Allele-Level Haplotype Frequencies and Pairwise Linkage Disequilibrium for 14 KIR Loci in 506 European-American Individuals

Cynthia Vierra-Green, David Roe, Lihua Hou, Carolyn Katovich Hurley, Raja Rajalingam, Elaine Reed, Tatiana Lebedeva, Neng Yu, Mary Stewart, Harriet Noreen, Jill A. Hollenbach, Lisbeth A. Guethlein, Tao Wang, Stephen Spellman, Martin Maiers

1 Center for International Blood and Marrow Transplant Research, Minneapolis, Minnesota, United States of America, 2 National Marrow Donor Program, Minneapolis, Minnesota, United States of America, 3 Departments of Oncology and Pediatrics, Georgetown University, Washington D.C., United States of America, 4 UCLA Immunogenetics Center, Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at UCLA, University of California Los Angeles, Los Angeles, California, United States of America, 5 American Red Cross, HLA Laboratory, Dedham, Massachusetts, United States of America, 6 University of Minnesota Medical Center, Fairview, Minneapolis, Minnesota, United States of America, 7 Children's Hospital Oakland Research Institute, Oakland, California, United States of America, 8 Stanford University, Stanford, California, United States of America, 9 Center for International Blood and Marrow Transplant Research, Milwaukee, Wisconsin, United States of America

Abstract

The immune responses of natural killer cells are regulated, in part, by killer cell immunoglobulin-like receptors (KIR). The 16 closely-related genes in the KIR gene system have been diversified by gene duplication and unequal crossing over, thereby generating haplotypes with variation in gene copy number. Allelic variation also contributes to diversity within the complex. In this study, we estimated allele-level haplotype frequencies and pairwise linkage disequilibrium statistics for 14 KIR loci. The typing utilized multiple methodologies by four laboratories to provide at least 2x coverage for each allele. The computational methods generated maximum-likelihood estimates of allele-level haplotypes. Our results indicate the most extensive allele diversity was observed for the KIR framework genes and for the genes localized to the telomeric region of the KIR A haplotype. Particular alleles of the stimulatory loci appear to be nearly fixed on specific, common haplotypes while many of the less frequent alleles of the inhibitory loci appeared on multiple haplotypes, some with common haplotype structures. Haplotype structures CA01 and/or TA01 predominate in this cohort, as has been observed in most populations worldwide. Linkage disequilibrium is high within the centromeric and telomeric haplotype regions but not between them and is particularly strong between centromeric gene pairs KIR2DL5-KIR2DS55 and KIR2DS35-KIR2DL1, and telomeric KIR3DL1-KIR2DS4. Although 93% of the individuals have unique pairs of full-length allelic haplotypes, large genomic blocks sharing specific sets of alleles are seen in the most frequent haplotypes. These high-resolution, high-quality haplotypes extend our basic knowledge of the KIR gene system and may be used to support clinical studies beyond single gene analysis.

Introduction

The natural killer (NK) cell immunoglobulin-like receptor (KIR) genes are clustered in a ~160 kilobase (kb) region in the leukocyte receptor gene complex on the long arm of human chromosome 19 [1,2,3]. The KIR gene complex consists of a centromeric region bordered by the genes KIR2DL3 and KIR3DP1 and a telomeric region bordered by KIR2DL4 and KIR3DL2. Within each region, the KIR genes lie less than 3 kb apart. The head to tail orientation of the KIR genes and the similarity of their gene structures and sequences suggest that the 16 KIR genes arose by duplication events [1]. Six genes (KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5, KIR3DS1) encode proteins on the surface of NK cells with short (S) intracytoplasmic tails that are thought to be activating receptors [4]. Seven genes (KIR2DL1, KIR2DL2, KIR2DL3, KIR2DL5, KIR3DL1, KIR3DL2, and KIR3DL3) encode cell surface receptors with longer (L) cytoplasmic tails whose signals inhibit NK cell activation. KIR2DL4 encodes a receptor that performs both inhibitory and activating functions. Two KIR are pseudogenes (KIR2DP1, KIR3DP1).

Studies of individuals, families, and populations have demonstrated that individuals vary in the number and type of KIR loci present in their genomes [5,6,7]. KIR2DL1, KIR2DL2, KIR2DL3, KIR2DL5, KIR3DL1, KIR2DS1, KIR2DS5, KIR3DS1 are found in some (but not all) individuals. In contrast, the four loci marking the boundaries of the centromeric and telomeric KIR regions, KIR3DL3, KIR3DP1, KIR2DL4, and KIR3DL2, are found in essentially all individuals and are termed ‘framework’ genes. The
variation in gene number and the gene positioning has been confirmed by long range genomic sequencing of 24 KIR haplotypes [1,2,9].

KIR gene-content haplotypes have historically been divided into two broad groups. Group A haplotypes, initially identified by the absence of a specific DNA restriction enzyme fragment, are currently defined by the presence of KIR2DS4 as the only stimulatory KIR gene in combination with KIR2DL1, KIR2DL3, KIR2DL4, KIR3DL1, KIR3DL2, KIR3DL3, KIR2DP1 and KIR3DP1 [9]. Group B haplotypes carry other KIR genes (inhibitory: KIR2DL2, KIR2DL3; stimulatory: KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5, KIR3DS1), sometimes including Group A haplotype-specific KIRs. Diversity within the Group A and Group B haplotypes has been noted with respect to gene content [6,10] as well as allelic variation [9,9,11,12]. This variation is found within both centromeric and telomeric regions. Since strong linkage disequilibrium is observed within the centromeric and telomeric regions, but not between the two [9,13], it has been suggested that a KIR haplotype consists of various permutations of centromeric and telomeric region variants [14].

