

## Effects of Preanalytic Variables on Circulating MicroRNAs in Whole Blood

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### Abstract

Research in the last decade suggests the clinical potential of circulating microRNAs in whole blood as biomarkers for cancer detection. However, before applying the identified circulating microRNAs clinically, biospecimen-focused research has to be performed to identify possible preanalytic variables that may significantly affect the levels of circulating microRNAs. In this study, using a unique resource of the Data Bank and BioRepository (DBBR) at Roswell Park Cancer Institute, we conducted a two-step analysis to identify internal control circulating microRNAs in whole blood and then to study how selected major preanalytic variables (namely, processing delay, storage condition, storage time, and freeze/thaw cycles) might affect the detection of circulating microRNAs. In the discovery phase of the first step, we identified three microRNAs, including *miR346*, *miR134*, and *miR934*, whose levels exhibited the smallest variation between the case-control groups, as well as within each group interindividually. In the further validation analysis, the consistency was validated for *miR346* and *miR134* but not for *miR934*. At the second step, using *miR346* and *miR134* as internal controls, we observed that as the numbers of freeze/thaw cycles increased, levels of both *miR346* and *miR134* were significantly decreased ( $P_{\text{trend}} < 0.0001$ ); varying other processing and storage conditions did not affect miRNA levels. In the paralleled analysis in plasma samples, levels of *miR16* were significantly decreased by increasing processing delay and increasing numbers of freeze/thaw cycles but not affected by storage condition and duration. The results from this study highlight the necessity of biospecimen-focused research on circulating microRNAs before clinical utilization.

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### Introduction

The discovery of microRNAs in the last decade, and the realization of their growing importance in carcinogenesis and cancer prognosis through regulation of

transcription of oncogenes and tumor suppressor genes, has led to an era of excitement and discovery regarding these small molecules (1–4). More recently, the occurrence of microRNAs in human circulation has been repeatedly observed in patients with cancer, as well as healthy controls (5–14). Intriguingly, the levels of microRNAs in circulation are more stable, reproducible, and consistent among individuals of the same species than are other circulating nucleotide acids. Because of the significance of microRNA in carcinogenesis, circulating microRNAs offer unique opportunities for studying these biomarkers for early and noninvasive diagnosis of human cancers.

For circulating microRNAs to be a possible early diagnostic cancer biomarker in the clinical setting, we have to understand how circulating microRNAs might be affected by different types of variations, including inter-/intraindividual variations, analytic variations, and preanalytic variations. Considering the process from blood collection to the analysis of the circulating microRNAs, there is a possibility for the occurrence of a significant amount of preanalytic variations. It has been shown that approximately 60% of laboratory errors may be due to preanalytic

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factors (15); but to date, our knowledge on how preanalytic variations might affect circulating microRNAs is still limited (16–20). Clearly, biospecimen research on circulating microRNAs to address preanalytic variations is urgently needed to optimize the potential use of these biomarkers for early detection of human cancers.

Using a unique resource of the Data Bank and BioRepository (DBBR) at Roswell Park Cancer Institute (RPCI; Buffalo, NY), we conducted biospecimen-focused research. We first identified internal control microRNAs for analysis of circulating microRNAs in whole bloods. Then, we applied the identified internal control microRNAs to investigate the effects of selected major preanalytic variables on whole-blood circulating microRNAs.

## Materials and Methods

### Study population

The study was approved by the Institutional Review Board at RPCI. Anonymous biospecimens and questionnaire data used in this study were made available through the DBBR, a Cancer Center Support Grant (CCSG) Shared Resource initiated in 2003, with an established and standardized infrastructure to collect biospecimens before surgery or cancer therapy and data (including epidemiologic, dietary, and clinical data; ref. 21). For the current study, 10 mL of whole blood was obtained from each study participant. To reduce the variation on blood drawn, we used PAXgene Blood RNA System (PreAnalytiX) to collect the whole-blood samples and extract total RNAs from whole-blood samples.

The discovery cohort for internal control microRNAs included 20 cancer cases and 20 healthy controls, all of whom were Caucasian. Among cancer cases, 12 were patients with breast cancer and 8 were patients with prostate cancer. The controls were matched with cases on age ( $\pm 5$  years old), race, and gender.

The validation cohort for internal control markers included 74 cancer cases and 90 healthy controls. Among cancer cases, 41 were patients with breast cancer, 26 were patients with prostate cancer, 3 were colorectal cancer cases, and 4 were lung cancer cases. Among healthy controls, 70 were females and 20 were males, all Caucasian.

