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Identification of reference microRNAs for quantitative expression analysis in porcine peripheral blood mononuclear cells treated with polyinosinic–polycytidylic acid

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Summary

Peripheral blood mononuclear cells (PBMCs) are clinically important cells. Detection of microRNAs (miRNAs) expression in PBMCs can be useful for miRNA biomarker discovery for various diseases. Quantitative real-time PCR (qRT-PCR) has become an important method used for measuring miRNAs expression. However, the reliability of qRT-PCR data critically depends on proper selection of reference genes. Here, we performed qRT-PCR to quantify the expression levels of nine miRNAs (Ssc-miR-16, Hsa-miR-25, Ssc-miR-34a, Hsa-miR-93, Bta-miR-92b, Ssc-miR-103, Ssc-miR-106a, Ssc-miR-128 and Ssc-miR-107) and one small nuclear RNA (U6) in PBMCs treated with polyinosinic–polycytidylic acid [poly (I:C)] that widely used for simulating viral infection. We used the four statistical algorithms (GeNorm 3.5, NormFinder, BestKeeper and comparative Δ Ct method) to evaluate gene expression stability and observed that Ssc-miR-34a was the best single reference gene and the pair of Ssc-miR-107 and Ssc-miR-103 was the best combination of reference miRNAs for porcine PBMCs treated with poly (I:C). Our study shows the first evidence of careful selection of reference miRNAs in porcine PBMCs and maybe helpful for discovering miRNA biomarkers for double-stranded RNA-induced disease.

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Introduction

MicroRNAs (miRNAs) are a family of small noncoding RNAs that regulate gene expression at the post-transcriptional level by translation repression or miRNA-mediated mRNA degradation. They exert an impact on a wide range of biological processes including cell proliferation and differentiation, apoptosis and metabolism (Huntzinger & Izaurralde, 2011). Previous studies have demonstrated that miRNAs are also involved in many pathological processes, such as inflammatory pathologies, neurological disorders and cancer (Jiang *et al.*, 2010; Esteller, 2011; Farazi *et al.*, 2011). Furthermore, it has been suggested that miRNAs could be promising biomarkers for pathologic diagnosis and prognosis (Sharkey *et al.*, 2012). It is therefore crucial to obtain accurate measurements of miRNA expression for the identification of candidate biomarkers for disease progression.

Polyinosinic–polycytidylic acid [poly (I:C)], a synthetic double-strand RNA (dsRNA) analogue commonly used for simulating viral infection, has been demonstrated to produce stimulatory function similar to viral dsRNA such as the strong induction of IFN-α/β (Alexopoulou *et al.*, 2001; Doyle *et al.*, 2002). Peripheral blood mononuclear cells (PBMCs) are clinically relevant cells commonly used in immunological assays, including noninvasive diagnostic and *ex vivo* stimulation (Lam *et al.*, 2012). Huang *et al.* (2006) confirmed that poly (I:C) challenge could elicit certain gene expression changes in human PBMCs by triggering a rapid innate immune response, which is similar to acute viral infection. However, the stimulatory effects of poly (I:C) stimulation on miRNAs expression have not been fully investigated. MiRNAs as key regulators of gene expression, accurate detection of changes in miRNAs expression induced by poly (I:C) stimulation is paramount for the identification of regulatory miRNAs involved in immune-related gene expression and

maybe helpful for discovering miRNA biomarkers for double-stranded RNA-induced disease.

Presently, quantitative real-time PCR (qRT-PCR) has been widely employed for measuring miRNAs expression both in miRNAs identification (Moyal *et al.*, 2013; Zumbrennen-Bullough *et al.*, 2014) and in validation of miRNAs expression profile performed by microarrays and by high-throughput sequencing (Baskerville & Bartel, 2005; Sun *et al.*, 2014; Yang *et al.*, 2014). However, several factors such as the amount of initial sample, RNA integrity and efficiency of cDNA synthesis may dictate the qRT–PCR results when comparing gene expression in different samples (Andersen *et al.*, 2004). To circumvent these potential biases, relative quantification is frequently used to process the qRT-PCR data. This approach normalizes the expression of target genes to one or more reference genes. Therefore, an optimal normalizer is essential for improving the accuracy and reliability of expression measurements. The chosen reference gene should be expressed abundantly and stably in the samples investigated and under the given experimental conditions (Dheda *et al.*, 2004). If the chosen reference gene shows fluctuations in gene expression when analysed in different samples, the significant difference between samples may be masked (Peltier & Latham, 2008).

