

Research Article

Blood Cell Origin of Circulating MicroRNAs: A Cautionary Note for Cancer Biomarker StudiesColin C. Pritchard¹, Evan Kroh³, Brent Wood¹, Jason D. Arroyo³, Katy J. Dougherty¹, Melanie M. Miyaji¹, Jonathan F. Tait¹, and Muneesh Tewari^{2,3,4}**Abstract**

Circulating, cell-free microRNAs (miRNAs) hold great promise as a new class of cancer biomarkers due to their surprisingly high stability in plasma, association with disease states, and ease of sensitive measurement. Yet little is known about the origin of circulating miRNAs in either healthy or sick people or what factors influence levels of circulating miRNA biomarkers. Of 79 solid tumor circulating miRNA biomarkers reported in the literature, we found that 58% (46 of 79) are highly expressed in one or more blood cell type. Plasma levels of miRNA biomarkers expressed by myeloid (e.g., miR-223, miR-197, miR-574-3p, and let-7a) and lymphoid (e.g., miR-150) blood cells tightly correlated with corresponding white blood cell counts. Plasma miRNA biomarkers expressed by red blood cells (e.g., miR-486-5p, miR-451, miR-92a, and miR-16) could not be correlated to red cell counts due to limited variation in hematocrit in the cohort studied but were significantly increased in hemolyzed specimens (20- to 30-fold plasma increase; $P < 0.0000001$). Finally, in a patient undergoing autologous hematopoietic cell transplantation, plasma levels of myeloid- and lymphoid-expressed miRNAs (miR-223 and miR-150, respectively) tracked closely with changes in corresponding blood counts. We present evidence that blood cells are a major contributor to circulating miRNA and that perturbations in blood cell counts and hemolysis can alter plasma miRNA biomarker levels by up to 50-fold. Given that a majority of reported circulating miRNA cancer biomarkers are highly expressed in blood cells, we suggest caution in interpretation of such results as they may reflect a blood cell-based phenomenon rather than a cancer-specific origin. *Cancer Prev Res*; 5(3); 492–7. ©2011 AACR.

Introduction

Since the initial description of circulating microRNAs (miRNAs) in 2008 (1–4), more than 200 articles have reported circulating miRNAs as biomarkers for a range of cancer types and other diseases (5, 6). At least 79 miRNAs have been reported as plasma or serum miRNA biomarkers of solid tumors (i.e., nonhematopoietic malignancies), including prostate, lung, breast, colon, ovarian, esophageal, melanoma, and gastric cancer (Supplementary Table S1). However, little attention has been given to the cellular origin of circulating miRNAs and what impact this has on biomarker specificity. We hypothesized that blood cells may

contribute significantly to circulating miRNA and that this could have important implications for interpretation of results from circulating miRNA cancer biomarker studies. In this study, we show that a majority of solid tumor-associated circulating miRNA biomarkers reported to date are highly expressed in blood cells and that plasma levels of these biomarkers are correlated to blood cell counts. We discuss the implications of these findings on the interpretation of circulating miRNA tumor biomarker results reported to date.

Materials and Methods**Clinical samples and plasma preparation**

Healthy volunteers. Blood was collected from adult healthy volunteer donors by standard antecubital vein phlebotomy at least 2 hours following the last meal. Four 10 mL K₂EDTA plasma tubes (BD Vacutainer 366643) were collected at each draw. All donors provided written informed consent.

Residual patient specimens. Forty-two consecutive unique patient specimens (study codes R1-R42) in which a complete blood count (CBC) with differential was ordered were collected from the hematology laboratory at the University of Washington Medical Center, Seattle, WA. All specimens were collected in 3 mL K₂EDTA plasma tubes (BD Vacutainer 367856) and complete differential blood

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Note: Supplementary data for this article are available at Cancer Prevention Research Online (<http://cancerprevres.aacrjournals.org/>).

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counts were determined with a Sysmex XE 2100 instrument. Plasma was isolated on the same day of blood draw. Details on these patients' specimens, including primary diagnosis, inpatient or outpatient status, age, gender, specimen characteristics (such as hemolysis determined by visual inspection), and CBC data are given in Supplementary Table S2. One patient (study code R46) had a total of 21 residual plasma specimens collected prospectively over a period of 33 days during the course of myeloablative chemotherapy and autologous hematopoietic stem cell transplant (Supplementary Table S3). This patient had a history of diffuse large B-cell lymphoma (DLBCL), with normal blood counts and no evidence of residual disease at the beginning of conditioning chemotherapy. The conditioning regimen did not include irradiation. All specimens were obtained in accordance with the declaration of Helsinki guidelines and with ethics approval from the local Institutional Review Board.

