# Identification of minor histocompatibility antigens based on the 1000 Genomes Project

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## ABSTRACT

Minor histocompatibility antigens are highly immunogeneic polymorphic peptides playing crucial roles in the clinical outcome of HLA-identical allogeneic stem cell transplantation. Although the introduction of genome-wide association-based strategies significantly has accelerated the identification of minor histocompatibility antigens over the past years, more efficient, rapid and robust identification techniques are required for a better understanding of the immunobiology of minor histocompatibility antigens and for their optimal clinical application in the treatment of hematologic malignancies. To develop a strategy that can overcome the drawbacks of all earlier strategies, we now integrated our previously developed genetic correlation analysis methodology with the comprehensive genomic databases from the 1000 Genomes Project. We show that the data set of the 1000 Genomes Project is suitable to identify all of the previously known minor histocompatibility antigens. Moreover, we demonstrate the power of this novel approach by the identification of the new HLA-DP4 restricted minor histocompatibility antigen UTDP4-1, which despite extensive efforts could not be identified using any of the previously developed biochemical, molecular biological or genetic strategies. The 1000 Genomes Project-based identification of minor histocompatibility antigens thus represents a very convenient and robust method for the identification of new targets for cancer therapy after allogeneic stem cell transplantation.

## Introduction

For several hematologic malignancies, allogeneic stem cell transplantation (allo-SCT) from an HLA-identical donor, alone or followed by donor lymphocyte infusion (DLI) is a potentially curative immunotherapeutic option.[1-4] Besides the rescue of the recipient's hematopoietic system, the infused graft can mediate a powerful graft-*versus*-tumor (GvT) reaction to induce long-term remissions. Despite this curative potential, allo-SCT is currently not recommended as the first-line treatment for several hematologic malignancies because it frequently causes a detrimental graft-*versus*-host-disease (GvHD). In an HLA-identical setting, both GvT and GvHD are predominantly driven by donor T cells, which are directed at allogeneic peptides presented on the HLA molecules of the recipient.[5-10] These highly immunogenic, non-HLA encoded allogeneic peptides are historically, perhaps mistakenly, designated as minor histocompatibility antigens (mHags). To date, after the identification of almost 50 autosomal mHags (*Online Supplementary Table S1*), it is evident that mHags are generated by intracellular degradation of polymorphic proteins. The genetic bases of these polymorphisms are either single nucleotide polymorphisms (SNPs), base-pair insertions or deletions (indels) or copy number variations (CNVs).[11] In the mid-1990s, the discovery that a specific set of mHags is solely expressed on malignant and non-malignant hematopoietic cells, shifted the paradigm on mHags and suggested that hematopoietic-restricted mHags could be ideal targets for separating GvT from GvHD.[1,12,13] Nonetheless, a thorough clinical evaluation of this attractive concept has not yet been realized, because the small number of genuinely hematopoietic system-specific mHags identified so far permits the inclusion of not even 20% of the transplant patients in the slow-running clinical trials. Thus, a timely clinical evaluation of mHag-based immunotherapy is highly dependent on the development of more rapid and robust strategies for identification of mHags.

The classical strategy to identify mHags is via the characterization of the epitope recognized by mHag-specific T-cell clones isolated from allo-SCT recipients. The first series of mHags were identified using demanding methods, such as peptide elution from HLA-molecules or screening of cDNA libraries derived from mHag-positive cells.[14-18] After the discovery that mHags are products of polymorphic genes, these techniques were replaced by genetic strategies that made use of the correlation of mHag phenotypes with large numbers of genetic variations, derived from databases such as the HapMap Project or self-assembled panels.[12,19-27] Although these genetic techniques proved to be successful and revealed the identity of more than 15 mHags, they all are still limited by the fact that the databases used do not include all existing genetic variations and variation types (i.e. indels and CNVs).

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Recently, the so-called 1000 Genomes Project created a nearly complete map of more than 40 million variations in 14 human populations by full-genome deep sequencing of 1092 individuals, including SNPs, indels and CNVs.[28] In order to overcome the drawbacks of all previous methods, we have now set out to develop a new genetic approach using the databases of the 1000 Genomes Project. Similar to earlier approaches, this new method is based on the functional mHag phenotyping of individuals by testing their antigen-presenting cells for recognition by mHag-specific T cells. Subsequently, these phenotypes are correlated to all the genotypic variations included in the 1000 Genomes Project.

