age of 30 days is an important stage for pig to develop the adaptive immunity [58]. We further evaluated the expression of *TLR6* and *TLR1*, which is a nearby gene of *TLR6* with only 4.2 kb far away, in different tissues of 30-days piglets between different ERV insertion genotypes. The qPCR analysis demonstrated that the mRNA expressions of *TLR6* and *TLR1* was enhanced significantly by ERV insertion in multiple tissues of 30-day piglets. TLRs play important roles in the innate immune response by interacting with adapter molecules, such as *MYD88*, *TIRAP*, *Rac1* and *Tollip*, which are downstream genes of the TLR pathway [59]. With the help of the qPCR analyses, we also confirmed that all/most detected downstream genes of TLRs were also upregulated by ERV insertion in multiple tissues of 30-day piglets, suggesting that the ERV insertion not only increases the expressions of *TLR6* and *TLR1*, but also triggers the expressions of their downstream genes.

The cytokines released by inflammatory cells are essential factors in resisting pathogen infection. When activated by pathogens, TLRs recruit adapter molecules and subsequently initiate downstream signaling pathway, resulting in the activation of transcription factor nuclear factor kappa B subunit (NF- κ B) and the production of downstream inflammatory cytokines [60, 61]. Higher *TLR1* expression suggested better prognosis in patients with pancreatic ductal adenocarcinoma (PDAC) [10]. The mRNA expressions of major TLR genes including *TLR1* and *TLR6* of Tibetan pigs were higher in most immune tissues than those of Yorkshires, which may attribute to stronger innate immunity for Tibetan pigs [62]. And higher expression of TLRs were also associated with stronger disease resistance [61]. Yorkshire × Landrace (YL) pigs exhibited more serious clinical symptoms when artificially infected with porcine circovirus type 2 (PCV2) virus compared with Laiwu, which is a China native pig breed, indicating YL and Laiwu pigs display different susceptibility to PCV2 infection and Laiwu pigs seem to be more resistant for PCV2 virus. The serum levels of IL-6, IL-8, IL-12 and transforming growth factor beta 1 (TGF- β 1) in the PCV2 relatively resisting pigs of Laiwu also increased significantly more pronounced at the early infection stages than in YL pigs [63]. Here, consistent to the increased expression of genes in the TLR pathway due to ERV insertion, we found that the expressions of the important inflammatory factors including IL-6, IL-8, and TNF α were also increased by ERV insertion in tissues of 30-day piglets, with significant increment of IL-6 and TNF α in the serum. Overall, these data strongly support that the 192 bp ERV insertions improves the expressions of *TLR6*, *TLR1*, and their downstream genes by acting as an enhancer and playing roles

in the regulation of TLR pathway, which may not only alter the gene activities in the TLR pathway and inflammatory factors, but also cause phenotype variations during the immune response.

Conclusion

By using bioinformatics analysis and PCR-based verification, five RIPs, located in the 3' flank of *TLR3* gene, 5' flank of *TLR5* gene, intron 1 of *TLR6* gene, intron 1 of *TLR7* gene, and 3' flank of *TLR8* gene, was identified respectively and uneven distribution in diverse pig breeds was observed. The 192 bp ERV insertion in the intron 1 of *TLR6* significantly increases the activity of the *TLR6* promoter and multiple mini-promoters acting as an enhancer. Furthermore, the ERV insertion also enhances the expression of *TLR6* and *TLR1*, the downstream genes (*MyD88*, *Rac1*, *Rac1*, *Tollip*) of TLR signaling pathway and the inflammatory factors (*IL-6*, *IL-8*, *TNFα*) in diverse tissues of 30-day piglets, as well as the serum concentrations of IL-6 and TNFα. Thus, the 192 bp ERV insertion allele is benefit for disease resistance and may be useful for molecular breeding of disease resistant animals.

Availability Of Data And Materials

The datasets supporting the conclusions of this article are included within the article.

Abbreviations

TLRs

Toll-like receptors

RIPs

Retrotransposon insertion polymorphisms

QTL

Quantitative trait locus

ERV

Endogenous retrovirus

PCR

Polymerase chain reaction

MyD88

Myeloid differentiation factor 88

Rac1

Rac family small GTPase 1

TIRAP

TIR domain containing adaptor protein

Tollip

Toll interacting protein

IL6

Interleukin 6

IL8
Interleukin 8
TNF α
Tumor necrosis factor alpha
IFN- α
Interferon alpha 1
LTR
Long Terminal Repeat elements
SINE
Short Interspersed Nuclear Elements
ELISA
Enzyme linked immunosorbent assay
IRAP
Inter-retrotransposon amplified polymorphism
REMAP
Retrotransposon-microsatellite amplified polymorphism
NF-Kb
Nuclear factor kappa B subunit
PDAC
Pancreatic ductal adenocarcinoma
PCV2
Porcine circovirus type 2
TGF- β 1
Transforming growth factor beta 1

Declarations

Funding

This work was supported by the Natural Science Foundation of China (31872977, 32002146), China Postdoctoral Science Foundation (2020M671630), and the Jiangsu Agriculture Science and Technology Innovation Fund [CX(19)2016]; and the Priority Academic Program Development of Jiangsu Higher Education Institutions and the High-end Talent Support Program of Yangzhou University to Chengyi Song.

