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High-throughput killer cell immunoglobulin-like receptor genotyping by MALDI-TOF mass spectrometry with discovery of novel alleles

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Abstract

The killer cell immunoglobulin-like receptors (KIR) interact with major histocompatibility complex (MHC) class I ligands to regulate the functions of natural killer cells and T cells. Like human leukocyte antigens class I, human KIR are highly variable and correlated with infection, autoimmunity, pregnancy syndromes, and transplantation outcome. Limiting the scope of *KIR* analysis is the low resolution, sensitivity, and speed of the established methods of *KIR* typing. In this study, we describe a first-generation single nucleotide polymorphism (SNP)-based method for typing the 17 human *KIR* genes and pseudogenes that uses analysis by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. It is a high-throughput method that requires minute amounts of genomic DNA for discrimination of *KIR* genes with some allelic resolution. A study of 233 individuals shows that the results obtained by the SNP-based *KIR*/MALDI-TOF method are consistent with those obtained with the established sequence-specific oligonucleotide probe or sequence-specific polymerase chain reaction methods. The added sensitivity of the *KIR*/MALDI-TOF method allowed putative novel alleles of the *KIR2DL1*, *KIR3DL1*, *KIR2DS5*, and *KIR2DL5* genes to be identified. Sequencing the *KIR2DL5* variant proved it was a newly discovered allele, one that appears associated with Hispanic and Native American populations. This *KIR*/MALDI-TOF method of *KIR* typing should facilitate population and disease-association studies that improve knowledge of the immunological functions of KIR–MHC class I interactions.

Keywords

KIR; HLA; MALDI-TOF; Genotyping; SNP

Introduction

Killer immunoglobulin-like receptors (KIR) are a family of inhibitory and activating major histocompatibility complex (MHC) class I receptors expressed on natural killer (NK) cells and subpopulations of T cells (Bottino et al. 2005; Moretta 2002). The inhibitory KIR, in partnership with CD94:NKG2A, ensure that NK cells are tolerant of healthy autologous cells and responsive to cells with compromised MHC class I expression (Carrington and Norman 2003), as occurs frequently in virus-infected and tumor cells. Although their ligands and

functions are less clearly defined, the activating KIR are hypothesized to contribute to the activation of NK cells in response to infection and malignancy.

The *KIR* gene family is part of the leukocyte receptor complex, located on human chromosome 19q13.4. The *KIR* complex comprises a tandem array of highly homologous genes, which exhibits haplotypic variation in gene content as well as polymorphism of the individual *KIR* genes. As a consequence of these variations, unrelated individuals usually differ in *KIR* genotype. Specific KIR, either alone or in combination with human leukocyte antigens (HLA) class I, have been correlated with a variety of diseases and syndromes. These include the outcome of bone marrow transplantation (Cook et al. 2004; Gagne et al. 2002; Parham and McQueen 2003; Ruggeri et al. 2002), the progress and severity of virally transmitted disease (Christiansen et al. 2003; Khakoo et al. 2004; Martin et al. 2002; Trachtenberg et al. 2004), the probability of preeclampsia during pregnancy (Hiby et al. 2004), and susceptibility to autoimmune diseases such as psoriatic arthritis, scleroderma, sarcoidosis, and type 1 diabetes (Mizuki et al. 2000; Momot et al. 2004; Nelson et al. 2004; van der Slik et al. 2003). Collectively, these studies make a convincing case that interactions between variable HLA class I ligands and variable KIR have considerable impact on human health. Consequently, better understanding of the underlying mechanisms could influence future clinical practice.

Limiting interpretation of the published studies on clinical associations and population comparisons of *KIR* are the small cohorts analyzed and the low resolution of the *KIR* genotyping, which has mainly involved determination of the presence and absence of *KIR* genes. The key problems are that the established methods of *KIR* genotyping are labor intensive and require more DNA (300 ng–5 µg) than is compatible with many of the samples that are available and informative to study. What are needed are new methods that combine speed, economical consumption of DNA, and a resolution that distinguishes all *KIR* alleles. To this end, we have explored the use of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS), as a technique that can accurately and rapidly detect single nucleotide polymorphisms (SNPs), the dominant feature of *KIR* variation. In this study, we describe the development of a MALDI-TOF MS-based method for highly accurate high-throughput *KIR* genotyping, its comparison to the established *KIR* typing methods, and its application to epidemiological studies and the discovery of novel *KIR* alleles.

Materials and methods

Assay design and development

The SEQUENOM™ MALDI-TOF MS-based homogenous mass extend (hME) platform was used to develop the high-throughput SNP-based *KIR*/MALDI genotyping assay. The instrumentation and related assay design and genotyping software comprise the MassARRAY™ MALDI-TOF system (San Diego, CA).

SNPs that differentiate the individual *KIR* genes were identified by inspection in silico of the 17 locus *KIR* sequence alignment in the Immuno Polymorphism Database (IPD; <http://www.ebi.ac.uk/ipd/kir>). A region of ~400 bp surrounding the SNP of interest was targeted for capture after evaluation for homology in the flanking regions and base composition of the extension primer site. The targeted sequence was uploaded to the SEQUENOM™ MassArray Assay Design software, which assisted in the design of primers, typically 17–21 bp in length, to capture a ~150–250 bp region surrounding the SNP (Table 1). The “capture primers” were designed with a 5′ 10-mer tag to keep them out of the 5–10 kDa window of detection used for this application; without the 10-mer tag, the capture primers would have similar mass to the extended and unextended hME products and confound the analysis. The 10-mer tag also helped to balance the primers so that they functioned well in multiplex reactions, which were initially identified using the SEQUENOM™ Design software, and then

optimized empirically. Because of the high degree of homology between the *KIR* loci, some assays could not be multiplexed.

