A Uniform Genomic Minor Histocompatibility Antigen Typing Methodology and Database Designed to Facilitate Clinical Applications

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Background. Minor Histocompatibility (H) antigen mismatches significantly influence the outcome of HLA-matched allogeneic stem cell transplantation. The molecular identification of human H antigens is increasing rapidly. In parallel, clinical application of minor H antigen typing has gained interest. So far, relevant and simple tools to analyze the minor H antigens in a quick and reliable way are lacking. Methodology and Findings. We developed a uniform PCR with sequence-specific primers (PCR-SSP) for 10 different autosomal minor H antigens and H-Y. This genomic minor H antigen typing methodology allows easy incorporation in the routine HLA typing procedures. DNA from previously typed EBV-LCL was used to validate the methodology. To facilitate easy interpretation for clinical purposes, a minor H database named dbMinor (http://www.lumc.nl/dbminor) was developed. Input of the minor H antigen typing results subsequently provides all relevant information for a given patient/donor pair and additional information on the putative graft-versus-host, graft-versus-tumor and host-versus-graft reactivities. Significance. A simple, uniform and rapid methodology was developed enabling determination of minor H antigen genotypes of all currently identified minor H antigens. A dbMinor database was developed to interpret the genomic typing for its potential clinical relevance. The combination of the minor H antigen genomic typing methodology with the online dbMinor database and applications facilitates the clinical application of minor H antigens anti-tumor targets after stem cell transplantation.

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INTRODUCTION

Minor Histocompatibility (H) antigens crucially affect the outcome of HLA-matched allogeneic stem cell transplantation (SCT) [1]. The influence of minor H antigen disparities between HLA identical SCT donor and recipient on graft-versus-host disease (GvHD), graft rejection, and on the graft-versus tumor (GvT) effect has been extensively reported. [2–4]. Recently, a new feature of minor H antigens was added comprising its potential to induce and maintain minor H antigen specific T regulator cells after an HLA identical but minor H antigen mismatched renal transplant [5]. The number of molecularly identified minor H antigens has significantly expanded recently, reaching a total of 10 autosomally encoded [6–14] and 10 Y-chromosome encoded antigens [15–25]. Genomic typing has been developed for some minor H antigens and implemented into the routine procedures of molecular HLA typing. The protocols used however vary and include: allele-specific PCR with sequence specific primers (PCR-SSP) [HA-1 [26], HA-2 [27], and HA-3 [12]], PCR-RFLP [HA-1 [28], HA-8 [9], and HB-1 [8]], gene specific PCR-SSP [UGT2B17 [11] and H-Y [29]], and the use of fluorogenic 3′-minor groove binding probes (ACC-1 [30]). The heterogeneity of these techniques as well as the lack of a molecular typing protocol for some of the molecularly identified minor H antigens prevent their clinical application. Additionally, the interpretation of minor H antigen typing data is relatively complex. Typically, a minor H antigen may differ with respect to tissue distribution and HLA restriction molecule. Some minor H antigen disparities are stimulating the beneficial GvT effect while others are involved in inducing GvHD [31]. Accordingly, pre-transplant information on minor H antigens may be of benefit to the patient. For example, typing of broadly expressed minor H antigen identifies those recipients who are at risk for GvHD. Information on minor H antigens with tissue distribution restricted to the hematopoietic system, e.g. HA-1, HA-2, HB-1, ACC-1, ACC-2, UGT2B17, PANEL1, and SP110 [8,10,11,13,14,32], is crucial for their potential use in the curative effect GvL of SCT. Likewise, minor H antigens with restricted tissue expression to solid tumors are of relevance to enhance the GvT effect after SCT [33,34].

Both the expansion of the genomic identification of minor H antigens and the growing interest in the application of minor H antigens as therapeutic tools require a simple and uniform allele specific PCR protocol, an up-to-date database, and tools that facilitate interpretation of the combined HLA and minor H antigen typing data. Here we report on the development of a uniform methodology for genotyping of all currently molecularly identified minor H antigens. Moreover, a minor H antigen database has been developed providing immediate information of the potential clinical relevance of the minor H antigen typing results for the relevant donor/recipient pair.