We here demonstrate the power of this new strategy by the rapid and unambiguous identification of the HLA-DP4 restricted mHag UTDP4-1, which, despite the great efforts made over the past 15 years, could not be identified with any of the previously developed strategies, including our previous HapMap-based strategy.

## Methods

### Cells

The CD4$^+$ mHag-specific cytotoxic T-cell (CTL) clone 3AB11 was generated as described previously.[29] In short, T cells were isolated from a multiple myeloma patient after allo-SCT. Several T-cell clones were generated of which 3AB11 displayed patient-specific cytotoxicity and was selected for additional experiments. T cells were cultured in RPMI 1640 supplemented with 10% human serum and standard antibiotics.

Epstein-Barr virus-transformed B cells (EBV-LCL) from an SCT-recipient, an SCT-donor and individuals from Caucasian and Mexican populations in the HapMap database (CEU and MXL) were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Integro, Zaandam, the Netherlands) and antibiotics. Written informed consent had previously been obtained from the patient and the donor. This study was approved by our local medical ethics committee.

### Peptides

Commercially synthesized and purified peptides (Peptide 2.0, Chantilly, VA, USA, and Pepscan, Lelystad, the Netherlands) and peptides kindly synthesized by Dr. H. Ovaa (The Netherlands Cancer Institute, Amsterdam, the Netherlands) were dissolved in DMSO and diluted in PBS or RPMI for use in functional assays.

### Flow cytometry

Cells labeled with fluorochrome-conjugated antibodies (BD and Beckman Coulter) were analyzed with a FACS Calibur flow cytometer (BD). Acquired data were analyzed with CellQuest software (BD).

### Enzyme-linked immunosorbent assay (ELISA)

The interferon gamma (IFNγ) content of cell-free supernatants was determined using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Sanquin, Pelipair) according to the manufacturer's instructions. At least duplicates were measured for all samples.

### Phenotyping HapMap individuals

The mHag phenotypes of the individuals from HapMap trios (father-mother-child) were determined with IFNγ ELISA using their EBV-LCLs as antigen-presenting cells for CTL clone 3AB11. If necessary, the EBV-LCLs were first retrovirally transduced with HLA-DPB1*0401, as described elsewhere.[30] EBV-LCLs were considered mHag+ if the mean absorbance value at 450 nm of triplicate tests was more than 0.300 (3-5 times the background absorbance value of EBV-LCLs or T cells). They were considered mHag- if the mean absorbance value at 450 nm of triplicate tests was less than 0.100 (comparable to background levels). Results between 0.100 and 0.300 were repeated or not used for analysis.

### Genome-wide zygosity-genotype correlation analysis

The correlation of experimental data with HapMap SNP genotypes was performed and analyzed as previously described.[12,24,25] In short, after mHag phenotypes had been determined, mHag zygosities of these individuals were deduced using the inheritance pattern within the family trios. These zygosities were correlated to the approximately 4 million SNPs included in the HapMap database (release #24), using ssSNPer software.[25]

### 1000 Genomes Project correlation analysis

The correlation between mHag phenotypes of individuals included in the 1000 Genomes Project and the more than 40 million variations of these individuals was analyzed using the free software environment R, v.2.15.2. Genotype files from the 1000 Genomes Project were downloaded from their ftp-server (*ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/*) and modified to allow analysis on a regular PC. The R-script runs a Fisher's Exact test for the mHag phenotypes versus the genotypes of every single SNP within the 1000 Genomes Project database. The script and the modified genotype files can be down-loaded at www.sourceforge.net. A single genome-wide run takes 20-30 h.

**Table 1.** Comparison of the genetic strategies used for mHag identification.

| | Classical pair-wise linkage analysis | Genome-wide association analysis – HapMap | Genome-wide association analysis – custom panel | 1000 Genomes Project-based analysis |
|---|---|---|---|---|
| Resource genetic data | CEPH reference family collections | HapMap database | SNP-arrays on a panel of individuals | 1000 Genomes Project database |
| Variation types | SNPs, microsatellite markers | SNPs | SNPs | SNPs, indels, CNVs |
| Variation numbers | 32,000 | ~4 million | ~1 million | ~40 million |
| Result | Large genetic locus | TagSNP(s) or encoding variation | Tag SNP(s) or encoding variation | Encoding genetic variation |

