Original Article

Analysis of miRNA-29a Expression in Porcine Peripheral Blood Mononuclear Cells Using Quantitative Reverse Transcription Polymerase Chain Reaction

Sunaree Nantakhruea Porntippa Lekcharoensuk Win Surachetpong *

Abstract

MicroRNAs (miRNAs) are small non coding RNA that regulate gene expression as post transcriptional regulators in mammalian tissues. One of the miRNAs that participates in mammalian immune response is miR-29a. Despite the limited information of miR-29a in porcine cells, it is interesting to determine whether miR-29a involves in immune regulation in pig. In this study, we developed a quantitative reverse transcription polymerase chain reaction using SYBR detection system to examine the expression of primary miR-29a (pri-miR-29a) in porcine peripheral blood mononuclear cells (PBMCs) in the presence of mitogen. PBMCs were stimulated with concanavalin A (Con A) at a concentration of 10 µg/ml for 12 and 24 hours. Results showed that the expression level of pri-miR-29a decreased significantly in Con A stimulated PBMCs, while interferon-gamma (IFN-γ) increased relatively to the untreated control cells at 12 and 24 hour. In conclusion, our findings demonstrate the inversed expression patterns of pri-miR-29a and IFN-γ in Con A stimulated porcine PBMCs. Future studies of the function of miR-29a should provide important insight into the roles of this microRNA in pig immune response.

Keywords: immunity, miR-29a, porcine, qRT-PCR

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บทคัดย่อ

การวัดระดับการแสดงออกของ miRNA-29a ในเซลล์เม็ดเลือดขาวสุกรด้วยเทคนิคพีซีอาร์เชิงปริมาณ

สุนารี นันต๊ะเครือ พรทิพภา เล็กเจริญสุข วิน สุรเชษฐพงษ์ *

ไมโครอาร์เอ็นเอจัดเป็นอาร์เอ็นเอขนาดเล็กที่ทำหน้าที่ควบคุมการแสดงออกของยีนในสัตว์เลี้ยงลูกด้วยนมหลังกระบวนการสังเคราะห์อาร์เอ็นเอจากดีเอ็นเอ หนึ่งในไมโครอาร์เอ็นเอที่มีความเกี่ยวข้องกับระบบภูมิคุ้มกันในสัตว์เลี้ยงลูกด้วยนม คือ miR-29a เนื่องจากปัจจุบันการศึกษาเกี่ยวกับ miR-29a ในสุกรยังมีจำกัด ทางคณะผู้วิจัยจึงได้พัฒนาเทคนิคพีซีอาร์เชิงปริมาณด้วยวิธี SYBR เพื่อตรวจหาระดับการแสดงออกของ primary-miR-29a (pri-miR-29a) ในเม็ดเลือดขาวสุกร ผลการทดลองกระตุ้นเม็ดเลือดขาวด้วยสาร concanavalin A (Con A) ที่ระดับความเข้มข้น 10 ไมโครกรัมต่อมิลลิลิตร เป็นเวลา 12 และ 24 ชั่วโมง พบว่าระดับการแสดงออกของ pri-miR-29a ลดลงอย่างมีนัยสำคัญ ในขณะที่ระดับไซโตคัยน์อินเตอร์เฟอรอนแกมม่า มีปริมาณเพิ่มขึ้นทั้งที่ 12 และ 24 ชั่วโมง ภายหลังเซลล์ถูกกระตุ้น การศึกษาครั้งนี้ได้แสดงให้เห็นถึงระดับการแสดงออกของ pri-miR-29a ที่แปรผกผันกับการสร้างไซโตคัยน์อินเตอร์เฟอรอนแกมม่า ในเซลล์เม็ดเลือดขาวสุกรที่ถูกกระตุ้นด้วย Con A ทั้งนี้การศึกษาเพิ่มเติมเกี่ยวกับกลไกการทำงานของ miR-29a จะช่วยเพิ่มความเข้าใจเกี่ยวกับบทบาทหน้าที่ของไมโครอาร์เอ็นเอชนิดนี้ต่อระบบภูมิคุ้มกันในสุกร

คำสำคัญ: ภูมิคุ้มกัน miR-29a สุกร พีซีอาร์เชิงปริมาณ

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Introduction

MicroRNAs are a group of small non-coding RNA with approximately 22 nucleotides in length that posttranscriptionally regulate gene expression. MicroRNA was first discovered in *Caenorhabditis elegans* and later identified to be evolutionally conserved across other species. (Lee et al., 1993; Chen and Rajewsky, 2007). Synthesis of miRNAs begins with transcription of primary miRNAs (pri-miRNAs) which are subsequently cleaved by RNase III enzyme Drosha to generate short hairpin structures called miRNA precursors (pre-miRNAs). The pre-miRNAs are then transported from the nucleus to the cytoplasm for further process by Dicer to create mature miRNA products. Upon processing, the guide strand of miRNA forms RNA-induced silencing complex with target mRNA genes, leading to targeted mRNA degradation or inhibiting their translation.