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MATERIALS AND METHODS

Development of a uniform genomic minor H antigen typing methodology

In total PCR-SSP for ten autosomally encoded minor H antigens and one for the Y-chromosome encoded H-Y minor H antigen were prepared. The design of the allele specific primers for PCR-SSP (Table 1) was based on the relevant minor H antigen genomic sequences and data from dbSNP [35]. Since typing for minor H antigens will generally be performed in HLA tissue typing laboratories, we developed the minor H antigen PCR protocol based on the most commonly used HLA typing protocols [36]. To establish specificity of the protocol [12,27,29]. The generated primers for the alleles of HA-3 and H-Y matched the proposed shortened by 2 nucleotides on the 5' end. The primers that were earlier described for HA-3 and H-Y matched the proposed primer sequences (shaded nucleotides). Single nucleotide polymorphisms (SNPs) are underlined. All sequences are depicted

Genomic DNA samples

EBV-LCL typed for HA-8, SP110, PANEL1, and UGT2B17 were provided by Dr. E. Warren, Seattle, USA. For ACC-1 and ACC-2, EBV-LCL from individuals from the CEPH (Centre d’Etude du Polymorphisme Humain) panel [10]. The CEPH cell lines have been used earlier by several researchers for identifying novel minor H antigens [10,30] and are generally available via the Coriell Cell Repositories (http://www.coriell.org; Coriell Institute for Medical Research, Camden, NJ). All cell lines were typed earlier by DNA sequencing, PCR-RFLP, PCR, CTL recognition, or by a combination of CTL recognition and genomic linkage analysis (Table 2). DNA of all samples was isolated using the Chemagic automated magnetic bead technology platform (Chromagen AG, Baeswiler, Germany) according to the recommendations of the manufacturer.

Genomic PCR

PCR mixes contained 80 ng genomic DNA, 0.25 U AmpliTaq (Perkin-Elmer, Norwalk, CT), 0.01% gelatin, 0.8 mM dNTP, 0.5 μM specific primers, 1.5 mM MgCl2, 50 mM KCl, 10 mM Tris HCl (pH 8.3), 6% sucrose, and 1 mM cresol red. To increase the yield of HA-1 and HB-1 products, primer concentrations were doubled. The PCR program started with 2 minutes at 94°C, followed by 10 cycles of 10 seconds at 94°C, and 60 seconds at 65°C. Subsequently, 20 cycles were run using the following conditions: 10 seconds at 94°C, 50 seconds at 61°C, and 50 seconds at 72°C. Samples were analyzed on a 2% agarose gel. Internal control primers for human platelet antigen (5’-ACCTAGATAGGTCGAGTCACCACGGGGATTTCC-3’ and 5’-AGGGCATATGTTCCACTTGCTT-3’), were designed. Nucleotide sequences of the primers are listed in Table 1. For HA-1, one 3’ nucleotide was removed from the previously published forward primer [37]. The reported 3’ primer sequences were introduced in the primer sequences (shaded nucleotides). The specificity of primers for HB-1 and ACC-1 was increased by introducing mismatches in the primer sequences (shaded nucleotides). Single nucleotide polymorphisms (SNPs) are underlined. All sequences are depicted

Table 1. PCR primers for allelic typing of minor H antigens.

