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BRIEF COMMUNICATION

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Characterization of a new *HLA-G* allele encoding a nonconservative amino acid substitution in the α3 domain (exon 4) and its relevance to certain complications in pregnancy

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The nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide sequence database and have been assigned the accession number AF312697. Based on the sequence of exon 2 to exon 4, the name *G*0106* has been officially assigned by the WHO Nomenclature Committee in October 2000. This follows the agreed policy that, subject to the conditions stated in the most recent Nomenclature Report names will be assigned to new sequences as they are identified. Lists of such new names will be published in the following WHO Nomenclature Report

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The HLA-G molecule is expressed on cytotrophoblast cells, which invade the maternal decidua and the spiral arteries during placentation. The human trophoblast does not express the classical HLA class I antigens, HLA-A and HLA-B, but does express HLA-C (reviewed in Le Bouteiller and Blaschitz 1999). Functions of HLA-G might be protection against natural killer (NK) cell-mediated lysis, modulation of cytokine secretion and inhibition of the transendothelial migration of human NK cells (e.g. Dorling et al. 2000; Maejima et al. 1997; Ponte et al. 1999). Although confusion existed for some years concerning the degree of *HLA-G* gene polymorphism, concensus has converged on the existence of only three well-described *HLA-G* gene polymorphisms that result in amino acid substitutions (Ishitani et al. 1999). One in exon 2 (α1 domain; codon 31, Thr–Ser) and one in exon 3 (α2 domain; codon 110, Leu–Ile) result in conservative amino acid substitutions. Furthermore, a deletion of a single nucleotide in exon 3 at codon 129/130 resulting in a frameshift mutation has been detected in several studies (e.g. Hviid et al. 1997; Ober et al. 1998; Suarez et al. 1997). In a study of *HLA-G* expression in first-trimester trophoblast samples, we detected a base substitution in codon 258 (ACG to ATG), which leads to a nonconservative amino acid substitution of threonine for methionine in the α3 domain (exon 4) (Hviid et al. 1998). The polymorphism is located near the transmembrane region, and other MHC class I molecules from human and other species all have threonine at this position. In Fig. 1, the location of the codon 258 polymorphism is illustrated based on the crystal structure of the complex between human CD8 and HLA-A2.

Interestingly, the woman in whom the new polymorphism was first detected had a history of infertility for several years and a spontaneous abortion. She later gave birth to two children after intrauterine insemination (information about homologue/heterologue insemination could not be obtained). Therefore,

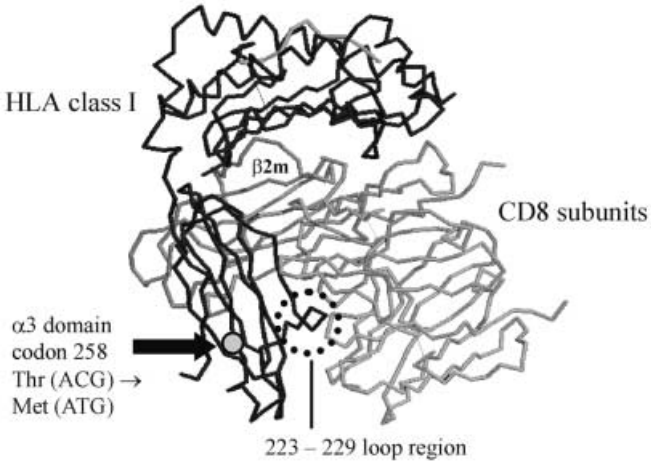

Fig. 1 Structure of the CD8/HLA-A2/peptide complex and the interaction surfaces. Residue 258 is marked with an *arrow*. Small changes of conformation in the residue 223–229 loop region (*dotted ring*) of the α3 domain seem to be important for contact with CD8 and may account for the differential binding of CD8 by both HLA class Ia and Ib molecules. Three-dimensional structure data from Gao and coworkers (1997)/NCBI/Molecular Modelling Database/MMDB Id: 6442/PDB Id: 1AKJ

we found it relevant to obtain the full sequence of the new allele and investigate its distribution in a population of healthy, fertile individuals and in couples with recurrent spontaneous abortions. Furthermore, we investigated the relevance of the new allele in pre-eclamptic cases because of the reported aberrant HLA-G expression in this condition (e.g. Goldman-Wohl et al. 2000).

