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A Novel Method for KIR-ligand Typing by Pyrosequencing to Predict NK Cell Alloreactivity

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Abstract

Studies have shown that KIR-ligand mismatching to predict NK cell alloreactivity may result in less relapse and better survival in patients with AML. KIR-ligands are distinguished by single nucleotide polymorphisms (SNPs) from HLA-B and HLA-C sequences. We hypothesized that pyrosequencing to determine KIR-ligand status by direct sequencing of the ligand epitope can be done as an alternative to high resolution HLA-typing. Pyrosequencing is rapid and would be particularly useful in analysis of retrospective cohorts where high resolution HLA-typing is unavailable or too expensive. To validate this assay, RNA and DNA from 70 clinical samples were tested for KIR-ligand by pyrosequencing. Primer binding to invariant regions without known SNPs was critical for KIR-ligand assignment by pyrosequencing to be in full concordance with high resolution HLA-typing. Pyrosequencing is sensitive, specific, high-throughput, inexpensive, and can rapidly screen KIR-ligand status to evaluate potential alloreactive NK cell or transplant donors.

Keywords

NK cells; human; killer immunoglobulin receptor; HLA-typing; pyrosequencing; alloreactivity

Introduction

Killer immunoglobulin receptor (KIR) ligands are defined by common epitopes of human leukocyte antigen (HLA)-B and HLA-C sequences. Transplant strategies first described by Ruggeri et al. [1], have resulted in less relapse, less GVHD and better overall survival in patients with AML where donor natural killer (NK) cell receptors are mismatched to recipient KIR-ligands [2–5]. Correct KIR-ligand assignment will impact on current strategies aimed at most

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accurately determining NK cell donor anti-host alloreactivity (KIR-ligand mismatch, receptor/ligand models) [5–7].

KIR-ligands can be grouped into 3 major categories based on the amino acid sequence determining the KIR-binding epitope in HLA-B and HLA-C alleles. Most HLA-B alleles can be classified as bearing either the Bw4 or Bw6 epitope, defined by a set of single nucleotide polymorphisms (SNPs) encoding amino acid positions 77 and 80–83 in the alpha 1 domain of the HLA-B molecule. Bw6 allele types, which are not KIR-ligands, are defined by the amino acid sequence G77 or S77, L78, R79, N80, L81, R82, G83, with no polymorphisms spanning this region [6,8,9]. In contrast, Bw4 allele types, which are KIR-ligands, can have multiple amino acid sequences at these positions [6,8]. Acceptable Bw4 motifs are N/I, D/T, S/T, N/T for amino acids 77/80 with other polymorphisms spanning this region [6,8,9]. HLA-C alleles can be categorized into 2 groups, C1 or C2 group defined by SNPs found at amino acid positions 77 and 80. Generally, C1 is designated S77 and N80, while C2 is designated N77 and K80 [6,10,11]. Studies using site-directed mutagenesis and crystal structure analysis of KIR bound to HLA-C molecules have shown that the footprint of KIR on the HLA molecule involves amino acid residues 73, 76, 80 and 90, where ligand specificity is imparted solely by amino acid 80; C1 group is N80 and C2 group is K80 [12–14]. Almost all HLA-B and -C alleles can be classified into one of the major KIR-ligand categories, although there are known rare ill-defined alleles that do not fit into the classical definition of Bw4, C1 or C2 group ligands [15,16]. Killer-immunoglobulin receptors KIR3DL1, KIR2DL2/L3 and KIR2DL1 bind KIR-ligands Bw4, C1 and C2 respectively, resulting in inhibition of NK cell mediated lysis [17–19].

Currently, allele level high-resolution HLA-typing is the standard for accurately determining KIR-ligand status [20,21]. As this may not be standard at some centers, unavailable from retrospective cohorts, or not practical for research samples, KIR-ligand specificity is being extrapolated from serologic or low-resolution HLA data. Although this may be accurate in most donor/recipient pairs, misclassifications based on the frequency of less common alleles can result in assignment to the opposite KIR-ligand group (Bw4 versus Bw6 or C1 versus C2). This assignment is only as accurate as class I HLA-typing and has been modified over time.

