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Improved accuracy of clinical HLA genotyping by next-generation DNA sequencing affects unrelated donor search results for hematopoietic stem cell transplantation

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ABSTRACT

Matching of human leukocyte antigen (HLA) genes is critically important in hematopoietic stem cell transplantation (HSCT). HLA genes are highly polymorphic and HLA matching has historically been limited by technologies that are unable to unambiguously determine HLA genotypes. Next generation DNA sequencing (NGS) overcomes these limitations by enabling near full-gene sequences with phase determination for heterozygous alleles. Here we examine the efficacy and utility of HLA-NGS in the clinical setting. In a 54-sample validation study and 955 patient samples subsequently tested using HLA-NGS, we observed significant improvement in the ability to unambiguously identify HLA genotypes in both the validation (97.3%) and clinical (97.4%) sample cohorts compared to previous standard-of care HLA genotyping methods. We modeled the clinical impact of this improved diagnostic ability by comparing National Marrow Donor Program (NMDP) search results for 56 patients using HLA-NGS genotypes and simulated standard-of-care HLA genotypes. Surprisingly, we observed significant differences in 7.1% of NMDP searches, with improved unambiguous HLA genotyping correlating with improved prediction of finding well-matched and partially-matched unrelated HSCT donors. These data demonstrate that HLA-NGS can provide highly accurate and unambiguous HLA genotyping that facilitates donor selection for allogeneic HSCT.

1. Introduction

Matching of human leukocyte antigen (HLA) genes is the most critical determinant of immunologic compatibility between donor and recipient for hematopoietic stem cell transplantation (HSCT) [1]. Mismatches of classical HLA genes *HLA-A*, *B*, *C*, *DRB1*, *DQB1*, and *DPB1* are well-established as risk factors for graft versus host disease (GVHD), non-engraftment, overall survival, and disease-free survival after HSCT [2–4]. The classical HLA genes encode human orthologs of the Major Histocompatibility Complex proteins, which present linear peptide antigens to T lymphocytes. The HLA genes are the most polymorphic genes in the human genome, with over 11,000 described protein-coding variants described [5]. Polymorphisms in HLA genes can have significant effects on interaction with T cells, presentation of self- and foreign antigens, and expression on the cell surface, underlying the ability of allogeneic HLA molecules to stimulate robust T cell-mediated immune responses such as GVHD [6].

Current donor selection criteria for standard matched unrelated allogeneic HSCT donors consider high-resolution allele-level (2-field) matching of *HLA-A*, *B*, *C*, and *DRB1* as essential for optimal outcomes, with compatibility for *DQB1*, *DRB3/4/5*, and *DPB1* as significant factors for consideration [1]. Accurately matching donor and recipient HLA genotypes depends on the precision of HLA typing methods, which have evolved significantly over the past 4 decades. Replacement of serology-based HLA phenotyping by DNA-based genotyping methods such as Sequence-Specific Oligonucleotide Probes (SSOP) and Sequence-Specific Primer PCR (SSP) enabled identification of serologically silent but functionally relevant polymorphisms. However, SSOP and SSP methods

Abbreviations: CWD, Common, well-documented; GVHD, Graft versus host disease; HLA, Human leukocyte antigen; NGS, Next-generation DNA sequencing; NMDP, National Marrow Donor Program; SBT, Sanger-based DNA sequence typing; SSO, Sequence-specific oligonucleotide probe; SSP, Sequence-specific primer PCR

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are limited to focused detection of known DNA sequences, inherently limiting their diagnostic ability. Implementation of Sanger Sequencing-Based Typing (SBT), the current standard of care, expanded the ability to identify both known and novel alleles, though it also has important limitations. SBT typically sequences exons 2, 3, and 4 for Class I genes, and exons 2 and 3 for Class II genes, precluding identification of potentially functional differences outside of those regions. In addition, DNA sequences generated by SBT have phase ambiguity; when heterozygous nucleotides are detected at more than one position, SBT cannot determine which nucleotides exist in the same allele, and which are on the homologous chromosome. Phase ambiguity affects as many as 53% of samples tested, providing a large source of potential error [7,8]. HLA genotype ambiguities often require significant additional testing to ensure accurate matching of patient and potential donor. However, ambiguities are often left unresolved when they include rare alleles or involve areas other than the antigen recognition, with unclear effects on clinical outcomes.

Next-generation DNA sequencing (NGS) methods have the potential to overcome these limitations by using parallel processing of millions of sequences to generate high-resolution HLA typing [9]. The high-throughput nature of NGS facilitates sequencing of full genes, enabling examination of polymorphisms outside of the traditionally examined exons. Importantly, the stranded nature of DNA sequences generated by NGS enables direct determination of phase for heterozygous nucleotides, reducing a significant source of HLA genotyping ambiguity. These benefits improve the accuracy of HLA genotyping and facilitate identification of rare or novel alleles [8,10–14]. However, the clinical utility of these improvements are not well-defined. Here, we examine data from our laboratory's clinical validation study as well as genotyping results and quality control data for the first 21 months of clinical use to evaluate the impact of NGS methods for HLA genotyping in HSCT.

