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Multiplex Genotyping of Human Minor Histocompatibility Antigens

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ABSTRACT: Minor histocompatibility antigens (mHAg) induce major histocompatibility complex–restricted, T cell–mediated immune responses that may contribute to increased risk of graft-versus-host disease and graft-versus-leukemia effects. Unlike human leukocyte antigen genes, mHAg are encoded by genetically and functionally unrelated genes located throughout the chromosome. The role of mHAg in stem cell transplantation and the population frequencies of mHAg alleles remain unknown due in part to the lack of suitable high throughput methods for genotyping these diverse genes. Here we describe the development and utility of a multiplexed Luminex assay for genotyping human mHAg, including HA-1, HA-2, HA-3, HA-8, HB-1, $CD31^{125}$, and $CD31^{563}$. The assay uses a multiplexed, allele-independent, gated amplification of mHAg genes followed by differential detection of allele-specific primer extension products using the MultiCode PL*x* system (EraGen Biosciences, Madison, WI). The alleles are interrogated using a multiplex allele-specific primer extension reaction using primers tagged with EraCodes. The products are hybridized to Luminex beads and the hybridization duplexes are detected using streptavidin-phycoerythrin. The assay resolved the mHAg genotypes of 259 Caucasian donors and provided population estimates of mHAg gene and phenotypic frequencies. All mHAg alleles evaluated in this study exhibited Hardy-Weinberg equilibrium, although some mHAg phenotypes were present in large majority of individuals tested (HA-2, HB-1). This assay will provide a valuable tool for determining mHAg frequencies in other ethnic populations, as well as for establishing the clinical importance of mHAg disparities in stem cell transplantation. *Human Immunology 66, 1174–1182 (2005).* © American Society for Histocompatibility and Immunogenetics, 2005. Published by Elsevier Inc.

KEYWORDS: Human minor histocompatibility antigen; genotyping; gene frequency

ABBREVIATIONS

GVHD	graft-versus-host disease
HLA	human leukocyte antigen
MFI	median fluorescence intensity
MMR	mean MFI ratio
mHAg	minor histocompatibility antigen(s)
PCR	polymerase chain reaction
SA-PE	streptavidin-phycoerythrin
SNP	single nucleotide polymorphism

INTRODUCTION

Minor histocompatibility antigens (mHAg) are immunogenic peptides derived from non–human leukocyte antigen (HLA) encoded gene products that can induce specific T-cell responses in individuals lacking the antigen [1–4]. Most mHAg represent allelic forms of normal proteins that arise due to single nucleotide polymorphisms (SNPs), although differential expression may also occur as a result of gene deletion [5]. A single amino acid change is sufficient to confer immunogenicity of a peptide when that peptide is presented in the context of class I or II HLA molecules to T cells from individuals who possess the alternate form of the protein. Much of the clinical interest in mHAg derives from their possible contribution to graft-versus-host disease (GVHD) and graft-versus-leukemia effects following bone marrow transplantation [6–12]. It is well established that transplants between related recipient donor pairs are associated with lower rates of GVHD and higher rates of engraftment compared to recipients of HLA-allele matched unrelated donors, yet HLA-identical sibling transplants still exhibit GVHD at rates of 20–50% [13]. These findings support a role for mHAg in bone marrow transplant outcomes.

Fewer than 20 human mHAg have been identified, and little is known about their population gene frequen-

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Received June 15, 2005; accepted August 31, 2005.

0198-8859/05/$–see front matter
doi:10.1016/j.humimm.2005.08.243

cies and their influence on bone marrow transplant outcomes [4, 14–16]. Clinical studies of mHAg disparities between donor and recipient transplant pairs have been restricted to evaluations of single mHAg or limited patient numbers, due in part to the lack of a suitable high throughout method for genotyping multiple mHAg.

We have developed a multiplex genotyping assay that allows multiple mHAg allele SNPs to be simultaneously genotyped in a single reaction vessel. The assay incorporates novel synthetic DNA bases (IsoBases, EraGen Biosciences, Inc., Madison, WI) that increase the specificity and efficiency of allele-specific hybridization reactions. This assay greatly increases throughput over conventional simplex (one genotype per reaction) SNP determination methodologies. In this report we describe the application of the assay to calculate allele and antigen frequencies for the human mHAg, HA-1, HA-2, HA-3, HA-8, HB-1, $CD31^{125}$, and $CD31^{563}$.