| minor H antigen | allele | 5’ primer | 3’ primer | length (bp) |
|-----------------|--------|-----------|-----------|-------------|
| HA-1            | +/H    | CTTAGGAGTGTTGCTGCA | ACTCTCATACATCCTGCA | 190 |
|                 | -/R    | CTTAGGAGTGTTGCTGCA | ACTCTCATACATCCTGCA | 190 |
| HA-2            | +/V    | ACAGTCTCTGAAGTGGCTCA | GCTCTGGTAGGGTTCAC | 271 |
|                 | -/M    | ACAGTCTCTGAAGTGGCTCA | GCTCTGGTAGGGTTCAC | 271 |
| HA-3            | +/T    | CCTCAGAGAGACCTGTCAC | GTCATGAGCAGGCTTAC  | 129 |
|                 | -/M    | CCTCAGAGAGACCTGTCAC | GTCATGAGCAGGCTTAC  | 129 |
| HA-8            | +/R    | TGACTAGCAGACCTCACG | CTTTCGGCCAAAAGATTGGA  | 187 |
|                 | -/P    | TGACTAGCAGACCTCACG | CTTTCGGCCAAAAGATTGGA  | 187 |
| HB-1            | +/-    | ATCTTTTTCTATAGGCTGGCT | AAGGGCATATGTTCCACTGCTT  | 211 |
|                 | -/Y    | GCCATCTTCTATTAGTCCTGCT | AAGGGCATATGTTCCACTGCTT  | 214 |
| ACC-1           | +/-    | GTCTGCTCTAACAGTGCTCAC | GTCTGCTCTAACAGTGCTCAC | 137 |
|                 | -/C    | GTCTGCTCTAACAGTGCTCAC | GTCTGCTCTAACAGTGCTCAC | 137 |
| ACC-2           | +/-    | GATGGAAAAAGGAGTTTTGGAAGGCA | CAGCCTCCTCTTTGTCCTATC  | 197 |
|                 | -/G    | GATGGAAAAAGGAGTTTTGGAAGGCA | CAGCCTCCTCTTTGTCCTATC  | 197 |
| SP110           | +/-    | AATGTGTTTAGGACAAAGACATG | CCTGTACTCTCATCTACTCTATC  | 185 |
|                 | -/G    | AATGTGTTTAGGACAAAGACATG | CCTGTACTCTCATCTACTCTATC  | 185 |
| PANEL1          | +/-    | AGGGCAAGTCCCAACTG | AATGCGGTAATAGGCTGTGCTG | 207 |
|                 | -/Stop | AGGGCAAGTCCCAACTG | AATGCGGTAATAGGCTGTGCTG | 207 |
| UGT2B17         | +      | TGTGTGGAGAATATCTGACTAA | CCCCCTACTGACACACTATACTATGCTT | 353 |
| H-Y (SRY)       | +      | TGGCGATTAAGTCAAAATCGC | CCCCCTAGTACCCAGCAATGATTT | 136 |

Differences between the primer for the immunogenic allele and the non-immunogenic allele are indicated in bold. The specificity of primers for HB-1 and ACC-1 was increased by introducing mismatches in the primer sequences (shaded nucleotides). Single nucleotide polymorphisms (SNPs) are underlined. All sequences are depicted from 5’ to 3’. H-Y PCR-SSP was based upon the SRY gene sequence.

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amplify a product of 504 bp. Internal control primers for UGT2B17 had the sequence 5'-TGCCAAGTGGAGCACC-CAA-3' and 5'-GCATCTTGCTCTGTGCAGAT-3'. These primers yield a 761 bp product derived from the HLA-DR locus.

**Minor H antigen database and applications**

All currently available information on the molecularly identified minor H antigens has been imported into the minor H antigen database (dbMinor: http://www.lumc.nl/dbminor). Data were retrieved from the relevant literature obtained via PubMed (http://www.ncbi.nlm.nih.gov/entrez), from abstracts in proceedings, or has been forwarded to us. Polymorphisms that have not been functionally characterized as encoding T cell epitopes, as well as minor H antigens that have only been defined at the cellular level, are not included. A 'submission' page has been constructed for submitting data on minor H antigen identification directly to the database. Algorithms and queries were developed for searching the dbMinor, for screening the expression of the immunogenic minor H antigenic allele(s) in an HLA-typed individual, and for screening HLA-typed and minor H antigen-typed donor/recipient pairs for putative immune reactivities in the specified combination. The dbMinor database and all applications are available via a web-based interface.

**RESULTS**

**Analysis and validation of the uniform genomic minor H antigen typing methodology**

Allele specific PCR-SSP on genomic DNA was established for all minor H antigens and their alleles known to date. Figure 1 displays representative results of the genomic minor H antigen typing by PCR-SSP of 2 EBV-LCL. Clear bands in the correct positions indicate the presence of a minor H antigen allele. Internal control primers demonstrate a band of 439 bp for the immunogenic allele and 504 bp for the non-immunogenic allele. The presence of a minor H antigen allele generally results in a weaker control band. In the absence of the minor H antigen allele, control bands are clearly visible, thus demonstrating correct amplification conditions.