Using genomic DNA extracted from a human first-trimester trophoblast biopsy homozygous for the codon 258 polymorphism and genomic DNA from a person heterozygotic for the codon 258 polymorphism, PCR products of 1928, 1724 and 1539 bp were generated. PCR primer sequences are listed in Table 1. For the 1928-bp PCR product, primers were NY11SEKHLAG5 located in the promoter region of *HLA-G* and SEKHLAGEX2 located in intron 2; for the 1724-bp PCR product, PCR primers were HLAGEX2A located in intron 1 and BHLAGEX4 located in intron 4, and for the 1539-bp PCR product, primers were HLAGEX4A located in intron 3 and LRHLAG3 located in exon 8 (the 3′ untranslated region). PCR conditions (1928 and 1539 bp): 200 ng of genomic DNA was made up to a final volume of 50 µl containing 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 2.0 mM $MgCl_2$, 300 µM dNTP, 25 pmol of each primer, and 2.5 units of *Taq* polymerase. Thermocycling conditions (1928 and 1539 bp): 94 °C for 2 min, 10 cycles of 94 °C for 10 s, 55 °C (1928 bp) or 60 °C (1539 bp) for 30 s, 68 °C for 3 min, 27 cycles of 94 °C for 10 s, 55 °C (1928 bp) or 60 °C (1539 bp) for 30 s, 68 °C for 3 min with a 20-s extension for each cycle, followed by 68 °C for 7 min. PCR conditions (1724 bp): 500 ng of genomic DNA was made up to a final volume of 100 µl containing 15 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dNTP, 50 pmol of each primer, and 2.5 units

Table 1 PCR and sequencing primers

PCR primers		Length of the PCR product (bp)
NY11SEKHLAG5	5′ ACTGGAGTGTTTTAGGTGGAGAAATGAC 3′	1928
SEKHLAGEX2	5′ ATGGAGGTGGGGGTCGTGATCT 3′	
HLAGEX2A	5′ GGGTCGGGCGGGTCTCAA 3′	1724
BHLAGEX4	5′ TGCTTTCCCTAAACAGACATGAT 3′	
HLAGEX4A	5′ CCATGAGAGATGCAAAGTGCT 3′	1539
LRHLAG3	5′ TGATTGGGGAAGGAATGCAGTTCAGCATGA 3′	
Sequencing primers		
HLAGKB3	5′ AACCTAAGAGTTCTGCTGCTTTGG 3′	
NY11SEKHLAG5	5′ ACTGGAGTGTTTTAGGTGGAGAAATGAC 3′	
3HLAG1KB	5′ AAATTTACCTTCATTCCGTAGCCC 3′	
PSHLAG5	5′ GTGAAGGGAGAGGGCCAGGGACCTT 3′	
5HLAGPRO	5′ GGCTCTCAGGGTCTCAGGCCCCAC 3′	
PROHLAG3	5′ AATGAGTCCGGGTGGGTGAGCGA 3′	
I1SEKHG3	5′ GGGCCCCTCCCTCCTCCGCGCAG 3′	
HLAGEX2A	5′ GGGTCGGGCGGGTCTCAA 3′	
5G28NA	5′ CACAGACTGACAGAATGAACCTGCA 3′	
BHLAGEX2	5′ TGCTTTCCCTAAACAGACATGAT 3′	
5HLGIN2	5′ CCCAGACCCTCTACCTGGGAGAACCCCA 3′	
TOG3E5	5′ TGCGGCTCAGATCTCCAAGCGCAAGTGT 3′	
INT3HLAG	5′ TGAAAGGAGAGTCAGAAAATTCAA 3′	
HLAGEX4A	5′ CCATGAGAAGATGCAAAGTGCT 3′	
BHLAGEX4	5′ TGCTTTCCCTAAACAGACATGAT 3′	
EX5HLAG3	5′ GGGATGGTGGGCAGGGAAGACT 3′	
FHG3	5′ GGCCTGGTTGTCCTTGCAGCTGTA 3′	
IN5HLAG	5′ ACCTTGATGATTGTAGTGATGGG 3′	
6INHLAG	5′ TCCTTCATACTTACTTGCAGCCT 3′	
GE14HLAG	5′ GTGATGGGCTGTTTAAAGTGTCACC 3’	

