Utilizing *Caenorhabditis elegans* microRNA-39 as an Exogenous Control for Quantitation of U6 snRNA in Microparticles

การใช้ *Caenorhabditis elegans* microRNA-39 สำหรับเป็นตัวควบคุม สำหรับการวิเคราะห์ปริมาณ U6 snRNA ในไมโครพาติเคิล

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ABSTRACT

Analysis of circulating miRNA is useful as biomarkers for several diseases. Reverse transcription quantitative PCR, commonly used for measuring the level of miRNA, is quantified by relative quantitation normalized with reference genes. Unlike cellular miRNA analysis, there is still no universal and reliable reference genes to use as a normalizer for circulating miRNA, especially miRNAs in microparticles (MPs). Here, *Caenorhabditis elegans* microRNA-39 (cel-miR-39) was used as exogenous control and spiked-in at RNA extraction step. The U6 small nuclear RNA was used as a model for small RNA in MP quantitation. Two cDNA synthesis approaches were also validated. The optimal protocol for quantitation of small RNA in MPs was using 100 fmol cel-miR-39 exogenous control spiked-in and performed cDNA synthesis by using fixed volume of total RNA.

บทคัดย่อ

การวิเคราะห์ไมโครอาร์เอ็นเอในกระแสเลือดมีประโยชน์ในการนำมาใช้เป็นตัวชี้วัดทางชีวภาพสำหรับโรคต่าง ๆ การวัดปริมาณของไมโครอาร์เอ็นเอทำโดยเทคนิค RT-qPCR โดยเปรียบเทียบกับยีนอ้างอิงซึ่งมีการแสดงออกคงที่ อย่างไรก็ตาม ยังไม่มียีนอ้างอิงที่เป็นมาตรฐานสำหรับตรวจวัดปริมาณไมโครอาร์เอ็นในกระแสเลือด โดยเฉพาะอย่างยิ่งไมโครอาร์เอ็นเอในไมโครพาติเคิล การศึกษานี้ จึงใช้ *Caenorhabditis elegans* microRNA-39 (cel-miR-39) เป็นตัวควบคุมภายนอก โดยเติมเข้าไปในขั้นตอนการสกัดอาร์เอ็นเอ แล้ววัดปริมาณของ U6 small nuclear RNA เป็นต้นแบบการวิเคราะห์ปริมาณอาร์เอ็นเอขนาดเล็กในไมโครพาติเคิล นอกจากนี้ยังเปรียบเทียบวิธีการสังเคราะห์ cDNA สองวิธี พบว่าวิธีที่เหมาะสมที่สุดสำหรับการหาปริมาณของอาร์เอ็นเอขนาดเล็กในไมโครพาติเคิล คือ การใช้ 100 fmol cel-miR-39 เป็นตัวควบคุมจากภายนอก และทำการสังเคราะห์ cDNA โดยควบคุมปริมาณของอาร์เอ็นเอให้คงที่

Keywords: Microparticles, MicroRNAs, Biomarkers
คำสำคัญ: ไมโครพาติเคิล ไมโครอาร์เอ็นเอ ตัวชี้วัดทางชีวภาพ

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Introduction

Microparticles (MPs) or medium extracellular vesicles are small membrane vesicles released from many cell types during cell activation or apoptosis. Circulating MPs are abundant in the plasma of normal individuals and can be derived from circulating blood cells such as platelets, red blood cells and leukocytes as well as from tissue sources, such as endothelial cells. Elevation of numbers and cellular origin of circulating MPs are associated with several diseases such as thalassemia, cancer, sepsis and diabetes (Kanazawa et al., 2003; Nieuwland et al., 2000; Pattanapanyasat et al., 2007; Sabatier et al., 2002). MPs contain a wide range of biomolecules such as microRNAs (miRNAs), mRNAs, DNA and proteins. Interestingly, depending on the stimulus, the content and characteristics of MPs derived from the same cell lineage are variety. MPs derived from stimulated THP-1 cells contained increased inflammatory miRNA (Diehl et al., 2012). MPs are important intercellular communicators. MPs transfer their components to target cells and mediate cell activation, phenotypic modification and reprogramming of cell function. As MPs are in the blood circulation, they can serve as intercellular communicators not only in their local environment but also at remarkable distance from their site of origin (Mause et al., 2010; VanWijk et al., 2003). MPs from activated platelets could transfer miRNA to macrophages and change gene expression and phagocytosis activity (Laffont et al., 2013). MPs from apoptotic endothelial cells could promote vascular endothelial cells repairing by transferred microRNA-126 (miR-126) to human coronary artery endothelial cells (HCAECs) (Jansen et al., 2013).

