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Journal

Journal of acquired immune deficiency syndromes (1999), 66(2)

ISSN

1525-4135

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Publication Date

2014-06-01

DOI

10.1097/qai.0000000000000146

Peer reviewed

Serum MicroRNAs in HIV-Infected Individuals as Pre-Diagnosis Biomarkers for AIDS-NHL

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Objective: To determine if changes in levels of serum microRNAs (miRNAs) were seen preceding the diagnosis of AIDS-related non-Hodgkin lymphoma (AIDS-NHL).

Design: Serum miRNA levels were compared in 3 subject groups from the Multicenter AIDS Cohort Study: HIV-negative men (n = 43), HIV-positive men who did not develop NHL (n = 45), and HIV-positive men before AIDS-NHL diagnosis (n = 62, median time before diagnosis, 8.8 months).

Methods: A total of 175 serum-enriched miRNAs were initially screened to identify differentially expressed miRNAs among these groups and the results validated by quantitative polymerase chain reaction. Receiver-operating characteristic analysis was then performed to assess biomarker utility.

Results: Higher levels of miR-21 and miR-122, and a lower level of miR-223, were able to discriminate HIV-infected from the HIV-

uninfected groups, suggesting that these miRNAs are biomarkers for HIV infection but are not AIDS-NHL specific. Among the HIV-infected groups, a higher level of miR-222 was able to discriminate diffuse large B-cell lymphoma (DLBCL) and primary central nervous system lymphoma (PCNSL) subjects from HIV-infected subjects who did not develop NHL, with area under the receiver-operating characteristic curve of 0.777 and 0.792, respectively. At miR-222 cutoff values of 0.105 for DLBCL and 0.109 for PCNSL, the sensitivity and specificity were 75% and 77%, and 80% and 82%, respectively.

Conclusions: Altered serum levels of miR-21, miR-122, and miR-223 are seen in HIV-infected individuals. Higher serum level of miR-222 has clear potential as a serum biomarker for earlier detection of DLBCL and PCNSL among HIV-infected individuals.

Key Words: HIV, lymphoma, miR-21, miR-223, miR-122, miR-222

(*J Acquir Immune Defic Syndr* 2014;66:229–237)

Received for publication March 11, 2014; accepted March 11, 2014.

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Supported by grants from the Margaret E. Early Trust (B-MEEMR 20113120) and the National Institutes of Health (R01-CA-168482, R01-CA-057152, NCI supplements to U01-AI-035040, and K07-CA-140360 to S.K.H.). The Multicenter AIDS Cohort Study includes the following: Baltimore—The Johns Hopkins University Bloomberg School of Public Health: Joseph B. Margolick (PI), Barbara Crain, Adrian Dobs, Homayoon Farzadegan, Joel Gallant, Lisette Johnson-Hill, Michael W. Plankey, Ned Sacktor, Ola Selnes, James Shepard, and Chloe Thio. Chicago—Feinberg School of Medicine, Northwestern University, and Cook County Bureau of Health Services: Steven M. Wolinsky (PI), John P. Phair, Sheila Badri, Maurice O'Gorman, David Ostrow, Frank Palella, and Ann Ragin. Los Angeles—University of California, University of California, Los Angeles, Schools of Public Health and Medicine: R.D. (PI), O.M.-M. (Co-PI), Aaron Aronow, Robert Bolan, Elizabeth Breen, Anthony Butch, Beth Jamieson, Eric N. Miller, John Oishi, Harry Vinters, Dorothy Wiley, Mallory Witt, Otto Yang, Stephen Young, and Zuo Feng Zhang. Pittsburgh—University of Pittsburgh, Graduate School of Public Health: Charles R. Rinaldo (PI), Lawrence A. Kingsley (Co-PI), James T. Becker, Ross D. Cranston, Jeremy J. Martinson, John W. Mellors, Anthony J. Silvestre, and Ronald D. Stall. Data Coordinating Center—The Johns Hopkins University Bloomberg School of Public Health: Lisa P. Jacobson (PI), Alvaro Munoz (Co-PI), Alison, Abraham, Keri Althoff, Christopher Cox, G.D., Priya Duggal, Elizabeth Golub, Janet Schollenberger, Eric C. Seaberg, Sol Su, and Pamela Surkan. NIH—National Institute of Allergy and Infectious Diseases: Robin E. Huebner; National Cancer Institute: Geraldina Dominguez. U01-AI-35042, U01-AI-35043, U01-AI-35039, U01-AI-35040, U01-AI-35041, and U01-RR025005 (NCATS, National Center for Advancing Translational Sciences). Website located at <http://www.statepi.jhsph.edu/mac/mac.html>. Specimens were provided by the AIDS and Cancer Specimen Resource, funded by the National Cancer Institute (UM1-CA-181255).