of AmpliTaq Gold (Applied Biosystems). Thermocycling conditions (1724 bp): 95 °C for 6 min, 35 cycles of 94 °C for 30 s, 60 °C for 60 s, 72 °C for 3 min, followed by 72 °C for 10 min. The PCR products were cloned into a pCR2.1 TOPO vector using the TOPO TA Cloning kit (Invitrogen). The cloned fragments and PCR products were DNA sequenced with an ABI Prism Big Dye Terminator cycle sequencing kit (Applied Biosystems) and an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

With primers HLAGEX4A and BHLAGEX4, a 364-bp PCR product was amplified from the *HLA-G* gene. PCR conditions: 100 ng of genomic DNA was made up to a final volume of 25 µl containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.8% Nonidet P40, 1.5 mM $MgCl_2$, 200 µM dNTP, 10 pmol of each primer, and 0.75 units of *Taq* polymerase. Thermocycling conditions: 94 °C for 2 min, 32 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, followed by 72 °C for 10 min.

A PCR-restriction fragment length polymorphism (RFLP) method was used to genotype the codon 258 polymorphism. All *HLA-G* alleles with ACG at codon 258 are cleaved with the restriction endonuclease *Eco*72I into PCR fragments of 209, 92, and 63 bp. In contrast, *Nsp*I cleaves PCR products with the ATG (codon 258) polymorphism into two PCR fragments of 272 and 92 bp. DNA fragments were analyzed by electrophoresis on agarose gels and stained with ethidium bromide. In a few samples, an extra band of 272 bp was seen after digestion with *Eco*72I. This reflects a known polymorphism at codon 188 (CAC to CAT) eliminating an *Eco*72I site.

First, 22 healthy, fertile women and 22 healthy, fertile men, who were *HLA-G* genotyped using direct sequencing, PCR-RFLP, and single stranded conformation polymorphism, were screened for the new allele using the enzyme *Eco*72I. Positive samples were checked using the enzyme *Nsp*I. Some of the PCR-RFLP results were confirmed by direct DNA sequencing of the PCR product. Then, a further 106 individuals with proven fertility were screened for the codon 258 polymorphism. Next, 31 women with a history of pre-eclampsia during a pregnancy and their partners were genotyped for the codon 258 polymorphism. Pre-eclampsia was defined as blood pressure $\geq$140/90 mmHg or a rise >20 mmHg in diastolic blood pressure and one or more of the following: proteinuria, edema, headache, or visual disturbance. Finally, 19 women who had experienced three or more spontaneous abortions and their partners were genotyped. The recurrent spontaneous abortions were unexplained, no abnormalities were revealed in a screening program consisting of hysterosalpingography or hysteroscopy, mid-luteal serum progesterone, serum thyroxine, and karyotyping of the couple.

The DNA sequence of the new *HLA-G* allele is summarized in Table 2 and compared to the originally published sequence of *HLA-6.0* (Geraghty et al. 1987). The new allele seems to be a subtype of the *HLA-G*01012* allele because it also contains CAT at codon 93. In 90 samples previously *HLA-G* genotyped, the codon 258 polymorphism (ATG) was in all cases found together with the CAT polymorphism at codon 93 and the 14-bp insertion polymorphism in the 3' untranslated region in exon 8. The allele frequency of *G*0106* in a Caucasian population of 150 fertile men and women was 4.0%. No homozygotes were detected.

In the study of pre-eclamptic women and their partners and of couples with recurrent spontaneous abortions, no statistically significant association with the new *HLA-G* allele was found (Table 3). The distribution of genotypes for the three groups with either pre-eclampsia, recurrent spontaneous abortions, or normal fertility were all in accordance with Hardy-Weinberg expectations using χ^2-contingency table analysis.