Plasma preparation. EDTA plasma was isolated by centrifugation of whole blood at 4,200 rpm ($3,298 \times g$) for 10 minutes at room temperature in an Allegra X-22 centrifuge (Beckman Coulter) using a SX4250 swinging bucket rotor with high brake. Plasma supernatant was recovered with a plastic transfer pipette, leaving at least 0.5 mL behind to avoid disturbing the buffy coat. All plasma processing was carried out in the specimen processing area of a large clinical laboratory by the same standardized plasma processing procedure applied to clinical specimens.

Fluorescence-activated cell sorting

A total of 4 independent fluorescence-activated cell-sorting (FACS) experiments were carried out on a Becton Dickinson ARIA cell sorter on separate days, using peripheral blood from the same healthy male volunteer donor (H1-1). Blood was drawn in K₂EDTA tubes as described earlier and run within 1 hour of collection. Automated complete differential blood counts were measured prior to each experiment with a Sysmex XE 2100 instrument. A total of 10 cell types were sorted: red blood cells (RBC; 5,000,000), platelets (5,000,000), neutrophils (2,500,000), eosinophils (1,000,000), basophils (10,000), B lymphocytes (1,000,000), T lymphocytes (1,000,000), natural killer (NK) cells (1,000,000), plasmacytoid dendritic cells (pDC; 10,000), and monocytes (1,000,000). Basophils and pDC were not used in additional experiments due to limited sample quantity. Detailed FACS methods are provided in Supplementary Methods.

RNA isolation

RNA was isolated from all samples by the miRNeasy Kit (Qiagen) according to the manufacturer's protocol with the following modifications. For cellular samples, 700 μ L of Qiazol were added. For liquid samples (plasma and whole blood), samples were mixed with 5 sample volumes of Qiazol reagent. All plasma RNA preparations were made from 200 μ L in duplicate to control for variation in the RNA preparation step. All plasma was processed fresh on the same day of blood draw (never frozen). Samples in Qiazol were incubated at room temperature for 5 minutes to

inactivate RNases and stored at -80°C . After thawing the samples on ice, we added 5 μ L of synthetic *Caenorhabditis elegans* miRNA oligonucleotides prepared in Qiazol to each sample, vortexed, and then added 0.2 volumes of chloroform. At that point, the manufacturer's protocol was followed, with the entire aqueous phase from each sample loaded onto a single affinity column.

Quantitative reverse transcriptase PCR (qRT-PCR)

miRNA profiling. Flow-sorted blood cells and corresponding plasma and whole-blood samples were profiled for the relative abundance of 369 miRNAs by miRNA Ready-to-Use PCR, Human panel I, V1.M qRT-PCR arrays (Exiqon). A total of 15 experimental arrays were applied to profile blood cells, plasma, and whole blood (plasma, 2; RBC, 2; platelets, 2; neutrophils, 2; monocytes, 2; B lymphocytes, 1; T lymphocytes, 1; NK cells, 1; and whole blood, 1). An additional 12 control arrays were applied to profile serial 2-fold dilutions of 30 μ L of plasma from a single healthy donor to determine the analytic measurement ranges for each quantitative PCR assay on the Exiqon array as previously described (7). Detailed miRNA profiling methods and data analysis methods are provided in Supplementary Methods.

Individual TaqMan assays. Individual miRNAs were measured by qRT-PCR as previously described (8). TaqMan assays for human miRNAs hsa-miR-451, hsa-miR-16, hsa-miR-92a, hsa-miR-486-5p, hsa-let-7a, hsa-miR-223, hsa-miR-150, hsa-miR-574-3p, hsa-miR-197, and hsa-miR-122 and *C. elegans* miRNAs cel-miR-39, cel-miR-54, and cel-miR-238 were obtained from Applied Biosystems. Oligonucleotides corresponding to the mature sequence of each miRNA were synthesized (Integrated DNA Technologies) and diluted in water for standard curves. We carried out absolute quantitation for each miRNA both in blood cells (Supplementary Fig. S3) and in plasma.

