

Increasing Levels of Serum Heat Shock Protein 70 Precede the Development of AIDS-Defining Non-Hodgkin Lymphoma Among Carriers of HLA-B8-DR3

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Background: We hypothesized that carriage of presumably high Hsp70-producing gene variants on a specific human major histocompatibility complex haplotype, the 8.1AH, may predispose HIV-infected individuals to AIDS–non-Hodgkin lymphoma (NHL).

Setting: We compared serum Hsp70 levels in the years preceding the diagnosis of AIDS–NHL in a matched case–control study (n = 151 pairs) nested in the Multicenter AIDS Cohort Study.

Methods: We tested the impact of 8.1AH-specific single-nucleotide polymorphism (SNP) and joint SNP–human leukocyte

antigen extended haplotypes previously associated with AIDS–NHL in the Multicenter AIDS Cohort Study on the circulating Hsp70 levels in mixed linear models.

Results: We report elevated serum levels of Hsp70 in the 4 years preceding the diagnosis of AIDS–NHL in cases that carry 8.1AH, but not in noncarrier cases and not in carrier- or non-carrier-matched controls. The strongest predictor of higher serum Hsp70 was the haplotype A-G-A-C formed by SNPs rs537160(A) and rs1270942(G) in the complement factor *CFB* gene cluster, and rs2072633(A) and rs6467(C) in nearby *RDBP* and *CYP21A2* located 70 Kb apart from the Hsp70 gene cluster. The association with A-G-A-C haplotype (beta = 0.718; standard error = 0.182; $P = 0.0002$) and with other 8.1AH-specific haplotypes including the high-producing tumor necrosis factor–alpha haplotype rs909253(G)–rs1800629(A) (beta = 0.308; standard error = 0.140; $P = 0.032$) were observed only with NHL identified as an AIDS-defining condition, but not as a post-AIDS condition, nor in combined AIDS and post-AIDS cases.

Conclusion: Our combined genetic and functional approach suggests that the altered level of Hsp70 is a correlate of 8.1AH-mediated AIDS–NHL. Further investigation of the Hsp70 gene cluster and nearby loci that are tagged by A-G-A-C could better elucidate the genetic determinants of the malignancy.

Key Words: HLA-B8, 8.1AH, lymphoma, Hsp70, MHC haplotypes, HIV, AIDS

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INTRODUCTION

Positive associations of B-cell non-Hodgkin lymphomas (NHLs) with lymphotoxin-alpha (*LTA*) +252G variant (rs909253 A>G) and tumor necrosis factor–alpha (*TNFA*) gene promoter variant –308A (rs1800629 G>A), alone or in combination with specific alleles of human leukocyte antigens (HLAs), have been reported in numerous epidemiologic studies.^{1–12} We have extended the findings initially reported on those 2 markers in classical NHL to HIV-associated NHL.¹³ Consistent with those previous reports on classical NHL,^{14,15} however, we have shown that the associations of the 2 NHL-associated alleles of these cytokine genes (and their biallelic G-A haplotype), as well as alleles of the linked

HLA and non-HLA class III genes, are not independent because all these alleles occur in tight linkage disequilibrium on the ancestral haplotype 8.1 (8.1AH). This conserved and extended haplotype is common among Caucasians and is tagged by HLA-B*0801 and specific alleles at many other polymorphic loci spanning up to 5–7 megabases across the central and extended human major histocompatibility complex (MHC).^{16–18} In our previous study, the 8.1AH-wide effect was the only conserved and extended haplotype associated with AIDS-NHL. The important corollaries of the foregoing observations are that 8.1AH haplo-specific gene variants so far associated with AIDS-NHL are not necessarily causal or even closely adjacent to the true causal variants and that any single variant or several of them jointly in the numerous immunomodulatory genes within 8.1AH are as likely putative candidates as the suspected *TNFA*-308.

In European Caucasians, 8.1AH is a common haplotype, and it is even more frequent among NHL cases. However, in the absence of a sufficient number of 8.1AH recombinants to support focused genetic mapping studies, the search for the causal 8.1AH variants by conventional genotyping alone will likely yield false-positive findings. Rather, a combination of genetic and functional studies will be a more effective approach to the identification of potentially causal loci. An appropriate test of functional specificity would be whether the expression of variants of suspected MHC-encoded candidates other than an HLA allele or the high-producing *TNFA*-308A is altered before the diagnosis of AIDS-NHL. Under this hypothesis, causal gene variants should occur jointly on 8.1AH in the cases, but not in the matched controls who potentially carry the G-A haplotype or haplotypes at other candidate loci on variant or recombinant 8.1AHs. If this hypothesis is valid, differences in the expression level of candidate MHC products would be expected between carriers and noncarriers of 8.1AH in the case group, but not in the control group.

We focused on 3 candidate MHC class III genes out of prior suspicion about their role in cancer, particularly in lymphoma. About 250 kilobases (Kb) centromeric to *LTA* and *TNFA* lies a cluster of 3 related heat shock protein 70 (Hsp70) genes (*HSPA1A*, *HSPA1B*, and *HSPAIL*). Dysregulated expression of these stress-inducible chaperone molecules is a hallmark of cancer cells.^{19–25} Involvement of Hsp70 in the development of NHL and AIDS-NHL or other B-cell lymphomas was suspected²⁶ and has recently been reported.^{27,28} We have studied the association of Hsp70 serum levels with the development of AIDS-NHL in a sample of treatment-naive, AIDS-NHL cases, and matched HIV-positive controls nested in the Multicenter AIDS Cohort Study (MACS). Here, we report an elevation of serum levels of Hsp70 among carriers of 8.1AH-specific haplotypes in the years preceding the diagnosis of AIDS-NHL.