2. Materials and methods

2.1. Patient samples

All samples were clinical specimens from potential allogeneic HSCT recipients or donors at University of California San Diego (UCSD) Moores Cancer Center and Rady Children's Hosptial. Validation studies were performed by blinded parallel testing of 54 samples (peripheral blood n = 48; buccal swab n = 6). Clinical utility of HLA-NGS was evaluated for 955 samples (peripheral blood n = 922; buccal swab n = 32; other n = 1) from 563 patients or their family members and 392 NMDP donors during the first 21 months after implementation. All clinical sample collection and testing was performed in accordance with standard-of-care procedures at UCSD.

2.2. HLA-NGS genotyping

HLA-NGS genotyping for *HLA-A*, *B*, *C*, *DRB1*, *DRB3/4/5*, *DQA1*, *DQB1*, *DPA1*, and *DPB1* was performed in the UCSD Immunogenetics and Transplantation Laboratory on DNA isolated from patient samples. Input DNA were normalized to 10 ng/μL with at least 400 ng per sample (peripheral blood) or 40–50 ng/μL (buccal swabs). HLA gene amplicons were generated by long-range polymerase chain reaction (PCR) using TruSight HLAv2 reagents (Illumina, San Diego, CA). Library preparation was performed from amplicons by bead-based purification, enzymatic fragmentation using Nextera XT, and addition of barcoded index adaptors (Illumina). Pooled libraries were sequenced using MiSeq Nano and Micro v2 300 cycle reagents on the MiSeq platform (Illumina). Phased exon sequence data were analyzed using Assign TruSight v2.1 (Illumina) and International ImMunoGeneTics project (IMGT) reference database [5]. Data were reported to 2-field allele-level resolution.

2.3. Reference method HLA genotyping

Validation studies were performed on samples genotyped using standard-of-care SeCore SBT reagents and uTYPE DX analysis software (Thermo Fisher Scientific, Waltham, MA), with LABType and Micro SSP reagents and HLA Fusion analysis software SSO (One Lambda, Canoga Park, CA) for ambiguity resolution. Data were reported to 2-field allele-level resolution, with ambiguous genotypes identified. Ambiguous genotypes with identical nucleotide sequences for peptide binding domains (exons 2 and 3 for class I and exon 2 for class II) were assigned HLA G group typing based on IMGT/HLA database designation [5]. Simulated SBT genotyping was performed by *in silico* de-resolution of HLA-NGS data using Assign TruSight v2.1 software, limiting DNA sequence analysis to non-phased DNA sequences for Class I exons 2, 3, and 4 and class II exons 2 and 3.

2.4. Data collection and analysis

Sequencing failure was defined as inability of the NGS software to generate any possible alleles at a particular locus. "No match" alleles were defined by successful NGS sequencing in which all possible alleles at a single locus demonstrated 1 or more nucleotide mismatches with known alleles. Ambiguity was defined as more than one possible HLA type with 0 nucleotide mismatch and at least 1 common, well-defined allele in the pair. When ambiguity occurred at a particular locus, it was quantified as affecting 1 or 2 alleles.

2.5. NMDP search comparisons

Genotyping data from HLA-NGS or de-resolved HLA-NGS data were entered into NMDP search engine (HapLogic [15]) and imputed [16] patient HLA genotypes were compared. Imputed HLA genotypes were subsequently used to evaluate the potential for identifying well-matched (8/8 for *HLA-A, B, C, DRB1* or 10/10 for *HLA-A, B, C, DRB1, DQB1*) or partially-matched (7/8 or 9/10) donors. Search analysis was performed using 2018 registry data, and the probability for identification of a matched donor among the top 3 identified donors was determined.

2.6. Statistical analysis

Statistical analyses were performed using Prism 7 software (GraphPad, San Diego, CA). Numerical data were analyzed non-parametrically using Kruskal-Wallis test with multiple comparisons. Categorical data were analyzed by Fisher's Exact test. All values are 2-tailed *P* values.

3. Results

3.1. Validation of genotyping accuracy by HLA-NGS

The analytic accuracy of HLA-NGS was evaluated by parallel testing of 54 clinical samples with standard-of-care SBT/SSO/SSP methods (Fig. 1A). Higher concentration DNA input was used for buccal swab samples based upon experience during assay development. All samples were successfully amplified and generated high-quality DNA sequence. Sequencing run metrics demonstrated an average cluster density of 873 K/mm^2 (range 528–1294 K/mm^2), with 92.8% of reads passing filtering (range 90.9–97.1%), and an average Q30 score of 95.5% (range 85–98%). Average read depth across all HLA loci was 234 reads/nucleotide, with Class I and Class II genes demonstrating equivalent read depth, and 99.4% (966/972) of alleles had read depth considerably above the 100 reads/nucleotide recommended for confident assignment of genotyping. For the 6 alleles with low coverage (average read depth 39–92 reads/nucleotide), samples were re-tested and genotyping results were consistent between runs with low coverage and re-

