Implications of Circulating microRNA Expression in HIV-PAH: Diagnosis, prognosis and pathobiology

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Abstract

HIV-related pulmonary arterial hypertension (HIV-PAH) is a deadly complication of HIV infection affecting approximately 0.5% of HIV patients. Recently, circulating microRNA have been implicated as biomarkers and putative long-distance signaling factors in human health and disease, and several miRNA serve established roles in PAH and HIV pathobiology. Here, we seek to identify circulating miRNA that may serve as markers in diagnosis and progression of HIV-PAH, and whose modulation in this devastating disease may result in better clinical outcomes. Using a previously validated network-biology based approach, we will identify miRNA likely involved in this disease. We will go on to test these hypotheses using a nested case-control study of 15 HIV-infected individuals with PAH and 15 HIV-infected individuals without PAH previously recruited in San Francisco between 2008 and 2012. Based on right heart catheter diagnosis of PAH, these patients will be compared with respect to their circulating miRNA expression profiles. MiRNA expression will also be correlated to clinical outcomes including 6 minute walk distance, *nef*-genotype and CD4 cell count. Lastly the molecular role of these miRNA will be explored by correlation with circulating levels of endothelin-1, a potent vasoconstrictor and regulatory of PAH pathology.

Central Hypothesis and Specific Aims

The expression of specific circulating miRNA can predict the development of pulmonary arterial hypertension in HIV infection.

Specific Aims

1) Using a unique network-biology based approach, predict miRNA important in HIV-PAH.
2) Combining network-based predictions with literature search, identify different circulating expression profiles of key miRNA in HIV-infected individuals with and without PAH.
3) Assess the predictive power of miRNA expression for clinical outcomes including pulmonary artery systolic pressure (PASP), 6-minute walk distance, and CD4 count amongst patients with HIV-PAH.
4) Investigate the molecular underpinnings of differential circulating miRNA expression in individuals with HIV-PAH, in particular by correlating miRNA expression to a) ET-1 expression in peripheral blood monocytes and b) HIV *nef*-genotype.

Background and Significance

HIV-related pulmonary arterial hypertension (HIV-PAH) is a deadly complication of HIV infection caused by destruction of mid-sized pulmonary arteries. The disease occurs in approximately 0.5% of HIV infected individuals, and although PAH therapies may extend life at the end-stages of disease ***(1)***, molecular predictors and modulators of disease development remain poorly understood.

Recently, microRNA have appeared at the forefront of research in the field of pulmonary arterial hypertension. These small non-coding RNA regulate gene expression by binding to mRNA transcripts and tagging them for translational repression or destruction ***(2)***. In PAH in particular, miRNA-21, and -204 are known important regulators of disease phenotype in the pulmonary vasculature ***(3,4)***. In addition, miR-210, -145, -155 and the cluster miR-17/92 are strongly implicated for their modulation by important molecular regulators in PAH and HIV including hypoxia, inflammation and TGF-β signaling ***(5,6,4,7)***.

While the expression of these miRNA in the pulmonary vasculature has been well characterized, it's use in diagnosis and prognostication in PAH is not feasible. The recent discovery of circulating (extracellular) miRNA and development of methods to measure them accurately affords access to an unique biomarker niche which can be used for such a purpose ***(8)***. MiRNA in the serum are bound to proteins and carried in vesicles. Their function as signaling factors and even endocrine molecules in the serum has yet to be elucidated, but should differences in their expression exist between HIV-infected individuals with and without PAH, such a discovery offers and important platform upon which to expand our diagnostic and prognostic power in this disease.

In addition, the pathogenesis of endothelial pathology in HIV-PAH is likely not dependent on viral infection of endothelial cells ***(9)***. This raises the question of how the virus manipulates native cellular pathways in other tissues to cause characteristic pulmonary vascular pathology observed in PAH. A few clues exist to this end. In particular, *nef*-genotype modifies the risk of PAH in HIV infected individuals ***(10)***. There exists some evidence that HIV infection changes cellular miRNA expression***(11)***. Additionally, circulating monocytes make more endothelin-1 (ET-1, a potent vasoconstrictor) in HIV-infected individuals with PAH than those without. MiR-21 is known to modulate ET-1 expression in mouse models of PAH ***(12)***. Identifying an association between *nef*-genotype, important vasoconstrictor expression and circulating miRNA expression will be an important first step in elucidating the mechanism by which the virus leads to the development of PAH.

Study Design and Methods

This pilot study will consist of a nested case-control design including 15 HIV-PAH affected patients and 15 HIV infected patients without PAH selected from a previously assembled database and blood bank of HIV-infected individuals collected over a period

of 5 years. As an added control for miRNA studies, 10 non-HIV, non-PAH affected healthy controls will be selected from a previous database (CIT).