Pyrosequencing is a relatively new method for sequencing DNA and is especially useful in detecting SNPs in known sequences. It is emerging as an innovative method that may outperform other common methods for SNP analysis such as the use of hybridization probes, endonuclease restriction digestion, discrimination of mismatched DNA ligases or polymerases, allele-specific enzymatic cleavage and DNA sequencing [22]. It is a method where nucleotides are incorporated sequentially from a sequencing primer, differing from other sequencing methods in the order of nucleotide incorporation. A pyrosequencer dispenses individual nucleotides according to a preprogrammed nucleotide dispensation order; each nucleotide is tested for its incorporation into a single strand DNA template. Subsequently, each nucleotide incorporation event is followed by the release of pyrophosphate, which is used in a series of enzymatic reactions that eventually generates visible light in a quantity equal to the amount of nucleotide incorporated. The light is detected by the pyrosequencer and displayed as a peak in a pyrogram. Unincorporated nucleotides and excess ATP are degraded before the next cycle of pyrosequencing begins [20].

Given the sensitivity and specificity of pyrosequencing, our aim was to apply this method as a rapid, high-throughput assay for accurately determining KIR-ligand status, by directly sequencing the actual ligand epitope, which did not rely on high resolution HLA-typing. This method could be used in a clinical setting to help predict NK cell or transplant donor alloreactivity and on banked RNA or DNA tissue samples for retrospective analysis when high resolution typing is not available or cost prohibitive.

Methods

Patients and Samples

Thirty-four samples from 17 donor/recipient pairs were tested for the presence or absence of KIR-ligands by pyrosequencing. All patients received haploidentical NK cell infusions as described on a clinical trial to treat patients with poor prognosis AML [4]. After assay development, an additional 36 samples from 18 donor/recipient pairs were also tested. Peripheral blood mononuclear cell pellets were disrupted in RLT buffer. RNA was extracted using RNeasy mini kit (Qiagen, Valencia, CA) and cDNA synthesized using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) from frozen peripheral blood mononuclear cell pellets.

Pyrosequencing

PCR primers were designed to amplify most consensus regions, such that the amplicon for HLA-B and HLA-C contained the polymorphic region of interest. HLA-B PCR reactions used the forward primer 5'-TTCGTGAGGTTCGACAGC-3' and the reverse primer 5'-biotinylated-CTCGTTCAGGGCGATGTAAT-3'. For HLA-C, the forward primer was 5'-TATTGGGACCGGGAGACACAG-3' and the reverse primer was 5'-biotinylated-GGCGATGTAATCCTTGCCGTC-3'. RT-PCR reactions were carried out using Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA). DNA-PCR reactions were carried out using AccuPrime™ Taq DNA Polymerase System (Invitrogen, Carlsbad, CA) with 10X AccuPrime PCR Buffer II and 1ul additional 10mM dNTP mix per reaction. PCR reactions were carried out in a PTC-100 Programmable Thermal Controller (MJ Research, Inc.) at 57° C for HLA-B and at 53° C for HLA-C over 40 cycles when using Platinum Taq DNA Polymerase and 35 cycles when using AccuPrime™ Taq DNA Polymerase System. PCR products were manipulated according to the Pyrosequencing Sample Preparation Guidelines [20]. The sequencing primer for HLA-B is 5'-CACAGACTGACCGAGAG-3' and for HLA-C is 5'-ACAGACTGACCGAGTGA-3'. Nucleotide dispensation order for HLA-B is TGAGACTAGCGTCGATCGTCGCGTCGC and for HLA-C is TGAGCTGCGACGT. Sequencing is carried out as instructed by the protocol using the PSQ 96MA (Biotage, Charlottesville, VA) pyrosequencing apparatus.