Contributions

XiaoYan Wang and Zixuan Chen performed animal trial, laboratory experiments, and statistical analysis, and drafted the manuscript; Eduard Murani and Enrico D'alessandro performed laboratory experiments; Yalong An and Cai Chen contributed to data collection and statistical analyses; Kui Li, Grazia Galeano

and Klaus Wimmers assisted in study design and reviewed manuscript; and Chengyi Song designed the study and reviewed the manuscript. The authors read and approved the final manuscript.

Ethics declarations

Ethics approval and consent to participate

All treatments and protocols involving animals in this study were strictly done in accordance with the guidelines of the Animal Experiment Ethics Committee of Yangzhou University (approval number: YZUDWSY2018-12).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. Genotype and allele frequency of five RIPs in the RIP-polymorphic breeds

RIP	Breed	N	Genotype/%			Allele/%		Hardy-Weinberg/P	PIC
			+/+	+/-	-/-	+	-		
<i>TLR3-SINE-RIP</i>	Duroc	24	0	16.67	83.33	8.33	91.67	0.656	0.141
	Erhualian	24	25.00	45.83	29.17	47.92	52.08	0.689	0.375
	Bama	30	3.33	60.00	36.67	33.33	66.67	0.552	0.346
	Tibetan	35	0	17.14	82.86	8.57	91.43	0.579	0.144
	Meishan	24	37.50	29.17	33.33	52.08	47.92	0.042	0.375
	Fengjing	23	47.83	39.13	13.04	67.39	32.61	0.599	0.343
<i>TLR5-LINE-RIP</i>	Duroc	24	4.17	62.50	33.33	35.42	64.58	0.073	0.353
	Yorkshire	24	8.33	41.67	50	29.17	70.83	0.967	0.328
	Wuzhishan	24	29.17	50.00	20.83	54.17	45.83	0.973	0.373
<i>TLR6-ERV-RIP</i>	Sujiang	163	52.76	20.86	26.38	63.19	36.81	1.89 ^{e-12}	0.357
	Erhualian	36	27.78	22.22	50.00	38.89	61.11	0.001	0.362
	Bama	43	44.19	39.53	16.28	63.95	36.05	0.350	0.355
	Fengjing	24	41.67	58.33	0	70.83	29.17	0.044	0.328
	Yorkshire (German)	31	100	0	0	100	0	1	0
	Sicilian black	30	100	0	0	100	0	1	0
	(Italy)								
	Landrace	32	100	0	0	100	0	1	0
<i>TLR7-ERV-RIP</i>	(German)								
	Landrace	18	0	83.33	16.67	41.67	58.33	0.002	0.368
	Wuzhishan	23	0	43.48	56.52	21.74	78.26	0.183	0.282
	Landrace	24	75.00	16.67	8.33	83.33	16.67	0.050	0.239
	Yorkshire	24	62.50	29.17	8.33	77.08	22.92	0.393	0.291
	Sujiang	24	50.00	45.83	4.17	72.92	27.08	0.432	0.317
	Wuzhishan	23	43.48	26.09	30.43	56.52	43.48	0.024	0.371

Eleven breeds (Duroc, Landrace, Yorkshire, Erhualian, Meishan, Fengjing, Bama, Tibetan,Wuzhishan, Diannan small-ear, and Sujiang) were used for all RIP evaluation, extra breeds of Sicilian black from Italy and Saddleback from Germany were used for *TLR6*-ERV-RIP evaluation. Only the breeds display polymorphic RIPs in Fig.1A were tested by increasing individuals except Diannan small-ear pig. Polymorphic information content (PIC) was measured by using the formula as described in materials and methods. The Hardy-Weinberg was detected by χ^2 test, and the $P<0.05$ indicates that the RIP distribution is deviated from the Hardy-weinberg equilibrium.

Figures

Figure 1

PCR identification and characteristic of RIP in TLRs. A: RIP identification in DNA pool by PCR; B: location of retrotransposon insertion in TLRs; C: characteristic of RIPs in TLRs.

Figure 2

Effect of ERV insertion on TLR6 promoter activity. A: sequence analysis of pig TLR6 and TLR1; B: a schematic diagram of the recombinant vector using pGL3-basic vector. En: SV40 enhancer; C: the luciferase activity assays. ** showed $P < 0.01$ between groups ($P < 0.01$).

Figure 3

Effect of ERV insertion on mini promoter activity. A: plasmid diagram of the recombinant vector using pGL3-basic vector; B: the luciferase activity assays of ERV on activity of β globin and Oct4 in PK15; C: the luciferase activity assays of ERV on activity of β globin and Oct4 in Hela cell. * showed $P < 0.05$; ** showed $P < 0.01$.

Figure 4

Effect of ERV insertion on expression of TLR6(A), TLR1(B) and downstream genes Myd88 (C) , Rac1 (D), TIRAP (E), TOLLip (F), IL6 (G), IL8 (H), TNFα (I) of TOLL like receptor signaling pathway in different organs or tissues of 30-day old piglets. * showed P<0.05; ** showed P<0.01.

Figure 5

The level of cytokine in blood of different TLR6-ERV-RIP genotypes; * showed $P < 0.05$; ** showed $P < 0.01$.

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