Although not evident during the initial naming of KIR genes, it became appreciated later that some genes are actually alleles at the same locus, determined by their similar positioning in the gene complex [1] and their negative linkage disequilibrium [6]. Thus KIR2DL2 and KIR2DL3 are variants of the same locus, as are KIR3DL1 and KIR3DS1, and KIR2DS3 and KIR2DS5; these multiple names are sometimes combined (e.g., KIR2D3S3S). KIR2DL5 and KIR2DS5 have been duplicated and appear in two different positions in KIR haplotypes [15,16]. KIR2DL5 is always associated with either KIR2DS3 or KIR2DS5. Specific combinations of alleles at each locus are commonly observed: KIR2DL52*002 is adjacent to KIR2DS3*00103 in the centromeric region, while KIR2DL52*001 (or *005) is adjacent to KIR2DS3*002 in the telomeric region [17]. Haplotypes carrying both copies of KIR2DL5 and of KIR2DS3 (or KIR2DS5) have been observed in studies of European families, although haplotypes with only one of each paralog have also been observed [18,19].

Other, less common haplotypes, apparently deriving from unequal recombination, have also been noted. A haplotype present in ~4.5% of a sample of European individuals carries a duplicated segment bearing KIR2DL4 and KIR3DL1 [20,21]. A chimeric gene formed at the site of recombination converts pseudogene, KIR3DP1, to an expressed locus [22]. Family and population studies and fosmid cloning have detected numerous other haplotypes with large deletions, duplications, and novel hybrid genes, such as a fusion of the KIR2DL1 and KIR2DL2 loci [19,23,24].

Previous work has demonstrated the important role of KIR in human health and disease. KIR participate in the interaction between maternal NK cells and fetal trophoblasts during fetal implantation [25]. Studies of disease susceptibility and resistance have noted associations with specific KIR loci [26]. In hematopoietic stem cell transplantation, specific KIR loci have been shown to have an impact on outcome, and adoptive therapy based on KIR/ligand reactivity has been used for the treatment of malignant relapse [27-29,30]. Given that KIR allelic products have been shown to differ in their specificity for and affinity of ligand binding [30,31], these allelic differences may translate into differences in disease progression [26,29,32].

In order to better understand the genetic diversity of KIR and to evaluate its role in transplantation, we have estimated allele-level haplotype frequencies and pairwise linkage disequilibrium for the 14 functional KIR loci in 506 individuals with European ancestry. These estimates provide the first view in a large cohort of haplotype associations of alleles of the KIR loci.

Materials and Methods

Subjects

DNA samples for a total of 506 unrelated individuals were obtained from the National Marrow Donor Program (NMDP) Research Repository. All individuals are of European ancestry and participated in a 10/10 HLA-matched hematopoietic stem cell transplantation (468 donors and 38 recipients) for acute lymphoblastic leukemia, acute myeloid leukemia, myelodysplastic syndrome, or chronic myelogenous leukemia. For each, KIR characteristics were unknown at the time of transplant. All subjects provided informed consent for participation in research. The NMDP Institutional Review Board approved the study.

KIR Allele Identification

Allele-level genotyping focused on 14 KIR loci (KIR2DL1–5, KIR2DS1–5, KIR3DL1–3 and KIR3DS1) and was performed in three independent laboratories. Participating laboratories utilized different genotyping methodologies, including sequence-specific-oligonucleotide (SSO) hybridization, sequence-specific-primer typing (SSP), and DNA sequencing [16,17,34,35,36,37,38,39,40,41,42,43]. Results were interpreted to a three-digit allele designation using the Immuno Polymorphism Database- KIR version 1.2.0 or higher [44]. The majority (85%) of the samples were typed in duplicate by two different laboratories and final calls were obtained by consensus. Results from a fourth laboratory were used to resolve ambiguities and discrepancies. Each laboratory reported results using the Standard Reporting Format for KIR Genotyping Data (IPD) (http://www.ebi.ac.uk/ipd/kir/standards.html). This is an open format that distinguishes between haploid and diploid ambiguities, accepts all levels of resolution, and reports the observed number of loci. This format is also easily translated into XML for downstream storage and analysis.

Physical linkage of loci and an assay for copy number variation were employed to define haplotypes for 7.5% of individuals whose haplotypes were unresolved in the first stage of analysis (see below, haplotype estimation). Methods have been previously described [12]. Briefly, haplotype specific extraction (HSE) (Qiagen, Valencia, CA) was used to isolate a DNA fragment carrying a single allele at a locus. Once isolated, the alleles were identified by DNA sequencing for expressed genes and sequence-specific amplification for the pseudogenes and some fusion genes. An extended PCR and sequencing was used to link some loci. Alleles for the second KIR haplotype in an individual were defined as those alleles not assigned by physical linkage to the first haplotype. Amplification reactions were used to identify the presence of KIR2DP1, KIR3DP1, and fusion genes [19,22,23,45,46]. TaqMan copy number assays were used to measure KIR2DL4 copy number variation (Applied Biosystems, Inc., Foster City, CA) [20].

Identification of Novel Alleles

Novel alleles (Table S1) were isolated for DNA sequencing using allele specific amplification, by cloning, or by using HaploPrep reagents (Qiagen, Valencia, CA) as previously described [39,40,42,43,47,48]. Two or three overlapping PCR amplicons were generated from each KIR gene covering the complete coding sequence. Sequences were compared to known KIR sequences obtained from the IPD-KIR database version 2.1.0 to determine allelic assignments. Allele designations for novel KIR alleles were assigned by the International Union of Immunological Societies.
and World Health Organization (IUIS/WHO) Subcommittee for KIR Nomenclature. Alleles have been deposited in GenBank and the Immuno Polymorphism Database.