METHODS

Cohort Characteristics

A case–control sample (n = 180 pairs) was nested in the MACS, a prospective investigation of the natural history of

HIV infection and AIDS²⁹ among 4954 homosexual men enrolled in 1984–1985, plus 668 men enrolled in 1987–1991 at 4 study centers in the United States (Baltimore, Chicago, Los Angeles, and Pittsburgh). Clinical information and blood samples were obtained at 6-month intervals. MACS protocols have been approved by the institutional review board at each center, and all participants gave informed consent; testing for genetic variants potentially related to HIV/AIDS outcomes was included.

Subphenotypic classification based on histologic data available in a subset of 96 NHL cases (63% of all eligible cases) identified diffuse large B cells (29.0%), large B-cell diffuse, immunoblastic (22%), Burkitt lymphoma and Burkitt lymphoma–like (17%), lymphoma/NHL, not otherwise specified (25%) and others (7%). An overlapping subset of NHL cases (n = 95) were further classified as either systemic (68%) or central nervous system (CNS) (32%).

Study Design

For our current NHL case–control analysis, cases were participants diagnosed with AIDS-NHL before 1996 (when highly active antiretroviral therapy was introduced), matched 1:1 with HIV+ NHL-free controls by (1) race and ethnicity, (2) duration of HIV infection based on the known date of seroconversion or date of entry to the study as HIV seroprevalent, (3) availability of serum samples at any of the 3 designated time points before NHL diagnosis: >3 years pre-NHL (closest to 4 years), 1–3 years pre-NHL (closest to 2 years), and/or 0–1 year pre-NHL (closest to 0.5 year), and (4) status of NHL diagnosis in the matched cases as either an AIDS-defining condition (henceforth AIDS-defining NHL) or one that developed subsequently to another AIDS-defining condition (henceforth post-AIDS NHL). For the AIDS-defining cases, the matched controls were HIV+ and AIDS-free; for the post-AIDS cases, controls (n = 99) were HIV+ individuals diagnosed earlier with another AIDS-defining condition. In both designs, the one-to-one matching was set to minimize the case–control differences in the duration of HIV infection.

Laboratory Methods

SNP and HLA Genotyping

The selected loci are those that showed significant associations with AIDS-related NHL in single-nucleotide polymorphism (SNP)-only or joint HLA-SNP haplotype tests.¹³ These are HLA-B, HLA-DRB1, and 6 MHC class III SNPs, 2 of which map to the *TNFA* gene cluster (rs909253 and rs1800629; coded as m34 and m39, respectively) and 4 to the complement factor *CFB* gene cluster and nearby *RDBP* and *CYP21A2* (rs537160, rs1270942, rs2072633, and rs6467; coded as m67, m70, m71, and m75, respectively). The SNPs were selected and genotyped on a bead array platform (BeadArray, Illumina Inc., San Diego, CA), as previously described.¹³

Portions of the subjects had previously been typed at HLA class I and class II loci for several different studies of HIV-related outcomes. In this study, we completed high-

resolution *HLA-B* ($n = 42$) and *HLA-DRB1* ($n = 62$) typing for additional samples predominantly from the control group, using polymerase chain reaction (PCR) with sequence-specific primers and automated sequencing-based typing. Considering that the markers included in this study do not extend beyond the central MHC region delimited by *HLA-B* and *HLA-DRB1*, the test 8.1AH haplotypes will, henceforth, be referred to as B8-DR3.

Hsp70 ELISA

Circulating Hsp70 levels were determined by an ultrasensitive ELISA assay (Hsp70 High Sensitivity ELISA Kit, Product #: EKS-715, Stressgen Bioreagents) specifically designed to quantify serum Hsp70 levels. This assay has a range of 0.20–12.5 ng/mL and is sensitive to 0.09 pg/mL, which is below the level typically observed in most people (1–7 ng/mL).³⁰ At most of the intervals designated for sampling, adequate amounts of serum (10 μ L required for duplicate determinations) were available to detect Hsp70.

Quality Control

Reliability in SNP typing was assessed through comparisons of duplicate data, as previously described.¹³ Whenever departure from Hardy–Weinberg equilibrium was observed, the genotypic call score was checked before including the test SNP in analyses. For immunoassays, we incorporated appropriate laboratory control sera as quality control (QC) standards in addition to those included by the assay manufacturer. Their inclusion with each assay run provided information on inter-assay variability. Expected ranges for QC controls were established according to the results of multiple independent determinations by the laboratory, expressed as the calculated mean value ± 2 SD. Assays were considered invalid and were repeated if plate controls were out of the expected range (mean ± 2 SD). Finally, in assays where duplicate determinations were performed, a difference of $>25\%$ between the 2 calculated values required repeated testing.

Statistical Analyses

Statistical tests were performed in SAS 9.3 (SAS Institute Inc., Cary, NC), unless otherwise indicated. The impact of extended SNP haplotypes and joint SNP-HLA haplotypes on serum levels of Hsp70 in the years preceding the date of AIDS-NHL diagnosis was evaluated separately in the cases and the matched controls. Extended MHC haplotypes were estimated through the expectation-maximization (EM) algorithm and were modeled as fixed, main, and interaction (with time) effects in mixed linear models with adjustment for covariates. A random intercept model was fitted to account for differential levels of Hsp70 at baseline.