Extension primers that query the chosen SNP were designed to terminate at the base immediately adjacent to the SNP (Table 1). Because of regions of high homology in the *KIR* genes, the designer software required extensive manipulation to obtain the primer designs necessary to create the specificity needed for a working *KIR* assay. Both capture primers and extension primers were chosen with an eye towards simultaneous identification of a particular locus (locus-specific assay) and a highly conserved region (positive control assay).

Sample populations and DNA preparation

Samples for validation of the method consisted of previously *KIR*-genotyped International Histocompatibility Working Group (IHWG) samples (Cook et al. 2003; Gomez-Lozano and Vilches 2002) and other controls from Stanford University (Uhrberg et al. 1997; Valiante et al. 1997 and unpublished). The *KIR*/MALDI method was further validated using two population-based sample groups, which were analyzed by both the *KIR*/MALDI and at least one other *KIR* genotyping method. These were 120 samples (59 donor/recipient pairs and two donors) from the National Marrow Donor Program (NMDP) repository and 98 samples from the Chicago Multicenter AIDS Cohort Study (MACS). All samples were genotyped in-house or previously genotyped by other laboratories using sequence-specific oligonucleotide probe (SSOP; Crum et al. 2000; Trachtenberg, unpublished) and/or sequence-specific polymerase chain reaction (PCR; SSP) methodologies (Gomez-Lozano and Vilches 2002; Uhrberg et al. 1997). Data from these previous analyses were used in the validation of the *KIR*/MALDI method. Whole blood or lymphocyte cell pellet samples were manually extracted using Qiagen's 96-well block extraction method (QIAamp 96 DNA Blood Kit) or robotically with Genovision's GenoM-6 magnetic bead-based extraction method. They were then quantitated using Picogreen (Invitrogen, Carlsbad, CA) fluorescence methodology and normalized to a final concentration of 2 ng/μL. Approximately 10.0% of the samples yielded concentrations below 2 ng/μL and were, therefore, typed using lower DNA concentrations.

KIR genotyping assay

Primary PCR for capture of the region surrounding the SNP was performed on an Applied Biosystems GeneAmp 9700 in a 384-well format in a volume of 5 μL with the following profile: denaturation at 95°C for 15 min, followed by 45 cycles of denaturation at 95°C for 20 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min, and a final extension at 72°C for 3 min. Final concentrations of each component were as follows: 1.25× Qiagen HotStar 10× PCR Buffer, 3.5 mM MgCl₂, 500 μM deoxyribonucleotide triphosphate (dNTPs; each), 100 nM PCR primers each, 0.15 U/reaction of Qiagen HotStar Taq, and 2 ng genomic DNA.

To inactivate the primary PCR by dephosphorylating any unincorporated dNTPs, a shrimp alkaline phosphatase (SAP) cocktail consisting of nanopure water, 10× hME buffer (SEQUENOM™), and 0.3 U SAP/reaction was distributed to the 384-well reaction plates using a Beckman Multimek 9600. Plates were then cycled on an Applied Biosystems GeneAmp PCR System 9700 with the following profile: dNTP terminal phosphate cleavage at 37°C for 20 min, followed by heat inactivation of the SAP enzyme at 85°C for 5 min, and a 4°C final hold.

To set up the primer extension reactions, 2 μL of hME cocktail was added directly to the PCR reactions using the Beckman Multimek 9600, bringing the final concentration of each reaction to ~1 μM each hME primer, 50 μM each d/ddNTP termination mixes, and 1.25 U Thermosequenase (Amersham). Extension reactions were then cycled on an Applied Biosystems GeneAmp PCR System 9700 according to the following profile: denaturation for 2 min at 94°C, followed by 99 cycles of denaturation at 94°C for 5 s, annealing at 52°C for 5

s, and extension at 72°C for 5 s, and a final hold at 4°C. After primer extension, 16 µL of nanopure water was distributed to each reaction using the Beckman Multimek 9600, followed by manual distribution of 6 mg of SpectroClean ion-exchange resin (SEQUENOM™). Plates were sealed with MJ Research (Hercules, CA) Microseal B adhesive sealers (#MSB-1001), rotated for approximately 15 min, and centrifuged at 3,000 rpm for 5 min. The reactions along with a three-point calibrant were spotted onto SpectroChip 384 chips with a Samsung MassArray NanoDispenser at a dispense speed of 65 mm/s.

The Assay Editor module within SEQUENOM™'s Mass-ARRAY Typer software was used to enter sample information and to map the chip in regards to name of assay, termination mix used, multiplex level, and genotyping rules for each hME.

MALDI-TOF MS analysis

Samples were analyzed on SEQUENOM™'s MassArray Compact MALDI-TOF MS, which processed each 384-well chip in approximately half an hour. Data was automatically retrieved by the SEQUENOM™ SpectroAC-QUIRE software and reviewed using the TrafficLights module of the MassARRAY Typer software.