The ribosomal RNA (5S) and small nuclear RNA (U6) are two commonly used reference genes in miRNAs expression studies (Takamizawa *et al.*, 2004; Feng *et al.*, 2010; Su *et al.*, 2010; Li *et al.*, 2011; Bai *et al.*, 2014). Accumulating evidence indicated that the expression level of some reference genes vary significantly under different experimental conditions (Guenin *et al.*, 2009; Song *et al.*, 2012; Timoneda *et al.*, 2012). Therefore, these genes could not be considered as the universal normalizers for all studies. A few studies focusing on the determination of reference miRNAs have highlighted the necessity of expression stability analysis of candidate reference genes under the given experimental conditions (Mestdagh *et al.*, 2009; Shen *et al.*, 2011; Wang *et al.*, 2013; Kagias *et al.*, 2014). In pigs, reference miRNAs for miRNA detection by qRT-PCR in adipose, muscle and reproductive tissues have been identified (Gu *et al.*, 2011; Wessels *et al.*, 2011; Timoneda *et al.*, 2012). Nevertheless, there is no report of stably expressed miRNAs in porcine PBMCs. We therefore performed an accurate selection of stably expressed miRNAs that could be effectively used as reference miRNAs for qRT-PCR analysis in porcine PBMCs. Our study will provide valuable information and insight to further studies aiming at miRNAs detection in PBMCs by qRT-PCR assays.

Materials and methods

Sample collection

Six clinically healthy pigs in 35 days old including three Landrace piglets (a Western breed) and three Dapulian piglets (an indigenous Chinese breed) were used in this study. All animals were raised under the same standard indoor conditions and did not receive any vaccinations except classical swine fever vaccine on day 21 after birth. For each piglet, 15 mL peripheral blood was collected via venipuncture into a vacutainer tube containing anticoagulant (EDTAK2). The peripheral blood samples collected from these animals were under veterinary supervision. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Shandong Academy of Agricultural Sciences.

PBMCs preparation and stimulation

Peripheral blood mononuclear cells were isolated from whole peripheral blood using Ficoll–Hypaque density-gradient centrifugation at room temperature following the manufacturer's instructions. In brief, the whole blood was firstly diluted by an equal volume of phosphate-buffer saline (PBS). Then 15 mL of diluted blood was carefully added on the top of 10 mL of Ficoll–Hypaque solution in a 50-mL conical tube and centrifuged at 460 *g* for 20 min at room temperature. After centrifugation, the middle whitish interface containing mononuclear cells was transferred to a new tube and washed by 25 mL PBS followed by centrifugation at 150 *g* for 10 min. Cells were counted using a hemocytometer, and the viability was confirmed by exclusion of the vital dye trypan blue. Isolated PBMCs were diluted into 50-mL RPMI-1640 medium (Hyclone, Logan, UT, USA) supplemented with 10% foetal calf serum, with the final cell concentration of $\sim 2 \times 10^6$ mL^{-1}. The cells were cultured in the presence of 20 μg mL^{-1} poly (I:C) or vehicle (PBS) for 24 h at 37°C with 5% CO_2 and then collected into a centrifuge tube and centrifuged at 3000 *g* for 5 min and stored at −80°C for mRNA isolation.

RNA extraction and miRNA cDNA synthesis

Total RNA was extracted from PBMCs using RNAiso Plus [Takara Biotechnology (Dalian) Co., Ltd., Dalian, China] following the manufacture's guidelines. RNA quality and quantity were measured by electrophoresis gel and NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). A total of 1 μg RNA was converted into miRNA cDNA with the use of miScript II RT Kit (Qiagen, Hilden, Germany) according to the manufacturer's directions.