**Determination of Reference Haplotypes**

An implementation of the expectation-maximization (E-M) algorithm in the Haplo-IHP software [49] was used to confirm that each individual's gene-content haplotypes could be explained by a pair from our set of reference haplotypes. The software defines gene content as zero or one copies for each gene. The Haplo-IHP algorithm also requires as input a list of reference haplotypes. The seven gene-content haplotypes described in Pyo et al. [8] were used as the reference set for the initial analysis (Figure 1). The genotypes of 38 individuals (7.5%) were unresolved given this set of reference haplotypes. Additional laboratory analysis was performed for these individuals to determine gene-content haplotypes. This analysis included physical linkage of neighboring alleles and clarification of copy number for the \( KIR2DL4 \) locus (selected to detect a common haplotype with a gene duplication [20,21,22]). Based on this analysis, five centromeric and six telomeric regions were added to the set of reference haplotypes (Figure 1).

Structural haplotype estimation was repeated with 15 reference haplotype structures, including 14 haplotypes consisting of previously reported centromeric and telomeric regions [8,13,15,19,21,22,23,24,50] and 1 novel haplotype (cA03–tB07) identified through molecular characterization of the haplotype as described above. \( KIR2DS3 \) and \( KIR2DS5 \) were treated as a single gene (\( KIR2DS3S5 \)) based on previous reports of their alternative and invariant linkage with \( KIR2DL5 \) [15,16].

The algorithm accepts constraint patterns for LD between loci and rejects individuals that do not meet the constraint patterns. Based on previous observations [6,10,13,15] that are consistent with our set of reference haplotypes, we applied five locus-level haplotype constraints to confirm all individuals could be explained by our reference haplotypes: (1) framework gene \( KIR3DL3 \) must be present, (2) \( KIR2DL5 \) and \( KIR2DS3S5 \) must be in complete linkage disequilibrium (LD) so that either both loci are present or both are absent, (3) \( KIR3DL1 \) and \( KIR2DS4 \) are in complete LD, and (4) \( KIR2DL2 \) and \( KIR2DL3 \) cannot both be present. It was not assumed that \( KIR3DS1 \) and \( KIR3DL1 \) are in complete negative LD based on [20,21,22]. All genotypes of all individuals were consistent with these constraints.

![Figure 1. Reference structures for KIR haplotype estimation.](https://example.com/fig1.png)

The reference haplotypes used in the prediction model are shown as centromeric and telomeric haplotype structures (e.g., cA01 indicates centromeric A haplotype 01). The colors represent KIR gene characteristics: framework (light blue), activating (pink), inhibitory (dark blue and green), pseudogene (yellow), and fusion gene (gradient). For fusion genes, the assigned locus name is in bold. Genes represented by a single allele are labeled with the allele name. Pseudogenes are omitted except when they occur in a duplicated region or as a fusion gene. All but one haplotype (cA03–tB07) have been previously reported [8,13,15,19,21,22,23,24,50].

doi:10.1371/journal.pone.0047491.g001
Allelic Haplotype Estimation

We estimated allelic haplotype frequencies using an E-M algorithm and assigned the most likely haplotype pair for each individual. After determining that each individual's genotype could be explained by at least two reference haplotypes, a custom E-M algorithm was used to estimate allelic haplotype frequencies, from which LD statistics were calculated. This implementation of the E-M algorithm does not support missing or unreported genotypes, but does support ambiguous allelic genotyping results and an arbitrary number of gene copies per haplotype (including explicit absence).

An overview of the process is depicted in Figure 2 and details follow. The first step fits individual allelic genotypes to all possible pairs of allelic haplotypes. Given the 506 allelic genotypes and the 15 reference haplotypes, each genotype was expanded into all possible haplotype pairs. The output explicitly details the allelic and haplotype ambiguities for each individual as a collection of allelic haplotype pairs. A decision matrix was created to address the possibility of gene duplications on a haplotype (Table S2). The matrix mapped KIR2DL1, KIR2DL3, and KIR2DS3S5 genotypes to their centromeric and telomeric locations based on the structure of known allelic haplotypes. A nomenclature system was used whereby paralogs were distinguished by appending a ‘C’ or ‘T’ to designate location in the centromeric or telomeric region, respectively; subsequently a number was added, (e.g., KIR2DL5T1 and KIR2DL5T2) to accommodate additional gene duplications. The numbers provide a unique identity, but do not imply any relative location or order. This nomenclature, used in the E-M, appears in the decision matrix (Table S2) and the haplotype frequencies table (Table S3). Haplotypes were named according to previously recommended nomenclature standards http://www.ebi.ac.uk/ipd/kir/standards.html [8].

The next step aggregated the individual information to produce haplotype frequency estimations for the entire cohort. Within each individual, all haplotype pairs were given equal weight; for example, if four haplotype pairs could exhaustively explain an individual's allelic genotype, each of the haplotype pairs would be given a fractional count of 0.25. These counts were aggregated per haplotype across all individuals and used as input to a custom E-M algorithm, which then estimated gene-content and allelic haplotype frequencies for the entire sample [51,52].

The final step made an allelic haplotype pair assignment for each individual, given the individual allelic genotypes and the estimated sample-level haplotype frequencies.

When multiple haplotype pairs could explain an individual's genotype, the pair with the highest total frequency was assigned. For homozygotes, the total frequency was calculated as the product of the two frequencies; for heterozygotes, the total frequency was twice the product of the two frequencies. This procedure resolved allele, copy number, and phase ambiguities on a per-sample basis.

Calculation of Linkage Disequilibrium (LD) Statistics

Custom software and the 'LDk' function of the Genetic Analysis Package (GAP) for the R language for statistical computing [R Core Development Team, 2009] were used to compute LD statistics between haplotype region (e.g., CA01, TB01, etc.), locus and allele pairs. Overall LD between loci was assessed using the Wn statistic (identical to r2 for biallelic loci or Cramer's V extension for multiple alleles) [53]; the D' statistic was used to measure LD between regions and alleles and significance testing for allele pairs was accomplished via a standard chi-squared measure [54,55,56,57].