A

Validation Sample Metrics		
	Peripheral Blood	Buccal Swab
Number of samples	48	6
Mean (range) DNA concentration	86.0 (14.4 - 230.0)	85.5 (43.0 -135.4)
Mean (range) read depth (reads/base)	144.3 (39 - 279)	227.7 (51 - 298)
Validation Run Metrics		
Mean (range) cluster density (K/mm^2)	873 (528 - 1294)	
Mean (range) cluster passing filter (%)	92.8 (90.9 - 97.1)	
Mean (range) Q30 (%)	95.5 (85 - 98)	

B

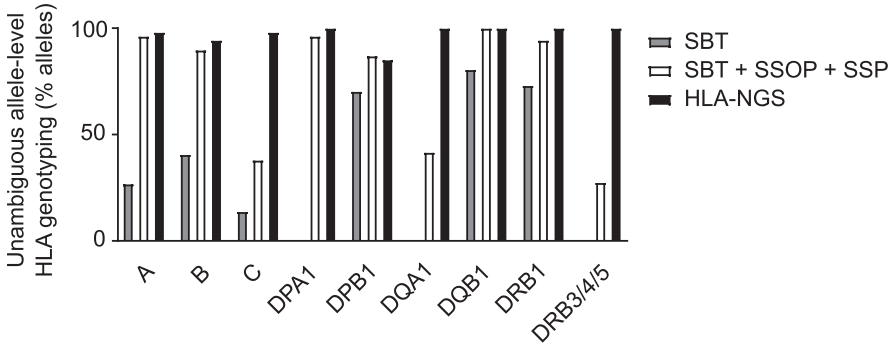

Fig. 1. Parallel testing of HLA-NGS with standard-of-care DNA sequencing. Validation of 54 clinical samples via parallel testing using HLA-NGS and standard-of-care SBT plus SSOP/SSP methods. A. Sample and run metrics for validation study. B. Rates of unambiguous typing defined for HLA-NGS as identification of a single 0 nucleotide mismatch for 2-field genotype. Unambiguous typing for SBT alone and SBT plus SSO/SSP defined as identification of a single 2-field allele genotype with at least 1 CWD allele as defined by IMGT.

testing with adequate coverage.

In parallel testing, HLA-NGS provided significantly higher rates of unambiguous typing compared to both SBT alone and previous standard-of-care SBT/SSO/SSP (Fig. 1B). Comparison of ambiguity rates for *HLA-A, B, C, DRB1, DQB1*, and *DPB1* (genes typed by SBT), identified a significantly lower ambiguity rate (2.7%, $P < 0.001$) for NGS as compared to 32.7% for SBT alone and 24.6% of SBT/SSO/SSP. HLA-NGS and standard of care typing were concordant in 99.4% of alleles examined. Genotypes were discordant in 2 samples; one sample involved *DRB1*, with HLA-NGS unable to identify a 0 nucleotide mismatch genotype for 1 allele genotyped by SBT, SSO, and SSP as DRB1*04:07:01G. The closest NGS-assigned genotype was DRB1*04:20, which is not included in the DRB1*04:07:01G group, with single non-synonymous nucleotide change in exon 2. Given the low confidence of HLA-NGS result, we considered the standard of care typing correct. The second case of discrepant genotyping was for *DRB4* in a sample where HLA-NGS did not detect an allele present, whereas standard of care SSO and SSP genotyping identified DRB4*01:01/03/06/08. Linkage analysis with *DRB1* (DRB1*07:01 and DRB1*09:01) and *DQB1* (DQB1*02:02 and DQB1*03:03) indicated that *DRB4* should be present and the discordant result was attributed to allele dropout by HLA-NGS. These discrepant results highlighted a potential for allele-dropout and low-confidence genotype results as potential sources of error in clinical testing.

3.2. Clinical performance of HLA-NGS

During the first 21 months after implementation, 955 clinical samples were tested by HLA-NGS (Fig. 2A). This included 563 patients or potential related donors and 392 NMDP donors. Genotyping was performed across 233 runs, which demonstrated run metrics (Fig. 2A) similar to those from validation. The majority of samples were peripheral blood (n = 922, 96.5%), with the remainder from buccal swabs (n = 32, 3.5%) or bone marrow aspirate (n = 1, 0.01%). DNA recovered from buccal swab samples had lower concentrations (11.4 ± 15.7 ng/μl) compared to peripheral blood samples (107.7 ± 32.4 ng/μl). The strategy of preemptively using increased DNA input from buccal swab samples facilitated consistent performance across sample types, with similar average read depth across all loci (excluding samples with amplicon generation failure) for peripheral blood and buccal samples (Fig. 2B). Average read depths less than 100 reads/nucleotide occurred in 14 total alleles from 11 samples (excluding 33 samples with identified non-amplification as described below). In these samples, read depth was considered sufficient across core exons (greater than 40 reads/base without loss of phasing across exon) to enable confident genotype identification based on experience from assay development and validation studies.