Detailed information on peak heights for each assay and a probability value for each call, based on signal-to-noise ratios and peak probability statistics, were reviewed for individual samples as necessary. In this system, probability was automatically calculated and parsed into three levels of stringency, resulting in conservative, moderate, and aggressive calls. Conservative calls resulted in the lowest rate of uncalled genotypes, while aggressive calls resulted in a higher error rate (<1%). Low probability calls were excluded. Spectral data were reviewed for individual samples (Fig. 1) when necessary. The Cluster Plot software module was used to visualize atypical heterozygous and homozygous SNP states (Fig. 1).

Genotype analysis

Because the *KIR* genotype of any one sample is based on results from many different SNP assays, a second level of automated calling was incorporated for high-throughput genotyping. Our genotyping program “*KIR* Genotype Caller” was developed to capture the output data from the SpectroAnalyzer module of the SEQUENOM™ software suite to generate a genotype from the individual SNP assays based on a hierarchical SNP-pattern table (Table 2) and simultaneously tag anomalous calls and problematic samples for user inspection. For each run, the *KIR* Genotype Caller created the following:

1. Conflict log—which checked for agreement between replicates of the same assay applied to the same sample in a given data set (duplicate check)
2. A partial match log—which checked for agreement between assays that typed for the same gene in different domains and generated a potential recombinants list
3. A control check—which checked the SNP profiles of previously characterized samples against an index of expected results for these samples

Tables 3 and 4 present the hME hit pattern used to distinguish between *KIR3DL1* and *KIR3DS1* as an example of how *KIR* Genotype Caller used the hit patterns from the targeted domains to determine genotype.

Locus-specific genotyping using SSOP

Samples were amplified at *KIR* domains D0, D1, and D2 and a transmembrane–cytoplasmic region using ~100 ng of genomic DNA per amplification and assayed in an SSOP format with 39 biotinylated probes designed to identify 14 *KIR* genes and some alleles (Trachtenberg, unpublished; Crum et al. 2000). The specific constellation of 39 SSO probes utilized did not

distinguish *KIR2DL5* subtypes A and B. Amplified PCR products were denatured and vacuum blotted onto replicate 96-sample nylon membranes. Replicate membranes were hybridized to SSO probes, washed under stringent conditions to remove unbound probe, and developed using nonradioactive detection methods. *KIR* probe hybridization patterns were individually decoded using a computer program developed in-house.

Cloning and sequencing of a novel *KIR2DL5* allele

Genomic DNA was extracted from the 10th International Histocompatibility Workshop (IHW) OLGA B cell line using the Qiagen Genomic-tip 20/G kit. Primers LFcon63 and LRg1769 were used to amplify the entire coding region of *KIR2DL5* (Vilches et al. 2000). OLGA is presumed homozygous consanguineous for *KIR2DL1*, *KIR2DL3*, *KIR3DL1*, and *KIR3DL2* loci (Garcia et al. 2004), but *KIR2DL5A* and *KIR2DL5B* have also been reported for this sample (Gomez-Lozano and Vilches 2002), and in this study, OLGA typed as *KIR3DL1* and *KIR3DS1* positive. A total of 100 ng of DNA was amplified in 20- μ L reactions containing 0.4 U Phusion DNA Polymerase (New England BioLabs, Beverly, MA), 1 $\times$ GC buffer, and 10 pmol of each primer. The following conditions were used for long-range PCR: initial denaturation at 98°C for 45 s, 35 cycles of 98°C for 10 s, 67°C for 30 s, and 72°C for 5 min, followed by a final extension at 72°C for 8 min and a 4°C hold. The PCR products were electrophoresed in a 0.8% agarose gel, and the DNA was visualized by crystal violet staining. The 9.3-kb fragment was purified using a SNAP column (Invitrogen, Carlsbad, CA). 3'-A overhangs were added to the purified fragment using 2.5 U Taq DNA polymerase (Roche) and 2'-deoxyadenosine 5'-triphosphate (dATP) and incubation at 72°C for 20 min. The fragment was subsequently inserted into the pCR-XL-TOPO cloning vector (Invitrogen, Carlsbad, CA). Plasmids were isolated from individual colonies using the Qiaprep Spin miniprep kit and sent to the UC Berkeley DNA Sequencing Facility (Berkeley, CA) for complete sequencing of exons 1 through 9. Three clones were sequenced in forward and reverse directions, including two clones with complete genomic sequence of all introns and exons and one clone sequenced at exons only. Primers were chosen based on genomic sequence for *KIR2DL5* (<http://genome.ucsc.edu>).

Results

A method for high-throughput *KIR* genotyping using MALDI-TOF mass spectrometry

Study of the functional and clinical consequences of *KIR* variation is currently limited by the sensitivity, resolution, and speed of the assays used for *KIR* genotyping. To address these limitations, we developed a high-throughput SNP-based MS method for *KIR* genotyping that is based on the SEQUENOMTM MALDI-TOF MS platform and hME chemistry (outlined in Fig. 2).

