Association of Killer Cell Immunoglobulin-Like Receptor Genes with Hodgkin’s Lymphoma in a Familial Study

Caroline Besson1,2,4,*, Sophie Roetynck5,6,7,*, Fionnuala Williams8, Laurent Orsi9, Corinne Amiel10, Catherine Lependevine10, Guillaume Antoni11, Olivier Hermine11, Pauline Brice12, Christophe Ferme13, Patrice Cardel13, Danièle Canioni14, Josette Brière15, Martine Raphael15,4, Jean-Claude Nicolas10, Jacqueline Clavel6,9, Derek Middleton16, Eric Vivier7,16, Laurent Abel1,2

1 Laboratoire de Génétique Humaine des Maladies Infectieuses, INSERM, U550, Paris, France, 2 Université Paris Descartes, Faculté de Médecine René Descartes, Paris, France, 3 Assistance Publique-Hôpitaux de Paris (AP-HP), Service d’Hématologie et Immunologie Biologiques, CHU Bicêtre, Le Kremlin-Bicêtre, France, 4 Université Paris Sud, Faculté de Médecine, Le Kremlin-Bicêtre, France, 5 Centre d’Immunologie de Marseille-Luminy, Université de la Méditerranée, Marseille, France, 6 INSERM, U631, Marseille, France, 7 CNRS, UMR6102, Marseille, France, 8 Northern Ireland Regional Histocompatibility and Immunogenetics Laboratory, City Hospital, Belfast, Northern Ireland, 9 INSERM, U754, Université Paris XI, Villejuif, France, 10 Laboratoire de Virologie, Hôpital Tenon, Paris, France, 11 Service d’Hématologie adultes, Hôpital Necker, Paris, France, 12 Service d’Onco-hématologie, Hôpital Saint-Louis, Paris, France, 13 Département d’Hématologie, Institut Gustave Roussy, Villejuif, France, 14 Service d’anatomopathologie, Hôpital Necker, Paris, France, 15 Service d’anatomo-pathologie, Hôpital Saint-Louis, Paris, France, 16 School of Biomedical Sciences, University of Ulster, Coleraine, Northern Ireland, 17 Assistance Publique-Hôpitaux de Marseille, Hôpital de la Conception, France

Background. Epstein-Barr virus (EBV) is the major environmental factor associated with Hodgkin’s lymphoma (HL), a common lymphoma in young adults. Natural killer (NK) cells are key actors of the innate immune response against viruses. The regulation of NK cell function involves activating and inhibitory Killer cell Immunoglobulin-like receptors (KIRs), which are expressed in variable numbers on NK cells. Various viral and virus-related malignant disorders have been associated with the presence/absence of certain KIR genes in case/control studies. We investigated the role of the KIR cluster in HL in a family-based association study. Methodology. We included 90 families with 90 HL index cases (age 16–35 years) and 255 first-degree relatives (parents and siblings). We developed a procedure for reconstructing full genotypic information (number of gene copies) at each KIR locus from the standard KIR gene content. Out of the 90 collected families, 84 were informative and suitable for further analysis. An association study was then carried out with specific family-based analysis methods on these 84 families. Principal Findings. Five KIR genes in strong linkage disequilibrium were found significantly associated with HL. Refined haplotype analysis showed that the association was supported by a dominant protective effect of KIR3DS1 and/or KIR2DS1, both of which are activating receptors. The odds ratios for developing HL in subjects with at least one copy of KIR3DS1 or KIR2DS1 with respect to subjects with neither of these genes were 0.44 (95% confidence interval 0.23–0.85) and 0.42 (0.21–0.85), respectively. No significant association was found in a tentative replication case/control study of 68 HL cases (age 18–71 years). In the familial study, the protective effect of KIR3DS1/KIR2DS1 tended to be stronger in HL patients with detectable EBV in blood or tumour cells. Conclusions. This work defines a template for family-based association studies based on full genotypic information for the KIR cluster, and provides the first evidence that activating KIRs can have a protective role in HL.

INTRODUCTION

Hodgkin’s lymphoma (HL) differs from other lymphomas in terms of both specific pathological and epidemiological features. HL is characterised by the presence of large tumour cells known as Hodgkin and Reed-Sternberg cells, derived from a germinal centre B cell [1]. The incidence of HL displays an unusual age distribution, with two peaks—between the ages of 15 to 34 years and over the age of 60 years in most Western countries, but in children and in the oldest age groups in developing countries [2]. HL is one of the most common forms of lymphoma occurring in young adults in developed countries, with an annual incidence of around 3 per 100,000 [2,3]. Both genetic and environmental factors are thought to be involved in the pathogenesis of HL [2]. There is growing evidence to suggest a genetic predisposition to HL, based on many reports of familial aggregation of the disease [4–6], including a twin study [7]. Interestingly, a review of these studies found that familial HL lacked the classic bimodal age distribution, with only one peak observed, between the ages of 15 and 34 years [6]. Several variants of the major histocompatibility complex (MHC) region have been reported to be associated with HL [8–10], but there is no consensus on the role of specific human leukocyte antigen (HLA) alleles or haplotypes in HL. In the only genome-wide scan by linkage analysis conducted to date,
suggestive evidence was obtained for a HL susceptibility locus on chromosome 4p16 [11]. The genetic basis of HL thus remains elusive.

Several findings strongly suggest that Epstein-Barr virus (EBV) is a major environmental factor contributing to oncogenesis in HL [12,13]. EBV clonal DNA is identified in the Reed-Sternberg cells in around 30% of cases of HL, and EBV infection is thought to provide survival signals for these abnormal B cells, leading to their proliferation [14]. Moreover, epidemiological studies have clearly shown that the risk of developing HL is up to three times higher in subjects with a previous history of infectious mononucleosis—the symptomatic form of primary EBV infection, particularly frequent in adolescence—than in other subjects [15]. HL patients have also been found to have high EBV antibody titres at the time of HL diagnosis, and years before and after diagnosis [16]. All these findings suggest that impairment of the immune response to EBV infection may contribute to the pathogenesis of HL. Natural killer (NK) cells are key actors of the innate immune response to viruses [17] [18], including EBV [19,20]. Their role is illustrated by the recent report of a child who developed an EBV-driven lymphoproliferative disorder associated with a novel specific NK cell deficiency [21]. Further support for an involvement of these cells in innate immunity to viruses has been provided by experimental models as susceptibility to murine cytomegalovirus (MCMV), another herpes-virus, is controlled by a single gene, Ly49H [22] (also called Kira7) encoding an NK activating receptor in mice [22,23].

