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Selection and Optimization of Circulating Exosomal Reference microRNAs for Quantitative Real-time PCR Studies of Porcine Body Fluids

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Abstract

Circulating exosomal miRNAs released into all body fluids have incredible functionality and stability. Their expression is associated with multiple pathological conditions, can be used as informative biomarkers when assessing and monitoring the body's physiopathological status. However, there is no consensus on reference miRNAs for circulating exosomal reference and abundance normalization. The present study aimed to quantify 16 potential reference miRNAs in ten porcine body fluids using qRT-PCR. Further, their stability was quantified by combining multiple gold-standard statistical tools, including BestKeeper, GeNorm, and NormFinder. The identified miRNAs were comprehensively ranked. The top-ranked miRNA was recommended as the optimal reference miRNAs for data normalization. To identify more stable genes, the body fluids were assigned into three groups based on the collection point, they are *in vivo* (bile, bladder fluid, and gastric juice), *in vitro* (colostrum, ordinary milk, semen, and urine) and in the blood (UVBP, UABP and PBS). The most stable optimal circulating exosomal reference miRNAs in the body fluids were let-7b-5p (miR-93) in bile, miR-92a in bladder fluid, miR-93 in gastric juice, let-7b-5p in colostrum, miR-92a in ordinary milk and urine, miR-25 in semen, let-7b-5p in UVBP, miR-25 in UABP and U6 in PBS. Overall, miR-93, miR-451 (miR-92a), and miR-25 are the bona fide reference miRNA for qRT-PCR data normalization of body fluids *in vivo*, *in vitro*, and blood, respectively. Across all body fluids, miR-451 was the most stable when determining the miRNA abundance in the circulating exosomes.

Keywords: Circulating miRNA • Exosomes • Reference genes • Porcine body fluids

Introduction

AmicroRNAs (MicroRNAs) found in exosomes in various body fluids have been considered promising diagnostic and prognostic biomarkers for diseases. This is due to their relatively high stability and role as post-transcriptional regulators of gene expression [1-4]. Exosomes are membrane-bound vesicles acting as biological cargo carriers of various molecules, including miRNAs, released from cells into the extracellular environment [5]. They are present in different body fluids with incredible stability [6]. The circulating exosomal miRNAs in various body fluids, including the amniotic fluid, breast milk, and blood have been linked to multiple pathological conditions [7,8]. Moreover, fluid-type-specific miRNAs have functional roles on the surrounding tissues [6]. Besides, there is a higher correlation between the clinical-pathological variables with miRNA level in exosomes than with the vesicle-free miRNAs in biofluids. Also, exosomal miRNAs are important biomarkers easily detected non-invasive from all body fluids [9]. However, their detection requires a reliable and quantitative assessment of the miRNA expression.

MiRNAs are short RNA molecules abundant and stable in biofluids, which regulate gene expression in eukaryotic cells. There has been heightened interest in the utility of circulating exosomal miRNAs as minimally invasive diagnostic and monitoring biomarkers for a wide range of human diseases. However, the quantification of circulating exosomal miRNAs is faced with specific challenges, including the relatively low RNA content in biofluids and lack well-established reference miRNAs [10]. Therefore, there is a need to develop protocols for detecting and quantifying miRNAs in biofluids. Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR) routinely used in detecting the levels of circulating miRNA remains the gold standard for specific detection of selected sets of miRNAs [11]. It is easily and with high accuracy quantifies the miRNA content in biological fluids [12]. Unfortunately, the RT-qPCR normalization procedures are sometimes disputable. Generally, the housekeeping molecules with stable expressions, including SNORD44 (RNU44), SNORD48 (RNU48), and nuclear RNA RNU6-1 (U6), are extensively used as reference elements during miRNA quantification in cell and tissue samples. However, there is considerable variation in their expressions in body fluids, such as plasma and serum; thus, it may not be suitable for normalizing the circulating miRNAs [13-16]. Therefore, the selection and optimization of circulating exosomal reference miRNAs in body fluids using RT-qPCR is extremely urgent.

