Chapter 5

HLA analysis in a classical Hodgkin lymphoma patient cohort from Brazil

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ABSTRACT

The B cell derived tumor cells of classical Hodgkin lymphoma (cHL) cases frequently have lost expression of HLA at the time of diagnosis. However, a considerable proportion of the Epstein Barr virus (EBV) positive cases retain expression of both HLA class I and class II. This enigmatic finding might be related to the observation that in Western Europe the HLA-A*01 allele increases and the HLA-A*02 allele decreases susceptibility to EBV+ cHL. In the Northern Chinese population, where HLA-A*01 is rare and HLA-A*02 has several common well-documented sub-alleles, we previously showed that HLA-A*02:07 is a risk allele for EBV+ cHL. The current pilot study focuses on EBV, HLA class I expression and associations with HLA-A alleles in a Brazilian cHL patient group.

Formalin fixed paraffin embedded tissue sections of 158 cHL Brazilian consultation cases were retrieved from the Consultoria em Patologia, Botucatu-São Paulo, Brazil. Tumor cell EBV status was determined by EBER *in-situ* hybridization and HLA expression was determined by immunohistochemistry using antibodies HC10 and β2-microglobulin for HLA class I and C3/43 for HLA class II. HLA-A*01 and HLA-A*02 qPCR was validated using known HLA typed samples to discriminate between non-carriers and heterozygous or homozygous carriers for HLA-A*01 and HLA-A*02. Control data on HLA typing was available for 46,044 blood bank donors from Brazil.

EBV positivity was observed in 34% of the cHL patients with the highest frequency in the mixed cellularity subtype. Membranous expression of HLA class I was significantly more often retained in EBV+ cHL compared to EBV- cHL (53% vs. 17%, p=0.0004). HLA class II was retained in 61% of all cHL cases with similar percentages in EBV stratified subgroups. The HLA-A*01 carrier frequency was significantly increased in EBV+ cHL compared to controls (32% vs. 18%, p=0.02) and the HLA-A*02 carrier frequency was significantly reduced in EBV+ cHL compared to controls (28% vs. 45%, p=0.02).

EBV positivity was associated with HLA class I expression by the tumor cells. The EBV status, HLA expression and HLA-A association were similar to those in the Western European cHL population.
INTRODUCTION

Classical Hodgkin lymphoma (cHL) is a rather complex disease with environmental, geographic and genetic factors contributing to its etiology. The highest incidence of cHL was reported among Caucasians, followed by African Americans and Hispanics, and the lowest incidence was found in Orientals (1). In general the age incidence follows a bimodal curve, with an early prominent age peak at third decade and a second peak above the age of 55 years. In a proportion of the cHL cases, the tumor cells harbor the Epstein Barr virus (EBV). The association with EBV varies with age, sex, histological subtype, geographical location and ethnic background (2, 3, 4). Up to 87% of Hispanic cHL patients have EBV+ tumor cells (5, 6), in Caucasians it is 20-40% (2, 7) and in Orientals the percentage is intermediate (7, 8). In general, EBV positivity associates with young age (9, 10), male gender and mixed cellularity subtype (9, 11).

The tumor cells of cHL, i.e. Hodgkin Reed Sternberg (HRS) cells, commonly show a loss of B cell identity, but often retain expression of components of the antigen presentation machinery including surface expression of HLA class I and class II (12). HLA plays a principle role in the adaptive immune system and presents intracellular or viral antigens to CD8+ T cells in the context of HLA class I and exogenous antigens to CD4+ T cells in the context of HLA class II. HLA class I restricted CD8+ cytotoxic responses are known to target and destroy EBV infected cells (13). Expression of HLA class I is frequently retained in EBV+ cHL whereas it is commonly lost in EBV- cHL at the time of diagnosis (14, 15). Retention of HLA class I in EBV+ cHL is remarkable, since HLA restricted immune responses should be detrimental to the EBV+ HRS cells, even considering the latency type II infection pattern that is characterized by expression of the less immunodominant LMP1, LMP2 and EBNA1 proteins. HLA class II is retained in 46-59% of tumor cells in the cHL population (14, 15).