Results

Allele alignments of all known HLA-B and HLA-C sequences from the IMGT/HLA Database as of June 2006 were analyzed and categorized according to their KIR-ligand assignment based on known sequences (Table 1) [15]. This data is now included as part of the IPD-KIR Database as a simple public web based KIR-ligand calculator (www.ebi.ac.uk/ipd/kir/ligand.html) which decodes both high and low resolution HLA typing into KIR-ligands as well as determining directional KIR-ligand mismatch between donor and recipient pairs (Fig. 1a and 1b) [23]. Most common serologic or low resolution typing groups stratify into either one or the other HLA-B or -C KIR-ligands, however KIR-ligand assignment by low-resolution HLA-typing leads to misclassifications as listed in Table 1 and the web-based KIR-ligand tool.

Pyrosequencing was used to directly determine KIR-ligand epitopes from both RNA and DNA of 34 donors and recipients from a recently reported allogeneic NK cell trial [4], and 36 additional samples from an ongoing allogeneic NK cell trial for the treatment of AML or breast cancer. After designing PCR reactions to amplify the HLA-B and -C segment containing the KIR-ligand epitope, sequencing primers were designed 1 base pair upstream of the first SNP of interest for direct KIR-ligand epitope sequencing. Pyrograms from patients or donors showing a Bw4/Bw4, Bw4/Bw6, Bw6/Bw6 or C1/C1, C1/C2 and C2/C2 assignment are shown

(Fig. 2 and 3, respectively). Allele level high-resolution HLA-typing was available on all samples to verify methods.

Initial test results showed some discrepancies between pyrosequencing and HLA typing data (data not shown). These samples were thoroughly investigated. Of the initial 34 samples tested, 3 were discrepant for the HLA-C assay. By high resolution HLA-typing, these samples were all heterozygous for C1/C2 group ligands (Cw*0102/Cw*1502, Cw*0702/Cw*1502, and Cw*0304/Cw*0602). Pyrosequencing results showed all samples were homozygous for the C1 group ligand (first two samples) or the C2 group ligand (third sample). PCR products from these reactions were sequenced by the Advanced Genetic Analysis Center at the University of Minnesota and showed that all were homozygous, in agreement with pyrosequencing data. To better understand these apparent discrepancies, a reanalysis of sequence alignments of HLA-C alleles was performed. We found that the initial primer set was designed over a polymorphic region, which did not amplify all known HLA-C alleles (Fig. 4a). It was evident that the initial set of HLA-C PCR primers were only amplifying Cw*0102, 0702 and 0602 and not Cw*0304 and 1502 in these discrepant samples, due to 2 mismatched bases located in relative close proximity to each other in the initial reverse primer (Fig. 4a). Although a single mismatched base was present in the initial forward primer for several HLA-C alleles, this did not affect proper PCR amplification. We revised the primers by moving the forward and reverse primer away from common polymorphic sites into better consensus regions where HLA-C allele mispriming would rarely be encountered (Table 2). All samples were retested using redesigned PCR primers (Fig. 4a); results were in full concordance with high resolution HLA data.

To further validate the assay, 36 additional samples were tested by pyrosequencing for their HLA-C KIR-ligand status using the revised set of HLA-C PCR primers. All samples were in concordance with high resolution HLA data when investigating both RNA and DNA templates, with the exception of 1 recipient sample. By high resolution HLA-typing, this sample was Cw*0102/Cw*0602 and assigned a C1/C2 KIR-ligand status. In contrast, pyrosequencing results from RNA/cDNA based template showed this sample as homozygous C2, while pyrosequencing results of this samples DNA showed that it was indeed C1/C2, in concordance with HLA-typing data. Allele alignments were performed but did not show any evidence of mispriming.