Pigs (Sus scrofa) are emerging as an attractive biomedical model for studying human diseases due to their similar anatomy, genetics, and physiological characteristics, unlike other commonly used mammals and mice [17,18]. Therefore, the present study aimed to determine the suitability of RT-qPCR in quantifying 16 potential reference miRNAs in ten pig body fluids. In addition, we aimed to analyze the differences in miRNAs content

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in vivo obtained in body fluids (bile, bladder fluid, and gastric juice) following a surgical procedure, *in vitro* obtained nonsurgically (colostrum, ordinary milk, semen, and urine) and in the blood (Umbilical Venous Blood Plasma (UVBP), Umbilical Arterial Blood Plasma (UABP) and Peripheral Blood Serum (PBS)). The findings in this study will be used in recommending the top-ranked miRNAs as reference miRNAs for data normalization in similar experimental systems.

Materials and Methods

All animal procedures were conducted in compliance with the institutional guidelines for the care and use of laboratory animals. The procedures were reviewed and approved by the Animal Care and Ethics Committee of Southwest University, Chong Qing, China (Permit number 20190723).

Sample collection

Animal experiments were organized by Southwest University and conducted at Chongqing Liu Jiu Animal Husbandry Technology Co., LTD. Ten fluid samples were collected from ten Yorkshire pigs, of which semen were come from breeding boar, colostrum and ordinary milk come from multiparous sows and the rest come from female commercial pigs. They were assigned into three groups based on the location of collection: body fluids *in vivo* (bile, bladder fluid, and gastric juice), body fluids *in vitro* (colostrum, ordinary milk, semen, and urine) and the blood (UVBP, UABP and PBS) (n=6). For UVBP and UABP collection, a 15% EDTA anticoagulant solution was prepared and 0.1ml was added in a 5 ml syringe before their collection at birth. Peripheral blood serum was collected using an EDTA-containing anticoagulant tube. Colostrum and ordinary milk were collected from multiparous sows on the first and seventh days after birth, respectively. Semen was artificially collected from adult boars in the boars' station, while urine entailed the morning urine of adult commercial pigs. Body fluids *in vivo* (bile, bladder fluid, and gastric juice) were collected during adult pigs' dissection, immediately frozen in liquid nitrogen then transferred to a freezer at-80°C for long-term preservation.

Exosome preparation, candidate Reference miRNAs Selectionand and RT-qPCR

The exosomes in the body fluids were isolated as previously described by Luo, et al. [19]. Multiple studies have been conducted to validate the stable expression of reference miRNA genes across different porcine tissues and organs [20,21]. Since circulating exosomal miRNAs in body fluids are mainly related to surrounding tissues and organs, the relatively stable miRNAs in tissues screened previously were used included 16 miRNAs: let-7b-5p, let-7a, miR-103, miR-17-3p, miR-106a, miR-191, miR-9, miR-25, miR-26a, miR-17-5p, miR-335, miR-148a, miR-451, miR-92a, miR-16 and U6 [22-24].

According to the manufacturer's instructions, the total exosomal RNA was extracted using the TRIzol LS reagent (Invitrogen). The purity of the extracted RNA was determined using a NanoDrop ND2000 spectrophotometer (hermo Scientific, Wilmington, DE, USA). Only RNA samples with a 260/280 ratio between 1.8 and 2.0 purity were selected for subsequent analyses. The obtained cDNA was diluted tenfold with ultrapure water to generate cDNA templates for qRT-PCR. According to the manufacturer's instructions, the reverse transcription of miRNAs was performed using a commercial kit (TaKaRa, China). Serially diluted cDNA templates were analyzed to generate standard curves for each reference gene. All analyses across all the samples were run in triplicate. Quantitative Real-time-PCR (qRT-qPCR) was performed using an SYBR Premix Ex Taq kit (TaKaRa, China) on a Bio-rad IQTM5 system (Bio-Rad, Hercules, CA, USA). The amplification conditions were as follows: initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 30s, annealing at 60°C for 40s, and extension at 72°C for 30s. The specificity of each primer pair was verified by melting curves analysis using a corresponding cDNA sample.

