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Risk of Breast Cancer with CXCR4-using HIV Defined by V3-Loop Sequencing

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Abstract

Objective—Evaluate the risk of female breast cancer associated with HIV-CXCR4 (X4) tropism as determined by various genotypic measures.

Methods—A breast cancer case-control study, with pairwise comparisons of tropism determination methods, was conducted. From the Women's Interagency HIV Study repository, one stored plasma specimen was selected from 25 HIV-infected cases near the breast cancer diagnosis date and 75 HIV-infected control women matched for age and calendar date. HIVgp120-V3 sequences were derived by Sanger population sequencing (PS) and 454-pyro deep sequencing

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(DS). Sequencing-based HIV-X4 tropism was defined using the geno2pheno algorithm, with both high-stringency DS [False-Positive-Rate (FPR 3.5) and 2% X4 cutoff], and lower stringency DS (FPR 5.75, 15% X4 cut-off). Concordance of tropism results by PS, DS, and previously performed phenotyping was assessed with kappa (κ) statistics. Case-control comparisons used exact P-values and conditional logistic regression.

Results—In 74 women (19 cases, 55 controls) with complete results, prevalence of HIV-X4 by PS was 5% in cases vs 29% in controls (P=0.06, odds ratio 0.14, confidence interval 0.003-1.03). Smaller case-control prevalence differences were found with high-stringency DS (21% vs 36%, P=0.32), lower-stringency DS (16% vs 35%, P=0.18), and phenotyping (11% vs 31%, P=0.10). HIV-X4-tropism concordance was best between PS and lower-stringency DS (93%, κ=0.83). Other pairwise concordances were 82%-92% (κ=0.56-0.81). Concordance was similar among cases and controls.

Conclusions—HIV-X4 defined by population sequencing (PS) had good agreement with lower stringency deep sequencing and was significantly associated with lower odds of breast cancer.

Keywords

Chemokine receptors; HIV; AIDS; breast cancer; parallel sequencing; women

Introduction

HIV must bind to both the CD4 protein and another transmembrane co-receptor to infect mononuclear leukocytes. HIV's predominant co-receptors are chemokine receptors CCR5 [1] and CXCR4 [2-4]. Preference in the utilization of these receptors by HIV to infect mononuclear cells, referred to as “R5 tropism” or “X4 tropism”, respectively, can be determined with recombinant phenotypic assays [original and enhanced-sensitivity Trofile® (ESTA), Monogram Biosciences, Inc.] or by genotypic methods based on analysis of the HIV envelope protein's third variable (V3) loop [5-7]. A large majority of incident HIV infections are initially R5-tropic, but approximately 50% of untreated HIV clade B (HIV_B) infections will switch from exclusively R5-tropic to X4-tropic, concomitant with CD4 decline and disease progression [8-10]. Effective suppression of HIV replication with antiretroviral therapy (ART) may also suppress the R5-to-X4 switch of HIV_B [11]. In contrast to HIV_B, X4-tropism is less well characterized for $HIV_{non\text{-}B}$ subtypes [12, 13].

CXCR4 is commonly expressed not only on mononuclear leukocytes but also on hyperplastic and malignant breast duct cells [14-16]. Noting that HIV envelope protein binding to CXCR4 induced apoptosis of breast cancer cells [17, 18], we previously conducted a case-control study with the original Trofile phenotype assay to test the hypothesis that HIV X4-tropic versus exclusively R5-tropic virus may, in part, account for the significantly reduced risk of breast cancer observed among women with AIDS in the United States [19, 20]. As postulated, we found that the odds of breast cancer was 90% lower with HIV-X4 compared to exclusive HIV-R5 [20].

The current project had two aims. The first aim quantified agreement on HIV-X4 versus –R5 tropism assignment between HIV V3-loop sequencing methods and the previous phenotype

data [20]. A second, exploratory aim tested whether the odds of breast cancer among HIV-infected women differed by tropism, as defined by genotypic methods, with the ultimate intent to study this in large numbers of prospectively followed HIV-infected women.

