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Definitive high resolution typing of HLA-E allelic polymorphisms: Identifying potential errors in existing allele data

Key words:
alleles; HLA-E; HLA class Ib; PCR-SSP; SNP

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Abstract: A set of robust PCR-SSP reactions were developed for each of the five polymorphic sites that define the five alleles of the HLA class Ib gene, HLA-E. This method was developed using 28 homozygous cell lines and further tested in a sample of African-Americans, a sample of Japanese, and a core panel of cell lines compiled for the 13th International Histocompatibility Workshop. Three alleles were found in each of these four sample groups, HLA-E*0101 (64.29, 50.00, 32.00 and 56.58%, respectively), *01031 (5.36, 20.65, 39.00 and 18.42%) and *01032 (30.35, 29.35, 29.00, and 25.00%). HLA-E*0102 was not detected in any of these samples nor in the cell line, LCL 722.221, in which this allele was originally described. HLA-E*0104 was not found either. This latter allele was originally reported in Japanese at a frequency of 1/22 (4.5%), which should have been high enough to have resulted in multiple occurrences of the *0104 allele in the samples tested in this study. We propose that the existence of the HLA-E*0102 and E*0104 alleles should be questioned.

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The nonclassical, or class Ib, HLA genes, HLA-E, -F and -G, are thought to code for functional proteins whose expression levels and pattern, and probably function, are different from that of the classical or class Ia genes, HLA-A, -B and -C. For example, HLA-G is mainly found in the trophoblast at the maternal–fetal interface and is thought to be relevant to the riddle of the fetal allograft (1, 2). HLA-E has been found to present class I leader peptides and to be recognized by natural killer cells (3, 4). This recognition is mediated by the interaction of HLA-E with the CD94/NKG2 receptor and can result in either inhibition or 'activation' of the natural killer cell, depending on the peptide presented and which NKG2 receptor it associates with (3, 5, 6). Although the precise functions of the class Ib genes have yet to be fully elucidated, it does appear that these gene products fulfill vital roles in immune function.

Also, unlike the class Ia genes, the class Ib loci have relatively low levels of allelic variation (7–13). However, few population genetics studies have been conducted for these genes, particularly for

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HLA-E and -F, and in only a limited number of populations. In addition, the importance of this genetic variation to functional differences between alleles, transplant rejection, and disease susceptibility has yet to be explored.

The goal of this study was to develop a high resolution typing method for one of the class Ib genes, HLA-E. There are five HLA-E alleles currently registered (at http://www.anthonynolan.org.uk/HIG/data.html) containing both synonymous and nonsynonymous substitutions, and designated HLA-E*0101, *0102, *01031, *01032, and *0104 (Table 1). The *0101 and *0103 alleles have been found in every population studied so far, using a variety of typing methods whereas the *0102 and/or *0104 alleles have not been found in all samples tested (9, 14, 15), and when they are detected using an SSO typing method their frequencies range from rare (14, 16) to frequent (17). In the present study, we set out to clarify the frequency and existence of the various HLA-E alleles by direct sequencing and by developing a PCR-SSP typing method for the reported HLA-E polymorphisms. These data and tools can in turn be used in new studies of HLA-E genetics.

Materials and methods

Samples

PCR-SSP typing methods were developed using the human genomic DNA derived from 28 human homozygous cell lines, of mainly Caucasian origin, compiled by the 10th International Histocompatibility Workshop (18), plus the cell line LCL 722.221. An additional 46 cell lines, part of the reference panel put together by the 13th International Histocompatibility Working Group (IHWG) (http://www.ihwg.org/shared/cbankservices.htm), were then typed for HLA-E polymorphisms. Six additional cell lines from the reference panel had already been typed as part of the original sample of 28 homozygous cell lines and were not included in the IHWG core panel so that these two sample groups did not overlap. Population samples analyzed by direct sequencing included DNA from 38 African-American individuals used in previous studies (12) and from 50 randomly selected, healthy, Japanese individuals.