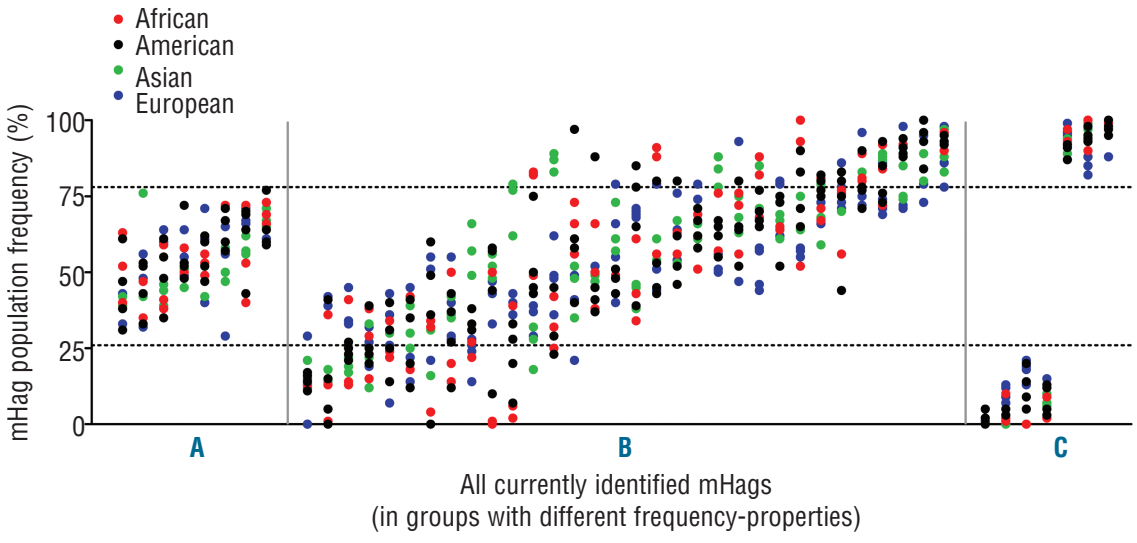


**Figure 1. Variation in distribution of mHag phenotypes between ethnic populations. The mHag-encoding basepairs and the corresponding variation numbers were derived from the original researchpapers (*Online Supplementary Table S1*). Based on this information the genotypes of all 1092 individuals were extracted from the 1000 Genomes Project datasets. Based on these data, the phenotype frequencies were calculated for each ethnic population separately. These frequencies, color-sorted by continent and divided in three groups. (A) the phenotype frequencies of all populations are between 26-78% (dashed lines), (B) the phenotype frequencies of several but not all populations are between 26-78%, and (C) none of the phenotype frequencies of the various populations are between 26-78%. *Online Supplementary Table S1* provides the exact values for each currently known mHag in each population in the same order.**

## Results

### *Implementation of the 1000 Genomes Project in mHag-specific GWAS*

For the new genetic approach, 1000 Genomes Project genotype files were down-loaded and used as input for an R-script specifically developed to determine the association between the mHag phenotypes and the genotypes of every single genetic variation within the 1000 Genomes Project database. Since several of the 1092 individuals present in the 1000 Genomes Project were also included in the HapMap project, we first validated the new strategy by re-identification of the mHag UTA2-1, which we had previously identified using the HapMap-based zygosity-phenotype analysis (*data not shown*).[24] Next, we confirmed that the genetic variations encoding for all known mHags are included in the 1000 Genomes Project, implying that all of them could have been identified using this new method (*Online Supplementary Table S1*).

### *Most previously identified mHags have a clinically relevant frequency in different ethnic populations*

The 1000 Genomes Project database contains genetic information on individuals from 14 ethnic populations from various continents. This comprehensive resource not only facilitates the identification of mHags, but it also enabled us to analyze various important characteristics of previously identified mHags. Most importantly, for each currently known mHag, we determined its frequency in different ethnic populations, revealing that the phenotype frequencies of all mHags display significant variation from population to population. This, first of all, has an impact on the mHag identification strategy. For instance, if too-high or too-low phenotype frequencies are observed in the primary test population, it will be beneficial to extend the correlation analysis to other populations (as exemplified by group B in Figure 1 and *Online Supplementary Table S1*).