Discrepancies were also initially found in the HLA-B assay. Among 3 of 34 samples, high-resolution HLA-typing showed they were homozygous (Bw6/Bw6) for HLA-B (B*1562/B*1563, B*1501/B*1801 and B*0702/B*4101), while Pyrosequencing showed they were heterozygous (Bw4/Bw6). Although sequence alignments of HLA-B alleles and position of PCR primers did not show any evidence of mispriming, new primers were designed (Fig. 4b). Using the initial primer set, the PCR product was 105 base pairs with the reverse primer located 5 bases from the last SNP site. With the redesigned PCR primers, a 180 base pair PCR product resulted with the reverse primer located 130 bases from the last SNP site. Comparison of initial PCR primers to newly designed primers by the Assay Design Software, Version 1.0.6, by Biotage, showed that the initial primer set was more likely to produce amplicons with template loops associated with the biotinylated strand. The initial PCR primer set received a maximum penalty score of 100 while the redesigned set had a penalty score of 39. Although most samples resulted in accurate results using our initial primers, it is likely that template loops interfered with sequencing reactions in these discrepant samples. Samples were retested by pyrosequencing and full concordance with HLA data resulted. An additional 36 samples were tested by pyrosequencing using the revised set of HLA-B PCR primers to further validate this assay. All samples were in concordance with high resolution HLA data.

Discussion

Allogeneic stem cell transplantation is a common treatment for advanced hematological and non-hematological malignancies. It has become increasingly apparent that definitive information about HLA allele identity will impact on current strategies. Although there have been many advances, HLA-typing is evolving and requires numerous primer pairs to reach the desired level of resolution. Currently, sequence based typing is the gold standard for HLA allele identification. However, this method incorporates nucleotides simultaneously along all DNA templates making it difficult to resolve ambiguities in heterozygous samples [20].

Pyrosequencing is a powerful and relatively new sequence-by-synthesis method for sequencing DNA. It has been used in a variety of applications such as genotyping of SNPs, microbial typing and screening for genetic mutations with great success. Pyrosequencing has been used to sequence segments of HLA with relative ease and greater resolution than traditional sequence based typing methodology. It can also be used for allele quantification in mixed samples, such as in hematopoietic chimerism studies to determine the proportion of DNA derived from two different populations of cells after allogeneic stem cell transplantation, with an accuracy of +1–5% in the range of 5–95% of mixed samples [24–26]. Such studies could provide insights into the relationship between the frequency of NK cell chimerism, NK cell receptor expression, acquisition and function in a patient post-transplant. Different segments of the DNA can easily be investigated by designing different primers spanning different regions of interest.

In this study, 70 samples were typed for their KIR-ligand status by high resolution HLA typing and by pyrosequencing. Complete concordance with high resolution HLA data depended on choice of PCR primers. Initial PCR primer pairs were found to be inefficient; HLA-C primers were not amplifying all alleles, while HLA-B primers resulted in products with template loops that interfered with sequencing reactions. As with any sequencing method, the quality of sequencing reactions was highly dependent on the purity of the PCR product. Results could be determined from RNA or DNA with equal resolution. Finding a discrepancy between RNA and DNA templates in one sample suggests either select HLA down regulation or a HLA-C nonexpressed allele, which has been reported [27]. This is important and may have functional implications, since one mechanism by which tumor cells evade the immune system is by down regulation of class I HLA [28,29]. This possibility suggests that KIR-ligand determination from RNA may be more functionally accurate.

Although this method has several advantages over traditional sequencing techniques, limitations with its short read length, typically 50–60 bases maximum, makes this method less useful for some applications such as genome sequencing [30]. Although it has been used to sequence portions of HLA genes, resolution of contiguous HLA gene sequences is not possible due to this limitation. In order to accomplish this task, the HLA gene must be pyrosequenced in segments and then assembled [20]. Improvements in pyrosequencing technology, reagent chemistry and automation will increase sensitivity, specificity and extended read lengths. However, for the purpose of KIR-ligand typing, pyrosequencing may be used for novel allele discovery since the read length of the defining sequence is relatively short. Modification of the nucleotide dispensation order, to a redundant cyclic order, could be used to ensure proper KIR-ligand classification and novel allele discovery. Although complications may arise with using pyrosequencing methodology for *de novo* sequencing in heterozygous samples, close examination of the known sequence and comparison of peaks for bases which serve as internal controls may help determine the sequence.

