molecular
human
reproduction

ORIGINAL RESEARCH

Allelic imbalance modulates surface expression of the tolerance-inducing HLA-G molecule on primary trophoblast cells

S. Djurisic[1], S. Teiblum[2], C.K. Tolstrup[3], O.B. Christiansen[4], and T.V.F. Hviid[1],*

[1]Centre for Immune Regulation and Reproductive Immunology (CIRRI), Department of Clinical Biochemistry, Copenhagen University Hospital (Roskilde) and University of Copenhagen, Denmark [2]Depatment of Obstetrics and Gynaecology, Copenhagen University Hospital (Roskilde) and University of Copenhagen, Denmark [3]Depatment of Obstetrics and Gynaecology, Herlev University Hospital, Denmark [4]Fertility Clinic 4071, Rigshospitalet, Copenhagen University Hospital, Blegdamsvej 9, DK-2100 Copenhagen, Denmark

*Correspondence address. Department of Clinical Biochemistry, Centre for Immune Regulation and Reproductive Immunology (CIRRI), Copenhagen University Hospital (Roskilde) and Roskilde Hospital, University of Copenhagen, 7-13 Køgevej, DK-4000 Roskilde, Denmark. Tel: +45-4732-5622; Fax: +45-4632-1615; E-mail: tvh@regionsjaelland.dk

ABSTRACT: The HLA-G molecule is expressed on trophoblast cells at the feto-maternal interface, where it interacts with local immune cells, and upholds tolerance against the semi-allogeneic fetus. Aberrant HLA-G expression in the placenta and reduced soluble HLA-G levels are observed in pregnancy complications, partly explained by *HLA-G* polymorphisms which are associated with differences in the alternative splicing pattern and of the stability of HLA-G mRNA. Of special importance is a 14 bp insertion/deletion polymorphism located in the 3′-untranslated region of the *HLA-G* gene. In the current study, we present novel evidence for allelic imbalance of the 14 bp insertion/deletion polymorphism, using a very accurate and sensitive Digital droplet PCR technique. Allelic imbalance in heterozygous samples was observed as differential expression levels of 14 bp insertion/deletion allele-specific mRNA transcripts, which was further associated with low levels of HLA-G surface expression on primary trophoblast cells. Full gene sequencing of *HLA-G* allowed us to study correlations between *HLA-G* extended haplotypes and single-nucleotide polymorphisms and HLA-G surface expression. We found that a 1:1 expression (allelic balance) of the 14 bp insertion/deletion mRNA alleles was associated with high surface expression of HLA-G and with a specific *HLA-G* extended haplotype. The 14 bp del/del genotype was associated with a significantly lower abundance of the G1 mRNA isoform, and a higher abundance of the G3 mRNA isoform. Overall, the present study provides original evidence for allelic imbalance of the 14 bp insertion/deletion polymorphism, which influences HLA-G surface expression on primary trophoblast cells, considered to be important in the pathogenesis of pre-eclampsia and other pregnancy complications.

Key words: pregnancy / HLA-G / polymorphism / 3′ untranslated region / allelic imbalance

Introduction

HLA-G is an immunomodulatory molecule with a limited tissue distribution, which, in non-pathological conditions, primarily extends to the extravillous trophoblast cells that infiltrate the decidua during pregnancy (Kovats et al., 1990; Ishitani et al., 2003; Hviid, 2006; Carosella, 2011). HLA-G is characterized by low polymorphism and an alternative splicing pattern which results in membrane-bound isoforms (HLA-G1, -G2, -G3 and -G4) and soluble isoforms (HLA-G5, -G6 and -G7), either secreted or shed from the surface (soluble HLA-G1) (Ishitani and Geraghty, 1992; Kirszenbaum et al., 1994; Hviid et al., 1998). Furthermore, several studies show an importance of HLA-G in relation to assisted reproduction and early pregnancy success (Pfeiffer et al., 2000; Fuzzi et al., 2002; Kotze

and Kruger, 2013). Low levels of soluble HLA-G (sHLA-G) in blood plasma during early pregnancy are associated with the subsequent development of pre-eclampsia, and abnormal HLA-G expression in pre-eclamptic placentas has been described in several studies (Yie et al., 2005; Hackmon et al., 2007).

HLA-G has been shown to inhibit natural killer and T-cell-mediated cell lysis through interaction with the receptors immunoglobulin-like transcript (ILT) 2, ILT4 and killer Ig-like receptor 2 DL4 (KIR2DL4) (Ponte et al., 1999; Rajagopalan and Long, 1999; Riteau et al., 2001; Menier et al., 2002). Also, HLA-G may—to some degree—induce a shift from a proinflammatory T-helper 1 (Th1) cell-mediated response toward a Th2 response (Kapasi et al., 2000; McIntire et al., 2004). Finally, HLA-G can inhibit an allocytotoxic T lymphocyte (CTL)

response, inhibit the proliferation of CD4$^+$ T-cells and induce CD4$^+$ T-cell anergy, and this may contribute to long-term immune escape or tolerance (LeMaoult et al., 2005).

The functional mRNA level of HLA-G is regulated by the rate of synthesis, mainly driven by the promoter region and the 5′-upstream regulatory region (5′-URR), in addition to the rate of degradation, stability, localization and translatability of the specific mRNA (Kuersten and Goodwin, 2003).

Also, the 3′-untranslated region (3′-UTR) of the HLA-G gene exhibits several regulatory elements including AU-rich motifs and a poly-A signal that influence mRNA stability, turnover, mobility and splicing pattern (Hviid et al., 2003; Donadi et al., 2011). The 5′-URR and 3′-UTR regions present a larger degree of variation compared with the coding region of the HLA-G locus, and may have functional significance for mRNA stability and the further processing of the HLA-G transcripts (Hviid et al., 2003; Rousseau et al., 2003; Hviid, 2006). In accordance, a reduced level of HLA-G mRNA has been observed in pre-eclamptic placentas, which correlated with the HLA-G genotype (O'Brien et al., 2001), and several potentially important polymorphisms have been described in the 5′-URR and the 3′-UTR of the HLA-G gene (Hviid et al., 1999; Ober et al., 2003; Larsen et al., 2010; Donadi et al., 2011). A 14 bp insertion/deletion (ins/del) polymorphism (rs 66554220) is the best studied in the 3′-UTR of the HLA-G gene. It was first described by Harrison et al. (1993) and has been shown to influence mRNA transcript size and stability (Hviid et al., 2003; Rousseau et al., 2003). In some cases, the presence of the 14 bp insertion allele generates a 92 bp deletion in the 3′-UTR of the HLA-G mRNAs (Fujii et al., 1994; Hviid et al., 2003). The 14 bp ins/del is associated with severe pre-eclampsia in some but not all studies (Hylenius et al., 2004; Hviid, 2006; Hviid et al., 2006; Iversen et al., 2008; Larsen et al., 2010).

The polymorphic sites at the 3′-UTR and at the 5′-URR might be in linkage disequilibrium (LD) and in combination with polymorphic sites in the coding regions, define specific promoter-, coding- and 3′-UTR haplotypes that could simultaneously influence the transcription and the stability of the HLA-G mRNA transcripts (Castelli et al., 2010). If this is the case, the haplotype should be evaluated instead of single polymorphisms to determine the significance of allelic variations of HLA-G on expression and function.

An emerging topic in the field of allele-specific expression is mRNA allelic imbalance, where regulatory polymorphisms are found in mRNA transcribed from one allele in heterozygous single-nucleotide polymorphism (SNP) sample pairs skewing the expression toward the other allele. This can give rise to higher abundance of mRNAs for one allele over the other, which contributes to variation of gene expression. Allelic imbalance is common in humans and has lately been described to be of clinical relevance in cancer (Shen et al., 2011).