Presented at the 20th Conference on Retroviruses and Opportunistic Infections, March 2013, Atlanta, GA.

Conceived and designed the study: D.R.T. and O.M.-M.; performed the experiments: D.R.T. and W.-C.T.; analyzed data: D.R.T., S.K.H., and O.M.-M.; provided access to MACS specimens, contributed to discussion, and revision of manuscript: S.K.H., G.D., J.H.B., C.J.A., V.A., and R.D.; wrote the paper: D.R.T. and O.M.-M.

The authors have no conflicts of interest to disclose.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.jaids.com).

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INTRODUCTION

The risk for non-Hodgkin lymphoma (NHL) is increased among HIV-infected (HIV+) individuals.^{1,2} AIDS-related NHL (AIDS-NHL) is typically of B-cell origin and include primary central nervous system lymphoma (PCNSL) and systemic lymphomas, including Burkitt lymphoma (BL) and diffuse large B-cell lymphoma (DLBCL).³ Despite a decrease in the incidence of NHL in the era of combination antiretroviral therapy (cART),^{4–6} HIV+ individuals continue to be at increased risk for NHL.^{7,8} In fact, in cART-treated populations, NHL is the most frequent AIDS-defining cancer,⁹ with PCNSL and systemic NHL accounting for 10% and 29% of AIDS-related causes of death, respectively.¹⁰

In recent years, microRNAs (miRNAs) have been shown to have critical roles in lymphoma pathogenesis and may serve as novel biomarkers for diagnosis or prognosis.¹¹ miRNAs are small noncoding RNAs that bind to the 3' untranslated region of messenger RNAs causing translational inhibition.¹² Numerous studies have identified the miRNA signatures of various B-cell cancers^{13–18} and have been used to distinguish BL from chronic lymphocytic leukemia (CLL)^{19,20} or DLBCL,¹⁵ DLBCL from PCNSL²¹ or follicular lymphoma,^{22–24} or between DLBCL subsets.^{25–28} Tumor-associated miRNAs have also been reported in the serum of DLBCL^{29,30} and CLL patients,³¹ cerebrospinal fluid of PCNSL patients,³² and in blood B cells of patients who later develop AIDS-NHL.³³ In CLL, several miRNAs have been identified for risk stratification,^{34–36} associated with disease progression,³⁷ and treatment-free and overall survival.^{37,38} Furthermore, in DLBCL patients undergoing immunochemotherapy, several miRNAs, including miR-21,^{25,29} miR-222,^{27,39} miR-18a, miR-181a,³⁹ an 8-miRNA signature,²² and a 9-miRNA signature,⁴⁰ have been shown to predict survival. However, studies on miRNAs as biomarkers for early detection of NHL are lacking.

The availability of banked specimens from the Multicenter AIDS Cohort Study (MACS) has allowed us to assess pre-NHL diagnosis protein biomarkers.^{41–45} Recently, we reported higher levels of miR-21 in circulating B cells up to 3 years before AIDS-NHL diagnosis.³³ However, serum miRNAs as early biomarkers for AIDS-NHL have not been explored, although a recent study by Witwer et al,⁴⁶ using a macaque/simian immunodeficiency virus (SIV) model, showed differential levels of plasma miRNAs during acute infection and a 6-miRNA signature that predicted central nervous system (CNS) disease. We hypothesized that serum miRNAs are dysregulated in HIV infection and that miRNAs are biomarkers for NHL. We screened the expression of 175 serum-enriched miRNAs in 3 subject groups from the MACS, HIV-negative men (HIV–), HIV-positive men who did not develop NHL (HIV+), and HIV-positive men who later developed NHL (pre-AIDS-NHL), to identify differentially expressed miRNAs, followed by verification using quantitative polymerase chain reaction (qPCR) assay.