**Minor H antigen genotyping of the CEPH Panel of the 13th IHWS**

Upon development and validation of the uniform minor H antigen typing methodology, 22 cell lines from the 13th International Histocompatibility Workshop Symposium (IHWS) reference panel were typed. These cell lines are generally available and have been used before by several researchers to identify novel minor H antigens [10,38]. The results are depicted in Table 3. These data may serve as standard quality controls for clinical and research laboratories.

**Minor H antigen database development and applications**

The minor H antigen database (dbMinor) supports 4 applications, i.e. the minor H antigen database (I), the minor H antigen search based upon HLA typing (II), a full donor/recipient minor H antigen and HLA typing analysis (III), and a data submission.
section (IV). All 4 components of the database and the applications are mutually linked (Figure 2).

I. The minor H antigen database

The minor H antigen database allows data searches for basic information on individual minor H antigens. The information includes the references to the relevant articles, the amino-acid sequence of the peptide, and the HLA restriction molecule. If available, links show the related clinical studies, the genes and the protein data available at the NCBI. Likewise, detailed information on tissue distribution and mRNA expression are included.

II. The minor H antigen search based upon HLA typing

This searching method has been designed to list the

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**Table 3.** Minor H antigen genotyping of 11 parental CEPH cell lines from the 13th IHWS.

| HA-1 | HA-2 | HA-3 | HA-8 | HB-1 | ACC-1 | ACC-2 | SP110 | PANE1 | UGT2B17 | H-Y |
|------|------|------|------|------|-------|-------|-------|-------|---------|-----|
| HW1003 | HR | VV | MM | RP | HY | CC | GG | RG | R- | + | -- |
| HW1010 | HH | VV | MM | RR | HH | CC | GG | RG | R- | + | + |
| HW1029 | RR | VV | MM | RP | HY | CC | GG | RR | RR | + | + |
| HW1031 | RR | VV | TM | RP | HY | CC | GG | RG | RR | + | -- |
| HW1045 | HR | VV | TM | PP | HY | CC | GG | RG | R- | + | -- |
| HW1046 | RR | VV | TM | RP | HH | YC | DG | GG | R- | + | + |
| HW1053 | HR | VV | MM | RR | HH | CC | GG | RG | R- | + | -- |
| HW1056 | HH | VV | MM | RR | HH | CC | GG | RG | R- | + | + |
| HW1074 | RR | MM | TM | RR | HH | CC | GG | RG | RR | + | -- |
| HW1077 | HR | VV | TT | RP | HH | CC | GG | RG | R- | + | -- |
| HW1088 | RR | VM | TT | PP | HH | CC | GG | RG | RR | + | -- |
| HW1091 | RR | VV | TT | RP | HH | YC | DG | RG | R- | + | + |
| HW1103 | RR | VV | TT | RP | HH | YC | DG | GG | R- | + | + |
| HW1108 | HR | VV | TT | RP | HH | YC | DG | GG | R- | + | -- |
| HW1116 | HR | VV | TM | RP | HH | YC | DG | GG | R- | + | + |
| HW1117 | HH | VV | TM | RP | HH | CC | GG | RG | R- | + | -- |
| HW1124 | HR | VV | TM | RP | HH | CC | GG | RG | R- | + | + |
| HW1135 | RR | VM | TM | PP | HH | CC | GG | RG | R- | + | -- |
| HW1141 | RR | VV | TM | RP | HH | YC | DG | RG | R- | + | + |
| HW1143 | HR | VV | TT | RP | HH | CC | GG | RG | R- | + | -- |
| HW1161 | RR | VV | TT | PP | HH | CC | GG | RG | RR | + | + |
| HW1166 | HH | VV | MM | RP | HH | CC | GG | RG | RR | + | -- |

Typing is depicted as the amino acid resulting from the genomic sequences. The stop codon for PANE1 is indicated by a “-“.

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potential expression of minor H antigens in an individual based upon the individuals’ HLA typing. HLA typing has to be entered in a two-digit format. The results of the query give an indication whether minor H antigens may play a role in post-transplant immune reactivity and for which minor H antigens typing should be performed. In case HLA sub-alleles have been reported to be unable to express the minor H antigen of interest, it is specifically listed in the results.

