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Research article

Next-generation sequencing and clinical histocompatibility testing

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ABSTRACT

Histocompatibility testing is essential for donor identification and risk assessment in solid organ and hematopoietic stem cell transplant. Additionally, it is useful for identifying donor specific alleles for monitoring donor specific antibodies in post-transplant patients. Next-generation sequence (NGS) based human leukocyte antigen (HLA) typing has improved many aspects of histocompatibility testing in hematopoietic stem cell and solid organ transplant. HLA disease association testing and research has also benefited from the advent of NGS technologies. In this review we discuss the current impact and future applications of NGS typing on clinical histocompatibility testing for transplant and non-transplant purposes.

1. Introduction

Comprising less than 0.15% of the human genome, the human leukocyte antigen (HLA) region on chromosome 6p21 contains over 224 annotated genes, with a span of over 3.6 Mbp and is one of the most diligently researched areas of the genome [1,2]. The HLA region is composed of vital genes for immune cell regulation and function [3,4]. Encoded by *HLA-A, -B, and -C* genes, the major histocompatibility complex (MHC) class I molecules are used by nearly all cells in the human body to express endogenous peptides on their surface for immune cell surveillance to identify defective and pathogen infected cells [5,6]. Class II MHC molecules are encoded by the HLA-*DPB1*, *DPA1*, *DM*, *DO*, *DQB1*, *DQA1*, *DRB1*, and *DRA* genes, among others. These MHC complexes are used by antigen presenting leukocytes to display peptides for CD4 T lymphocyte examination and recruitment [7]. While this is typically the case for antigen processing and presentation by the class I and II MHC molecules, due to cross-presentation and other processes, it is possible for differing peptides to be displayed in the context of class I and class II molecules. Other important immune gene products encoded in the HLA region include the complement system proteins which are vital for the innate immune response in the opsonization and neutralization of pathogens [8].

HLA allele variants are associated with a plethora of human diseases, including autoimmune disorders such as systemic lupus erythematosus, psoriasis, multiple sclerosis, ankylosing spondylitis, and sarcoidosis among others. For some of these autoimmune diseases, HLA associated risk alleles have been identified as strong genetic predictors of disease development. Additional diseases associated with different HLA alleles include Parkinson's disease, type II diabetes, schizophrenia, and coronary artery disease [9]. Further, there are several HLA alleles correlated with adverse drug reactions such as HLA-B*57:01 and abacavir hypersensitivity and adverse drug reactions to carbamazepine and oxcarbazepine associated with the *HLA-B*15:02, HLA-B*15:11*, or *HLA-A*31:01* alleles [10].

HLA typing is vital in the assessment and treatment of many medical conditions including rheumatologic, autoimmune, hematologic, and cardiologic diseases[11]. With the prevalence of hematologic malignancy now greater than 63 cases per 100,000 individuals, there is increasing demand for hematopoietic cell transplant (HCT), which is often the best long term cure for many of these diseases[12]. Some patients may be able to receive an autologous transplant, however, many patients need an HCT allotransplant for which HLA typing is required to find an appropriate HLA matched donor [13-15]. In solid organ transplant, HLA typing plays a role in organ allocation and monitoring patients for the development of donor specific antibodies which could lead to adverse outcomes[16]. Inaccurate HLA typing can put these patients at a higher risk of transplant rejection and potential chronic systemic disease. In modern day medical practice and as evidenced by numerous studies available in peer reviewed literature, HLA typing is essential for the diagnosis of disease and a critical part of transplant medicine [17,18].

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2. Clinical histocompatibility typing

Multiple methodologies have been utilized for HLA typing including serologic[19], restriction fragment length polymorphism (RFLP)[20,21], polymerase chain reaction (PCR) - sequence-specific oligonucleotide probe typing (SSOP)[22,23], PCR - sequence-specific primer (SSP)[24-27], and Sanger sequence-based typing (SBT) (Table 1). Many clinical laboratories use a form of SSP best known as real-time PCR (RT-PCR) or real-time SSP typing [28]. Currently RT-PCR typing is most commonly performed on deceased donors to obtain a low to intermediate resolution of typing in a short period of time, allowing for timely organ allocation.