Covariates

The decline in CD4 count over the course of HIV infection is well documented in association with variants in HLA genes in general,^{31,32} and with the 8.1AH in particular.³³ Although the HLA effect on CD4 count may simply be secondary to its

influence on HIV viremia, it seemed prudent to control for the effect of this known predictor of HIV progression as follows: Up to 39 serial measurements of CD4 count were used to estimate the cumulative exposure to low CD4 counts. This was summarized by the trapezoidal method³⁴ as the area under the curve defined by a CD4 count cutoff of less than 200, and this measurement was included as a covariate. Although the control for immunosuppression was not necessary for analyses restricted to post-AIDS NHL, we did include this covariate in the analysis of this subset for the sake of comparability in the estimates between AIDS- and post-AIDS NHL subsets.

The resulting area under the curve covariate was included, along with the age at diagnosis, in mixed linear models to evaluate the associations of 8.1AH-specific SNP haplotypes and SNP-HLA joint haplotypes with circulating Hsp70 levels at 4-, 2-, and 0.5-year intervals before the diagnosis of NHL in the case and control pairs.

Population Structure

Because most of the sampled cases and controls were of European American descent and matched 1:1 by race and ethnicity, no adjustment for population structure was performed. Moreover, a number of the main analyses were conducted separately in cases and matched controls.

Haplotype Analysis

Haplotypes were inferred through the EM algorithm³⁵ implemented in the haplotype trend regression (HTR) approach.³⁶ This approach, assuming an additive model, estimates posterior probabilities for each subject for all EM-inferred haplotypes. These posterior probabilities were treated as independent variables in the HTR model with the weights in the design matrix reflecting various alternative inferences about haplotypes. A mixed linear model containing the weighted haplotypes as predictor variables was used to accommodate our longitudinal design and quantitative outcome with control for the time-varying CD4 count covariate. Haplotypes with a frequency $< 2\%$ were aggregated as a single term in the HTR models.

Only crude sensitivity analysis of NHL subphenotypes could be conducted in a study of this size and degree of histologic heterogeneity. Nonetheless, we report analyses stratified by the type of NHL outcome, ie, whether NHL occurred as an AIDS-defining condition or as a post-AIDS condition. To ensure that our results are not method-dependent, we tested the relationship between serum levels of Hsp70 and carriage of B8-DR3 under 2 different designs, under a main design in which the trajectory of the serum Hsp70 level was compared between carriers and noncarriers of B8-DR3 separately in NHL cases and NHL-free controls, and under an alternative design comparing the levels of Hsp70 in cases versus controls separately in carriers and noncarriers of B8-DR3.

RESULTS

Of the original sample from our earlier study,¹³ 133 cases and 144 controls were available for analyses. Hsp70 was detected in most of their longitudinal samples, but not at one or more designated time points for 27 subjects because either the result was inconclusive or the volume of available serum at a given time point was not sufficient.

In the light of our previously reported data indicating 8.1AH-wide effect in AIDS-NHL and the corollary hypothesis that multiple MHC loci are involved,¹³ we compared the influence of B8-DR3-associated haplotypes vs all remaining haplotypes on the serum Hsp70 level in NHL cases and matched controls. None of the B8-DR3 haplotypes that predicted all AIDS-related NHL¹³ impacted serum Hsp70 levels in either the case or the control group ($P > 0.05$) in analyses of the pooled AIDS-defining NHL plus post-AIDS NHL subsets (see Table S1, Supplemental Digital Content, <http://links.lww.com/QAI/B301>).

When the analyses were restricted to the AIDS-defining NHL subset, all B8-DR3 haplotypes predicted significant increases in Hsp70 serum levels (beta coefficients ≥ 0.308 , $P \leq 0.05$) among the cases (Table 1). Hsp70 serum levels were not especially higher or lower (coefficients close to zero) in

TABLE 1. Association of HLA-B8-DR3-Specific Haplotypes With Serum Heat Shock Protein 70 in the Years Preceding the Diagnosis of AIDS-Defining NHL in the MACS

Extended MHC Haplotype, Haplotype (Total Number*)	Controls (n = 59)†			Cases (n = 52)†		
	Beta‡	SE	P§	Beta‡	SE	P§
1) m34-m39 (3)						
G-A	-0.032	0.099	0.748	0.308	0.140	0.032
2) hla_B-m34-m39 (14)						
B*0801-G-A	-0.006	0.104	0.953	0.448	0.176	0.014
3) m67-m70-m71-m75 (7)						
A-G-A-C	-0.041	0.102	0.692	0.718	0.182	0.0002
4) m67-m70-m71-m75-hla_DRB1 (8)						
A-G-A-C-DRB1*0301	-0.014	0.110	0.895	0.690	0.199	0.0010
5) m34-m39-m67-m70-m71-m75 (10)						
G-A-A-G-A-C	-0.040	0.103	0.695	0.689	0.185	0.0005
6) hla_B-m34-m39-m67-m70-m71-m75-hla_DRB1 (3)						
B*0801-G-A-A-G-A-C-DRB1*0301	-0.014	0.110	0.895	0.610	0.199	0.0034

Mixed models with random intercept for variation in the serum level of heat shock protein 70 (Hsp70) in carriers versus noncarriers of HLA-B8-DR3-specific haplotypes in the 4 years preceding the diagnosis of AIDS-defining NHL (AIDS-NHL).

Haplotypes formed by SNP m34 in lymphotoxin- α (*LTA*); m39 in *TNFA*; m67 and m70 in complement factor B (*CFB*); m71 in RD RNA binding protein (*RDBP*); and m75 in cytochrome P450, family 21 or steroid 21 hydroxylase (*CYP21A2*). Only haplotypes marking the HLA-B8-DR3 lineage are shown.