Multiple microRNAs including miR-29, miR-181, miR-155 and miR-17~92 appeared to be important regulators of both innate and adaptive immune response (Xiao and Rajewsky, 2009). Among these, recent findings suggested that miR-29 family was implicated in regulation of bacterial response, T cell differentiation and B cell oncogenic transformation (Ma et al., 2011; Liston et al., 2012). In mice, miR-29a suppressed host immune response through downregulation of IFN-γ production in natural killer cells and T lymphocytes (Ma et al., 2011). It was shown that miR-29a inhibited IFN-γ mRNA expression by complementarily binding to 3′ untranslated regions (UTRs) of IFN-γ mRNA (Ma et al., 2011; Savan et al., 2011). Additional study indicated that miR-29a targets T-bet and Eomes which regulate IFN-γ gene expression in CD4 helper T cells (Steiner et al., 2011). Interestingly, sequence analysis of miR-29 revealed that all miR-29 members shared identical mature microRNA sequences with highly conserved across multiple species including dog, mouse, rat and human. Based on the sequence similarity, identification of the expression level and functional studies of miR-29a could provide an important insight into the role of this small RNA in pig.

One of the methods that are commonly used to quantify gene expression in living cells is quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Roa et al., 2011). Advantages of qRT-PCR include high sensitivity and specificity, small volume of samples, cost-effective and non-radioactive procedure. Indeed, the qRT-PCR has been applied to study microRNA expression in various systems (Schmittgen et al., 2008). Therefore, the aim of present study was to develop qRT-PCR and use this technique to quantify expression level of pri-miR-29a. Furthermore, level of IFN-γ transcripts and pri-miR-29a were investigated to determine their expression patterns in unstimulated and Con A stimulated porcine peripheral blood mononuclear cells.

Materials and Methods

Primer design: Specific forward and reverse primers were designed from *Sus scrofa* miR-29a accession number NR_038524.1 using Primer-BLAST, NCBI (www.ncbi.nlm.nih.gov/tools/primer-blast/). All

other primers were designed according to previous publications; GAPDH (Zhang et al., 2012) and IFN-γ (Kametani et al., 2012). All primer sequences are shown in table 1.

Cloning of miR-29a and plasmid DNA extraction: Porcine miR-29a was PCR-amplified using genomic DNA from Porcine Kidney (PK-15) cells as a template. The PCR products were cloned into pGEM-T easy vector (Promega, USA), later called pGEM-miR29a and transformed into DH5 alpha *E. coli* competent cells. To confirm the sequence of *Sus scrofa* miR-29a, purified plasmid DNA pGEM-miR29a was isolated using QIA prep® SpinMiniprep Kit (Qiagen, Germany) and was sent out for DNA sequencing (Macrogen, Korea). Nucleotide sequences were compared to *Sus scrofa* miR-29a sequence available on GenBank database.

Plasmid copy number calculation and standard curve analysis: To optimize a qPCR protocol, a standard curve was generated using purified plasmid pGEM-miR29a with ten-fold serial dilution. The plasmid was diluted to six concentrations. Plasmid copy number was calculated according to the following equation:
Copy number = plasmid concentration (g/µl) x 6.022x10^{23} / insert size (bp) x 650
The calculated plasmids ranged from 2.99x10^{7} to 2.99x10^{2} copy number/reaction. Each concentration was run in triplicate.

Isolation and stimulation of peripheral blood mononuclear cells: To separate PBMCs, heparinized blood samples were collected from three healthy individual pigs ages between 2-2.5 years. PBMCs were separated using Ficoll-Hypaque Plus (GE healthcare, USA) density gradient centrifugation. Briefly, blood samples were diluted in phosphate buffer saline (PBS : blood = 1 : 1) and were then overlay on Ficoll-Hypaque Plus. Samples were centrifuged at 2500 rpm for 20 min at 20-24^{0}C. PBMCs layer was collected and resuspended in RPMI medium containing 10% fetal bovine serum, 100 U/ml penicillin/streptomycin, and 50 µg/ml gentamicin (Katial et al., 1998). Subsequently, isolated PBMCs were plated in 24-well plate with 5x10^{5} cells/ml per well. To stimulate cells, concanavalin A (Sigma, USA) at 10 µg/ml (Suradhat et al., 2003) was mixed in culture medium and incubated at 37^{0}C, 5% CO_2 for 12 and 24 hour. Non treated PBMCs were used as a control group.