The cohort for assessing preanalytic variables included 28 cancer cases and 28 healthy controls matched on age ( $\pm 5$  years old), race, and gender. Both cases and controls were equally distributed into 4 preanalytic variable testing groups: processing delay time, storage condition, storage duration, and freeze/thaw cycles. Thus, each group included 7 cancer cases and 7 healthy controls.

### Selection of preanalytic variables and study schemes

We selected 4 preanalytic variables. Below are detailed descriptions of each variable and our study schema.

**Processing delay time (no delay vs. 24-hour delay).** In this study, we intended to compare no delay versus 24-hour delay. Two PAXgene tubes were used for each study

subject. When the PAXgene tubes arrived in the DBBR laboratory, one tube was randomly selected to be processed immediately for RNA extraction. Another tube was purposely left at room temperature (25°C) for another 24 hours. After the delay, the blood was processed for RNA extraction using the same protocol.

**Storage condition (no storage,  $-20^{\circ}\text{C}$  vs.  $-80^{\circ}\text{C}$  at freezer).** In this study, 3 PAXgene tubes were used for each study subject. When the tubes arrived in the DBBR laboratory, one tube was randomly selected to be processed immediately for RNA extraction, the second tube was selected to be stored at  $-80^{\circ}\text{C}$ , and another PAXgene tube was stored at  $-20^{\circ}\text{C}$ . For the tube stored at  $-80^{\circ}\text{C}$ , it was first frozen at  $-20^{\circ}\text{C}$  for 24 hours and then transferred to  $-80^{\circ}\text{C}$ . After 1 month of storage, both tubes were removed from storage and processed for RNA extraction.

**Storage duration (no storage vs.  $-20^{\circ}\text{C}$  for 6 months).**

Two PAXgene tubes were used for each study subject. When the tubes arrived in the DBBR laboratory, one tube was randomly selected to be processed for RNA extraction immediately and the other tube was stored at  $-20^{\circ}\text{C}$  for 6 months. After 6 months of storage, the tube was pulled out from the storage and processed for RNA extraction.

**Freeze/thaw cycles (0 vs. 1 vs. 2).** Three PAXgene tubes were used for each study subject. When the tubes arrived in the DBBR laboratory, one tube was randomly selected to be processed for RNA extraction immediately and two tubes were stored at  $-80^{\circ}\text{C}$  for 2 weeks. After 2 weeks, both tubes were pulled out and thawed. The tubes were placed upright in a wire rack at room temperature (25°C) for approximately 2 hours. After reaching room temperature, one tube was randomly selected to be processed for RNA extraction and the other one refrozen for an additional 2 weeks. After that, the stored blood was pulled out and thawed and processed for RNA extraction.

### MicroRNA profiling

To identify potential internal control microRNAs, we profiled microRNA expression in the whole-blood samples of the discovery cohort using Exiqon mercury LNA Universal RT microRNA PCR Technology (Exiqon A/S), a platform that includes a total of 742 human microRNAs. Briefly, 40 ng total RNAs were reverse transcribed using the Exiqon Universal RT enzyme. The manufacturer's recommended protocol was strictly followed. Quantitative reverse transcriptase PCR (qRT-PCR) was carried out on an Applied Biosystems 7900HT real-time PCR instrument using the manufacturer's recommended cycling conditions.

### TaqMan-based microRNA qRT-PCR assays

TaqMan-based microRNA qRT-PCR was performed to validate the candidate internal control microRNAs in whole-blood samples and assess the impact of selected preanalytic variables. For each sample, 10 ng RNAs were used as input into the reverse transcription reaction.

For generation of standard curves, chemically synthesized RNA oligonucleotides corresponding to known microRNAs were included in the analysis. Real-time PCR was carried out on an Applied BioSystems 7900HT thermocycler. Data were analyzed with SDS Relative Quantification Software version 2.2.2.

### Statistical analysis

In the discovery cohort, we used 3 criteria to prioritize a list of candidate internal control microRNAs: (i) the  $C_t$  value is less than 38 in each of the 40 samples; (ii) the fold change is less than 1.2 for each of the 3 case-control comparisons (all, breast, and prostate); and (iii) the distribution of  $C_t$  value across the samples has small coefficient of variation (CV). The panel of candidate internal control microRNAs was evaluated in the independent validation cohort using the same 3 criteria. In addition, only circulating microRNAs whose expression levels were consistent across different cancer types were eligible to be considered as the potential internal control circulating microRNAs. For the list of validated internal control microRNAs, 2-way ANOVA was performed to assess whether their  $C_t$  values were affected by different pre-analytic variables in the assessment cohort. All reported  $P$  values were 2-sided. All statistical analyses were carried out using the program R.