III. Full donor/recipient minor H antigen and HLA typing analysis

The combined HLA and minor H antigens typing results of the donorrecipient pair under study subsequently provides information on putative minor H antigen reactivity. An automatic query has been designed to analyze these putative immune reactivities. Upon submission of the locally obtained minor H antigen typing results in combination with the 2-digit HLA typing data (Figure 3a) the algorithm filters all minor H antigens for the HLA restriction molecules expressed in the donorrecipient pair and provides the direction of the potential immune reactivity per individual minor H antigen (Figure 3b). In case the donor and recipient are matched for a relevant minor H antigen, a “±” symbol appears. In case of mismatches, the direction of the potential immune reactivity/reactivities is/are indicated with an arrow from recipient to donor in the case rejection and from donor to recipient in the case of GvHD/GvT reactivity. Since a link is created to the tissue distribution of each minor H antigen, clinically relevant information is subsequently provided. If HLA sub-alleles have been reported to be unable to express the minor H antigen of interest, this is specifically listed in the results.

IV. Data submission section

New minor H antigen data can be submitted directly to the dbMinor (http://www.lumc.nl/dbminor/submit.html). Submission of information includes local minor H antigen designation, amino acid sequence of the peptide of the immunogenic allele and, if relevant, its allelic counterpart, the HLA restriction molecule, phenotype frequency of the minor H antigen, name of the gene encoding the T cell epitope as assigned by the HUGO Gene Nomenclature Committee, links to Entrez Gene and Homologene, tissue distribution on the cellular and/or RNA level. Optional data that can be submitted are: reference(s) to relevant publications and to clinical studies. If requested, peptide sequence data and protein/gene information can be treated as confidential until publication. The submissions will be generally screened on correctness and made public via the database after final permission of the submitting author. The responsibility of the accuracy of the submitted data remains with the submitting author(s).

DISCUSSION

Routine typing for minor H antigens of stem cell and solid organ transplant donor and recipient is a prerequisite for their potential clinical use and provides insights in the minor H antigen alloimmune reactivities [39]. A universal and simple methodology enabling genomic minor H antigen typing was, as yet, non-existing. We here describe a uniform protocol for the genomic typing of the currently molecularly identified minor H antigens. Our basic requirements for the development of the method were: 1) easy implementation in routine HLA typing laboratories, 2) high reliability, and 3) optimal reproducibility. To further facilitate implementation in routine HLA tissue typing, PCR primers were designed to run on the most common temperature profile for HLA typing [36]. Choosing separate temperature profiles for each individual minor H antigen might increase the yield of the PCR product. Particularly the PCR-SSP for HA-1 and HB-1 may benefit from such an adaptation. This however also implicates that screening of a single DNA sample would require different PCR runs. Since the yield of HA-1 and HB-1 PCR products is sufficiently high for reliable detection, a single methodology approach is preferred over the optimal amplification. To check its reproducibility, all experiments in this study were performed at least twice, yielding identical results in all experiments (data not shown). Subsequent typing of previously otherwise typed DNA materials demonstrated perfect matching with earlier results (Table 2). Once established, 22 parental CEPH reference cell lines were minor H antigen typed with the developed methodology (Table 3). These typing results can function as reference for the laboratories implementing the minor H antigen typing methodology.

Today, information on minor H antigen typing may have relevant spin-offs in the area of stem cell and renal transplantation. Regarding the former, typing of donorrecipient pairs prior to SCT provides information on two crucial GvH reactivities; i.e.; GvHD and GvT. Broadly expressed minor H antigens are prone to causing GvHD. Pre-SCT information on the disparities for the broad minor H antigens is relevant enabling early anticipation on putative development of GvHD. Minor H antigens with tissue distribution restricted to the hematopoietic system play a role in the graft-versus-leukemia (GvL) effect post-SCT [40]. Typical examples of the latter group are; HA-1, HA-2, HB-1, ACC-1, ACC-2, PANE1 and SP110 [8,10,13,14,32]. Disparities for these minor H antigens in the GvH direction facilitate the GvL effects of SCT. These GvL effects can be boosted by adoptive immunotherapy using hematopoietic system specific minor H peptides; similarly in the case of SCT for solid tumors the GvT effect can be boosted using minor H peptides expressed on the carcinoma cells but absent on their healthy epithelial counterparts [40]. Beside the development of a uniform genomic typing method for minor H antigens, quick and easy interpretation of the potential clinical consequences of the minor H antigen typing.