Results

We used qRT-PCR to examine expression of 79 miRNAs reported as circulating solid tumor biomarkers in purified subpopulations of blood cells and in matched healthy donor plasma (9–13; see Supplementary Table S1 for comprehensive list of miRNA references; Supplementary Fig. S1). We found that 58% (46/79) of these literature-reported cancer biomarkers are highly expressed in one or more blood cell type. Most of the blood cell-expressed biomarkers (42 of 46, 91%) were also present at considerable basal levels in healthy donor plasma (>50th percentile among all miRNAs detected), suggesting that blood cells could be a major source for these plasma miRNAs (Fig. 1 and Supplementary Fig. S1). Comparing expression profiles of miRNAs detectable in plasma to miRNA profiles of blood cells showed that the distribution of miRNA expression values in plasma mirrors that of blood cells (Fig. 1).

If circulating miRNAs are derived from blood cells, we expected levels of these miRNAs to vary as a function of blood cell counts. To test this hypothesis, we collected a cohort of 42 plasma samples from a hospital clinical

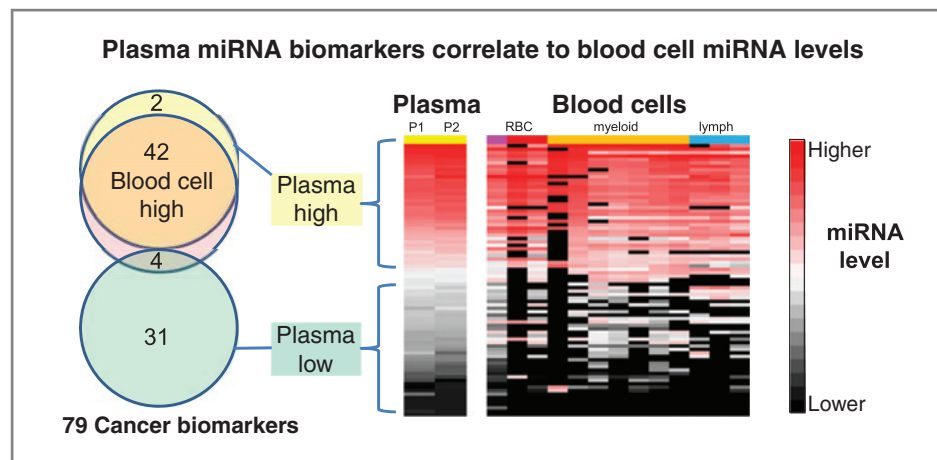


Figure 1. Relationship between blood cell and plasma miRNA expression among published circulating cancer biomarkers. Left, the Venn diagram depicts the distribution of 79 miRNAs published as biomarkers of non-hematopoietic cancers in healthy donor plasma and matched blood cells. "Plasma high" (yellow circle) refers to expression above the 50th percentile among 292 reliably detectable miRNAs by quantitative PCR; "Plasma low" (green circle) refers to miRNAs detected below the 50th percentile or not detected. "Blood cell high" (red circle) refers to miRNAs detected in the top 50th percentile in whole blood and at least one blood cell class as described in detail in the Supplementary Methods. Right, a heat map showing side-by-side comparisons of plasma and blood cell expression of the 79 reported biomarkers shows the close correlation between plasma and blood cell miRNA expression. P1 and P2 represent 2 independent plasma specimens drawn on different days. For blood cells, the columns represent multiple replicates and/or cell types as detailed in the methods. Circulating miRNA biomarkers are sorted in descending order of expression in healthy donor plasma and are not clustered.

hematology laboratory, in which a complete differential blood count (CBC with differential) was conducted prior to preparing plasma. The cohort consisted of consecutive residual patient plasma samples collected at a single academic medical center, including both inpatients and outpatients with a wide variety of underlying diseases (Supplementary Table S2). We selected 10 miRNAs to measure in plasma and correlate with blood cell counts. Eight of these were selected by virtue of being published circulating miRNA cancer biomarkers which we found in our blood cell miRNA profiling studies to be expressed in blood cells in a cell type-enriched manner (for RBC, miR-451, miR-92a, miR-16, and miR-486-5p; for myeloid blood cells, miR-223, let-7a, miR-197, and miR-574-3p; Fig. 2A). One additional miRNA, miR-150, was chosen that showed strong lymphoid cell-enriched expression (Fig. 2A) but has not yet been reported as a circulating cancer biomarker. As a negative control, we selected the liver-specific miRNA, miR-122, that is not expressed in blood cells (14; Fig. 2A).

