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Promoter CpG methylation status in porcine *Lyn* is associated with its expression levels

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ABSTRACT

Resistance to disease and improvement of product quality are important goals in pig farming. Tyrosine Protein Kinase Lyn (LYN) is one of several Src-family tyrosine kinases in immune cells. This protein functions both as a positive and negative regulator of B cell activation, and regulates signaling pathways through phosphorylation of inhibitory receptors, enzymes and adaptors, which suggested that LYN could be correlated with immunity and can be considered as a candidate gene to study in disease resistance. Until now, the profiles of expression and transcriptional regulation of the *LYN* gene in pig breeds different in immune capacity remain unclear. Using real-time PCR, it indicated that porcine *LYN* mRNA expressed mainly in immune organs including the spleen, duodenum and liver. Furthermore, Dahuabai pigs (a kind of Chinese indigenous pig breeds with higher immune capacity) showed significant higher *LYN* mRNA expression levels than that in Landrace. Methylation analysis indicates that *LYN* expression levels were associated with the methylation status of the *LYN* promoter, and methylation of the novel CpG site at −1268C/−1267G generated by transposition at −1267 (A→G) results in up-regulating transcriptional activity of this gene. Interestingly, the base A located in −1267 mainly exhibited in landrace while the base G mainly in Dahuabai pigs. These results might contribute to study the function of this gene in pig breeding for disease resistance.

1. Introduction

DNA cytosine methylation in the genomes of higher eukaryotes has been associated with gene regulation, genomic imprinting, the timing of DNA replication, aging, determination of chromatin structure, X-inactivation and carcinogenesis (Constancia et al., 1998; Jablonka et al., 1985; Razin, 1998; Razin and Cedar, 1991). Methylation of CpG clusters or CpG islands within gene promoters is generally thought to silence gene expression (Bird, 2002; Jaenisch and Bird, 2003), although methylation of gene silencers, suppressors and spacers can on occasion be conducive to transcription. Identifying changes in DNA methylation at CpG islands is therefore expected to lead to a clearer understanding of the differentiation of normal tissues and the development of complex diseases including cancer (Jones and Takai, 2001).

DNA methylation regulates gene expression at two levels, namely transcriptional gene silencing (TGS) and posttranscriptional gene silencing (PTGS). The former involves the inhibition of transcription and is associated with the hypermethylation of promoter sequences. The latter mechanism does not affect the rate of transcription but rather is involved in the posttranscriptional degradation of RNA species, and is associated with the hypermethylation of transcribed or coding sequences (Paszkowski and Whitham, 2001). In higher plants, promoter regions of silent genes have been found to be more highly methylated than actively transcribed sequences (Finnegan et al., 1993; Pikaard, 1999). In pigs, however, related reports are relatively few. Although it has been reported that DNA methylation may play a role in the regulation of tissue-specific gene expression (Ching et al., 2005; Futscher et al., 2002), differential DNA methylation patterns among tissues in adult pigs were until now not confirmed.

In humans, LYN is a Src-family kinase (SFK) that is predominantly expressed in B cells in the immune system, and has both positive and negative regulatory roles in B cell receptor (BCR)-induced signal transduction(Ingley, 2012; Xu et al., 2005). LYN participates in regulating intracellular signaling pathways through phosphorylation of inhibitory receptors, enzymes and adaptors (Ingley, 2012; Xu et al., 2005) and plays key roles in cell morphology, proliferation, motility, and survival in megakaryocytopoiesis (Kamińska et al., 2008). LYN

Abbreviations: BSP, Bisulfite sequencing PCR; BCR, B cell receptor; cDNA, Complementary DNA; CpG, Cytosine-guanine dinucleotide; dNTP, Deoxy-ribonucleoside triphosphate; LYN, v-yes-1 Yanaguchi sarcoma viral related oncogene homolog; PCR, Polymerase chain reaction; PTGS, Posttranscriptional gene silencing; RT-PCR, Reverse transcription Polymerase Chain Reaction; SFK, Src-family kinase; SNP, Single nucleotide polymorphism; TGS, Transcriptional gene silencing; TPKL, Tyrosine-protein kinase Lyn; TSS, Transcription Start Site.

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is an important regulator of autoimmune disease due to its profound ability to influence immune cell signaling (Choi et al., 2010; Ingley, 2012; Wu et al., 2008). To date, however, no paper has been reported regarding the tissue expression profiles and regulation of *LYN* gene in pig breeds different in immune capacity.

In this study, the expression patterns in different pig breeds were performed using real time PCR, and the 5′-flanking sequence of porcine *LYN* was cloned to study the mechanism of differential expression in different pig breeds through methylation analysis, which provided an insight into transcriptional regulation of the *LYN* gene and the potential biological roles of its gene product.

2. Materials and methods

2.1. Extraction of DNA and RNA

Eleven tissues (heart, liver, spleen, lung, kidney, duodenum, cerebrum, skeletal muscle, black fat, lymph and stomach) from five adult healthy Dahuabai pigs and four adult healthy Landrace pigs were harvested for DNA and RNA extraction. Total genomic DNA was extracted according to the manufacturer's protocol using the E.Z.N.A.™ Tissue DNA Kit I (Omega). Total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's instructions. DNA and RNA from the same tissues were pooled from different individuals of the same breeds. cDNA was then reverse-transcribed from normalized RNA using oligo (dT) primers and M-MLV reverse transcriptase (Promega).