The polymorphisms that define the reported HLA-E alleles

	Exon 2		Exon 3		
Allele	69	294	309–310	382	532
*0101	C	C	CG	A	A
*0102[a]	C	C	GC	A	A
*01031	C	C	CG	G	A
*01032	C	T	CG	G	A
*0104	C/T[b]	C	CG	G	G

[a]Original and official *0102 allele (10)
[b]T reported in (16)

Table 1

PCR-SSP

The primers used for each PCR-SSP reaction are given in Table 2. The C at position 69 was detected by combining primer 69-ALL-as with 104x2N5, and T69 by combining 69-ALL-as with 104x2Y5, yielding a product of 230 bp. The single nucleotide polymorphism (SNP) at location 294 was detected by combining E25 with 1031x23S for T and with 1032x23S for C, giving a product of 269 bp. For positions 309–310, there were two primer sets. One, 294-ALL-as and 102Y3b, detected the sequence reported for the official 0102 allele, GC at 309–310 (10), and the second pair, 294-ALL-as and 102N3, detected the sequence reported for all other HLA-E alleles, and CG at 309–310. All gave a product of 171 bp. The A at position 382 was detected by combining primer 382-ALL-as with 2E-382-A-F, and G382 by combining 382-ALL-as with 2E-382-B-F yielding a product of 160 bp. Finally, A at position 532 was detected using primers 532-ALL-s and 1043N3, and G was detected using 532-ALL-s with 104Y3, giving a band at 137 bp.

For this PCR-SSP method, sequence specific products were amplified using a two step PCR program with 10 cycles (94°C for 10 s, 65°C for 1 min) and 20 cycles (94°C for 10 s, 61°C for 1 min, 72°C for 30 s) proceeded by a 2-min 94°C denaturation step. PCR reactions included 50 ng genomic DNA, 2.5 pmol detection primers, 0.5 pmol positive control primers, 1 × PCR buffer, 0.1 mM nucleotides, 1.5 mM $MgCl_2$ and 0.4 Units Taq polymerase in a 10-μL reaction.

Additional primer combinations and protocols were originally designed for two of the polymorphic sites, 382 and 532. The primers for position 382 (Eg2.21 and 2E-382-A-F; Eg2.21 and 107G) and 532 (Eg1.12 and 1043N3; Eg1.12 and 1043Y3) are given in Table 2. The two step PCR program described above was modified for each of these reactions, first by increasing the second denaturation step from 10 to 30 s. For 382, the second annealing temperature was raised to 63°C, and for 532 the first and second annealing temperatures were decreased to 63°C and 59°C, respectively. Otherwise the PCR reaction was the same as that described for the previous methods. Although these are robust primers and protocols, the other set of primers were designed in order that the SSP protocols for each polymorphism would use the same PCR conditions. The original primers are included here to provide an alternative typing protocol for SNP positions 382 and 532.

Positive control DNA

As we had no genomic DNA that contained HLA-E*0102 and *0104 alleles, as demonstrated by sequencing, we constructed positive control DNA for the three reported polymorphisms using standard methods. Briefly, positive control DNA was constructed using primers containing the appropriate mutation and the nonspecific 'ALL' primer for the same position (Table 2). For position 69, 69-ALL-as and MUT-69-s were used, generating a product which was 230 base pairs long, and for position 532, primers 532-ALL-s and MUT-532-as were used, generating a product of 140 base pairs. One construct was made for positions 309–310 which represented the official HLA-E*0102 sequence (primers 294-ALL-s and MUT-310-as). This product was 171 bp in length. The PCR conditions were as described above. The resulting PCR products were ligated into the pGem plasmid (Promega, WI, USA) and sequenced to verify the appropriate altered sequence. These cloned DNA were subsequently used as positive control templates for the appropriate SSP reaction.