In Western Europe, HLA-A*01 is predisposing and HLA-A*02 is protective for the development of EBV+ cHL (16, 17, 18). In a Chinese cHL population, HLA-A*02:07 (common among Chinese but not among West Europeans) was found to be a risk allele for developing EBV+ cHL (19). These susceptibilities are most likely driven by the effectiveness of different HLA-A alleles to present antigenic EBV derived peptides to cytotoxic T cells (CTLs). Indeed, HLA-A*01 and HLA-A*02:07 have no or low affinity for EBV latency type II proteins (20, 21) whereas HLA-A*02:01 can efficiently present such proteins resulting in effective EBV specific CTL responses (22, 23, 24). These studies demonstrate a relationship between ethnicity and HLA risk pattern and contribute to our understanding of the role of HLA in the pathogenesis of EBV+ cHL.

Studies on HLA and disease predisposition in cHL patients from South America are lacking. We investigated a Brazilian cHL population to explore a possible
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HLA-A type susceptibility in EBV+ cHL. In addition, we also investigated HLA expression by the tumor cells in EBV stratified subgroups.

MATERIALS AND METHODS

Study Population

A total of 158 cHL consultation cases (2005-2007) obtained from throughout Brazil were available at the Consultoria em Patologia (Botucatu-São Paulo, Brazil). Data on sex and age were retrieved for all the cases. Hematoxilin & Eosin (H&E) stained sections from formalin fixed paraffin embedded tissue blocks were used to determine the histological subtypes according to the WHO classification (25). DNA was isolated from paraffin tissue sections for all the patients. HLA-A*01 and HLA-A*02 frequencies from 46,044 blood donors were retrieved from the Hospital Amaral Carvalho-Jaú, São Paulo and the INCA (National institute of cancer) bank, Rio de Janeiro, Brazil.

In-Situ Hybridization

Detection of EBV was performed by in-situ hybridization (ISH) on paraffin tissue sections with a fluorescein-conjugated PNA probe specific for EBV-encoded RNAs (EBERs), (DAKO, Glostrup, Denmark). Presence of tumor cells was confirmed by CD30 and H&E staining. A known EBV+ tissue section was used as a positive control.

Immunohistochemical staining and scoring of HLA class I and class II

Tissue Microarray (TMA) slides were obtained for all the cases and were deparaffinized by xylene and rehydrated through a graded ethanol series into water. Antigen retrieval was performed with Tris-EDTA solution (10mM Tris Base, 1mM EDTA Solution, pH 9.0) in a microwave. Endogenous peroxidase activity was blocked in 3% H2O2. Expression of HLA class I was detected using the monoclonal antibody HC-10, which recognizes HLA-B and HLA-C molecules, as well as a few HLA-A molecules, at a dilution of 1:200 (kindly provided by Prof. dr. J. Neefjes, the Netherlands Cancer Institute, Amsterdam). The polyclonal rabbit anti-human β2-microglobulin (DAKO, Glostrup Denmark) was used at a dilution of 1:200. For detection of HLA class II, we used the CR3/43 monoclonal antibody (DAKO, Glostrup Denmark) that binds to a specific monomorphic epitope in the β chain of HLA-DP, HLA-DQ and HLA-DR. Antibody binding was visualized with Avidin Biotin Complex (ABC) Using Diaminobenzidine (DAB) as the chromogen. Tissue sections were counterstained with haematoxylin. The TMAs were scanned (Aperio Scanscope CS2, Leica Biosystems) and analyzed using Aperio ImageScope software v11.1.
For both HLA class I and class II a similar method of scoring was used. HLA class I heavy chain (HC-10) staining was scored for each case simultaneously with B2-microglobulin staining. The surrounding lymphocytes were used as internal positive control and also as a reference for assessing the intensity of HLA expression by HRS cells. A strong membranous staining on at least 50% of the tumor cells was identified as positive. In case the staining intensity on the tumor cells was similar to the intensity on the surrounding reactive cells, membranes in between adjacent tumor cells were evaluated. Staining could be evaluated in 105 (HLA class I) and 90 (HLA class II) cases. In the remaining cases there were not enough tumor cells or a bad tissue morphology prohibited scoring.

