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REVIEW AND PERSPECTIVES

HLA testing in the molecular diagnostic laboratory

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Abstract

The human leukocyte antigen (HLA) system is a highly polymorphic family of genes involved in immunity and responsible for identifying self versus non-self. HLA typing is essential for solid organ and bone marrow transplantation as well as in non-transplant settings such as disease association and pharmacogenomics. Typing of HLA genes differs from most molecular testing as, rather than evaluating differences from an accepted "wild-type" gene, it must distinguish between thousands of similar, but distinct alleles. This article will describe the HLA system and nomenclature. We will then discuss clinical uses of HLA typing including solid organ transplantation, hematopoietic stem cell transplantation, evaluation of platelet refractory patients, disease association, and pharmacogenetics. Finally, we describe common molecular methods of HLA typing.

Keywords Molecular · HLA · Transplantation · Immunogenetics · SSO · SSP

Introduction

The human leukocyte antigen (HLA) system plays a crucial role in the regulation of immune function in the determination of self from non-self. Consequently, accurate HLA typing is of utmost importance in solid organ transplantation (SOT), in hematopoietic stem cell transplantation (HSCT), in transfusion medicine for platelet refractory patients, and in the diagnostic workup of various disease associations and pharmacogenomics applications.

The HLA genes are located within the most polymorphic region of the human genome, the major histocompatibility complex (MHC), which is located on chromosome 6p21.3. There are over 20,000 distinct, but closely related alleles in the MHC system, encoding the Class I HLA-A, HLA-B, and HLA-C loci, and Class II HLA-DR, HLA-DQ, and HLA-DP loci. HLA molecules interact with T cell receptors in the thymus to modulate the immune response and determine what cells are recognized as self.

MHC Class I includes the HLA-A, HLA-B, and HLA-C loci. Class I proteins are expressed, to varying degrees, on the surface of all nucleated cells and are comprised of one transmembrane heavy chain with three extracellular domains, designated α1, α2, and α3; and a β2-microglobulin light chain that anchors the heavy chain to the cytoplasmic membrane. Class I genes include eight exons. The α1 and α2 segments, coded for by exons 2 and 3, form a peptide binding groove, which presents peptide antigens to CD8+ T lymphocytes [1, 2].

MHC Class II includes the HLA-DP, HLA-DQ, and HLA-DR loci. Expression of these proteins is limited to specific immune cells, including B cells, activated T cells, macrophages, dendritic cells, and thymic epithelium. Class II proteins are comprised of an alpha and a beta chain. The α and β chain genes are separately designated; for example, the two genes coding for the HLA-DQ protein are HLA-DQA1 and HLA-DQB1. Each α and β chain protein includes two extracellular domains designated respectively α1 and α2, and β1 and β2. In HLA-DR, the β chains of the class II proteins are more polymorphic than the α chains, which are relatively preserved. In HLA-DQ and -DP, both the α and β chains are polymorphic. The peptide binding groove is formed by the α1 and β1 segments, which are coded for by exon 2 of the α and β genes. MHC Class II proteins present peptides to CD4+ T lymphocytes [1, 2].

Both Class I and Class II molecules present short peptides to T lymphocytes; Class I MHC presents endogenously produced peptides, including native proteins as well as mutated, damaged, degraded, or misfolded proteins, as well as viral proteins to CD8-positive T lymphocytes [1]. Class II MHC presents exogenously produced peptides, such as bacterial proteins, to CD4-positive T lymphocytes.

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HLA alleles are expressed in a codominant fashion. The significant diversity of the HLA alleles is secondary to amino acid substitutions within the regions coding for peptide-binding sites in the second and third exons of the class I genes or the second exon of the class II genes. Changes in these regions alter the peptide antigens, and the HLA molecule is able to present. Most alleles differ by multiple single nucleotide substitutions; this is suggestive of segmental exchange of nucleotides between alleles at the same locus [1, 2]. The HLA alleles from a given parent are typically inherited as a haplotype, or set of HLA genes at contiguous loci. An individual inherits one haplotype from each parent. The haplotypes are inherited in Mendelian fashion; with more siblings, the likelihood of two sharing one haplotype or being HLA identical increases. Linkage disequilibrium, or the non-random association of alleles at different loci, commonly occurs in the HLA system and may occur between HLA classes or over an entire HLA haplotype. Common alleles and haplotype frequencies vary between population groups [3].