### Results

In the discovery cohort, we selected a panel of candidate internal control microRNAs that had detectable expression in all tested samples, did not have significant expression differences between the cancer cases and the healthy controls, and belonged to those expression-invariant microRNAs (showing low interindividual variance). Among the 742 microRNAs profiled, 6 microRNAs were detected in all 40 samples and had less than 1.2-fold change in all 3 case-control comparisons (Table 1). Three of these 6 microRNAs (*miR134*, *miR346*, and *miR934*) had consistently smallest CVs in the 3 case-control groups and were included for the validation analysis.

In the independent validation cohort, we found these 3 microRNAs (*miR134*, *miR346*, and *miR934*) were ubiquitously expressed ( $C_t < 33$ ) in whole blood of all 164 study subjects. To be consistent with the discovery cohort analysis, we evaluated the consistency of their expression level between overall cases and controls, breast cancer cases and controls, and prostate cancer cases and controls. As shown in Table 1, *miR934* had fold change of 1.5, 1.47, and 1.57 in the 3 case-control comparisons and was therefore excluded from further analysis. *miR346* had fold change less than 1.2 in all 3 case-control comparisons. *miR134* had fold change less than 1.2 in both overall and breast comparisons, and its fold change (1.28) in prostate comparison

**Table 1.** The identification of internal control microRNAs in whole bloods

Discovery cohort						
ID	Overall cancer cases vs. controls		Breast cancer cases vs. controls		Prostate cancer cases vs. controls	
	(20 vs. 20)		(12 vs. 12)		(8 vs. 8)	
	Fold change	CV	Fold change	CV	Fold change	CV
<i>miR134</i>	-1.046	0.034	1.023	0.034	-1.159	0.034
<i>miR934</i>	1.025	0.034	1.035	0.037	1.009	0.027
<i>miR346</i>	-1.028	0.036	-1.053	0.031	1.009	0.042
<i>miR409.3p</i>	-1.083	0.048	-1.103	0.039	-1.053	0.056
<i>miR485.3p</i>	-1.014	0.053	-1.029	0.053	1.009	0.049
<i>miR144</i>	1	0.086	1.023	0.092	-1.035	0.079
Validation cohort						
ID	Overall cancer cases vs. controls		Breast cancer cases vs. controls		Prostate cancer cases vs. controls	
	(74 vs. 90)		(41 vs. 70)		(26 vs. 20)	
	Fold change	CV	Fold change	CV	Fold change	CV
<i>miR346</i>	-1.054	0.068	-1.006	0.069	-1.113	0.066
<i>miR134</i>	-1.149	0.051	-1.036	0.05	-1.276	0.052
<i>miR934</i>	1.504	0.047	1.464	0.046	1.568	0.052

**Table 2.** The effects of selected preanalytic variables on miR346 and miR134 in whole-blood samples

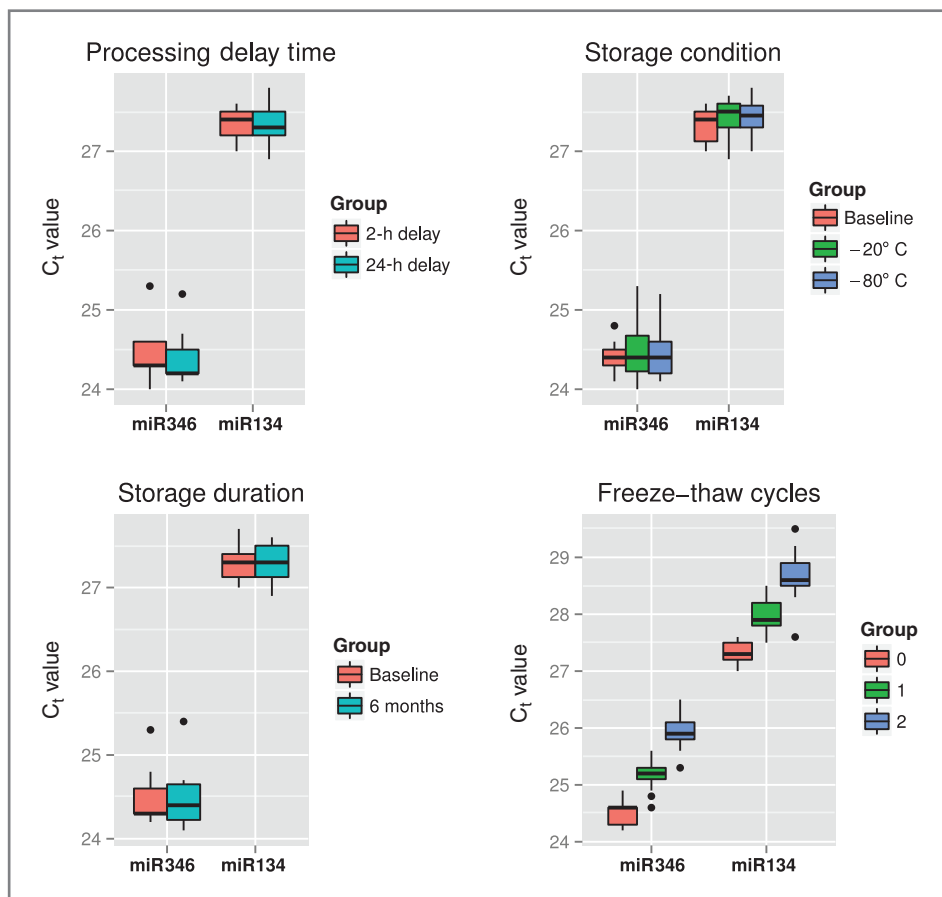
	Processing delay time		Storage condition		Storage duration		Freeze/thaw cycle	
	miR346	miR134	miR346	miR134	miR346	miR134	miR346	miR134
All study subjects	0.34	0.64	0.78	0.09	0.83	0.84	$1.78 \times 10^{-15}$	$2.73 \times 10^{-13}$
Cancer cases only	0.61	0.66	0.14	0.19	0.77	0.28	$1.50 \times 10^{-6}$	$4.22 \times 10^{-6}$
Healthy controls only	0.45	0.31	0.61	0.36	1.00	0.36	$1.07 \times 10^{-7}$	$9.44 \times 10^{-6}$