Analysis of Allele and Genotype Frequency Distributions

Watterson’s homozygosity statistic (F, used to examine allele frequency distributions relative to expectations under neutrality) and the fit of genotype frequencies to expectations under Hardy-Weinberg Equilibrium proportions were performed with the PyPop software framework for population genetics analysis [58,59].

Results

We estimated pairs of allelic full-length haplotypes for each individual. This allowed fine-grained analysis of genotypes, allele frequencies, haplotype frequencies, conserved haplotype blocks, and LD at the region, locus, and allele levels.

Genotype Assignment

Genotyping for the 14 functional KIR loci in the study cohort yielded three-digit allele assignments for each locus present in each of the 506 individuals. Heterozygous results (two alleles identified at a locus in an individual) allowed confirmation of the presence of that locus on both chromosomes in that individual. However, for loci where only one allele was identified, the genotyping methods were unable to distinguish between homozygosity at the locus (present on both chromosomes), or, alternatively, absence of the locus on the second chromosome in that individual. In order to estimate copy number for each locus, posterior probabilities derived from the estimated haplotype frequencies were used to assign the most likely gene-content haplotypes (and therefore, full genotypes) for each individual.

![Figure 2. Overview of methods for estimation of haplotypes consisting of fully resolved alleles at each locus](image-url)
KIR Allele Frequency Distributions

Gene and allele frequencies for each KIR locus tested are shown in Figure 3 (details are in Table S4). The framework genes as well as the telomeric A genes (KIR3DL1 and KIR2DS4) are most likely to be present on gene-content haplotypes and exhibit the most diversity. For example, KIR3DL3 is found in 100% of KIR centromeric haplotypes; 24 alleles were found at frequencies varying from ~1–21% at this locus. In general, the inhibitory loci (and framework genes in particular) exhibit more allelic variability than the stimulatory loci; however, the stimulatory loci are more variable in gene content. While not statistically significant, there is a strong trend toward more even distributions for KIR2DL1, KIR2DL2L3 (KIR2DL2 and KIR2DL3), KIR2DL4 and KIR3DL1S1 (KIR3DL1 and KIR3DS1), all with normalized deviates of the F statistic ~(-1). For the stimulatory loci, particular alleles appear to be nearly fixed on specific haplotypes, with less common variants appearing generally only on less common haplotypes in a haplotype-specific manner. For example, KIR2DS1*005 (frequency = 0.004) is found only in haplotypes tB02 and tB07 while KIR2DS1*002 (frequency = 0.209) is found on tB01, tB03, and tB04 (Figure 3c). In contrast, many of the less frequent alleles of the inhibitory loci (e.g., KIR2DL4*002, KIR3DL1*007, KIR3DL1*008, KIR3DL2*011 and KIR3DS3*003) appear on multiple haplotypes, including both the common and less common (Table S3). This is particularly evident at the framework loci, which exhibit the highest degree of overall variability.

KIR Haplotype Frequencies

Table 1 details the estimated frequencies of the centromeric region, telomeric region, and full-length gene-content haplotypes. Haplotype frequencies in this cohort are very similar to those observed for a European white population [8]. Compared to those frequencies, our estimate for the Group A full-length haplotype is lower (54.1% vs. 62.5%), although the percentage of A haplotype centromeric or telomeric blocks is similar but distributed among haplotypes combining A and B structures (i.e., cA0x~tB0x and cB0x~tA0x). Haplotype frequencies within the centromeric or telomeric regions are also similar to those reported by Hou et al. [12], whose data set is a subset of that used here.

Haplotype frequencies with allelic resolution are given in Table S3. The most common centromeric and telomeric allele-level haplotypes observed in this cohort are cA01- KIR3DL3*001-KIR3DL1*002- KIR2DL3*002- KIR2DL1C*002 (14%) and tA01-KIR2DL4*008- KIR3DL1S1*001- KIR2DS4*003- KIR3DL2*001 (14.0%), respectively.

Within the centromere, the cA01 structure dominates, with fifteen of the twenty most common haplotypes; the remaining five haplotypes are cB01 (n = 2) and cB02 (n = 3). Likewise, tA01 dominates in the telomere, with sixteen of the twenty most common haplotypes; the remaining four are tB01. When full-length (centromeric and telomeric) haplotypes are considered, thirteen of the top twenty are cA01~tA01, three are cB02~tA01, and there are two each of cA01~tB01 and cB01~tB01. These results are consistent with the findings of Hou et al. 2011 [12]. cA01~tA01 is the only haplotype with a frequency (54.1%) greater than 15%; seven haplotypes have frequencies less than 1%.

LD statistics (D') between the centromeric and telomeric regions are given in Table 1 (details are in Table S5). Complete LD between the centromeric and telomeric regions is observed in seven haplotypes, each of which has a frequency below 2%: cA01~tB04, cA01~tB05, cA01~tB06, cA02~tB02, cA02~tB07, cB03~tA01 and cB04~tB03. The remaining eight full-length haplotypes have much lower D' values, between negative and
positive 0.5; these include the six most common haplotypes in this cohort, observed at frequencies ranging from 2.8–54.1%.

Overall LD between loci across the entire KIR region is depicted in Figure 4. As mentioned above, alleles of KIR2DL2 and KIR2DL3 are considered to be from a single locus, and thus were combined into KIR2DL2L3 for simplicity of depiction. Similarly, KIR2DS3 and KIR2DS5 were combined as KIR2DS3S5. Strong LD is observed within the centromere or telomere regions and minimal LD between the centromere and telomere regions; overall linkage disequilibrium between the genes flanking the intervening region is much lower (e.g., Wn = 0.17 for KIR2DL1~KIR2DL4) than between the genes flanking each region (e.g., Wn = 0.46 for KIR3DL3~KIR2DL1 and 0.58 for KIR2DL4~KIR3DL2). The highest overall LD is found between KIR2DL5~KIR2DS3S5 (in both the centromeric and telomeric regions), KIR2DS3S5~KIR2DL1 in the centromeric region, and KIR3DL1~KIR2DS4 in the telomeric region.