While the proteins coded for by the HLA genes are similar in structure, each allele has a unique DNA sequence. Polymorphisms in the HLA genes are secondary to point mutations, meiotic recombination events between different alleles of the same genes, and gene conversion events where recombination occurs between different genes. Conversion events may be seen up to 1% per meiosis between HLA-A and HLA-B and HLA-B and HLA-DR, up to 0.6% between HLA-A and HLA-C, and up to 0.2% per meiosis between HLA-B and HLA-C [4].

Historically, HLA antigens were described based on reactivity in serologic assays. Serum from sensitized patients, typically women who were sensitized during pregnancy to paternal HLA antigens expressed by their fetuses, was incubated with lymphocytes from various donors in the presence of complement and a vital dye. Cytotoxicity was then assessed. All HLA loci were therefore originally defined by antibody responses, numbered in order of their discovery. As testing methods were refined, it was discovered that some typings included multiple proteins that were previously indistinguishable. These antigens were "split" from their broad parent group. For example, HLA-A23 and HLA-A24 are splits of the originally described HLA-A9 parent antigen. Additional splits were developed as molecular typing identified distinct alleles that code for serologically similar proteins.

Serologic methods can only resolve a very small fraction of HLA alleles. Alleles with different DNA sequences can encode for proteins with similar serologic reactivity. Starting in the 1980s, sequencing of HLA genes resulted in a rapid increase in newly identified alleles and eventually created a need to further refine HLA nomenclature. Molecular typing allows the definition of HLA type based on DNA sequence in addition to the amino acid sequence and serologic reactivity.

The current nomenclature includes four numeric fields based on DNA sequencing result: the allele or antigen group, which often corresponds to the serologic designation; the specific amino acid sequence of the allele; presence of synonymous polymorphisms; and differences in non-coding regions. An asterisk in an allele name indicates that the allele was typed using molecular methods. As an example, consider the typing A*68:02:01:02. The first field, 68, indicates that this allele belongs to the broad protein group 68. The second field, 02, indicates that this is the second gene sequence described in that group. The third field, 01, shows that there are no synonymous substitutions in the coding region compared to the first sequence described. The fourth field, 02, indicates that this allele differs from the first described sequence in a non-coding region. When present, a letter suffix at the end of an allele name is used to note changes in gene expression; for example, an "L" indicates low expression and "N" designates a null allele that is not expressed [5] (Fig. 1).

Molecular typing methods allow typing of the HLA allele to different levels of resolution. Traditionally, these levels are described as low- or high-resolution typing. Low resolution, or "two-digit" typing, generally, but not always, corresponds to the serologic typing result and provides the first field in the molecular-based nomenclature (example: HLA-A*01). Low-resolution typing is typically sufficient for solid organ transplantation, transfusion support, and some disease association and pharmacogenomic testing. High-resolution or "four-digit" typing distinguishes alleles based on the sequence of the peptide-binding region of the HLA molecule, corresponding to the first two fields of the molecular nomenclature (example: HLA-B*57:01) [6]. This level of typing does not distinguish between alleles that differ in synonymous substitutions or in non-coding regions. High-resolution typing typically requires gene sequencing and is required for bone marrow transplant typing and some disease association and pharmacogenomic testing [7]. Resolution of the entire four field description is referred to as allele-level typing.

An additional category of "intermediate resolution" is occasionally used. Intermediate resolution typing includes resolution of some, but not all, of the possible amino acid sequences for a given allele group depending on the specific testing technique and sometimes the allele in question [8].