Determination of reference gene expression stability

The initial qRT-PCR data were obtained from the StepOnePlus™ software (Applied BioSystems, Foster City, CA, USA) and put into a Microsoft Excel 2010 datasheet. The mean quantification cycle (Cq) value was converted into the relative expression level using the $2^{-\Delta\Delta Ct}$ method during miRNA expression stability analysis. Next, GeNorm, NormFinder and BestKeeper were used to evaluate the gene expression stability [25-27]. The Cq values averages run in triplicate were used to compare the stability of each candidate house-keeping miRNA gene.

Statistical analysis

The statistical analysis of the expression levels of the reference miRNA genes was performed using Duncan multiple comparisons using the SPSS11.0 software. $P<0.05$ was regarded as a level of statistical significance. The stability of miRNAs in the body fluids was quantified by combining multiple gold-standard statistical tools, including GeNorm, NormFinder and BestKeeper [25-27].

Results

Expression levels of the candidate reference miRNA

The Cycle threshold (Ct) value for each reference miRNA is presented in Supplementary Table 1. There were significant differences across the 16 reference miRNAs in the ten body fluids. The mean Ct value of the candidate genes ranged from 22 to 40 cycles, with miR-148a having the highest transcript levels (22.73 ± 0.02) in ordinary milk and miR-16 with the lowest (39.49 ± 0.14) in urine. Based on the geNorm calculations of the preliminary Ct values and stability sequencing (Supplementary Table S2), ten miRNAs were selected for subsequent stability analysis including, miR-92a, miR-93, let-7a, miR-451, miR-106a, U6, miR-17-5P, miR-26a, miR-25, let-7b-5p.

GeNorm analysis of candidate reference miRNAs in body fluids

The geNorm analysis revealed that all candidate genes had an M value below 1.5, which is the maximum value for acceptable gene expression stability [25]. The gene with the lowest M value has the most stable expression. However, the optimal candidate miRNA stability expressed in each body fluid differed (Supplementary Table 2). The most stable miRNA were miR-25 and let-7b-5p in URBP, let-7b-5p and miR-93 in UVBP, miR-26a and miR-335 in PBS, miR-17-5P and miR-148a in ordinary milk, miR-451 and miR-16 in colostrum, miR-103 and miR-451 in semen, miR-191 and miR-92a in urine, let-7b-5p and miR-93 in bile, miR-191 and miR-16 in bladder fluid, and miR-17-5p and miR-93 in gastric juice. Further analyses of the M value ranking in all body fluids *in vivo*, *in vitro*, and the blood revealed the first five ranking of stability in all body fluids as miR-451>miR-92a (let-7b-5p)>miR-106a (U6). The average stability rank in the blood, *in vitro* and *in vivo* was let-7b-5p>miR-25>U6, miR-451>miR-92a>miR-106a, and U6>miR-92a>let-7a, respectively.

Analysis of candidate reference miRNAs in blood

To explore the stability of miRNA expression, the stability of miRNA in URBP, UVBP, and PBS were analyzed (Table 1). A comprehensive ranking of the miRNA expression stability revealed that let-7b-5p expressed in URBP was the most stable, ranking 1, 2, and 1 with GeNorm, NormFinder, and BestKeeper, respectively. Moreover, miR-25 expression was the most stable in UVBP and PBS. The U6 expression was also stable in PBS (Table 1).

Table 1. Ranking and best combination of candidate reference genes in blood.