In order to examine the association of short haplotype blocks within the most common full-length haplotypes, the entire distribution of two-allele haplotypes was filtered on LD statistics ($D' > 0.9; p < 0.0001$) and frequency ($f > 0.1$), yielding a total of 26 common two-allele haplotypes that are observed in strong and significant LD (Figure 5a). These two-allele haplotypes were aligned with neighboring two-allele haplotypes to form longer allelic haplotype blocks. Contiguous two-locus haplotypes meeting these criteria are similarly colored in Figure 5a; two-allele haplotypes that do not meet the filtering criteria are represented in black. Figure 5b depicts the 16 most common (each with a frequency greater than 1%) full-length allelic haplotypes with allele pairs color-coded as in Figure 5a; these 16 haplotypes have a combined frequency of 28%. The haplotypes are numbered according to frequency, starting with ‘01’ for the most common haplotype. Figure 5c arranges the haplotypes based on centromeric allele content while in Figure 5d haplotypes are arranged

Table 1. Gene-content haplotype frequencies.

| Centromeric/Telomeric Haplotype | Frequency | Full Haplotype | Frequency | $D'$ |
|---------------------------------|-----------|----------------|-----------|------|
| cA01                            | 66.5%     | cA01~tA01      | 54.1%     | 0.22 |
| cB02                            | 17.1%     | cB02~tA01      | 14.3%     | 0.33 |
| cB01                            | 14.0%     | cB01~tB01      | 9.5%      | -0.26|
| cB04                            | 1.5%      | cB04~tA01      | 7.2%      | -0.32|
| cB05                            | 0.5%      | cB05~tB01      | 6.8%      | 0.36 |
| cA02                            | 0.3%      | cA02~tB01      | 2.8%      | -0.16|
| cB03                            | 0.1%      | cB03~tB05      | 1.8%      | 1    |
| tA01                            | 75.9%     | cB04~tB03      | 1.5%      | 1    |
| tB01                            | 19.4%     | cA01~tB06      | 0.8%      | 1    |
| tB05                            | 1.8%      | cA01~tB04      | 0.4%      | 1    |
| tB03                            | 1.5%      | cB05~tB01      | 0.3%      | 0.50 |
| tB06                            | 0.8%      | cA02~tB07      | 0.2%      | 1    |
| tB04                            | 0.4%      | cB05~tA01      | 0.2%      | -0.47|
| tB07                            | 0.2%      | cA02~tB02      | 0.1%      | 1    |
| tB02                            | 0.1%      | cB03~tA01      | 0.1%      | 1    |

Estimated frequencies for each centromere, telomere, and full reference haplotype. LD ($D'$) is included for each pairing of centromere and telomere. Haplotypes with frequencies less than 1% where determined by physical linkage and gene copy number of KIR2DS4.

doi:10.1371/journal.pone.0047491.t001
based on telomeric allele content, illustrating the relative independence and likely frequent recombination between the centromeric and telomeric intervals of the KIR region. The haplotype blocks use the nomenclature and are consistent with those identified by Hou et al. [12]. In the centromere, the three most frequent full-length allelic haplotypes occur in different consensus haplotypes (identical centromeric allele blocks excluding KIR2DL3) and contribute 10% of all haplotypes. These haplotypes are boxed and bolded in Figures 5c and 5d.

The 506 individuals have 489 unique pairs of full-length allelic haplotypes; 17 haplotype pairs were seen twice (i.e., they occurred in exactly two individuals) and the rest are unique. Of the 17 pairs that occurred twice, 6 were structurally cA01~tA01+cA01~tA01, 4 were cA01~tA01+cB02~tA01, and the remaining 7 were unique.

205 of the 506 individuals have genotypes that are haplotypically ambiguous. Details are in Table S6 and Figure 6 provides a graphical example of such ambiguity.

Discussion

This detailed study of KIR diversity, with the discovery of new alleles and haplotypes and the ascertainment of haplotype and allele frequencies using a multiplexed bioinformatics approach in a large European-American cohort, extends our understanding of the strength and extent of LD across the KIR region. To our knowledge, this is the first study of its kind to utilize multiple genotyping methods employed by different laboratories in order to verify allele calls for each individual in a cohort of this size. To ensure accurate results, each individual was genotyped to at least the three-digit allele resolution in multiple laboratories. 94.5% of the final genotypes were unambiguous at the three-digit level. Our careful laboratory analysis and consistent confirmation between laboratories further allowed identification of forty-seven novel alleles at nine loci in 63 individuals. While the sample cohort derives from successful matching for HLA in the context of hematopoietic stem cell transplantation and is therefore likely enriched for common HLA haplotypes, the large sample size and ethnic homogeneity, coupled with our redundant genotyping protocol, permit an unprecedented view of allelic variation across the KIR region. Assessment of the conformation of allele frequencies to expectations under Hardy-Weinberg equilibrium was performed for KIR2DL2L3 – a locus expected to be carried by all haplotypes and one with the least ambiguous allele assignments. Due to ambiguities, alleles containing KIR2DL2*001 or KIR2DL2*005 were collapsed to a common allele; under this scenario, the proportions did not deviate from expectations under Hardy Weinberg Equilibrium (data not shown).

There are three sources of ambiguity in our data: structural, allelic, and phase (the knowledge of which genes occur together on a chromosome). The active recombination in the KIR region creates multiple large segments of unusual similarity, and thereby creates structural ambiguity by complicating gene identity and determination of physical location. Regions must often be physically linked with other regions of known location. The refinement over time of gene names in KIR nomenclature parallels this ambiguity. Allelic ambiguity is the result of typing with primers and/or oligonucleotides whose range and specificity don’t match all the regions or base pair combinations in a gene. Finally, current KIR typing methods don’t separate chromosomes, and therefore don’t specify phase.