Subjects and Recruitment

Subjects and controls will be HIV-infected men and women selected from a pre-existing database and sample bank previously constructed by Dr. Priscilla Hsue and the vascular biology research group at San Francisco General Hospital. Subjects in this database were recruited between 2008 and 2010 via flyers and other advertisements (e.g., Craigslist). Responders to advertisements were screened for PAH with an initial echocardiogram to measure PASP. For those subjects whose PASP was greater than 30, a formal echocardiogram was performed to confirm elevated pulmonary pressures before proceeding to right heart catheterization (RHC) to confirm the presence of PAH (defined as mean pulmonary artery pressure >25mmHg and pulmonary capillary wedge pressure <12mmHg.) All patients with PASP >30mmHg were enrolled in the study. Longitudinal data including blood collection, echocardiograms and 6-minute walk test were collected every 6 months to 2 years depending on loss to follow up. Patients who were screened but not enrolled were compensated $25. Patients who underwent RHC were reimbursed $250. Patients are compensated $50 for each follow up visit.

For this particular study, 15 cases and 15 age- and sex- matched controls were selected from this database (β= 0.77 to detect an effect size of 50%). Inclusion criteria were age between 30 and 60 years and HIV infection. Subjects were excluded for PASP <30mmHg on RHC, and transgender and transsexual patients were also excluded. Healthy controls for miRNA studies were age- and sex-matched from a separate database including non-HIV infected, non-PAH affected subjects.

The institutional review board at UCSF approved these recruitment, screening, diagnostic and reimbursement methods.

Clinical Measures

<u>Predictor variables: PAH diagnosis, 6-minute walk test and *nef*-genotype</u>
Echocardiography was performed at San Francisco General Hospital by a consistent group of trained echocardiography technicians. PASP was measured based on the tricuspid regurgitation jet by standard procedure.

RHC was also performed by a small group of trained interventional cardiologists using the same equipment manufacturer, and the same catheterization lab in order to create consistency amongst measurements.

Six minute walk test was performed in a standardized fashion with the patient walking for 6 minutes around a 100-ft-long course. Heart rate and oxygen saturation were taken by pulse-oximetry before and after the 6-minute walk and distance walked in 6 minutes was recorded.

Blood collection was achieved with percutaneous venipuncture achieved by trained phlebotomists. Approximately 5 ml of blood was collected at each follow up visit.

Nef-genotyping was accomplished using sequencing methods previously described ***(10)***.

Outcome variables: circulating miRNA expression and ET-1 expression
Quantification of circulating miRNA will be achieved using quantitative reverse polymerase chain reaction. Extraction and concentration of miRNA from plasma will be achieved using previously described methods ***(13)***. ET-1 expression in peripheral blood monocytes will be quantified via QRT-PCR as previously described ***(12)***.

Potential Confounders: CD4 count, ARV compliance, and PAH-treatment
CD4 cell count is recorded from blood drawn at each follow up visit. If no blood was drawn, a CD4 count within 6 months of the follow up visit date will be acquired from patient records.

ARV compliance, cocaine and methamphetamine use, and PAH-treatment were recorded from patient surveys mailed to the patient after each follow up visit. These are recorded as binary variables (yes/no).

Planned Statistical analyses

Network-based analyses will be completed with previously described methods ***(4)***. Briefly, using a previously described tool for miRNA target prediction as well as developed databases of genes and pathways important to HIV and PAH, a hypergeometric (enrichment) analysis will be used to identify miRNA highly likely to regulate crucial genes in HIV-PAH.

ANOVA will be used to detect variance in miRNA expression in HIV-PAH, HIV alone and healthy subjects. Post-hoc student's T-tests will be used to test differences between pairs of these groups. Student's T-test will also be used to detect differences in miRNA expression between different *nef*-genotypes.

Within the group of subjects with HIV-PAH, miRNA expression correlation with clinical variables including 6-minute walk distance, CD4 count and PASP will be accomplished using an R statistic.

As important potential confounders including anti-retroviral adherence, cocaine and methamphetamine use and PAH-treatment could also represent important effect modifiers of clinical outcomes and miRNA levels in this population, we have chosen not to match cases and controls based on these variables and instead will employ a regression analysis to detect effect modification by these variables.

Quality control, data management and administrative issues

As this nested case-control study uses data previously coded, it relies on previous quality control measures used in data-base construction. For example, only patients are included in whom elevated PA pressures by echocardiography correlate with RHC data.

miRNA and ET-1 expression data will be generated by Stephen Chan's laboratory at Brigham and Women's hospital. A material's transfer agreement has already been processed between San Francisco General Hospital and Brigham and Women's Hospital.

All data are coded only by subject number and are kept on secure password-enabled encrypted devices to ensure subject privacy. The custodians of the database are trained in epidemiology research methods and are employed by the Hsue lab and Vascular Group at San Francisco General Hospital.

The timetable for this project will extend from November 2012 through March 2013. During November and December, samples will be sent to the Chan lab and miRNA measurements will take place. During the month of January, data will be analyzed. This preliminary data will be used to write a grant for a multi-year project during the months of April and March.

