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B-cell activation induced microRNA-21 is elevated in circulating B cells preceding the diagnosis of AIDS-related non-Hodgkin lymphomas

Dharma R. Thapa^{a,b}, Kishor Bhatia^d, Jay H. Bream^e, Gypsyamber D'souza^f, Charles R. Rinaldo^g, Steven Wolinsky^h, Roger Detels^c, and Otoniel Martinez-Maza^{a,b,c}

^aDepartment of Obstetrics & Gynecology David Geffen School of Medicine at UCLA

^bDepartment of Microbiology, Immunology & Molecular Genetics, David Geffen School of Medicine at UCLA

^cDepartment of Epidemiology, UCLA School of Public Health, UCLA AIDS Institute and Jonson Comprehensive Cancer Center, University of California Los Angeles, Los Angeles, CA

^dDepartment of National Cancer Institute, Bethesda, MD

^eDepartment of Molecular Microbiology and Immunology Johns Hopkins Bloomberg School of Public Health, Baltimore, MD

^fDepartment of Epidemiology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD

^gDepartment of Infectious Diseases and Microbiology, University of Pittsburgh Graduate School of Public Health, Pittsburgh, PA

^hDivision of Infectious Diseases, The Feinberg School of Medicine, Northwestern University, Chicago, IL.

Abstract

We show that microRNA-21 is significantly elevated in peripheral B cells of HIV infected individuals who go on to develop AIDS-NHL (n=13, < 3 yrs prior to diagnosis) when compared to HIV negative (n = 18) or HIV positive controls (n = 21) (p < 0.01). Moreover, miR-21 is overexpressed in activated B cells, and can be induced by IL4 alone, or with CD40 or IgM costimulation, and lipopolysaccharides (LPS), suggesting that miR-21 may help maintain B-cell hyperactivation, contributing to lymphomagenesis.

Most AIDS related non-Hodgkin lymphomas (AIDS-NHL) are of B cell origin, and include Burkitt's lymphoma (BL), diffuse large B cell lymphoma (DLBCL), primary central nervous system lymphoma (PCNSL), primary effusion lymphoma (PEL), and plasmablastic lymphomas [1]. The availability of specimens from the Multicenter AIDS Cohort Study (MACS), a large prospective longitudinal observational cohort study, allowed us to assess pre-diagnostic specimens for biomarkers that precede the development of such NHL. microRNAs (miRNA) have been found to be dysregulated in most cancers [2]. Tumor-specific miRNAs have been detected in body fluids such as serum/plasma, urine, saliva

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Correspondence to Otoniel Martínez-Maza, UCLA AIDS Institute, 173 BSRB, 615 Charles E. Young Drive South, Los Angeles, CA 90095–7363. Tel: +310 825 2542; fax: +310 206 5387; omartinez@mednet.ucla.edu.

Conflicts of Interest

The authors declare no competing financial interests.

(reviewed in [3]) and cerebrospinal fluids [4], making them attractive candidates as biomarkers that might be perturbed early in the course of disease development.

miR-21 is the most commonly overexpressed miRNA in cancers. It is highly expressed in tumors arising from diverse tissue types, including brain, ovary, liver, breast, colon, lung, prostate, and stomach [5,6], as well as those of hematological origin, such as chronic lymphocytic leukemia [7], DLBCL [8], acute myeloid leukemia [9], and Hodgkin lymphoma [10]. A recent meta-analysis of 17 independent studies assessing the prognostic role for miR-21 in various cancer types concluded that high miR-21 expression in tumors correlated with poorer prognosis, especially in digestive system and head and neck squamous cell carcinomas [11]. In contrast, higher tumor miR-21 levels were associated with better prognosis in DLBCL [8]. miR-21 can also be a diagnostic marker for PCNSL [4] or a differential diagnostic marker for DLBCL subtypes [8]. In mouse models, overexpression of miR-21 in cells of the hematopoietic lineage led to pre-B-cell lymphoma [12], confirming the potential of miR-21 as an oncogene in B cell malignancies.

Viably frozen peripheral blood mononuclear cells (PBMC) from HIV infected men who went on to develop NHL (AIDS-NHL, $n = 22$), as well as from HIV uninfected ($n = 18$) and HIV infected ($n = 21$) men who did not develop NHL, were obtained from the MACS. AIDS-NHL subjects were separated into two groups based on whether samples were obtained < 3 years prior ($n = 13$) or > 4 years prior ($n = 9$) to NHL diagnosis. CD19+ B cells were isolated from PBMCs, followed by total RNA extraction and miR-21 quantification by qPCR, as described [13]. Because all clinical samples were anonymized, the UCLA medical IRB determined that the study was eligible for exemption from IRB review.

We found significant upregulation of miR-21 in CD19+ B cells from AIDS-NHL patients in the 3 years prior to NHL diagnosis, when compared to either HIV infected or uninfected groups ($p < 0.01$ for both comparisons), but not in AIDS-NHL patient samples obtained > 4 years prior to NHL diagnosis (Fig. 1a). The pre-diagnosis AIDS-NHL samples comprised of subtypes BL ($n = 3$), PCNSL ($n = 4$), and DLBCL ($n = 6$) in the < 3 years group, and BL ($n = 1$), PCNSL ($n = 2$), and DLBCL ($n = 6$) in the > 4 years group. We observed no correlation between B cell Epstein-Barr virus (EBV) positivity and miR-21 overexpression in these samples (data not shown). These observations indicated that miR-21 is overexpressed in circulating B cells from HIV positive individuals who go on to develop NHL. Previously, miR-21 was also found to be elevated in serum and cerebrospinal fluid of DLBCL [14] and PCNSL [4] patients. Our observations indicate that the source of this elevated miR-21 may also include circulating B cells.