SBT is the final technique to be used to provide HLA typing for patients. In principle all sequenced based typing utilizes a single primer post DNA amplification and fluorescently labeled-dideoxy nucleotides which act as chain terminating molecules to end PCR amplification during sequencing[29,30]. Different sequencing technologies can then be used to determine the nucleotide at the place of PCR termination. This method of typing provides the advantage of higher resolution results with less ambiguity than SSP or SSOP as sequencing can identify every base pair in the HLA gene being typed. Unfortunately compared with PCR based methods, Sanger SBT is both more labor intensive and time consuming. In addition, typing ambiguities resulting from the inability to phase heterozygous base positions necessitated follow-up testing to resolve the actual HLA type. However, various methods of next generation sequencing (NGS) technologies have begun to mitigate these disadvantages for HLA typing.

NGS sequencing studies of the HLA locus were conducted as soon as NGS technology became economically reasonable for research with some of the earliest studies being performed by pyrosequencing[1,31]. It was not long thereafter that several clinical histocompatibility laboratories received approval from the American Society for Histocompatibility and Immunogenetics (ASHI) to apply PCR amplification enrichment NGS-based methods for clinical HLA typing purposes[32]. Since the initial implementation of NGS for HLA typing, the number of histocompatibility laboratories using NGS, and the number of novel HLA alleles has continued to increase[33,34]. NGS typing, while still labor intensive and time consuming, is less so than Sanger sequencing and results in higher resolution, phasing and thus fewer ambiguities [35].

With the increased genomic clarity gained via SBT methods, including NGS, it is important to understand the nomenclature for HLA typing for high and ultra-high resolution NGS. In 2010, it was decided to separate fields HLA fields by colons that denote differences in alleles. Currently HLA alleles are defined by the HLA Nomenclature Committee guidelines that classify each gene followed by an asterisk and four fields separated by colons[36]. Each of these fields are numeric and designate with the first field identifying the serologic assignment or allele family and the second field designating the order of discover or amino acid identifier. The third field denotes the synonymous variation in the allele exons with the final and forth field designation indicating differences in the non-coding region of the HLA allele. Some alleles have a suffix added to the end of the HLA assignment which normally denote changes in expression, such as N, which denotes a null allele.

Current NGS approaches to HLA typing typically utilize a method to enrich the patients isolated DNA for the HLA loci to ensure that the depth of coverage (DOC) is acceptable for high resolution typing results. Obtaining appropriate DOC can be challenging in HLA particularly when patient genomic DNA must be extracted from buccal swabs which can often yield fragmented and low concentration genomic DNA due to poor collection. DOC is an important indicator that HLA loci of interest for typing have enough coverage to provide a reliable typing for clinical utility. Enrichment protocols rely on one of several methods including short-range PCR[37,38], long range PCR[39-41], or hybrid capture [42,43]. For these NGS library enrichment protocols, multiplex primers are used to amplify the *HLA-A, -B, -C, -DPA1, -DPB1, -DQB1, -DQA1*, and *-DRB1/3/4/5* loci [44,45]. With short-range PCR based enrichment comes the advantage of faster sequencing and higher DOC for enriched regions, however, it suffers from the potential loss of phasing over longer stretches of DNA. In turn, long-range PCR exchanges a longer library prep time to gain amplification of entire HLA genes including introns, upstream and downstream flanking sequences, and in some cases, untranslated regions. Some commercial kit protocols using long-range PCR have been able to streamline the library preparation so that the PCR amplification time can be accomplished in about 3 h [42,46,47]. These enrichment methods for NGS HLA typing have been in practice for many years now and have demonstrated reliable performance. Although it is rare (~1–4%), these enrichment strategies have the potential to suffer from allele dropout or imbalance as a consequence of where the primers anneal and the sequence composition in these genomic areas[414849]. As allelic dropout is not specific to HLA haplotypes and dropout can be lot specific to NGS reagents, it is important to use appropriate quality control and assurance methods to detect when this is occurring[48]. Additionally, it has been recommended that homozygous alleles or typing suspect to be influenced by drop-out should be typed by another methodology to ensure correct typing[48]. These PCR based NGS methods provide high quality typing results with the appropriate quality control by verifying HLA loci and allele associations and utilizing sequence run metrics to ensure appropriate quality for typing[49-52]. Allele balance percentage, overall quality scores, and depth of coverage assessment are just several of the metrics which can be utilized to judge the quality of a sequencing run to ratify the condition of the sequencing data or identify samples or entire sequencing runs that require further investigation.