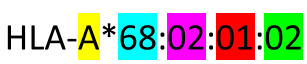

Fig. 1 The different fields used in standard HLA nomenclature. The yellow field indicates the gene name or locus, A in this example. The asterisk separator indicates that this is molecular typing. Field one (blue) gives the allele group, field two (pink) indicates the specific HLA protein, field three (red) synonymous polymorphisms, and field four (green) differences in non-coding regions. "Low-resolution" typings include only the first field (blue), while "high-resolution" typings include the first and second (blue and pink) fields

Applications of molecular HLA typing

Solid organ transplantation

Solid organ transplant activities are typically coordinated by country-specific umbrella organizations that create procedures and requirements for transplantation, including molecular typing of samples, in a given region. In the USA, the Organ Procurement and Transplantation Network (OPTN) is the network linking all professionals involved in solid organ transplantation. Under the OPTN bylaws, histocompatibility testing for deceased donors must be performed by molecular methods, with results reported to the level of serologic splits. Typing results must be provided for deceased kidney and pancreas donors and are provided as requested by the transplanting facility for deceased liver, heart, and lung donors. Kidney and pancreas transplant recipients, unless transplanted with any other organs, are required to have HLA typing by molecular methods performed when listed for the organ [9]. Typing for living donors can be done on a routine basis, while typing for deceased donors typically requires rapid typing results to facilitate timely transplantation of organs.

Accurate donor and recipient typing is important to determine the degree of HLA mismatch between the donor and recipient. In the majority of antibody-mediated transplant rejection, the antibodies are directed against donor HLA antigens expressed by the transplanted organ but not present in the recipient [10]. Therefore, knowledge of the donor and recipient HLA types is needed to monitor recipients for development of donor-specific HLA antibodies and antibody-mediated rejection post-transplant.

Although advances in immunosuppression modalities have been shown to improve transplanted graft outcomes, accurately determining both the donor and recipient HLA types and minimizing HLA mismatches is also of utmost importance to maximizing graft and patient survival [11]. Studies have demonstrated that highly sensitized patients, in particular, have improved outcomes with fewer or acceptable HLA mismatches [12, 13]. This further emphasizes the importance of obtaining accurate HLA typing results using molecular methods prior to transplantation.

Historically, HLA typing of solid organ donors was limited to the HLA-A, HLA-B, and HLA-DR loci. Most transplant organizations currently recommend that all six loci be typed for renal transplant cases.

Hematopoietic stem cell transplant (HSCT)

HSCT involves the replacement of a recipient's blood forming cells with stem cells from a healthy donor. These cells may be derived from donor bone marrow, peripheral blood, or cord blood. It is most often used as a treatment option for patients with hematopoietic neoplasms such as leukemia and is recommended at the time of diagnosis of acute leukemia or intermediate- and high-risk myelodysplastic syndrome, among other conditions, in order to facilitate identification of an appropriate donor [14]. HSCT can also be used in other types of neoplasms, bone marrow failure syndromes, immunodeficiency syndromes, and in other constitutional disorders such as sickle cell disease or metabolic disorders. Regardless of the reason for transplant, the process of bone marrow transplant effectively replaces the recipient's immune system with that of the donor. Differences in HLA types can lead to graft failure, disease recurrence, and graft-versus-host disease, where the donor immune system attacks recipient cells particularly in the skin, gut, and liver.

Bone marrow transplant graft survival has been highly dependent on the degree of HLA matching. Matching is assessed at the HLA-A, HLA-B, HLA-C, and HLA-DRB1 loci at minimum, but most transplant programs also assess the HLA-DQB1 and HLA-DPB1 loci. HLA-DPB1 matching in particular has been shown to significantly impact outcomes. Certain HLA mismatches are better tolerated than others and are referred to as "permissive." Non-permissive HLA-DPB1 mismatches are associated with increased mortality and increased incidence of graft-versus-host disease [15].

Donor-recipient pairs with higher numbers of matched HLA loci show improved survival, transplant engraftment, and decreased risk of graft-versus-host disease [16, 17]. Donor identification has generally therefore focused on finding HLA-matched-related donors such as siblings. In patients without a related matched donor, identification of an HLA-matched unrelated donor from a donor registry is pursued. This process is time consuming, and the likelihood of identifying a matched donor varies widely depending on the ancestry of the recipient, ranging from 75% in white patients of European descent to 16% in black patients of Central and South American descent [18]. Because of these difficulties, transplantation from an unmatched donor may be considered, including mismatched unrelated donors, cord blood products, and haploidentical matched donors [19]. Transplantation with haploidentical related donors, who share a single HLA haplotype, or half of their HLA genes, with the recipient, is becoming more widespread. These donors typically include siblings or parents. Use of haploidentical donors increases the donor pool and is often used in patients with high-risk disease who cannot wait for identification of an unrelated matched donor. Haploidentical transplant regimens rely on increased immunosuppression and T cell depletion to improve engraftment and decrease the risk of graft versus host disease. Haploidentical transplants show higher rates of graft failure and disease relapse compared to matched transplant, but can improve outcomes in patients with high-risk disease without a matched donor [20]. Potential recipients of haploidentical transplants must be screened for anti-HLA antibodies prior to transplant.