URBP	Genormer (M value)	Normfinder stability value	Bestkeeper CV (%CP)	UVBP	Genormer (M value)	Normfinder stability value	Bestkeeper CV (%CP)	PBS	Genormer (M value)	Normfinder stability value	Bestkeeper CV (%CP)
miR-92a	0.12 (10)	0.11 (10)	0.27 (8)	miR-17-5P	0.10 (10)	0.09 (10)	0.03 (2)	miR-451	0.11 (10)	0.11 (10)	0.33 (10)
miR-93	0.11 (9)	0.10 (9)	0.32 (9)	miR-92a	0.09 (9)	0.08 (9)	0.31 (8)	miR-106a	0.09 (9)	0.10 (9)	0.29 (9)
let-7a	0.10 (8)	0.09 (8)	0.34 (10)	miR-26a	0.08 (8)	0.08 (8)	0.31 (7)	miR-93	0.08 (8)	0.08 (8)	0.24 (8)
miR-451	0.08 (7)	0.07 (7)	0.23 (7)	miR-106a	0.07 (7)	0.04 (5)	0.19 (5)	miR-92a	0.06 (7)	0.04 (6)	0.12 (6)
miR-106a	0.06 (6)	0.05 (6)	0.12 (5)	miR-451	0.07 (6)	0.04 (6)	0.31 (9)	let-7b-5p	0.06 (6)	0.05 (7)	0.13 (7)
U6	0.06 (5)	0.04 (5)	0.11 (4)	miR-93	0.06 (5)	0.02 (1)*	0.20 (6)	miR-26a	0.04 (5)	0.01 (3)	0.03 (2)
miR-17-5P	0.04 (4)	0.01 (1)*	0.19 (6)	let-7b-5p	0.06 (4)	0.02 (2)	0.16 (4)	let-7a	0.03 (4)	0.01 (2)	0.10 (3)
miR-26a	0.03 (3)	0.04 (4)	0.10 (3)	U6	0.04 (3)	0.05 (7)	0.15 (3)	U6*	0.03 (3)	0.01 (1)*	0.02 (1)*
miR-25	0.02 (1)*	0.02 (3)	0.09 (2)	miR-25*	0.03 (1)*	0.03 (3)	0.01 (1)*	miR-25*	0.01 (1)*	0.04 (5)	0.11 (4)
let-7b-5p*	0.02 (1)*	0.01 (2)	0.08 (1)*	let-7a	0.03 (1)*	0.03 (4)	0.38 (10)	miR-17-5P	0.01 (1)*	0.04 (4)	0.12 (5)

Note: * represents the ranking and the best combination of candidate reference genes. The numbers in brackets represent the stability sort.

Abbreviations: URBP: Umbilical Arterial Blood Plasma; UVBP: Umbilical Venous Blood Plasma; PBS: Peripheral Blood Serum.

Analysis of the candidate reference miRNAs in body fluids *in vitro*

Except for blood, commonly used to detect disease, body fluids *in vitro* are more readily available than *in vivo*. Here, the stability of miRNA expression in body fluids *in vitro* (ordinary milk, colostrum, semen, and urine) was analyzed. The findings are presented in Table 2. A comprehensive ranking presented miR-106a, let-7b-5p, miR-25, and miR-92a as the most stably expressed in ordinary milk (ranks 1, 1 and 3), colostrum (ranks 1, 1 and 1), semen (ranks 5, 1 and 3), and urine (ranks 1, 2 and 1) using GeNorm, NormFinder, and BestKeeper, respectively (Table 2).

Analysis of the candidate reference miRNAs in body fluids *in vivo*

The stability of miRNA expressed in body fluids *in vitro*, including in bile, bladder fluid, and gastric juice, is presented in Table 3. A comprehensive ranking of the miRNA expression stability revealed that miR-93 (let-7b-5p), miR-92a, and miR-93 were most stably expressed in bile (ranks 1, 2, and 1), bladder fluid (ranks 5, 2, and 2), and gastric juice (ranks 1, 2 and 2) based on the GeNorm, NormFinder and BestKeeper analyses, respectively (Table 3).

Comprehensive ranking of the reference miRNAs

Given that each body fluid has its distinct characteristics, it is necessary to identify the optimal reference miRNAs in each fluid for a better practical application. The comprehensive comparative analysis revealed that miR-25, let-7b-5p, miR-25, and U6 were the optimal reference miRNA in blood, URBP, UVBP, and PBS, respectively (Table 4). The optimal reference miRNA in body fluids obtained *in vitro* was miR-451 and miR-92a, with miR-92a, let-7b-5p, miR-25, and miR-92a as the optimal reference miRNA in ordinary milk, colostrum, semen, and urine, respectively. In addition, the optimal reference miRNA in body fluids *in vivo* was miR-93, with let-7b-5p (miR-93), miR-92a, and miR-93 as the optimal reference miRNA in bile, bladder fluid, and gastric juice, respectively. Overall, miR-25, miR-92, and miR-93 were the most common in blood, *in vivo*, and *in vitro*, respectively (Table 4).