The most recent enrichment method, and an alternative to enrichment by PCR amplification, employs HLA locus complementary probes which hybridize to the locus of interest. These probes are composed of oligonucleotide sequences bound to magnetic beads, which allow for the selection and concentration of HLA genes during library preparation. This not only decreases the time required for library preparation while maintaining a DOC sufficient to provide quality results, but it also mitigates the potential problem of allele dropout[53,54]. This process removes a large portion

Table 1
Clinical Histocompatibility Typing Methods.

Typing Method	Compared to NGS						
	Resolution	Throughput	Cost	Protein Expression	Time	Sensitivity	Other Disadvantages
Serology	Serological/Low	–	+	+	++	–	No genotype, no novel alleles, & limited by serum panels.
PCR-SSOP	Low-Intermediate	+	+	=	++	–	Lower resolution and limited by probe sets used
PCR-SSP	Low-Intermediate	–	+	=	+++	–	Limited to the primer sets utilized
Sanger SBT	High	–	–	=	+	–	More typing ambiguities

of the HLA non-specific reads, improving post sequencing data analysis and allowing for more coverage of the areas of interest for typing.

3. Hematopoietic stem cell transplant

Prior to NGS-based HLA typing, Sanger SBT was the standard approach for typing recipients and donors for hematopoietic cell transplant (HCT). This technique is still used but a major disadvantage is the requirement for a significant amount of follow up testing (ie. additional HLA typing) to resolve typing ambiguities due to its limited ability to phase heterozygous base calls. NGS typing methods are having a significant impact on HCT by improving typing resolution, accuracy and efficiency. In addition, NGS is allowing examination of higher stringency HLA matching effects based on its ability to provide up to 4-field typing results and identification of polymorphisms outside of the core exonic sequences[5556].

Current donor selection criteria for matching allogeneic HSC donors includes comparing a high resolution (2-field) and/or allele level typing for *HLA-A, -B, -C*, and *-DRB1*. Current guidelines require either unambiguous assignment of a single allele pair at these loci or ambiguous combinations of alleles as long as only one pair has a common or well documented allele pair (ie all other ambiguous pairs are rare allele combinations). Additional loci including *HLA-DQB1, -DRB3/4/5*, and *-DPB1* are commonly typed to a 2-field resolution[56]. In addition HLA typing is focused on the core exons encoding the antigen recognition sequence of the HLA proteins to achieve this requirement.

Due to the difference in typing methods, several studies have been able to highlight the performance of NGS based typing against other methods for HSC donor selection. A study performed by the DKMS group in Germany found that when they compared NGS typing to Sanger SBT methods, NGS typing demonstrated a 'very low error rate' with decreased costs and less ambiguities[57]. Their findings corroborated other studies and also emphasized the clinical utility of NGS in improving the ease of identifying and verifying novel HLA alleles[56,57]. Further, using high resolution unambiguous NGS HLA typing has shown to result in a higher probability of identifying well matched HSC matched and partially-matched donors from the National Marrow Donor Program (NMDP)[56]. Further, NMDP requirements for confirmatory typing have been updated this year to reflect the newest Common, Intermediate and Well-Documented HLA Alleles in World Populations (CWID), making typing by NGS important to meet these requirements. Additional groups have concurred in the demonstrating the accuracy and reduced ambiguities with NGS typing[41].