*Total number of common (frequency $\geq 2\%$) haplotypes.

†Total numbers with available SNP and HLA genotypes.

‡Beta coefficient.

§P value for difference in logarithm of Hsp70 (ng/ μ L) between carriers and noncarriers of HLA-B8-DR3-specific haplotypes in models adjusted for immune suppression and age at diagnosis.

tests of the B8-DR3 vs other haplotypes in the control group. The strongest effect [$\beta = 0.718$; standard error (SE) = 0.182; $P = 0.0002$] was observed with the A-G-A-C haplotype (haplotype #3). The differential distribution of serum Hsp70 between carriers and noncarriers of the B8-DR3-specific A-G-A-C haplotype is illustrated in the box plots separately for AIDS-defining NHL cases (Fig. 1A) and controls (Fig. 1B). Continuous increases of serum Hsp70 before the diagnosis of AIDS-defining NHL are evident only among cases that carry the B8-DR3-specific A-G-A-C haplotype. Extending this haplotype further to the telomeric *TNFA* gene cluster (haplotype #5) or to the centromeric *HLA-DRB1* (haplotype #4) resulted in diminished effects (Table 1), suggesting that the effect of the Hsp70 loci was independent of the others. Notably, the effect of the LTA-TNF G-A haplotype alone (haplotype #1) was less than half of that

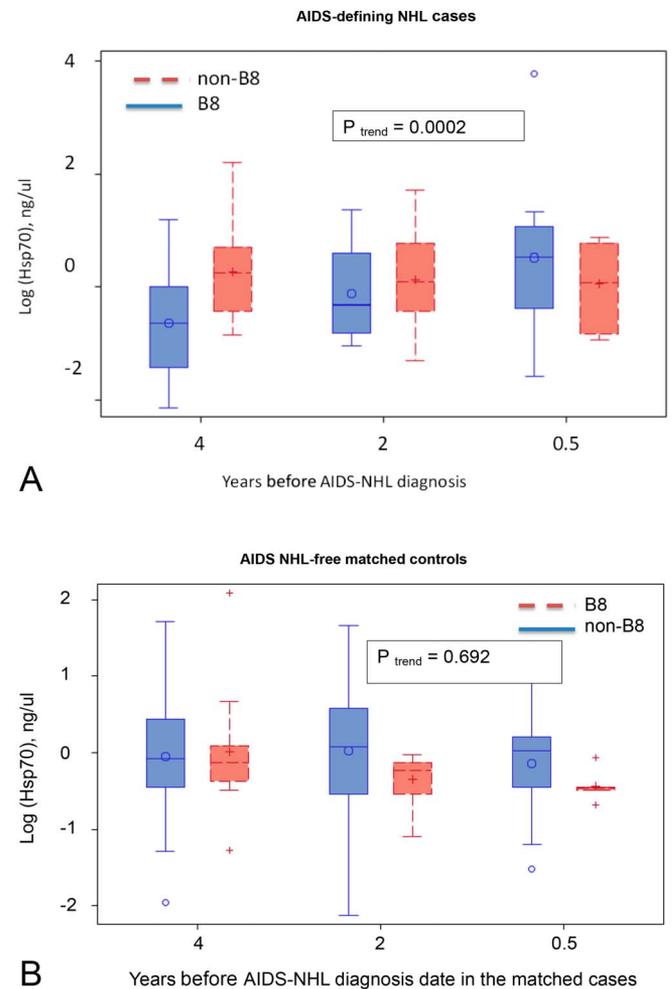


FIGURE 1. Box plots of Hsp70 serum levels in the years preceding the diagnosis of AIDS-related NHL (AIDS-NHL). The box plots are shown separately for AIDS-defining NHL cases (A) and matched HIV-infected controls (B) stratified by carriage status for the strongest HLA-B8-DR3 haplotype predictor of serum Hsp70 increase in the years preceding the development of AIDS-defining NHL in the MACS. An overall test of significance (P for trend) is reported for each comparison.

observed when it was combined with the A-G-A-C haplotype (beta coefficients = 0.308 versus 0.689).

Restricting the analyses to the post-AIDS NHL subset showed no significant associations ($P > 0.20$) in either the case or the control group (see Table S2, Supplemental Digital Content, <http://links.lww.com/QAI/B301>). However, this subset of cases accounted for the nonsignificant trends toward negative associations with Hsp70 observed in the entire case and control sample.

We re-evaluated the impact of B8-DR3 haplotypes in analyses stratified by carrier status, with the control group serving as a reference for estimating the model parameters. When both AIDS-defining and post-AIDS NHL samples were considered, slight increases in the coefficients were observed among the carriers, but not among the noncarriers of B8-DR3; however, the observed increases were not significant, except for the G-A haplotype ($P = 0.039$) (see Table S3, Supplemental Digital Content, <http://links.lww.com/QAI/B301>).

When the tests were performed for the AIDS-defining NHL subset alone, strong associations peaking with A-G-A-C were observed among the carriers of B8-DR3, but not among the noncarriers (Table 2). The smaller coefficients estimated for the B8-DR3 group compared with those obtained with the entire case group (Table 1) can be explained by the presence of causal variants on non-B8-DR3 HLA backgrounds in some cases. By contrast, in the post-AIDS NHL subset, none of the tested haplotypes were associated with significant changes to serum Hsp70 in either carriers or noncarriers of B8-DR3 (see Table S4, Supplemental Digital Content, <http://links.lww.com/QAI/B301>).