Nucleotide variation in the non-coding parts of the HLA-G gene seem to influence the expression of HLA-G, and a range of studies have reported abnormal levels of HLA-G in the maternal blood and in the placenta in pre-eclampsia and in spontaneous abortions (Colbern et al., 1994; Hara et al., 1996; Yie et al., 2005; Hackmon et al., 2007; Steinborn et al., 2007; Rizzo et al., 2009; Darmochwal-Kolarz et al., 2012; Zhu et al., 2012). However, no studies have investigated correlations between HLA-G genetics, mRNA expression and the expression levels of membrane-bound HLA-G on trophoblast cells simultaneously.

To further understand the influence of the gene polymorphism on levels of transcripts and cell surface expression of HLA-G, we comparatively analyzed the presence of the 14 bp ins/del polymorphism and a range of SNPs defining specific haplotypes in the HLA-G gene, and the surface HLA-G protein expression on primary trophoblast cells from first trimester of pregnancy. We found that allelic imbalance is evident for the 14 bp ins/del polymorphism, and this is associated with significant differences in HLA-G protein expression. This is the first large and extensive study of correlations between the levels of HLA-G protein expression on the surface of trophoblast cells and the HLA-G genotype. A given correlation may prove to have functional significance, and may affect the final outcome of pregnancy.

Materials and Methods

Overview of the experimental approach

The overall experimental approach is presented in Fig. 1A. Droplet digital PCR (ddPCR, Fig. 1B) is a very sensitive and precise method for quantification of specific cDNA copies (mRNA transcripts) or specific genomic DNA sequences using a water−oil emulsion droplet technology (Huggett et al., 2013).

First trimester placental tissue

First trimester placental tissue ($n = 46$ in total) was obtained from women undergoing elective termination of normal pregnancies (gestational age week 7–11) at Copenhagen University Hospital (Roskilde and Herlev), Copenhagen, Denmark. All placental samples were analyzed for membrane-bound HLA-G1 expression and full gene sequencing of HLA-G. Forty-one samples were included in the assessment of HLA-G allelic imbalance. The Ethical Committee of Region Zealand approved the study and written informed consent was obtained from all participants. Placental tissue was either fixed in RNAlater for ddPCR studies, snap-frozen on dry ice for genotyping or used immediately to isolate trophoblast cells for flow cytometric analysis. In most cases, maternal EDTA-stabilized whole blood was obtained for reference genotyping and assessment of cross-contamination between maternal and placental (fetal) tissue (Supplementary data, Fig. S1).

Isolation of trophoblast cells from first trimester placental tissue

First trimester placental tissue was obtained within 1 h post-elective termination of pregnancy. Primary trophoblast cells were isolated as follows. Placental tissue was dissected, scraped from the membranes and washed with cold Hanks' balanced salt solution (HBSS; Gibco-Invitrogen, Carlsbad, CA, USA) to remove excess blood. The cells were transferred to 0.25% trypsin−EDTA digestion buffer (Gibco-Invitrogen) and incubated at 37°C for 10 min with shaking. Trypsin digestion was inactivated by adding 10% fetal bovine serum (FBS). This step was repeated twice. Contaminating red blood cells, tissue debris and granulocytes were removed by resuspending the cellular pellet in 20 ml HBSS, and carefully layering this over 10 ml of Lymphoprep (AXIS-SHIELD PoC AS, Copenhagen, Denmark), centrifuging at 700g for 20 min with no brake. The cellular interface containing the trophoblast cells was harvested and further enriched by depleting leukocytes with CD45 Dynabeads (Life Technologies Europe BV, Nearum, Denmark) according to the manufacturer's instructions. Enriched trophoblast cells were resuspended in fluorescence-activated cell sorter (FACS) buffer for flow cytometric analysis.

Flow cytometric analysis of trophoblast cells

Trophoblast cells were analyzed for surface HLA-G expression by flow cytometric analysis using the BD FACSCanto II flow cytometer, and the data were analyzed using BD FACSDiva™ software version 6.0 (BD Biosciences, San Jose, CA, USA). In brief, isolated trophoblasts were washed twice with ice-cold buffer phosphate-buffered saline/1% bovine serum albumin (1% BSA). The cells were incubated at 4°C for 30 min in the dark with selected monoclonal antibodies (mAb): anti-HLA-G-PE (MEM-G, Exbio, Praha, Czech Republic),
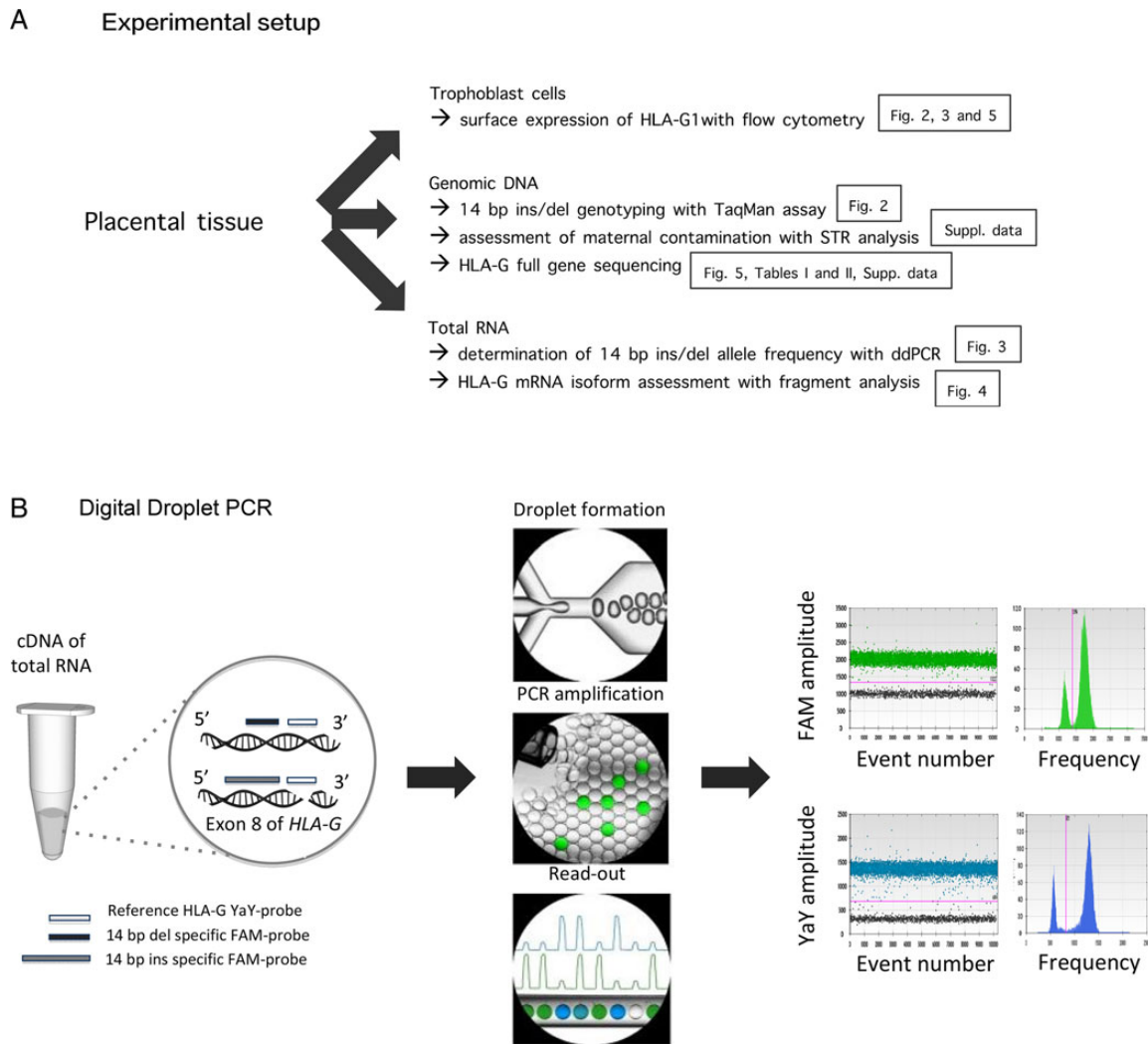
Figure I (**A**) Schematic representation of the overall experimental setup. (**B**) Depiction of the methodological approach for determining specific allele frequencies with ddPCR. ddPCR is a very sensitive and precise method for quantification of specific cDNA copies (mRNA transcripts) or specific genomic DNA sequences using a water−oil emulsion droplet technology. A PCR sample is fractionated into 15−20 000 droplets in a microtiter well. PCR amplification of the specific template molecules occurs in each individual droplet with a template. The template cDNA/DNA is diluted to an extent, where only a fraction of the droplets include a template copy. Following PCR, each droplet is analyzed in a flow cytometer to determine the fraction of PCR-positive droplets that are droplets with a specific fluorescence signal above background, in the original sample.