A recent retrospective study investigated the impact of ultra-high resolution typing on transplant outcomes[58]. Ultra-high resolution refers to typing HLA alleles to the third field of resolution and beyond, which compares sequences of all the class I and II extracellular domains as well as the non-coding region. In this study they found that ultra-high resolution typing resulted in improved survival outcomes. However, one of the major weaknesses of their study was the extremely small group of 12 donor and recipient pairs that were ultra-high resolution matched. Larger ultra-high resolution matched cohorts will be needed to truly verify if matching at that resolution really does improve patient outcomes[59]. However, their study does indicate that high resolution ARS mismatches impact transplant outcomes as 98% of their patient cohort had ARS mismatches, highlighting the importance of matching at HLA ARS loci[59]. A follow up study recently published investigating ultra-high resolution typing of HCT patients and their outcomes while unable to confirm the findings of the previous study, did demonstrate that ultra-high resolution matching decreases the risk of acute graft-versus-host disease in T cell replete HCT and that ultra-high resolution mismatches for T cell depleted HCT was associated with increased transplant-related mortality[60]. This is an area of interest for HCT and will require more studies to fully understand the impact and extent on patient outcomes that ultra-high resolution matching actually could provide.

4. Solid organ transplant

Current guidelines require HLA typing of the *HLA-A, -B, -C, -DRB1, -DRB3, -DRB4, -DRB5, -DQA1, -DQB1, -DPA1*, and *-DPB1* loci using molecular typing methods[61]. Typing in solid organ transplant serves two main purposes. The first is for donor organ allocation. In the United States, allocation points are provided for better HLA-DR matches between donors and candidates. In addition, candidates that are 0 *HLA-A, -B, -DRB1* mismatched with a potential donor are also afforded some priority for that donor organ. Currently, this application of HLA typing in solid organ transplant is used for kidney allocation. The second reason for typing is to facilitate assignment of unacceptable HLA antigens. Solid organ transplant candidates are tested for HLA specific alloantibodies and, based on program specific criteria, this information is used to assign unacceptable antigens to avoid allocation of organs that possess these antigens. This is a critical application of HLA typing [62].

In solid organ transplant and kidney transplant in particular, a recent emphasis has been placed on the importance of high resolution typing[63]. Several studies have demonstrated that typing by NGS is important for generating the HLA 2-field typing that is vital to identify donor specific antibodies, both pre- and post-transplant [64,65]. Low resolution typing can limit the accurate detection of donor specific alleles and corresponding donor specific antibodies (DSA). This limitation can have clinical consequences as lack of recognition of donor allele-specific antibodies can result in antibody mediated rejection (AMR) and poor transplant outcomes. NGS based typing methods offer the highest resolution available for donor typing which should be utilized to identify potential DSA pre-transplant[66].

In past studies utilizing other methods of HLA typing, mismatching at the *HLA-A, -B, -C, -DRB1, -DQB1*, and *–DPB1* loci in kidney transplant patients has been shown to be immunogenic and associated with increased risk of de novo DSA[67,68]. Mismatches at some of these loci are considered to be more immunogenic than others. For example, mismatching at *HLA-DRB3/4/5* was associated with more DSA generation than mismatches at *HLA-DRB1* and *HLA-DP*[68] and mismatches at *HLA-A* and *-B* are associated with more patients developing DSA than mismatching at *HLA-C* [69]. Of course the number of mismatches at these loci also play a role in immunogenicity.

These findings have been corroborated using NGS methods. Additionally, more accurate molecular analysis can be performed with the high resolution typing offered by NGS compared to Sanger SBT methods. This is evidenced in several recent studies using NGS and molecular mismatch eplet analysis. On molecules, chains of amino acids that can potentially provoke a specific immune response or be recognized by antibodies are called epitopes. The functional component of an epitope has been referred to as an eplet. Eplet mismatch loads are of considerable interest in transplant medicine as several studies have demonstrated that HLA eplet mismatch loads can predict risk for graft rejection[7071]. These studies found that their patient cohorts with varying eplet mismatches loads at the *HLA-DQ* and *HLA-DP* loci result in increased risk for *de novo* DSA formation, graft rejection, and kidney failure[72,73]. These and other studies provide evidence that NGS typing results paired with molecular scoring of donor mis-

matches could be used to guide donor selection. This may further allow personalization of immunosuppression to reduce the risk of associated complications[73].