Figure 1 shows a schematic drawing of an HLA class I molecule together with CD8, and the amino acid position 258 is marked. The CD8 molecule binds to the HLA class Ia molecule primarily interfacing with the α3 domain stabilizing the interaction of the T-cell receptor with the HLA/peptide complex (Gao et al. 1997, 2000). Interestingly, CD8 has been reported to bind to HLA-G (Sanders et al. 1991). Gao and co-workers (2000) have shown that HLA-G is able to bind CD8 with an affinity similar to classical HLA/CD8αα affinities. Finally, HLA-G has been shown to bind peptides in vitro (Diehl et al. 1996; Lee et al. 1995). So, HLA-G might be able to interact with the T-cell receptor. Furthermore, a recent study suggests that soluble HLA-G1 triggers CD95/CD95 ligand-mediated apoptosis in activated $CD8^+$ cells by interacting with CD8, and this CD8-mediated apoptosis induction of soluble HLA-G was shown to be T-cell receptor independent (Fournel et al. 2000). Although residue 258 in classical HLA class I antigens does not seem to be directly involved in the binding of CD8, it is near residue 262, which has been shown to be in the interaction surface (Gao et al. 1997). Even polymorphism outside these direct contact residues has been shown to change rather dramatically the binding of CD8 to a classical HLA class I allele (Salter et al. 1989). This might affect the recognition by alloreactive and peptide-specific cytotoxic T lymphocytes. Therefore, we found it interesting to characterize the HLA-G allele further and study its possible implications in disorders of pregnancy such as pre-eclampsia and spontaneous abortion. Aberrant expression of HLA-G in pre-eclamptic placentas has been described in several studies (e.g. Goldman-Wohl et al. 2000; Hara et al. 1996), indicating that marked HLA-G polymorphism could be important as well, maybe in connection with certain viral infections. For example, human cytomegalovirus gene products have been shown to down-regulate HLA-G (Jun et al. 2000), and the HLA-G polymorphism could be of

Table 2 Polymorphism of the *G*0106* allele in comparison with the published sequence of the *G*01011* allele (*HLA-6.0*) (Geraghty et al. 1987). Nucleotide position number after the start of exon 1 is according to Geraghty and co-workers (1987). DNA sequencing started at nucleotide position –1380; DNA sequencing stopped at nucleotide position 3850; the sequence between –900 and –543, intron 6, and intron 7 were not DNA sequenced (*UTR* untranslated region)

Nucleotide sequence position/codon		*G*0106*	*G*01011* (*HLA-6.0*)
–1305		a	g
–1301		g	a
–1178		g	a
–1139		t	a
–963		a	g
–485		c	a
–475		g	c
–370		a	c
–200		a	g
–69		ag	a
15	Exon 1	GCA (Ala)	GCG (Ala)
36		CTA (Leu)	CTG (Leu)
929	Intron 1	c	t
970		t	c
Codon 57	Exon 2	CCA (Pro)	CCG (Pro)
1264	Intron 2	c	t
1276		c	a
1292		cc	c
1313		c	g
1418		t	c
Codon 93	Exon 3	CAT (His)	CAC (His)
1801	Intron 3	c	t
1846		c	t
1999		g	a
2096		a	g
2147		a	g
2334		g	a
Codon 258	Exon 4	ATG (Met)	ACG (Thr)
2714	Intron 4	c	g
Codon 290	Exon 5	GGT (Gly)	GGC (Gly)
Codon 309		AGG (Arg)	AGA (Arg)
2943	Intron 5	c	t
3060		c	t
3094		t	c
3112		g	a
3200		a	g
3301		g	a
3741	Exon 8 (3′ UTR)	14-bp insertion	
3761		g	a
3777		c	g

importance in a physical association with such gene products.