Evaluation of platelet refractoriness

Some patients receiving donor platelets do not show expected increase in platelet count following transfusion. This can be due to a number of factors, including recipient immune-mediated destruction of donor platelets. The majority of these cases are secondary to anti-class I HLA antibodies. This is most commonly seen in women who have been sensitized to HLA through pregnancy but can also occur in patients sensitized through blood transfusion or transplant. Patients with poor response to platelet transfusion at 10 min or 1 h post-transplant should be screened for anti-HLA antibodies. If antibodies are present, identification of antigen negative units should be pursued. Some patients with HLA-associated platelet refractoriness are likely to require many transfusions, such as leukemia patients. These patients may be HLA typed and transfused with HLA-matched units when possible to decrease the risk of additional sensitization [21].

Disease association testing

Numerous diseases, particularly those with an autoimmune component, are associated with certain HLA alleles. Perhaps the best known is the association of HLA-B27 with ankylosing spondylitis (AS); however, numerous other conditions such as Behçet disease, celiac disease, and narcolepsy also have HLA associations. These associations have been refined, as HLA testing improves and becomes more widespread. For example, high-resolution typing has shown that while most B27 alleles, such as the common B*27:05, are associated with increased risk of AS, other alleles such as B*27:06 and B*27:09 are not associated with this disease [22]. In addition, association between risk of AS and other HLA loci and alleles have been identified, including B*40:01 and DRB1*04:04 [23]. Although the association of B27 and AS is strong, only a small subset of individuals who carry the B27 allele develop AS. In general, the inheritance of a disease-associated HLA allele may increase a patient's likelihood of developing the disease but is only a risk factor and does not guarantee that the disease will occur (Table 1).

The mechanisms of these associations are not clearly understood and may be due to immune response to self-antigens due to aberrant T cell selection or cross reactivity between self and foreign antigens. There may also be HLA presentation of altered self-antigens or aberrant interaction of part of the HLA molecule with its receptor [24]. In some cases, HLA genes have been mistakenly associated with diseases due to linkage disequilibrium of HLA genes with the actual causative gene. For example, hemochromatosis was originally described in association with the A3 and A29 alleles because these alleles are found in linkage disequilibrium with the mutated *HFE* gene [25].

Pharmacogenomics

A subset of HLA alleles are associated with adverse events including hypersensitivity reactions, Stevens-Johnson Syndrome/toxic epidermal necrolysis (SJS/TEN), and increased drug toxicity following treatment with specific medications. For example, HIV patients who carry the B*57:01 allele are at increased risk of a potentially fatal hypersensitivity reaction when treated with abacavir, a nucleoside analog reverse transcriptase inhibitor. All candidates for abacavir treatment should be screened for the presence of B*57:01 prior to initiating therapy, and the drug should not be given in positive patients. Other HLA-associated adverse drug reactions include B*58:01 and allopurinol-induced hypersensitivity, B*15:02, and A*31:01 and carbamazepine-induced SJS/TEN, and B*57:01 and flucloxacilin-related toxic liver injury [26–29] (Table 2).

Molecular typing of HLA

Typing HLA alleles is a unique challenge due to the large number of very similar genes which must be distinguished from one another. Most methods focus only on the sequence of exons 2 and 3 for Class I alleles and exon 2 of Class II alleles, as these regions determine the sequence of the peptide binding region. Many clinical laboratories utilize multiple typing methodologies to resolve ambiguous typings. Although HLA typing for solid organ and stem cell transplant purposes is subject to additional regulatory oversight through organizations like the American Society for Histocompatibility & Immunogenetics (ASHI), the European Federation for Immunogenetics (EFI), Eurotransplant, and the Organ Procurement and Transplantation Network (OPTN) in many regions and is therefore typically performed in specialized laboratories, HLA typing for other purposes such as disease association testing may be performed by general molecular laboratories. While investigation of polymorphisms in other genes requires comparison to an accepted "wild-type" sequence, reference sequences are not present in the highly polymorphic HLA genes. HLA sequence analysis requires comparison of results to extensive lists of possible alleles, typically requiring interpreter expertise and the use of specialized software packages (Table 3).