The human Killer cell Immunglobulin-like receptor (KIR) genes correspond functionally to the murine Ly49 gene family, providing a potential example of convergent evolution [24]. KIRs are inhibitory or activating transmembrane receptors, present on the surface of NK cells [25]. The length of their intracytoplasmic domains—long (KIR-L), or short (KIR-S)—determines whether they are inhibitory (KIR-L) or activating (KIR-S). Inhibitory KIRs are essential for the regulation of NK cell function via interactions with MHC class I molecules [26]. The ligands of activating KIRs are less well known, although recent results have suggested that activating and inhibitory receptors recognise the same sets of peptide-MHC class I complexes, but differ in binding affinities [27]. The common Human Genome Organization (HUGO) nomenclature for KIRs will be used throughout this report (Figure 1). The HUGO nomenclature accounts for KIR protein structure and consists of four major subdivisions based on two features: the number of extracelluar Ig domains (2D or 3D) and the length of the cytoplasmic tail (L or S). The KIRs are encoded by a cluster of genes (Figure 1) varying considerably between individuals, resulting in the expression of between seven and 12 receptors [26]. KIR gene polymorphism adds further variability, with multiple alleles identified for each KIR locus, but the principal source of diversity in the KIR region remains the presence/absence of KIR genes. Several case/control studies have reported an association between the presence/absence of specific KIRs and disease progression following infection with some oncogenic viruses [25]. For example, the presence of activating KIR3DS1 in combination with its HLA class I ligand (the Bc4IBo allele) has been shown to protect against progression to acquired immuno-deficiency syndrome in HIV-infected patients [28], and against the development of hepatocellular carcinoma in hepatitis C virus-infected patients [29]. However, the presence of this gene has also been associated with an increase in the risk of human papilloma virus-induced cervical cancer [30]. Consistent with this finding, patients with EBV-associated nasopharyngeal carcinoma (NPC) tend to have more activating KIRs than controls [31].

Alltogether these data suggest that KIRs may be involved in the pathogenesis of virus-induced malignancies, although the role of activating and inhibitory receptors remains unclear. In the present work, we investigate the role of KIRs in the occurrence of HL. Our main study was based on a familial design, presenting two major advantages. First, we were able to use full genotypic information for KIR loci (number of gene copies), with the corresponding haplotypes, rather than the phenotypic information (presence/absence of the gene) used in previously published case/control designs investigating the association of KIRs with other virus-induced diseases. Second, family-based association studies are not subject to the possible confounding of gene-phenotype associations due to inappropriately chosen controls or population substructures. Our results for this familial sample show that some activating KIRs have a protective effect against HL, although this association was not reproduced in a tentative replication case/control study.

RESULTS

KIR gene content in HL patients

Our sample consisted of 90 nuclear families, including 345 subjects (90 index HL cases, 2 affected parents, 143 unaffected parents, 3 affected sibs and 107 unaffected sibs). The mean age at diagnosis of the 90 index cases of HL was 26.2 years (range 16 to 35 years). The index cases comprised 51 men (57%) and 39 women (43%). The main morphological group was nodular sclerosis (87%), followed by mixed cellularity (10%). Figure 2 shows the distribution of the presence of the variable KIRs, as determined by KIR gene content analysis for the HL index cases (n = 90). The proportion of HL cases with a positive KIR phenotype varied from 23% for KIR2DS3 to more than 95% for KIR2DL1. Standard KIR gene content analysis provides only binary phenotypic results for each KIR (absent or present), with no information about the number of copies/alleles (1 or 2) when the KIR is present. As our analysis focuses on the genotypes (number of copies) of each of the nine variable KIR genes shown in figure 1 (KIR2DS2, KIR2DL2/2DL3, KIR2DL1, KIR3DS1/3DL1, KIR2DL5, KIR2DS3, KIR2DS5, KIR2DS1, KIR2DS4), we first reconstructed the genotypes from the KIR gene content results.

Genotype reconstruction in families

The procedure used for genotypic reconstruction is described in detail in Figure 3. In the first step, genotypes could be unambiguously determined for all negative KIR phenotypes, and for the two KIR genes with known allelic forms, KIR2DL2/2DL3 and KIR3DS1/3DL1. Allele typing for KIR2DS4 distinguished between the two forms—KIR2DS4del and KIR2DS4del—making it possible to reconstruct most of the genotypes for this receptor. Finally, an analysis of familial segregation provided additional genotypic determination, resulting in 60% to 100% of the genotypes being known (Table 1), for all KIRs except KIR2DL1. The very small number of negative subjects (10/345) and the absence of additional information for KIR2DL1 made it impossible to distinguish between heterozygous KIR2DL1 (+,−) and homozygous KIR2DL1 (+,+) in most families. Based on both genotypic reconstitution and additional allele typing of the anchor gene KIR2DL4, two families with Mendelian inconsistencies were excluded. Four additional families were also excluded, as individuals did not fit the pattern of familial segregation at two adjacent loci, KIR2DL4 and KIR3DS1/3DL1, due either to Mendelian incompatibilities or to possible deletion of these loci in one haplotype. Finally, the sample subsequently used for linkage disequilibrium (LD) and association studies consisted of 84 families, comprising 322 subjects and including a total of 88 HL patients.