A set of 38 SNPs that distinguish the 17 *KIR* genes and pseudogenes was identified by inspection in silico of the aligned *KIR* sequences from the IPD (<http://www.ebi.ac.uk/ipd/kir>). The two pseudogenes (*KIR2DP1* and *KIR3DP1*) were included in the analysis to help identify recombinants and to assist with haplotype analysis. Primer pairs were developed (Table 1) to 'capture' these SNPs in ~100–250-bp PCR products amplified from genomic DNA samples. After SAP treatment, the amplification products were subjected to a primer-extension reaction (called an hME) using a primer (Table 1) that terminates adjacent to the targeted SNP, which is extended through the SNP by incorporating deoxy and/or dideoxy nucleotides. The particular termination mixes used are dictated by the targeted SNP and its surrounding sequence. The rationale for a particular mixture being that by choosing an appropriate mixture of deoxy and dideoxy nucleotides, one can determine not only which extension products will be produced but which product will be extended by one, two, or three nucleotides. The MALDI-TOF MS is sensitive enough to distinguish between these nucleotide

extensions. Because recombination within these tandemly arrayed homologous genes is a source of novel alleles (Rajalingam et al. 2004; Shilling et al. 1998; Witt et al. 1999), the assay was designed to enable identification of recombinants or novel alleles. To accomplish this, the hME reactions for each functional *KIR* gene were designed to be redundant: SNPs from a minimum of two different domain-coding exons were targeted. The primer–extension products from the hME reactions were analyzed by MALDI-TOF MS, which measures the mass of the extension products and uses the mass differences to determine which nucleotides were incorporated to identify gene presence and absence and to detect novel variants.

This method for *KIR* genotyping provides two levels of specificity. The capture reaction limits the subsequent analysis to the inclusion of a single queried *KIR* gene and the conserved region in a framework or common gene, which provides a positive control for the PCR reaction (Fig. 3a). In the hME reaction, the choice of SNP, and the design of extension primers and dideoxy/deoxy nucleotide reaction mixes provide the second level of specificity and the potential for distinguishing allelic variants as well as the presence or absence of the targeted gene (Fig. 3b). The SNP-based *KIR*/MALDI-TOF assay uses 38 capture primer sets and 38 SNP extension primers multiplexed into 16 reactions to distinguish all 15 functional *KIR* genes and two pseudogenes, as well as certain *KIR2DL1*, *KIR2DS4*, and *KIR2DL5* variants. For the MALDI-TOF MS analysis, the hMEs are distributed on 384-array microchips that are analyzed in less than 30 min. The data generated is automatically deposited into an Oracle database, which can be accessed to visualize the spectra and assess the SNP calls. Data from the Oracle database is then sent to our *KIR* Genotype Caller program for automated *KIR* genotyping and data archiving. Using the *KIR*/MALDI system, ~384 *KIR* genotypes per technologist can easily be determined in 1 week.

Concordance between *KIR*/MALDI-TOF gene-content typing and established methods

In developing the SNP-based *KIR*/MALDI-TOF *KIR* genotyping assay, we analyzed a panel of 15 individuals who encompass most of the published variability at the *KIR* locus, as shown by analysis with the established SSOP and/or SSP methods. The set of 38 hME assays proved necessary and sufficient to define the presence or absence of the 15 expressed *KIR* genes and the two *KIR* pseudogenes. At this level of resolution, there was complete agreement between the results obtained with *KIR* /MALDI-TOF genotyping and those obtained by SSOP and SSP (Table 5).

A panel of 98 individuals from the Chicago MACS population that we had previously genotyped for *KIR* using the SSOP and/or SSP methods was analyzed using the *KIR*/MALDI-TOF method. Again, there was complete concordance in the determination of the *KIR* gene content. This analysis shows that in terms of its precision and resolution, the *KIR*/MALDI-TOF is the equal of the established SSOP and SSP methods. It combines the advantage of allowing high-throughput analysis with no loss in either accuracy or sensitivity.

Discovery of novel *KIR* alleles by *KIR*/MALDI-TOF typing

Certain *KIR* allele differences have profound functional consequences. For example, common *KIR2DS4* alleles are inactivated by a deletion of 18 nucleotides. Our *KIR*/MALDI-TOF typing system was designed to distinguish the *2DS4* deletion variants from the full-length forms and gave results concordant with those obtained by the SSOP and SSP methods (Table 5).

A more complicated situation is presented by *KIR2DL5*, for which the gene can variably be found in either the centromeric or telomeric parts of *KIR* haplotypes or in both parts. Although these genes have been designated as *KIR2DL5B* and *KIR2DL5A* according to their centromeric or telomeric position, respectively, their sequences show no A-specific or B-specific character and form a single lineage of seven ‘alleles.’ Consequently, to distinguish the two *KIR2DL5A*

subtypes from the five *KIR2DL5B* subtypes, it was necessary to perform allele-specific typing. We designed hME subtyping assays to distinguish the *KIR2-DL5A* and *KIR2DL5B* subtypes, based on six SNPs, previously used in SSP typing to discriminate *KIR2DL5* variants (Gomez-Lozano and Vilches 2002). In the 15-member panel, seven samples were previously subtyped for *KIR2DL5A* and *KIR2DL5B*, and these included individuals who had only *KIR2DL5A* or only *KIR2DL5B*. For six of the donors, there was concordance between *KIR*/MALDI-TOF and SSP typing (Table 5). The seventh donor, OLGA, had previously been characterized as having both *KIR2DL5A* and *KIR2DL5B* (Gomez-Lozano et al. 2002). Only one nucleotide was detected at each of the five SNP positions analyzed at *2DL5* for OLGA using the *KIR*/MALDI system. Furthermore, this combination of SNPs did not correspond to any of the known *KIR2DL5A* or *KIR2DL5B* alleles. Moreover, WT47 (IHWG sample) had also been typed as *KIR2DL5A* and *KIR2DL5B* (Gomez-Lozano et al. 2002) and did show the expected SNP patterns for the previously reported type. These data indicated that OLGA had a novel variant of *KIR2DL5*.