Ethical considerations

Certainly the inclusion of human subjects in this research poses ethical consequences due not only to major invasive procedures used in generating the database including RHC, but also due to the sensitive information regarding the social stigma of HIV diagnosis.

Because of this, we have not only completely de-identified patient data save for one database which associates subject number to subject identifying information, which is kept under physical lock, password an encryption.

Because of the significant risk of measurements collected in this study, there must be a benefit offered to the patient. Certainly, screening and diagnosis of PAH are benefits to those patients who are cases. In addition, patients received the monetary compensation outlines in the Subjects and Recruitment Section.

All methods for data collection were approved by the SFGH and UCSF IRB prior to initiation of database building.

References

1. Janda S, Quon BS, Swiston J HIV and pulmonary arterial hypertension: a systematic review. HIV Med 11 (10):620-634. doi:HIV829 [pii]
10.1111/j.1468-1293.2010.00829.x
2. Ambros V (2004) The functions of animal microRNAs. Nature 431 (7006):350-355. doi:10.1038/nature02871
nature02871 [pii]
3. Courboulin A, Paulin R, Giguere NJ, Saksouk N, Perreault T, Meloche J, Paquet ER, Biardel S, Provencher S, Cote J, Simard MJ, Bonnet S (2011) Role for miR-204 in human pulmonary arterial hypertension. J Exp Med 208 (3):535-548. doi:jem.20101812 [pii]
10.1084/jem.20101812
4. Parikh VN, Jin RC, Rabello S, Gulbahce N, White K, Hale A, Cottrill KA, Shaik RS, Waxman AB, Zhang YY, Maron BA, Hartner JC, Fujiwara Y, Orkin SH, Haley KJ, Barabasi AL, Loscalzo J, Chan SY (2012) MicroRNA-21 Integrates Pathogenic Signaling to Control Pulmonary Hypertension: Results of a Network Bioinformatics Approach. Circulation 125 (12):1520-1532. doi:CIRCULATIONAHA.111.060269 [pii]
10.1161/CIRCULATIONAHA.111.060269
5. Bockmeyer CL, Maegel L, Janciauskiene S, Rische J, Lehmann U, Maus UA, Nickel N, Haverich A, Hoeper MM, Golpon HA, Kreipe H, Laenger F, Jonigk D Plexiform vasculopathy of severe pulmonary arterial hypertension and microRNA expression. J Heart Lung Transplant 31 (7):764-772. doi:S1053-2498(12)01043-1 [pii]
10.1016/j.healun.2012.03.010
6. Chan SY, Zhang YY, Hemann C, Mahoney CE, Zweier JL, Loscalzo J (2009) MicroRNA-210 controls mitochondrial metabolism during hypoxia by repressing the iron-sulfur cluster assembly proteins ISCU1/2. Cell Metab 10 (4):273-284
7. Satoh M, Minami Y, Takahashi Y, Tabuchi T, Nakamura M A cellular microRNA, let-7i, is a novel biomarker for clinical outcome in patients with dilated cardiomyopathy. J Card Fail 17 (11):923-929. doi:S1071-9164(11)01048-7 [pii]
10.1016/j.cardfail.2011.07.012
8. Turchinovich A, Weiz L, Langheinz A, Burwinkel B (2011) Characterization of extracellular circulating microRNA. Nucleic Acids Res 39 (16):7223-7233. doi:gkr254 [pii]
10.1093/nar/gkr254
9. Mette SA, Palevsky HI, Pietra GG, Williams TM, Bruder E, Prestipino AJ, Patrick AM, Wirth JA (1992) Primary pulmonary hypertension in association with human immunodeficiency virus infection. A possible viral etiology for some forms of hypertensive pulmonary arteriopathy. Am Rev Respir Dis 145 (5):1196-1200
10. Almodovar S, Hsue PY, Morelli J, Huang L, Flores SC Pathogenesis of HIV-associated pulmonary hypertension: potential role of HIV-1 Nef. Proc Am Thorac Soc 8 (3):308-312. doi:8/3/308 [pii]
10.1513/pats.201006-046WR
11. Klase Z, Houzet L, Jeang KT MicroRNAs and HIV-1: Complex interactions. J Biol Chem. doi:R112.415448 [pii]
10.1074/jbc.R112.415448

12. Ehrenreich H, Rieckmann P, Sinowatz F, Weih KA, Arthur LO, Goebel FD, Burd PR, Coligan JE, Clouse KA (1993) Potent stimulation of monocytic endothelin-1 production by HIV-1 glycoprotein 120. J Immunol 150 (10):4601-4609
13. Baggish AL, Hale A, Weiner RB, Lewis GD, Systrom D, Wang F, Wang TJ, Chan SY (2011) Dynamic Regulation of Circulating MicroRNA during Acute Exhaustive Exercise and Sustained Aerobic Exercise Training Journal of Physiology Epublication ahead of print