One pathway that has been suggested to be critical in lymphomagenesis is B cell activation. In HIV+ individuals, several B-cell stimulatory factors such as IL6 [15,16], IL10 [16,17], B-lymphocyte stimulator (BLyS) [18], LPS [19] and the B cell chemokine CXCL13 [20] are elevated in the serum. Additionally, increased surface expression of TNF α on T lymphocytes [21], and the CD40 ligand on T lymphocytes [17] and HIV virions [22,23], also contribute to B cell activation. B cell activation also occurs in secondary lymphoid organs such as lymph nodes, spleen, or tonsils following B cell antigen encounter, and maturation via the germinal center (GC) reaction.

To explore whether miR-21 levels were differentially modulated in activated B cells, we measured miR-21 levels in tonsillar and peripheral B cell populations, obtained from tonsillectomies ($n = 4$) or healthy donors ($n = 7$), respectively. We observed a 10.8 fold higher signal for miR-21 in tonsillar B cells when compared to peripheral B cells ($p < 0.0001$) (Fig. 1b). Within tonsillar B cell subsets, we found significantly higher miR-21 expression in the GC ($p < 0.0001$) and memory ($p = 0.027$) subsets compared to naïve

subsets (Fig. 1c). Thus, miR-21 is overexpressed in activated B cells, particularly in the GC and memory subsets.

To further explore possible pathways for miR-21 overexpression in B cells, we utilized several molecules to activate B cells *in vitro*. We found significant miR-21 upregulation by stimulation with IL4 alone ($p = 0.002$), or in combination with anti-CD40 ab ($p = 0.024$) or anti-IgM ab ($p = 0.021$), but not by IL6 ($p = 0.720$) or IL10 ($p = 0.181$) (Fig. 1d). Although anti-CD40 or anti-IgM ab augmented IL4 induced miR-21 expression, they were insufficient for miR-21 induction, suggesting that the helper T cell produced cytokine IL4 is important for miR-21 upregulation.

Previously, it was shown that B cells express the LPS receptor CD14, and are functionally responsive to LPS [24]. Direct stimulation of B cells by LPS did not change miR-21 levels (Fig. 1d, LPS, $p = 0.340$), however, when LPS was added to whole PBMC (Fig. 1d, LPS(PBMC)), it significantly induced miR-21 overexpression in B cells ($p = 0.021$), suggesting a role for activated monocytes and T cells in B cell miR-21 upregulation.

Overall, these data suggest that miR-21 overexpression is a consequence of B cell activation seen in HIV infection, with a shift to a GC phenotype among circulating B cells. Increased appearance of circulating B cells with GC or immature/transitional phenotype [25] is a feature of HIV disease progression. The phenotype of cells contributing to increased miR-21 expression in AIDS-NHL remains to be defined. In summary, elevated levels of the B-cell activation induced miR-21 in circulating B cells are seen in those persons who go on to develop AIDS-NHL, suggesting that miR-21 may be an important biomarker for assessing NHL risk in HIV infected individuals.

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Fig. 1. miR-21 levels in peripheral B cells from MACS subjects, in normal B cell subpopulations, and upon *in vitro* stimulation

(a) miR-21 level is significantly elevated in circulating B cells preceding AIDS-NHL diagnosis. Relative miR-21 levels were normalized to RNU48 and calculated using the $2^{-\Delta Ct}$ method. miR-21 levels were compared across HIV-, HIV+, and pre-AIDS-NHL subjects grouped into < 3 yrs or > 4 years prior to NHL diagnosis. The box represents the median, 25th and 75th percentile; and the whiskers, the range of values. *P* values were obtained by one way ANOVA with Bonferroni's multiple comparison test. **signifies *p* < 0.01 (b) Comparison of miR-21 expression in CD19⁺ B cells isolated from peripheral blood (*n* = 7) and tonsils (*n* = 4). Error bars represent the mean $\pm$ SD of the biological replicates. *P* value was calculated using two tailed *t*-test. (c) miR-21 expression in tonsillar CD19⁺ B cell

subsets ($n = 3$). The subsets were separated by FACS based on the following immunophenotype: naïve (IgD^+ , CD38^{lo}), germinal center (IgD^- , CD38^{hi}), and memory (IgD^- , CD38^{lo}) as described [13]. Bars represent mean $\pm$ SD. P values were calculated using two tailed t -test. (d) Effect on miR-21 levels by B-cell stimulatory molecules. B cells were incubated for 48 hrs with IL6 (200pg/ml), IL10 (10ng/ml), IL4 (10ng/ml), anti-CD40 (500ng/ml), anti-IgM (1ug/ml), or LPS (100ng/ml). All stimulants (except LPS(PBMC)) were directly added to CD19^+ B cells isolated from PBMC. For LPS(PBMC), LPS was added to whole PBMC, and 48 hrs later CD19^+ B cells were subsequently isolated for analysis. miR-21 expression values were normalized to control stimulation (set to 1). Error bars represent mean ($\pm$ SD) of at least three independent experiments. P values were calculated by two tailed paired t -test in comparison to control stimulation. (*, $p < 0.05$)