The miRNAs are small non-coding RNA that act as a negative regulator at the post-transcriptional level by either degraded targeted mRNA or suppressed protein translation. miRNAs play importance roles in biological process such as cell proliferation, cell survival, hematopoiesis and oncogenesis (Etheridge et al., 2011; Samanta et al., 2016). Currently, miRNAs have become a potential biomarker of several diseases such as cancer and cardiovascular disease, due to the alteration expressing pattern of miRNAs could reflect the pathological process that occurring (Ardekani et al., 2010). In addition, miRNAs are stable in blood circulation in form of encapsulation in extracellular vesicles such as MPs and exosomes or complexing with argonaute 2 protein complex, which protect them from degradation (Alexandru et al., 2016). Circulating miRNAs have been shown to be associated with many vascular diseases and served as potential biomarkers such as pulmonary hypertension, myocardial infarction and pulmonary embolism (Kessler et al., 2016; Wei et al., 2013).

Reverse transcription quantitative PCR (RT-qPCR) is commonly used for measure the level of miRNA and quantifying by relative quantitation normalized with reference gene (Schwarzenbach et al., 2015). This would be relatively easy for analysis of cellular miRNA. However, there is still no universal and reliable reference gene to use as a normalizer for circulating miRNA, especially miRNAs carried by MPs. To overcome the limitation of unreliable internal reference gene, several external small RNA spike-in during RNA extraction have been employed such as miRNA from *Caenorhabditis elegans* (cel-miR-39, cel-miR-54 and cel-miR-238) or *Arabidopsis thaliana* (ath-miR-159a) (Kuhlmann et al., 2014; Marabita et

al., 2016; McDonald et al., 2011). Nevertheless, various amount of these external miRNA normalizer control has been reported, for example, 25 fmol cel-miR-39 and 25 fmol cel-miR-54 have been used as spiked-in exogenous control (Kuhlmann et al., 2014; Sourvinou et al., 2013).

Therefore, this study aims to explore the use of cel-miR-39 as an exogenous control. In addition, two cDNA synthesis methods, fixed amount of total RNA and fixed volume of total RNA, were evaluated. The U6 small nuclear RNA (U6 snRNA), a constitutive expression small RNA, in MPs was quantified as a model for analysis of miRNA in MPs. Herein, we found that 100 fmol cel-miR-39 was an optimal spiked-in exogenous control for quantification of small RNA in circulating MPs.

Objective of the study

This study aims to develop a method for quantification of miRNA in MPs by determining the optimal amount of cel-miR-39 exogenous control and cDNA synthesis method using U6 snRNA in MPs as a model.

Methodology

Subjects

This study was performed in accordance with the Helsinki declaration and was approved by the Mahidol University Central Institutional Review Board, approval number 2014/013.0502. Written informed consent was obtained from all individual participants included in the study. Peripheral blood samples were collected from 3 healthy donors at ages ranging from 25- to 35-year-old. All subjects had no evidence of concurrent infection, history of vaso-occlusive episode or atherosclerotic vascular disease. All blood samples were collected into 3.2% sodium citrate anticoagulant tubes by using two syringes venipuncture with sterile technique and processed within 3 hours.

Microparticle analysis

Diluted whole blood samples were stained with fluorochrome conjugated annexin V (phosphatidylserine marker). Data was acquired and analyzed by FACScan flow cytometer (BD Biosciences) (Figure 1). The absolute number of MPs was calculated by using TruCount™ beads (BD Biosciences) (Figure 1A). The MP population was characterized by size that compared to a 1-μm in diameter BlankBeads (Spherotech) (Figure 1B).

Microparticle isolation

MPs were isolated by using sequential centrifugation steps. Six mL peripheral blood samples were centrifuged at 1,500×g for 15 min at 25°C to collect platelet-poor plasma (PPP) and re-centrifuged at 14,000xg at 4°C for 2 min to obtained platelet-free plasma (PFP). MPs were collected by re-centrifuged at 14,000xg at 4°C for 45 min. MPs pellet were washed once with 1x phosphate buffer saline.