Sequencing

A 1135 nucleotide portion of HLA-E encompassing all of the polymorphic sites was amplified using primers HE01F and HE01R, details of which are given in Table 2, for each of the 28 cell lines, as well as for cell line LCL 722.221. All PCR reactions were carried out using 50 ng of genomic DNA with 2.5 mM Mg^{2+}, 100 nM dNTPs (Promega, WI, USA), 5 pmol each forward and reverse primer, 1 × PCR buffer (Invitrogen, CA, USA), 1 × Q-solution (Qiagen Inc., CA, USA), and 1.25 Units Taq DNA polymerase (Roche Diagnostics Corporation, IN, USA) in a 25-μL total PCR volume. All PCR reac-

Primers used for SSP, sequencing and for constructing positive control DNA for alleles *0102 and *0104

Name	Sequence	Position	Base	Allele
SSP Primers				
69-ALL-as	ATCTGTGCGGTGTCCCTGGC	69		
104x2N5	TCCTCGCCCCCAGGCTCC	69	C	*0101, *0102, *01031, *01032
104x2Y5	TCCTCGCCCCCAGGCTCT	69	T	*0104
E25	GATCTCAGCCCCTCCTCG	294		
1031x23S	CGCAGCGTCCGCAGG	294	C	*0101, *0102, *01031, *0104
1032x23S	CGCAGCGTCCGCAGA	294	T	*01032
294-ALL-s	CAGTTCGTGCGCTTCGACAA	309–310		
102N3	GCTCTGATTGTAGTAGCCG	309–310	CG	*0101, *01031,*10132, *0104
102Y3b	GCTCTGATTGTAGTAGCGC	309–310	GC	*0102-official
382-ALL-as	CCGCCTCAGAGGCATCATTTG	382		
2E-382-A-F	GCGAGCTGGGGCCCGTCA	382	A	*0101, *0102
2E-382-B-F	TGCGAGCTGGGGCCCGGCG	382	G	*0103, *0104
532-ALL-s	CCTACGACGGCAAGGATTATCTCA	532		
1043N3	GTCTTCCAGGTAGGCTCT	532	A	*0101, *0102, *01031, *01032
104Y3	GTCTTCCAGGTAGGCTCC	532	G	*0104
Eg2.21	GGAGGAGTGGTATTCTGG	382		
107G	GCGAGCTGGGGCCCGACG	382	G	*01031, *01032, *0104
Eg1.12	CGGGACTGACTAAGGGGC	532		
Sequencing				
HE01F	TCCTGGATACTCATGACGCAGACTC	1135 bp		
HE01R	CCTCTTACCCAGGTGAAGCAGCG			
Positive Control				
MUT-69-s	TCCTCGCCCCCAGGCTCTCACTCC	69	T	*0104
MUT-310-as	GCTCTGATTGTAGTAGCGCCGCA	309–310	GC	*0102-official
MUT-532-as	CTCTCCAGGTAGGCTCCCTGGTG	532	G	*0104

Table 2

tions were carried out in either a Model 9600 or 9700 Thermocycler (Applied Biosystems, CA, USA) using the following cycling protocol: initial denaturation of 94°C for 5 min; 94°C for 20 s, 65°C for 30 s, 72°C for 45 s for 10 cycles; 94°C for 20 s, 55°C for 30 s, 72°C for 45 s for 25 cycles; along with a final extension of 72°C for 5 min. PCR products were enzymatically purified before sequencing by treating 90 ng PCR product with 10 Units Exonuclease I and 1 U Shrimp Alkaline Phosphatase for 15 min at 37°C followed by 15 min at 80°C.

Direct sequencing of PCR products was conducted on both strands using Perkin Elmer Big Dye Terminator chemistry (Perkin Elmer), following the manufacturer's protocols, and run on ABI 377 automated sequencers. Sequences were assembled and analyzed using PhredPhrap, Consed viewing software (19, 20).