NOTE: The *P* value of 2-way ANOVA is displayed.

was only slightly larger than 1.2. The CV of *miR134* was smaller than *miR346* in all 3 study groups. Therefore, *miR346* and *miR134* were included in the final panel of internal controls to assess the impact of preanalytic variables.

Using the 2 identified microRNAs identified above, we analyzed whether their levels in whole blood were affected by the 4 different preanalytic variables, namely, processing delay time (no delay vs. 24-hour delay), storage condition (no storage,  $-20^{\circ}\text{C}$  vs.  $-80^{\circ}\text{C}$ ), storage duration (no storage vs. 6 months), and freeze/thaw cycles (0, 1 vs. 2).

The results are summarized in Table 2. As shown in Fig. 1, we did not observe significant difference for the expression level of either *miR134* or *miR346* in processing delay time, storage condition, and storage duration. On the other hand, significant differences were observed for the levels of both *miR134* and *miR346* among freeze/thaw cycles (0, 1 vs. 2). Specifically, the levels of *miR346* and *miR134* decreased significantly when the number of freeze/thaw cycles increased. We observed the same trends when the analysis was performed using case (or control) samples only (Supplementary Figs. S1–S4).



**Figure 1.** The effects of preanalytic variables on the expression level of miR346 and miR134 in whole blood. The preanalytic variables include processing delay time (no delay vs. 24-hour delay), storage condition (baseline,  $-20^{\circ}\text{C}$  vs.  $-80^{\circ}\text{C}$ ), storage duration (baseline vs. 6 months), and freeze/thaw cycles (0, 1 vs. 2).

## Discussion

In the current study, we identified 2 circulating microRNAs in whole-blood samples, namely, *miR134* and *miR346*, whose levels were consistent across 3 case-control comparison groups, as well as within the groups. Then, we applied them as internal control microRNAs to assess the effect of 4 preanalytic variables on the quality of circulating RNAs in whole-blood samples. Our results show that the differences in processing delay time, storage condition, and storage duration did not affect the levels of *miR134* and *miR346* in whole-blood samples. However, increasing number of freeze/thaw cycles seems to have a significant negative effect on whole-blood levels of *miR134* and *miR346*.

Because RPCI's DBBR applies the "best practice" (22) to blood collection, processing, and biospecimen storage, using DBBR to recruit study subjects and obtain whole-blood samples decreased the possibility of preanalytic variations during the entire process. In addition, with the linked epidemiologic and clinical data, it provided the opportunity for us to explore the impact of demographic, lifestyle, and clinical variables on the quality of biospecimens. Unfortunately, with the relatively small sample size, we did not explore the effect of those variables on the levels of *miR134* and *miR346* in whole-blood samples. However, those variables will be considered in future larger biospecimen studies.