RNA extraction

Total RNA form MPs was isolated using TRIzol (Invitrogen). A synthetic cel-miR-39 (Invitrogen) was spiked-in to MPs before incubation with Trizol at different amount (0.1, 1, 10 and 100 fmol). The process of RNA extraction was performed as manufacturer's instructions. RNA was dissolved in diethyl pyrocarbonate (DEPC)-treated water (Sigma). Concentration of RNA samples were quantified by using NanoDrop ND-1000 Spectrophotometer analysis (NanoDrop Technologies).

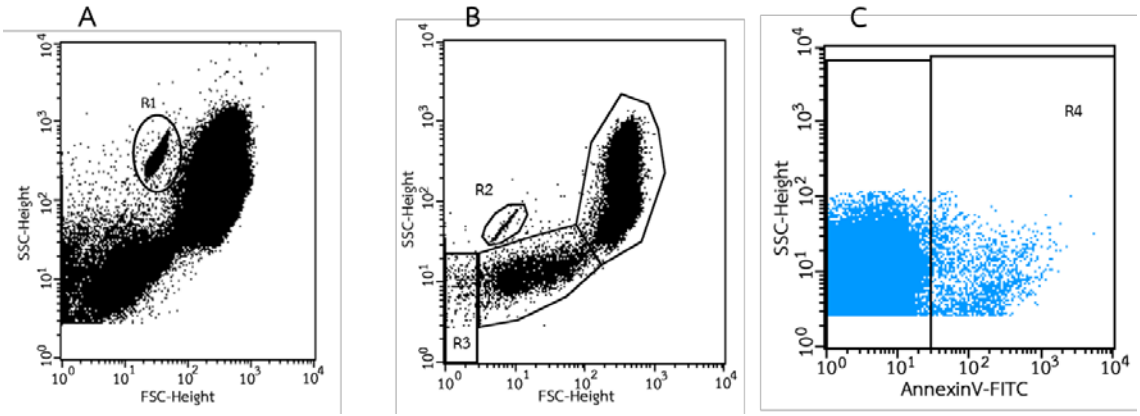

Figure 1 Flow cytometric analysis of MPs. Dot plot analysis of FSC-H and SSC-H was analyzed (A) the TruCount™ beads in region 1, (B) a 1-µm in diameter BlankBeads (range 1.0 – 1.4 µm) in region R2 and MPs in region R3. (C) MPs in R3 region were gated to analysis of total MPs as defined as annexin V^+ in R4 region.

cDNA synthesis

cDNA synthesis of the U6 snRNA and spiked-in cel-miR-39 was performed by using the TaqMan Small RNA Assays kit (Applied Biosystems) and TaqMan Advanced miRNA Assays kit (Applied Biosystems), respectively. The two approaches for cDNA synthesis were used. Method A, fixed amount of total RNA used for cDNA synthesis, 250 ng for U6 snRNA and 40 ng for cel-miR-39. Method B, fixed volume of total RNA used for cDNA synthesis, 5 µL for U6 snRNA and 2 µL for cel-miR-39.

Quantitative RT-PCR analysis of U6 and cel-miR-39

The U6 snRNA and cel-miR-39 levels were determined by quantitative PCR (qPCR) using TaqMan™ Small RNA Assays Kit (Applied Biosystems) and TaqMan™ Advanced miRNA Assays Kit (Applied Biosystems), respectively. The relative expression of U6 normalized with cel-miR-39 was calculated as $2^{-\Delta Ct}$. Duplication experiments were performed in each sample.

Statistical analysis

Data were analyzed using SPSS Version 18.0 (IBM, Chicago, USA.). The total number of MPs was analyzed using the Mann-Whitney U test. The coefficient of determination of the amount of spiked-in cel-miR-39 was determined by using Pearson's correlation. The comparison of the mean $2^{-\Delta Ct}$ value of U6 and cel-miR-39 between condition groups was evaluated with the Mann-Whitney U test. The statistical significance for all comparisons is *P*-value < 0.05.

Results

Circulating MPs in healthy donors

MPs from three healthy donors were analyzed by a flow cytometer. The total number of AnV^{+}MPs in healthy donors was 1.6±4.5 x 10^4 particles/µL (Table 1).