Methods

Subjects and specimens

Breast cancer cases were identified from January 1993 through November 2010 from the Women's Interagency HIV Study (WIHS). The WIHS is a national, multi-site, longitudinal study of women with and at-risk for HIV and is representative of HIV-infected women in the USA. Study methodology, standardized data collection, and repository requirements have been previously reported.[21, 22] Cases were HIV-infected women who had stored plasma specimens that were collected within 24 months (plus or minus) of their breast cancer diagnosis (because HIV co-receptor tropism can change over time). Three HIV-infected women in the control group, without breast cancer, were matched to each case based on age and plasma collection date. All cases and controls had an HIV viral load ≥500 copies/mL, which is the minimum needed for the tropism assays. Participant characteristics considered in the analyses included demographic, behavioral, reproductive, and clinical factors. ART was classified in accordance with the guidelines of the Department of Health and Human Services.[23]

Laboratory methods

V3-loop amplification, population sequencing (PS), deep sequencing (DS), and bioinformatic analysis methods have been described previously [5, 6]. Briefly, HIV RNA was extracted from 500μL plasma, with which reverse-transcriptase PCR was used to amplify sequences, including the 105bp V3 loop, of HIV cDNA in triplicate. These amplicons were sequenced independently on an ABI 3730 sequencer (for PS) and a Roche/454-GS Jr platform (for DS), the latter in pools of 12 with barcode sequence tags.

Sequence analysis and co-receptor assignment

Sequences were aligned to the HXB2 reference strain of HIV_B. Co-receptor use for PS was predicted by automated geno2pheno analysis of the V3 loop sequences, with assignment to HIV-X4 with a false-positive rate (FPR) cutoff of 5.75 [6]. Co-receptor usage from DS was determined using the geno2pheno algorithm applied to each V3 loop sequence; specimens were assigned to HIV-X4 with high stringency (FPR 3.5) and a highly sensitive, previously defined cutoff (2% of sequences) [6]. We further assessed HIV-X4 assignment by DS at lower stringency (FPR 5.75) and higher cutoff (15% of sequences) designed to approximate the PS assay. Assignment of co-receptor use was performed blindly to the several methods.

Statistical analysis

Primary analysis was restricted to women with complete data for all four HIV-X4 tropism determination methods. To assess possible bias, secondary analyses were performed that included women with partial as well as complete HIV-X4 tropism results. Contingency table analyses were conducted to compare the distribution of participant characteristics by case-control status, and Mantel-Haenszel chi-square or Fisher exact tests measured two-sided

statistical significance. Paired t-tests were used to measure equality of means for normally distributed continuous variables. Proportions of CXCR4-using sequences, presented as medians and interquartile range (IQR), were compared with Wilcoxon rank-sum tests. Pairwise agreement [Kappa (κ) statistic]and concordance were measured between the four assays (three genotypic and one phenotypic). Unadjusted and adjusted exact conditional, matched-pair, logistic regression was performed and odds ratios (OR) and 95% confidence intervals (CI) for breast cancer were estimated. The following continuous variables were transformed for the regression analyses: body mass index was divided by 10, $CD4^+$ cell count per mm^3 was divided by 100, and HIV viral load was $\log_{10}$ transformed. Variables with associations at the P-value <0.10 level in the unadjusted regression models were included in the adjusted analysis. Improvement in model fit was assessed with the likelihood ratio test. Statistical analyses were performed using SAS® software version 9.3 [24].

Results

For HIV V3-loop sequencing, plasma specimens from 25 breast cancer cases and 75 matched controls were selected from the WIHS repository. Of these 100 specimens, six cases were excluded due to missing data for at least one of the three tropism assays, and 20 matched controls were also excluded because the data were missing for tropism assays or because the matched case was missing tropism data. Analysis of HIV-X4 prevalence (including dual/mixed R5-X4 tropism) and pairwise concordance of the assay results was restricted to the 74 women (19 cases [mean age 46, mean CD4+ cell count 360]and 55 matched controls [mean age 46, mean CD4+ cell count 336]) with complete data. In these 19 breast cancer cases and 55 controls, there was no difference in demographic, behavioral, or conventional HIV/AIDS characteristics (at the time of the plasma collection) among the cases compared to the controls (all P>0.15, Table 1). For cases who had a pre-cancer specimen tested, the median number of months from tropism assay to cancer diagnosis was 8.1 months, and it was 1.9 months for cases who had a post-cancer specimen tested.

HIV-X4 prevalence among all 74 women was 23% with PS, 32% with high-stringency DS, 26% with lower-stringency DS, and 26% by phenotype. All 19 women classified as X4-using by phenotype were reported as dual/mixed R5-X4; dual/mixed was not determined by PS or DS. As shown in Table 2, most pairwise concordances were around 90% (88%-92%, κ=0.70 – 0.81). Concordance was 93% between PS and lower-stringency DS (κ=0.83); but these had only 82-84% concordance with phenotype (κ=0.56). Concordance was similar among breast cancer cases and controls.