HLA Match Maker, a software program, was designed to calculate these potential molecular mismatches as a better way to judge recipient immune risk for transplant from a given donor[70]. NGS typing provides the unambiguous identification of HLA alleles, a requirement for eplet analysis, allowing for more accurate assessment of eplet mismatch load. Without the use of NGS methods to provide a high resolution typing, inferences must be utilized to estimate the most likely high resolution typing to provide an estimate of the likely eplet mismatch load.

An alternate program to predict immunogenicity of HLA mismatches, requiring high resolution HLA typing, is the PIRCHE algorithm[74]. PIRCHE analysis identifies potential donor immunogenic peptides that can be presented by recipient T cells. A recent study using NGS typing to generate PRICHE-II molecular mismatch scores in a kidney transplant cohort found that the scores correlated well with alloreactivity and could predict de novo donor-specific T cell alloimmune responses[75].

A further example is provided by a recent study conducted using NGS high resolution typing of a cohort of lung transplant patients found that as the count of eplet mismatches increased, the severity of primary graft dysfunction (PGD) also significantly increased[76]. Study results recorded that in their lung patients both eplet mismatch load and HLA-DQ mismatch were associated with increased risk of PGD and acute graft rejection. This study underlines the potential importance in performing molecular mismatch analysis and encourages DSA identification in recipients prior to lung transplant[76].

Obtaining the best possible matched organ for a recipient is preferred in solid organ transplant and with the help of NGS SBT this process is improving. However, it is important to note that patients in critical conditions may not have the ability to wait to receive the best match possible, and it is appropriate for them to receive a compatible organ as soon as possible[61]. For this reason it is important to continue improving HLA typing methods in solid organ transplant. By continuing to provide higher resolution typing in a timelier manner, particularly donor typing, it may be possible to improve a patient's opportunity to receive a better matched organ rather than simply a minimally compatible organ.

A recent study authored by Huang et al. described and gave case examples of two additional ways that high resolution typing impacts solid organ transplant[63]. High resolution typing has the ability to aid in the identification of allele specific self-anti-HLA antibodies and assist in the interpretation of cross-match results. The identification of allele specific antibodies can be achieved for most alleles only with the use of SBT methods. Self-anti-body identification can be confirmed using auto and surrogate cross-matches, requiring the use of a high resolution NGS to provide the genotype for both the recipient and/or surrogates to confirm suspected self-anti-HLA antibodies[63]. Further, NGS high resolution typing can aid in the clarification of HLA cross-match results. Unexplained positive cross-match results using serologic or ambiguous SSOP or RT-PCR based typing, can be clarified by providing a higher resolution typing to identify DSA that could be causing the unexplained positive cross-match results.

High resolution typing by NGS can also assist in identifying alleles that are not serologically defined. These novel alleles can arise by recombination events, as a result of point mutations, or single nucleotide exchanges[7778]. An example of this is highlighted by the study identifying the novel HLA-B*14:53 allele[79]. Obtaining HLA-B locus typing using high resolution SBT, they were able to identify that the novel allele was likely due to intralocus gene conversion events, a type of recombination mechanism, between B*08:01 and B*14:01:01. When serologically typed, the novel B*14:53 allele carrying cells failed to react with the B64 (B*14:01) and B65 (B*14:02) anti-sera as other B*14 alleles would and only reacted with the B*14 specific anti-sera, which gives it a short or weak B14 serological profile[79]. NGS high resolution typing will continue to be instrumental in the continued identification of novel alleles that will require serological profiling and defining.

5. Disease association

A additional clinical application of HLA typing is identification of genetic variants that are associated with increased risk or resistance to autoimmune and infectious diseases[9,80,81]. This susceptibility or increased protection conferred by the HLA region is the result of several possible molecular processes involving the MHC molecule including alternate docking, low-affinity-mediated thymocyte escape, weak peptide-HLA stabilization, hybrid peptide generation, and molecular mimicry among others[80]. NGS methods were utilized in this area of clinical importance years before NGS SBT started being used for clinical HLA typing, as a result, the medical community has benefited from many more years of high resolution data generated in this area of clinical interest.