Finally, in similar tests of association stratified by histologic types, the A-G-A-C association held only for the

group that developed AIDS-defining NHL as a systemic condition (Table 3). On the basis of limited information available for the histological and phenotypic types (see Table S5, Supplemental Digital Content, <http://links.lww.com/QAI/B301>), we also examined the specificity of the A-G-A-C association for aggressive histologic type of NHL (large B-cells and diffuse large B-cell lymphoma) versus other known and unknown NHL types. The association with the latter types appeared considerably more significant than with the former ones ($P = 0.0006$ versus $P = 0.069$) (Table 4).

DISCUSSIONS

Until recently, virtually nothing was known about the association of Hsp70 with the development of NHL or other B-cell cancers. Because specific haplotypes comprising variants of MHC-encoded Hsp70 genes carried on B8-DR3 chromosomes were shown previously in the MACS to be associated with AIDS-NHL,¹³ we examined potential effects of those haplotypes on serum levels of Hsp70 and whether higher levels were associated with the development of AIDS-NHL. We measured Hsp70 levels in serum taken at successive intervals before the development of AIDS-NHL and then determined whether higher levels were associated with the haplotypes implicated in the occurrence of AIDS-NHL.

Using an improved case and matched control study design to account for the differential timing of NHL development in models with appropriate adjustment for low CD4 count, we showed that increasing serum levels of Hsp70 predicted AIDS-defining NHL in individuals with B8-DR3 haplotypes. Specifically, in analyses stratified by timing of NHL diagnosis (as either AIDS-defining or developing after the diagnosis of AIDS), elevated serum Hsp70 levels were detected during the few years before AIDS-defining NHL in cases that carried B8-DR3-specific haplotypes, but levels were not elevated in post-AIDS cases, cases that were non-B8-DR3 carriers, or controls. These results are consistent with our previous report of genetic associations between B8-DR3 haplotypes and AIDS-NHL¹³ and suggest that AIDS-NHL is a heterogeneous disease.

We also found that carriage of the B8-DR3-specific A-G-A-C haplotype in *CFB* and adjacent loci was the strongest predictor of serum Hsp70 increases among the cases, but not among the matched controls. These results suggested that genetic variants located at or close to the Hsp70 gene cluster (see Figure S1, Supplemental Digital Content, <http://links.lww.com/QAI/B301>) and carried on a B8-DR3-specific chromosome could be the causal variants. The highly suspected TNF-producing G-A haplotype in AIDS-NHL (and in classical NHL) was also associated with elevated serum Hsp70 levels, but to a lesser degree; this previously reported association is potentially due to mere co-occurrence (ie, tight linkage disequilibrium) of the G-A and A-G-A-C haplotypes on the same B8-DR3 chromosomes. However, until the expression of TNFA and other suspected MHC markers is tested between carriers and noncarriers of B8-DR3, we cannot exclude the possibility of coregulated expression of one or more other MHC-encoded factors in AIDS-NHL.

TABLE 2. Association of Serum Heat Shock Protein 70 With AIDS-Defining NHL by HLA-B8-DR3 Carriage Status Among HIV-Infected Men in the Multicenter HIV Cohort Study

Extended MHC Haplotypes, Haplotype (Total Number*)	Mixed model*			
	B8-DR3 (n = 21–45)		Non-B8-DR3 (n = 63–84)	
	Beta†	P†	Beta	P
1) m34-m39 (3) G-A	0.216	0.007	-0.058	0.606
2) <i>HLA_B</i> -m34-m39 (15) B*0801-G-A	0.307	0.011	0.040	0.434
3) m67-m70-m71-m75 (7) A-G-A-C	0.465	0.0002	0.0001	0.985
4) m67-m70-m71-m75- <i>HLA_DRB1</i> (10) A-G-A-C-DRB1*0301	0.446	0.004	0.036	0.936
5) m34-m39-m67-m70-m71-m75 (12) G-A-A-G-A-C	0.445	0.0001	0.011	0.871
6) <i>HLA_B</i> -m34-m39-m67-m70-m71- m75- <i>HLA_DRB1</i> (4) B*0801-G-A-A-G-A-C-DRB1*0301	0.421	0.005	0.030	0.626

*Adjusted for immunosuppression and age at diagnosis.

†Beta coefficients [logarithm of Hsp70 (ng/ μ L)] estimated in mixed models with the control groups serving as references. Note that non-B8-DR3 means any haplotype other than those listed under the "Extended MHC Haplotypes" column.

TABLE 3. Stratified Analysis of HLA-B8-DR3 Association With Serum Heat Shock Protein 70 by Anatomical Location (Systemic Versus CNS) of NHL in the MACS

NHL Outcome Associated With m67(A)-m70(G)-m71(A)-m75(C)	Mixed Models							
	Systemic NHL				CNS NHL			
	n	Beta*	SE	P†	n	Beta	SE	P
AIDS-defining and post-AIDS NHL								
Cases	86	0.328	0.202	0.107	47	-0.230	0.160	0.157
Controls	94	-0.050	0.106	0.638	49	-0.143	0.278	0.941
AIDS-defining NHL								
Cases	43	0.691	0.186	0.0005	9	1.777	1.127	0.166
Controls	49	-0.051	0.103	0.622	10	—	—	—
Post-AIDS NHL								
Cases	43	-0.459	0.415	0.275	39	-0.266	0.161	0.107
Controls	45	0.222	0.605	0.715	39	0.002	0.288	0.994

Mixed linear models with random intercept for variation in the serum level of heat shock protein 70 (hsp70) predicted by carriage of HLA-B8-DR3-specific haplotype # 3 in the 4 years preceding the diagnosis of AIDS-related NHL (AIDS-NHL).