Figure 2. Structure of the minor H antigen database (dbMinor). Data from dbMinor are obtained via direct submission and from literature screening. The data in dbMinor can be analyzed via an HLA-based query, a full typing query and a minor H antigen query. Results from these queries are linked to external data from the NCBI database. doi:10.1371/journal.pone.0000042.g002
Figure 3. Analysis of donor/recipient HLA and minor H antigen typing.
A. Query section: Minor H antigen typing data can be entered by marking the checkboxes per allele per minor H antigen. HLA typing data has the 2-digit format. Full typing for all HLA loci is not required. After submitting the query, a page with results will appear.
B. Results: The results section lists minor H antigens relevant in the particular donor-recipient combination based upon the HLA typing. In case donor and recipient are minor H antigen matched, as is the case here for HA-1, the sign “=“ will appear between donor and recipient. In case recipient and donor are minor H antigen mismatched, the disparities are indicated by an arrow. The direction of the arrow indicates the direction of the immune responses. In this example immune reactivity can occur in the recipient-to-donor (host-versus-graft, rejection) direction for HA-2 and HA-3 and in the donor-to-recipient (graft-versus-host) direction for HA-8 and for the various H-Y antigens. The tissue distribution of each relevant minor H antigen is listed as “broad” or “restricted”. By clicking these terms, a detailed list of target cells expressing the minor H antigen will be displayed.
Minor H antigens are presented by selected HLA molecules. The HLA alleles expressed by recipient and donor that are not able to present a minor H antigen are listed at the bottom of the results section. Information will appear in case a particular minor H antigen cannot be presented by a specific HLA sub-allele (4-digit format; in this example HLA- DQB1*0503/0504 for DQ5/H-Y).

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results are desired. Interpretation of the typing results in relation to GvH reactivities in SCT is rather complex; the recipient requires to be homozygous or heterozygous for the immunogenic minor H allele, while the donor should be homozygous for the non-immunogenic allele. Minor H antigens are peptides encoded by polymorphic proteins. In general, only one polymorphic allele of the protein is immunogenic. Some exceptions are reported, e.g. the HLA-B44 restricted HB-1 and the HLA-B60 restricted HA-1 can be immunogenic bidirectionally [41,42]. Furthermore, minor H antigens are presented in the context of a certain HLA allele. Moreover, as discussed earlier, minor H antigens have different and distinct tissue expression patterns resulting in differential GvH reactivities. Thus, the complex combination of these various characteristics hampers rapid interpretation of the minor H antigen typing results. We therefore developed algorithms to facilitate the analysis of the minor H antigen typing results. This novel algorithm supports in interpreting the typing data with regard to putative minor H antigen specific allo-immune responses and in selecting the correct minor H antigens for adoptive immunotherapy in the relevant donor/recipient pairs.

In all algorithms implemented in dbMinor, the two-digit HLA typing format is used instead of the high resolution 4-digit format. The rationale behind this strategy is lack of complete data regarding this issue for most minor H antigens. For some minor H antigens sub-alleles have been reported that are unable to present the minor H antigen of interest. For example the HLA-DQ5/H-Y minor H antigen can be presented by individuals who carry either the HLA-DQB1*0501 or the HLA-DQB1*0502 allele. However, the HLA-DQB1*0503 and HLA-DQB1*0504 alleles are unable to present this particular H-Y peptide (Laurin et al. in press). If such exceptions have been reported, they are specifically listed. As soon as more information on this issue becomes available for other minor H antigens, 4-digit-based queries will be implemented.

In summary, we here describe a universally applicable genomic PCR-based minor H antigen typing protocol. It offers a simple, uniform, and rapid methodology to determine minor H antigen genotypes of all currently molecularly identified minor H antigens. The methodology can be combined with the online applications for the interpretation of minor H antigen typing results identifying donor/recipient pairs eligible for adoptive immunotherapy and patients with increased risk for developing GvHD. The minor H antigen genomic methodology combined with the online applications will further facilitate the clinical application of minor H antigens in transplantation.

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Author Contributions
Conceived and designed the experiments: EG ES JD. Performed the experiments: ES JD MH JP MS. Analyzed the data: EG ES FC JD JP MS. Contributed reagents/materials/analysis tools: ES JD MH JP MS. Wrote the paper: EG ES.

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