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RNA and protein expression of HLA-A*23:19Q

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ABSTRACT

The assignment of null alleles is clinically relevant in stem cell transplantation, in particular for donor selection. It is unclear how questionable (Q) alleles, having an unknown expression profile, should be considered in matching criteria. In this study we analyzed the RNA and protein expression profile of a questionable allele encountered in a sample of the Guadeloupe population: GD23Q, HLA-A*23:19Q, 29:02:01. Full-length DNA sequencing of HLA-A*23:19Q revealed a single polymorphism at position 619 (G > A) compared to HLA-A*23:01:01. Serological typing showed only the presence of HLA-A29; HLA-A*23:19Q was not detected on the cell surface. The absence of HLA-A*23:19Q surface expression was shown by flow cytometry using a directly labeled monoclonal antibody and a panel of five indirectly labeled polyclonal antibodies all directed against HLA-A23 (HLA-A9) molecules. Allele specific amplification revealed the absence of intact full-length mRNA, but the presence of two major alternatively spliced mRNAs: sequencing identified that in one variant exon 3 is missing and in the other variant introns 2 and 3 are retained. Based upon the lack of HLA-A*23:19Q surface expression and the presence of aberrant mRNA transcripts only, this study shows that HLA-A*23:19Q is non-expressed.

1. Introduction

Immune recognition of infectious pathogens is mediated through the presentation of foreign peptides by the Human Leukocyte Antigen (HLA) molecules. The HLA class I (A, B and C) and class II (DR, DQ and DP) genes are the most polymorphic loci in the human genome with 12,242 different HLA alleles currently known (IMGT/HLA v3.18) [1]. The high level of HLA polymorphism reflects its biological importance i.e. having the potential to present a large diversity of peptides to the immune system and trigger an effective immune response [2]. Most polymorphism is located in exons 2 and 3 for HLA class I and in exon 2 for class II, which encode the peptide binding groove involved in binding and presentation of peptides to T-cells [3].

In the transplantation setting, HLA polymorphism leads to strong adverse reactions when transplanted between HLA mismatched individuals [4]. In the cases where a mismatch search is necessary, the interval between diagnosis and transplantation should be kept to a minimum decreasing the risk of a disease progression caused by a prolonged search for a more suitable donor [5]. HLA-A and particularly HLA-B mismatches were shown to be strongly associated with an adverse outcome, compared to a HLA-C mismatch which showed a low impact on the overall survival [6]. Furthermore, certain mismatched HLA class I alleles have shown to lead to less severe complications. This is due to specific key residues present in the peptide binding groove that are associated with severe acute graft-versus-host disease and mortality, therefore donor-recipient pairs having certain mismatches at these positions should be avoided [7]. A strong effect of HLA-DRB1 mismatching was seen on transplant-related mortality, whereas an HLA-DQB1 mismatch is acceptable [6,8]. High resolution HLA typing leads to a more accurate matching of the HLA-A, -B, -C, -DRB1 and -DQB1 alleles and better patient survival [8,9].

Abbreviations: BSA, Bovine Serum Albumin; CDC, Complement Dependent Cytotoxicity; CREG, Cross-Reactive Group; EBV, Epstein–Barr Virus; EMBL, European Molecular Biology Laboratory; FCS, Fetal Calf Serum; HLA, Human Leukocyte Antigen; IMGT/HLA, ImMunoGeneTics–HLA database; MFI, mean fluorescence intensity; PBMC, Peripheral Blood Mononuclear Cells; RT-PCR, Reverse Transcription Polymerase Chain Reaction; SNP, Single Nucleotide Polymorphism; UTR, untranslated region; WHO, World Health Organization.

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In HLA matching procedures it is important to determine and take into account the expression patterns of abnormally expressed HLA variants [10]. Null (N) alleles are not expressed on the cell surface, secreted (S) alleles encode for proteins that are expressed as soluble molecules and lowly expressed (L) alleles are characterized by a weak surface expression of an intact HLA molecule [1]. For the questionable (Q) HLA alleles it remains unclear how they should be considered in the matching criteria, because their expression has not been confirmed. This could lead to a mismatch between donor and recipient caused by the questionable allele being a possible null allele. The only option is to search for a donor having the exact same haplotype, which is difficult due to the low frequency of the questionable alleles.

HLA-A*23:19Q was first assigned in 2007 as a questionable allele due to its unknown surface expression. The allele has been found in 7 different individuals and was confirmed in 4 laboratories by sequence analysis, although only partial allele sequences have been submitted (exons 2, 3 and 4) [1]. We encountered this allele, GD23Q: HLA-A*23:19Q, 29:02:01, in a sample of the Guadeloupe population [11,12]. In the current study we used this sample to determine the RNA and protein expression profiles of HLA-A*23:19Q by RNA sequencing, serology and flow cytometry.

2. Materials and methods

2.1. Cell lines

HLA-A*23:19Q was encountered in a Peripheral Blood Mononuclear Cells (PBMC) sample of an individual from Guadeloupe (GD23Q: HLA-A*23:19Q, 29:02:01) and an EBV B-lymphocyte cell line was established. In addition, B-cell lines were selected as negative or positive controls (Table 1). All cell lines were cultured in RPMI-1640 (Gibco, Life Technologies, Bleiswijk, the Netherlands) containing 15% FCS (Gibco) and 2% Penstrep (Gibco).