METHODS

Study Population, Tissues, and Ethics Statement

Serum samples from HIV-negative men (HIV–, *n* = 43), HIV+ men who did not develop NHL (HIV+,

n = 45), and HIV+ men who later developed NHL (pre-AIDS-NHL, *n* = 62) were obtained from the MACS repository. For pre-AIDS-NHL cases, the median time before NHL diagnosis at the time of serum collection was 8.8 months, ranging from 2.5 to 21.5 months. The pre-AIDS-NHL group included 3 NHL subtypes: BL (*n* = 10), DLBCL (*n* = 32), and PCNSL (*n* = 20). All samples, except 2, were obtained from the pre-cART use era. The MACS is a prospective cohort study of gay/bisexual men (www.statepi.jhsph.edu/macs).⁴⁷ Pertinent data, peripheral blood mononuclear cells (PBMC), and serum/plasma are collected at each semi-annual visit. Information on hepatitis B virus (HBV) and hepatitis C virus (HCV) coinfection and Epstein–Barr virus (EBV) tumor infection status for many of the NHL cases were provided by the MACS. The MACS protocols have been approved by the institutional review board at each MACS center. Anonymized tonsils were obtained from the University of California, Los Angeles, Translational Pathology Core Laboratory. AIDS-related tumors were provided by the AIDS and Cancer Specimen Resource, funded by the National Cancer Institute.

RNA Extraction

Serum RNA was extracted using TRIzol LS reagent from Life Technologies (Carlsbad, CA). Each 250 μ L of serum was extracted with 750 μ L of TRIzol LS (this ratio was always maintained) as per the manufacturer's protocol. Forty micrograms of glycogen (Life Technologies) was added to the aqueous phase to aid in nucleic acid precipitation. RNA was resuspended in 25 μ L of water and quantified using Quant-iT RiboGreen RNA Reagent and kit (Molecular Probes, Eugene, OR). The average yield ($\pm$ SD) of total RNA was 102 ± 27 ng/mL of serum. For tonsillar B cells, tonsils were first minced and the mononuclear cells were separated using Ficoll-paque (GE Healthcare, Uppsala, Sweden). B cells were separated from the mononuclear population using CD19⁺ magnetic beads (Life Technologies). B cells from PBMC obtained from the MACS were also separated in a similar manner. Total RNAs from these B cells and tumor specimens were extracted using the mirVana miRNA isolation kit (Ambion, Austin, TX).

miRNA Screening to Identify Differentially Expressed miRNAs

Exiqon's Serum/Plasma Focus miRNA PCR panel (Exiqon, Vedbaek, Denmark) was used to identify differentially expressed serum miRNAs among HIV– (*n* = 8), HIV+ (*n* = 7), and pre-AIDS-NHL (*n* = 7) subject groups. This panel consists of two 96-well PCR plates containing a total of 175 predefined serum/plasma-enriched miRNAs with additional 7 miRNAs (miR-451, miR-16, miR-103, miR-425, miR-423-5p, miR-93, and miR-191) as potential references. First, complementary DNA (cDNA) synthesis was performed using miRCURY LNA Universal reverse transcription (RT) miRNA PCR kit (Exiqon). Each reaction consisted of 4 μ L of 5 $\times$ reaction buffer, 2 μ L of enzyme mix, 1 μ L synthetic spike, 4 ng total RNA, and water to a final volume of 20 μ L. This

mixture was incubated for 1 hour at 42° C and 5 minutes at 95° C and stored at –20° C. For real-time PCR amplification, the cDNA was diluted 50× with water and then mixed 1:1 with 2× SYBR Green master mix (Exiqon). A 10-μL aliquot of this mixture was dispensed to each well of 96-well PCR panels. Real-time PCR was performed using ABI 7300 real-time machine (Applied Biosystems, Foster City, CA) using the following protocol: 95° C for 1 minute and 40 cycles of 95° C for 10 seconds and 60° C for 1 minute. RNA spike-in control (UniSp6 CP) added during the RT step was used to monitor optimal cDNA synthesis across samples. Readings between plates were normalized using interplate calibrators (UniSp3). Spurious amplifications were monitored using the no-template control wells. Threshold cycles (Ct) were obtained, and levels of all miRNAs were normalized to miR-16 using $dCt = Ct_{miRNA} - Ct_{miR-16}$. Differentially expressed miRNAs were identified using significance analysis of microarray feature of the MultiExperiment Viewer software v4.8 (<http://mev.tm4.org>)⁴⁸ with false discovery rate set to 0%.