We then estimated pairwise LD between KIRs based on the genotypes determined in this first reconstruction step (Figure 4). As
previously reported, the KIR region was in strong LD, and two pairs of KIRs were found to be in perfect LD ($\phi^2 = 1$). KIR2DS5 and KIR2DS4 were in perfect LD—the presence of one gene in a given haplotype excluding the presence of the other, with only two haplotypes, KIR2DS5+/KIR2DS4- and KIR2DS1-/KIR2DS4+, observed. KIR2DL2 and KIR2DS2 were in perfect LD—with only the KIR2DL2+/KIR2DS2+ and KIR2DL2-/KIR2DS2- haplotypes observed. These findings, which are fully consistent with previously reported haplotypes [26], made possible the complete genotypic determination of these four KIRs. Three of the four remaining KIRs that were not fully reconstructed, KIR2DL3, KIR2DS3 and KIR2DS5, presented complete LD ($|D'| = 1$) with several other KIRs (Figure 4).

Based on these observations, genotypes at these three KIRs could be determined for most subjects. Reconstruction of the few remaining unknown KIR2DL3, KIR2DS3 and KIR2DS5 genotypes was based on previously reported haplotypes [26], and led to the genotypic determination of all but two individuals for KIR2DL5 and all but one individual for KIR2DS5. By contrast, substantial genotypic reconstruction was not possible for KIR2DL, which was not in strong LD with other KIRs. As KIR2DL1 was not expected to be informative for the association study because of its very high phenotypic frequency (>95%), this receptor was excluded from further analysis.

After these two reconstruction steps, studies subjects had known genotypes for eight KIR gene systems—KIR2DS2, KIR2DL2/2DL3,
Association study of HL with KIR genotypes

The results of the family-based study of association between HL and the eight KIR gene systems are presented in Table 2, assuming a dominant mode of inheritance, which gave the best fit in conditional logistic regression analysis. Alleles KIR3DS1+, KIR2DL5+, KIR2DS5+ and KIR2DS1+ were significantly associated (p<0.05) with a protective effect against HL, whereas allele KIR2DS4del was associated with an increase in the risk of HL. The strongest effects were observed for alleles KIR3DS1+/p<0.006, and KIR2DS1+ (p<0.01). Under a model of dominant inheritance, the odds ratio (OR) for developing HL in subjects homozygous KIR3DS1+/+ or heterozygous KIR3DS1+/− with respect to subjects homozygous KIR3DS1−/− was 0.44 [95% confidence interval: 0.23–0.85]. Similarly, the OR for KIR2DS1+/+ or KIR2DS1−/− subjects with respect to KIR2DS1−/− was 0.42 [0.21–0.85].

As the five KIR genes significantly associated with HL were in strong LD (Figure 4), the next step was to carry out both multivariate and haplotype analyses. Multivariate conditional logistic regression demonstrated that the association between HL and the KIR region was supported by a single signal. Once one of the five associated KIR genes was entered in the regression model, particularly for the most significant system, KIR3DS1+/3DL1, the other four genes ceased to have a significant effect. Haplotype analysis provided further detail. It showed that, due to LD, the five associated KIR genes were organised into only five common haplotypes (frequency>0.02), accounting for 96% of the haplotypes observed in our population (Table 3). The two haplotypes significantly associated with a protective effect against HL (H2 and H5 in Table 3) contained the same alleles for KIR3DS1 (+), KIR2DL5 (+), KIR2DS1 (+), and KIR2DS4 (−), but different alleles for KIR2DS5 (+ and −, respectively). We also noted that the two protective haplotypes H2 and H5 did not carry KIR2DS4, whereas the risk-associated haplotype (H1) carried the deleted form, resulting in the production of a truncated protein predicted to have no functional effect [34,35]. These observations made KIR2DS5 and KIR2DS4 unlikely candidates for the observed effect. Finally, haplotypes H3 and H4 which carried different KIR2DL5 alleles, seemed to have opposite effects, although this difference was not significant. Thus, these results suggest that the protective effect against HL is mediated by the presence of KIR3DS1 or KIR2DS1 or both genes.

We then investigated whether the association between KIR3DS1, KIR2DS1 and HL was influenced by the HLA class I status of the patients, according to their known ligand specificity. For KIR3DS1, we considered the HLA-B genotype, as determined by the Bw4I80 allele and, for KIR2DS1, we considered the HLA-C genotype, as determined by the C2 allele. No significant heterogeneity (p>0.79) was found in the association between KIR3DS1 and HL, according to the presence or absence of the Bw4I80 allele (Table 4). For KIR2DS1, the protective effect seemed to increase with the number of HLAC2 alleles, although the heterogeneity test was not significant (p = 0.35) (Table 4). The OR decreased from 0.52 [0.17–1.65] for C1/C1 homozygotes to 0.37 [0.15–0.90] for subjects with at least one C2 allele. Interestingly, when we considered the presence of both ligands (i.e. patients with at least one Bw4I80 allele and one C2 allele), the protective effect of KIR2DS1 and KIR3DS1 tends to be stronger with an OR of 0.21 [0.04–1.07] for both KIR alleles. However, the number of informative families in this latter analysis was small (seven families), precluding to draw definitive conclusion from this result. Finally, we investigated the role of some of the characteristics of EBV-infected patients, focusing on the EBV status of the tumour (positive/negative) or EBV load in peripheral blood mononuclear cells (detectable/undetectable). No significant heterogeneity in the association between HL and KIR3DS1 or KIR2DS1 was found for any of these characteristics (Table 4). However, the protective effect seemed to be stronger in patients with EBV-positive tumours or detectable viral load. This effect was clearest for KIR3DS1, with an OR of 0.27 [0.08–0.87] for patients with detectable viral load (40% of patients) versus 0.61 [0.27–1.37] for those with no detectable viral load (60%).

We tried to replicate these results by comparing KIR phenotype frequencies in the independent sample from the ENGELA study consisting of 68 HL patients and 60 controls. In this sample, no significant association was observed between any KIR phenotype and HL. Further analyses stratified for age (e.g. restricted to the 15–35 age class), HLA class I genotype, and the EBV status of tumour tissues identified no HL subgroup associated with KIR3DS1 or KIR2DS1. In this sample, the frequencies of positive phenotypes for KIR3DS1 and KIR2DS1 among HL cases were 43% (29/68) and 41% (28/68), respectively. These frequencies were higher than for the 84 index HL cases of the familial study (33% for both KIR phenotypes), although these differences were not significant (p = 0.27 and 0.24 respectively).