Table 2. Ranking and best combination of candidate reference genes of body fluids *in vitro*.

Ordinary milk	Genormer (M value)	Normfinder stability value	Bestkeeper CV (% CP)	Colostrum	Genormer (M value)	Normfinder stability value	Bestkeeper CV (%CP)	Semen	Genormer (M value)	Normfinder stability value	Bestkeeper CV (%CP)	Urine	Genormer (M value)	Normfinder stability value	Bestkeeper CV (%CP)
let-7a	0.06 (10)	0.07 (10)	0.36 (9)	let-7a	0.09 (10)	0.08 (9)	0.37 (10)	let-7b-5p	0.12 (10)	0.12 (10)	0.27 (8)	U6	0.11 (10)	0.15 (10)	0.38 (9)
miR-25	0.05 (9)	0.06 (9)	0.25 (8)	miR-25	0.08 (9)	0.08 (10)	0.22 (8)	let-7a	0.10 (9)	0.08 (9)	0.25 (7)	miR-25	0.08 (9)	0.10 (9)	0.29 (7)
U6	0.05 (8)	0.05 (8)	0.2 (7)	miR-93	0.07 (8)	0.08 (8)	0.22 (9)	miR-93	0.09 (8)	0.08 (8)	0.24 (5)	let-7a	0.07 (8)	0.08 (8)	0.30 (8)
miR-26a	0.04 (7)	0.03 (6)	0.07 (1)*	miR-26a	0.06 (7)	0.06 (7)	0.13 (7)	miR-92a	0.08 (7)	0.04 (4)	0.10 (2)	miR-106a	0.06 (7)	0.05 (7)	0.10 (4)
miR-93	0.04 (6)	0.03 (7)	0.12 (6)	miR-17-5P	0.05 (6)	0.03 (5)	0.08 (4)	miR-451	0.08 (6)	0.03 (3)	0.08 (1)*	miR-26a	0.05 (6)	0.03 (6)	0.05 (2)
let-7b-5p	0.03 (5)	0.02 (5)	0.10 (4)	miR-106a	0.04 (5)	0.01 (2)	0.07 (3)	miR-25*	0.07 (5)	0.01 (1)*	0.11 (3)	let-7b-5p	0.04 (5)	0.02 (5)	0.11 (6)
miR-17-5P	0.02 (4)	0.01 (3)	0.37 (10)	miR-92a	0.04 (4)	0.05 (6)	0.11 (6)	miR-26a	0.06 (4)	0.01 (1)*	0.14 (4)	miR-93	0.04 (4)	0.02 (4)	0.11 (5)
miR-451	0.02 (3)	0.02 (4)	0.11 (5)	U6	0.03 (3)	0.03 (4)	0.09 (5)	miR-17-5P	0.03 (3)	0.08 (7)	0.32 (10)	miR-451	0.03 (3)	0.01 (1)*	0.09 (3)
miR-106a*	0.01 (1)*	0.01 (1)*	0.08 (3)	miR-451	0.02 (2)	0.02 (3)	0.02 (2)	miR-106a	0.02 (1)*	0.05 (5)	0.24 (6)	miR-92a*	0.02 (1)*	0.01 (2)	0.01 (1)*
miR-92a	0.01 (1)*	0.01 (2)	0.07 (2)	let-7b-5p*	0.02 (1)*	0.01 (1)*	0.02 (1)*	U6	0.02 (1)*	0.06 (6)	0.32 (9)	miR-17-5P	0.02 (1)*	0.01 (3)	0.38 (10)

Note: * Represents the ranking and the best combination of candidate reference genes.

Table 3. Ranking and best combination of candidate reference genes of body fluids *in vivo*.