Amplicon generation was evaluated for all samples by gel electrophoresis. Samples not generating DNA sequence for at least 1 allele (n = 33, 3.5%) were identified as possible amplification failures with lack of an identifiable band on gel visualization (Fig. 3A). Amplification failures were disproportionately associated with buccal swab samples, affecting 16/32 (50%) of all buccal swab samples tested, despite using increased concentration of DNA. Only 6/16 buccal swab (37.5%) cases of non-amplification were resolved by re-amplification using increased DNA concentration, compared to successful re-amplification of all peripheral blood samples that initially failed. Conversely, absence of a visualized band on gel electrophoresis was not predictive of a failure to generate high-quality DNA sequence sufficient for genotyping. This indicated that low concentrations of amplicons (less than the 3 ng considered to be sufficient for visualization) are sufficient for preparation of NGS-HLA libraries.

In addition to confirmed cases of non-amplification described above, potential allele dropout was investigated in 57 samples with extensive homozygosity or deviation from common HLA gene linkage. Additional testing using SSOP or SSP confirmed dropout in 2 samples (3.5% of suspected samples and 0.2% of total samples tested) (Fig. 3B). Both of these samples had dropout of DRB3/4/5 identified by linkage analysis, confirmed by SSP. NGS-HLA produced correct results with re-amplification and sequencing. No cases of allele dropout due to homozygosity were confirmed.

3.3. Diagnostic utility of HLA-NGS

Using an analysis strategy of phased analysis for all exons sequenced, HLA-NGS produced unambiguous typing in 97.0% of alleles

A

Implementation Sample Metrics			
	Peripheral Blood	Buccal Swab	Bone Marrow
Number of samples	922	32	1
Mean (range) DNA concentration	107.7 (3.3 - 208.5)	11.4 (3.1 - 92.1)	102
Mean (range) read depth (reads/base)	243.7 (43 - 324)	228.8 (20 - 298)	256
Problematic samples (n, %)	18 (2.0%)	13 (40.6%)	0 (0%)
Implementation Run Metrics			
Mean (range) cluster density (K/mm^2)	856.0 (528 - 1294)		
Mean (range) cluster passing filter (%)	91.4 (75.2 - 98.0)		
Mean (range) Q30 (%)	91.8 (84.4 - 97.4)		
Runs failing QC (n, %)	10 (4.6%)		

B

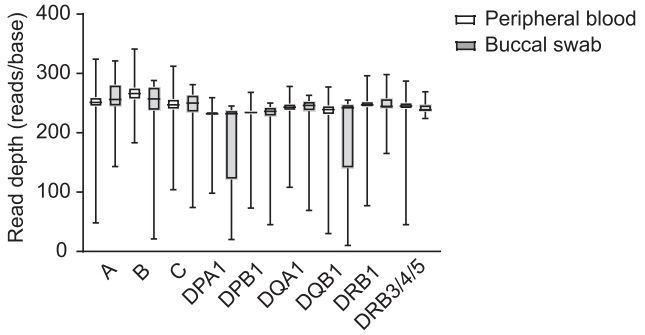

Fig. 2. Implementation of HLA-NGS. During clinical implementation, 955 samples were tested using HLA-NGS. A. Sample and run metrics for clinical samples. B. Average read depth for all loci (mean, quartile, min to max).

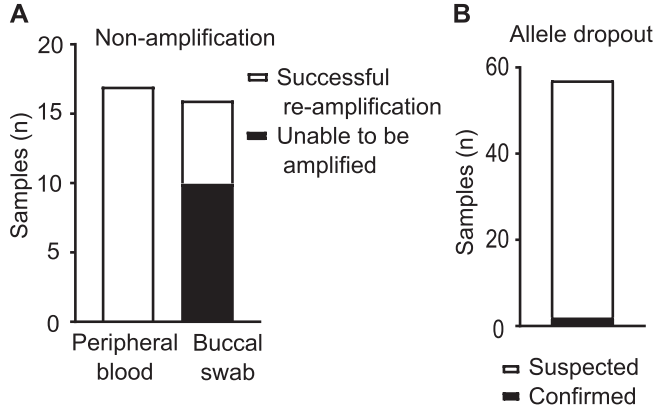

Fig. 3. Investigation of suspected technical errors in HLA-NGS. During clinical implementation, 955 samples were tested using HLA-NGS, with 126 samples investigated for suspected technical errors. Samples with suspected technical errors were re-tested using HLA-NGS or using secondary methods including SSOP and SSP. A. Non-amplification was suspected in 17 peripheral blood samples and 16 buccal swab samples due to absence of DNA sequence generated by NGS. Samples were re-tested with increased DNA concentration, resulting in successful amplification of 20/33 samples. B. Allele dropout was suspected in 57 samples based on homozygosity or atypical linkage analysis. Suspected allele dropout was confirmed in 2 cases using a second method.

tested (Fig. 4A). Rates of unambiguous typing for *A*, *B*, *C*, *DRB1*, *DRB3/4/5*, *DQA1*, *DQB1*, and *DPA1* ranged from 96.6% to 99.9%, with only *DPB1* being significantly lower (79.6%, $P < 0.001$). Ambiguity rates for clinical samples was similar to rates from validation testing (Fig. 1). Ambiguous genotypes typically resulted from informative nucleotide positions outside of the sequenced region (Fig. 4B). All ambiguous allele genotypes were included within G groups, providing genotype information sufficient for HSCT donor selection.