Sequence-specific oligonucleotide probes (SSO)

One of the first PCR-based HLA typing methods developed was the sequence-specific oligonucleotide probe assay, often abbreviated SSO to clearly distinguish it from sequence-specific primer assays (SSP). This utilizes short (around 20 nucleotides) probes complementary to a limited number of known HLA alleles. After DNA is extracted from a patient

Table 1 This table provides examples of some HLA-associated diseases, but is not comprehensive. The presence of a disease-associated allele in a patient indicates increased risk of a disease but should not be used alone for diagnosis of a disease without the appropriate clinical correlation

Disease	HLA associations	Notes
Ankylosing spondylitis	B*27, B*14:03, B*38, B*40:01, B*52, DRB1*04:04	Up to 95% of AS patients have B*27. B*27:02 and B*27:05 have the highest increased risk. Of note, B*27:06 and B*27:09 are not associated with AS
Behçet disease	B*51	40–80% of patients have B*51
Birdshot retinochoriodopathy	A*29	98% of patients have A*29
Celiac disease	DQA1*05:01/DQB1*02:01 (DQ2), DQA1*03/DQB1*03:02 (DQ8)	90% of celiac patients carry DQ2 and most of the remaining 10% express DQ8
Narcolepsy	DQA1*01:02-DQB1*06:02	Both homozygosity for DQB1*06:02 and heterozygosity for DQB1*03:01 and DQB1*06:02 are associated with increased risk compared to a single DQB1*06:02 allele
Reactive arthritis (Reiter syndrome)	B*27, B*40:01	Up to 80% of patients are B*27-positive
Rheumatoid arthritis	DRB1*04:01	Multiple DRB1 alleles carry an increased risk, although the risk with DRB1*04:01 is greatest

sample, generic primers are selected that will amplify most alleles of a particular locus, and polymerase chain reaction is performed. In traditional, or "forward" SSO, the amplified product is denatured into single strands and is bound to a membrane. The membrane is then hybridized with different SSO probes, usually in a dot blot setup. If the amplified DNA sequence is complimentary to the oligonucleotide probe, hybridization occurs. Hybridized product/probe is detected using chemiluminescent or colorimetric methods, and the HLA type can be determined [30]. In practice, reverse SSO is more commonly used. In the reverse method, the amplified product is added to a solid matrix to which the probes have been bound.

The most common adaptation of the SSO methodology in current use is the flow cytometric bead assay. Sample DNA is amplified and incubated with fluorescent labeled beads impregnated with specific SSO probes. If the sample DNA is complementary to the probe sequence, it will bind to the bead. The bound product is labeled with a detection tag such as phycoerythrin (PE), and a flow cytometer or fluorocytometer is then used to detect binding. The flow cytometer detects the specificity of the bead label and the strength of the PE signal [31, 32].

This method is advantageous for several reasons. Numerous kit-based commercial assays are available and provide highly reproducible results. Turn-around-times are relatively short, compared to sequence based testing. SSO is scalable and can be used for single sample testing as well as batch testing large numbers of patients at once. Although previous SSO assays provided only low resolution typing, newer kits are available that include hundreds of probes. These can reliably provide intermediate- or high-resolution types. Due to the large number of HLA alleles, however, SSO may provide ambiguous typing results that require follow-up testing. Because the most commonly used, commercially available kits only include probes that bind to relatively short sequences in the peptide binding regions, additional testing is required to distinguish alleles that differ outside of these areas. Stringent adherence to washing procedures is required to prevent non-specific binding.