Table 3 presents HIV-X4 prevalence and association with breast cancer for each of the four assay methods. By PS, prevalence of HIV-X4 was 5% in cases vs 29% in controls (P=0.06, odds ratio 0.14, confidence interval 0.003-1.03). With lower-stringency DS (FPR=5.75, and a detection threshold of 15% designed to mimic the PS assay), HIV-X4 prevalences were 16% in cases vs 35% in controls (P=0.18). With the other methods, HIV-X4 case-control prevalence differences were: 21% vs 36% with high-stringency DS (P=0.32); and 11% vs 31% by phenotype (P=0.10). Case-control comparisons were similar when based on all available data for each of the four assays (Table 4).

Proportions of sequences predicted to use CXCR4 were estimated by deep sequencing. With lower-stringency DS, median HIV-X4 sequences were 0.5% (IQR, 0% – 2.3%) in cases versus 1.0% (0% – 41.2%) in controls (P=0.25). With high-stringency DS, median HIV-X4 sequences were 0% (IQR, 0% – 1.7%) in cases versus 0.2% (0% – 9.6%) in controls (P=0.15).

As previously reported [20], with an adjusted exact conditional regression model both HIV-X4 by phenotype and menopause were independently associated with lower odds of breast cancer. In the 74 women with complete data, adding menopausal status to the regression model did not significantly change the odds of breast cancer with HIV-X4 defined by PS (OR 0.14 without menopause vs OR 0.17 with menopause added to the model, P=0.1; Table 5). In contrast, menopause status reduced the odds of breast cancer with phenotype-defined HIV-X4 (OR 0.22 without considering menopause vs OR 0.12 with menopause in the model, P=0.02). Lastly, we addressed the possible effects of lead time bias and competing risk. When adjusted for CD4 cell count, use of ART, or HIV viral load, the significance level for CXCR4-tropic HIV was not attenuated (data not presented).

Discussion

This study found greater than 80% concordance between sequencing-based tropism prediction and phenotypic characterization of co-receptor usage of HIV_B infections. This is almost identical to the genotype-phenotype concordance reported previously [6]. To efficiently address our concordance objective, we re-evaluated our previous tropism phenotype data, deliberately overlapping 80% of the subjects in the current study and the previous study [20]. Considering both studies, HIV-X4 was detected by either phenotype or population sequencing (PS) in 9% of the breast cancer cases compared to 28% of the controls.

Two aspects of the deep sequencing (DS) associations with breast cancer are noteworthy. First, breast cancer's association with optimized high-stringency DS was markedly attenuated compared to the cancer association with either PS or phenotype. The attenuated association with high-stringency DS, which is much more sensitive than PS, raises the possibility that there may be a threshold below which CXCR4-using HIV is too rare to affect breast cancer risk. Second, although data were sparse, the level of HIV-X4 sequences tended to be lower in cases than controls (median 0% versus 0.2%, P=0.15) with high-stringency DS, but this was not seen with lower-stringency DS. We would speculate that neoplastic breast cells are affected by a particular subset of HIV-X4 sequences that are detected with high-stringency methods.

With its natural ligand, CXCL12, CXCR4 is an important mediator of breast cancer metastasis [25]. How HIV-X4 might reduce development of breast cancer is unknown. In vitro, Endo and colleagues have observed that breast cancer cells undergo apoptosis when HIV envelope protein binds to CXCR4 [17, 18]. Perhaps in vivo, incipient malignancy is aborted if hyperplastic pre-malignant breast duct cells that express CXCR4 (but not CD4) enter apoptosis when HIV-X4 envelope is bound [16]. It must be noted that patients with

HIV-X4 may also have accelerated mortality, such that competing risk could contribute to the observed lower incidence of breast cancer among women with HIV.[19]

The current study largely overlapped with, and thus is not an independent validation of, our study of phenotype-defined HIV-X4 and breast cancer [20]. Herein we used state-of-the-art sequencing methods, as well as specimens and data from the WIHS, a population that is representative of HIV-infected women in the USA. We observed a lower risk of breast cancer associated with early menopause in a population with a mean age of 46. However, none of the other clinical, demographic, behavioral, or HIV/AIDS variables was associated with breast cancer. Importantly, none of these variables confounded the association of PS-defined HIV-X4 with breast cancer. Thus, additional case-control matching was unnecessary and was not performed. However, the current study is small, and it characterized HIV co-receptor tropism at only one time point. HIV co-receptor tropism can change over time, usually from exclusive HIV-R5 to HIV-X4 or dual HIV-R5/X4 [26].