Analysis software was unable to identify any phased 0 nucleotide mismatch genotype for 36 alleles; the best alignments to IMGT reference sequences had 1 or more nucleotide mismatches within exons (Fig. 4C). Sequencing coverage and mismatch location were manually evaluated to identify potential inappropriate base calls by the analysis software, however no changes to base calls for mismatched positions were made. Additional testing with SSO or SSP confirmed allele-level genotypes consistent with HLA-NGS results, either the same allele or within the same G group. Among HLA genes, *DRB3/4/5* (13 alleles) and *DPA1* (9 alleles) were most frequently affected. In total, HLA-NGS was able to successfully produce HLA unambiguous genotype information without repeat or additional testing in 884/955 (92.6%) of samples tested. Repeating HLA-NGS testing resolved problems for 71.4% of samples re-tested, leaving only 10 (1.0% of total samples) samples requiring additional testing by other methods. Clinical testing with HLA-NGS provided results in an average of 5.4 days (range 3–15 days), with a majority of samples (94.3%) meeting the goal of 7 days, demonstrating the robustness of HLA-NGS for clinical use.

3.4. Clinical impact of improved genotyping accuracy by HLA-NGS

To evaluate potential benefits in HLA genotyping from HLA-NGS, we estimated the ambiguity rate for the first 199 clinical samples as if they had been genotyped using only SBT methods. SBT-predicted genotypes were generated from HLA-NGS sequencing data by examining only “Core” DNA sequence (exons 2, 3, and 4 for class I genes, and exons 2 and 3 for class II genes) without use of phasing information. Possible genotype combinations containing at least 1 common, well-documented (CWD) allele were considered as informative. Using Core sequence-derived genotyping generated unambiguous genotyping in 41.5% of alleles (Fig. 4A), similar though lower than the 67.3% observed for SBT alone and the 75.4% observed using SBT/SSOP/SSP during parallel testing validation studies (Fig. 1) highlighting a limitation of this approach in estimating changes in HLA genotyping accuracy.

We modeled the clinical implications of the improvement in unambiguous HLA genotyping using HLA-NGS by examining unrelated HSCT donor selection. HLA typing results using HLA-NGS and *in silico*

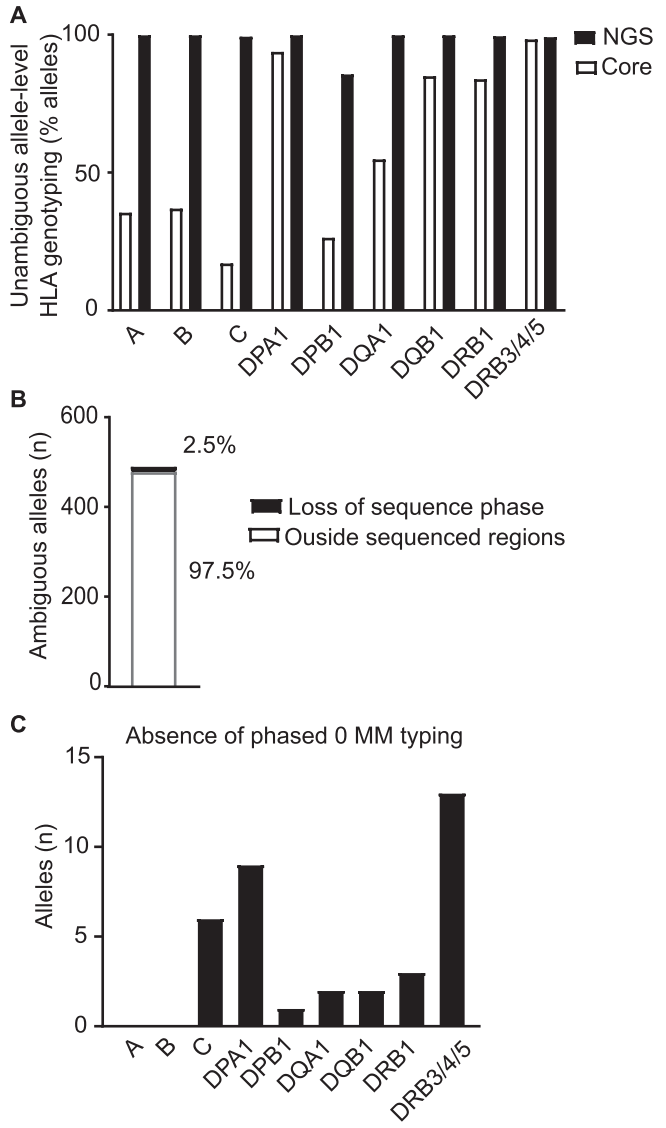

Fig. 4. Comparison of unambiguous typing rates using HLA-NGS versus simulated standard of care SBT. A. Rates of unambiguous HLA genotyping of clinical samples tested with HLA-NGS (n = 199) were compared with phase-unresolved sequencing of Core exons. Unambiguous typing defined for HLA-NGS as identification of a single 0 nucleotide mismatch for 2-field genotype. Unambiguous typing for Core typing was defined as identification of a single 2-field allele genotype with at least 1 CWD allele as defined by IMGT. B. Ambiguous genotypes generated from HLA-NGS were examined to identify location of potentially informative nucleotides using IPD-IMGT/HLA Sequence Alignment Tool. Ambiguous genotypes were reported as indicated. C. Analysis of DNA sequence generated by HLA-NGS failed to identify a 0 nucleotide mismatch genotype for 13 alleles.