Previous methods of HLA typing have identified associations with HLA loci in diseases such as multiple sclerosis, rheumatoid arthritis, type 1 diabetes, good pasture disease, celiac disease, systemic lupus erythematosus, ankylosing spondylitis, Behçet disease, Birdshot retinochoriodopathy, Narcolepsy, Reiter syndrome, and Crohn's disease among numerous other autoimmune and infectious diseases[81-84]. A number of studies comparing NGS typing for disease association to other methods including SSOP and real-time PCR have confirmed that NGS is the most accurate clinical method for identifying alleles associated with disease risk and drug hypersensitivity[85,86]. Clinically, the differentiation of the various B*27 alleles is important as several are not associated with disease, such as the B*27:06 and B*27:09, while others play a distinct role in disease pathogenesis, including B*27:04 and B*27:05. A recent study highlighting this point found that for B*27 associated alleles with ankylosing spondylitis, NGS was best able to differentiate between pathogenic and non-pathogenic B*27 alleles in all tested cases when compared to RT-PCR melt curve analysis and flow cytometry assays[87]. Additionally, NGS can be fiscally advantageous for some disease association testing in comparison to other methods when sample volume is high enough[85].

An early NGS study investigating HLA allele association with Parkinson's disease (PD) using high resolution HLA typing results for nearly 3,000 PD patients along with over 2,000 control cases found that risk association changed when the cohort subjects had certain single nucleotide polymorphisms (SNPs) [88]. The study identified the risk haplotype *HLA-B * 07:02 ~ C * 07:02 ~ DR B5 * 01 ~ DRB1 * 15:01 ~ DQA1 * 01:02 ~ DQB1 * 06:02*. However, when the risk haplotype was influenced by the presence of specific SNPs located in non-coding regions involved in regulating expression of the HLA loci, the risk association was negated. Even single nucleotide changes to these regulatory elements can drastically impact transcription. These findings indicate that that HLA gene expression likely plays a role in PD development and further highlights additional elements that can be identified to ascertain disease risk using NGS typing[88] given its ability to sequence entire genes .

A variety of recent research investigations using NGS based typing have confirmed results from previous studies and been able to further evaluate disease risk alleles in several autoimmune diseases as a result of utilizing NGS typing. A type 1 diabetes (T1D) study demonstrated the utility of using NGS typing with previously identified risk alleles[89]. Previous studies investigating T1D and disease association using lower resolution techniques found that

DR17-DQ2 and DR4-DQ3 haplotypes were associated with risk for disease specific autoantibody production[8990]. This study found that *DRB1*03:01:01* was affected by both *DRB3*01:01:02* and *DRB3*02:02:01* alleles in risk association for their T1D patients. Further, the *DRB1*13:01:01* allele, which normally has been associated with a lack of risk for T1D, was influenced by the *DRB3*01:01:02* allele resulting in increased risk. However in patients with *DRB1*13:01:01* and *DRB3*02:02:01* haplotype, the negative association was maintained. Additionally, the study indicated that there was a strong association with T1D and the *DRB4*01:03:01* loci. However, this association was dependent on the *DRB1* alleles from the patient population with the *DRB1*04:03:01* conferring a protective haplotype, *DRB1*04:05:01* associated with a low risk haplotype and *DRB1*04:01:01* indicating a high risk haplotype[89]. As a result of high resolution 3 field typing, the study was able to identify the risk association, previously undefined, for T1D between *HLA-DRB1*, *-DRB3*, and *-DRB4* loci.