Total RNA extraction and cDNA synthesis: Total RNA was extracted from porcine PBMCs using Trizol reagent (Invitrogen, USA) following manufacturer's instructions. Subsequently, RNA pellets were resuspended with DNase and RNase free water. Amount and quality of RNA were determined by NanoDrop spectrophotometer 2000 (ThermoScientific, USA). The first strand cDNA was synthesized by reverse transcriptase enzyme, SuperscriptIII (Invitrogen, USA). Five hundred ng of total RNA in each group was reverse transcribed by OligodT primer (Promega, USA).

Quantitative polymerase chain reaction (qPCR) : To determine miR-29a expression, 2 µl of cDNA was amplified in 20 µl reaction with SYBR green mastermix (Biorad, USA). The reactions were performed on a real-time PCR detection system CFX96 (BioRad, USA). The PCR conditions included an activation step at 95^{0}C for 2 min and 30 sec in initial step, followed by 40 cycles of 95^{0}C for 5 sec, 55^{0}C for 30 sec, and 72^{0}C for 30 sec. To determine the specificity of PCR products, melting curve analysis and running of PCR products on NuSieve 3 : 1 agarose gel (Lonza, USA) were performed. GAPDH gene was used as an internal control to normalized gene expression. Expression of miR-29 was calculated by $2^{-\Delta\Delta Cq}$ method for relative quantification (Livak and Schmittgen, 2001). The expression level was shown in the format of log 2 graph.

Data and statistical analysis: ΔC_q value was calculated for expression analysis. Statistical significance was assessed using paired sample *t* tests by GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). *P*-values ($p < 0.05$) was used to evaluate significant value.

Results

Development of qRT-PCR to determine pri-miR-29a expression: To determine level of pri-miR-29a expression in porcine cells, we developed qRT-PCR using SYBR green technology. Firstly, we designed a primer pair specific to porcine miR-29a based on the genomic sequence available on GenBank database and other primers were reference from previous published papers (Kametani et al., 2012; Zhang et al., 2012). To evaluate the sensitivity and specificity of the developed method and primers, we constructed the recombinant plasmid (pGEM-T easy vector) containing a fragment of miR-29a (later called pGEM-

Table 1 Primer sequences

Target	Primer name	Primer sequence (5′ to 3′)	Size (bp)	GenBank Accession number
miR-29a	miR29A_F miR29A_R	CCCCTTAGAGGATGACTGAT CCCCAATCATTATAACCGAT	86	NR_038524.1
GAPDH	GAPDH_F GAPDH_R	AGGTCATCCATGACAACTTCGGCA AGCACCAGTAGAAGCAGGGATGAT	155	AF017079
IFN-γ	IFNG_F IFNG_R	GCTCTGGGAAACTGAATGACTT TATTGCAGGCAGGATGACAA	199	NM_213948.1

miR29a). The insert of pGEM-miR29a was confirmed with 100% sequence similarity to *Sus scrofa* miR-29a sequence on GenBank database (data not shown). The amplification efficiency of miR-29a primer was determined by serially ten-fold dilution of pGEM-miR29a. The C_q-values were plotted against the log of the pGEM-miR29a copy number to obtain a standard curve (Fig 1a). The correlation coefficient (R^2 value) was 1.00 with a slope of the reaction of -3.700 and an amplification efficiency of 86.3% (Fig 1b).

To test the specificity of qRT-PCR product, we performed melting curve analysis at the end of PCR cycle. As shown in Figure 2a, all of the melting curves revealed a specific product at 74°C resembling the expected size of miR-29a PCR amplicon (86 bp). Further, the PCR products were run on NuSieve gel 3 : 1, yielding a single band at 86 bp (Fig 2b). Taken together, these findings confirm the specificity of newly developed qRT-PCR protocol.