Bile	Genormer (M value)	Normfinder stability value	Bestkeeper CV (%CP)	Bladder fluid	Genormer (M value)	Normfinder stability value	Bestkeeper CV (%CP)	Gastric juice	Genormer (M value)	Normfinder stability value	Bestkeeper CV (%CP)
miR-451	0.12 (10)	0.11 (10)	0.40 (10)	miR-93	0.11 (10)	0.11 (10)	0.28 (10)	miR-26a	0.07 (10)	0.09 (10)	0.27 (10)
miR-25	0.11 (9)	0.10 (9)	0.28 (9)	miR-17-5P	0.10 (9)	0.10 (9)	0.13 (3)	let-7b-5p	0.05 (9)	0.08 (9)	0.20 (9)
miR-17-5P	0.09 (8)	0.07 (8)	0.10 (3)	let-7b-5p	0.08 (8)	0.08 (7)	0.17 (6)	let-7a	0.04 (8)	0.03 (7)	0.10 (6)
miR-92a	0.09 (7)	0.03 (3)	0.15 (6)	miR-25	0.08 (7)	0.08 (8)	0.25 (9)	miR-25	0.04 (7)	0.03 (8)	0.13 (8)
miR-26a	0.08 (6)	0.06 (4)	0.15 (7)	U6	0.07 (6)	0.03 (3)	0.04 (1)*	U6	0.03 (6)	0.02 (6)	0.07 (4)
miR-106a	0.07 (5)	0.07 (7)	0.15 (5)	miR-92a*	0.06 (5)	0.02 (2)	0.06 (2)	miR-92a	0.03 (5)	0.02 (5)	0.08 (5)
U6	0.05 (4)	0.06 (6)	0.15 (4)	let-7a	0.04 (4)	0.01 (1)*	0.19 (8)	miR-451	0.02 (4)	0.01 (1)*	0.02 (1)*
let-7a	0.04 (3)	0.06 (5)	0.22 (8)	miR-451	0.01 (3)	0.03 (4)	0.15 (4)	miR-106a	0.02 (3)	0.01 (3)	0.04 (3)
let-7b-5p*	0.04 (1)*	0.03 (2)	0.04 (1)*	miR-106a	0.01 (1)*	0.04 (5)	0.17 (5)	miR-93*	0.02 (1)*	0.01 (2)	0.04 (2)
miR-93*	0.04 (1)*	0.02 (1)*	0.05 (2)	miR-26a	0.01 (1)*	0.04 (6)	0.18 (7)	miR-17-5P	0.02 (1)*	0.01 (4)	0.12 (7)

Note: * represents the ranking first and the best combination of candidate reference miRNAs.

Table 4. The Optimal reference gene in each body fluids calculated by geNorm, NormFinder and Bestkeeper algorithms.

Type of fluid	URBP	UVBP	PBS	Blood	Ordinary milk	Colostrum	Semen	Urine	Body fluids *in vitro*	Bile	Bladder fluid	Gastric juice	Body fluids *in vivo*
Optimal reference gene	let-7b-5p	miR-25	U6	miR-25	miR-92a	let-7b-5p	miR-25	miR-92a	miR-451	let-7b-5p	miR-92a	miR-93	miR-93
									miR-92a	miR-93			

Abbreviations: URBP: Umbilical Arterial Blood Plasma; UVBP: Umbilical Venous Blood Plasma; PBS: Peripheral Blood Serum

Discussion

Exosomes are nanovesicles released from almost all cell types under physiological and pathological conditions. Due to their unusually high stability in biofluids, circulating exosomal miRNAs have emerged as promising non-invasive biomarkers for human disease [28]. MicroRNA expression profiling in body fluids, specifically the plasma and the serum, is key to identifying miRNA biomarkers informing early disease diagnosis and predicting response to therapy [29]. However, ribonucleases in body fluids degrade RNA, including the extracellular miRNA; thus, obtaining miRNAs expression profiles in biofluids is technically challenging [30]. Several reference genes for miRNA expression profiles have been identified in tissues [31] and blood in the umbilical cord [32] using different analysis strategies [33]. Besides, the reference genes in exosomes have been screened [34]. However, the miRNA stability in the specific body fluids exosomes remains unknown. In addition, there are no reference genes identified in the circulating exosomal miRNA in body fluids. Given the protective effect of exosomes on RNA, the circulating exosomal RNA can be used as biomarkers for biological humoral diseases [30,35,36]. This has increased the interest in extracellular RNA, specifically in circulating exosomal miRNA. As a result, there is a growing interest in identifying these circulating exosomal miRNAs as non-invasive biomarkers for the early detection of diseases or selection of treatment options.