anti-c-erbB2-APC (eBioscience, Aarhus, Denmark), anti-CD45-FITC (BD Pharmingen, Albertslund, Denmark) and isotype-matched controls (BD Pharmingen). Following two washes, the cells were resuspended in 300 µl ice-cold FACS-buffer supplemented with BSA and analyzed. The HLA-G expression on trophoblast cells is expressed as the mean of relative fluorescence intensity determined as follows: the mean fluorescence intensity obtained with specific mAb minus the mean fluorescence intensity obtained with irrelevant isotype-matched mAb.

RNA isolation and cDNA preparations for the identification and quantification of HLA-G mRNA levels

The total RNA was extracted from first trimester placental tissue fixed in RNAlater. The concentration was determined with a Multiscan[TM] GO

Microplate Spectrophotometer (Thermo Scientific, Slangerup, Denmark), and 600 ng was used to synthesize cDNA for quantification and identification of HLA-G mRNA isoforms. In most cases, RNA integrity (RIN-values) was obtained with a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The total RNA was reverse-transcribed using the SuperScriptIII RT cDNA synthesis-kit (Invitrogen, Waltham, MA, USA), according to the manufacturer's protocol.

Extraction of genomic DNA for genotyping

Genomic DNA was extracted from EDTA-stabilized whole blood or placental tissue using the Maxwell[®] 16 Instrument (Promega, AB Branch Office, Sweden). For DNA extraction, the Maxwell[®] 16 Blood DNA Purification Kit (Promega) was used for whole blood and the Maxwell[®] 16 Tissue DNA Purification Kit for placental tissue, in both cases strictly according to the

manufacturer's protocols. DNA concentration and optical density (A260/A280) was determined using the MultiScan apparatus.

Microsatellite analysis for the detection of cross-contamination

Maternal cross-contamination of placental (fetal) tissue was assessed with Promega's Powerplex ESI 17 Pro System (Promega, Fitchburg, WI, USA) for microsatellite detection. In brief, the reaction mixture consisted of the PowerPlex® ESI 5X Master Mix, the PowerPlex® ESI 17 Pro 10X Primer Pair Mix and genomic DNA (0.5 ng) extracted from placental tissue and from maternal whole blood; the latter was used as a control. Water (amplification grade) was added to a final reaction volume of 25 μl. The thermal cycling protocol comprised an initial single-cycle denaturation step at 96°C for 2 min, then 30 cycles of 94°C for 30 s, 59°C for 2 min and 72°C for 90 s, followed by a final cycle at 60°C for 45 min before soaking at 4°C. One microliter of each amplified fragment was added to a loading cocktail containing 1 μl of CC5 Internal Lane Standard 500 Pro and 10 μl of water to a final volume of 12 μl. The samples were denatured at 95° for 3 min and immediately chilled on ice. Amplified fragments were detected using a 3500 Genetic Analyzer (Life Technologies) and analyzed with GeneMarker HID software V2.4.0 (SoftGenetics, State College, PA, USA).

HLA-G real-time PCR end-point genotyping assays

The *HLA-G* 14 bp ins/del polymorphism was assessed according to a recently published real-time RT−PCR genotyping assay using the LightCycler480 (Roche) (Djurisic et al., 2012). The primer set was as follows: 5′ GTG ATG GGC TGT TTA AAG TGT CAC C 3′ (HLA-G14-forw), and 5′ GGA AGG AAT GCA GTT CAG CAT GA 3′ (HLA-G14-rev). The 14 bp deletion allele was detected with a Cy5-probe: 5′ Cy5-GAG TGG CAA GTC CCT TTG TG-BHQ-3 3′ (HLAGdelCY5) and the 14 bp insertion allele with an FAM probe: 5′ FAM-CAA GAT TTG TTC ATG CCT TCC C-BHQ-1 3′ (HLA-G14-FAM). The +3142 polymorphism was assessed with the same primer set and thermal cycling protocol as the 14 bp ins/del polymorphism. However, probe designs were adopted from a recent publication (Bortolotti et al., 2012).

Full-gene DNA sequencing of HLA-G

Four PCR primer sets were used to amplify the coding region of *HLA-G* and upstream and downstream regions (app. 2000 bases long) that flank the coding region (Table I). The reaction mixture consisted of the GoTaq Hotstart Mastermix (Promega, Fitchburg, WI, USA) and extracted genomic DNA. The thermo cycling protocol was as follows: hot start (95°C for 2 min) for one cycle and then 40 cycles of denaturation (90°C for 30 s), annealing (66°C for 60 s), amplification (72°C for 120 s) and a final extension step (72°C for 5 min). For the full gene sequencing, a total of 17 primers (a−q) were used on the four PCR fragments (Table I). The PCR product was directly sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA) and a 3500 Genetic Analyzer (Life Technologies). The DNA sequence analyses were performed with Mutation Surveyor V4.0.9 (SoftGenetics, State College, PA, USA). As no consensus sequence for the full *HLA-G* gene exists, the reference sequence used for these analyses was assembled using the coding sequence found in the IMGT/HLA database and the 5′-URR and 3′-UTR regions presented in the NG_029039 sequence from NCBI, which also has a reference in Ensemble, ENSG00000204632.

Table I PCR primers and sequencing primers for full gene sequencing of *HLA-G*.