Table 2 This table provides examples of some HLA-associated adverse drug reactions, but is not comprehensive. These adverse reactions can be severe and potentially fatal. Regulatory agencies such as the United States Food and Drug Administration have provided warnings against treatment with many drugs in patients who carry the associated HLA alleles. *SJS/TEN* Stevens Johnson Syndrome/toxic epidermal necrolysis

Drug	HLA association	Adverse event
Abacavir	B*57:01	Hypersensitivity reaction
Allopurinol	B*58:01	Hypersensitivity reaction
Carbamazepine	A*31:01, B15:02	SJS/TEN
Flucloxacillin	B*57:01	Hepatotoxicity
Lapatinib	DQA1*02:01, DRB1*07:01	Hepatotoxicity
Oxcarbazepine	B*15:02	SJS/TEN
Pazopanib	B*57:01	Hepatotoxicity
Phenytoin	B*15:02	SJS/TEN

Sequence-specific primer assay (SSP)

Sequence-specific primer-based typing utilizes primers that only amplify a limited number of alleles. HLA typing by SSP was first used to type the HLA-DR genes; advancements were quickly made to allow for typing of the remaining HLA Class I and Class II genes [33]. Primers complimentary only to specific HLA allele sequences or to a parent HLA allele at the 3′ end are used; under specified amplification conditions,

Table 3 Comparison of the common molecular HLA typing techniques. Allele level typing is possible by any of these methods; however, this gives the typical level of resolution obtained in routine clinical use. *SSO* sequence-specific oligonucleotide probes, *SSP* sequence-specific primer assay, *NGS* next-generation sequencing, *SOT* solid organ transplantation, *HSCT* hematopoietic stem cell transplantation

Method	Typical resolution	Common applications	Turnaround times	Test volume
SSO	Low to intermediate	SOT Transfusion support Disease association Pharmacogenomics	Short	Low to moderate
SSP	Low to high	SOT Transfusion support Disease Association Pharmacogenomics Resolution of ambiguous results from other method	Short	Low to moderate
Real-time PCR	Low to high	SOT	Most rapid	Low
Sanger sequencing	High	HSCT	Long	Low
NGS	Full allele	HSCT	Long	High

these are not extended by Taq polymerase if there is a DNA mismatch.

In typical SSP assays, prepared primer sets are provided in trays. DNA is extracted from a patient sample, added to a mixture of buffer, nucleotides (dNTPs), and polymerase, and aliquotted into the trays with the primers. In addition, internal control primers are included to ensure that the reaction occurs appropriately. The sample is then subjected to PCR. The result then undergoes a detection method such as gel electrophoresis to identify the presence of amplification product. Mismatches between the 3′ end of the primer and the template DNA prevent amplification. Based on the presence or absence of appropriately sized product, the HLA type is assigned [32].

SSP assays are often used for resolution of ambiguous typing calls made by other methods as they provide highly accurate results [34]. A number of commercially prepared trays for specific alleles are available. SSP testing tends to be more susceptible to errors caused by variation in test conditions, as inappropriate test conditions can cause nonspecific primer binding or lack of specific binding. Large numbers of PCR cycles are required for accurate results, so this test is not as useful for simultaneous HLA typing for large numbers of patients [32].

Real-time PCR

Traditional real-time PCR is an extension of SSP typing methods. Patient DNA is isolated and undergoes PCR with allele specific-primers. The creation of product is measured as it is amplified [35, 36]. However, this is not well suited to the identification of specific HLA alleles, and modifications are required to improve performance.

For HLA typing, most real-time assays use a modified method incorporating high-resolution melting analysis. Patient DNA is isolated, mixed with fluorescent dye, and added to a 384 well plate with allele-specific primers. The plate is sealed and undergoes PCR. After PCR, the product is heated and the release of the dye is used to detect the melting characteristics. Software is used to analyze the shape of the melting curves and assign specific allele calls [37].

TaqMan real-time assays are also used for HLA typing. These methods utilize polymerase with exonuclease activity along with fluorescently labeled, sequence-specific probes that provide enhanced specificity without the use of melting curve analysis. Patient DNA is amplified using unlabeled primers specific for a known set of HLA genes. Sequence-specific probes with a fluorescent reporter on one end and a quencher on the other are added. During PCR, these probes bind to denatured DNA. The proximity of the quencher to the reporter suppresses fluorescence. During amplification, the exonuclease activity of the polymerase separates the reporter and quencher, resulting in a detectable fluorescent signal [38].