Selection of candidate reference miRNAs

According to previous studies regarding analysis of miRNAs expression stability (Gu *et al.*, 2011; Shen *et al.*, 2011; Wessels *et al.*, 2011; Timoneda *et al.*, 2012; Wang *et al.*, 2013), nine miRNAs (Ssc-miR-16, Hsa-miR-25, Ssc-miR-34a, Hsa-miR-93, Bta-miR-92b, Ssc-miR-103, Ssc-miR-106a, Ssc-miR-128 and Ssc-miR-

Table 1. Candidate reference genes and primers used for qRT-PCR assays

miRNA	Accession	miRNA-specific primer	Annealing temperature (°C)	Amplification efficiencies (%)
Ssc-miR-34a	MIMAT0007757	GCAGTGTCTTTGCTGGTTGT	55	103.6
Ssc-miR-107	MIMAT0002155	CCGCATTCTACAGGGCTATC	55	96.4
Ssc-miR-103	MIMAT0002154	GCCTTGAACAGGGCTATG	55	101.9
Hsa-miR-25	MIMAT0000081	CATAGCACTTGTCTCGGTCTGA	55	102.8
Hsa-miR-93	MIMAT0000093	CGTTACGCAAAGTGCTGTTC	55	104.9
Bta-miR-92b	MIMAT0009384	TATCGCACTCGTCTCGGC	55	95.8
Ssc-miR-128	MIMAT0002157	GCACAGTGAATCGGTCTCTTT	55	103.3
Ssc-miR-16	MIMAT0007754	GCAGCAGGTAACTATTGGCG	55	99.8
Ssc-miR-106a	MIMAT0002118	TCGTGACCACAGTGCTTACA	55	104.7
U6	EU520423	CTTCGGCAGCACACATACTAA	55	95.5

107) and one commonly used small nuclear RNA (U6) were selected as candidate reference genes for this study (Table 1).

Quantitative real-time PCR

Quantitative real-time PCRs were prepared using miScript SYBR Green PCR kit (Qiagen) with a final volume of 15 μL including 8 μL of 2× QuantiTect® SYBR Green PCR Master Mix, 1 μL of 10× miScript Universal Primer, 1 μL of miRNA-specific primer (10 pM μL^{-1}) (Table 1), 1 μL of 20-fold diluted cDNA and 4 μL of RNase-free water. The thermal cycling conditions were 95°C for 15 min, 40 cycles of 94°C for 15 s, 55°C for 30 s, 70°C for 30 s and 60–95°C at a rate of 0.1°C per second for melting curve generation. All qRT-PCR assays were performed using the LightCycler® 480 Real-Time PCR System (Roche, Hercules, CA, USA). Standard curves were generated using a fourfold serial dilution from a pool of cDNA of all samples. After the generation of curves, qRT-PCR efficiency was calculated for each gene using the LightCycler 480 software. In addition, melting curve analysis was carried out in each assay to ensure specific amplifications. For all qRT-PCR assays, crossing point (Cp) values in triplicate were used for further analysis.

Gene expression data for each candidate reference gene was also analysed by a two-way analysis of variance with the mixed model procedure of SAS (SAS9.0; SAS Institute, Inc., Cary, NC, USA). The main fixed effects included in the model were poly (I:C) stimulation, breed and poly (I:C) stimulation by breed interaction. The significance of difference in gene expression between poly (I:C)-treated group and control group was determined by paired *t*-test. It was declared statistically significant if the P value < 0.05.