de-resolved Core typing from 56 sequential patients evaluated for HSCT were entered into the NMDP registry search algorithm for comparison of the probability of identifying a well-matched donor. HLA-NGS generated 100% unambiguous *HLA-A, B, C, DRB1*, and *DQB1* genotyping for all 56 patients. De-resolving HLA genotypes introduced ambiguity in 40/56 (71.4%) of samples. Parsing of HLA genotypes for registry search by the NMDP algorithm resulted in 4 (7.1%) samples where de-resolved Core HLA genotypes produced differences in the imputed HLA genotype used for NMDP search, and 4 (7.1%) samples where de-resolved HLA genotypes were unable to generate imputed genotypes. This likely reflects the effects of HLA genotype ambiguity existing within the NMDP database and the subsequent ability of the search algorithm to identify potential donor haplotypes likely to contain the alleles in question. Importantly, these discrepant imputed HLA genotypes affected the likelihood of identifying well-matched (8/8 or 10/10) or partially-matched (7/8 or 9/10) donors in the NMDP registry (Table 1). While the use of ambiguous allele combinations for unrelated donor searches is well-accepted and produces good clinical outcomes, the effects of HLA genotyping ambiguity demonstrated here suggests that improvements in unambiguous HLA genotyping may facilitate identification of well-matched donors.

We also examined the potential converse effect, comparing the increased unambiguous typing ability of HLA-NGS on rates of allele-level matching for potential matched unrelated donors (n = 75) based upon NMDP search predictions. Allele-level match rates were compared for *HLA-A* (n = 150 alleles), *B* (n = 148 alleles), *C* (n = 124 alleles), *DRB1* (n = 150 alleles), and *DQB1* (n = 106 alleles) with NMDP match prediction (Fig. 5). All NMDP registry typing results were confirmed. Unsurprisingly, 100% of all alleles predicted to be allele-level ("A") matches were correctly matched, and 92.3% (12/13) of predicted mismatches ("M") were not matches. Rates of matching for alleles with possible ("P") match prediction varied widely between genes; 94.8% of HLA-A, 89.4% of –B, 95.6% of –C, 97.8% of -DRB1, and 100% of DQB1 alleles with a P prediction were accurately matched. All of the mismatched alleles had nucleotide differences in the Core exons (encompassing the antigen recognition domains) as compared to patient alleles, indicating a high likelihood for these mismatches to be clinically-relevant.

4. Discussion

Application of NGS to clinical HLA genotyping is being embraced by the transplantation community as a potential solution to previous technical limitations of standard-of-care Sanger-based DNA sequencing. Several studies, including data presented here, demonstrate the ability of NGS methods to improve unambiguous HLA genotyping. In our experience, the rate of unambiguous 2-field genotyping by HLA-NGS (97.0%) was significantly higher than that of current standard-of-care methods (Figs. 1 and 4). While HLA-NGS was highly effective in unambiguous HLA genotyping, the assay has limitations that must be considered. Additional testing was performed in 13.2% of samples for suspected technical problems, including non-amplification, allele dropout, and inability of the analysis software to confidently identify a 0 mismatch genotype. However, only 33 (3.4%) of samples had issues with non-amplification and 2 (0.2%) of samples demonstrated allele dropout (Fig. 3). Our continued experience has reaffirmed the patterns presented here; non-amplification and allele dropout are exceedingly rare, problematic samples are disproportionately found among buccal swab samples with low concentration and degraded DNA, and ambiguities that do occur are predictable (79.0% of ambiguities were *DPB1*) and definable within NMDP G groups. While the limited incidence of these problems highlights the reliability of the NGS approach, they remain relevant and require consideration.

The diagnostic utility of NGS methods for HLA genotyping is reasonably well-established. However, we wanted to evaluate the potential clinical impact of NGS-based HLA genotyping. We attempted to model the clinical impact of this improved diagnostic ability by comparison of NMDP search algorithm results using HLA-NGS data and the *in silico* generated Core data. It is important to note that de-resolved HLA genotypes had increased rates of ambiguity (58.5%) compared to actual SBT data from the validation study (32.7%), which could possibly overestimate the effect on NMDP search algorithm comparisons. However, the finding that 7.1% of samples had significant differences of imputed HLA genotypes between HLA-NGS and Core typing data entered into the NMDP algorithm. This provides evidence for the clinical benefit of increased accuracy in HLA genotyping, regardless of the magnitude of the effect. The higher probability of identifying well-matched and partially-matched unrelated HSCT donors using the unambiguous HLA-NGS data (Table 1) provides a clear demonstration for

Table 1
Differences in NMDP search results from discrepant imputed HLA genotype from HLA-NGS and de-resolved HLA typing.