Other recent studies investigating HLA alleles associated with multiple sclerosis (MS) found that *HLA-DRB1*15:01:01:01* was associated with risk for developing MS[91,92]. For MS it has been known for some time that HLA-DR4 and DR15 are associated for risk of MS, likely due to molecular mimicry, weak peptide stabilization by the TCR, and/or alternate docking of the TCR[80]. In this recent study, the haplotype *DRB1*15:01 ~ DQB1*06:02* had a particular strong association. The *HLA-DQB1*03:01:01:01 ~ HLA-DQA1*03:03:01:01* or *HLA-DQB1*03:02:01 ~ HLA-DQA1*03:01:01* haplotypes were identified as being highly protective for disease development with the haplotype *HLA-DQB1*03:02:01 ~ HLA-DQA1*03:01:01 ~ HLA-DRB1*04:01:01:01SG ~ HLA-DRB4*01:03:01:01* demonstrating a predisposing effect for MS[92]. With the use of NGS high resolution typing, this study was able to identify allele specific *HLA-DRB1*, *-DQB1*, and *-DQA1* haplotypes that were associated more specifically with disease risk.

A final study that emphasized the use of NGS typing with respect to disease association previously confirmed that *HLA-B*53*, *-B*78*, and *-A*74* conferred protection from P. falciparum infection by non-NGS SBT methods[81]. Using NGS, all of the previous samples were retyped and analyzed. They found that the low-resolution *HLA-B*53* allele that conferred protection was, in nearly all cases, the *HLA-B*53:01:01* and that disease resistance was strongly associated with the *B*53:01:01 ~ C*04:01:01:01* haplotype. Additionally, *HLA-B*78* typed at low resolution that conferred protection was the *B*78:01:01* allele. The new data generated by NGS further allowed for further evaluation of the *HLA-A*74* alleles and it was determined that they were not associated with protection from malaria[81]. These studies are but a few that demonstrate both the clinical and research utility of using NGS HLA typing in identifying HLA disease associations.

6. Pharmacogenomics

A number of HLA alleles are associated with an interesting array of adverse events such as delayed hypersensitivity reactions (DHR), Steven-Johnson Syndrome/toxic epidermal necrosis (SJS/TEN), and risk for increased drug toxicity when patients with these alleles use certain medications. Many studies have shown that this is likely the consequence of a combination of factors including drug metabolism, drug interaction with T cell receptors, and drug interaction with the HLA molecule[82,93]. Due to the specificity of drug reactions, a two-field resolution is preferable to establish risk of drug adverse reactions with HLA loci, as such, the use of NGS becomes immensely helpful as it provides this high resolution of typing.

The use of NGS has proved an extremely powerful tool in identifying HLA alleles associated with drug reactions in patient populations the world over. In a study published this year by Wang et al, utilized NGS sequencing to provide whole genome analysis of 151 patients from Taiwan, Thailand, and Malaysia that suffered severe cutaneous adverse drug reactions in response to Co-trimoxazole [94]. Their study found the SNP *rs41554616* and the *HLA-B*13:01* allele correlated well with Co-trimoxazole adverse drug reactivity.

There are a variety of other HLA alleles that are associated with adverse drug events. An example includes *HLA-B*57:01* which is associated with Abacavir induction of DHR. Years after the discovery of this association it became standard of care to type patients for the HLA-B locus prior to treatment with Abacavir[9596]. Some additional HLA loci associated with adverse drug reactions include *HLA-B*58:01* (Allopurinol SJS/TEN), *-B*15:02* (Carbamazepine SJS/TEN), *-B*13:01* (Dapsone DHR), *-C*04:01* (Nevirapone SJS/TEN), *-C*05:01* (Nevirapone SJS/TEN), *-C*18:01* (Nevirapone SJS/TEN) and *-A*32:01* (Vancomycin)[94,97-101].

7. Future advances in typing with Next-Generation sequencing

While NGS typing of transplant recipients and donors has contributed to refined patient matching, it also offers great opportunity to continue improving patient outcomes. It is possible that the 3rd field or higher resolution offered by NGS typing could further identify better matches and as a result and improve clinical outcomes with the ability to use lower dosages of pharmacologic therapeutics for transplant patients[56]. Current software, such as PIRCHE, paired with NGS data currently offer the potential to decrease alloreactivity by providing better matching based on nucleotide sequence and the inferred proteomic result[102]. Additionally, NGS facilitates the exploration of non-traditional HLA loci that might have an impact on transplant outcomes, which is an important area of clinical importance as polymorphisms in non-coding DNA regions have been shown to influence T cell stimulation affecting transplant outcomes and infection[103-105].