DNA Isolation

After deparaffinization, two to three 10μm tissue sections were incubated overnight at 55°C in 240μL of PK1 lysis buffer (10mM Tris pH 8.0, 50mM KCl, 2.5mM MgCl2, 0.45% NP40, 0.45% Tween 20, 0.01% gelatin) with 7.5μL Proteinase K (20mg/ml, Invitrogen). Proteinase K was heat inactivated for 5 min at 100°C and the supernatant was collected after a centrifugation step. The DNA concentration was determined by nanodrop-1000 spectrophotometer and diluted to a concentration of 5 and 10ng/μL for the PCR analysis.

HLA-A*01/A*02 quantitative PCR

Primers were designed for the least polymorphic regions in exons 2 and 3 of the HLA-A gene (Table 1). The HLA-A*01 forward primer (GP405) was partially matching with HLA-A*11 and A*36 alleles and the reverse primer (GP406) was also matching with the HLA-A*36 allele. As HLA-A*36 is a very rare allele (0-2%), the chances of false positive results are limited. Primer concentrations used for the amplification reactions were 1.5μM for GP405 and 2μM for GP406. For HLA-A*02, both primers specifically recognized HLA-A*02:01 and were used at a final concentration of 3μM.

Quantitative PCRs were carried out on an ABI PRISM 7900HT (Applied Biosystems) with SYBR-green in a 384-well microtiter plate and primers specific for the PTP4A1 gene were used as a quality and quantity control as described previously (19). Briefly, all reactions were performed in triplicate in a final reaction volume of 20μl consisting of 10μl SYBR® Green PCR master mix (Applied Biosystems), 2μl of each primer, 1μl milliQ and 5μl (5ng/μl) DNA. PCR conditions consisted of an initial 2-min AmpErase UNG activation step at 50°C and a 10-min hot start at 95°C, followed by 40 cycles of denaturation at 95°C for 15s and combined annealing/extension at 60°C for 1min. A melting curve was generated (95°C for 15s, 60°C for 15s, and 95°C for 15s) to verify specificity of the PCR products. Cycle
threshold (Ct) values were determined by using the default baseline setting of 3 to 15 cycles. Samples with poor DNA quality were excluded based on lack of a specific peak in the melting curve or a Ct value higher than 36 for PTP4A1. Relative abundance of HLA-A*01/A*02 was calculated by using the formula $2^{-\Delta Ct}$. Based on 30 previously HLA-A typed samples, the cut-off level for positivity was set at a $2^{-\Delta Ct}$ value of 0.1 and the cut-off level for negative at a value <0.05.

### Statistical Analysis

EBV+ cHL was compared with EBV- cHL for gender using chi-square test, median age using Mann Whitney U test and histological subtype using Fisher’s exact test. HLA class I and class II expression status was compared between EBV+ cHL and EBV- cHL using a chi-square test. To compare the frequencies of HLA-A*01 and HLA-A*02 between patients and controls, a chi-square test was used. P-values of less than 0.05 were considered significant. For all statistical tests Graph pad Prism5 software was used.