Expression of pri-miR-29a and IFN-γ in Con A stimulated porcine peripheral blood mononuclear cells

To determine expression pattern of miR-29a in porcine PBMCs, we used the new qRT-PCR method to determine expression level of pri-miR-29a and IFN-γ in stimulated PBMCs. The PBMCs were treated with a mitogenic compound Con A to stimulate IFN-γ expression. As shown in Figure 3, cells treated with Con A showed upregulation of IFN-γ to 6.42 folds (log2) at 12 hours and 6.17 folds (log2) at 24 hours with statistical significance at 24 hours ($p < 0.05$) relatively to the control cells. Interestingly, the expression of pri-miR-29a significantly decreased at 12 and 24 hours with 76% and 96% reduction (-2.17 and -6.24 log2 fold change, respectively) in Con A stimulated cells. However, the expression of pri-miR-29a and IFN-γ were not statistically different between each time point.

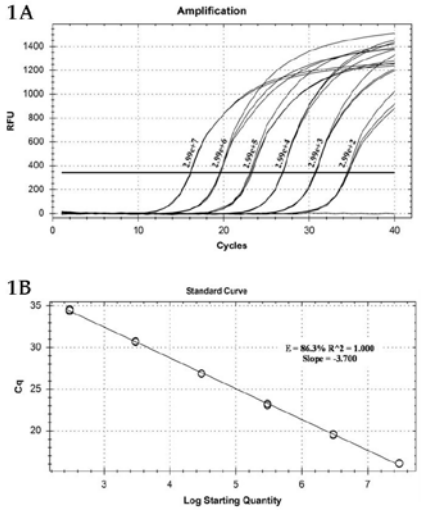

Figure 1 Standard curve for ten-fold serial dilutions of pGEM-miR29a. Amplification plots of qRT-PCR with DNA template from plasmid (pGEM-miR29a) with ten-fold serial dilution ($2.99x10^7$, $2.99x10^6$, $2.99x10^5$, $2.99x10^4$, $2.99x10^3$, $2.99x10^2$ copy number). C_q values were plotted against corresponding log concentration (Fig 1a). Slope of standard curve was -3.700 and R^2 was 1.00 with 86.3% efficiency (Fig 1b).

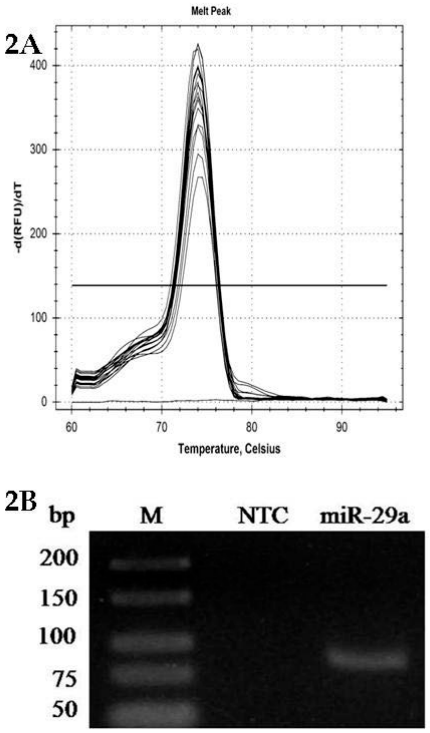

Figure 2 Melting curve analysis of PCR products revealed a specific product (Tm = 74°C),which was related to the expected amplicon size of miR-29a (Fig 2a). PCR products were run on low melting agarose gel 5% Nuseive 3 : 1 agarose gel (M: 10 bp DNA marker, NTC: no template control, PCR products of miR-29a.

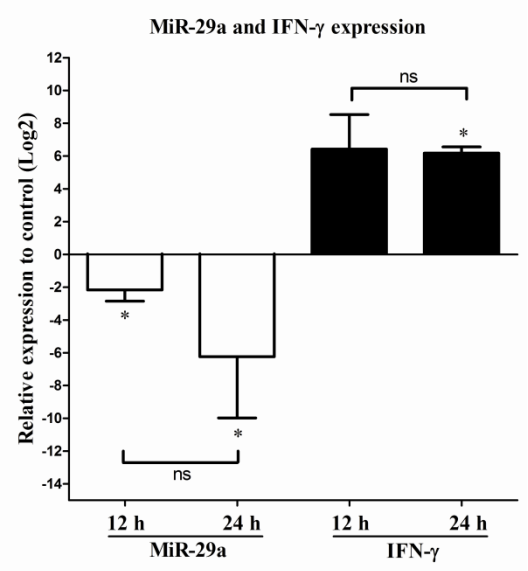

Figure 3 Expression of pri-miR-29a and IFN-γ in Con A treated PBMCs. $5x10^5$ PBMCs isolated from three individual pigs were incubated with Con A at 10 µg/ml for 12 and 24 hours. Expression of pri-miR-29a and IFN-γ in Con A stimulated PBMCs were measured by qRT-PCR. Log2 value represents relative expression level compared to untreated control cells at each time point. * : $p < 0.05$ and ns : not statistically significant.