PCR				Sequencing	
PCR fragment	Primer name	PCR primer	Fragment size	Primer name	Sequencing primer
Fragment 1	HLAGFwd1	5′ ACA TTC TAG AAG CTT CAC AAG AAT GAG GTG G 3′	1317 bp	SEQA, fwd	5′ AGCTTCACAAGAATGAGGTGG 3′
	HLAGRev1	5′ GTA TCC AGG AAG AAG GAC CCG ACA CAG G G 3′		SEQB, fwd	5′ CCTGGTTGCAACATATAGTAAC 3′
				SEQC, rev	5′ CGTAGCCCTAAGTTTCCGTGTG 3′
				SEQD, rev	5′ CAGGAAGAAGGACCCGACACAGGTTAGG 3′
Fragment 2	HLAGFwd2	5′GAC AGA ACG CTT GGC ACA AGA GTA GC 3′	1252 bp	SEQE, fwd	5′ GAACGCTTGGCACAAGAGTAGCGG 3′
	HLAGRev2	5′ CTT CCC GTT CTC CAG GTA TCT GTG G 3′		SEQF, fwd	5′ TAAAGTCCTGCTCACCCAC 3′
				SEQG, fwd	5′ GAGTCTCCGGGTCTGGGATCCA 3′
				SEQH, rev	5′ TCTCCCAGGTAGAGGGTCTGGG 3′
				SEQI, rev	5′ TCTCCAGGTATCTGTGGAGCCAC 3′
Fragment 3	HLAGFwd3	5′ CTA CGA TGG CAA GGA TTA CCT CGC 3′	1587 bp	SEQJ, fwd	5′ CTGAACGAGGACCTGCGCTC 3′
	HLAGRev3	5′ CCCTCCTAAGGTCTGTCCTTAGCCAG 3′		SEQK, fwd	5′ CACAGCAGCCTTGGCACCAGGA 3′
				SEQL, fwd	5′ CTTAGGATGGTCACATCCAGGTGCTGC 3′
				SEQM, fwd	5′ GATGGAGTAAGGAGGGAGGATGGAGGC 3′
				SEQN, rev.	5′ CTAAGGTCTGTCCTTAGCCAGGGA 3′
Fragment 4	HLAGFwd4	5′ CAC CAT CCA CAC TCA TGG GCC 3′	1402 bp	SEQO, fwd	5′ CATCCACACTCATGGGCCTAC 3′
	HLAGRev4	5′ ATT GAA AGA GAC CTG GAA GGA GGG 3′		SEQP, fwd	5′ TTCCTCTAGGACCTCATGGCC 3′
				SEQQ, rev	5′ CTGGAAGGAGGGGAAGAGGTG 3′

Real-time RT–PCR and ddPCR for the detection and quantification of allele-specific 14 bp insertion and deletion mRNA

Initial quantification of the 14 bp ins/del allele frequencies was performed with an RT–PCR assay using the LightCycler480, with the same master mixture under the same cycling conditions as described for the previously published end-point genotyping assay but with cDNA as template. The forward primer was 5′ TACTCTCAGGCTGCAATGTGAAAC 3′ (FwdEx6-Ex8cDNA), and the reverse primer was 5′ CTGGAACAGGAAAGGT GATTGG 3′ (RevEx8cDNA). The 14 bp deletion was detected with an FAM probe: 5′ FAM-GAG TGG CAA GTC CCT TTG TG-BHQ-3 3′ (HLAGdelFAM), and the 14 bp insertion allele with another FAM probe: 5′ FAM-CAA GAT TTG TTC ATG CCT TCC C-BHQ-1 3′ (HLA-G14-FAM). Each allele-specific probe was run separately, but both reactions included a reference Yakima Yellow (YaY) probe, which detected all HLA-G transcripts: 5′ Yakima Yellow-CACCATGACCCTCTTCCTCATG CTG-BHQ-1 3′. Another more sensitive assay for which data are presented was performed using the QX100 Droplet Digital PCR system (Bio-Rad, Hercules, CA, USA), to consolidate the results. Each reaction mix consisted of the following: $2\times$ ddPCR Supermix, $20\times$ specific target primers, $20\times$ specific target FAM probes, $20\times$ reference Yay-probe, $1-3$ µl cDNA template and the mixture was adjusted with PCR grade water to a final volume of 20 µl. The thermal cycling conditions were as follows: enzyme activation, $95°C$ for 10 min (1 cycle); denaturation, $94°C$ for 30 s. (40 cycles); annealing/extension, $57°C$ for 1 min (40 cycles); and hold $98°C$ for 10 min (1 cycle). The ddPCR data were analyzed with QuantaSoft analysis software (Bio-Rad), and the quantification of either the deletion or the insertion allele was presented as the number of copies per microliter of PCR mixture.

Fragment analysis for detection of HLA-G isoforms

Primers were designed to amplify all HLA-G isoforms as follows: forward primer 5′-Fam-CACAGACTGACAGAATGAACCTGCA-3′ (Fam-5G2RNA), and reverse primer 5′-GAAGGAATGCAGTTCAGCATGA-3′ (RHG4). Each reaction mixture consisted of 13.5 µl Go Taq qPCR Master Mix (Promega, USA), 8 µl nuclease-free water, 0.5 µl forward primer and 0.5 µl reverse primer (100 pmol/µl), and 2.5 µl cDNA synthesized from 600 ng/µl RNA. The thermal cycling conditions were as follows: one cycle of $95°C$ for 2 min, and 40 cycles of $94°C$ for 30 s, $62°C$ for 1 min and one last cycle of $72°C$ for 10 min. The amplified products were heated for 3 min on a heating block and put on ice. For each analysis, 2 µl PCR product was added to 0.5 µl GeneScan-1200 Liz Size Standard (Life Technologies) and 10 µl nuclease-free water. The fragment analysis was performed with a 3500 Genetic Analyzer, and analyzed with GeneMarker software V2.4.0. Predicted sizes are depicted in Fig. 4 and in Supplementary data, Table SII.

Statistical analyses

Variables were tested for Gaussian distribution. For data assuming Gaussian distribution, an unpaired t-test (with Welch's correction, when appropriate) or one-way analysis of variance (and one-way ANOVA Welch, when the assumption of homogeneity of variance was not met), were performed. A non-parametric Kruskal–Wallis test, including the Dunn's multiple comparison *post hoc* test, was performed when Gaussian distribution was not assumed. A P-value below 0.05 was considered statistically significant. Genotype/haplotype frequencies were compared with the Fisher's exact test. Genotype frequencies were compared with the Hardy–Weinberg expectations using a χ^2 test. Clear *a priori* hypotheses were made

before performing the experiments and statistical analyses. All analyses were performed and figures prepared in GraphPad Prism v5.04 (GraphPad Software, Inc., La Jolla, CA, USA) and IBM SPSS v22 (SPSS Inc., Chicago, IL, USA).

Results

No maternal cross-contamination of the first trimester trophoblast tissue

The presence or absence of maternal cross-contamination was assessed with microsatellite analysis in 15 placental samples, focusing on samples that showed a high degree of allelic imbalance. No evidence of maternal contamination was present in any of the tested samples (Supplementary data, Fig. S1).

HLA-G1 surface expression in relation to the 14 bp ins/del genotype groups

All samples were genotyped for the 14 bp ins/del polymorphism and analyzed for HLA-G1 surface expression via flow cytometry (Fig. 2A–D). Enrichment of trophoblast cells was performed before flow cytometry analysis by depleting the CD45-positive leukocytes from the cell suspension. The gating strategy for trophoblast cells isolated from first trimester placentas in the flow cytometry analyses were as follows: initially, trophoblast cells were gated based on forward scatter and side scatter; however, the isolated pool of trophoblast cells also contain the villous type, and only the extravillous trophoblast fraction expresses HLA-G (Fig. 2A). Therefore, extravillous trophoblast cells were identified by co-expression of HLA-G (with anti-MEM-G/9 mAb) and c-erbB-2 (Fig. 2C and D).