Furthermore, this analysis revealed that the frequency of only eight mHags was between the 26-78% boundaries in all populations (Figure 1 and *Online Supplementary Table S1, group A*). We have previously reported that this boundary defines the clinical applicability of an mHag, because for mHags with a frequency beyond these boundaries, the odds for an mHag mismatch between donor and patient are very small.[12] Hence, only eight "universally important" therapeutic mHags have been identified so far. On the other hand, however, we also found that there are only seven "universally non-relevant" mHags with a frequency beyond the 26-78% boundaries in all populations (Figure 1 and *Online Supplementary Table S1, group C*). Thus, the majority of mHags that have been identified up till now are clinically relevant in at least one ethnic population.

### *Identification of the new HLA class II restricted mHag UTDP4-1 with the 1000 Genomes Project-based approach*

After establishing that this new strategy had sufficient power to identify all previously discovered mHags, we evaluated its value in identifying a new mHag. For this, we chose the HLA-DP*0401-restricted mHag recognized by the $CD4^+$ T-cell clone 3AB11, because we could not unravel its target peptide, despite our great efforts over the past 15 years. Notably, our earlier attempts including a cDNA-screening and pair-wise genetic linkage studies had failed (*data not shown*). Recently, using our genome-wide zygosity-genotype correlation analysis, based on family trios of the HapMap Project,[12] we mapped this mHag with a low confidence $r^2$=0.68 to a genetic locus on chromosome 9 defined by rs12551834 (Figure 2A). Still we could not iden-