Circulating exosome miRNA studies using qRT-PCR depends on an endogenous reference miRNA to normalize the abundance of the miRNAs [37]. In the present study, we analyzed the stability of miRNA in ten common body fluids, including those obtained *in vivo*, *in vitro*, and the blood. To identify the most suitable reference exosomal miRNA for stable expression in the different body fluids, a sequencing analysis of miRNA present in all body fluids was conducted to screen out the optimal circulating exosomal miRNAs. We performed a preliminary analysis on all the 16 candidate miRNAs using GeNorm. We selected the top ten stably expressed in the body fluids for subsequent stability analysis. The most suitable candidate reference miRNAs in the different body fluids were revealed by geNorm analysis. There were huge differences in miRNAs stability between the different body fluids. For example, the most stable miRNA in all body fluids was miR-451. However, let-7b-5p was the most stable in the blood, miR-451 in body fluids obtained *in vitro* and U6 in body fluids obtained *in vivo*. This is consistent with previous studies, which revealed that miR-451had strong stability; thus, it could be detected at all detection points [38]. In contrast, U6 has been recommended as the reference gene for the relative quantification of miRNA expression levels [39].

Based on the GeNorm analysis, the most stable miRNA in blood, body fluids obtained *in vitro* and *in vivo* were let-7b-5p>miR-25>U6, miR-451>miR-92a>miR-106a, and U6 >miR-92a>let-7a, respectively. Moreover, let-7b-5p was the most stably expressed in URBP and miR-25 in UVBP and PBS in blood. This finding is consistent with previous studies where let-7b-5p and miR-25 were identified as stably expressed genes in blood [40,41]. Moreover, miR-106a, let-7b-5p, miR-25, miR-92a, MiR-93 (let-7b-5p), miR-92a, miR-93 were the most stably expressed miRNA in ordinary milk, colostrum, semen, urine, bile, bladder fluid, and gastric juice, respectively. This provides a reference for selecting marker genes during specific physiological conditions.

A comprehensive analysis revealed that the optimal reference miR-25, miR-92, and miR-93 were common in body fluids obtained in the blood, *in vivo*, and *in vitro*, respectively. This result is consistent with previous findings where miR-92 was identified as an internal reference for stable expression in body fluids and miR-93 as a suitable reference gene for serum miRNA analysis in gastric cancer patients [33,42]. The preliminary findings in this study proved a huge potential in body fluid-based miRNAs used as references in diagnosing diseases and predicting results following a therapeutic intervention.

Conclusion

In the present study, we analyzed the stability of 16 miRNAs obtained from exosomes in ten body fluids and optimized the reference miRNAs. Based on the comprehensive ranking, the optimal reference genes *in vivo* were let-7b-5p (miR-93) in bile, miR-92a in bladder fluid, and miR-93 in gastric juice, while let-7b-5p in colostrum, miR-92a in ordinary milk and urine, and miR-25 in semen were the optimal reference genes in body fluids *in vitro* and let-7b-5p in UVBP, miR-25 in UABP and U6 in PBS in the blood. Across all body fluids, miR-451 was the most stable circulating exosomal reference miRNA.

Statements and Declarations

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Author contribution

The authors contribute are as follows: the conception and design of the study (QiangPu, Lin Wang), acquisition of data (QiangPu, Lin Wang, Guojun Kang, Changbao Liu), analysis and interpretation of data (QiangPu, Lin Wang, Changfeng Yang), drafting the article (QiangPu, Lin Wang), final approval of the version to be submitted (JiaLuo, Yongfu Huang).

Ethics approval

This study was granted by the Ethics Committee of Southwest University (No.20190723).

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