DISCUSSION

We report here the first family-based association study to show an association between a disease and genes of the KIR cluster. Familial studies have been conducted to determine the structure of the KIR region [26,36], but the previously reported associations between the presence/absence of KIRs and various disease phenotypes were identified in case/control studies [25]. Family-based association studies have important advantages over case/control studies in that they can make use of full genotypic information at KIR loci (number of gene copies) and make it possible to avoid the possible confounding of gene-phenotype associations due to inappropriately chosen controls or population...
**Figure 3. Genotypic reconstruction.** KIR gene content analysis provides only binary phenotyping results for each KIR (absent or present), rather than complete genotypic information. Thus, for genotype analysis (i.e. the number of copies of each KIR gene), genotypic reconstruction in two successive steps was required. The first step combined the results of KIR gene content analysis, knowledge of allelic forms of KIRs, the additional allele typing carried out for three KIR genes, and the analysis of intra-familial segregation. KIR gene content made it possible to determine genotype unambiguously only in individuals with a negative phenotypic result. For example, an individual with a negative KIR2DS2 phenotype has no copy of the gene, and its genotype can be deduced and denoted KIR2DS2(2,2). Conversely, an individual with a positive phenotype for KIR2DS2 may have either one copy of the gene, denoted KIR2DS2(+,−), or two copies of the gene, denoted KIR2DS2(+,+). Two of the nine variable KIR genes have known allelic forms: KIR2DL2/2DL3 and KIR3DS1/3DL1. We defined as (+,+), the KIR3DS1/3DL1 genotype of subjects homozygous for the presence of KIR3DS1 (phenotype KIR3DS1+, KIR3DL1−) and as (−,−) the genotype of subjects homozygous for the absence of KIR3DS1 (phenotype KIR3DS1−, KIR3DL1+). An individual with the KIR3DS1+, KIR3DL1− phenotype has one copy of each gene and is therefore heterozygous at the locus, with genotype KIR3DS1/3DL1(+,−). The genotypes of KIR2DL2/2DL3 followed the same principle. Additional KIR allele typing was especially useful for the analysis of familial gene segregation. In particular, for KIR2DS4, it made it possible to distinguish between the two common forms, 2DS4wt and 2DS4del (Figure 1). The second step of the reconstruction was based on the KIR haplotype determination of individuals in each family. We first estimated the pairwise linkage disequilibrium (LD) pattern between KIR genes in our families, using the genotypic information obtained from the first step of reconstruction (see statistical analysis). Perfect and complete LD between pairs of KIRs made it possible to infer certain genotypes. For the few remaining unsolved KIR genotypes, haplotypic reconstruction was based on previously described haplotypes [26,33].

doi:10.1371/journal.pone.0000406.g003

**Table 1. Summary of the genotypic reconstruction results for the nine variable KIR genes in 322 subjects.** The procedure of reconstruction is detailed in figure 3.

| KIR       | 2DS2 | 2DL2/2DL3 | 2DL1 | 3DS1/3DL1 | 2DL5 | 2DS3 | 2DS5 | 2DS1 | 2DS4 |
|-----------|------|-----------|------|-----------|------|------|------|------|------|
| Patients with known genotype: |      |           |      |           |      |      |      |      |      |
| - after the 1st step N (%) | 216 (67) | 322 (100) | 27 (8) | 322 (100) | 231 (72) | 285 (89) | 280 (87) | 268 (83) | 193 (60) |
| - after the 2nd step N (%) | 322 (100) | 322 (100) | 27 (8) | 322 (100) | 320 (99) | 321 (100) | 322 (100) | 322 (100) | 322 (100) |
| Allele frequency* | 0.33 | 0.33/0.67 | 0.25 | 0.25/0.75 | 0.29 | 0.15 | 0.19 | 0.23 | 0.77 |

*estimated from all the reconstructed genotypes after the 2nd step.
*excluded after the second step of reconstruction.

doi:10.1371/journal.pone.0000406.t001
admixture. A first phase of genotypic reconstruction is required, based principally on a combination of information from the familial data. Published data were required for the full determination of only a minority of genotypes (for \textit{KIR2DL5}, \textit{KIR2DS3} and \textit{KIR2DS5}), and \textit{KIR2DL1} was the only locus that could not be reconstructed, due to its high frequency. We propose a method of \textit{KIR} genotypic reconstruction in familial samples that could be applied to other family-based studies, as already demonstrated for a sample of African origin (S.R. \textit{et al}., unpublished data).

Host genetic factors are known to be important in HL pathogenesis [4–7,11], and many association studies have suggested a possible association between HL and HLA genes [8–10]. Given the strong link between EBV and HL and the role of HLA in the presentation of viral antigens, EBV specificities in the role of HLA genes in HL have recently been investigated. An association was found between EBV-positive HL and markers located in the HLA class I region, whereas EBV-negative HL was found to be associated with markers of the HLA class III region [10]. However, no consensus has yet emerged on the role of any specific HLA molecule [8–10]. \textit{KIR}s were considered as candidate genes for HL predisposition in this study since they are alternative receptors for HLA class I molecules.