Our observed >80% concordance between genotype and phenotype for HIV co-receptor use corroborates previous findings that detection of exclusive HIV-R5 by sequencing may support the clinical use of maraviroc, a CCR5 antagonist [6]. Irrespective of clinical applications, sequencing-based HIV co-receptor determination can be used for epidemiologic research. This has practical implications, because sequencing can be cheaper and less resource-intensive than the phenotype assay. The findings from the current small study suggest that sequencing can be employed to examine whether HIV-X4 is associated with a range of malignant and non-malignant conditions [27-29], including in populations with $HIV_{non\text{-}B}$ clades [12, 13]. The next steps must employ specimens from larger numbers of well characterized subjects who have been followed over time. If supported by these additional investigations, then functional studies, including physiochemical characteristics of the HIV sequences, peptide statistics, and heterogeneity across HIV-X4 envelope sequences, might be used to identify targets for prevention of breast cancer.

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Table 1

Characteristics of the 19 breast cancer cases and 55 controls in the Women's Interagency HIV Study cohort with complete HIV co-receptor test data.

Participant characteristic*	Cases N (%)	Controls N (%)	P-value†
Age, years [mean]	46.2	45.9	0.92
31-40	4 (21.1)	18 (32.7)	0.64
41-50	8 (42.1)	18 (32.7)	
> 50	7 (36.8)	19 (34.5)	
Race/ethnicity White, non-Hispanic	1 (5.3)	10 (18.2)	0.50
White, Hispanic	2 (10.5)	3 (5.5)	
African American	12 (63.2)	32 (58.2)	
Other	4 (21.1)	10 (18.2)	
HIV RNA viral load [mean $\log_{10}$ copies/mL]	4.1	4.4	0.24
500 – 4,000 copies/mL	4 (21.1)	11 (20.0)	0.94
4,000 – 49,000 copies/mL	9 (47.4)	24 (43.6)	
≥50,000 copies/mL	6 (31.6)	20 (36.4)	
CD4+ count (cells/μL) [mean]	360.2	335.6	0.68
<200	3 (15.8)	20 (36.4)	0.18
200 – 499	13 (68.4)	25 (45.5)	
≥500	3 (15.8)	10 (18.2)	
Current HIV therapy¶			
None	14 (73.7)	30 (54.5)	0.58
Mono therapy	1 (5.3)	7 (12.7)	
Combination	0 (0)	3 (5.5)	
HAART	4 (21.1)	15 (27.3)	
Ever used ART			
Yes	12 (63.2)	42 (76.4)	0.26
No	7 (36.8)	13 (23.6)	
Ever used HAART			
Yes	10 (52.6)	31 (56.4)	0.78
No	9 (47.4)	24 (43.6)	
Self-reported clinical AIDS diagnosis			
Yes	8 (42.1)	33 (60.0)	0.18
No	11 (57.9)	22 (40.0)	
Educational attainment			
< High school	8 (42.1)	20 (36.4)	0.93
High school	8 (42.1)	21 (38.2)	
Some college	2 (10.5)	10 (18.2)	
≥ 4 years college	1 (5.3)	4 (7.3)	
Body mass index			
Underweight <19.8	1 (5.6)	4 (7.8)	0.56

Participant characteristic*	Cases N (%)	Controls N (%)	P-value†
Normal 19.8-26.0	6 (33.3)	25 (49.0)	
Overweight 26.1-29.0	4 (22.2)	10 (19.6)	
Obese >29.0	7 (38.9)	12 (23.5)	
Missing	1	4	
Age at menarche [mean]	12.5	13.3	0.17
<12	3 (21.4)	8 (17.0)	0.66
12-13	8 (57.1)	22 (46.8)	
>13	3 (21.4)	17 (36.2)	
Missing	5	8	
Number of term births [mean]	2.9	2.3	0.29
0	4 (21.1)	11 (20.0)	0.94
1-2	7 (36.8)	23 (41.8)	
≥3	8 (42.1)	21 (38.2)	
Ever used oral contraceptives			
Yes	15 (78.9)	39 (70.9)	0.56
No	4 (21.1)	16 (29.1)	
Ever used hormone replacement therapy			
Yes	2 (16.7)	11 (33.3)	0.46
No	10 (83.3)	22 (66.7)	
Missing	7	22	
Menstrual period in the last 12 months			
Yes	14 (73.7)	32 (58.2)	0.23
No	5 (26.3)	23 (41.8)	
History of hysterectomy			
No	15 (78.9)	51 (92.7)	0.19
Yes	4 (21.1)	4 (7.3)	
Current cigarette smoker			
No	6 (31.6)	21 (38.2)	0.61
Yes	13 (68.4)	34 (61.8)	
Alcohol use in past six months			
None	7 (36.8)	29 (52.7)	0.38
<3 drinks per week	6 (31.6)	17 (30.9)	
3-13 drinks per week	5 (26.3)	8 (14.5)	
≥14 drinks per week	1 (5.3)	1 (1.8)	
History of injection drug use			
No	8 (42.1)	30 (54.5)	0.35
Yes	11 (57.9)	25 (45.5)	
HCV positive at baseline			
No	6 (33.3)	24 (44.4)	0.41
Yes	12 (66.7)	30 (55.6)	
Missing	1	1	
CXCR4-tropic (population sequencing)	1 (5.3)	16 (29.1)	0.05