Quantitative Real-Time PCR

Individual miRNAs were quantified using TaqMan miRNA Reverse Transcription kit and TaqMan miRNA Assay kit (Applied Biosystems). Briefly, total RNA was reverse transcribed in a reaction mix containing 1.5 μL of 10× RT buffer, 0.19 μL of RNase inhibitor, 0.15 μL of deoxynucleotide triphosphate (dNTP) mix, 5 μL of 3× primer mix (of 11 miRNAs), 5 μL RNA (equivalent RNA in ~50 μL serum volume), 1.0 μL of Multiscribe RT enzyme, and H₂O to a final volume of 15 μL and subjected to the following thermal protocol: 30 minutes at 16° C, 30 minutes at 42° C, and hold at 4° C. The RT product was then diluted to 225 μL. Each qPCR reaction consisted of 10 μL of Taqman 2× Universal PCR Master Mix (No AmpErase UNG), 1 μL of 20× miRNA-specific assay primers/probe mixture, 5 μL of the RT product, and H₂O to a final volume of 20 μL. qPCR was performed using ABI 7300 qPCR machine with the following protocol: 10 minutes at 95° C followed by 40 cycles of 15 seconds at 95° C (denature) and 60 seconds at 60° C (anneal/extend). Each sample was assayed in triplicate. The levels of all serum miRNAs were normalized to miR-16 (to be consistent with the screening protocol), whereas cellular miRNAs were normalized to RNU 48 (small nucleolar RNA), using the following expression: $dCt = Ct_{miRNA} - Ct_{miR-16}$ (or RNU 48). The relative expression of miRNAs was calculated using 2^{-dCt} .

Statistical Analyses

Groupwise miRNA levels were compared using Kruskal–Wallis test with multiple comparison test as indicated. Receiver-operating characteristic (ROC) curves were generated to assess the diagnostic value of serum miRNAs to discriminate among HIV–, HIV+, and pre-AIDS-NHL groups. *P* value <0.05 was considered statistically significant. All analyses were performed using GraphPad Prism 5.0 software (GraphPad, La Jolla, CA).

RESULTS

Subject Characteristics

Characteristics of the subject population are described in Table 1. Although there was no significant difference in the median age of subjects in the HIV–, HIV+, and pre-AIDS-NHL groups (43.4, 36.4, and 40.2 years, respectively) (*P* = 0.064), the HIV– group was about 5 years older than the HIV+ group. As expected, there were lower numbers of CD4+ cells in the HIV+ group compared with the HIV– group (424 vs 995 cells/μL) and of the pre-AIDS-NHL group compared with the HIV+ group (160 vs 424 cells/μL) (*P* < 0.001, both comparisons). There was no significant difference in the duration of HIV positivity between the HIV+ and pre-AIDS-NHL groups (5.2 vs 5.5 years) (*P* = 0.672).

Screening for Differentially Expressed Serum miRNAs

Initial screening for differentially expressed serum miRNAs was performed using the Exiqon Serum Focus miRNA PCR panel, using a subset of samples from the 3 subject groups (see Methods). The raw Ct values obtained for all 175 miRNAs are attached in the **Supplemental Digital Content**, <http://links.lww.com/QAI/A511>. The average Ct (±SD) for the highest expressing miR-451 was 25.03 ± 1.99 and the lowest expressing miR-182 was 37.65 ± 1.20. miR-16 level was 28.13 ± 1.94 and was used as our reference. Heatmap of 10 miRNAs identified by significance analysis of microarray (at false discovery rate of 0%) as being differentially expressed is shown in Figure 1.

Validation of Screening Results by qPCR

Validation of screening results was performed by qPCR quantification of miRNAs from our entire sample set, which included the HIV– group (*n* = 43), HIV+ group (*n* = 45), and the pre-AIDS-NHL group (*n* = 62), which consisted of lymphoma subtypes BL (*n* = 10), DLBCL (*n* = 32), and PCNSL (*n* = 20). Out of the 10 miRNAs identified in our screening, 4 miRNAs (miR-22, miR-99a, miR-22*, and miR-29a) were detected at low levels and 2 miRNAs (miR-29c and miR-130a) showed no significant differences in the larger group. To increase confidence in our findings, these miRNAs were excluded from further analysis. The differential expression of the remaining miRNAs (miR-21, miR-223, miR-122, and miR-222) was validated by qPCR (Figs. 2A–D).