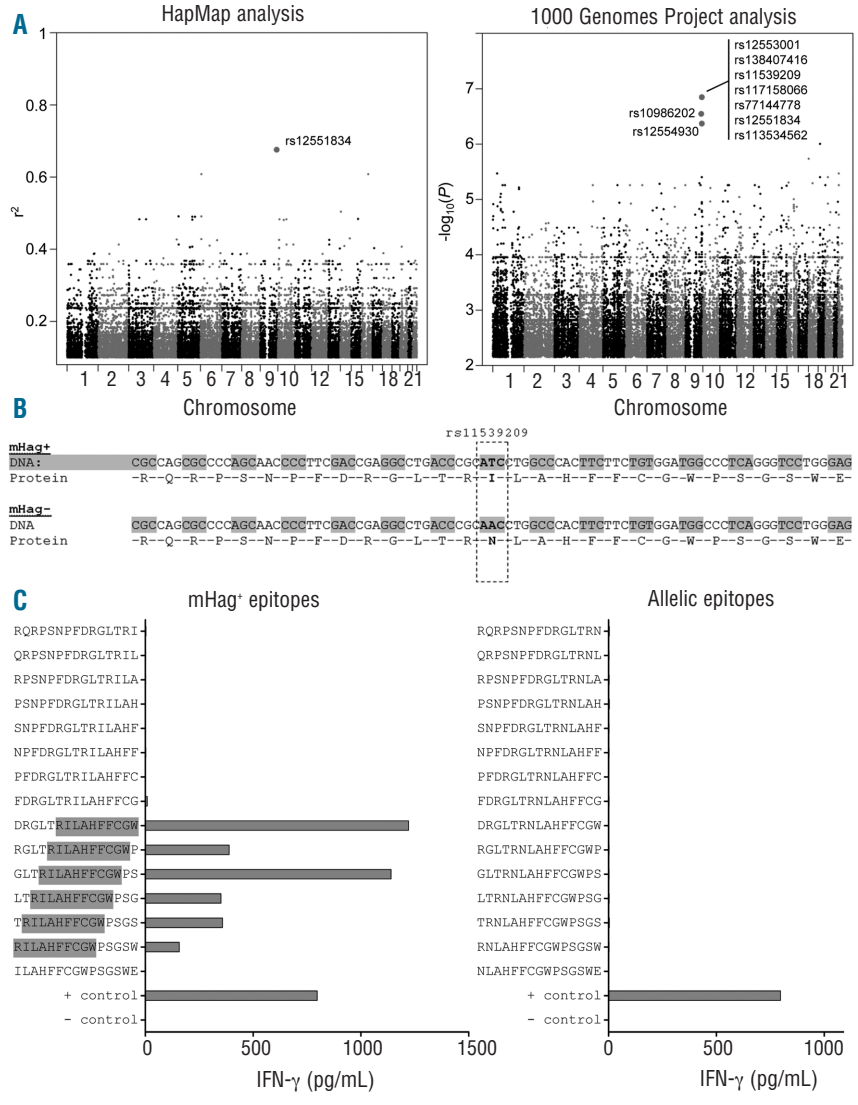


**Figure 2. Successful identification of mHag UTDP4-1 with 1000 Genomes Project-based GWAS. (A) Manhattan plots showing the genome-wide correlation values ($r^2$) from the HapMap correlation analyses (left panel) and association *P*-values from the 1000 Genomes Project analyses (right panel; the minus logarithm of the *P*-value of each SNP is plotted). Only SNPs with an $r^2 > 0.05$ or $P<0.01$ are shown and ordered by chromosomal position on the x-axis. The SNPs with the highest $r^2$-value and the lowest *P*-value are emphasized by larger red dots. (B) SNP rs11539209 was identified as the polymorphism responsible for the presence of this mHag. This genetic locus was found on gene ZDHCC12 encoding the epitope recognized by CTL clone 3AB11. Here both the nucleotide (chromosome 9, position 131.483.594-131.483.508) and amino acid sequences of the immunogenic and allelic variants are shown. (C) Epitope screening of the overlapping mHag+ (left graph) and allelic (right graph) 15-mer peptides, containing polymorphic amino acids I or N respectively, at shifting positions, reveals recognition of a number of the mHag-positive 15-mer epitopes with the immunogenic amino acid I at positions 2 to 7. The minimally required sequence is, therefore, identified as RILAHFFCGW. The peptides were loaded onto an mHag-negative EBV-LCL in a concentration of 100 nM and after overnight incubation screened for recognition by CTL 3AB11 in an IFNγ ELISA experiment.**

tify it, even after an extensive analysis of all missense SNPs in a large genetic region (0.5 Mbp) around rs12551834. For both genetic analyses together, we phenotyped 146 individuals in total but were not able to pin point the mHag encoding variation. Since 43 of these are included in the 1000 Genomes Project, we executed a new genetic analysis, this time correlating the mHag phenotypes with the comprehensive dataset of the 1000 Genomes Project. Remarkably, and consistent with our previous findings, we found a very strong correlation, with significant *P*-values of around $10^{-7}$, between the mHag phenotype and the locus on chromosome 9 (Figure 2A). In contrast to our earlier analyses, however, the locus was now defined by nine genetic variations of which seven were 100% correlated with the phenotype data of the 43 individuals. Retrospective analyses revealed that these variations were not included in the databases of the HapMap Project and are located just 9600 basepairs outside of the large region previously analyzed based on the HapMap Project correlation analysis. Interestingly, according to the current gene annotations of Ensembl, rs11539209 is the only one of these seven polymorphisms encoding for a missense variation, suggesting that this SNP could be responsible for our long-sought mHag.

### *The newly identified mHag UTDP4-1 is encoded by ZDHHC12*

SNP rs11539209 gives rise to an A-to-T nucleotide switch, leading to an amino acid substitution from asparagine (N) to isoleucine (I) in ZDHHC12 (zinc finger, DHHC-type containing 12) (Figure 2B). By testing the recognition of a 29-mer peptide with the altered amino acid in the middle, covering all possible 15-meric or under T-cell epitopes, we confirmed that this SNP indeed encoded the mHag targeted by clone 3AB11 (*data not shown*). Subsequently, all 15-mer peptides containing the isoleucine at shifting positions were screened for recognition by 3AB11 and several peptides indeed stimulated IFNγ production (Figure 2C). We designated this new mHag UTDP4-1. The minimal amino acid sequence required for a positive response was RILAHFFCGW. Also, the allelic counterparts containing the asparagine amino

acid were screened, but none of these peptides was recognized by clone 3AB11.

## Discussion

In this study, we provide proof of the feasibility, accuracy and efficacy of a new strategy for the identification of mHags based on the comprehensive genomic variation data collected by the 1000 Genomes Project. Our study design and results are in concordance with the fundamental idea of the 1000 Genomes Project, which was initiated to provide a complete overview of all human sequence variations, and widen understanding of the relationship between genotype and phenotype.[28] Importantly, the successful and rapid identification of mHag UTDP4-1 illustrates that this new 1000 Genomes Project-based strategy can overcome many of the drawbacks encountered so far in the identification of mHags.