Figure 5. KIR gene haploblocks with fully resolved alleles at each locus. Allele pairs with high significance (p < 0.0001), LD (D’ > 0.9), and frequency (freq > 0.1) are shown in column 1 (5a). Pairs found on the same haplotype are combined in column 2 and are represented by larger colored blocks in column 3. Pairs that don’t meet at least one of the three criteria are represented by black boxes. Full-length KIR3DL3 genes are highly polymorphic and all alleles are represented as a single grey block. These blocks were mapped onto the 16 most frequent (freq > 1%) allelic haplotypes and visually clustered by

---

**Table:**

| Allele Pair | Frequency | LD Score | Significance |
|------------|-----------|----------|--------------|
| cA01~tA01  | 0.05      | 0.80     | 0.001        |
| cA01~tA01  | 0.10      | 0.90     | 0.001        |
| cA01~tA01  | 0.15      | 0.95     | 0.001        |
| cA01~tA01  | 0.20      | 0.98     | 0.001        |
| cA01~tA01  | 0.25      | 0.99     | 0.001        |

---

**Figure 5:** KIR gene haploblocks with fully resolved alleles at each locus. Allele pairs with high significance (p < 0.0001), LD (D’ > 0.9), and frequency (freq > 0.1) are shown in column 1 (5a). Pairs found on the same haplotype are combined in column 2 and are represented by larger colored blocks in column 3. Pairs that don’t meet at least one of the three criteria are represented by black boxes. Full-length KIR3DL3 genes are highly polymorphic and all alleles are represented as a single grey block. These blocks were mapped onto the 16 most frequent (freq > 1%) allelic haplotypes and visually clustered by
In our study cohort, each of five full-length reference gene-content haplotypes occurs with a frequency over 6.8% and they collectively contribute 92% of the estimated haplotypes. The full-length reference haplotype frequencies largely conform to the frequencies reported by Pyo et al. [8], where six full-length reference haplotypes resolve 99% of genotypes in their sample of 96 European-American individuals. It is more difficult to compare our results with earlier studies [7,11,16,56,60,61,62,63,64,65,66], whose data were from dissimilar populations or were derived from lower resolution KIR genotyping results. However, we believe our results are consistent with the emerging understanding of content and diversity in the KIR region in its broadest context.

KIR haplotype estimation is complicated by gene-content and allelic variability and the inability of current typing methods to define this variability unambiguously. A primary structural complication is the occurrence of varying copy number of the same gene on a haplotype due to duplications or deletions. Haplotypes can be ambiguous when this copy number variation is not considered. Although most of the haplotypes are structurally unambiguous, ambiguity increases when haplotype pairs are considered. A primary factor is the apparent history of frequent recombination between centromeric and telomeric regions. For example, cA01−tA01+cB01−tB01 individuals (with an expected frequency of 7.4%) are identical for gene-content with cA1−tB01+cB01−tA01 individuals (1.4%). Gene copy number and insertions/deletions of varying length contribute more ambiguity: cA01−tB01+cB02−tA01 (predicted frequency of 2.7%) is indistinguishable from cA01−tB05+cB02−tB01 (0.10%). Similarly, a cA01−tB01 homozygote containing two copies of the 3DS1−2DL5−2DS3S5 region has a structural genotype identical to the haplotype pair cA01−tB01+cA01−tB04, which contains three copies of the same region or the haplotype pair cA01−tB01+cA02−tB02, which contains one copy of the region (Figure 6). In fact, there are seven haplotype pairs that may explain the genotype that produces the cA01−tB01 homozygous haplotype. A full list of haplotype pair ambiguities seen within our cohort can be found in Table S6. Given the parsimonious approach to full haplotype assignment and the fact that 92.5% of the samples did not undergo explicit linking and copy number analysis, our set of reference haplotypes might underrepresent the true haplotype diversity of our cohort.

Figure 6. Structural genotypic ambiguity. Panel a depicts the presence or absence of specific loci in a particular genotype. Panel b illustrates three of the seven haplotype pairs that are consistent with the presence/absence genotype in panel a. The haplotype pairs contain 2, 3, and 1 copies of the KIR3DS1, KIR 2DL5, and KIR2DS3S5 regions respectively. doi:10.1371/journal.pone.0047491.g006
Due to copy-number variation for the KIR loci, allele-level haplotype estimates permit better characterization of allele frequencies than from genotypes alone. Analysis of KIR allele frequencies in our cohort reveals that the telomere is more diverse in terms of both gene-content and allelic variation than the centromere, with the exception of KIR3DL3, which is located in the centromeric region and is substantially diverse. The B haplotypes generally exhibit more structural (gene-content) variation but less allelic variation than the A haplotypes, as has been shown previously [9,67].

This study serves as an example of how laboratory and computational methods can partner to illuminate details of a somewhat intractable genetic region. Initially, laboratories determine the major structural characteristics of the region; this produces a collection of structural reference haplotypes. Then, algorithmic approaches can fit sample genotypes into these reference haplotypes and flag individuals with potentially unknown structures. These two steps continue until all samples can be explained from a structural perspective. Subsequently, computational methods can leverage information inherent at the sample or population levels to resolve structural, allelic, and phase ambiguities in a maximum likelihood fashion. We anticipate that as genotyping methods for the KIR continue to mature, the reliance on computational methods will fall away. We expect this progression to continue as haplotype resolution expands to samples from other populations.

**Supporting Information**

Table S1 Novel alleles identified. For each novel allele, the new number, and number of individuals with the new allele is shown.

**References**

1. Wilson MJ, Torkar M, Haude A, Milne S, Jones T, et al. (2000) Plasticity in the organization and sequences of human KIR/ILT gene families. Proc Natl Acad Sci U S A 97: 4770–4773.

2. Martin AM, Freitas EM, Witt CS, Christiansen FT (2004) Comparative genomic analysis, diversity and evolution of two KIR haplotypes A and B. Gene 335: 121–131.