When HLA-G surface expression on trophoblast cells was analyzed based on one-way ANOVA and according to the HLA-G 14 bp ins/del (I/D) genotype, there was no significant difference between genotype groups (Fig. 2E). However, the HLA-G del/del genotype showed the highest expression, ins/del an intermediate expression and ins/ins showed the lowest expression. Comparing the homozygous genotypes using an unpaired t-test, the ins/ins genotype was shown to express a significantly lower HLA-G level than the del/del genotype ($P = 0.046$). Samples that were heterozygous for the null allele (depicted as unfilled data points on Fig. 2E) were included. The argument for including the four heterozygous samples with the null allele in the two homozygous groups (del/del and ins/ins) is based on the assumption that the null allele does not contribute to HLA-G1 protein on the cell surface. A relatively low level of surface expression of the four null-heterozygous samples was also observed. Moreover, the three N:D samples exhibited a higher surface expression than the N:I sample, which further supports the approach. One sample (Sample 9) was considered an outlier and omitted from the analysis.

Defining HLA-G haplotypes and genotypes

Based on the gene polymorphisms in the 5′-URR, the coding region and 3′-UTR of the HLA-G gene, 26 different extended haplotypes were defined and named according to previous studies (Castelli *et al.*, 2011) (Supplementary data, Table SIA and B). Furthermore, haplotypes defining the promoter/5′-URR, the coding region or the 3′-UTR were also determined for all samples following previous studies by Castelli *et al.* (Castelli *et al.*, 2011; Martelli-Palomino *et al.*, 2013) (Table III and
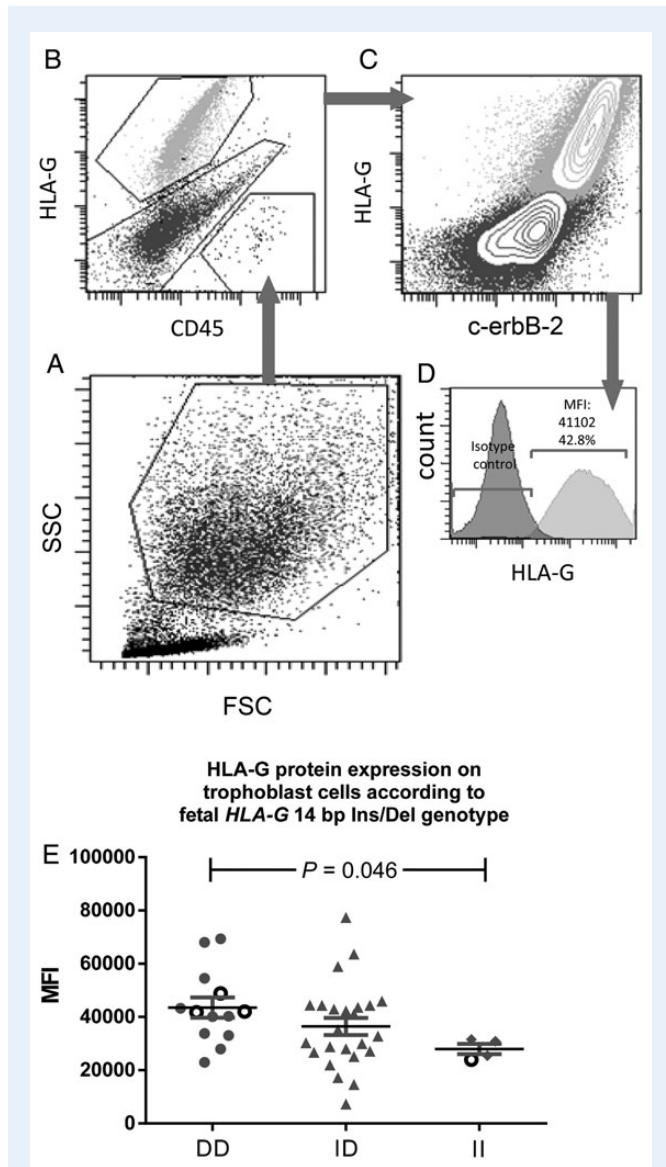
Figure 2 Gating strategy for trophoblast cells isolated from first trimester placentas. (**A**) Trophoblast cells were initially gated based on forward scatter and side scatter; however, the isolated pool of trophoblast cells also contain the villous type, and only the extravillous trophoblast fraction expresses HLA-G. (**B**) Enrichment of trophoblast cells was performed before flow cytometry analysis by depleting the CD45 leukocytes; the CD45-positive cells are shown for reference. (**C** and **D**) Extravillous trophoblast cells were identified by co-expression of HLA-G (with MEM-G/9 mAb) and c-erbB-2. (**E**) HLA-G surface expression according to the 14 bp *HLA-G* ins/del (I/D) genotype (one-way ANOVA, not significant). Comparing the homozygous genotypes, the *HLA-G* ins/ins genotype was shown to express a significantly lower HLA-G level than the del/del genotype ($P = 0.046$, unpaired *t*-test). Samples heterozygous for the null allele (depicted as unfilled data points) were included. Data are shown as mean $\pm$ SEM. One sample (Sample 9, not depicted) was considered an outlier and omitted from the analysis. MFI, mean fluorescence intensity.

Supplementary data, Tables SIA and SII). The null allele was defined by a deletion at position $+814$ in exon 3 leading to a premature stop codon that prevents translation of the full-length HLA-G1.

Allelic balance/imbalance of the 14 bp ins/del HLA-G polymorphism is associated with HLA-G1 surface expression on trophoblast cells

To investigate the effect of the 14 bp ins/del on allelic expression or frequency at the mRNA-level, we performed allele-specific ddPCR on cDNA synthesized from total RNA extracted from 41 individual placental tissues. The concentration of the specific HLA-G 14 bp allele was acquired as copies/µl and a ratio between the 14 bp ins/del allele-specific probe and a reference probe could be calculated. Each sample was analyzed in duplicate or triplicate with an allele-specific probe ('Ins' for 14 bp insertion or 'Del' for 14 bp deletion) in combination with a reference probe that covered all transcripts, and a ratio between the two alleles was calculated after adjustment with the reference. This adjustment was conducted to ensure that the proportion of allele-specific transcripts would sum up to 1 and that a true ratio was reflected in the end concentration of total transcripts. The samples categorized with balanced allelic expression have a mean of 0.50 and a standard deviation of 0.01. A 50:50 allelic expression was considered for single allele ratios 0.50 $\pm$ SD 0.03. Allelic ratios that strayed from a mean of 0.50 $\pm$ SD 0.03 were considered imbalanced, and the samples were grouped accordingly (Fig. 3A). The ddPCR results revealed different degrees of expression. An interesting pattern was observed regarding the relative levels of all the 14 bp ins HLA-G transcripts (the -92 bp transcripts excluded) versus all the 14 bp del transcripts when analyzing the trophoblast samples heterozygous for the 14 bp ins/del polymorphism (Fig. 3A). One group of 14 bp ins/del heterozygous trophoblast samples had an allelic frequency very close to 1:1 ($n = 8$), while another group had a higher fraction of the 14 bp del allele transcript compared with the 14 bp ins allele transcript ($n = 12$). In only three trophoblast samples, a slight deviation in favor of the 14 bp ins allele was observed (Fig. 3A). The differences were statistically significant ($P = 0.004$, paired *t*-test). The same was observed for the HLA-G1 mRNA isoform, when data from the HLA-G RT−PCR fragment analysis were analyzed ($P = 0.006$, paired test; Fig. 3B). The allelic expression of the 14 bp ins/del alleles was confirmed with real-time RT−PCR, although the method was less sensitive than ddPCR (data not shown).

When subdividing the heterozygous group according to the specific 14 bp ins/del allele frequency, a significantly higher surface expression was observed for samples with an allele frequency of 1:1, compared with samples where the allele frequency was skewed towards a higher prevalence of either ins or del ($P = 0.0005$, one-way ANOVA; Fig. 3C).