Analysis of expression stability

The expression stability of each candidate reference gene was assessed using four statistical algorithms including GeNorm 3.5 (Vandesompele *et al.*, 2002), NormFinder (Andersen *et al.*, 2004), BestKeeper (Pfaffl *et al.*, 2004) and comparative Δ Ct method (Silver *et al.*, 2006). The RefFinder (http://www.leonxie.com/referencegene.php), a Web-based comprehensive tool which integrates these four programs to compare and rank the tested candidate reference genes, was also used in this study. The expression data obtained through qRT-PCR were transformed into formats suitable for import into the statistical algorithms. The GeNorm algorithm can work out the gene expression stability value (M) for each gene according to the average pairwise variation between all genes investigated. The NormFinder algorithm calculates the stability value for each candidate reference gene and provides the intra- and intergroup variation of the sample set. For both algorithms, genes with the lowest stability value are considered as the most stably expressed reference genes. The BestKeeper establishes the best suited standards and determines the stable reference genes using pairwise correlations. The comparative Δ Ct method compares the relative expression of pairs of genes within each sample and then ranks the stability of the candidate reference genes as per the repeatability of differences in gene expression among different samples.

Results

Analysis of qRT-PCR efficiency

Quantitative real-time PCR efficiency was calculated for each candidate reference gene using the standard curve which was generated by a fourfold serial dilution of a cDNA pool. The linear correlation coefficient (R^2) of all standard curves was greater than 0.99 (Fig. S1 in Supporting Information). All qRT-PCR amplification efficiencies ranged from 95.5% to 104.7% based on the slope of corresponding standard curves (Table 1). Melting curve analysis indicated that each qRT-PCR assay had specific amplified product with a single melting peak for each primer pair (Fig. S2 in Supporting Information).

Expression of candidate reference genes

Each candidate reference gene was successfully amplified by qRT-PCR, making it feasible to accurately quantify the gene expression. The Cp values of the ten candidate reference genes are shown in Fig. 1. The

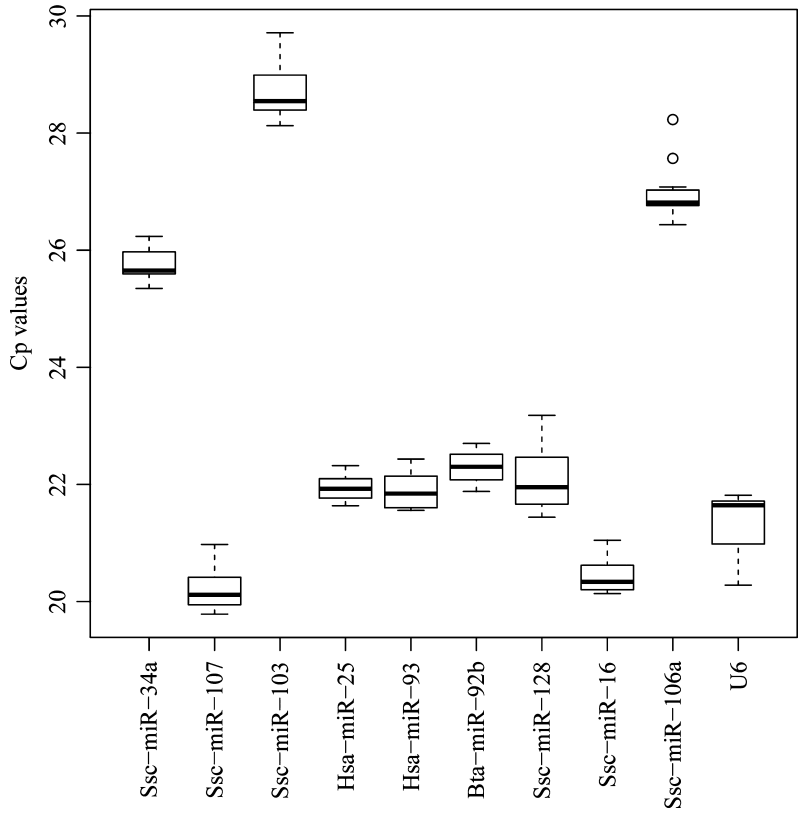

Figure 1. Quantification of crossing point (Cp) values of candidate reference genes in all Peripheral blood mononuclear cells samples. Boxes indicate the interquartile range between the first and third quartile, and the bold line denotes the median. Whiskers represent the minimum and maximum within 1.5 times of the interquartile range between the first and third quartiles. Outliers outside the whiskers are exhibited as circles.

mean Cp values of these genes ranged from 20.20 to 28.68. Ssc-miR-107 was the most highly expressed gene in all samples with a median Cp value of 19.12, whereas Ssc-miR-103 showed a relatively low expression level with a median Cp value of 28.55.