Compared with the HIV– group, HIV+ group had significantly higher median levels of miR-21 (0.051 vs 0.105, *P* < 0.001) and miR-122 (0.012 vs 0.034, *P* < 0.01) and a lower level of miR-223 (1.131 vs 0.501, *P* < 0.001) (Figs. 2A–C). Likewise, compared with the HIV– group, there was a higher level of miR-21 seen in the BL, DLBCL, and PCNSL subgroups (0.051 vs 0.151, 0.160, and 0.146, respectively; *P* < 0.01 for all), higher miR-122 levels in DLBCL and PCNSL subgroups (0.012 vs 0.029 and 0.043, respectively; *P* < 0.001 for both), higher miR-222 levels in the DLBCL and PCNSL subgroups (0.046 vs 0.127 and 0.150, respectively; *P* < 0.001 for both), and a lower level of miR-223 in the DLBCL and

TABLE 1. Select Characteristics of HIV-Negative (HIV−), HIV-Positive Without NHL (HIV+), and HIV-Positive With Subsequent NHL Diagnosis (Pre-AIDS-NHL) Subject Groups

	N	Age in Years	CD4 Count	HIV Positivity Duration in Years	Months Preceding NHL Diagnosis
		Median (Range)	Average ± SD	Average ± SD	Median (Range)
HIV− (HIV negative)	43	43 (24–70)	995 ± 314	NA	NA
HIV+ (HIV positive without NHL)	45	36 (27–58)	424 ± 308	5.2 ± 3.0	NA
HIV positive with subsequent NHL					
BL	10	39 (25–44)	316 ± 205	4.4 ± 2.4	7.2 (3.1–20.6)
DLBCL	32	41 (28–58)	160 ± 139	5.9 ± 3.5	8.4 (2.5–21.5)
PCNSL	20	40 (24–55)	82 ± 153	5.5 ± 3.5	10.3 (3.2–19.3)
Pre-AIDS-NHL	62	40 (24–58)	160 ± 172	5.5 ± 3.3	8.8 (2.5–21.5)

All subjects are gay/bisexual men from the MACS.
NA, not applicable.

PCNSL subgroups (1.131 vs 0.634 and 0.712, respectively; $P < 0.05$ for both) (Figs. 2A–D). Only miR-222 levels were seen to be significantly different between the HIV+ and pre-AIDS-NHL groups. Compared with the HIV+ group, miR-222 expression was higher in the DLBCL and PCNSL subgroups (0.063 vs 0.127 and 0.150, respectively; $P < 0.001$ for both) (Fig. 2D). Because CD4 counts varied between the subject groups, we assessed whether levels of miR-222 were correlated with CD4 counts. We found no such correlation within the HIV+ group, which had a wide range of CD4 levels ($r = -0.133$, $P = 0.389$) (Fig. 2E).

Serum miRNAs as Potential Biomarkers for HIV Infection and AIDS-NHL Diagnosis

The ability of miRNAs (miR-21, miR-223, and miR-122) to discriminate between HIV− and HIV+ groups was demonstrated with ROC plot analysis yielding area under curves (AUC) of 0.773 [95% confidence interval (CI):