Allelic balance of the 14 bp ins/del HLA-G polymorphism is highly represented in samples containing the G*01:01:03:01 allele

The question whether the observed allelic imbalance was associated with a specific *HLA-G* genotype was addressed by whole-gene sequencing, and samples heterozygous for the 14 bp polymorphism were further evaluated for specific SNPs and extended promoter-, coding- and 3′-UTR haplotypes. No significant differences between the two ins/del groups (subdivided according to allelic balance/imbalance) were found for the 5′-URR haplotype (Table II), and all except one sample was characterized by the PROMO-010102a promoter haplotype as described by
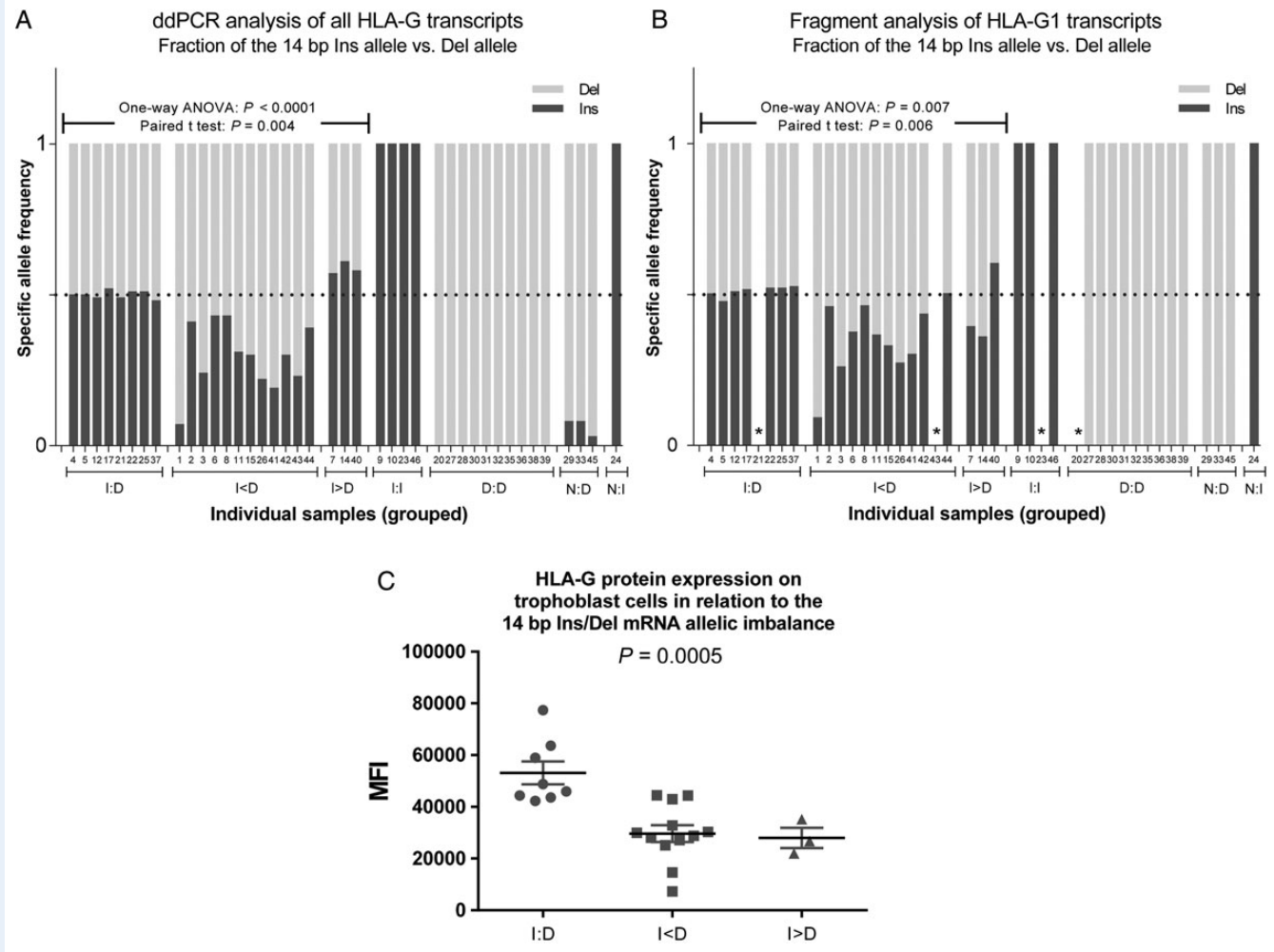
Figure 3 Specific 14 bp ins/del allele frequencies in individual samples determined in all isoform transcripts and in HLA-G1 transcripts only. All samples were grouped first on the basis of 14 bp ins/del heterozygosity/homozygosity, and next, heterozygous samples (with exception of the null allele) were grouped according to allelic balance (I:D) or imbalance (I>D and I < D). Dotted lines represent an exact 1:1 mRNA allele expression. (**A**) Determination of 14 bp ins/del allelic frequencies in all transcripts by ddPCR. Differences in the ins/del allele ratio were significant between the three heterozygous groups ($P < 0.0001$, one-way ANOVA), and the 14 bp deletion allele was significantly more abundant than the insertion allele overall ($P = 0.004$, paired t-test). Low abundance of ins transcripts is evident in samples with the null allele. (**B**) Determining the 14 bp ins/del allelic frequencies in HLA-G1 transcripts by semi-quantitative fragment analysis consolidated the ddPCR results, although the ins transcripts are absent in the G1 pool for the null allele. Quantification was performed by estimating peak heights, and missing data are indicated with asterisks. (**C**) Subanalysis of samples heterozygous for the 14 bp ins/del. A significantly higher surface expression was evident in samples with allelic balance (I:D) compared with samples exhibiting allelic imbalance (I > D and I < D) ($P = 0.0005$, one-way ANOVA; data are presented as the mean $\pm$ SEM); D, 14 bp deletion; I, 14 bp insertion; N, null allele.

Castelli *et al.* (2011). This finding indicated that the 14 bp ins/del allelic imbalance was not a result of different transcriptional rates of the two alleles. Instead, three SNPs (+188 C/T located in intron 1, +748 A/T in exon 3 and the 3027 C/A in the 3′-UTR) were significantly different between the allelic balanced and imbalanced groups ($P = 0.025$, 0.021 and 0.021, respectively, Table II). When analyzing the data according to promoter, coding and 3′-UTR haplotype, the 3′-UTR haplotype UTR-7 was significantly more abundant in the balanced group ($P = 0.021$, Table II). These results may merely be a reflection of the extended haplotype H16 (12), which is represented in the G*01:01:03:01 allele, as the presence of this allele is significantly different between the two subgroups ($P = 0.021$, Table II).

Estimation of HLA-G isoform mRNA distribution reveals inter-sample variations and a higher G3 abundance in 14 bp deletion transcripts

Seven transcript isoforms are described for HLA-G. To further investigate whether the ddPCR analysis performed on all transcripts reflects primarily the G1 transcript, or the sum of transcripts, a full isoform profiling was performed with RT–PCR fragment analysis on 37 placental samples. In this assay, RT–PCR primers were designed to amplify all HLA-G mRNA isoforms (Fig. 4A). Most samples were shown to express all alternatively spliced HLA-G mRNA isoforms that corresponded to predicted sizes

TABLE II Differences in *HLA-G* genotype/haplotype frequencies found between subgroups with differential expression of the 14 bp ins/del HLA-G mRNA transcripts: I:D (samples exhibiting allelic balance) and I>D or I<D (samples exhibiting allelic imbalance).