To test this hypothesis, the *KIR2DL5* gene from OLGA was isolated and characterized. Long-range PCR amplification of genomic DNA was used to obtain a fragment spanning the 5' untranslated region (UTR) through to the 3'UTR. This 9.3-kb fragment, including exons 1 through 9, was cloned and sequenced (GenBank accession #EF102434). The sequence analysis showed that OLGA had a *KIR2DL5* allele with a combination of nucleotide substitutions in exons 3 and 5 not seen in previously characterized *KIR2DL5* subtypes (Table 6). The OLGA *KIR2DL5* novel allele was also observed in a Hispanic individual from the National Marrow Donor Program study and in one sample from the MACS cohort.

Additional novel *KIR* alleles were identified from analysis of 120 individuals from the sample repository of the NMDP. At the level of the *KIR* gene content, *KIR*/MALDI-TOF typing gave results that were in full agreement with the *KIR* types we had previously obtained using the SSOP method. The increased resolution of the *KIR*/MALDI-TOF analysis identified four novel combinations of SNPs that correspond to putative novel alleles at the *KIR2DL1*, *KIR3DL1*, *KIR2DS5*, and *KIR2DL5* genes (Tables 7 and 8). The average putative novel allele frequency for the MACS and NMDP populations and IHW samples was 4% (9/233) using the *KIR*/MALDI method.

Discussion

To date, most *KIR* genotypes have been obtained using sequence-specific priming of PCR, the first method developed for *KIR* genotyping (Dupont et al. 1997; Selvakumar et al. 1997a, b; Shilling et al. 2002; Shilling et al. 2003; Uhrberg et al. 1997). In this assay, genomic DNA is amplified using a battery of primer pairs in separate reactions and the products visualized by gel electrophoresis. The requirement for many different and independent PCRs makes this method both machine and labor intensive. It also requires relatively large quantities (~3–5 µg) of good quality DNA. For high-throughput *KIR* genotyping, the SSOP blot method has offered some advantage over SSP because it is amenable to analysis of hundreds of samples concurrently and requires less DNA (<1 µg), an important factor for the study of rare archival samples.

In this study, we describe a first generation assay for high-throughput SNP-based *KIR* genotyping using primer extension PCR and MALDI-TOF MS. This method detects the presence of 17 *KIR* genes and pseudogenes and has some capacity for discriminating *KIR* alleles and for discovering new *KIR* alleles. The method uses 38 capture primer sets and 38 SNP extension primers multiplexed into 16 reactions that are carried out under identical conditions in a 384-well format. In this configuration, 24 samples can be run per 384-well SpectroChip. The method also has the flexibility to run each of the 16 multiplexed reactions on separate

chips, allowing a total of 384 samples to be genotyped per 16 chips. The latter format easily allows the addition of new assays and is ideal for higher-throughput studies. The MALDI-TOF analyzes two 384-well chips (48 samples) in ~1 h.

Comparative analysis of 233 samples shows excellent agreement between the results obtained by SSP, SSOP, and MALDI-TOF. However, *KIR* typing by the SNP-based MALDI-TOF method has major advantages over the SSP and SSOP methods. Mass spectrometric analysis uses at least an order of magnitude less DNA (<40 ng), with much higher throughput and greater sensitivity and accuracy than these other methods. The inclusion of a primer extension step after PCR also increases precision and accommodates DNA samples of poorer quality. The primer extension's advantage is that the primer that queries a SNP anneals not to the polymorphic position but to the adjacent conserved sequence. Consequently, an extension product is made regardless of the nucleotide at the SNP. It is thus possible to determine all the nucleotides present at a given SNP position from one primer extension assay. As a result, the SNP-based MALDI-TOF approach can give greater precision with fewer assays than SSP and SSOP. This property is illustrated by the new *KIR2DL1*, *KIR3DL1*, *KIR2DS5*, and *KIR2DL5* alleles identified during MALDI-TOF analysis of the 233 samples.

Mounting evidence suggests that interactions between KIR and their HLA ligands influence bone marrow transplant outcome (Gagne et al. 2002; Parham 2003; Parham and McQueen 2003; Ruggeri et al. 2002), viral disease outcome (Christiansen et al. 2003; Khakoo et al. 2004; Martin et al. 2002; Trachtenberg et al. 2003), and the etiology of several autoimmune diseases (Mizuki et al. 2000; Momot et al. 2004; Nelson et al. 2004; van der Slik et al. 2003). Because both KIR and HLA are so highly polymorphic, large cohorts will be necessary to have power sufficient to define many of these interactions. Such studies demand a methodology for *KIR* typing that is efficient in the use of genomic DNA and combines high resolution with high-throughput. The *KIR*/MALDI-TOF method described here has all these attributes. This rapid and sensitive method is currently being refined for allelic level resolution of all the *KIR* genes.

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Fig. 1.