2.2. DNA/RNA isolation and cDNA synthesis

Approximately 1×10^7 cells were used for DNA isolation following manufacturer's protocol (QIAamp DNA Blood Mini Kit, Qiagen, Hilden, Germany). Total RNA isolation was performed using about 5×10^6 cells according to protocol (RNeasy minikit, Qiagen). DNA and RNA concentration and quality were determined by measuring the A260/280 ratio using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA). To ensure removal of all DNA in the RNA samples, a DNase step was applied using the deoxyribonuclease I kit (Life Technologies). For the transcription of RNA into cDNA, the Superscript III first-strand synthesis system kit for RT-PCR (Life Technologies) was used.

2.3. Sequence-based typing on DNA and RNA templates

Full-length RNA and DNA sequencing of the coding and intron sequences was performed by using HLA-A*23 specific amplification primers located in the 5′ and 3′ UTR as described previously [13,14]. The region from the promoter to exon 1 has been amplified with a 5′ primer upstream: 5′-GTGGACTCACACAGAAACTC-3′ (pos. −859 to −840), and a 3′ primer in exon 1: 5′-CCCCGAGAGTAGCAGGAC-3′ (pos. 28 to 45). The promoter, including the class I regulatory complex (CRC), is located at −219 to −149 upstream the start codon [15]. Sequencing this region was performed with the amplification primers, and 2 forward and 2 reverse sequencing primers. The 3′ untranslated region (UTR) was amplified using a 5′ primer located in intron 7, 5′-CTCACTGTGACAGATATGAAG-3′, and a 3′ primer, 5′-CAAATCAGCATAAGCAACACG-3′, located 450 bp downstream of the TGA stop codon. Sequencing the 3′UTR was performed using the amplification primers as sequencing primers. All PCR amplification and sequencing reactions were carried out as previously described [13,14].

PCR products were visualized on a 1.5% agarose gel (Life Technologies) with a final concentration of 0.5 μg/ml ethidium bromide (Sigma–Aldrich, Zwijndrecht, The Netherlands). In case of multiple alternatively spliced RNA products, separation was achieved on a 0.6% low melting point agarose gel for 2.5 h at 50 V in 1× TAE buffer (Life Technologies). The MinElute gel extraction kit (Qiagen) was used to extract the products from the gel which were subsequently used for sequence analysis. Resulting sequences were analyzed using Lasergene software (DNASTAR Lasergene 9 Core Suite, Madison, USA).

Table 1
HLA class I typing of GD23Q and control cell lines.

Cell ID	HLA-A	HLA-B	HLA-C
GD23Q	*23:19Q, 29:02:01	*08:01:01, 35:01:01	*03:04:01, 08:02:01
TER241	*23:01:01	*07:02:01, 41:01:01	*07:02:01, 08:02:01
TER364	*29:02:01, 68:01:01	*27:05:02, 44:03:01	*02:02:02, 16:01:01
TER174	*02:01:01, 02:07:01	*46:01:01	*01:02:01
K562	–	–	–

2.4. Serological typing

Serological typing of GD23Q was performed by the complement-dependent cytotoxicity test (CDC). Antisera routinely used for serological detection were obtained locally, commercially and by exchange with national and international colleagues. In brief, 1 μl antiserum and 1 μl lymphocyte-suspension at a concentration of 4×10^6 cells/ml were incubated for 30 min at 20 °C. Five μl rabbit complement (Life Technologies) was added and incubation was prolonged for 60 min at 20 °C. After addition of 5 μl FluoroQuench (One lambda, Canoga Park, USA) and 10 min incubation at room temperature, cell lysis was assessed by automated fluorescence ratio measurements for intact and lysed cells, respectively.

2.5. Flow cytometric analysis of HLA-A*23:19Q surface expression

Protein expression of the GD23Q and control cell lines (Table 1), was determined by flow cytometry using a FITC-conjugated monoclonal anti-HLA-A9 (HLA-A23 & HLA-A24) IgG2b antibody (FH0964, One Lambda) and 5 patient sera containing polyclonal antibodies directed against HLA-A23 or A9.

Approximately 5×10^4 cells were incubated on ice for 30 min with an increasing amount of undiluted monoclonal antibody ranging between 0.1 and 5 μl. As an isotype control, cells were incubated with 1 μl of FITC-conjugated mouse IgG2b isotype control antibody (Immunotools GmbH, Friesoythe, Germany). Cells were washed twice with PBS buffer containing 1% FCS and analyzed by flow cytometry (FACSCanto II, Becton–Dickinson, New Jersey, USA). Stainings were performed in triplicate.

For the polyclonal antibodies, about 3×10^5 cells were incubated for 30 min at room temperature with 50 μl of the anti-HLA-A9 patient sera. Subsequently, cells were washed 3 times with PBS/1% BSA (Bovine Serum Albumin) followed by labeling for 30 min at 4 °C with an anti-human IgG FITC-conjugated antibody (Sigma–Aldrich). Upon staining, cells were washed with PBS buffer containing 1% BSA and suspended in 200 μl of PBS/1% BSA. Positive and negative control sera used were those that routinely are used for diagnostic flow cytometry crossmatch analysis. Stainings were performed in duplicate. Analysis was performed using BD FACSDiva™ software (Becton–Dickinson, version 7).