The comparable expression level of each candidate reference gene between the samples and groups is a precondition for analysis of expression stability using above-mentioned statistical algorithms. We thus analysed the expression level of each candidate reference gene between poly (I:C)-treated and control groups. No significant differences in gene expression were observed between these two groups ($P > 0.05$). Therefore, the ten candidate reference genes were all included in subsequent expression stability analysis.

Expression stability analysis

We evaluated expression stability of all candidate reference genes using four statistical algorithms. According to the rankings from each program, the RefFinder provides the final expression stability ranking by assigning a weight to each gene and calculating the geometric mean of their weights. The final rankings for each candidate reference gene are shown in Table 2.

The GeNorm calculates the expression stability measure (M) for each candidate reference gene among the samples under study (Table 2). Genes with the lowest M values have the most stable expression. With the stepwise exclusion of genes with the highest M value, the GeNorm provides the average expression stability value of remaining reference genes, ending with the two most stable genes in the tested samples. As shown in Fig. 2, Ssc-miR-107 and Ssc-miR-103 were the most stable genes, and Ssc-miR-106a was the least stable gene. To determine the optimal number of reference genes, we calculated the pairwise variation $V_{n/n+1}$ between the two sequential normalization factors for all samples using GeNorm (Fig. S3 in Supporting Information). The pairwise variation $V_{2/3} = 0.085$ was smaller than the cut-off value of 0.15 recommended by GeNorm (Vandesompele *et al.*, 2002), indicating the inclusion of an additional reference gene is not required. Therefore, the combination of Ssc-miR-107 and Ssc-miR-103 would be sufficient for a reliable normalization.

The NormFinder calculates both overall expression variations of candidate reference genes and variations between subgroups of investigated samples. According the rankings of expression stability, Ssc-miR-34a and Ssc-miR-107 were the top two stably expressed genes

Table 2. Expression stability values of candidate reference genes

Rank	Gene symbol	RefFinder (Geomean)	GeNorm (*M* value)	NormFinder (stability value)	BestKeeper [SD (±CP)]	Δ Ct (average of SD)
1	Ssc-miR-34a	1.86	0.249	0.074	0.22	0.17
2	Ssc-miR-107	2.11	0.164	0.097	0.28	0.17
3	Ssc-miR-103	3.13	0.164	0.108	0.35	0.18
4	Hsa-miR-25	3.50	0.287	0.117	0.18	0.19
5	Hsa-miR-93	4.36	0.230	0.101	0.25	0.18
6	Bta-miR-92b	5.66	0.364	0.180	0.21	0.23
7	Ssc-miR-128	6.19	0.331	0.168	0.43	0.21
8	Ssc-miR-16	6.48	0.307	0.154	0.25	0.21
9	Ssc-miR-106a	7.95	0.446	0.215	0.32	0.25
10	U6	9.00	0.403	0.197	0.42	0.24

The overall final ranking is based on the geomean values calculated by RefFinder. Lower geomean values indicate genes that are stably expressed.
Cp, crossing point; SD, standard deviation.