2.2. Isolation of the 5′-flanking region of the pig LYN gene

To isolate the 5′-flanking region of pig *Tyrosine-protein Lyn*, the following gene specific primers were used: 5′-ACCAATCGTCCCCAAACAT-3′ (sense primer) and 5′-CTTGACCCCATAACAGCCC-3′ (antisense primer), generating a product of 2153 bp from −2024 nt to +129 nt (relative to the ATG initiation codon). Each 50 μl of PCR reaction mixture contained 5 μl of 10×10×*LA* PCR buffer II(Mg^{2+} Plus), 12 μl of dNTP (2.5 M),2 μl of forward primer (10 μM), 2 μl of reverse primer (10 μM), 25.5 μl of water, 2 ng of genomic DNA, and 0.5 μl of *LA* Taq Polymerase. The reactions were performed in a PTC-200 Peltier Thermocycler (BIO-RAD, USA). The reaction conditions were as follows: 94 °C for 5 min, then 32 cycles of 94 °C for 30 sec, 66 °C for 50 sec, and 72 °C for 2 min, and finally, at 72 °C for 5 min. Amplicons were cloned using PMD18-T vector TA cloning kits (Takara, Japan) according to the manufacturer's instructions and inserts were subsequently sequenced completely in both directions.

2.3. Determination of the transcription start site (TSS) for the LYN

In order to shed light upon the transcriptional regulation of porcine *LYN*, we characterized the transcription start site (TSS) of the gene using RT-PCR (Her et al., 1998; Lee et al., 2005; Wang et al., 2007), a procedure that employed an antisense primer located in −56 position (position is relative to the translation start codon) and seven various overlapping sense primers ranging from nt −222 to nt −757 (see Additional File A). RT-PCRs were performed using both pooled DNA and pooled cDNA templates isolated from eleven tissues. If PCR products were obtained using two templates and same primers, it indicated that PCR fragments amplified should be from the 5′-UTR region of the *LYN* mRNA.

2.4. Construction of promoter reporter

A series of plasmids containing fragments of various sizes from the 5′-flanking region of the porcine *LYN* gene was generated by PCR using forward primers for sequences located at varying distances from the transcription start site and including a *Mlu* I recognition sequence to facilitate ligation. The primers pR containing a *Hind* III recognition sequence were used as the common reverse primers and had 5′ ends located within the first intron (Additional File A). The amplified DNA fragments were digested with *Mlu* I and *Hind* III, and immediately inserted into the multiple cloning sites of the pGL3-basic vector (Promega) and sequenced.

2.5. Cell culture and transient transfection assay

The MARC-145 cell line, derived from African green monkey kidney, was purchased from the Shanghai Cell Bank of the China Academy of Sciences. Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO, USA)/high glucose supplemented with 10%(v/v) fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin and maintained at 37 °C in 5% CO_2. Transient transfection of MARC-145 with *LYN* promoter/firefly luciferase reporter plasmids was done with Lipofectamine™ 2000 transfection reagent (Invitrogen). At 48 h after transfection, cells were lysed and assayed for promoter activity using the dual luciferase reporter assay system. The enzymatic activity of luciferase was measured with a Microplate Luminometer LB 96V (Berthold). To normalize for transfection efficiency, the cells were co-transfected with 2 ng of *Renilla* luciferase reporter plasmid (pRL-TK vector, Promega).

2.6. Methylation analysis of CpG island and core promoter

The methylation status of individual CpG motifs in the 5′-flanking region of *LYN* was determined using bisulfite genomic pyrosequencing. Genomic DNA was modified according to the manufacturer's protocol using the EzWay™ DNA Methylation Detection Kit (Komabiotech, Korea). A CpG island (nucleotides −1365 to −1145) was predicted with three software applications (Methyl Primer Express software v1.0, CPG Islands 2 (http://ccnt.hsc.usc.edu/cpgislands2/cpg.aspx) and Urogene (http://www.urogene.org/meth-Primer)) in the promoter region of *LYN*. Parameters for CpG island prediction were: size ≥200 nt; GC content = 55% and CpG island observed/expected = 65% (Gardiner-Garden and Frommer, 1987). Average methylation level was carried out using 15 CpG sites (12 sites from CPG Islands, 3 sites from core promoter) of LYN gene isolated from 11 tissues.

A pair of primers was designed to amplify wild-type genomic *LYN* DNA sequences in the CpG island and core promoter, and PCR amplicons were sequenced after being cloned into the PMD18-T vector (Takara, Japan). In addition, two pairs of nested BSP (bisulfite sequencing PCR) primers were designed based on bisulfite-modified DNA sequences to amplify the CpG island and core promoter, respectively. After nested PCR was performed using inner primers labeled with biotin, the nested PCR products were sequenced by Pyrosequencing (Biotage, Sweden). Analysis of gene-specific DNA methylation patterns was performed by pyrosequencing technology (Tost and Gut, 2007a,b). In this study, three sequencing primers were used to analyze methylation in CpG island, and two sequencing primers were designed for the core promoter (see Additional File A). The bisulfite-modified DNA sequences were compared with the wild-type sequences to find the mutation sites and methylation sites.

2.7. Real-time PCR

Real-time PCR was performed using the SYBR Premix Ex Taq Kit (TaKaRa) with cycling conditions consisting of an initial 1 min at 95 °C, then 40 cycles comprising 15 sec at 95 °C, 15 sec at 55 °C, 40 sec at 72 °C, and followed by 95 °C for 1 min, 55 °C for 15 sec, 95 °C for 30 sec. Purified PMD-18T vectors (TaKaRa) containing the target gene cDNA were serially diluted (undiluted, 1/4, 1/16, 1/64 and 1/256) to construct standard curves for determining the optimal amplification conditions. PCR was then performed in triplicate and the gene expression levels were quantified relative to the expression of β-actin using LightCycler 480 Real-Time PCR System (Roche), employing an optimized comparative Ct (ΔΔCt) value method.