Several quality control tools have been developed recently to assess the quality of certain biospecimens (23–25). For example, protein S is used to assess plasma storage duration (24). MMP9 activity is used to assess the effects of freeze/thaw on serum samples (25). However, for the majority of possible analytes, including circulating microRNAs in whole-blood samples, quality control tools are not yet available. Our results show that both *miR134* and *miR346* have the potential to serve as internal control markers in whole blood. The functions of both microRNAs have been studied previously in a variety of human tissue specimens, but there is a lack of report about their expression and function in whole blood. Thus, further exploration of the origins of those 2 microRNAs in whole blood is warranted.

Among all the possible variations, including interindividual, intraindividual, analytic (during analysis), and preanalytic variations (handling of the sample), preanalytical variations are the most difficult to manage. It has been estimated that more than 60% of laboratory errors are due to preanalytic factors (15). Little is known about preanalytic variation on circulating microRNAs in whole blood. The main reason for selecting these 4 preanalytic variables to study is because they are commonly occurring in our daily biospecimen handling so studying their impacts on circulating microRNAs has practical implication. In addition, compared with many other preanalytic variables, the variations from them are relatively easier to control, so the knowledge from this study can be quickly translated in practice. In addition, studies have shown that they may affect the quality of other types of biospeci-

mens (22, 26–30). Our finding on freeze/thaw cycles is expected. Theoretically, the more the samples are frozen, thawed, and frozen again, the higher the likelihood of product degradation. The observation that the levels of *miR134* and *miR346* in whole-blood samples are not affected by processing delay time, storage condition, and storage duration is probably due to the fact that we used PAXgene Blood RNA System to collect blood samples. PAXgene Blood RNA system is designed to minimize possible preanalytic variations during the blood collection and storage.

One limitation about this study is the limited generalization, as biospecimens were collected by PAXgene Blood RNA system. Different blood collection systems may warrant consideration of different preanalytic variables. Also, the results from whole bloods may not be applied to serum/plasma samples, which are banked by most of existing molecular epidemiological studies. In parallel with the whole-blood study, we have attempted to study the effects of same 4 preanalytic variables on circulating microRNAs in plasma samples from the same study subjects. We have evaluated the plasma-level expression consistency of *miR346* and *miR134*, the 2 internal controls identified from the whole-blood study, as well as *miR16*, one of the most widely used internal controls for circulating microRNA studies, determine the suitable internal control for assessing the impact of preanalytic variables in plasma. While none of them met our criteria for internal control, *miR16* was chosen as it was ubiquitously detected in all plasma subjects of the evaluation cohort and showed a relatively more stable plasma expression pattern than *miR346* and *miR134* (Supplementary Material and Supplementary Table S1). In further analysis, we assessed whether the plasma levels of *miR16* were affected by the 4 different preanalytic variables, namely, processing delay time (no delay vs. 18-hour delay), storage condition (cryovial vs. straw), storage duration (no storage vs. 6 months), and freeze/thaw cycles (0, 1, 2 vs. 4). The results are summarized in Supplementary Table S2 and Supplementary Figs. S5–S9. Similar to the whole-blood study, we did not observe significant difference for the plasma level of *miR16* in storage condition and storage duration, and we observed significant differences for the plasma levels of *miR16* among freeze/thaw cycles (0, 1, 2 vs. 4). On the other hand, unlike the whole-blood study, significant differences were observed for the plasma levels of *miR16* in processing delay time (no delay vs. 18-hour delay). Specifically, the plasma levels of *miR16* decreased significantly when comparing the 18-hour delay with no delay ( $P = 3.03 \times 10^{-7}$ ). In addition, the relatively small sample size limits our power to detect the effects of demographic, lifestyle, and clinical variables. We only studied 4 preanalytic variables.

Nevertheless, our results show the impact of preanalytic variables on circulating microRNAs in whole-blood and plasma samples. It highlights the need and

importance of biospecimen-focused research for circulating microRNAs.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Authors' Contributions

**Conception and design:** H. Zhao, J. Shen, W. Davis, C.B. Ambrosone, S. Liu

**Development of methodology:** H. Zhao, J. Shen, W. Davis, Y. Guo, C.B. Ambrosone, S. Liu, M. Nesline

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** W. Davis, L. Medico, Y. Guo, C.B. Ambrosone, M. Nesline

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** H. Zhao, J. Shen, Q. Hu, D. Wang, L. Yan, B. Liu, M. Qin, Q. Zhu, S. Liu

**Writing, review, and/or revision of the manuscript:** H. Zhao, J. Shen, Q. Zhu, S. Yao, C.B. Ambrosone, S. Liu, M. Nesline

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** H. Zhao, J. Shen, W. Davis, Y. Guo, S. Liu, M. Nesline

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