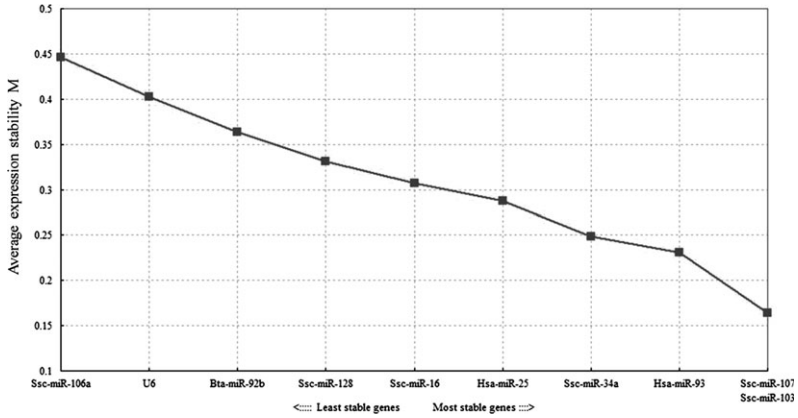

Figure 2. Average expression stability values of remaining reference genes during stepwise exclusion of the least stable gene. The values were calculated using GeNorm algorithm.

in the samples investigated. U6 and Ssc-miR-106a were the least stable genes (Fig. 3). In addition, the intra- and intergroup variation of these genes are depicted in Fig. 4.

Gene expression variability of candidate reference genes was calculated using Cp values and shown as the standard deviation and coefficient of variance by BestKeeper. Among these genes, Ssc-miR-25 was seen as the most stably expressed gene, followed by Bta-miR-92b, Ssc-miR-34a, Ssc-miR-16 and Ssc-miR-107 (Fig. 3).

Compared with the aforementioned specialized programs, the comparative Δ Ct method is a relatively simple approach which compares relative expression of pairs of genes within each sample to determine the suitable reference gene. Briefly, when analysed in different samples if the Δ Ct value between two genes keeps constant, it indicates either both genes are stably expressed among those samples or coregulated. In contrast, if the Δ Ct value varies, then one or both genes are unstably expressed. Based on the repeatability of gene expression differences among the tested samples, expression stability of candidate reference genes will be ranked and the most suitable reference gene(s) could be selected. Based on this method, Ssc-miR-34a, Ssc-miR-107 and Ssc-miR-103 showing the least amount of variability were considered as the most suitable reference miRNAs (Fig. 3).

As using different statistical models, the aforementioned programs provide different expression stability rankings. To further confirm the most stable reference miRNAs, we made use of RefFinder which integrates these four programs by weighting the rankings of each program. Results showed that Ssc-miR-34a and Ssc-miR-107 were the top two stably expressed miRNAs, coinciding with the rankings of GeNorm, NormFinder and comparative Δ Ct method. U6 was the most unstable candidate reference gene because of its high variability (Table 2).

Discussion

Quantitative real-time-PCR has recently become a widely used method for quantifying miRNA expression

