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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.

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 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

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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-immunoglobin 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 out perform 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′-TATTGGGACCGGGAGA-CACAG-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 10× AccuPrime PCR Buffer II and 1 μl additional 10 mM 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 it is 5′-ACAGACTGACCGAGTGA-3′. Nucleotide dispensation order for HLA-B is TGAGACTAGCGTCGATCGTCGCGTCGC and for HLA-C it 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