Spectral data (*left*) and cluster plots (*right*) illustrating single nucleotide and double nucleotide calls with the hME assay "3DS1.3DL1.D1.S," which discriminates between *KIR3DS1* and *KIR3DL1*. **a** and **c** show spectral data and cluster plots for the single nucleotide call T (3DL1) and G (3DS1), respectively. **b** shows data for the double nucleotide call T/G (*KIR3DL1* and *KIR3DS1* both present). Arrows indicate specific data points illustrated in spectral graphs. For all panels, the lowest mass peak (6447.2 Da) represents the unextended primer. The peak produced when the polymerase pauses leading to incorporation of an unexpected dNTP (pausing peak) is also shown—Cluster plots (*right*) illustrate the intensity of the high mass product (T) peak vs intensity of the low mass product (G) peak for a given sample. The separation between clusters and the tightness within a cluster are indicative of the accuracy and specificity of the assay

Fig. 2.

A high-throughput SNP-based MS method for KIR genotyping that is based on the SEQUENOM™ MALDI-TOF MS platform and homogenous MassExtend (hME) chemistry

Fig. 3.

Two tiers of specificity are built into the *KIR*/MALDI assay. **a** The first tier of specificity is created by the “capture” primers used in the initial PCR. In this example, allelic forms containing an “A” fail to amplify (⊗) while those containing “T” are captured (and amplified) by the primers during the PCR. One of the *KIR* genes that is present in all haplotypes (“framework” gene) or another common *KIR* gene will also be captured with the same set of primers, thereby serving as an internal control for the PCR. **b** The design and directionality of the extension primer, along with the chosen deoxy (*d*)/dideoxy (*dd*) mixture provide the second tier of specificity. In this example, ddG, ddA, dC, and ddT are used in the extension reaction. When ddG is incorporated during the primer extension reaction using the capture product from the targeted locus (the uppermost product shown in both **a** and **b**) and framework gene, the reaction is terminated by the incorporation of the dideoxynucleotide. When dC is incorporated during primer extension using the capture product from the targeted locus and framework gene, the extension reaction proceeds until a dideoxy nucleotide is incorporated. The choice of the extension primer and deoxy/dideoxy mixes allows the creation of extension products that differ by length as well as composition. The extension products are measured by the MALDI-TOF MS, which can easily differentiate between products that differ in length by one or more bases

Table 1Primer sequences and plex level and composition for the *KIR*/MALDI genotyping assay