Currently, with the HLA antigen recognition site (ARS) being the only required domain for HLA typing, the other domains in the HLA region could be typed to provide additional medically relevant information. There is gathering evidence that genomic regions outside of the HLA ARS can influence clinical outcomes[58,106-109]. A recent study example that provides evidence for this found that the leader peptide of HLA-B, coded in exon 1, was associated with risk of graft-versus-host disease in HSC patients and based on the genomic sequence, patients could be risk stratified[108]. An earlier study that compared the HLA-DRB1 sequence to the HLA-DRβ amino acid motifs allowed the research group to assess the shared structural properties of HLA-DR molecules that correlate with mortality risk in HSC patients. Their study found that the germline HLA-DRB1 alleles encoding certain amino acid substitutions influenced the variety of HLA-DRβ peptides, predisposing transplant patients for increased risks of morbidity post-transplantation[109].

This later study also emphasizes the clinical utility of measuring mRNA HLA expression in transplant patients that could help inform clinical decisions based on those levels. Another example of this is a recent study identifying specific SNPs within the HLA-DPB1*15:01 allele non-coding region that have regulatory functions and can impact clinical outcomes, likely by altering the DNA methylation status of the HLA-DRB1 loci[110]. Another study has further suggested that varying levels of expression caused by the SNP impacts B cell flow cytometric crossmatches[111]. It is possible that in the future, obtaining both genotype and transcriptome analysis of the HLA loci of interest for donors and recipients could provide additional information for transplant teams to better inform them on patient immunologic risk and expected transplant outcomes.

Another area that we expect to improve dramatically in the coming years as a result of NGS is the quality and amount of reference sequences available. As described in previous publications, the deficiency of full length IMGT/HLA reference sequences poses a large challenge for trying to provide unambiguous typing with such high resolution methods[11]. Many individuals have realized this demand by histocompatibility laboratories and are working to increase the high definition reference sequences available. The Anthony Nolan Research Institute, for example, has aided by extending reference sequences for 95 HLA class I alleles[112]. Additionally, this same group, who maintains the IMGT/HLA database, are now obtaining more full-length HLA class I submissions than partial gene sequences with the continued adoption of HLA typing by NGS by HLA laboratories[113].

Improved NGS methods is a final future improvement that is approaching clinical utility in HLA typing. These include third-generation sequencing methods such as the Oxford Nanopore Technologies (ONT) and Molecular Real Time DNA sequencing (SMRT). These technologies utilize long-read, often greater than 20 kb, sequencing enabling laboratory staff to sequence DNA or RNA in shorter amounts of time due to less library preparation time, faster sequencing speeds, and decreased bioinformatics computation requirements[114]. This allows for sequencing of the entire HLA class I and II loci[115,116]. Several recent publications demonstrate how use of these technologies can provide high resolution HLA typing in 4 to 5 h[108]. Rapid advanced sequencing technologies would allow for the easy use of RNA sequence based HLA typing and molecular mismatched based allocation for deceased organ allocation[63,117-119].

Typing by NGS is being readily accepted by the transplant community for both solid organ and hematopoietic stem cell transplant. While one of its current limitations is the time consuming nature of the process, several upcoming technologies and procedures offer potential solutions to mitigate this disadvantage. This method of HLA NGS typing has become increasingly cost effective with the batching and multiplexing of patient samples and the continued monetary decrease in NGS reagents, supplies, and equipment. NGS typing has been extremely effective at decreasing or eliminating ambiguous typing results. Depending on NGS methods, non-amplification and allele drop out can still be problematic. However, these occurrences are rare and less frequent when utilizing NGS compared to other typing methods. Additionally, good quality assurance practices in HLA laboratories as well as possible advances in NGS methods can also be used to further minimize these occurrences.

Conflicts of Interest

EW is a Scientific Advisory Board Member for CareDx and a One Lambda Consultant to One Lambda. JS and CC have no conflicts of interest to disclose at the time of this publication.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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