0.673 to 0.873], 0.804 (95% CI: 0.712 to 0.897), and 0.726 (95% CI: 0.662 to 0.830), respectively ($P < 0.0001$, for all) (Fig. 3A). These same miRNAs were also able to discriminate between HIV− and pre-AIDS-NHL groups with an AUC of 0.876 (95% CI: 0.804 to 0.948) for miR-21, 0.740 (95% CI: 0.643 to 0.836) for miR-223, and 0.777 (95% CI: 0.686 to 0.868) for miR-122 ($P < 0.001$, for all) (Fig. 3B). miR-222 could not distinguish HIV− from HIV+ group [AUC: 0.588 (95% CI: 0.468 to 0.707), $P = 0.154$] but could discriminate HIV− from pre-AIDS-NHL group [AUC: 0.848 (95% CI: 0.773 to 0.922), $P < 0.0001$] (Figs. 3A, B). A separate ROC analysis of miR-222 showed that it was able to discriminate HIV+ group from the DLBCL [AUC: 0.777 (95% CI: 0.670 to 0.884)] and PCNSL groups [AUC: 0.792 (95% CI: 0.663 to 0.920)] ($P < 0.001$ for both), but not the BL group [AUC: 0.624 (95% CI: 0.469 to 0.779), $P = 0.221$] (Fig. 3C). At cutoff values of 0.105 for DLBCL and 0.109 for PCNSL, miR-222 levels were predictive of NHL diagnosis in HIV+ subjects with a sensitivity and specificity of 75% and 77%, and 80% and 82%, respectively. Overall, a classification tree based on cutoff values of miR-21, miR-223, and miR-122 correctly distinguished all HIV-infected subjects, but one, from HIV-uninfected subjects (99%), whereas higher levels of miR-222 identified up to 85% of PCNSL and 78.1% of DLBCL from among the HIV-infected subjects (Fig. 3D).

miR-222 Levels Are Elevated in AIDS-DLBCL and AIDS-PCNSL Tumors

We explored possible cellular sources of serum miR-222 that may contribute to the higher levels seen preceding AIDS-NHL diagnosis. First, we examined the levels of miR-222 in circulating blood cells, finding that levels of miR-222 were not significantly different in the B-cell or the non-B-cell fraction (B-cell-depleted PBMC) among HIV−, HIV+, or pre-AIDS-NHL subjects, suggesting a non-blood-cell source for miR-222 (Fig. 4). Consequently, we examined miR-222 levels in various primary AIDS-NHL tumors and tonsillar B cells. Compared with the overall level in blood cells or tonsillar B cells, miR-222 expression was significantly higher in DLBCL ($P < 0.01$) and PCNSL tumors ($P < 0.001$) but not in BL tumors ($P = 0.121$) (Fig. 4). This trend is in agreement with our results on serum miR-222 levels among the NHL subtypes (Fig. 2D).

FIGURE 1. Differentially expressed miRNAs identified during screening. Heatmap of miRNAs identified as differentially expressed among HIV−, HIV+, and pre-AIDS-NHL groups by significance analysis of microarray test (false discovery rate = 0%). Relative expression levels are depicted in red (high) and green (low).

FIGURE 2. Validation of serum miRNA levels by qPCR. Levels of (A) miR-21, (B) miR-223, (C) miR-122, and (D) miR-222 were assessed by qPCR in HIV-, HIV+, and pre-AIDS-NHL subjects. Comparisons that yielded significant differences are identified by asterisk(s) (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). miRNA levels were normalized to levels of miR-16. Horizontal lines represent median values. P values were from Kruskal–Wallis test with Dunn multiple comparisons. E, Spearman correlation analysis of miR-222 levels with CD4⁺ counts in the HIV+ group.

Association of Serum miRNA Levels and HBV, HCV, and EBV Infection Status

We also looked at the association of miR-21, miR-223, miR-122, and miR-222 levels with the HBV and HCV status of our study subjects and with the EBV status of the NHLs. Among pre-NHL subjects, no significant association of these miRNAs with EBV status was seen (Fig. S1). Among all HIV-infected subjects, higher miR-122 and miR-21 were associated with HBV infection (Fig. S2), whereas none of these miRNAs were associated with HCV status (Fig. S3). Overall, these results suggest serum miR-222 to be a predictive biomarker for NHL subsets irrespective of tumor EBV status, whereas HBV infection is associated with elevated serum levels of miR-21 and the liver-enriched miR-122.

DISCUSSION

Circulating miRNAs have shown promise as cancer biomarkers.^{49,50} However, studies exploring serum miRNAs as precancer diagnosis biomarkers in high-risk populations

are lacking. In this study, we demonstrate that levels of serum miRNAs are altered in HIV-infected individuals. Three miRNAs (miR-21, miR-223, and miR-122) were able to discriminate HIV+ from uninfected subjects. However, these miRNAs were not able to discriminate HIV+ subjects who did not develop NHL from those who did, suggesting that HIV-associated changes, rather than nascent NHL tumors, contributed to their dysregulation. We, however, found that levels of miR-222 were able to discriminate HIV-infected subjects who did not develop NHL (HIV+ group) from those who later developed DLBCL or PCNSL, with a sensitivity and specificity of 75% and 77%, and 80% and 82%, respectively. We further showed that miR-222 was overexpressed in primary DLBCL or PCNSL tumors.