Another limitation of pyrosequencing is the accurate determination of the number of incorporated nucleotides in homopolymeric regions, due to nonlinear light response following

incorporation of several identical nucleotides [30]. In the pyrograms for the HLA-C, the “A” peak at the second SNP site for all three possible outcomes (C1/C1, C2/C2, C1/C2) is consistently the height of a triple peak. Interpretation of this information alone would translate to a predicted sequence of AAA, or a Lysine at amino acid position 80, indicative of C2 group. However, analysis of the relative height of the next peak, in comparison to peaks of non SNP sites or internal controls, help distinguish whether this is truly a run of 3 “A”s or a false signal. Given the known sequence of this region there are three possible outcomes: AACC, AAAC, or AACC/AAAC. C1/C1 peaks show a strong double C peak (sequence of AACC), whereas C2/C2 shows a single C peak (sequence of AAAC), and C1/C2 shows a C peak which is intermediate in intensity (AACC/AAAC). Although the pyrosequencing program has an analysis function that can call bases for individual SNP sites, based on preprogrammed information and computer generated algorithms, interpretation of pyrograms should always be interpreted with care. In addition, the pyrosequencing program generates examples of pyrograms for all possible outcomes and can determine the ratio of one base over another at that particular SNP site to help distinguish the sequence in heterozygote samples.

Due to the high homology of HLA-B and -C sequences it is possible that both HLA loci are amplified during PCR amplification resulting in possible interference. However, the location of the sequencing primer, the nucleotide dispensation order, and the differences in SNP frequency between the HLA-B and -C loci insure specificity. The HLA-B sequencing primer overlaps the HLA-C sequencing primer with its 3’ end located 1 base pair upstream from that of the 3’ end of the HLA-C primer. At amino acid position 76 there is a 1 base mismatch in HLA-B and -C sequences at the location of the 3’ end of the sequencing primers, which helps distinguish the difference between the two assays by causing differences in primer binding efficiencies for each assay. It is possible that the HLA-C sequencing primer may bind to HLA-B alleles and vice versa, but the nucleotide dispensation order determined by the SNP frequency further helps distinguish between the two possibilities.

Due to the highly polymorphic nature of HLA it is difficult to design PCR primers in true consensus regions spanning all alleles. Although the current PCR primers worked in all cases tested, it is possible that they may not amplify some alleles. Sequence alignments of all known HLA-B and -C alleles show that there are alleles which may not amplify due to polymorphisms within the sequence of the primer binding site. However, these situations would be encountered rarely based on the known frequencies of these alleles [31].

In conclusion, determination of KIR-ligand status by pyrosequencing may be useful for evaluating potential alloreactive NK cell or transplant donors. We acknowledge that the critical mismatch for determining NK cell alloreactivity is between the recipient KIR-ligands and the donor KIR, therefore accurate prediction of NK-mediated GVL may still require donor KIR genotyping. Pyrosequencing is specific, sensitive, high-throughput, inexpensive, and can rapidly screen to determine KIR-ligand status. Based on batch testing of 30 samples, we estimate that a single sample would cost approximately \$20, 1/10 the cost of the current research charge of high resolution HLA-typing at our institution. Since pyrosequencing is a nearly fully automated system and capable of processing and analyzing up to 96 samples at once, batch testing will increase this cost differential.

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IPD - KIR Database

KIR Ligand Calculator

Search for Mismatches Between KIR Ligands				
Patient	HLA-B* 07	HLA-B* 15	HLA-Cw* 03	HLA-Cw* 14
Donor	HLA-B* 40	HLA-B* 44	HLA-Cw* 01	HLA-Cw* 04
<input type="button" value="Submit Typings"/> <input type="button" value="Reset the form"/>				