Haplotype # 3 is formed by SNPs m67 and m70 in complement factor B (*CFB*); m71 in RD RNA binding protein (*RDBP*) and m75 in cytochrome P450, family 21 or steroid 21 hydroxylase (*CYP21A2*).

*Beta coefficient and SE.

†P value for difference in logarithm of Hsp70 (ng/μL) between carriers and noncarriers of the best haplotype predictor of AIDS-NHL in models adjusted for immune suppression and age at diagnosis. Note that the number of individuals n is the total number in each of the outcome and anatomical site categories and not the number of individuals with the associated A-G-A-C haplotype.

Our data were also consistent with the CNS location of many post-AIDS NHL cases in MACS. NHL in the CNS is believed to be associated with Epstein-Barr virus (EBV) infection³⁷ and, therefore, may not share full genetic etiology with NHL that develops outside the CNS. Of course, these novel results of our stratified analyses in relatively small population samples should be interpreted with caution until confirmed. However, independent corroboration of these findings in studies of similar depth and phenotypic specificity

will be difficult to conduct because of the fortunate paucity of large treatment-naïve HIV-infected populations under study.

Given that our results were obtained in a cohort study of HIV-infected men (men who have sex with men), we cannot exclude the possibility of gender differences in susceptibility to B8-DR3-associated AIDS-NHL because genes encoding sex hormones are present in MHCs and sexual dimorphism has been reported in HIV progression to AIDS-defining lymphoma.³⁸

TABLE 4. Analysis of HLA-B8-DR3 Association With Serum Heat Shock Protein 70 in the Years Preceding the Development of NHL, Stratified by Histological Types in the MACS

NHL Outcome Associated With m67(A)-m70(G)-m71(A)-m75(C)	Mixed Models							
	LBC and DLBCL*				Unknown Types			
	n‡	beta§	SE	P†	n	Beta	SE	P
AIDS-defining and post-AIDS NHL								
Cases	46	-0.027	0.275	0.918	87	0.023	0.131	0.859
Controls	47	0.017	0.156	0.915	96	-0.253	0.150	0.095
AIDS-defining NHL								
Cases	22	0.435	0.230	0.069	30	1.085	0.278	0.0006
Controls	24	-0.067	0.123	0.591	35	-0.214	0.234	0.367
Post-AIDS NHL								
Cases	24	-0.755	0.483	0.130	58	-0.125	0.157	0.430
Controls	23	2.14	1.221	0.092	61	-0.245	0.192	0.665

Mixed models with random intercept for variation in the serum level of heat shock protein 70 (hsp70) predicted by carriage or noncarriage of HLA-B8-DR3 test haplotype in the 4 years preceding the diagnosis of AIDS-defining NHL (AIDS-defining NHL). The test haplotype is formed by SNPs m67 and m70 in *CFB*; m71 in RD RNA binding protein (*RDBP*); and m75 in cytochrome P450, family 21 or steroid 21 hydroxylase (*CYP21A2*).

*DLBCL, diffuse large B-cell lymphoma; LBC, large B cells.

†P value for difference in logarithm of Hsp70 (ng/μL) between carriers and noncarriers of the best haplotype predictor of AIDS-defining NHL in models adjusted for immune suppression and age at diagnosis.

‡Note that the numbers of AIDS and post-AIDS NHL cases do not add to n = 49 (29% DLBCL and 22% LBC of a total of 96 cases with available histology data) because of missing genotypes or covariate data. Note also that the number of individuals (n) is the total number in each of the outcome and histologic categories and not the number of individuals with the associated A-G-A-C haplotype.

§Beta coefficient and SE.

There is growing evidence that Hsp70 may be involved in the pathogenesis of NHL. Hsp70 is a member of a family of B-cell stimulatory molecules and a candidate lymphomagenic molecule.^{39–42} Hsp70 is an inducible protein whose expression may be upregulated under conditions of stress. The molecule is a chaperone that prevents protein aggregation and is involved in the repair of damaged proteins. It also appears to play a role in B-cell activation. Hsp70 is expressed on the surface of germinal center B cells, which undergo both IgH class switch recombination and somatic hypermutation.⁴³ Interestingly, Hsp70 appears to function as a natural alternative ligand for CD40,⁴⁴ which is the receptor for CD154 (CD40 ligand). CD154, in turn, is an immune stimulatory molecule that plays a central role in B-cell activation and is normally expressed on the surface of helper T cells. Therefore, Hsp70 may be involved in homotypic interactions among activated germinal B cells, promoting their continuing activation.

In recent preliminary work, we found that recombinant human Hsp70 induces expression of activation-induced cytidine deaminase (AICDA) (a DNA-modifying enzyme) in normal B cells (Martinez-Maza, unpublished observation), indicating that it can function in a manner analogous to the CD40 ligand and potentially promote lymphomagenic molecular lesions in these cells. AICDA is essential for somatic hypermutation and class switch recombination of the immunoglobulin (Ig) genes in B cells.⁴⁵ Thus, development of NHL may be potentiated by Hsp70-induced AICDA.

Overall, our data indicated that variant loci at the complement factor gene cluster region that is located about 70 Kb and 300 Kb centromeric to the Hsp70 and TNFA gene clusters, respectively, are associated with the production of circulating Hsp70 molecules in the years preceding the development of AIDS-defining NHL. Of note, because this study used a genotype data set that was generated in a previous study of genetic association of MHC alleles and haplotypes with AIDS-NHL in MACS (MHC-wide approach),¹³ typing data for Hsp70 gene isoforms or nearby regulatory sequences were not available in the present candidate gene approach to allow for evaluation of the relationship between the predictor A-G-A-C haplotype and polymorphisms known to affect Hsp70 transcript abundance.⁴⁶ Thus, deeper genotyping of Hsp70 polymorphisms more directly responsible for the expression of these 3 Hsp70 isoforms is needed to establish the precise mechanistic links between functional and genetic data.