		Balance *I:D*	Imbalance *I > D or I < D*	Total	*P*-value
+188 SNP	CC	4	1	5	0.025
	CT	3	13	16	
	Total	7	14	21	
+748 SNP	AA	3	14	17	0.021
	AT	4	1	5	
	Total	7	15	22	
+3027 SNP	CC	3	14	17	0.021
	CA	4	1	5	
	Total	7	15	22	
3′-UTR haplotype	UTR-7/OTHER	4	1	5	0.021
	Other	3	14	17	
	Total	7	15	22	
Coding haplotype	G*01:01:03:01/OTHER	4	1	5	0.021
	OTHER	3	14	17	
	Total	7	15	22	
PROMO haplotype	PROMO-G010102a/OTHER	7	14	21	1
	OTHER	0	1	1	
	Total	7	15	22	

The comparison was performed with Fisher's exact test, and exact *P*-values are presented.
SNP, single-nucleotide polymorphism; 3′ UTR, 3′-untranslated region.

TABLE III A schematic overview of the grouping of extended *HLA-G* haplotypes, which includes 54 SNPs and the 14 bp ins/del polymorphism. Extended haplotypes (H1–H25) were combined from promoter, coding and 3′-UTR haplotypes.

| Position	Promoter 5′URR haplotype -13 -1179 -1155 -1140 -1138 -1121 -762 -725 -716 -689 -666 -646 -633 -540 -533 -509 -486 -483 -477 -443 -400 -391 -369 -201 -56	Coding haplotype 15 36 99 126 130 147 188 292 297 372 706 748 755 814 871 902 1016 1019 1054 1590 1799 1827	3′UTR haplotype 14-bp 3003 3010 3027 3035 3142 3187 3196	*HLA-G* lineage
H01	G A G A A C C C T A G A G A A C A A C G G G C G C PROMO-G010101a	G G G C T T C A G G C A C C G T C T G C C G 01:01:01:01	D T G C C C G C UTR-1	HG010101 (HG010101a)
H03	G A G A A C C C T A G A G A - C A A C G G G C G C PROMO-G010101f	G G G G C T C A G G C A C C G T C T G C C G 01:01:01:04	D T G C C C A C UTR-6	HG010101 (HG010101b)
H02	G A G A A C C G T A G A G A A C A A C G G G C G C PROMO-G010101b	G G A G C T C A G G C A C C G T C T G C C G 01:01:01:05	D C G C C C A C UTR-4	HG010101 (HG010101c)
H04	G A G A A T C G T A G A G A A C A A C G G G C G C PROMO-G010101c			
H10	A G G T A C T C G G T A A A A C C A G G G G A A C PROMO-G010102a	A A G G C C T A G A T A C C G T C C G C C G 01:01:02:01	I T C C C G A G UTR-2	HG010102
H11		A A G G C C T A G A T A C C G T C C G C T G 01:06		
H13		A A G G C C T A G A T A C C G T T C G C C G 01:01:02:02		
H15		A A G G C C T A G A T A C C G T C C G C T G 01:06	I T G C C G A G UTR-8	
H16	A G G T A C T C G G T A A A A C C A G G G G A A C PROMO-G010102a	A A G G C C C A G A C T C C G T C C G C C G 01:01:03:01	I T C A T G A C UTR-7	HG010103
H20	G G G A G C C T T A G A G I A G A A G G A A A G T PROMO-G0103a	G G G G C C C T G G C A C C G T C C T T C G 01:03	I T C C T G A C UTR-5	HG0103
H21	G G G A G C C T T A G G G G A A G A A G G A A A G T PROMO-G0103e			
H23	A G A A A C T C G G T A A A A C C A G G G G A A C PROMO-G0104a	A A G G C C T A G A C A A C G T C C G C C G 01:04:01	D T C C C G A C UTR-3	HG0104
H24		A A G G C C T A G A C A A C G T C C G C C A 01:04:04		
H25	A G A A A C T C G G T A A A A C C A G A G G A A C PROMO-G0104b	A A G G C C T A G A C A A C G T C C G C C G 01:04:01		
H07	G A G A A C C C T A G A G A A C A A C G G G C G C PROMO-G010101a	G G G C T T C A G G C A C C G T C T G C C G 01:01:01:01	D T G C C C A C UTR-6	-
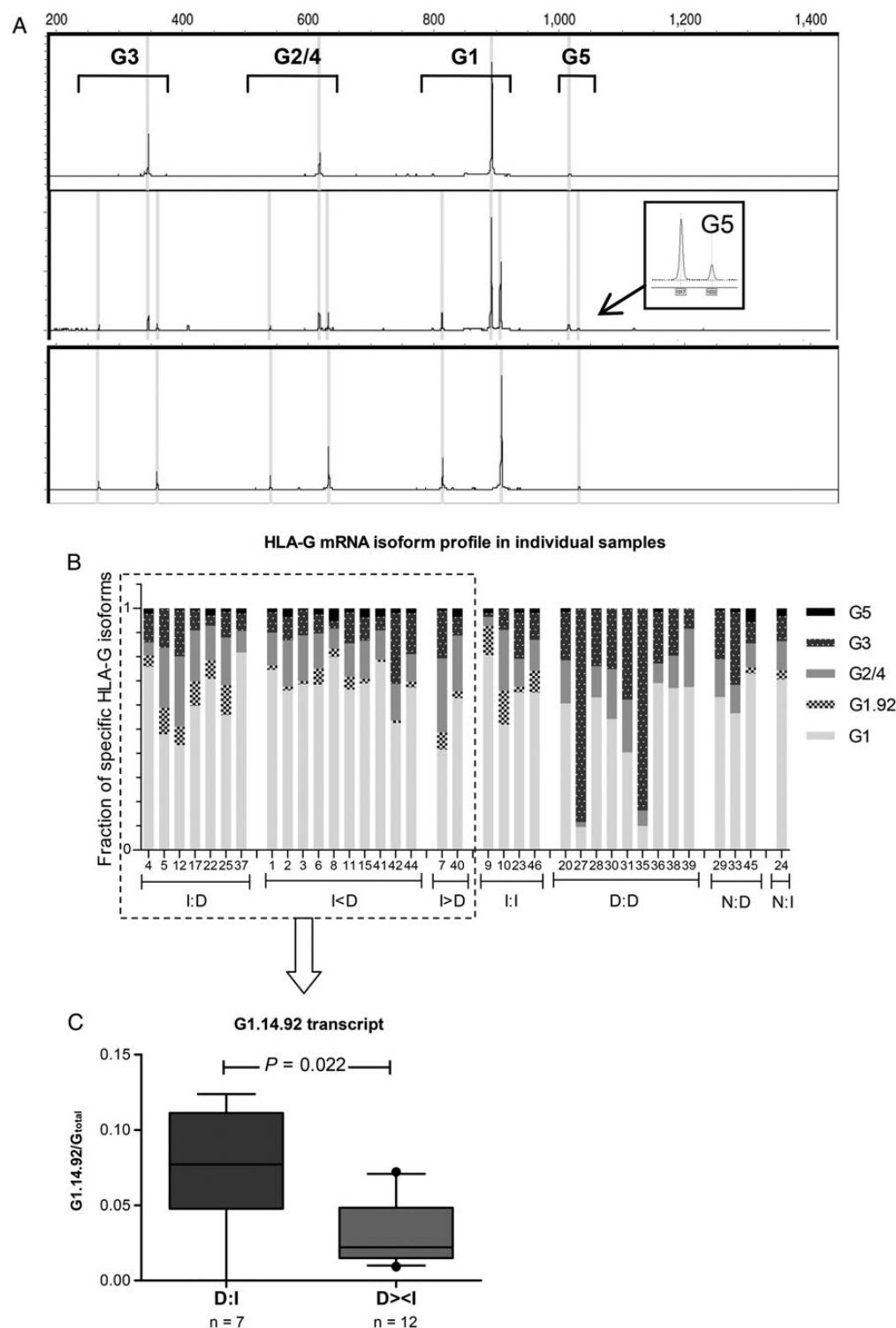
Figure 4 Determination of HLA-G isoform distribution with RT–PCR fragment length analysis. (**A**) Examples of electropherograms of HLA-G RT–PCR fragments representing different HLA-G mRNA isoforms of different length. Quantification was performed by estimating peak height. (**B**) Individual samples show a high variation in the distribution of HLA-G isoforms. (**C**) The 92 bp transcript is significantly more abundant in the allelic balanced group, compared with the imbalanced group ($P = 0.022$, Mann–Whitney), which supports the presence of a lesser amount of insertion transcripts in the latter. The median with 10–90 percentiles is depicted. (**D**) HLA-G isoform transcripts G1–G5 according to the 14 bp ins/del genotype. The G2 and G4 isoforms are grouped due to similar expected sizes. Isoform distribution was assessed based on the Kruskal–Wallis test; asterisks mark P-values determined with Dunn's post-test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). The median with 10–90 percentiles is depicted. D, 14 bp deletion; I, 14 bp insertion; N, null allele. G1.14.92, HLA-G1 isoform containing the 14 bp insertion and the −92 bp alternative transcript.
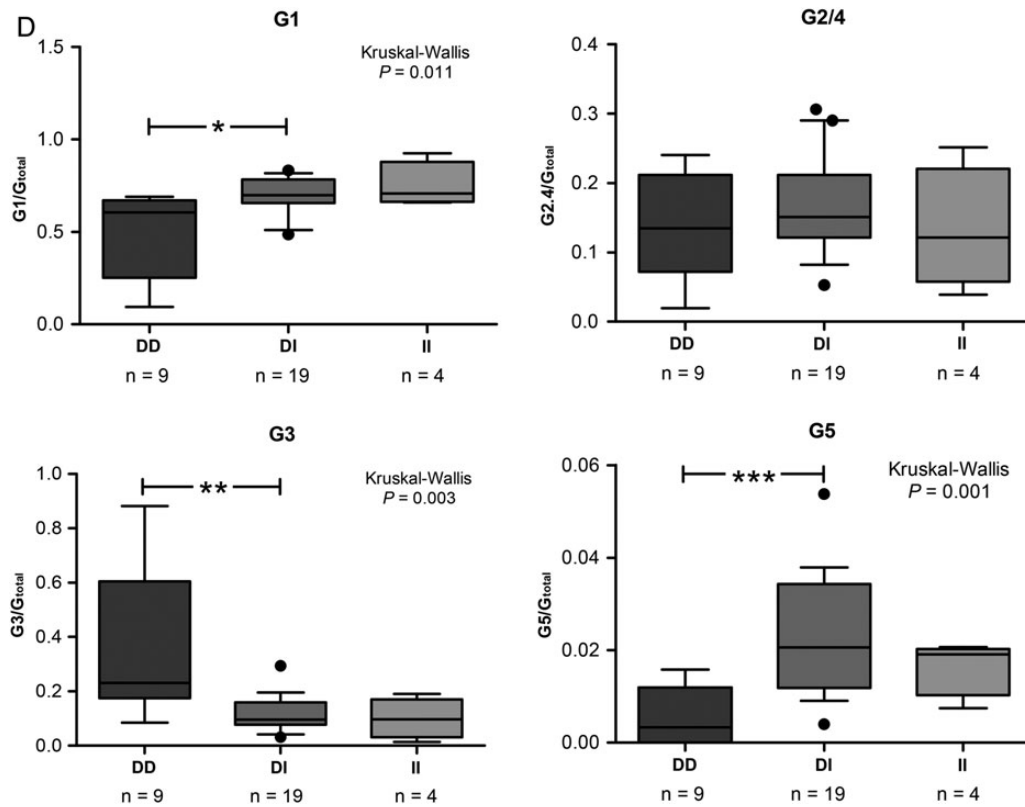
Figure 4 *Continued.*