Well number	SNP assay name	5' Capture primer (5' 10-mer tag not shown)	3' Capture primer (5' 10 mer tag not shown)	Extend primer
W1 4 plex	2DS3.D1.G	CTGTGATCACGATGTCCAG	AAGGCCAACTTCTCCATCGG	CACTCCCCCTATCAGTT
	2DL2.D2.S	GAGCTCCTATGACATGTACC	GCCTGGAATGTTCCGTTGACCTTG	CCCTGCAGAGAACCTAC
	3DL3.D1.G	GATGACTAAGGACCCCTTGC	TCATGGGACCCATGGAATAG	AATAGTTGACCTGGGAACCC
	3DL3.D2.G	AGAATGTGACCTTGTCTCTGC	CAGTGAGCCTAAGTTCACCG	GGATAGATGGTAAATGTCAA
W2 3 plex	2DL4.DO.G	TGCCGACCACTCAGTGGG	CCCTGAGCTCTACAACAGAA	TGGAACAGTTTCTCAT
	3DL2.TC.G	GATGAACAAGACCCTCAGGAGGTG	TACACGCTGGTATCTGTT	GCCTCTGAGAAGGGCGA
	3DS1.3DL1.D1.S	CAAGGCCAATTTCTCCATCG	GGGAGCTGACAACTGATAGG	CTGTAGGTCCCTGCAAGGCC
W3 4 plex	2DL5.D2.G	TGACAGAAACAAGCAGTGGG	GACTTTCTCTGGGCCCTG	CCACGGAGGGACCTACA
	2DL5.TC.G	CTTGGGCTCTGAGAAGGG	CAAGACCCTCAGGAGGTGAC	CACTGCGTTTTACACAGA
	2DS4.D1.G	AGAGACAGTCATCCTGCAATG	ATGGAGAAGTTGGCCTTGGA	GAAGTGCTCAAACATGACAT
	3DL2.D1.G	GGAGCTGACAACTGATAGGG	CCAAGGCCAACTTCTCCATC	CTTGCAAGAACCTACAGATG
W4 3 plex	2DL1.D2.G.no004	GACTTTGACCACTCGTAT	CAGGGCCCAAGGTCAACG	ATGCTTCGGCTCTTTCC
	2DL4.TC.G	ATCTGTTGAGGTCTCTTGC	AGGTGACATACGCACAGTTG	CACAGTTGGATCACTGC
	2DL2.004.TC.G	GGCCGAGGAGTACCTACCT	GTAATGGACCAAGAGTCTGC	GAAACAGAACAGCGAATA
W5 2 plex	2DS2.D1.G	AGAAGTTGGCCTTGAGACC	CCTGCAATGTTGGTCAGATG	GCACAGAGAGGGGGAAGT
	3DL1.TC.S	ATGGGACAGGAGACAACCTTG	CACTGCGTTTTACACAGAG	GAGGCCCAAGACACCCCC
W6 2 plex	2DS5.D2.G	AGGCCCATGAACGTAGGCTCC	AAGAGCCGAAGCATCTGTAG	CTCCGTGGGTGGCAGGG
	2DL1.no005.2DL2. 004.TC.S	GTAATGGACCAAGAGTCTGC	CGGGCCGAGGAGTACCTACCT	CGCTATTGCTGTCTGTCT
W7 uniplex	2DL3.2DL2.D1.S	GAGTCCACAGAAAACCTTCCCTCC	AGTGTCCTTAAACTTCCCTTCTC	CTTCTGATTTCACCAGG
W8 2 plex	2DS3.D2.S	AGGTCAACGGAACATTCCAGGCCG	AAGAGCCGAAGCATCTGTAG	CATCTGTAGGTTCTCTCC
	3DS1.TC.G.INT	AACTGCTATGATTAGCTTC	GATGAAGGAGAAAGAAGAGGAGGA	GAATGTGCAGGTGTCTG
W9 5 plex	3DP1.D2.G	GAGCTGCAGGACAAGGTCAC	TGGGAAACCTTCTCTCTCAGCC	CTCTCTCAGCCCAGCCG
	3DS1.DO.S	TCATGCTATACAAAGAAGAC	TGTGTAGTTCCCTGCATGTG	AGGGCTCATGTTGAAGC
	2DL5sub5	GATCTTGGCTTAGCATTTGG	CTGCGTTTTACACAGAC	CTTCTCAGAGGCCCAAG
	2DL5sub4	CCACGGAGGGACCTACAC	GTGACAGAAACAAGCAGTGG	GTGAGTCATGGAGAGAGC
	2DL5sub1	AGGACAAGCCCTTGCTGTCT	CAAGACGAGAGCGACACA	GTCTCTCTCGAGGCACCACA
W10 2 plex	2DS5.D1.G	ACACTTTGCGCTCATTTGGAG	GTGAGTAACAGAACCGTAG	GACCGATGGAGAAGTTG
	2DP1.D0.G	GGGTTTAACTTCACTCTGTGT	TGTGCTGGGGTCACAGGGCC	ATTCTGTTGTAGAGCTCAG
W11 2plex	2DS2.D2.G	GTCTATATGAGAAACCTTC	GGACAAGGTCACGCTCTCTC	CACGCTCTCTCCTGCCA
	2DL1.2DS1.D1.S.tri	AAGGCCAACTTCTCCATCA	GTGAGTAACAGAACCGTAGC	GGTCCCTGCCAGGTCTTGC
W12 2 plex	2DL5sub3	GACATGAGTCCTCTGACCTG	CCCTGAGCTCTACAACAA	GCAACCCCCTGGTGATC
	2DL5sub2	GACATGAGTCCTCTGACCTG	CCCTGAGCTCTACAACAA	CGCTCCCCATTGAGTGGTC
W13 2 plex	2DS4del.sub	TTGACCACTCGTAGGGAGC	CGGTTGAGGAGGAGAGAGAT	CCTGTCTGTCAGCTCC
	2DL5sub6	TAAGGTGGCGCTCTTCTC	CAAGACGAGAGCGACACA	AGCAAGGGCTTGTCTCTG
W14 2plex	2DS4.D2.S	GAGCTCTGTGACGGAAACAA	GCATCAACGGAACATTCCAGGCC	TCGGCTCTTTCCGTGAC
	2DL3.TC.S.INT	CTGCTTCGTGAGACTTACTT	GTAACCCAGACACCTGCATG	TCTCTTCATCGCTGGTGCT
W15 uniplex	2DL2.001.2.3.D1.G	GGAGCTGACAACTGATAGGG	CCTGCAATGTTGGTCAGATG	CATGATGGGGTCTCCAA
W16 uniplex	2DS1.D2.G	ACTTGACTTTGACCACTCGT	CCTATGACATGTACCATCTA	CAACGGAACATTCCAGGCC

The assays are named according to the following convention: “Targeted Locus.Targeted Domain.Specificity (G or S).comment.” If more than one locus is targeted, the convention is “Targeted Locus. 2nd Targeted Locus.Targeted Domain.Specificity (G or S)”. A “universal” 10-mer tag (ACGTTGGATG) is added to the 5' end of each of the capture primers to keep the capture primers out of the “mass window” where the extension primers are measured.

W Well, e.g., *W1* well no. 1; *INT* intronic; *G* generic, i.e., primers are designed to capture more than the targeted locus; *S* specific, i.e., primers were designed to exclude most of the *KIR* genes; *D0* domain 0; *D1* domain 1; *D2* domain 2; *Trans/Cyt* transmembrane/cytoplasmic domain; *sub* subtyping primer; *tri* triallelic choice

[illegible]

D2		Trans/cytoplasmic										subtyping									
2DL1.	2DL2.	2DL5.	2DL1.	2DL2.	2DL3.	2DL3.	2DL4.	2DL5.	3DL1.	3DL2.	3DL1.	3DL2.	3DL1.	3DL2.	3DL1.	3DL2.	3DL1.	3DL2.	3DL1.	3DL2.	
D2.G.	D2.S	D2.G	D2.G	D2.G	D2.S	D2.S	D2.S	D2.S	D2.G	D2.G	D2.G	D2.G	D2.G	D2.G	D2.G	D2.G	D2.G	D2.G	D2.G	D2.G	
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C																					

[illegible]

Table 3

hMEs used to distinguish *KIR3DL1* from *KIR3DS1*

Domain allele	D0	D1	D2	Trans/cyt	No. SNPs
3DL1		3DS1.3DL1, D1.S		3DL1.TC.S	2
3DS1	3DS1, D0.S	3DS1.3DL1, D1.S		3DS1.TC, G.INT	3

This table presents the hME pattern used to distinguish between *KIR3DL1* and *KIR3DS1* as an example of how the SNP patterns are used in our genotyping program “*KIR* Genotype Caller”.