### Table 1. Primers used for the HLA-A*01/A*02 quantitative PCR

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Primer</th>
<th>Sequence* (5’ –3’ )</th>
<th>Location</th>
<th>Size</th>
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<tbody>
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<td>HLA-A*01</td>
<td>GP405</td>
<td>F: TCCGCGGGTACCGGCAGGAC</td>
<td>Exon 3</td>
<td>164bp</td>
</tr>
<tr>
<td></td>
<td>GP406</td>
<td>R: GCACCGGCCCCCTCAGGGTAAG</td>
<td>Exon 3</td>
<td></td>
</tr>
<tr>
<td>HLA-A*02</td>
<td>GP343</td>
<td>F: GAGCCCCGCTTCATCGCA</td>
<td>Exon 2</td>
<td>132bp</td>
</tr>
<tr>
<td></td>
<td>GP344</td>
<td>R: CCCGTCCCAATACTCCCGGA</td>
<td>Exon 2</td>
<td></td>
</tr>
<tr>
<td>PTP4A1</td>
<td>PTP4A1F</td>
<td>F: GCACAGCAGACCTCTATGC</td>
<td>Exon 2</td>
<td>142bp</td>
</tr>
<tr>
<td></td>
<td>PTP4A1R</td>
<td>R: CCAGGTCAGAAGCTTCTGAATGC</td>
<td>Exon 2</td>
<td></td>
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*F: forward primer, R: reverse primer

### RESULTS

#### Patient characteristics

The male to female ratio of the patient cohort was 1.2:1. The median age was 25 years (range 3-83). Nodular sclerosis (NS) was the most common subtype (78.1%) followed by mixed cellularity (MC) (18.1%). Lymphocyte depleted (LD) and lymphocyte rich (LR) subtypes were relatively rare with 0.6% and 3.1% respectively (Table 2). For three cases the subtype could not be determined and these were classified as not otherwise specified (NOS) cHL. EBER positivity was observed in tumor cells in 54 of the 158 cases (34%). EBV positivity was significantly higher in males compared to females (68.5% vs. 31.5%, P=0.02). EBV positive cases were more
commonly MC subtype and younger age patients, whereas EBV negative cases were more commonly of the NS subtype.

Table 2. Patient characteristics and comparison between EBV+ and EBV- cHL cases.

<table>
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<tr>
<th></th>
<th>All patients (n=158)</th>
<th>EBV+ cHL (n=54)</th>
<th>EBV- cHL (n=104)</th>
<th>p-value</th>
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<tbody>
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<tr>
<td>Male/female</td>
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<td>2.17</td>
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<td></td>
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<tr>
<td>Male</td>
<td>88</td>
<td>55.7%</td>
<td>37</td>
<td>68.5%</td>
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<td>Female</td>
<td>70</td>
<td>44.3%</td>
<td>17</td>
<td>31.5%</td>
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<td><strong>Median age (range)</strong></td>
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<td></td>
</tr>
<tr>
<td>All patients</td>
<td>25 (3-83)</td>
<td>25 (3-82)</td>
<td>27 (4-83)</td>
<td>0.92b</td>
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<td>24 (3-71)</td>
<td>25 (4-71)</td>
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<tr>
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<td>33 (6-82)</td>
<td>29 (11-83)</td>
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<tr>
<td><strong>Subtype</strong></td>
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<tr>
<td>Nodular sclerosis</td>
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<td>78.1%</td>
<td>30</td>
<td>56.6%</td>
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<tr>
<td>Mixed cellularity</td>
<td>28</td>
<td>18.1%</td>
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<td>41.5%</td>
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<td>0</td>
<td>0.0%</td>
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<tr>
<td>Lymphocyte rich</td>
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<td>3.2%</td>
<td>1</td>
<td>1.9%</td>
</tr>
<tr>
<td>Not otherwise specified</td>
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<td>1</td>
<td>1</td>
<td>2</td>
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<tr>
<td><strong>HLA I expression</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>30</td>
<td>28.5%</td>
<td>18</td>
<td>53%</td>
</tr>
<tr>
<td>Negative</td>
<td>75</td>
<td>71.5%</td>
<td>16</td>
<td>47%</td>
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<tr>
<td><strong>HLA II expression</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>55</td>
<td>61%</td>
<td>18</td>
<td>64%</td>
</tr>
<tr>
<td>Negative</td>
<td>35</td>
<td>39%</td>
<td>10</td>
<td>36%</td>
</tr>
</tbody>
</table>