Results

Searching for HLA-E*0102 and *0104 alleles

In order to develop a rapid typing method allowing for the examination of HLA-E alleles in large numbers of samples, PCR-SSP typing protocols were developed for each of the reported HLA-E polymorphic sites. However, as no DNA samples positive for HLA-E*0104 and *0102 (either the official or corrected version of this allele) could be found, we constructed recombinant DNA containing the appropriate sequences to optimize our PCR-SSP typing reactions for these alleles. Positive control DNA samples were constructed for those positions (69, 309-310, and 532) that define the *0102 and *0104 alleles using standard methods. Using these constructs, we attempted to determine whether our typing protocol for these sites would detect those polymorphisms when present and distinguish them from alternative allelic sequences. Constructs were diluted to concentrations including the molarity of genomic DNA (0.0001 ng), and were examined with the appropriate PCR-SSP assay (Fig. 1). From these analyses it was apparent that each of the specific PCR-SSP assays did distinguish the appropriate sequence at a representative concentration, although there was one false positive at position 69. However, this result was at DNA concentrations that were 20 times greater than that used in the genomic DNA samples and was therefore considered adequate for the purposes of this study.

PCR-SSP and sequencing

Using what we considered to be a complete PCR-SSP toolkit for distinguishing the five HLA-E alleles, we first tested this typing method for HLA-E on DNA from 28 cell lines derived from the 10th HLA workshop. From this three alleles were found, HLA-E*0101 (64.52%), *01031 (6.45%) and *01032 (29.03%). Representative examples of SSP gels for the polymorphic sites defining the three alleles (294 and 382) are shown in Fig. 2. These typing results were confirmed for each cell line by sequencing a region of HLA-E that encompassed all of the polymorphic sites. However, the *0102 and *0104 alleles were not detected in this sample group, including the LCL 722.221 cell line in which the *0102 allele had been reported originally. Upon resequencing HLA-E in .221, it was discovered that the HLA-E sequence was consistent with the *0101 allele and did not contain the substitutions reported for either the original or corrected *0102 allele.

This analysis was expanded by examining DNA from three additional groups. Allele frequencies for HLA-E in the IHWG core sample, the African-American collection, and Japanese are shown in Table 3. Again, all samples were tested with the complete HLA-

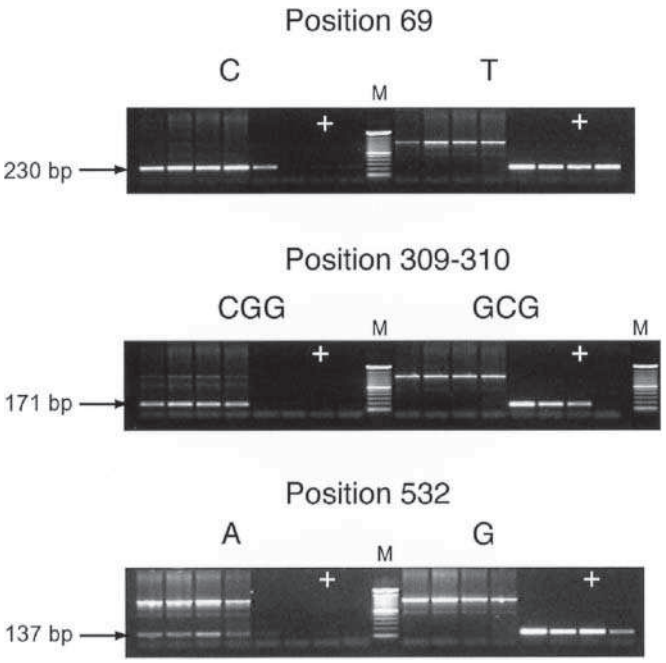

Fig. 1. **Distinguishing HLA-E allelic variants *0102 and *0104 with PCR-SSP. SSP gels are shown for positions 69, 309–310, and 532, those that define the HLA-E*0102 and *0104 alleles.** The base detected by each reaction is indicated above the set of eight samples that were typed. The first four lanes of each set show the results using genomic DNA samples. The final four samples typed for each allele are dilutions of the appropriate positive control DNA (0.002 ng, 0.0002 ng, 0.0001 ng, 0.00002 ng). A plus sign is located over the lane that represents the molarity of this locus in genomic DNA (0.0001 ng). The smaller PCR product is the band of interest while the larger product is from the positive control primer set. The sizes of DNA products are indicated to the left of each gel picture. The marker lane identified with an M contains the 100 bp Ladder, DNA Molecular Weight Marker (Invitrogen, CA, USA).