Table 1 Absolute number of MPs

Parameters	Normal 1	Normal 2	Normal 3	Mean ± S.D.
Total number of MPs ($x10^4$ particles/µL)	2.0	1.1	1.6	1.6 ± .45

Optimization of cel-miR-39 exogenous control for RNA extraction and small RNA quantification in MPs

Vary amounts of synthetic cel-miR-39 (0.1, 1, 10 and 100 fmol) were spiked-in to MPs in Trizol reagent before RNA extraction as an exogenous control. After obtaining total RNA, two cDNA synthesis methods were employed, fixed amount of total RNA (method A) and fixed volume of total RNA (method B). Amount of cel-miR-39 was quantified by RT-qPCR. The Ct cycle of qPCR analysis of cDNA template form both cDNA synthesis methods was decreased with the increased amount of cel-miR-39 spiked-in. A linear plot analysis of Ct of cel-miR-39 amplification at different spiked-in concentration shown linear range of detection at 1-100 fmol cel-miR-39 for both cDNA synthesis methods, while cDNA synthesis using fixed volume of RNA method had higher coefficient of determination (R^2 = 0.933) than that of cDNA synthesis using fixed amount of RNA method (R^2 = 0.779) (Figure 2). In addition, the lower amount of cel-miR-39 spiked-in had the higher Ct cycle variance. The Ct cycle of 0.1 fmol cel-miR-39 spiked-in was higher than 35 cycles in both cDNA synthesis methods (Figure 2). The Ct cycle of 100 fmol spiked-in cel-miR-39 with cDNA synthesis using fixed volume of RNA method (17.06 ±0.59 cycles) was significant higher compared to cDNA synthesis using fixed amount of RNA method (23.24±5.06 cycles) (Figure 3).

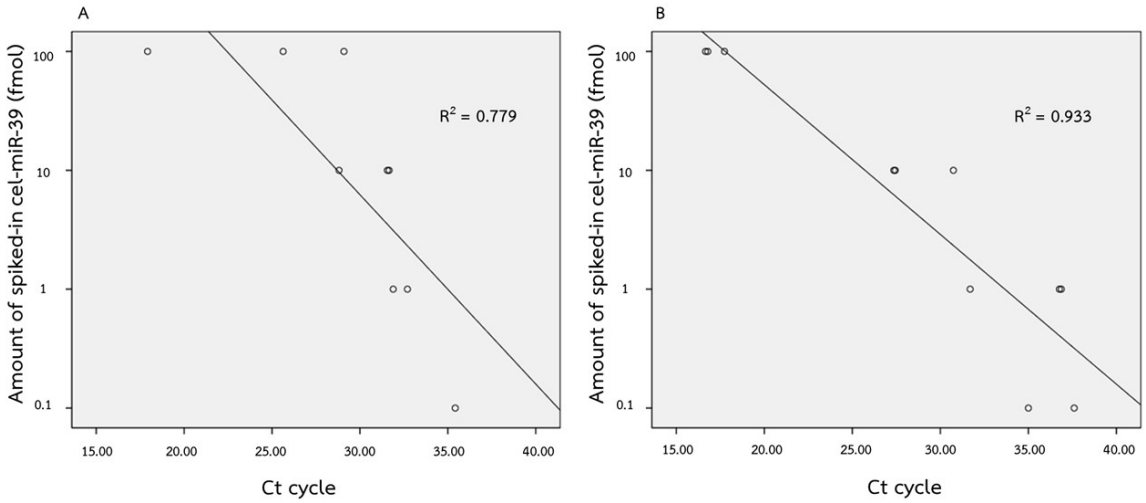

Figure 2 Linear plot analysis of the threshold cycles of spiked-in cel-miR-39 qPCR analysis. Amplification of cel-miR-39 cDNA obtained from two cDNA synthesis methods, (A) fix amount of total RNA (method A) and (B) fixed volume of total RNA (method B).

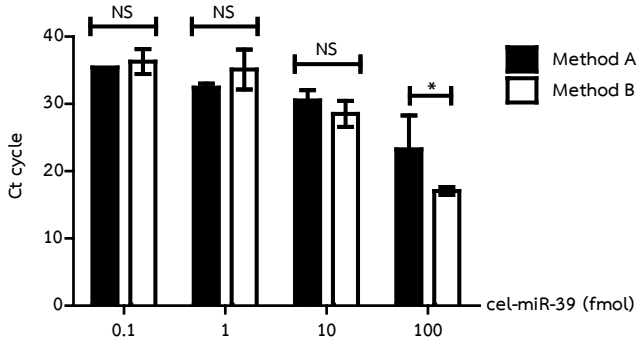

Figure 3 Threshold cycle of cel-miR-39 RT-qPCR analysis. Amplification of cel-miR-39 cDNA obtained from two cDNA synthesis methods, fix amount of total RNA (method A) and fixed volume of total RNA (method B). Data presents as mean ± S.D. *Significant difference between groups at *P-value* < 0.05. NS; not significant difference.

