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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-122, miR-222

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 cancers13-18 and have been used to distinguish BL from chronic lymphocytic leukemia (CLL),19-21 or DLBCL,12 23-25 DLBCL from PCNSL26 or follicular lymphoma,27-29 or between DLBCL subsets.30-33 Tumor-associated miRNAs have also been reported in the serum of DLBCL34,35 and CLL patients,36 cerebrospinal fluid of PCNSL patients,37 and in blood B cells of patients who later develop AIDS-NHL.38 In CLL, several miRNAs have been identified for risk stratification,39-43 associated with disease progression,44,45 and treatment-free and overall survival.46,47 Furthermore, in DLBCL patients undergoing immunochemotherapy, several miRNAs, including miR-21,25,26 miR-222,27,39 miR-18a, miR-181a,48 an 8-miRNA signature,49 and a 9-miRNA signature,50 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.43 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 with addi-

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). Pertinent data, peripheral blood mononuclear cells (PBMC), and serum/plasma are collected at each semiannual 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 μL of serum was extracted with 750 μL 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 μL of water and quantified using Quant-iT RiboGreen RNA Reagent and kit (Molecular Probes, Eugene, OR). The average yield (±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 miCURY LNA Universal reverse transcription (RT) miRNA PCR kit (Exiqon). Each reaction consisted of 4 μL of 5× reaction buffer, 2 μL of enzyme mix, 1 μL synthetic spike, 4 ng total RNA, and water to a final volume of 20 μL. 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) 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).
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 discrim-
inate 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).
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. 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, CD4+ T cells, Jurkat cells, HeLa cell lines, PBMC of elite suppressors compared with viremic patients, and CD4+ T cells of elite long-term nonprogressors compared with multiply exposed uninfected patients. Studies have also implicated miRNAs in host defense against...
HIV, such as in the susceptibility of monocytes/macrophages to infection,\textsuperscript{57} maintaining latency in infected T cells,\textsuperscript{58} and suppression of viral replication.\textsuperscript{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.\textsuperscript{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,\textsuperscript{58} activated germinal center B cells,\textsuperscript{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.\textsuperscript{61}

Also given that variations within the blood-cell subpopulations can alter serum miRNA levels,\textsuperscript{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\textsuperscript{63,64} or T lymphocytes seen during HIV infection, although we did not find as significant correlation with CD4\textsuperscript{+} T-cell subsets (data not shown).

miR-122 is a liver-specific miRNA\textsuperscript{65} 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,\textsuperscript{66} have made liver disease the most common non–AIDS-related cause of death among HIV-infected persons.\textsuperscript{67}

Numerous studies have implicated miR-122 in liver diseases, such as in hepatocellular carcinoma,\textsuperscript{68–70} chronic hepatitis,\textsuperscript{68} HCV-induced fibrosis,\textsuperscript{71} or drug-induced liver injury.\textsuperscript{72}
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, higher serum levels correlated with longer relapse-free survival in DLBCL patients, and higher plasma levels were predictive of CNS disease in an SIV model. 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. Additionally, miR-21 is overexpressed in a wide variety of other cancer types (both in tissues and serum, 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.

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 immunotherapy, higher miR-222 level correlated with poorer overall survival or progression-free survival either as a sole biomarker or in combination with other miRNAs. In CLL, higher miR-222 levels were seen in plasma, with higher cellular miR-222 levels associated with disease progression and resistance to fludarabine treatment.

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. Despite the molecular heterogeneity of DLBCLs, it retains an miRNA signature distinct from BL, including higher miR-222 level in DLBCL compared with BL. 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.

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