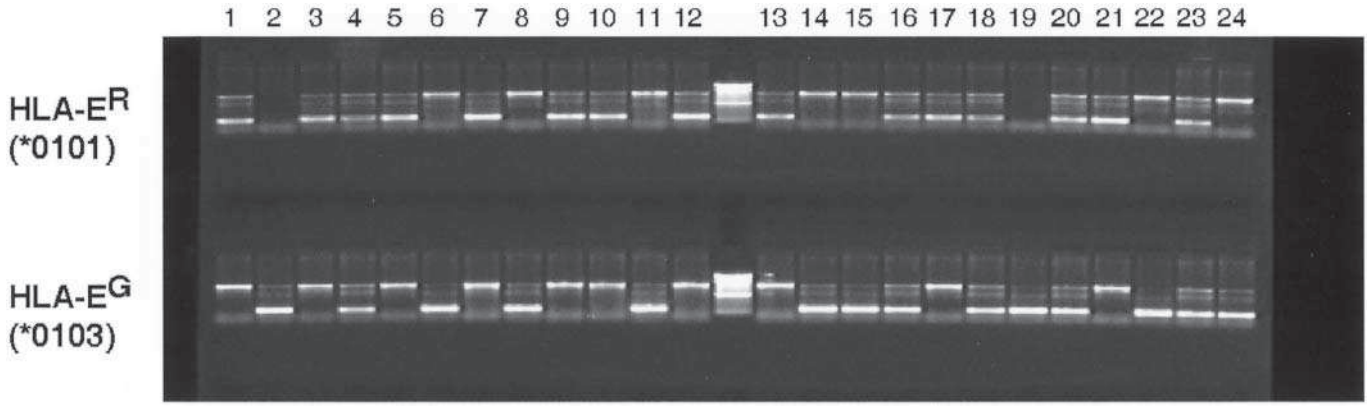

Fig. 2. **Examination of genomic DNA with the HLA-E PCR-SSP reaction detecting nonsynonomous alleles HLA-E^R (*0101) and -E^G (*01031 and *01032).** Representative SSP gels examining HLA-E at position 382 in genomic DNA are shown (see Materials and methods section). The genomic DNA tested for the presence of either the HLA-E^R (primer 2E-382-A-F) or the HLA-E^G (primer 2E-382-B-F) alleles are indicated above each lane. The presence of either allele is indicated by the presence of the lower band at 160 bp and the discrimination of heterozygotes and homozygotes by that band in both or only one of the allele specific reactions, respectively. The larger band predominant in negative lanes is derived from a primer set added to control for effective PCR conditions (see Materials and methods section). Numbers above each lane correspond to the designation of cell lines from the IHWG SSOP Reference Cell Panel as indicated at http://www.ihwg.org/shared/cbankservices.htm

E PCR-SSP typing kit and results were confirmed by sequence analysis. Detailed results from HLA-E typing for the homozygous cell lines and the IHWG core sample are currently available upon request and will be made available through the IHWG cell and gene bank (http://www.IHWG.org). Notably, both typing protocols for positions 382 and 532 were tested and yielded identical results. As found in the first group of samples tested, no occurrences of the *0102 or *0104 alleles were found.

Discussion

It has been suggested that the allele frequencies of the two HLA-E alleles defined by an amino acid substitution, HLA-E*0101 and *0103, are the result of balancing selection which maintains these alleles in various populations (9). This would indicate that there must be functional differences between the molecules encoded by each of these alleles upon which the selection is acting. If this finding is correct, this could have implications towards human health in general and transplant success in particular.

As HLA-E is ubiquitously expressed (3) it could possibly influence the success of transplant of bone marrow or other organs. For example, although genetic matching of the classical HLA antigens is clearly a major determinant of successful marrow transplant outcome, the frequency of graft versus host disease (GVHD) in HLA-matched donor transplants indicates that other polymorphic genetic factors must be also involved (21). HLA-E is a prime candidate for such a genetic factor. It fits the criteria of exhibiting polymorphism: two alleles, which may not be in strong linkage disequilibrium with the HLA classical class I loci (13). Although one study found evidence for low levels of linkage disequilibrium between HLA-E alleles and those of the class Ia loci (9), those levels were lower than those seen between the alleles of other class I loci (see (11) for example). Therefore a significant percentage of unrelated marrow transplant donor-recipient pairs matched for HLA-A and -B would likely be mismatched at the HLA-E locus.