Quantification of U6 snRNA in MPs

The level of U6 in MPs was determined by RT-qPCR and normalized with exogenous control, spiked-in cel-miR-39. The U6 snRNA is a housekeeping gene and anticipated less amount variable among healthy donors. Higher variation of U6 snRNA levels was found in samples when normalized with 0.1-10 fmol cel-miR-39 spike-in (Table 2). The U6 snRNA levels normalized with 100 fmol spiked-in cel-miR-39 with cDNA synthesized by using fixed volume of total RNA showed less deviation than cDNA synthesized by using fixed amount of total RNA (Table 2).

Table 2 Level of U6 in MPs

Amount of spiked-in cel-miR-39 (fmol)	Level of U6 snRNA (folds, $2^{-\Delta Ct}$)					
	Method A			Method B		
	Normal 1	Normal 2	Normal 3	Normal 1	Normal 2	Normal 3
0.1	56.1028	0.0000	0.0000	445.7219	0.0000	95.6704
1	6.5887	0.0000	1.8532	1428.2175	1.0943	380.0380
10	21.4068	0.0967	1.0718	1.9453	1.2746	0.0442
100	1.2834	0.0187	0.0003	0.0005	0.0001	0.0002

U6 snRNA and cel-miR-39 cDNA were obtained from two cDNA synthesis methods, fix amount of total RNA (method A) and fixed volume of total RNA (method B).

Discussion

Circulating miRNAs are promising biomarkers for several diseases. In blood, circulating miRNAs can withstanding degradation through their inclusion in MPs. Quantification of miRNA is mostly performed by qPCR, using different reference genes for normalization. Typically, analysis of cellular miRNA is required the housekeeping genes used as the normalization for data from qPCR technique. Because they represent endogenous controls. Stable small RNAs are currently used as reference RNA controls for quantifying of cellular miRNAs such as small nucleolar RNA (SNORD44 and SNORD48) and small nuclear RNA (U6 snRNA) (Marabita et al., 2016). Importantly, the lack of a recognized and reliable reference gene for circulation miRNA, especially miRNA in MPs, is still a significant problem that can lead to misinterpretation of (Schwarzenbach et al., 2015). Herein, we investigated the use of synthetic-cel-miR-39 as an exogenous control to determine levels of miRNA in MPs. U6snRNA was used as a model of small RNA quantification as it is constitutively expressed.

Spiked-in of exact amounts of exogenous control have the potential to be a better normalizer than endogenous control for quantification of miRNA in MPs. With the known-amount, this is convenient to normalize the data and also eliminate factors about RNA degraded or technical variability. In this study, 100 fmol cel-miR-39 spiked-in showed the low Ct cycle and more reproducibility among samples, providing more reliability on data than other three concentrations examined. There is various amount of these external miRNA normalizer control have been reported, for example, 25 fmol cel-miR-39 and 25 fmol cel-miR-54 have been used as spiked-in exogenous control (Kuhlmann et al., 2014; Sourvinou et al., 2013).

Another factor-related to the quantification of miRNA in MPs was also examined. Two cDNA synthesis methods were evaluated. We found that the amount and volume of total RNA in the cDNA synthesis could affect U6 snRNA and cel-miR-39 quantification by RT-qPCR. U6 snRNA is a constitutive express gene. One would anticipate consistence amount among normal individuals. In the cDNA synthesis condition using a fixed volume of total RNA had less variations among individual samples when compared to fixed amount of total RNA. Our previous small RNAs in MPs analysis by small RNA sequencing also showed no significant differences of U6

expression among normal individuals (Unpublished data). Consistent with this study, that U6 expressed level in MPs seems consistence among individual samples.

Conclusion

We recommended 100 fmol of cel-miR-39 be used as exogenous control for quantification of miRNAs in MPs. Fixed volume of total RNA as manufacturer's recommendation could be used for cDNA synthesis, and then, determine miRNA expression by RT-qPCR.

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