Several questions remain regarding the significance of miRNAs in blood. An altered PBMC miRNA profile has been observed in HIV infection,^{51,52} CD4⁺ T cells,⁵³ Jurkat cells,⁵⁴ HeLa cell lines,⁵⁵ PBMC of elite suppressors compared with viremic patients,⁵⁶ and CD4⁺ T cells of elite long-term nonprogressors compared with multiply exposed uninfected patients.⁵³ Studies have also implicated miRNAs in host defense against

FIGURE 3. ROC curve analysis. A, HIV- group vs HIV+ group. The AUC (95% CI) was 0.773 (0.673 to 0.873) for miR-21, 0.804 (0.712 to 0.897) for miR-223, 0.726 (0.662 to 0.830) for miR-122, and 0.588 (0.468 to 0.707) for miR-222. $P < 0.001$ for all except miR-222 ($P = 0.154$). B, HIV- group vs pre-AIDS-NHL group. AUC (95% CI) was 0.876 (0.804 to 0.948) for miR-21, 0.740 (0.643 to 0.836) for miR-223, 0.777 (0.686 to 0.868) for miR-122, and 0.848 (0.773 to 0.922) for miR-222. $P < 0.001$ for all. C, HIV+ group vs pre-AIDS-NHL subgroups. AUC (95% CI) was 0.777 (0.670 to 0.884) for DLBCL, 0.792 (0.663 to 0.920) for PCNSL, and 0.624 (0.469 to 0.779) for BL. $P < 0.001$ for DLBCL and PCNSL, $P = 0.221$ for BL. D, miRNA-based classification of the 150 serum samples. Cutoff values were 0.094 (miR-21), 0.920 (miR-223), 0.024 (miR-122), and 0.091 (miR-222) and were determined based on the average of the median (relative expression levels) of the groups being compared. Numbers in parentheses denote percentages.

HIV, such as in the susceptibility of monocytes/macrophages to infection,⁵⁷ maintaining latency in infected T cells,⁵⁸ and suppression of viral replication.^{54,59} In an SIV model, a 45-miRNA plasma signature (including higher miR-21 and miR-222 and lower miR-223, as was seen by us) was associated with acute infection.⁴⁶ In summary, the complex interaction of host and virus involves miRNAs and together might explain dysregulation of serum miRNAs in HIV-infected individuals.

The lower level of serum miR-223 that we see in HIV-infected subject groups is likely the result of lymphocyte activation in the milieu of chronic immune stimulatory environment. miR-223 is expressed at lower levels in activated T cells,⁵⁸ activated germinal center B cells,^{20,27,60} and in vitro interleukin 4/CD40-stimulated B cells (Dharma R. Thapa and Otoniel Martinez-Maza, 2009, unpublished data). Lower serum miR-223 (and miR-146a) has also been observed in patients with systemic inflammatory response syndrome, with a further

significant decrease seen in patients with sepsis.⁶¹ Also given that variations within the blood-cell subpopulations can alter serum miRNA levels,⁶² lower serum miR-223 level may also be associated with age or with the decline in the number of miR-223-rich cells, such as neutrophils^{63,64} or T lymphocytes seen during HIV infection, although we did not find a significant correlation with CD4⁺ T-cell subsets (data not shown).

miR-122 is a liver-specific miRNA⁶⁵ and is not associated with normal B-cell biology or tumorigenesis. Several HIV-related factors, such as coinfection with HCV or HBV, liver toxicity from cART, and alcohol abuse, among others,⁶⁶ have made liver disease the most common non-AIDS-related cause of death among HIV-infected persons.⁶⁷ Numerous studies have implicated miR-122 in liver diseases, such as in hepatocellular carcinoma,^{68–70} chronic hepatitis,⁶⁸ HCV-induced fibrosis,⁷¹ or drug-induced liver injury.⁷²