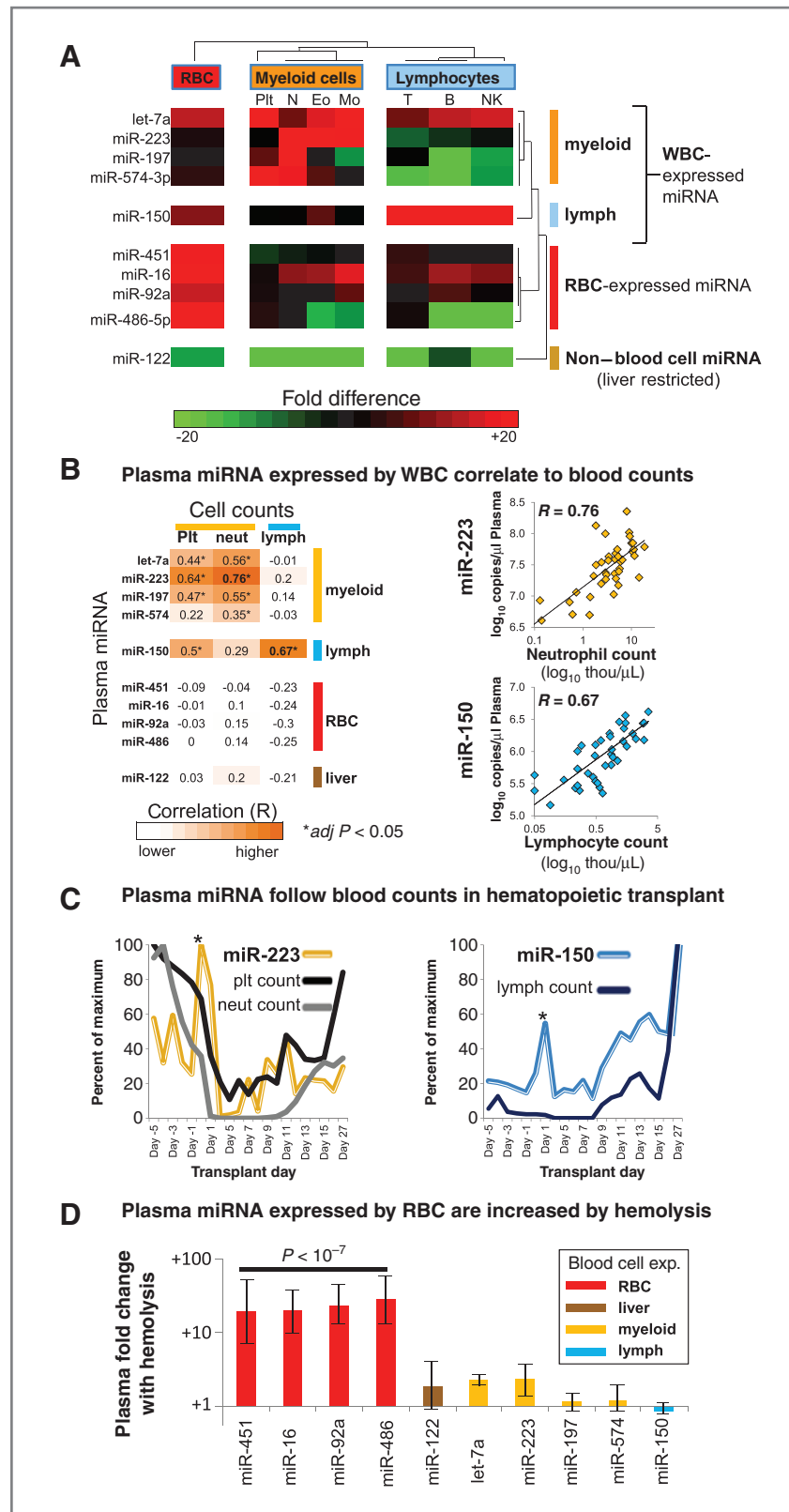
Plasma levels of the myeloid-expressed miRNAs let-7a, miR-223, miR-197, and miR-574-3p showed significant positive correlations with myeloid blood cell counts (neutrophils and platelets; Fig. 2B, left), with greater than 50-fold differences in plasma miRNA biomarker levels between patients with the highest and lowest overall cell counts (Fig. 2B, right). Also consistent with our hypothesis, levels of the lymphoid-enriched miRNA, miR-150, were most highly correlated with lymphocyte count (Fig. 2B). Importantly, plasma levels of a non-blood cell-expressed miRNA (miR-122) did not strongly correlate with blood cell counts, and RBC-expressed miRNAs we had selected did not show correlations with white blood cell or platelet counts (Fig. 2B, left). Furthermore, when these 10 miRNAs were sub-

jected to unsupervised hierarchical clustering based on their expression in plasma across the 42 patient samples, they clustered into separate groups corresponding to the 4 RBC-expressed miRNAs, the 5 white blood cell-expressed miRNAs, and the non-blood cell-expressed miRNA (Supplementary Fig. S2A), consistent with the notion that plasma expression patterns for many miRNAs are reflective of their blood cell origin.