3. Liu L, Chen M, Ng J, Hurley CK (2011) Conserved KIR allele-level haplotypes and their frequencies, along with the structural haplotype assignment of each. (XLSX)

4. Gourraud PA, Meenagh A, Cambon-Thomsen A, Middleton D (2010) Linkage disequilibrium organization of the human KIR superlocus: implications for KIR data analyses. Immunogenetics 62: 729–740.

5. Hsu KC, Chida S, Geraghty DE, Dupont B (2002) The killer cell immunoglobulin-like receptor (KIR) genomic region: gene-order, haplotypes and allele polymorphism. Immunol Rev 180: 40–52.

6. Gonzalez A, Meenagh A, Sleator C, Middleton D (2008) Allelic polymorphism synergizes with variable gene content to in-

7. Du Z, Sharma SK, Spellman S, Reed EF, Rajalingam R (2008) KIR2DL5 alleles mark certain combination of activating KIR genes. Genes Immun 9: 470–480.

8. Ordonez D, Meenagh A, Gomez-Lozano N, Castano J, Middleton D, et al. (2008) Duplication, mutation and recombination of the human orphan gene KIR2DS3 contribute to the diversity of KIR haplotypes. Genes Immun 9: 431–437.

9. Shilling HG, Guethlein LA, Cheng NW, Gardiner CM, Parham P, et al. (2002) Allelic polymorphism synergizes with variable gene content to in-

10. Martin MP, Bashirova A, Traherne J, Trowsdale J, Carrington M (2003) Cutting edge: expansion of the KIR locus by unequal crossing over. J Immunol 171: 2192–2195.

11. O'Reilly RJ, Hsu KC, Chida S, Geraghty DE, Dupont B (2002) The killer cell immu-

12. Hou L, Chen M, Ng J, Hurley CK (2011) Conserved KIR allele-level haplotypes and their frequencies, along with the structural haplotype assignment of each. (XLSX)

13. Gourraud PA, Meenagh A, Cambon-Thomsen A, Middleton D (2010) Linkage disequilibrium organization of the human KIR superlocus: implications for KIR data analyses. Immunogenetics 62: 729–740.
Allele-Level KIR Haplotype Estimation

26. Bashirrova AA, Martin MP, McVicar DW, Carrington M (2006) The killer immunoglobulin-like receptor gene cluster: tuning the genome for defense. Annu Rev Genomics Hum Genet 7: 277–300.

27. Grzybowski B, Miller JS, Vernesi MR (2008) Use of natural killer cells as immunotherapy for leukemias. Best Pract Res Clin Haematol 21: 467–483.

28. Cooley S, Weidorf DJ, Guenthlein LA, Klein JP, Wang T, et al. (2010) Donor selection for natural killer cell receptor genes leads to superior survival after unrelated transplantation for acute myelogenous leukemia. Blood.

29. Witt KS (2009) The influence of NK allotype matching on mismatched unrelated donor and HLA identical sibling hematopoietic stem cell transplantation. Curr Opin Immunol 21: 531–537.

30. Sharma D, Bastard K, Guenthlein LA, Norman PJ, Yawata N, et al. (2009) Dimorphic motifs in D0 and D1+D2 domains of killer cell Ig-like receptor (KIR) 3D1 combine to form receptors with high, moderate, and no avidity for the complex of a peptide derived from HIV and HLA-A2402. J Immunol 183: 4569–4582.

31. Moesta AK, Norman PJ, Yawata M, Yawata N, Gleimert M, et al. (2008) Synergetic polymorphism at two positions distal to the ligand-binding site makes KIR2DL2 a stronger receptor for HLA-C than KIR2DL3. J Immunol 180: 3969–3979.

32. Martin MP, Qi Y, Gao X, Yamada E, Martin JN, et al. (2007) Innate partnership of HLA-B and KIR3DL1 subtypes against HIV-1. Nat Genet 39: 733–740.

33. Khakoo SI, Thio CL, Martin MP, Brooks CR, Gao X, et al. (2004) HLA and NK-cell inhibitory receptor genes in resolving hepatitis C virus infection. Science 305: 872–874.

34. Ashouri E, Farjadjan S, Reed EF, Ghaderi A, Rajalingam R (2009) KIR gene content diversity in four Iranian populations. Immunogenetics 61: 483–492.

35. Do Z, Gertzon DW, Reed EF, Rajalingam R (2007) Receptor-ligand analyses define minimal killer cell Ig-like receptor (KIR) in humans. Immunogenetics 59: 1–15.

36. Levinson RD, Du Z, Luo L, Monnet D, Tabary T, et al. (2008) Combination of KIR and HLA gene variants augments the risk of developing birdshot chorioretinopathy in HLA-A29-positive individuals. Genes Immun 9: 249–258.

37. Luo L, Du Z, Sharma SK, Cullen R, Spellman S, et al. (2007) Chain-terminating natural mutations affect the function of activating KIR receptors. KIR3DS1 and 2DS1. Immunogenetics 59: 779–792.

38. Lebedeva TV, Ohashi G, Zannelli G, Cullen R, Yu N, et al. (2007) Comprehensive approach to high-resolution KIR typing. Hum Immunol 68: 789–796.

39. Hou I, Steiner NK, Chen M, Belle I, Kubit AL, et al. (2008) Limited allelic diversity of stimulatory two-domain killer cell immunoglobulin-like receptors. Hum Immunol 69: 174–178.

40. Belle I, Hou L, Chen M, Steiner NK, Ng J, et al. (2008) Investigation of killer cell immunoglobulin-like receptor gene diversity in KIR3DL1 and KIR3DS1 in a transplant population. Tissue Antigens 71: 434–439.

41. Hou I, Steiner N, Chen M, Belle INJ, Hurley C (2007) KIR3DL1 Allelic Diversity: Four New Alleles Characterized in a Bone Marrow Transplant Population and Three Families. Tissue Antigens 69: 250–254.