Participant characteristic*	Cases N (%)	Controls N (%)	P-value†
CXCR4-tropic (deep sequencing, 15%)	3 (15.8)	19 (34.5)	0.15
%HIV-X4 (DS-15% cutoff) [mean]	9.4%	23.1%	0.12
CXCR4-tropic (deep sequencing, 2%)	4 (21.1)	20 (36.4)	0.27
%HIV-X4 (DS-2% cutoff) [mean]	7.8%	14.6%	0.36
CXCR4-tropic (phenotype)	2 (10.5)	17 (30.9)	0.13

*Status as of the plasma collection for current study.

†For categorical variables, Fisher's exact test if any cell had <5 observations, else Mantel-Haenzel Chisquare; equality of mean for continuous variables.

¶The four categories of current HIV therapy are defined as follows: none=no reported HIV antiviral medications, monotherapy=only one reported HIV antiretroviral medication (non-HAART), combination therapy=more than one HIV antiretroviral medication but does not meet the DHHS guidelines for HAART, and HAART=meets the DHHS guidelines for HAART.

Table 2

Prevalence, concordance and agreement beyond chance (kappa) for classification of HIV-X4 (CXCR4 use) in 74 HIV-infected participants in the Women's HIV Interagency Cohort Study.

HIV-X4 prevalence, by assay	Concordance (Kappa), by assay			
	PS	$DS_{high\ stringency}$	$DS_{lower\ stringency}$	Phenotype
PS: 23%	1	88% (0.70)	93% (0.83)	84% (0.56)
$DS_{high\ stringency}$: 32%	88% (0.70)	1	92% (0.81)	88% (0.71)
$DS_{lower\ stringency}$: 26%	93% (0.83)	92% (0.81)	1	82% (0.56)
Phenotype: 26%	84% (0.56)	88% (0.71)	82% (0.56)	1

Table 3

Association of breast cancer with HIV-X4 tropism by exact conditional logistic regression analysis in 74 women with complete data.

	HIV-X4 prevalence			
Assay	**Breast cancer cases N=19**	**Controls N=55**	**Odds ratio (95% confidence interval)**	**P-value***
PS	5%	29%	0.14 (0.003, 1.03)	0.06
$DS_{high\ stringency}$	21%	36%	0.44 (0.09, 1.76)	0.32
$DS_{lower\ stringency}$	16%	35%	0.33 (0.05, 1.46)	0.18
Phenotype	11%	31%	0.22 (0.02, 1.22)	0.10

*Two-sided exact tests.

Table 4

Association of breast cancer with HIV-X4 tropism by exact conditional logistic regression analysis, using all available data.

	HIV-X4 prevalence			
Assay (N_{cases}, $N_{controls}$)	**Breast cancer cases**	**Controls**	**Odds ratio (95% confidence interval)**	**P-value***
PS (22, 64)	9%	28%	0.27 (0.03, 1.25)	0.12
$DS_{high\ stringency}$ (24, 70)	29%	31%	0.85 (0.24, 2.70)	0.98
$DS_{lower\ stringency}$ (24,70)	21%	31%	0.55 (0.14, 1.89)	0.44
Phenotype (23, 69)[20]	9%	28%	0.20 (0.02, 1.1)	0.06

*Two-sided exact tests.

Table 5

Effect of menopause status on breast cancer association with HIV-X4 assigned by PS or Phenotype in 74 women with complete data.

Parameter	Exact Odds Ratios (95% confidence interval)		Likelihood ratio P-value
Parameter	Univariate*	Bivariate	
PS	0.14 (0.003, 1.03)	0.17 (0.004, 1.19)	0.1
Menopause	Not included	0.22 (0.004, 2.22)	
Phenotype	0.22 (0.02, 1.22)	0.12 (0.003, 1.03)	0.02
Menopause	Not included	0.09 (0.001, 1.36)	

* As in Table 3.