O'Brien and co-workers (1999) investigated the inheritance of *HLA-G* polymorphisms in trios of mother, father and offspring in first pregnancies complicated with pre-eclampsia and in control trios. In one-third of the pre-eclamptic pregnancies the offspring inherited the polymorphisms CAT codon 93 and the 14-bp insertion in exon 8, corresponding with the *G*01012* allele, from the father, and the polymorphism CAC codon 93 together with no 14-bp insertion in exon 8 from the mother, compared with no such cases in the trios with uncomplicated pregnancies. This is interesting, because the *G*0106* allele is coupled with the same polymorphisms. In the present study, we also genotyped maternal and paternal samples from pre-eclamptic cases, but we did not find any differences between the distribution of the codon 258 polymorphism in the pre-eclamptic cases and the controls. The possibility of an association of *HLA-G* polymorphism and recurrent spontaneous abortions has been proposed (van der Ven et al. 2000), but no association with the codon 258 polymorphism could be detected in the present study.

With the use of known X-ray structures of other HLA class I molecules and a protein structure modulating programme (ProteoMine™, Structural Bioinformatics, San Diego, Calif.), prediction of any structural changes caused by the codon 258 polymorphism was attempted, especially concerning the 223–229 loop region. No changes were detected, indicating that the polymorphism might not have any structural importance. However, it should be noted that this approach might not yet be as sensitive as required.

Although the *HLA-G* polymorphism reported in this study is the most marked single amino acid substitution detected so far, no obvious association with either recurrent spontaneous abortion or pre-eclampsia was observed. If HLA-G has an important function during pregnancy, more substantial polymorphisms/mutations may be lethal. *HLA-G*0105N* with a frameshift mutation in exon 3 is causing a dilemma in this

Table 3 Frequencies of the *HLA-G* codon 258 polymorphism (*G*0106* allele) in fertile women, women with an uncomplicated pregnancy, and women with pre-eclampsia and recurrent spontaneous abortions, and their partners. No significant association of the codon 258 (ATG) allele with any of the four groups of samples or females and males in each group was found using Fisher's exact test (*F* female, *M* male, *N* number of alleles, *n* number of couples, + ATG, – ACG)

	Uncomplicated pregnancy		Pre-eclampsia		Fertile		Recurrent spontaneous abortions	
	F (*N*=44)	M (*N*=44)	F (*N*=62)	M (*N*=62)	F (*N*=70)	M (*N*=70)	F (*N*=38)	M (*N*=38)
*G*0106*	3 (6.8%)	4 (9.1%)	4 (6.5%)	3 (4.8%)	0 (0.0%)	5 (7.1%)	1 (2.6%)	1 (2.6%)
Other alleles	41 (93.2%)	40 (90.9%)	58 (93.5%)	59 (95.2%)	70 (100.0%)	65 (92.9%)	37 (97.4%)	37 (97.4%)
Distribution of genotypes								
F	M	(*n*=22)*		(*n*=31)*		(*n*=35)**		(*n*=19)**
+/–	+/–[a]	0 (0.0%)		1 (3.2%)		0 (0.0%)		0 (0.0%)
+/–	–/–	4 (18.2%)		3 (9.7%)		0 (0.0%)		1 (5.3%)
–/–	–/–	15 (68.2%)		29 (93.5%)		30 (85.7%)		17 (89.5%)
–/–	+/–	3 (13.6%)		2 (6.5%)		5 (14.3%)		1 (5.3%)

*P=0.41 and **P=0.34 using Fisher's exact test on unordered $r \times c$ contingency tables (Mehta and Patel 1986)
[a] Nine combinations of genotypes exist but only those observed are listed

regard, because several healthy homozygotes have been reported (Castro et al. 2000; van der Ven et al. 2000); however, a functional soluble HLA-G2 molecule may exist in these persons, or HLA-C or HLA-E might compensate for the loss of HLA-G function. Finally, if certain *HLA-G* polymorphisms are important in certain complications in pregnancy, they are likely to have implications for the levels of expression of membrane-bound and soluble HLA-G rather than structural implications. Interesting in this regard is a recent study by Kapasi and co-workers (2000) who found that high HLA-G concentrations suppressed an allo-cytotoxic T lymphocyte (CTL) response whereas low concentrations of HLA-G augmented the allo-CTL response compared to the control without HLA-G.

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