To test whether there is a causal relationship underlying correlations observed between blood cell count and plasma miRNA biomarkers, we examined miR-223 (myeloid cell expressed) and miR-150 (lymphoid cell expressed) miRNAs in the plasma of a patient over a time course of myeloablative chemotherapy and hematopoietic stem cell transplant engraftment (Supplementary Table S3). We collected serial plasma samples and corresponding blood count data at 25 time points over a 32-day period (Fig. 2C). We found that plasma levels of the myeloid-enriched miRNA, miR-223, closely tracked with changes in myeloid blood counts (platelets and neutrophils), whereas plasma levels of the lymphoid-enriched miRNA, miR-150, mirrored lymphoid counts (Fig. 2C), showing that white blood cell counts can significantly influence plasma levels of blood cell-expressed miRNAs.

We could not assess correlations between RBC-expressed miRNAs and RBC counts because there was limited variation in RBC count among individuals in the cohort we analyzed. However, 3 of the 42 plasma specimens were noted to be hemolyzed. Comparing expression of the 10 miRNAs detailed earlier in hemolyzed versus non-hemolyzed specimens, we found that all 4 RBC-associated miRNAs were increased by 20- to 30-fold in hemolyzed plasma, including the published colon cancer plasma biomarker miR-92a (15, 16). In contrast,

Figure 2. Circulating miRNA biomarkers are influenced by blood cell counts and hemolysis. **A**, a heatmap depicts the relative expression in blood cells of the 10 selected plasma miRNAs, 8 of which are published circulating miRNA cancer biomarkers. Let-7a, miR-223, miR-197, and miR-574-3p had the highest expression in myeloid blood cells (Plt, platelets; N, neutrophils; Eo, eosinophils; and Mo, monocytes). miR-150 was most abundant in lymphocytes (T, T cells; and B, B cells), whereas miR-451, miR-16, miR-92a, and miR-486-5p were enriched in RBC. A liver-specific miRNA selected as a negative control (miR-122) was not appreciably expressed in any of the blood cells. Blood cell expression levels were determined by Exiqon v1 qRT-PCR arrays and confirmed in independent samples with TaqMan qRT-PCR assays as described in the Supplementary Methods. **B**, Left: results of plasma miRNA correlation to blood cell counts in 42 consecutive plasma samples from an academic hospital clinical laboratory are shown. Blood cell expression levels of the 10 miRNAs were inferred on the basis of qRT-PCR C_t values as described in the Supplementary Methods. Pearson correlation coefficients of plasma miRNA levels and blood cell counts in the 42 clinical samples are shown. Statistically significant correlations after correcting for multiple comparisons (i.e., adjusted *P* value < 0.05) are highlighted by black boxes. Right: data corresponding to correlations for miR-223 with neutrophil count and miR-150 with lymphocyte count are plotted. **C**, in a patient undergoing myeloablative chemotherapy and autologous hematopoietic stem cell transplant, plasma miR-223 tracked with changes in myeloid blood counts (neutrophils and platelets) and plasma miR-150 correlated with changes in lymphocyte counts. Notably, spikes in plasma miR-223 and miR-150 were observed following infusion of hematopoietic stem cells (asterisks). **D**, shown are the differences in mean expression of plasma miRNAs in hemolyzed specimens (*n* = 3) compared with nonhemolyzed specimens (*n* = 39). Error bars represent the standard error of the difference of means. The biomarkers that were most highly expressed in RBC (miR-451, miR-16, miR-92a, and miR-486-5p) were 20- to 30-fold higher in hemolyzed specimens, which was highly statistically significant (*P* < 10⁻⁷, 2-tailed *t* test).



none of the 6 non-RBC-associated miRNAs were significantly increased with hemolysis (Fig. 2D). We also found that plasma levels of RBC-expressed miRNAs were tightly intercorrelated even among non-hemolyzed specimens but not substantially correlated to 6 non-RBC-expressed miRNAs we examined (Supplementary Fig. S2B and S2C). The results suggest that RBC can contribute significant levels of reported cancer biomarkers into plasma and have important implications for biomarker interpretation. For example, a greater propensity for RBC hemolysis in patients with colon cancer (17) could explain the relatively modest (<5 fold) increase in plasma miR-92a that has been reported in patients with colon cancer (15, 16).