Discussion

MicroRNAs are small non-coding regulatory RNAs that play important roles in controlling gene expression. To date, hundreds to thousands of microRNAs have been characterized in mammals including pigs (Timoneda et al., 2013). It has been shown that microRNAs participate in multiple cellular mechanisms including organ and tissue development, host pathogen interaction and cancer progression (Xiao et al., 2007; Zhang et al., 2007; Cullen, 2013). In the present study, we developed the qRT-PCR to detect pri-miR-29a expression in porcine cells using SYBR technology. The SYBR qRT-PCR offers a sensitive, reproducible and cost-effective method to quantify target gene expression. The specificity of the new protocol was confirmed by melting curve analysis and testing of PCR products on low melting temperature agarose gel, yielding a single specific band. Additionally, we successfully cloned the miR-29a into the plasmid vector. Such plasmid could be used as a positive control for future study of this microRNA in pig.

Several studies had highlighted the role of miR-29a in innate and adaptive immune responses during intracellular bacterial and viral infection. For example, inhibition of miR-29a enhanced IFN-γ production and T_H1 responses, resulting in lowering of *Listeria monocytogenes* burdens in mice (Ma et al., 2011). Specifically, sequence analysis of miR-29a and IFN-γ mRNA revealed that miR-29a targeted IFN-γ mRNA at 3′ UTRs, leading to post transcription inhibition (Ma et al., 2011; Savan et al., 2011). Moreover, miR-29a downregulated IFN-γ production through inhibition of two transcription factors, T-bet and Eomes in T lymphocytes (Steiner et al., 2011). In addition, miR-29a specifically targeted human immunodeficiency virus (HIV) replication in human T lymphocytes (Nathans et al., 2009). Such findings emphasized the role of miR-29a in the host immune response. Based on the conserved sequence of microRNA, it is interesting to examine miR-29a expression and function in the pig immune system.

In this study, we used concanavalin A, a mitogenic compound to stimulate IFN-γ expression in porcine PBMCs. We found that IFN-γ was upregulated at 12 and 24 hours which is similar to other reports (Verfaillie et al., 2001; Suradhat et al., 2003). Upon activation, the expression level of IFN-γ increased significantly at 24 hour in stimulated PBMCs while the expression of pri-miR-29a decreased significantly at both time points. These findings are in accordance with recent observations in human and mice (Ma et al., 2011; Steiner et al., 2011; Yamada et al., 2011).

The basic understanding of microRNAs could be applied in veterinary medicine in the area of host pathogen interaction, antiviral therapy, biomarker and vaccine design. Currently, at least two microRNAs, miR-181 and miR-125b, have been shown to regulate porcine reproductive and respiratory syndrome virus (PRRSV) replication in porcine cells. MiR-181 dose dependently reduced PRRSV viral load in MARC-145 cells through specific binding of open reading frame (ORF) 4 of the viral genomic RNA (Guo et al., 2013). Likewise, miR-125b inhibited PRRSV replication through down regulation of NF-kB activation in MARC-145 and pulmonary alveolar macrophage (Wang et al., 2013). Other applications of microRNAs include antiviral strategy and usage of microRNA as biomarkers for disease severity and prognosis in infection. For example, artificial miRNA targeting ORF5 and ORF6 of PRRSV inhibited viral replication without inducing interferon production in MARC-145 cells (Xiao et al., 2011). Moreover, the miRNA expression profile could be used as biomarkers for disease diagnosis in rabies virus infected mice (Han et al., 2011).

In summary, the qRT-PCR protocol was developed to quantify pri-miR-29a expression in porcine cells. Our preliminary data indicated that the expression of pri-miR-29a massively decreased in Con A stimulated PBMCs, while the expression level of IFN-γ increased. Given to the molecular roles of microRNAs in mammalian immune system, it is interesting to determine whether miR-29a participates in host-pathogen interaction and vaccine response in pig. This newly developed protocol will provide a valuable tool to evaluate miR-29a expression in the future studies.

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