	HLA gene					Probability of NMDP match*			
	A	B	C	DRB1	DQB1	8/8	7/8	10/10	9/10
NGS	11:02	35:01	04:01	11:01	03:01	0.999	0.999	0.999	0.999
	24:02	51:01	15:02	13:01	06:03				
Core	11:CJB	35:AZCRC	04:FEX	11:AZCRA	03:01	0.295	0.999	0.029	0.295
	24:BG	51:AZCRD	15:EUF	13:AZCRB	06:03				
NGS	01:01	08:01	07:01	03:01	02:01	0.995	0.999	0.995	0.999
	30:02	52:01	12:02	11:01	03:01				
Core	01:01	08:01	07:WTR	03:AE	02:AC	0.870	0.999	0.867	0.988
	30:02	52:01	12:02	11:AD	03:AD				
NGS	01:01	08:01	03:03	03:01	02:01	0.999	0.999	0.999	0.999
	02:01	15:01	07:01	11:01	03:01				
Core	01:BAFH	08:FXBX	03:03	03:AE	02:AC	0.999	0.999	0.999	0.999
	02:NMV	15:HVHV	07:WTR	11:AD	03:AD				
NGS	02:01	08:01	02:02	11:01	03:01	0.991	0.999	0.991	0.999
	68:01	51:01	07:01	13:01	06:03				
Core	02:EXG	08:AC	02:02	11:AZCRB	03:01	0.887	0.999	0.887	0.997
	68:GRH	51:BKWR	07:WTR	13:01	06:03				

* Probability of identification of 8 allele (*A, B, C, DRB1*) or 10 allele (*A, B, C, DRB1, DQB1*) potentially matched donors from NMDP registry.

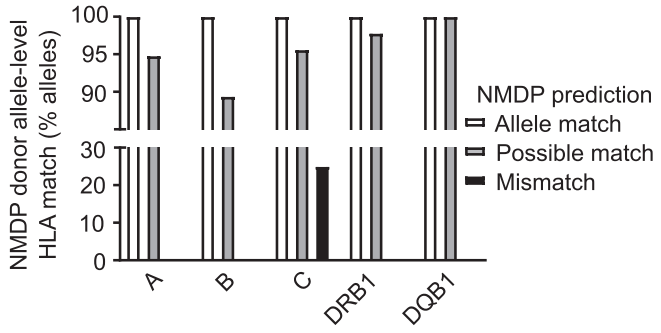

Fig. 5. Evaluation of HLA matching for potential unrelated HSCT donors. Potential unrelated HSCT donors identified by NMDP search results (n = 75) were genotyped for *HLA-A* (n = 150 alleles), *B* (n = 148 alleles), *C* (n = 124 alleles), *DRB1* (n = 150 alleles), and *DQB1* (n = 106 alleles) using HLA-NGS. Results were compared to potential recipient HLA genotyping results using HLA-NGS and considered a match if identical for 2-field genotype. Donor alleles are separated into groups based on NMDP matching algorithm prediction of allele-level match (A), possible match (P), and mismatch (M).

the potential translation of this effect into clinical practice. We also observed a converse effect, where NMDP was unable to parse rare alleles definitive identified by HLA-NGS (DRB1*09:31 and DQB1*06:74) into imputed haplotypes to perform donor searches, and instead required us to input the genotypes as ambiguous allele strings containing the rare allele and lowest numerical member of the G group. While HSCT using donors matched to G and P group alleles is well-accepted and clinically tolerated [17,18], it is unclear whether more stringent matching that could be enabled by NGS methods would improve clinical outcomes and larger clinical outcomes studies are necessary.

It is possible and even likely that genetic variation beyond that captured by 2-field genotyping will influence HSCT compatibility and outcomes. Indeed, expression variants of HLA, attributed to polymorphisms in non-coding DNA, have been demonstrated to affect HLA function and the ability to stimulate T cells in infection and transplantation [19–21]. NGS methods are well-suited to examine these variants, as many methods, including the HLA-NGS method described here, sequence non-coding regions. However, current analysis of this data is constrained by limitations in the well-curated public IMGT database, where description of DNA sequences for novel alleles has previously focused on Core exons with predicted function related to antigen presentation, frequently causing ambiguous results when aligning intron or untranslated region sequences to reference sequence. Potential matching of HLA genes beyond currently considered criteria, including considerations such as polymorphisms in non-coding regions or synonymous nucleotide mismatches, likely presents the most significant opportunity for improvements for matching donors and recipients in HSCT. Evaluating clinical effects of matching non-coding regions of HLA genes, much like the effects of matching/mismatching beyond G and P groups, will require large cohort analyses. Retrospective studies comparing HSCT outcomes for patients who are matched with their donors according to current standard-of-care criteria to those that are more accurately matched using newer methodologies, including for polymorphisms in regions outside of the antigen presentation region or in non-coding regions, will be essential to fully evaluate the clinical benefit from increasing our ability to examine HLA genes.

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Conflict of interest disclosure

The authors have no relevant conflicts of interest to declare. The results presented have not been published previously except in abstract form.