Discussion

Taken together, our results indicate that a majority of miRNAs reported in the literature as circulating cancer biomarkers may originate in large part from blood cells. This finding is supported by 2 recent studies in this area (18, 19). Importantly, our studies in the setting of myeloablative chemotherapy and hematopoietic stem cell transplant engraftment provide direct, *in vivo* evidence that blood cell abundance can influence circulating miRNA levels. Among the miRNA biomarkers we examined, we observed variation attributable to blood cell effects that was greater in magnitude than many of the differences reported between patients with cancer and controls (13, 15, 20). This raises the concern that many miRNAs reported as circulating cancer biomarkers reflect a secondary effect on blood cells rather than a tumor cell-specific origin. For example, elevated neutrophil counts are associated with shortened progression-free and overall survival in several cancers including non-small cell lung carcinoma (NSCLC) and breast cancer (21, 22). A recent study published in *PNAS* found that increased ratios of neutrophil-expressed plasma miRNAs (miR-197, miR-142-3p, miR-140-5p, miR-17, and miR-21) compared with RBC-expressed miRNAs (miR-92a, miR-486-5p, miR-16, and miR-451) were associated with poorer outcomes in patients with NSCLC (13). Although blood cell counts were not available in that report, our findings suggest the possibility that the reported plasma miRNA ratios primarily reflect differences in blood counts that could be more readily measured with a routine CBC. A similar concern may be raised for many other studies where circulating miRNAs that are expressed highly in blood cells have been reported as cancer biomarkers (10, 15, 16, 20, 23–31).

In contrast to blood cell-expressed miRNAs, plasma levels of liver-restricted miR-122 that we selected as a negative control were not significantly correlated to blood cell counts or influenced by red blood cell hemolysis. This suggests that organ-restricted miRNAs may escape the problem of blood cell interference.

Our blood cell findings should be interpreted in the context of other preanalytical and analytical sources of variation that may influence plasma miRNA levels. For example, differences in collection procedures and specimen

processing conditions have been shown to contribute to plasma miRNA variability (18, 19). Imprecision attributable to specific methods of RNA extraction, miRNA measurement, data acquisition, and data normalization is also likely to have meaningful impact on plasma miRNA biomarker assessments, especially when fixed biases exist.

This study does not directly assess the relative contribution of different blood cell types to plasma miRNA or examine mechanisms of miRNA release into plasma. Here, we provide a framework and rationale for future investigations into these questions. A more detailed understanding of the cellular origin of circulating miRNA will inform the appropriate use of this exciting new class of analyte as a cancer biomarker.

In conclusion, we show that blood cell counts can substantially influence plasma miRNA biomarker levels. For studies of circulating miRNA biomarkers that are expressed in blood cells, we propose that CBC data be collected and that miRNA expression levels be interpreted in light of blood cell counts. Acceptable ranges for blood cell counts might be established for specific miRNA biomarkers that are particularly vulnerable to blood cell effects. In the future, deeper quantitative understanding of the contributions of specific blood cell types to circulating miRNAs may enable correction for variation in blood cell number in some cases. That said, it is important to note that a significant minority of literature-reported solid tumor biomarkers were not highly expressed in blood cells. In clinical contexts where highly specific circulating miRNA biomarkers of cancer are sought, efforts may be most effective if focused on such miRNAs that are not blood cell expressed.

Disclosure of Potential Conflicts of Interest

M. Tewari has had employment as miRNA technology consultant/SAB service for CombiMatrix, Inc. and Wafergen Biosystems, Inc. during the last 3 years; has received an honorarium for invited miRNA lecture at a Genentech conference; and has ownership interests (pending patent applications related to circulating miRNA). No potential conflicts of interest were disclosed by the other authors. The content is solely the responsibility of the authors and does not necessarily represent the official views of the funders. The funders had no role in study design, data collection, analysis, or interpretation, in writing of the manuscript, nor in the decision to submit the report for publication.

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Cancer Prevention Research

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