Predicted Ligands for Patient				
Typing	B*07	B*15	Cw*03	Cw*14
Alleles	<u>Allele listing</u>	<u>Allele listing</u>	<u>Allele listing</u>	<u>Allele listing</u>
Ligand	Bw6	Bw6	C1	C1
Exceptions	B*0711 (Unclassified) B*0727 (Bw4) B*0736 (Bw4) B*0738 (Bw4)	B*1513 (Bw4) B*1516 (Bw4) B*15170101 (Bw4) B*15170102 (Bw4) B*151702 (Bw4) B*1523 (Bw4) B*1524 (Bw4) B*1536 (Bw4) B*1543 (Bw4) B*1557 (Unclassified) B*1567 (Bw4) B*1587 (Unclassified) B*1589 (Bw4) B*1595 (Bw4) B*9515 (Bw4)	Cw*0307 (C2) Cw*0310 (C2) Cw*0315 (C2) Cw*0329 (C2)	
Predicted Ligands for Donor				
Typing	B*40	B*44	Cw*01	Cw*04
Alleles	<u>Allele listing</u>	<u>Allele listing</u>	<u>Allele listing</u>	<u>Allele listing</u>
Ligand	Bw6	Bw4	C1	C2
Exceptions	B*4013 (Bw4) B*4019 (Bw4) B*4037 (Unclassified) B*4047 (Bw4)	B*4409 (Bw6) B*4446 (Bw6)		Cw*0411 (C1)
Mismatching in the GvH Direction				
HLA-B	KIR ligands are (mis)matched in the GvH Direction (Bw4)			
HLA-C	KIR ligands are (mis)matched in the GvH Direction (C2)			
Mismatching in the HvG Direction				
HLA-B	KIR ligands are matched			
HLA-C	KIR ligands are matched			
In summary, these ligands will be (mis)matched in the GvH direction and matched in the HvG direction.				
Use the KIR Ligand Calculator again				

Figure 1.

a) IPD-KIR Database: input screen of KIR-ligand calculator (www.ebi.ac.uk/ipd/kir/ligand.html). HLA-B and -C alleles of patient and donor is entered into the appropriate fields. In this example, the patient has low resolution typing HLA-B*07/15 and HLA-Cw*03/14, while the donor has HLA-B*40/44 and HLA-Cw*01/04. Click "Submit Typings" after entering HLA information. b) IPD-KIR Database: output screen of KIR-ligand calculator. Results decode high or low resolution HLA typing into KIR-ligands and determine KIR-ligand mismatch in the GvH or HvG directions.

Figure 2.

Pyrograms of known Bw4/Bw4, Bw6/Bw6 and Bw4/Bw6. Highlighted area is the region of interest (SNPs), areas not highlighted are known sequences and serve as internal controls. The presence and intensity of peaks seen in the pyrogram determine the sequence. For example, pyrogram of Bw4/Bw4 is read as follows: double peak A, double peak C, single peak T, G, C, double peak G, single peak A, T, C, G, C, G, C, T, double peak C, single peak G and C: resulting in a sequence of **AACCTGCGGATCGCGCTCCGC** (polymorphic nucleotides are denoted in bold and underlined). For each SNP site the pyrosequencing analysis program assigns bases according to a computer generated algorithm determined by preprogrammed information of the known sequence, SNP sites and nucleotide dispensation order. They appear over the region of the pyrogram which contain the SNP site. For example, the second SNP site with an A/A present is interpreted as homozygous A at this position and therefore Bw4/Bw4. When there is a G/G present it signifies homozygous Bw6 and a G/A indicates Bw4/Bw6. Often times the presence of a heterozygous sample will result in a less intense peak at that SNP site because the light emitted is generally proportionate to the amount of nucleotide incorporated. This may cause some confusion especially if the SNP site contains a possible nucleotide that is identical or adjacent to a homopolymeric region. Close examination of the first and second SNP site for the three different pyrograms will show this effect due to nonlinear light response following incorporation of several identical nucleotides.

Figure 3.

Pyrograms of known C1/C1, C2/C2, C1/C2. Highlighted area is the region of interest (SNPs), areas not highlighted are known sequences and serve as internal controls. The presence and intensity of peaks seen in the pyrogram determine the sequence. For example, pyrogram of C1/C1 is read as follows: single peak G, double peak C, single peak T, G, C, double peak G, double peak A, double peak C, single peak T: resulting in the sequence **GCCTGCGGAACCT** (polymorphic nucleotides are denoted in bold and underlined). Interpretation of these pyrograms is based on 2 SNP sites. For the first SNP site, when a sample is homozygous C1, the pyrogram will display a G/G above that peak, for homozygous C2, an A/A and for heterozygous samples, a G/A.