References

[1] C.A. Howard, M.A. Fernandez-Vina, F.R. Appelbaum, et al., Recommendations for donor human leukocyte antigen assessment and matching for allogeneic stem cell transplantation: consensus opinion of the Blood and Marrow Transplant Clinical Trials Network (BMT CTN), Biol. Blood Marrow Transplant. 21 (1) (2015) 4–7.
[2] N. Flomenberg, L.A. Baxter-Lowe, D. Confer, et al., Impact of HLA class I and class II high-resolution matching on outcomes of unrelated donor bone marrow transplantation: HLA-C mismatching is associated with a strong adverse effect on transplantation outcome, Blood 104 (7) (2004) 1923–1930.
[3] P. Loiseau, M. Busson, M.L. Balere, et al., HLA Association with hematopoietic stem cell transplantation outcome: the number of mismatches at HLA-A, -B, -C, -DRB1, or -DQB1 is strongly associated with overall survival, Biol. Blood Marrow Transplant. 13 (8) (2007) 965–974.
[4] C. Kollman, S.R. Spellman, M.J. Zhang, et al., The effect of donor characteristics on survival after unrelated donor transplantation for hematologic malignancy, Blood 127 (2) (2016) 260–267.
[5] J. Robinson, J.A. Halliwell, J.D. Hayhurst, et al., The IPD and IMGT/HLA database: allele variant databases, Nucl. Acids Res. 43 (database issue) (2015) D423–D431.
[6] N.J. Felix, P.M. Allen, Specificity of T-cell alloreactivity, Nat. Rev. Immunol. 7 (12)

(2007) 942–953.
[7] S.D. Adams, K.C. Barracchini, D. Chen, et al., Ambiguous allele combinations in HLA Class I and Class II sequence-based typing: when precise nucleotide sequencing leads to imprecise allele identification, J. Transl. Med. 2 (1) (2004) 30.
[8] E.T. Weimer, M. Montgomery, R. Petraroia, et al., Performance characteristics and validation of next-generation sequencing for human leucocyte antigen typing, J. Mol. Diagn. 18 (5) (2016) 668675.
[9] C. Lind, D. Ferriola, K. Mackiewicz, et al., Next-generation sequencing: the solution for high-resolution, unambiguous human leukocyte antigen typing, Hum. Immunol. 71 (10) (2010) 1033–1042.
[10] K. Hosomichi, T.A. Jinam, S. Mitsunaga, et al., Phase-defined complete sequencing of the HLA genes by next-generation sequencing, BMC Genom. 14 (2013) 355.
[11] M. Danzer, N. Niklas, S. Stabentheiner, et al., Rapid, scalable and highly automated HLA genotyping using next-generation sequencing: a transition from research to diagnostics, BMC Genom. 14 (2013) 221.
[12] V. Lange, I. Bohme, J. Hofmann, et al., Cost-efficient high-throughput HLA typing by MiSeq amplicon sequencing, BMC Genom. 15 (2014) 63.
[13] Y. Ozaki, S. Suzuki, K. Kashiwase, et al., Cost-efficient multiplex PCR for routine genotyping of up to nine classical HLA loci in a single analytical run of multiple samples by next generation sequencing, BMC Genom. 16 (2015) 318.
[14] J.L. Duke, C. Lind, K. Mackiewicz, et al., Determining performance characteristics of an NGS-based HLA typing method for clinical applications, HLA 87 (3) (2016) 141–152.
[15] J. Dehn, M. Setterholm, K. Buck, et al., HapLogic: a predictive human leukocyte antigen-matching algorithm to enhance rapid identification of the optimal unrelated hematopoietic stem cell sources for transplantation, Biol. Blood Marrow Transplant. 22 (11) (2016) 2038–2046.
[16] A. Madbouly, L. Gragert, J. Freeman, et al., Validation of statistical imputation of allele-level multilocus phased genotypes from ambiguous HLA assignments, Tissue Antigens 84 (3) (2014) 285–292.
[17] Y. Xiao, A.M. Lazaro, C. Masaberg, et al., Evaluating the potential impact of mismatches outside the antigen recognition site in unrelated hematopoietic stem cell transplantation: HLA-DRB1*1454 and DRB1*140101, Tissue Antigens 73 (6) (2009) 595–598.
[18] A. Pasi, R. Crocchiolo, M. Bontempelli, et al., The conundrum of HLA-DRB1*14:01/*14:54 and HLA-DRB3*02:01/*02:02 mismatches in unrelated hematopoietic SCT, Bone Marrow Transplant. 46 (7) (2011) 916–922.
[19] R. Apps, Y. Qi, J.M. Carlson, et al., Influence of HLA-C expression level on HIV control, Science 340 (6128) (2013) 87–91.
[20] E.W. Petersdorf, T.A. Gooley, M. Malkki, et al., HLA-C expression levels define permissible mismatches in hematopoietic cell transplantation, Blood 124 (26) (2014) 3996–4003.
[21] E.W. Petersdorf, M. Malkki, C. O'hUigin, et al., High HLA-DP expression and graft-versus-host disease, N. Engl. J. Med. 373 (7) (2015) 599–609.