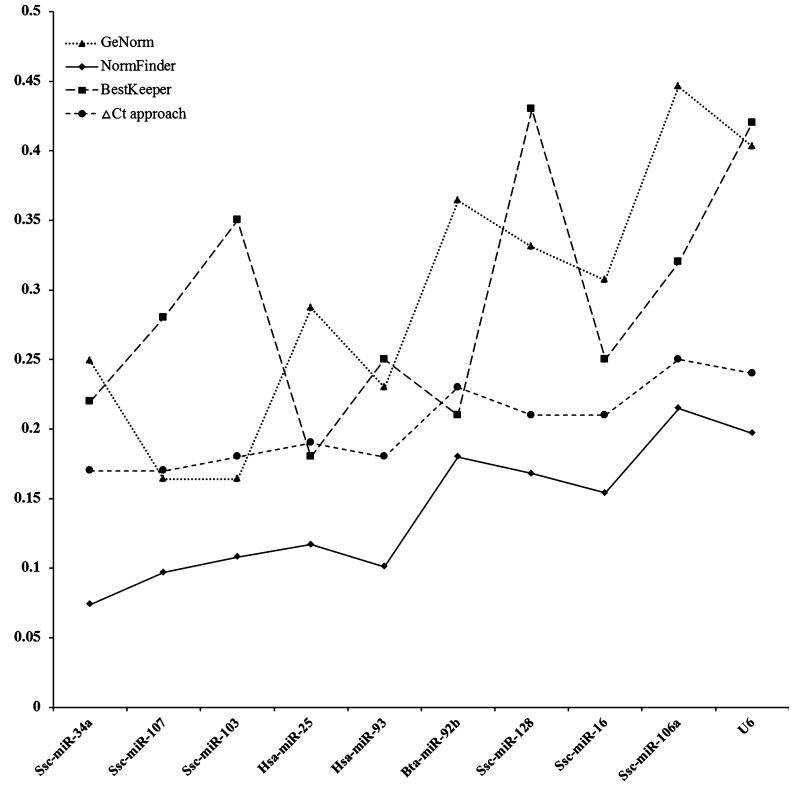

Figure 3. Expression stability values of the candidate reference genes. Genes that are stably expressed are indicated by lower values of expression stability.

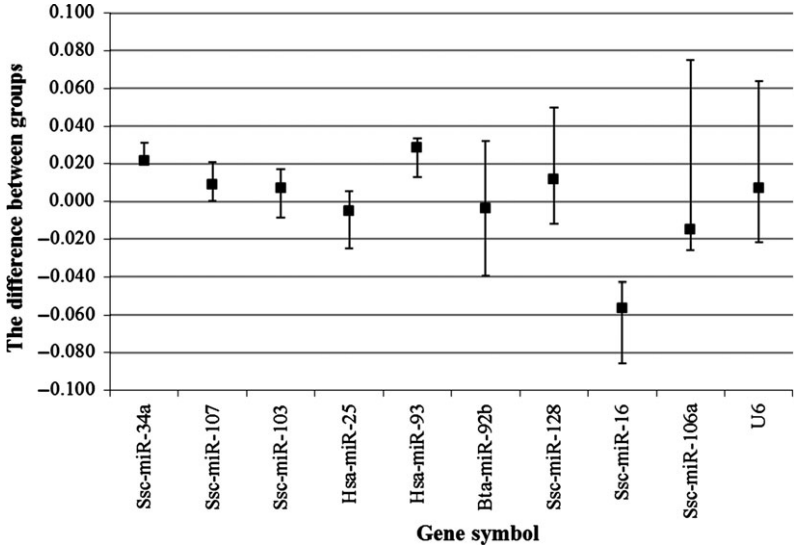

Figure 4. Intra- and intergroup variation of candidate reference genes. The intragroup variation is denoted by vertical bars that give a confidence interval for the difference. The intergroup variation is indicated by small black squares showing the expression difference between the two groups. As the average of the intergroup variations is close to 0, the intergroup variation of >0 suggests that the gene is systematically expressed higher in the untreated control group than in the polyinosinic–polycytidylic acid-treated group and the opposite if the value <0. The candidate reference gene with an intergroup variation as close to 0 as possible and the same time having as small errors bars as possible will be ranked as the optimal one by NormFinder.

in tissues or cells (Andersen *et al.*, 2004; Timoneda *et al.*, 2012; Sperveslage *et al.*, 2014; Tratwal *et al.*, 2014). Previous reports indicated that inappropriate use of reference genes can significantly alter the results of target miRNAs quantification (Peltier & Latham, 2008; Chang *et al.*, 2010). As subtle differences in miRNAs

expression may cause biologically meaningful changes (Calin & Croce, 2006), it is pivotal to identify suitable normalizer for miRNAs expression studies. In addition, researchers have developed several statistical algorithms to analyse qRT-PCR data for appropriate selection of the most suitable reference gene (Vandesompele *et al.*, 2002). Using these statistical algorithms, a number of suitable reference miRNAs have been identified for quantifying miRNAs expression in humans (Doyle *et al.*, 2002; Pfaffl *et al.*, 2004; Guenin *et al.*, 2009; Shen *et al.*, 2011; Lamba *et al.*, 2014) and in livestock species (Gu *et al.*, 2011; Wessels *et al.*, 2011; Timoneda *et al.*, 2012; Li *et al.*, 2014).