Compared to gel-based product detection, real-time PCR reduces potential contamination as products are not manipulated after amplification. Of the commonly used typing methods, real-time PCR has the fastest turn-around-time and has excellent discrimination between HLA types. In addition, it does not require post-PCR processing and can be automated.

Sequence-based typing

Sequencing is considered the gold standard for identifying unambiguous HLA typings across loci and is required when typing for bone marrow transplantation. Sequencing provides the exact DNA sequence in the region analyzed, and it is possible to design assays that sequence across gene regions not typically analyzed in routine HLA typing. Therefore, sequencing is required to identify novel HLA alleles. Most sequencing methods begin with a PCR step to increase the

amount of DNA available for sequencing. This step may utilize generic primers that amplify most alleles of a specific HLA locus, group-specific primers that amplify a limited number of alleles, or specific primers that amplify only single allele. The product is then subjected to further sequencing techniques, most commonly either Sanger sequencing or massively parallel "next-generation" sequencing (NGS).

Sanger sequencing

Sanger is a chain termination sequencing technique that generates a single sequence trace from input DNA [39]. Numerous commercial kits for Sanger sequencing of HLA alleles are available. Typically, these provide sequence for exons 2, 3, and 4 of the class I genes and exon 2 of the class II genes. The sequence collected is compared to a database of described HLA alleles to identify the alleles present. Sanger provides high-resolution, or four-digit results.

Because patients are often heterozygous across the HLA alleles, Sanger provides a single result including base sequence from both the paternally and maternally derived genes. It is not possible to determine which allele includes a specific base at heterozygous positions, an issue referred to as a phase or cis/trans ambiguity [40]. This leads to ambiguous typings, where multiple heterozygous pairs have the same Sanger sequencing results. Additional testing, such as with SSP, is