Our family-based analysis provides the first evidence for an association between HL and certain \textit{KIR} alleles in strong linkage disequilibrium. Refined haplotype analysis results were consistent with a dominant protective effect of \textit{KIR3DS1} and/or \textit{KIR2DS1} against HL. The validity of our familial analysis is also strengthened by the observation of random transmission of the \textit{KIR} alleles considered in the 100 unaffected children of our sample (i.e. the healthy siblings of HL patients). We tried to replicate this association, using a case/control sample collected in the context of a French survey on adult lymphoma and including 68 HL patients. No significant association between HL and the presence/absence of any \textit{KIR}s was found. The HL patients did not differ between the two samples in terms of ethnic origin and clinical features. EBV viral load in peripheral blood mononuclear cells was not determined in the case/control study, whereas we found that the protective effect of \textit{KIR3DS1} and/or \textit{KIR2DS1} in familial sample

\textbf{Figure 4.} Pairwise linkage disequilibrium between \textit{KIR} genes in the family sample. This analysis was based on the genotypes reconstructed after the first step, without using any information about LD pattern between \textit{KIR} genes. Each square represents the magnitude of pairwise LD between \textit{KIR} genes, as measured by D' (panel A) or r² (panel B). Red squares represent a |D'| or r² value of 1.0, corresponding to complete and perfect LD, respectively. Orange, yellow, and white squares indicate |D'| or R² values of [0.75-1[, [0.5-0.75[, and <0.5, respectively.

doi:10.1371/journal.pone.0000406.g004

\begin{table}[h]
\centering
\caption{Results of the family-based association study between \textit{KIR} genes and Hodgkin’s lymphoma using a dominant model}
\begin{tabular}{|l|c|c|c|c|c|}
\hline
\textit{KIR} & frequency present allele & N informative families\(^a\) & p-value\(^b\) & Odds Ratio [95\% CI]\(^c\) & Effect \\
\hline
\textit{2DL2} & 0.33 & 37 & 0.89 & 1.13 [0.55–2.34] & \_ \\
\textit{2DL2/2DL3}\(^d\) & 0.33/0.67 & 37 & 0.89 & 1.13 [0.55–2.34] & \_ \\
\textit{3DS1/3DL1}\(^e\) & 0.25/0.75 & 41 & 0.006 & 0.44 [0.23–0.85] & protective \\
\textit{2DL5} & 0.29 & 41 & 0.02 & 0.56 [0.30–1.04] & protective \\
\textit{2DS3} & 0.15 & 37 & 0.11 & 0.65 [0.34–1.25] & \_ \\
\textit{2DS5} & 0.19 & 31 & 0.05 & 0.49 [0.23–1.04] & protective \\
\textit{2DS1} & 0.23 & 37 & 0.01 & 0.42 [0.21–0.85] & protective \\
\textit{2DS4del}\(^f\) & 0.58 & 30 & 0.03 & 2.22 [0.94–5.27] & at risk \\
\textit{2DS4wt}\(^f\) & 0.19 & 30 & 0.67 & 1.02 [0.50–2.1] & \_ \\
\hline
\end{tabular}
\begin{flushleft}
\(\text{\(^a\)Informative families are those with at least one heterozygous (+,−) parent for the corresponding \textit{KIR} gene.}\) \\
\(\text{\(^b\)computed using the FBAT software.}\) \\
\(\text{\(^c\)computed by conditional logistic regression.}\) \\
\(\text{\(^d\)association results for allelic forms of the same \textit{KIR} locus are provided for the first variant of the pair.}\) \\
\(\text{\(^e\)deleted form}\) \\
\(\text{\(^f\)wild type}\) \\
\end{flushleft}
\end{table}
tended to be stronger in patients with detectable viral load. We also restricted the analysis to the 34 patients belonging to the same age class (15–35 years) as our familial patients, corresponding to the first peak of HL incidence in developed countries, but no significant association was observed in this small sample. In any case, a larger sample is certainly required for a more powerful replication study.

Due to strong linkage disequilibrium, it was almost impossible to distinguish between the effects of KIR2DS1 and KIR3DS1. The observed trend for the protective effect of KIR2DS1 to increase in the presence of its putative ligand, HLA-C2, may be more consistent with a role for this receptor although the effectives of each strata are small. We cannot exclude the possibility that both these KIRs are responsible for the observed protective effect, as different cognate KIR-HLA class I pairs may have the same biological effects, since, for example, all activating KIR-S share the same KARAP/DAP12-dependent signalling pathways [37]. This hypothesis is supported by the trend toward a stronger protective effect in presence of at least one allele of each HLA class I ligands of KIR3DS1 and KIR2DS1, and the even greater trend in presence of both ligands. Our results are consistent with those of a study showing the protective effect of KIR3DS1 against HCV-related hepatocarcinoma [29], and with the general idea that activating KIRs are beneficial for the response to infectious diseases and tumours. However, immune activation may not be beneficial at all stages of these disorders and could, for example, increase the risk of other tumours associated with local inflammation [25]. This may account for two other reports of activating KIRs being associated with an increase in the risk of virus-related localised cancers, such as NPC [31] and cervical cancer [30].

We observed an interesting trend towards a stronger protective effect of KIR2DS1 and/or KIR3DS1 in the presence of EBV,

### Table 3. Association study based on haplotypes constructed from five KIR genes

| Haplotype | 3DS1 | 2DS5 | 2DS1 | 2DS4 | Estimated frequency | N fam | p-value | Effect |
|-----------|------|------|------|------|---------------------|-------|---------|--------|
| H1 | - | - | - | - | del* | 0.55 | 31 | 0.04 | at risk |
| H2 | + | + | + | + | - | 0.15 | 29 | 0.03 | protective |
| H3 | - | - | - | - | wt* | 0.15 | 30 | 0.54 | _ |
| H4 | - | + | - | - | del* | 0.06 | 16 | 0.56 | _ |
| H5 | + | + | - | + | - | 0.05 | 11 | 0.03 | protective |