Figure 4a. HLA C allele alignments for discrepant samples

Figure 4b. HLA B allele alignments for discrepant samples

Figure 4.
a) HLA-C alignment for discrepant samples. Shown are the positions of initial and redesigned PCR primers (denoted by dotted box and solid lined box respectively), as well as sequencing primers (denoted by bold solid lined box) and SNP sites for HLA-C assay, amino acid 77 and 80. b) HLA-B alignment for discrepant samples. Shown are positions of initial and redesigned PCR primers (denoted by dotted box and solid lined box respectively), as well as sequencing primers (denoted by bold solid lined box) and SNP sites for HLA B assay, amino acid 77, 80–83.

Table 1

KIR-ligand Assignment with Discrepancies based on Low-resolution HLA-typing

Low Resolution HLA-type	Main KIR-ligand Assignment	Exceptions (assigned to opposite group)	Unclassified alleles
B*14, 42, 45, 46, 48, 50, 54, 67, 73, 78, 81, 82, 83	Bw6		
B*38, 49, 51, 57, 58, 59	Bw4		
B*07	Bw6	*0727, 0736, 0738	*0711
B*08	Bw6	*0802, 0803	*0806, 0817
B*13	Bw4	*1309	
B*15 and *95	Bw6	*1513, 1516, 15170101, 15170102, 151702, 1523, 1524, 1536, 1543, 1567, 1589, 1595	*15010102N, 1557, 1587, 1594N
B*18	Bw6	*1809	
B*27	Bw4	*2708, 2712, 2718, 2726, 2733	
B*35	Bw6		*3527, 3552, 3556
B*37	Bw4	*3711	*3705
B*39	Bw6		*3920
B*40	Bw6	*4013, 4019, 4047	*4037
B*41	Bw6		*4105
B*44	Bw4	*4409, 4446	
B*47	Bw4	*4702, 4703	
B*52	Bw4		*5209
B*53	Bw4		*5305
B*55	Bw6		*5512
B*56	Bw6	*5607	
Cw*01, 14	C1		
Cw*02, 05, 17, 18	C2		
Cw*03	C1	*0307, 0310, 0315	
Cw*04	C2	*0411	
Cw*06	C2	*0611	
Cw*07	C1	*0707, 0709	
Cw*08	C1	*0810	
Cw*12	C1	*120401, 120402, 1205, 1209	
Cw*15	C2	*1507	
Cw*16	C1	*1602	

Table 2Alleles that may not amplify and frequencies as reported by www.allelefrequencies.net [31]

HLA type	KIR-ligand type	Position of polymorphism from the 3' end	Frequency of allele
Cw*030404	C1	4 th base of forward primer	Rare (~0%)
Cw*1206	C1	5 th base of reverse primer	Rare
B*080103	Bw6	4 th base of forward primer	Rare
B*150104	Bw6	12 th base of forward primer	Rare
B*1560	Bw6	6 th base of forward primer	Rare
B*4426	Bw4	16 th base of forward primer	0.3% Kenya
B*5401	Bw6	12 th base of forward primer	Relatively high in Japanese populations, 6.3%, see www.allelefrequencies.net for more detail
B*5402	Bw6	12 th base of forward primer	0.2% China Beijing Shijiazhuang Tianjian Han
B*5403	Bw6	12 th base of forward primer	Rare
B*5404	Bw6	12 th base of forward primer	Rare
B*5405N	Bw6	12 th base of forward primer	Rare
B*5406	Bw6	12 th base of forward primer	Rare
B*5407	Bw6	12 th base of forward primer	Rare
B*5408N	Bw6	12 th base of forward primer	Rare
B*5515	Bw6	At 3' end of forward primer	Rare
B*4433	Bw4	9 th base of reverse primer	Rare
B*5138	Bw4	9 th base of reverse primer	Rare
B*150103	Bw6	17 th base of reverse primer	Rare
B*1805	Bw6	17 th base of reverse primer	2% Portugal Central