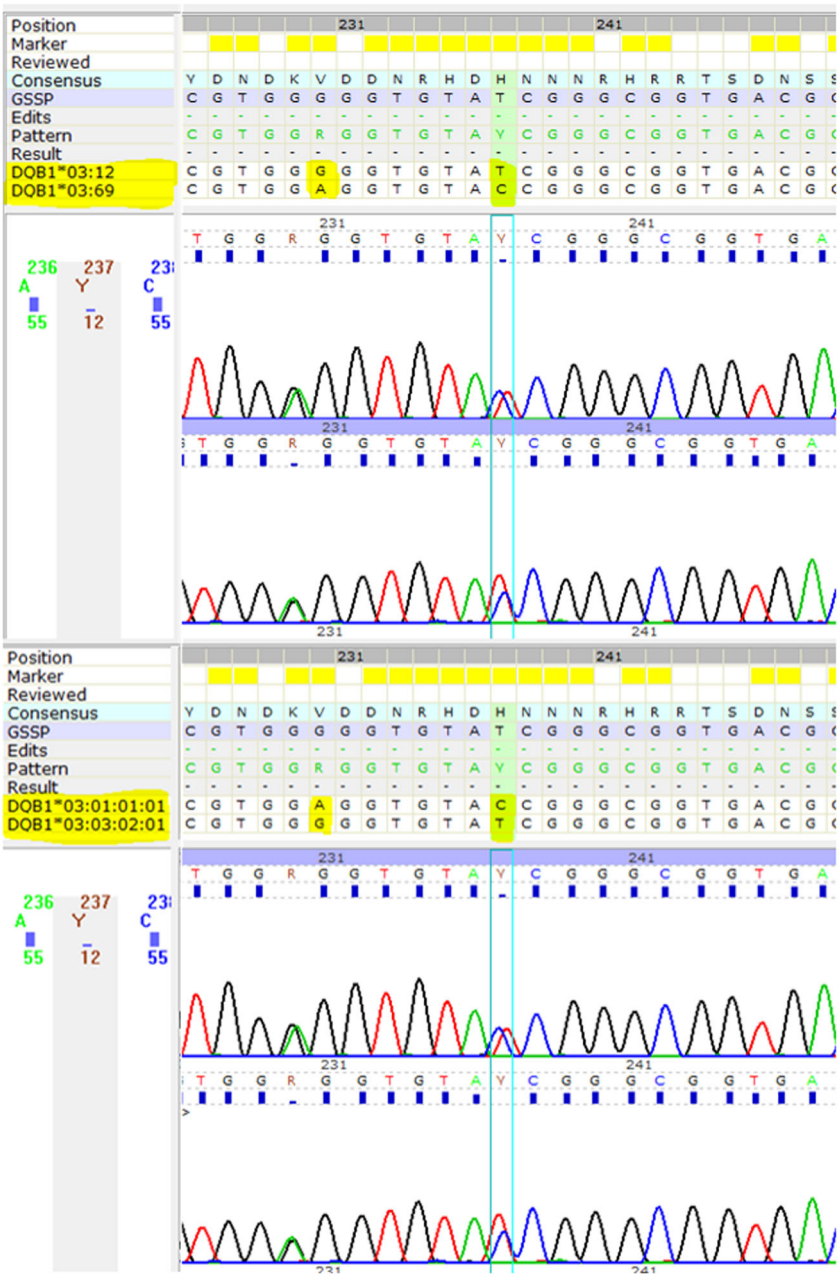

Fig. 2 Example of a phase ambiguity by Sanger sequencing. In this case, Sanger sequencing from location 228 through 239 gives a sequence of GG*R* GGT GTA *Y*CG, representing the combined sequences of the paternal and maternal alleles. The calls R and Y represent heterozygous calls of G and A (highlighted in yellow) and C and T (highlighted in green), respectively. Sanger sequencing cannot assign these heterozygous calls to individual strands. If the allele sequences are GG*G* GGT GTA *T*CG and GG*A* GGT GTA *C*CG, the correct alleles are DQB1*03:12 and DQB1*03:69. If the allele sequences are GG*A* GGT GTA *C*CG and GG*G* GGT GTA *T*CG, however, the correct typing is DQB1*03:01 and DQB1*03:03. Additional testing, for example, by allele specific primers, is needed to resolve this ambiguity. Next-generation sequencing methods do not have this pitfall. This image is courtesy of Brianna Bodo

required to resolve these ambiguities. Sanger sequencing has limited scalability and is typically used for a single sample at a time. Turn-around-times are long (Fig. 2).

Next-generation sequencing

NGS methods allow separate, parallel sequencing of multiple single strands of DNA. This allows base calls at heterozygous positions to be correctly assigned to the paternal or maternal allele, decreasing the phase ambiguity seen with Sanger sequencing. There are several different NGS platforms available for HLA typing that use different methods for sequencing detection. In all methods, the HLA genes are amplified and then fragmented into random segments, which are individually sequenced. Many sequences are generated from each allele present; however, each sequence is derived from a single strand. This allows for complete characterization of both alleles and accurate typing, often to the allele level.

NGS is well suited to typing all HLA loci across large numbers of samples simultaneously; however, testing and resulting can take several days. Instrumentation is expensive. Testing and analysis can be complex. Once NGS has been implemented, however, it is a highly reproducible, cost-effective, and efficient typing method. Consolidation of routine typing for all indications to NGS may streamline laboratory operations. Because it includes more gene regions than typically done by Sanger sequencing, NGS provides the highest possible resolution typing, potentially including third and fourth nomenclature field information, and is more likely to identify novel alleles [41].

Summary

The human leukocyte antigen system is a highly complex set of genes which govern the immune system and distinguish self from non-self. Typing of all or a subset of a patient's HLA genes can be used in several health care settings, including solid organ and bone marrow transplantation, transfusion support, to determine disease risk, and to guide treatment with certain medications. Due to the need to identify specific polymorphisms to identify alleles, HLA typing utilizes special methods and interpretation. Laboratories participating in transplantation testing typically perform other important assays such as HLA antibody screening and identification and pre-transplant donor/recipient crossmatching. HLA typing for non-transplant purposes such as disease association may be performed in a general molecular laboratory. In addition, other related testing such as stem cell engraftment monitoring may be done in either a transplant-focused or general molecular laboratory with appropriate accreditation.

Authors contributions Both authors contributed significantly to the writing and preparation of this manuscript.

Compliance with ethical standards

The manuscript is an original work of all authors. All authors made a significant contribution to this study. All authors have read and approved the final version of the manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

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