*computed by conditional logistic regression

*computed by the FBAT software using a dominant model

*deleted form

*wild type

doi:10.1371/journal.pone.0000406.t003

### Table 4. Effect of KIR3DS1 and KIR2DS1 stratified on HL patients characteristics

| HL cases | KIR3DS1 | | KIR2DS1 | |
|----------|---------|-----------------|---------|-----------------|
| N informative families | Odds-Ratio [95% CI]* | p* | N informative families | Odds-Ratio [95% CI]* | p* |
| All | 41 | 0.44 [0.23–0.85] | 0.006 | 37 | 0.42 [0.21–0.85] | 0.01 |
| HLA-B | | | | |
| No Bw4I80 allele | 30 | 0.46 [0.21–1.01] | 0.01 | - | - | - |
| At least one Bw4I80 | 11 | 0.38 [0.11–1.31] | 0.24 | - | - | - |
| HLA-C | | | | |
| C1/C1 | - | - | - | 13 | 0.52 [0.17–1.65] | 0.23 |
| At least one C2 | - | - | - | 24 | 0.37 [0.15–0.90] | 0.02 |
| HLA-B and -C combined | | | | |
| C1/C1 or no Bw4I80 | 34 | 0.51 [0.25–1.06] | 0.04 | 30 | 0.52 [0.23–1.10] | 0.10 |
| At least one Bw4I80 and one C2 | 7 | 0.21 [0.04–1.07] | 0.03 | 7 | 0.21 [0.04–1.07] | 0.03 |
| EBV in situ | | | | |
| EBV+ | 8 | 0.24 [0.05–1.24] | 0.09 | 7 | 0.28 [0.05–1.51] | 0.14 |
| EBV– | 22 | 0.37 [0.14–0.97] | 0.02 | 19 | 0.37 [0.13–1.04] | 0.04 |
| EBV load in PBMCs | | | | |
| detectable | 16 | 0.27 [0.08–0.87] | 0.02 | 15 | 0.28 [0.09–0.92] | 0.03 |
| undetectable | 24 | 0.61 [0.27–1.37] | 0.15 | 22 | 0.54 [0.22–1.23] | 0.17 |

*computed by conditional logistic regression

*computed by the FBAT software under a dominant model

*at least one present allele. Only two HL patients are Bw4I80 homozygotes.

*missing in 11 cases

*missing in 1 case

*PBMCs corresponds to peripheral blood mononuclear cells

doi:10.1371/journal.pone.0000406.t004
detected in either HL tumour cells or in peripheral mononuclear blood cells. This suggests that the protective role of activating KIRs may be more pronounced in HL cases associated with EBV. NK cells were recently shown to limit lytic EBV infection through the down-regulation of MHC class I molecules that bind to inhibitory KIRs, and up-regulation of activating ligands such as ULBP-1 and CD112 [30]. An indirect control of virus replication by cytokines produced by NK cells may also be involved in this protective effect, as shown for mouse cytomegalovirus [39]. We favor another model for the potential role of NK cells in the control of HL via activating KIRs. The malignant Hodgkin/Reed-Sternberg (HRS) cells of EBV-associated HL display high-level expression of MHC class I molecules [12,13,41]. The up-regulation of MHC class I peptide complexes in HRS cells would then trigger NK cell effector functions via activating KIRs, leading to the participation of NK cells in the control of HL. This hypothesis is consistent with previous biological findings as KIR2DS1 has been shown to recognize peptide-HLA class I complexes that are up-regulated on EBV human infected B cells [27]. Similarly, the control of mouse cytomegalovirus infection in certain strains of mice involves the recognition of peptide-MHC class I complexes through the activation of Ly49 receptors [40]. To further confirm our hypothesis, it will be of particular interest to follow the activation of NK and T cells subsets according to the presence of KIR2DS1 and/or KIR3DS1 when the detection of these receptors will be possible. In any case, the recognition of HL cells by KIRs warrants further investigation, and we are currently carrying out an immunohistological study, comparing the presence of NK cells in EBV-positive and EBV-negative HL specimens.

METHODS

Study population

HL index cases were recruited from three haematology units in the Paris area. Patients were included at diagnosis (n = 21) or whilst in complete remission, at least one year after diagnosis (n = 69). Inclusion criteria were age between 15 and 35 years and negative serological tests for HIV at diagnosis. Tumour biopsies were reviewed by expert haematological pathologists [JB, DC, MR]. Intratumoral EBV expression was assessed by mRNA in situ hybridisation for Epstein-Barr virus-encoded small non polyadenylated RNA (EBER1) sequences or by indirect immunofluorescence staining for latent membrane protein 1 (LMP1). First-degree relatives (parents and siblings) of the patients were asked to participate in the study. All participating subjects were interviewed to determine their medical history, and all gave a blood sample for DNA extraction and EBV measurements. EBV viral load was measured in peripheral blood mononuclear cells, as previously described [42]. All recruited families (n = 90) included the index HL case and at least two other individuals (either both parents or one parent and one unaffected sibling). Five of the 90 families were multiplex (three families with two affected siblings, and two families with an affected parent and an affected child).

In addition to this family sample, we also studied a case-control sample recruited in the framework of the ENGELA (Environnement et Génétique des Lymphomes de l’Adulte) study on adult lymphoma. This study was conducted in six French cities (Brest, Bordeaux, Caen, Lille, Nantes and Toulouse), and included newly diagnosed HIV-negative HL cases confirmed by histology. Controls and cases were matched for centre, sex, age (±3 years) and ethnic origin, with the controls hospitalised or consulting at the same hospitals as the cases, but for reasons other than cancer, occupational diseases or injuries, and tobacco-or alcohol-related diseases. DNA samples were available for 68 Caucasian cases (52 with nodular sclerosis, 11 with mixed cellularity, 5 with HL of another or an unspecified type), including 40 men and 28 women, and 60 Caucasian controls (36 men and 24 women), mostly from orthopaedic departments. The subjects included were aged 18 to 71 years, and 32 cases and 23 controls were less than 35 years old. The EBV status of tumour tissue was available for 46 cases, 13 (28%) of which were positive.

Both studies were approved by the French Consultative Committee for Protecting Persons in Biomedical Research (CCPPRB) of Paris Necker and Kremlin-Bicêtre, respectively. Human experimentation guidelines were followed in the conduct of the research. Informed consent was obtained from adults or from parents of minors.