Table 4SNP patterns used to distinguish *KIR3DL1* from *KIR3DS1*

Domain	D0	D1	TC	TC
	3DS1.	3DS1.3DL1.	3DL1.	3DS1.TC.G.
	D0.S	D1.S	TC.S	INT
3DL1				
3DL1*001		T	T	
3DL1*002		T	T	
3DL1*004		T	T	
3DL1*005		T	T	
3DL1*006		T	T	
3DL1*007		T	T	
3DL1*008		T	T	
3DL1*009	G	T	T	
3DS1				
3DS1*010	G	G		T
3DS1*011	G	G		T
3DS1*012	G	G		T
3DS1*013	G	G		T
3DS1*014	G	G		T

This table presents the SNP pattern used to distinguish between *KIR3DL1* and *KIR3DS1* as an example of how the SNP patterns are used in our genotyping program “*KIR* Genotype Caller”.

Concordance between *KIR*/MALDI genotyping and established methods: results for 15 samples at 17 *KIR* loci

[illegible]

		2DL1	2DL2	2DL3	2DL4	2DL5 generic	2DL5 A&B	3DL1	3DL2	3DL3	2DS1	2DS2	2DS3	2DS4 generic	2DS4 *del	2DS5	3DS1	2DP1	3DP1
Number of probes/locus		3	4	2	2	2	5	2	2	2	2	2	2	2	1	2	3	1	1
FC	Obs		+		+	+	A		+	+	+	+				+			+
	Exp	+			+			+	+	+		+	+	+	+			+	+
	Obs	+	+		+			+	+	+		+	+	+	+			+	+

Abbreviations : Exp = expected; Obs = observed

A “+” in the expected row signifies that the locus is expected to be present; blank signifies that locus is not expected to be present. Filled gray indicates that the sample had not been previously typed at allelic level resolution. “NEW” is a putative novel allele found for “OLGA” at KIR 2D L 5 A/B .

Expected KIR genotypes for BM16, PITOUT, BM92, OLGA, RML, T7527 and HOR are as shown in Cook et al. 2003. Expected KIR genotypes for RML, WT47, OLGA, HOR, NV, and DU145 are as shown in Gomez-Lazano and Vitches 2002 and 2004 . Expected KIR genotypes for HS, WC, RR, YW and FC are unpublished results from PP.

A generic assay for the presence of KIR 2DL 5 is combined with a higher resolution assay capable of resolving KIR 2DL 5A and KIR 2DL 5B . A generic assay for KIR 2DS 4 is combined with an assay for detection of the deletion variants KIR 2DS 4*003 /*004/*006.

Table 6

Nucleotides that distinguish *KIR2DL5*OLGA* from other *KIR2DL5* alleles

Nucleotide	Exon 3					Exon 5		
	139	173	364	385	410	581	647	
	Sub6	Sub1		Sub2	Sub3	Sub4	Sub4	
2DL5A	001	T	G	A	A	G	A	G
2DL5B	002	A	G	G	G	G	G	A
	003	T	G	A	A	A	A	G
	004	T	A	A	A	G	A	G
	005	A	G	G	G	G	G	A
	006	T	G	A	A	G	A	G
	007	T	G	A	A	A	A	G
OLGA		T	G	A	G	G	A	G

Nucleotides positions shown in bold were queried by *KIR*/MALDI method, using indicated *KIR2DL5* subtyping primers. Numbering system follows Vilches et al. (2000).

Table 7

Expected/observed MALDI-TOF MS SNP patterns for putative novel alleles of *KIR2DL1*, *KIR3DL1*, and *KIR2DS5*: unexpected SNP patterns observed through locus-specific resolution two-SNP/two-domain strategy

Domain	D0	D1	D2	Trans/cyt
2DL1 expected SNP pattern	+	+		+
Putative novel 2DL1 allele	+		missing	missing
3DL1 expected SNP pattern	+			+
Putative novel 3DL1 allele	+			missing
2DS5 expected SNP pattern	+	+		
Putative novel 2DS5 allele	+		missing	

⁺ indicates expected hME SNP pattern, and *missing* indicates aberrant hME SNP pattern.

Table 8
Expected SNP pattern for known *KIR2DL5* alleles with intermediate resolution subtyping hME's

Probe	2DL5sub1	2DL5sub2	2DL5sub3	2DL5sub4	2DL5sub5	2DL5sub6
Expected SNP pattern for all known <i>KIR2DL5</i> alleles with six subtyping assays						
Nr position	173	385	410	947	1325	139
2DL5A*001	G	A	G	G	A	T
2DL5B*002	G	G	G	A	A	A
2DL5B*003	G	A	A	G	A	T
2DL5B*004	A	A	G	G	A	T
2DL5A*005	G	G	G	A	A	A
2DL5B*006	G	A	G	G	A	T
2DL5B*007	G	A	A	G	A	T
Novel SNP pattern found in OLGA that identified a putative new allele						
2DL5* <i>novel</i>	G	G	G	G	A	T