MATERIALS AND METHODS

Study Populations

Genotypes and allele frequencies for mHAg included in the test panel (Table 1) were determined using genomic DNA obtained from a population of 259 healthy Caucasian blood donors. Initial assay validation was performed using 25 samples derived from individuals of unknown ethnic origin. The samples utilized in this study cannot be identified directly or through identifiers linked to the study subjects. As a result, this study does not qualify as human subjects research and hence does not require Institutional Review Board (IRB) review.

Genomic DNA Preparation

Genomic DNA was isolated from anticoagulated whole blood using a DNA Blood Mini Kit (Qiagen, Valencia, CA). Briefly, red cells were lysed using ammonium chloride lysing buffer and DNA was extracted from the remaining leukocytes according to the manufacturer's instructions. DNA concentrations were adjusted to 40 ng/μl in 10 mM Tris-HCl, pH 9.0, and stored at −20 °C. DNA used in larger population studies was not quantitated prior to use.

Liquid Bead Array

The liquid bead array utilizes novel synthetic nucleotide bases (IsoBases) that specifically recognize each other based on hydrogen bonding patterns that are distinct from those used by natural base-pairing [17]. Primers used for mHAg locus-specific amplification and allele-specific extension reactions were synthesized by EraGen Biosciences (Madison, WI), and their sequences are provided in Table 2.

The assay was performed in multiple steps involving multiplex polymerase chain reaction (PCR) amplification of target mHAg loci, allele-specific primer extension reactions, hybridization of extension products to specifically tagged Luminex™ beads, and fluorescent detec-

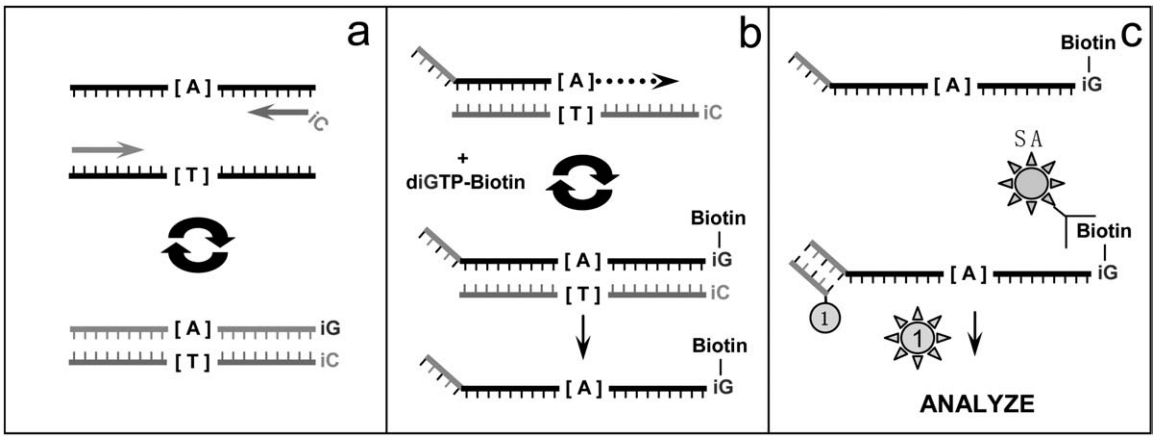

FIGURE 1 (A–C) The multiplex minor histocompatibility antigen (mHAg) genotyping system involves three major steps: (A) initial target gene amplification (1 hour), (B) allele-specific primer extension (15 minutes), and (C) hybridization, labeling, and analysis (20 minutes). The first stage (A) involves allele-independent polymerase chain reaction (PCR) amplification of the target mHAg gene using gene-specific primers, one of which contains the IsoBase, isocytosine (iC), at its 5′ terminus. Multiplexed allele-specific primer extension (B) is performed on the amplified target using primers specific for the alternate mHAg alleles. Each allele-specific primer contains a 5′ EraCode tag, a unique sequence comprised of both natural bases and IsoBases. The allele-specific extension is carried out in the presence of biotinylated 2′-deoxy-isoguanosine triphosphate (diGTP). In the final stage (C), the extension products are denatured, hybridized at room temperature to beads tagged with sequences complementary to the 5′ allele-specific primer tails, the products detected using phycoerythrin-conjugated avidin, and analyzed without washes in a Luminex 100 cytometer.