Molecular methods

KIR gene content

KIR gene content was determined with an updated version of the previously reported PCR-SSOP technique [43], which consists in oligonucleotide primers and probes selected from the first KIR gene typing system, as described in [44]. Briefly, we used two separate locus-specific PCR to amplify the D1, D2 and transmembrane/cytoplasmic tail regions. The D1 and D2 regions were amplified together, with primers flanking exons 4 and 5; 5’AGAGAXXGTCATCCTGCA/A/GTGTTGCTGGCT 3’ (exon 4, nucleotides 56-82) and 5’TCTACCTXTGAGC/G/AAAGG- AAGGAGTGGG 3’ (intron 5-273 exon 5). The transmembrane/cytoplasmic tail region was amplified as previously described. PCR products were blotted onto nylon membranes and hybridised with 19 digoxigenin-labelled sequence-specific oligonucleotide probes, using hybridisation and chemiluminescence procedures, as previously described. Internal control samples, identified with a unique probe pattern, were also included in each experiment.

Additional KIR allele typing

KIR2DL4, KIR2DS4 and KIR3DS1/3DL1 alleles were identified using previously described SSOP techniques, based on a gene-specific PCR product [36,45,46]. KIR2DL4 allele typing consists involves the PCR amplification of a 1440 bp region of KIR2DL4 exons 3/5 (D0/D2 domains), followed by hybridisation, using 16 digoxigenin-labelled probes for allele discrimination. The KIR2DS4 allele typing system is based on the specific amplification of the 199/221bp region of KIR2DS4 exon 5 (D2 domain) and 11 probes were used for allele identification. Finally, the KIR3DS1/3DL1 allele typing strategy uses three separate specific amplifications of the extracellular D0 (exons2/3), D1 and D2 domains (exons 4/5) and a section of the cytoplasmic tail (exons 8/9), respectively. The third amplification concerned a single region of KIR3DS1 only, and was required for discrimination between certain KIR3DS1 alleles. The amplified DNA was subjected to SSOP typing, using a total of 28 oligonucleotide probes, as previously reported. In each system, a set of nine internal control samples with a unique pattern of probe reactivity was included among the samples investigated. Computational analysis was carried out on the data obtained with the various probes, to identify the alleles of each gene present.

HLA class I KIR ligand typing

The study was focused on the identification of HLA-Bw4 and HLA-C1/C2 KIR ligands, that were typed using updated methods, as described in [47]. Briefly, we used four probes (BL20, BL21, BL22, BL23) to define HLA-Bw4, with the same PCR conditions as the previously report typing system for HLA-B [48]. These probes are able to distinguish threonine or isoleucine present at position 80 of the HLA-Bw4 molecules. Similarly, HLA-C1 and C2 groups were defined using only two probes from the full HLA-C allele typing system [49], which differentiated nucleotides at positions 77 and 80. These probes were C293 for C1 group and C291 for C2 group.
Statistical analysis
Hardy-Weinberg equilibrium was tested after genotypic reconstruction for each KIR gene, using all the parents of the families. Measures of pairwise linkage disequilibrium (LD) between KIR genes, such as D^2 and r^2 [50], were estimated using Haploviz (http://www.broad.mit.edu/personal/jcbarrett/haplo/). |D'| and r^2 vary between 0 and 1. Complete LD in a pair, defined as |D'| = 1, is obtained when one of the four possible haplotypes is not observed. Perfect LD between two KIR genes, defined as r^2 = 1, is reached when two of the four possible haplotypes are not observed, i.e |D'| = 1 and the frequencies of the (+, +) alleles of the two KIR genes are equal.

The main association study was conducted on the family sample, using specific methods developed in this context. Family-based association studies avoid the possible confounding of gene-phenotype associations due to inappropriately chosen controls that can occur in case/control studies. The general principle of these studies is to search for a distortion of the transmission of alleles from parents to affected offspring, by means of the “transmission/disequilibrium test” (TDT) [51]. Families with one missing parent (30/84 in our sample) can be analysed either by reconstructing parental genotypes from children (RC-TDT) [52], or by using unaffected siblings as controls (Sib-TDT) [53]. The family-based association study was carried out mainly according to the method implemented in the FBAT program [54], which combines these three different methods (TDT, RC-TDT, and Sib-TDT). This method also allows the use of an empirical variance-covariance estimator for the statistic, which is consistent when sibling marker genotypes are equal. [e.g. when the analysis includes multiple affected sibs] [55]. Finally, alleles for which evidence of association was obtained were also analysed by conditional logistic regression, as previously described [56]. This analysis generated odds-ratio (OR) estimates and made it possible to compare different models of dominance (recessive, dominant or additive). It also made it possible to test for heterogeneity of the sample according to a qualitative criterion (e.g. HLA class I genotypes) by performing the analysis on the whole sample, and separately on subsamples, as described elsewhere [57]. For the association study conducted on the case/control sample, KIR phenotype distributions were compared between HL patients and controls, using classical chi-squared tests. Conditional logistic regression analyses (proc PHREG) and chi-squared tests (proc FREQ) were carried out with SAS software (version 0.2, SAS Institute, Cary, NC).

ACKNOWLEDGMENTS
We would like to thank J.L. Casanova, A. Alcaín, S. Plancoûlaine, and all members of the Laboratory of Human Genetics of Infectious Diseases for helpful discussions. We are grateful to C. Mulet (INSERM U490, Paris, France), P.Rinçé (Hôpital Bicêtre, Le Kremlin-Bicêtre, France) and F. Zatla (Hôpital Ténon, Paris, France) for assistance with the management of biological and pathological samples and to all staff of the Northern Ireland Histocompatibility and Immunogenetics Laboratory for precious help in this study. We also thank the patients and their families for their trust and willingness to participate in this study, and R. Delarue and J. Vangafit (Hôpital Necker, Paris, France) for identifying patients. Thanks are also due to J.L. Bresson, A. Mogent and all members of the Centre d'Investigation Clinique de l'Hôpital Necker (Paris, France) for the cordial reception of their patients and their families.