42. Godil MA, Steiner NK, Hurley CK (2007) KIR3DL2 diversity in a hematopoietic stem cell transplantation population. Tissue Antigens 70: 228–232.

43. Hou J, Chen M, Steiner NK, Belle I, Turino G, et al. (2007) Seventeen novel alleles add to the already extensive KIR3DL3 diversity. Tissue Antigens 70: 469–474.

44. Robinson J, Mistry K, McWilliam H, Lopez R, Marsh SG (2010) PIPP—the Immuno Polymorphism Database. Nucleic Acids Res 38: B1863–869.

45. Vilches C, Panda MJ, Rajalingam R, Gallinari CM, Parham P (2000) Discovery of two novel variants of KIR2DS5 reveals this gene to be a common component of human KIR‘B’ haplotypes. Tissue Antigens 56: 453–456.

46. Vilches C, Castano J, Gomez-Lozano N, Estefania E (2007) Facilitation of KIR genotyping by a PCR-SSP method that amplifies short DNA fragments. Tissue Antigens 70: 413–422.

47. Hou JH, Steiner NK, Chen M, Belle I, Ng J, et al. (2007) KIR2DL1 allelic diversity: four new alleles characterized in a bone marrow transplant population and three families. Tissue Antigens 69: 250–254.

48. Shulke C, Steiner NK, Hurley CK (2007) Allelic diversity in KIR2DL4 in a bone marrow transplant population: description of three novel alleles. Tissue Antigens 70: 157–159.

49. Yoo YJ, Tang J, Kaslow RA, Zhang K (2007) Haplotype inference for present-absent genotype data using previously identified haplotypes and haplotype patterns. Bioinformatics 23: 2399–2406.

50. Ordonez D, Gomez-Lozano N, Rosales L, Vilches C (2011) Molecular characterisation of KIR2DS2*005, a fusion gene associated with a shortened KIR haplotype. Genes Immun 12: 544–551.

51. Excoffier L, Slatkin M (1995) Maximum-likelihood estimation of molecular haplotype frequencies in a diploid population. Mol Biol Evol 12: 923–927.

52. Do CB, Batzoglou S (2008) What is the expectation maximization algorithm? Nat Biotechnol 26: 897–899.

53. Cramer H (1996) Mathematical Models of Statistics. Princeton: Princeton University Press.

54. Lewontin RC (1964) The Interaction of Selection and Linkage. I. Optimum Models. Genetics 50: 757–782.

55. Klitz W, Stephens JC, Gore M, Carrington M (1995) Discordant patterns of linkage disequilibrium of the peptide-transporter loci within the HLA class II region. Am J Hum Genet 57: 1436–1444.

56. Single RM, Martin MP, Meyer D, Gao X, Carrington M (2008) Methods for assessing genetic content diversity of KIR with examples from a global set of populations. Immunogenetics 60: 711–725.

57. Thomson G, Klitz W (1987) Disequilibrium pattern analysis. I. Theory. Genetics 116: 623–632.

58. Lancaster AK, Single RM, Solberg OD, Nelson MP, Thomson G (2007) PyPop update—a software pipeline for large-scale multilocus population genomics. Tissue Antigens 69 Suppl 1: 192–197.

59. Watterson GA (1978) The homozygosity test of neutrality. Genetics 88: 405–417.

60. Martin MP, Single RM, Wilson MJ, Trowsdale J, Carrington M (2008) KIR haplotypes defined by segregation analysis in 39 Centre d’Etude Polymorphisme Humain (CEPH) families. Immunogenetics 60: 767–774.

61. Majorczyk E, Luszczek W, Nowak I, Pawlik A, Wisniewski A, et al. (2008) Distribution of killer cell immunoglobulin-like receptor genes in Poles. Int J Immunogenet 35: 405–407.

62. Velickovic M, Velickovic Z, Panigoro R, Dunckley H (2009) Diversity of killer cell immunoglobulin-like receptor genes in Indonesian populations of Java, Kalimantan, Timor and Irian Jaya. Tissue Antigens 73: 9–16.

63. Holienbach JA, Meenaugh A, S neger C, Alaez G, Bengoche M, et al. (2010) Report from the killer cell immunoglobulin-like receptor (KIR) anthropology component of the 15th International Histocompatibility Workshop: worldwide variation in the KIR loci and further evidence for the co-evolution of KIR and HLA. Tissue Antigens 76: 9–17.

64. Gerztekadhze K, Norman PJ, Abi-Rached L, Layrisse Z, Parham P (2006) High KIR diversity in Americans is maintained using few gene-content haplotypes. Immunogenetics 58: 474–480.

65. Norman PJ, Carrington CV, Byng M, Maxwell LD, Curran MD, et al. (2002) Natural killer cell immunoglobulin-like receptor (KIR) locus profiles in African and South Asian populations. Genes Immun 3: 86–95.

66. Jiang K, Zhu FM, Lv QF, Yan LX (2005) Distribution of killer cell immunoglobulin-like receptor genes in Indonesian populations of Java, Kalimantan, Timor and Irian Jaya. Tissue Antigens 73: 9–16.

67. Majorczyk E, Luszczek W, Nowak I, Pawlik A, Wisniewski A, et al. (2008) Distribution of killer cell immunoglobulin-like receptor genes in Poles. Int J Immunogenet 35: 405–407.

68. Do CB, Batzoglou S (2008) What is the expectation maximization algorithm? Nat Biotechnol 26: 897–899.

69. Watterson GA (1978) The homozygosity test of neutrality. Genetics 88: 405–417.

70. Martin MP, Single RM, Wilson MJ, Trowsdale J, Carrington M (2008) KIR haplotypes defined by segregation analysis in 39 Centre d’Etude Polymorphisme Humain (CEPH) families. Immunogenetics 60: 767–774.