The existence of B8-DR3 heterogeneity (as suggested by the apparently distinct B8-DR3 chromosomes in AIDS-NHL cases and matched controls) will continue to cloud our understanding and interpretation of genetic associations involving this extraordinarily extended and conserved MHC haplotype. Further deciphering of the nucleotide sequence variation across B8-DR3 chromosomes in populations of different ancestries will be a key step to gaining insights into the true genetic determinants not only of AIDS-defining and non-AIDS NHL, but also of the numerous other disorders associated with this haplotype and its variants.

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REFERENCES

1. Warzocha K, Ribeiro P, Bienvenu J, et al. Genetic polymorphisms in the tumor necrosis factor locus influence non-Hodgkin's lymphoma outcome. *Blood*. 1998;91:3574–3581.
2. Juszczynski P, Kalinka E, Bienvenu J, et al. Human leukocyte antigens class II and tumor necrosis factor genetic polymorphisms are independent predictors of non-Hodgkin lymphoma outcome. *Blood*. 2002;100:3037–3040.
3. Spink CF, Keen LJ, Mensah FK, et al. Association between non-Hodgkin lymphoma and haplotypes in the TNF region. *Br J Haematol*. 2006;133:293–300.
4. Rothman N, Skibola CF, Wang SS, et al. Genetic variation in TNF and IL10 and risk of non-Hodgkin lymphoma: a report from the InterLymph Consortium. *Lancet Oncol*. 2006;7:27–38.
5. Purdue MP, Lan Q, Krickler A, et al. Polymorphisms in immune function genes and risk of non-Hodgkin lymphoma: findings from the New South Wales non-Hodgkin Lymphoma Study. *Carcinogenesis*. 2007;28:704–712.
6. Cerhan JR, Liu-Mares W, Fredericksen ZS, et al. Genetic variation in tumor necrosis factor and the nuclear factor-kappaB canonical pathway and risk of non-Hodgkin's lymphoma. *Cancer Epidemiol Biomarkers Prev*. 2008;17:3161–3169.
7. Wang SS, Cozen W, Cerhan JR, et al. Immune mechanisms in non-Hodgkin lymphoma: joint effects of the TNF G308A and IL10 T3575A polymorphisms with non-Hodgkin lymphoma risk factors. *Cancer Res*. 2007;67:5042–5054.
8. Gao S, Zhu G, Lin Y, et al. Tumor necrosis factor-308 polymorphism with the risk and prognosis of non-Hodgkin lymphomas: a meta-analysis study. *Onco Targets Ther*. 2016;9:1657–1670.
9. Hosgood HD III, Au WY, Kim HN, et al. IL10 and TNF variants and risk of non-Hodgkin lymphoma among three Asian populations. *Int J Hematol*. 2013;97:793–799.
10. Bi X, Zheng T, Lan Q, et al. Genetic polymorphisms in IL10RA and TNF modify the association between blood transfusion and risk of non-Hodgkin lymphoma. *Am J Hematol*. 2012;87:766–769.
11. Fernberg P, Chang ET, Duvefelt K, et al. Genetic variation in chromosomal translocation breakpoint and immune function genes and risk of non-Hodgkin lymphoma. *Cancer Causes Control*. 2010;21:759–769.
12. Skibola CF, Bracci PM, Nieters A, et al. Tumor necrosis factor (TNF) and lymphotoxin-alpha (LTA) polymorphisms and risk of non-Hodgkin lymphoma in the InterLymph Consortium. *Am J Epidemiol*. 2010;171:267–276.