FIGURE 4. miR-222 is overexpressed in DLBCL and PCNSL tumors. Relative level of miR-222 was assessed in cells from blood (B-cell and non-B-cell fraction from PBMC from the 3 subject groups) and tissues (tonsils and primary AIDS-related BL, DLBCL, and PCNSL tumors). miR-222 expression was normalized to small nucleolar RNU 48. Significant differences by Dunnett multiple comparison (to blood cells) are identified by asterisk(s) (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

The potential association of these 4 serum miRNAs with HBV or HCV status, or with tumor EBV status, was examined. No significant association of these miRNAs with EBV status was seen (Fig. S1). Among HIV+ subjects, higher miR-122 and miR-21 were associated with HBV infection (Fig. S2), whereas none of these miRNAs were associated with HCV infection (Fig. S3). These results suggest that serum miR-222 is a predictive biomarker for AIDS-NHL, irrespective of EBV status. The association between chronic HBV infection and elevated miR-21 and miR-122 levels suggests that liver infection may result in elevated serum levels of these miRNAs.

The miRNAs identified in our study have been reported as being useful biomarkers for B-cell malignancies. Higher miR-21 levels in PBMC correlated with lower overall survival in CLL, higher levels in cerebrospinal fluid was diagnostic of PCNSL,³² higher serum levels correlated with longer relapse-free survival in DLBCL patients,^{25,29} and higher plasma levels were predictive of CNS disease in an SIV model.⁴⁶ Here, we saw an increased serum miR-21 in both the HIV+ and pre-AIDS-NHL groups compared with the HIV- group, although the levels were not higher in the pre-AIDS-NHL group compared with the HIV+ group. Thus, given the increased levels in HIV+ individuals, serum miR-21 may not be a biomarker for early detection of AIDS-NHL, although miR-21 levels obtained at diagnosis might have prognostic value. The design of our study is in contrast to that of previous studies, where serum miRNA levels were measured at or post-DLBCL diagnosis and compared with healthy controls.^{29,30} Additionally, miR-21 is overexpressed in a wide variety of other cancer types (both in tissues and serum),^{73,74} making it a diagnostic biomarker with

good sensitivity but poor specificity. This feature makes miR-21 undesirable for use as an early diagnostic marker for NHL in HIV-infected individuals, as they are at increased risk not only for NHL but also for other non-AIDS-defining cancers.^{10,75}

Only miR-222 was able to discriminate between HIV-infected subjects who did or did not develop NHL, suggesting a role for this miRNA in B-cell malignancies. In support of this, several studies in the non-HIV setting have implicated B-cell tumor-expressed miR-222 as a prognostic marker. For example, in DLBCL patients undergoing immunochemotherapy, higher miR-222 level correlated with poorer overall survival^{27,40} or progression-free survival,^{27,39,40} either as a sole biomarker²⁷ or in combination with other miRNAs.^{39,40} In CLL, higher miR-222 levels were seen in plasma,³¹ with higher cellular miR-222 levels associated with disease progression³⁶ and resistance to fludarabine treatment.⁷⁶

It is notable that we found higher levels of miR-222 in pre-diagnosis DLBCL and PCNSL, but not BL serum or tumor tissues. PCNSLs are DLBCLs with immunoblastic features presenting in the CNS.³ Despite the molecular heterogeneity of DLBCLs, it retains an miRNA signature distinct from BL,^{13,15} including higher miR-222 level in DLBCL compared with BL.¹⁵ Additionally, we found no difference in the level of miR-222 in circulating cells, either in the B-cell or in the non-B-cell compartment, among the 3 subject groups. Given these findings and the relative proximity to the time of diagnosis that our samples were obtained (median of 8.8 months pre-diagnosis), it is probable that the cellular source for serum miR-222 preceding NHL diagnosis is pre-malignant cells and/or as yet undiagnosed DLBCL.

In conclusion, serum miRNAs are promising biomarkers for early detection of AIDS-NHL. Future expanded studies, including work with other cohorts, in conjunction with global miRNA screening, will be needed for the identification and verification of circulating miRNAs as bona fide biomarkers for NHL.

ACKNOWLEDGMENTS

The authors would like to thank Larry Magpantay, Jose Merino, and Lupita Pena from the Martinez-Maza laboratory for their support; Christopher Coley and Janet Schollenberger for providing the relevant laboratory data on the MACS subjects; and all the MACS participants whose extraordinary commitment and dedication over the years have made studies like this possible.

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