Among the many mHag identification strategies developed so far, those non-genetic strategies were the most cumbersome. In fact, these methods were originally developed for the identification of HLA class I restricted T-cell epitopes and did not take genetic polymorphisms into account. Since their introduction, the superiority of genetic approaches in identification of mHags was clearly demonstrated by the identification of several mHags within a fraction of the time required to identify the first series of classically characterized mHags. The main advantage of genetic methods over the classical T-cell epitope identification strategies is that they make specific use of the fact that mHags are immunogenic reflections of genetic polymorphisms. For instance, the earlier "pair-wise genetic linkage analysis", which was successful in the identification of mHags LRH-1 and ACC1/2, is based on correlation of mHag phenotypes of individuals of the CEPH reference family collections with genomic markers (Table 1).[19,20] Since this method could not identify the precise locus of several mHags, we moved on to implement genome-wide association studies (GWAS) to identify mHags. By correlation of the mHag phenotypes of father-mother-child trios of CEPH pedigrees included in this project with the approximately $4x10^6$ SNPs included in the HapMap databases, we rapidly identified the mHags CD19L, SLC19A1R and UTA2-1.[12,24,25] At the same time, other investigators developed similar approaches, either based on the HapMap databases or using mHag-phenotyped custom panels of EBV-LCLs, which they genotyped for up to one million SNPs (Table 1).[23,26,31]

Nonetheless, even the most comprehensive approach could not facilitate the identification of all mHags as, for example, the HapMap database did not contain the SNP encoding mHag UTDP4-1. The extensive analysis of a large adjacent region of SNP rs12551834 based on a rather weak correlation revealed by the HapMap approach was in this case not sufficient to identify the true mHag-encoding SNP. Now, knowing the exact location of the SNP encoding the mHag UTDP4-1, we worked out that it is located just outside of the arbitrary region of 0.5 Mbp that we had thoroughly analyzed. These findings clearly indicate that the incompleteness of the SNP dataset in HapMap was the main reason for our failure, as this was probably also the case for the failure of other investigators to identify mHags using dedicated databases (*Oostvogels et al. and M Griffioen, personal communication, 2014*). Thus, a great advantage of exploiting the 1000 Genomes Project is the thoroughness of the database in which approximately 99.5% of the variations with a 5% frequency are included.[28] Furthermore, the option to use various ethnic populations (which according to our analyses may display even totally different mHag prevalences) increases the likelihood for identification.

Another potentially important advantage of using 1000 Genomes Project over the other applied variation databases is that it contains not only SNPs but also CNVs and indels. The determination, or at least estimation, of the frequency of mHags arising as a consequence of indels and/or CNVs is not only of practical value, but can significantly improve our understanding of the fundamental requirements for the generation of mHags. The allelic counterparts of mHags generated as a consequence of indels or CNVs will never be expressed at all, whereas the allelic variants of SNPs may lead to the presentation of peptides with a single amino acid difference. Since T-cell receptors are frequently unable to discriminate a single amino acid difference, the expression of both allelic peptides in the context of HLA may frequently lack immunogenic potential, due to negative selection of the peptide-specific T cells in the thymus. In fact, differential cell surface presentation of polymorphic peptides seems to be a key requirement for mHag generation because allelic counterparts of several important mHags, like HA-1, HA-2 and HA-3, are not expressed on the cell surface, and of all autosomal mHags identified so far, only 2 are known to be bilaterally immunogenic.[16,19] Previously we reported that identification of mHags beyond 10-85% prevalence would not be useful, since such mHags will make little contribution to the broad application of mHag-based therapies.[12] While this idea is still valid, the analysis of the diverse populations in the 1000 Genomes Project revealed that mHags with seemingly unfavorable frequencies in a certain population may still be broadly applicable in other ethnic populations. Ultimately, this improved knowledge of mHags may lead to superior identification strategies through the design of algorithms directed towards the computational prediction of true immunotherapeutic mHags.[11,32,33]

In conclusion, the new approach integrating all variations of the 1000 Genomes Project currently represents the most attractive method for highly efficient mHag identification. This methodology will conceivably facilitate the identification of the broad set of mHags that are not only necessary for the optimal setup of mHag-based immunotherapy trials, but also for a better understanding of mHag immunobiology.

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