Author Contributions
Conceived and designed the experiments: EV LA DM CB JC. Performed the experiments: EV JB CA JN CL MR SR FW DC. Analyzed the data: LA CB GA LO SR. Contributed reagents/materials/analysis tools: OH JA CB GA LO SR. Contributed to the writing of the paper: EV JB CA JN CL MR SR FW DC. Analyzed the data: OH. Wrote the paper: EV LA CB GS. Reviewed and approved the final version of the manuscript: EV DM CB PB JB CA JN CL MR SR FW DC.

REFERENCES
1. Re D, Kuppers R, Diehl V (2005) Molecular pathogenesis of Hodgkin’s lymphoma. J Clin Oncol 23: 6579–6636.
2. Cartwright RA, Watkins G (2004) Epidemiology of Hodgkin’s disease: a review. Hematol Oncol 22: 11–26.
3. Hjalgrim H, Amland J, Pukkala E, Hansen S, Munksgaard L, et al. (2001) Incidence of Hodgkin’s disease in Nordic countries. Lancet 357: 297–298.
4. Grufferman S, Cole P, Smith PG, Lubes R (1977) Hodgkin’s disease in siblings. N Engl J Med 296: 240–250.
5. Shugart YY, Hemminki K, Vaittinen P, Kingman A, Dong C (2000) A genetic study of Hodgkin’s lymphoma: an estimate of heritability and anticipation based on the familial cancer database in Sweden. Hum Genet 106: 533–536.
6. Ferraris AM, Racchi O, Raperzi D, Gaetani GF, Boletto P (1997) Familial Hodgkin’s disease: a disease of young adulthood? Ann Hematol 74: 131–134.
7. Mack TM, Cozen W, Shihata DK, Weiss LM, Nathwein BN, et al. (1995) Concordance for Hodgkin’s disease in identical twins suggesting genetic susceptibility to the young-adult form of the disease. N Engl J Med 332: 413–418.
8. Hors J, Dassa J (1983) HLA and susceptibility to Hodgkin’s disease. Immunol Rev 70: 167–192.
9. Harty LC, Lin AY, Goldstein AM, Jaffe ES, Carrington M, et al. (2002) HLA-DQ, HLA-DQ, and TAP genes in familial Hodgkin disease. Blood 99: 690–693.
10. Diez-Zauro A, Nens M, Vellenga E, van Imhoff GW, Nolte IM, et al. (2005) Association with HLA class I in Epstein-Barr-virus-positive and with HLA class III in Epstein-Barr-virus-negative Hodgkin’s lymphoma. Lancet 365: 2216–2224.
11. Goldin LR, McMaster ML, Ter-Minassian M, Saldemirre S, Harmes B, et al. (2005) A genome screen of families at high risk for Hodgkin lymphoma: evidence for a susceptibility gene on chromosome 4. J Med Genet 42: 595–601.
12. Gandhi MK, Tellam JT, Khanna R (2004) Epstein-Barr virus-associated Hodgkin’s lymphoma. Br J Haematol 125: 267–281.
13. Thomas RK, Re D, Wolf J, Diehl V (2004) Part I: Hodgkin’s lymphoma—molecular biology of Hodgkin and Reed-Sternberg cells. Lancet Oncol 5: 11–18.
14. Thorley-Lawson DA, Gross A (2004) Persistence of the Epstein-Barr virus and the origins of associated lymphomas. N Engl J Med 350: 1320–1337.
15. Hjalgrim H, Amland J, Rostgaard K, Hamilton-Dutoit S, Frisch M, et al. (2003) Characteristics of Hodgkin’s lymphoma after infectious mononucleosis. N Engl J Med 349: 1239–1243.
immunoglobulin-like receptor 3DS1 gene against the development of hepatocellular carcinoma in patients with hepatitis C virus infection. J Infect Dis 192: 162–163.

30. Carrington M, Wang S, Martin MP, Gao X, Schiffman M, et al. (2005) Hierarchy of resistance to cervical neoplasia mediated by combinations of killer immunoglobulin-like receptor and human leukocyte antigen loci. J Exp Med 201: 1069–1075.

31. Butsch Kowacic M, Martin M, Gao X, Fukunoko T, Chen Cj, et al. (2005) Variation of the killer cell immunoglobulin-like receptors and HLA-C genes in nasopharyngeal carcinoma. Cancer Epidemiol Biomarkers Prev 14: 2673–2677.

32. Denis I, Sivula J, Gourraud PA, Kerdudou N, Chout R, et al. (2005) Genetic diversity of KIR natural killer cell markers in populations from France, Guadeloupe, Finland, Senegal and Reunion. Tissue Antigens 66: 267–276.

33. Uhrberg M, Parham P, Wernet P (2002) Definition of gene content for nine common group B haplotypes of the Causcasoid population: KIR haplotypes contain between seven and eleven KIR genes. Immunogenetics 54: 221–229.

34. Hu K, Liu X, Selvakumar A, Mickelson E, O'Reilly RJ, et al. (2002) Killer Ig-like receptor haplotype analysis by gene content: evidence for genomic diversity with a minimum of six basic framework haplotypes, each with multiple subsets. J Immunol 169: 5118–5129.

35. Maxwell LD, Wallace A, Middleton D, Curran MD (2002) A common KIR2DS4 deletion variant in the human that predicts a soluble KIR molecule analogous to the KIR1D molecule observed in the rhesus monkey. Tissue Antigens 60: 254–258.

36. Halfpenny IA, Middleton D, Barnett YA, Williams F (2004) Investigation of killer cell immunoglobulin-like receptor gene diversity: IV. KIR3DL1/S1. Hum Immunol 65: 613–621.

37. Vivier E, Nunes JA, Vely F (2004) Natural killer cell signaling pathways. Science 306: 1517–1519.

38. Pappworth I, Wang S, Rowe M (2007) The Switch from Latent to Productive CMV infection. J Immunol 169: 5118–5129.

39. Loh J, Chu DT, O'Guin AK, Yokoyama WM, Virgin HWt (2005) Natural killer cell immunoglobulin like receptor gene diversity: II. KIR2DS4. Hum Immunol 65: 613–621.

40. Parham P (2005) MHC class I molecules and KIRs in human history, health and survival. Nat Rev Immunol 5: 201–214.