13. Aissani B, Ogwaro KM, Shrestha S, et al. The major histocompatibility complex conserved extended haplotype 8.1 in AIDS-related non-Hodgkin lymphoma. *J Acquir Immune Defic Syndr*. 2009;52:170–179.
14. Nowak J, Kalinka-Warzocha E, Juszczynski P, et al. Association of human leukocyte antigen ancestral haplotype 8.1 with adverse outcome of non-Hodgkin's lymphoma. *Genes Chromosomes Cancer*. 2007;46:500–507.
15. Nowak J, Kalinka-Warzocha E, Juszczynski P, et al. Haplotype-specific pattern of association of human major histocompatibility complex with non-Hodgkin's lymphoma outcome. *Tissue Antigens*. 2008;71:16–26.
16. Stewart CA, Horton R, Allcock RJ, et al. Complete MHC haplotype sequencing for common disease gene mapping. *Genome Res*. 2004;14:1176–1187.
17. Degli-Esposti MA, Leaver AL, Christiansen FT, et al. Ancestral haplotypes: conserved population MHC haplotypes. *Hum Immunol*. 1992;34:242–252.
18. Price P, Witt C, Allcock R, et al. The genetic basis for the association of the 8.1 ancestral haplotype (A1, B8, DR3) with multiple immunopathological diseases. *Immunol Rev*. 1999;167:257–274.
19. Kumar S, Stokes J III, Singh UP, et al. Targeting Hsp70: a possible therapy for cancer. *Cancer Lett*. 2016;374:156–166.
20. Murphy ME. The HSP70 family and cancer. *Carcinogenesis*. 2013;34:1181–1188.
21. Nylandsted J, Brand K, Jäättelä M. Heat shock protein 70 is required for the survival of cancer cells. *Ann N Y Acad Sci*. 2000;926:122–125.
22. Tang D, Khaleque MA, Jones EL, et al. Expression of heat shock proteins and heat shock protein messenger ribonucleic acid in human prostate carcinoma in vitro and in tumors in vivo. *Cell Stress Chaperones*. 2005;10(1):46–58.
23. Cornford PA, Dodson AR, Parsons KF, et al. Heat shock protein expression independently predicts clinical outcome in prostate cancer. *Cancer Res*. 2000;60:7099–7105.
24. Clark PR, Ménoret A. The inducible Hsp70 as a marker of tumor immunogenicity. *Cell Stress Chaperones*. 2001;6:121–125.
25. Chatterjee S, Burns TF. Targeting heat shock proteins in cancer: a promising therapeutic approach. *Int J Mol Sci*. 2017;18:E1978.
26. Aissani B, Martinez-Maza O, Shrestha S, et al. Elevated serum levels of heat shock protein 70 precede the development of AIDS-non-Hodgkin lymphoma in carriers of the common and highly conserved HLA-B8-DR3 haplotype. *Infect Agent Cancer*. 2010;11:5.
27. Magangane P, Sookhayi R, Govender D, et al. Determining protein biomarkers for DLBCL using FFPE tissues from HIV negative and HIV positive patients. *J Mol Histol*. 2016;47:565–577.
28. Wang WF, Yan L, Liu Z, et al. HSP70-Hrd1 axis precludes the oncorepressor potential of N-terminal misfolded Blimp-1s in lymphoma cells. *Nat Commun*. 2017;8:363.
29. Kaslow RA, Ostrow DG, Detels R, et al. The Multicenter AIDS Cohort Study: rationale, organization, and selected characteristics of the participants. *Am J Epidemiol*. 1987;126:310–318.
30. Multhoff G, Hightower LE. Distinguishing integral and receptor-bound heat shock protein 70 (Hsp70) on the cell surface by Hsp70-specific antibodies. *Cell Stress Chaperones*. 2011;16:251–255.
31. Lazaryan A, Song W, Lobashevsky E, et al. Human leukocyte antigen class I supertypes and HIV-1 control in African Americans. *J Virol*. 2010;84:2610–2617.
32. Peterson TA, Kimani J, Wachihi C, et al. HLA class I associations with rates of HIV-1 seroconversion and disease progression in the Pumwani Sex Worker Cohort. *Tissue Antigens*. 2013;81:93–107.
33. Kaslow RA, Duquesnoy R, VanRaden M, et al. A1, Cw7, B8, DR3 HLA antigen combination associated with rapid decline of T-helper lymphocytes in HIV-1 infection. A report from the Multicenter AIDS Cohort Study. *Lancet*. 1990;335:927–930.
34. Cole SR, Napravnik S, Mugavero MJ, et al. Copy-years viremia as a measure of cumulative human immunodeficiency virus viral burden. *Am J Epidemiol*. 2010;171:198–205.
35. Excoffier L, Slatkin M. Maximum-likelihood estimation of molecular haplotype frequencies in a diploid population. *Mol Biol Evol*. 1995;12:921–927.
36. Zaykin DV, Westfall PH, Young SS, et al. Testing association of statistically inferred haplotypes with discrete and continuous traits in samples of unrelated individuals. *Hum Hered*. 2002;53:79–91.
37. Breen EC, Hussain SK, Magpantay L, et al. B-cell stimulatory cytokines and markers of immune activation are elevated several years prior to the diagnosis of systemic AIDS-associated non-Hodgkin B-cell lymphoma. *Cancer Epidemiol Biomarkers Prev*. 2011;20:1303–1314.
38. Jarrin I, Geskus R, Bhaskaran K, et al. Gender differences in HIV progression to AIDS and death in industrialized countries: slower disease progression following HIV seroconversion in women. *Am J Epidemiol*. 2008;168:532–540.
39. Gaudio E, Paduano F, Ngankeu A, et al. Heat shock protein 70 regulates Tc11 expression in leukemia and lymphomas. *Blood*. 2013;121:351–359.
40. Leu JI, Pimkina J, Frank A, et al. A small molecule inhibitor of inducible heat shock protein 70. *Mol Cell*. 2009;36:15–27.
41. Briones J. Heat-shock proteins: a c-Myc lymphoma target? *Blood* 2015; 125:1685–1686.
42. Zappasodi R, Ruggiero G, Guarnotta C, et al. HSPH1 inhibition downregulates Bcl-6 and c-Myc and hampers the growth of human aggressive B-cell non-Hodgkin lymphoma. *Blood*. 2015;125:1768–1771.
43. International Collaboration on HIV, Cancer. Highly active antiretroviral therapy and incidence of cancer in human immunodeficiency virus-infected adults. *J Natl Cancer Inst*. 2000;92:1823–1830.
44. Dalla-Favera R, Ye BH, Lo Coco F, et al. BCL-6 and the molecular pathogenesis of B-cell lymphoma. *Cold Spring Harbor Symp Quant Biol*. 1994;59:117–123.
45. Gaidano G, Pasqualucci L, Capello D, et al. Aberrant somatic hypermutation in multiple subtypes of AIDS-associated non-Hodgkin lymphoma. *Blood*. 2003;102:1833–1841.
46. Mauger N, Radhakrishnan J, Knight JC. Genetic determinants of HSP70 gene expression following heat shock. *Hum Mol Genet*. 2010;19:4939–4947.