Previous studies suggested that different mRNA reference genes are required for mRNA gene expression studies in porcine PBMCs at different stages or with different immunological stimulants (Ju *et al.*, 2011; Cinar *et al.*, 2013). While many reference genes suitable for mRNA expression studies have been identified in porcine PBMCs, no well-established reference miRNAs have been investigated. In this study, we performed qRT-PCR to detect suitable reference genes in porcine PBMCs treated with poly (I:C). Results indicated that Ssc-miR-34a was the most stably expressed gene among all tested samples. It has been suggested that the use of geometric mean of two or more carefully selected reference genes can lead to relatively reliable normalization for qRT-PCR analysis (Vandesompele *et al.*, 2002). We thus carried out pairwise variation analysis and found that the pair of Ssc-miR-107 and Ssc-miR-103 was the best combination of reference miRNAs. To our knowledge, this is the first study to aim at identifying suitable reference miRNAs for miRNAs quantification by qRT-PCR in porcine PBMCs.

Of the nine candidate reference miRNAs tested in this study, six have been analysed in porcine tissues. Gu *et al.* (2011) reported that Ssc-miR-107 and Ssc-miR-103 were stably expressed in 47 different tissues, and Ssc-miR-106a and Ssc-miR-16 were less stable, which is concordant with the findings presented here. When multiple tissues from different pig breeds were considered, Ssc-miR-93 was shown as the most stable miRNA, followed by Hsa-miR-25, Ssc-miR-106a, Ssc-miR-16 and Ssc-miR-103 (Calin & Croce, 2006). Out of these miRNAs, Ssc-miR-103 was found to be the most stable miRNA in uterus when analysed by tissues. Furthermore, Wessels *et al.* (2011) demonstrated that RNU1A was the most stable reference gene for porcine maternal–foetal tissues. The discrepancy in miRNAs expression stability among these studies was predominantly caused by the differences in the tested samples, such as the type of tissues, developmental stages and pig breed. Previous studies have shown U6 to be an unsuitable/unstable reference gene because of its variability (Gu *et al.*, 2011; Wessels *et al.*, 2011; Wotschofsky *et al.*, 2011; Zhu *et al.*, 2012). Our data also indicated that U6 was the least stable candidate reference gene due to its high variability. Therefore, there is currently no universal reference gene suitable for all miRNAs expression studies in pigs.

In this study, we used several statistical algorithms to assess the expression stability of candidate reference genes. First, the GeNorm was applied to calculate expression stability for each gene, and Ssc-miR-34a was found to be the most stable one. With the stepwise exclusion of the gene with the highest M value, the pair of Ssc-miR-107 and Ssc-miR-103 was shown as the best combination of reference miRNAs. As the GeNorm ranks the genes based on the similarity of their expression profiles, genes coregulated may affect the stability rankings. To exclude the coregulation effects, we next used other three statistical models to evaluate the expression stability. As shown in Table 2, the NormFinder and comparative Δ Ct approach confirmed that Ssc-miR-34a was the most stable gene and Ssc-miR-107 and Ssc-miR-103 were also stably expressed among the samples investigated. According to the BestKeeper, Ssc-miR-34a, Ssc-miR-107 and Ssc-miR-103 were intermediately stable miRNAs, which was slightly different with the results of other statistical algorithms. Instead of the use of relative quantities transformed from Cp values for stability evaluation, the BestKeeper directly uses Cp values, which may result in different rankings of expression stability among these statistical algorithms (Scharlaken *et al.*, 2008).

The careful selection of reference miRNAs is paramount for accurate expression measurement by qRT-PCR in miRNA expression studies. We herein performed comprehensive expression stability analysis of a subset of candidate reference genes and observed that Ssc-miR-34a was the best single reference gene and the pair of Ssc-miR-107 and Ssc-miR-103 was the best combination of reference genes for qRT-PCR quantification of miRNAs. Our study shows the first evidence of careful selection of reference miRNAs in porcine PBMCs and maybe helpful for discovering miRNA biomarkers for double-stranded RNA-induced disease.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Figure S1 qRT-PCR efficiency plots of candidate reference genes.
Figure S2 Melting peaks of candidate reference genes.
Figure S3 Determination of the optimal number of reference genes.