*aChi-square test, bMann Whitney U test, cFishers exact test for NS and MC subtypes. Significant p-values are shown in bold (p<0.05)

**HLA class I and class II surface expression on tumor cells**

Cell surface expression of HLA class I heavy chain was consistent with β2-microglobulin in HRS cells of all cases. Thirty out of 105 (28.5%) cases were HLA class I positive. The EBV+ cHL group more frequently retained HLA class I expression compared to EBV- cHL (53% vs. 17%, p=0.0004). Fifty five out of 90 (61%) cases were positive for HLA class II expression with a similar percentage of positive cases for the EBV+ cHL and EBV- cHL subgroups (Table 2).
Validation of HLA-A*01/A*02 qPCR

Analysis of nine previously HLA-A genotyped control samples revealed a clear separation between samples lacking HLA-A*01 alleles and samples with one or two HLA-A*01 alleles. The relative expression values ($2^{-\Delta CT}$) varied between 0.0006 and 0.027 for negative and between 0.12 and 0.47 for positive samples. A known HLA-A*36 carrying sample had a $2^{-\Delta CT}$ value of 0.09, which was considered negative (Figure 1A). For the HLA-A*02 qPCR, analysis of 21 known samples revealed a clear separation between HLA-A*02 negative ($2^{-\Delta CT}$, 0.002-0.027), heterozygous ($2^{-\Delta CT}$, 0.63-0.1.3) and homozygous samples ($2^{-\Delta CT}$, 1.4-4.26) (Figure 1B).

HLA-A*01/A*02 carrier frequency

A total of 118 cases for HLA-A*01 and 130 cases for HLA-A*02 qPCR were successfully analyzed, the remaining cases were rejected due to bad quality of DNA in combination with low values for the housekeeping gene. Two cases had $2^{-\Delta CT}$ values falling outside the cut-off values and could not be classified as positive or negative (Figure 1 C-D). In total, 22% of all cases were positive for HLA-A*01. The HLA-A*01 carrier frequency was significantly higher in EBV+ cHL compared to the control population (32.4% vs. 18%, $p=0.02$). A similar trend was observed when we compared EBV+ cHL with EBV- cHL, however, the difference did not reach significance probably due to the low number of samples (Figure 2A).

In total, 36% of all cases were positive for HLA-A*02. The HLA-A*02 carrier frequency in the EBV+ cHL group was significantly lower compared to controls (28% vs. 45%, $p=0.02$). The HLA-A*02 carrier frequency was not statistically different between the EBV+ cHL compared to the EBV- cHL group (28% vs. 40%) probably due to a low number of samples (Figure 2B).

DISCUSSION

Epidemiological studies in cHL have shown significant variations in age and EBV positivity in relation to ethnic background and geographic location. We detected EBV in 34% of the cHL cases, which is much lower compared to other studies from Brazil that has shown EBV positivity in 55-66% in adult cHL and up to 87% in pediatric cHL cases (5, 6).

We showed downregulation of membranous HLA class I and class II expression on tumor cells in 71% and 39% of the cases, respectively. Out of 83 cases, 16 cases retained expression of both HLA class I and class II, whereas 26 cases lost expression of both HLA class I and class II. EBV positive tumor cells more frequently retained HLA class I expression. The association of EBV positivity with HLA class I
expression is consistent with findings in other studies of West European and Northern Chinese cHL populations and HLA class II expression is similar between the three populations (14, 15). Downregulation of HLA expression by the HRS cells is a possible mechanism to evade anti-tumor immune responses in part of the cases, but apparently is unusual in EBV+ cHL.