HLA-E variation could also play a role in human health. Not only could it be directly involved in disease susceptibility or resistance, but the SNP located in this gene could also be used as markers for disease association at neighboring, perhaps as yet undiscovered, loci. Again, the relatively low levels of linkage disequilibrium reported between the alleles of HLA-E and the alleles of class Ia loci suggest that this is an ideal region for study. The variation of HLA-

Frequencies of the HLA-E alleles in the different samples

	Allele frequency (%)				
Sample	*0101	*0102	*01031	*01032	*0104
Homozygous cell lines ($n = 28$)	64.29	0.00	5.36	30.35	0.00
13th IHWG core sample ($2n = 92$)	50.00	0.00	20.65	29.35	0.00
Japanese ($2n = 100$)	32.00	0.00	39.00	29.00	0.00
African-American ($2n = 76$)	56.58	0.00	18.42	25.00	0.00

Table 3

E is not completely associated with that of the rest of the MHC and would be more independent of other SNP in this region.

For any studies, many individuals would need to be typed for the various HLA-E polymorphisms, especially if the effect of HLA-E on health or transplant outcome is relatively small or complicated by other factors, as would be expected. It is therefore necessary to have a reliable, high resolution typing method that can easily be used for large numbers of samples. For this reason, we developed a PCR-SSP typing method for each of the polymorphisms reported for HLA-E.

Using this method, we tested for the presence of five previously described HLA-E alleles in two sets of cell lines, mainly of Caucasian origin, as well as random samples of African-American and Japanese individuals. In these samples, we found only three alleles, HLA-E*0101, *01031 and *01032 (Table 3). The similar frequencies of HLA-E*0101 and *0103 (*01031 and *01032) in African-Americans and the greater frequency of *0103 in the Japanese sample are similar to previous reports of allele frequencies in African-Americans and Asians (9).

Although alleles HLA-E*0102 and *0104 were not detected using this method, we determined, by using constructed positive control DNA samples diluted to genomic levels, that this method could indeed detect these alleles if they had been present (Fig. 1). In addition, the cell line in which HLA-E*0102 was initially described, LCL 722.221, was also typed and found to have the *0101 allele. This finding was confirmed by sequencing HLA-E in this cell line. This discovery questions the designation of the HLA-E*0102 allele, and therefore, the termination of this variant as an official allele may be prudent.

HLA-E*0104 was also not found in any of the samples examined and was absent from an additional 200 samples in a further study (data not shown). This is particularly interesting as this allele was initially reported at a frequency of 1/22 (4.5%) in a small sample of Japanese individuals. This frequency is high enough to expect multiple occurrence and detection of this allele in the Japanese samples used in this research. Allele HLA-E*0104 has subsequently been found, using an SSO typing method, in other populations and at higher frequencies (17). The fact that this allele was not found in any of samples in this study or other studies (14, 15) suggests that the frequency of this allele is actually lower than previously reported or it may not exist. Further investigation is needed to determine which is correct. However, as we were unable to obtain any DNA containing this allele, even from the labs originally reporting the allele, we suggest that the designation be removed until compelling evidence of its existence can be presented.

In summary, a PCR-SSP typing method for the reported HLA-E polymorphisms was developed to study the HLA-E alleles and their frequencies and ultimately to determine the utility of the HLA-E polymorphisms as SNP markers, as well as the importance of HLA-E matching to transplant outcome. As no HLA-E*0102 allele was found in this sample, and the cell line in which this allele was originally reported was found to have the *0101 allele instead, the assignment of the *0102 allele needs to be re-evaluated. In addition, because no examples of the HLA-E*0104 polymorphism were found, the existence and frequency of the *0104 allele should also be reconsidered.

These findings have been lodged with the HLA Nomenclature Committee to consider the implications.

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