**Figure 1.** Validation and results of the HLA-A*01 and HLA-A*02 qPCR. Validation of qPCR was performed on known samples. A clear separation between non carriers (A*01 neg), heterozygous (A*01/-) and homozygous (A*01/A*01) carriers of HLA-A*01 allele and specificity of HLA-A*01 primers to HLA-A*36 is also shown (A). A clear separation between HLA-A*01 positive and negative samples is shown and for two cases 2^ΔCT values are within the cut-off values (HLA-A*01 unknown) (B). Similarly a clear separation between non carriers (A*02 neg.), heterozygous (A*02/-) and homozygous (A*02/A*02) carriers of HLA-A*02 allele is shown in C, HLA-A*02 positive and negative samples are shown in D.

With regards to HLA-A types, both HLA-A*01 and HLA-A*02 show variable allele frequencies among different ethnicities. The HLA-A*01 allele frequency is highest in the European Caucasian population (21%), intermediate in Hispanics from Brazil (10%) and low in Chinese (2-4%). HLA-A*02 has multiple suballeles and HLA-A*02:01 is by far the most frequent suballele in Caucasians (allele frequency of 29%) and
Hispanics (21%). In the Chinese other HLA-A*02 suballeles are more common with allele frequencies of 5%, 11%, 4% and 9% for HLA-A*02:01, A*02:03, A*02:06, and A*02:07 respectively (27, 28).

We previously observed a striking difference between Western European and Northern Chinese populations in HLA-A type risk pattern for EBV+ cHL. In Western Europeans HLA-A*01 is found to be a risk and HLA-A*02 to be a protective allele for developing EBV+ cHL (16, 17, 18). In the Chinese population the HLA-A*02:07 was found to be a risk allele for EBV+ cHL, while HLA-A*01 that is rare in the Chinese population did not have a significant effect.

Figure 2. HLA-A*01 and HLA-A*02 carrier frequency compared between EBV+ cHL, EBV- cHL and controls. Percentage of HLA-A*01 or A*02 carriers (black) and non-carriers (white) are indicated in bars. The HLA-A*01 carrier frequency is significantly increased in EBV+ cHL compared to controls (A). The HLA-A*02 carrier frequency is significantly decreased in EBV+ cHL compared to controls (B).

Additionally the frequency of non HLA-A*02:07 (Combined A*02 sub alleles other than A*02:07) was decreased in EBV+ cHL consistent with the Western population, albeit not significant (19). These findings indicate that the variability of HLA association is influenced by common and well defined alleles in that particular ethnic population. In Taiwanese nasopharyngeal carcinoma patients, in whom the tumor has a similar EBV latent infection as EBV+ cHL, a similar pattern was shown with HLA-A*02:07 being a risk allele, whereas no association was observed for HLA-A*02:01 (26).

Consistent with this pattern of susceptibility, earlier studies have shown that the HLA-A*02 sub alleles, HLA-A*02:01, HLA-A*02:02, HLA-A*02:03 and HLA-A*02:06 are more effective in binding and presentation of EBV LMP1 antigens than HLA-A*02:07 (21, 22). We now show that in cHL patients from Brazil the HLA-A*01
frequency was increased in the EBV+ group and the HLA-A*02 frequency was reduced compared to controls similar to the Western European cHL population (16, 17, 18) and different from the Chinese population (19).

Results in our Brazil cHL study cohort are similar to Western European cHL patients in terms of HLA susceptibility and HLA expression pattern. However, the low percentage of EBV+ cHL cases raises some concerns with respect to the ethnicity of our Brazil cHL population, which may in fact be a mix of Hispanics and Caucasians. Inclusion of consultation cases may have resulted in a selection bias towards Caucasian ethnicity. It will be essential to extend this study with a well-defined cohort of cHL patients with true Hispanic ancestry.
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REFERENCES