(Fig. 4A and B and Supplementary data, Table SII). Estimating the peak height enabled a semi-quantitative evaluation of the relative isoform distribution. For simplicity, only the G5, G3, G2/4 and G1 isoforms (including the 92 bp splice-out transcripts) were evaluated, although additional peaks, possibly representing other isoforms such as G6 and G7, were readily noticed (Supplementary data, Table SII). First, the 14 bp ins/del allele frequencies in the full-length G1 isoform transcripts were assessed, showing a significantly higher expression of the 14 bp del allele, which confirmed the findings from the complementary ddPCR specific for the 14 bp insertion and deletion allele in all transcripts ($P = 0.006$, Fig. 3B). Interestingly, no significant difference was found in the total amount of any of the isoforms, G1, G2/4, G3 and G5 between the allelic balanced and imbalanced heterozygous groups (data not shown).

In addition to allelic imbalance for the 14 bp insertion/deletion being negatively associated with the insertion transcript, we found that this was also the case for the alternative transcript lacking 92 bp ($P = 0.022$, Mann–Whitney test) (Fig. 4C). HLA-G1 represented the majority of transcripts in most samples. In a few cases, HLA-G3 was the dominant isoform (Samples 27 and 35, Fig. 4B). Interestingly, the HLA-G RT–PCR fragment length analysis revealed that in samples heterozygous for the null allele the 92 bp transcript was still present. A previous study has suggested that out-splicing of the 92 bp from G2/4 only occurs for the G*01:01:03 allele; however, the presence of this alternative transcript was also observed for other alleles in this study (Supplementary data). Second, the proportions of G1, G3, G2/4 and G4 were evaluated according to the 14 bp ins/del genotype. Notably, a

higher relative abundance of G3 was associated with the del/del genotype ($P = 0.003$, Kruskal–Wallis test, Fig. 4D), while G5 and G1 (including the 92 bp transcripts) were associated with the ins/del genotype ($P = 0.001$ and 0.011, respectively, Kruskal–Wallis test; Fig. 4D). No significant difference was observed for the G2/G4 transcript.

Analysis of SNPs spanning the 5′-URR and 3′-UTR shows single effects on protein level

The *HLA-G* gene was DNA sequenced and evaluated for 54 SNPs and the 14 bp ins/del polymorphism for all samples. All of the genotyping results were confirmed by DNA sequencing during haplotype profiling. Where sample size allowed it, each SNP was confirmed to be in Hardy–Weinberg equilibrium (data not shown). The four genotypes, −725, +3010, +3142 and +3187 with more than three samples in each genotype group were tested using the one-way (between groups) ANOVA. The statistical comparison was further based on a comparison between the two homozygous genotypes for each of the four SNPs (unpaired test with Welch's correction when appropriate).

For the other SNPs, the two represented genotypes for each SNP were compared (unpaired test with Welch's correction when appropriate). The P-values were adjusted by multiplying with the number of possible genotypes (Pc = corrected P-value).

Of the *a priori* selected *HLA-G* SNPs, four could be evaluated for all three genotypes. Three of these showed an overall significant difference
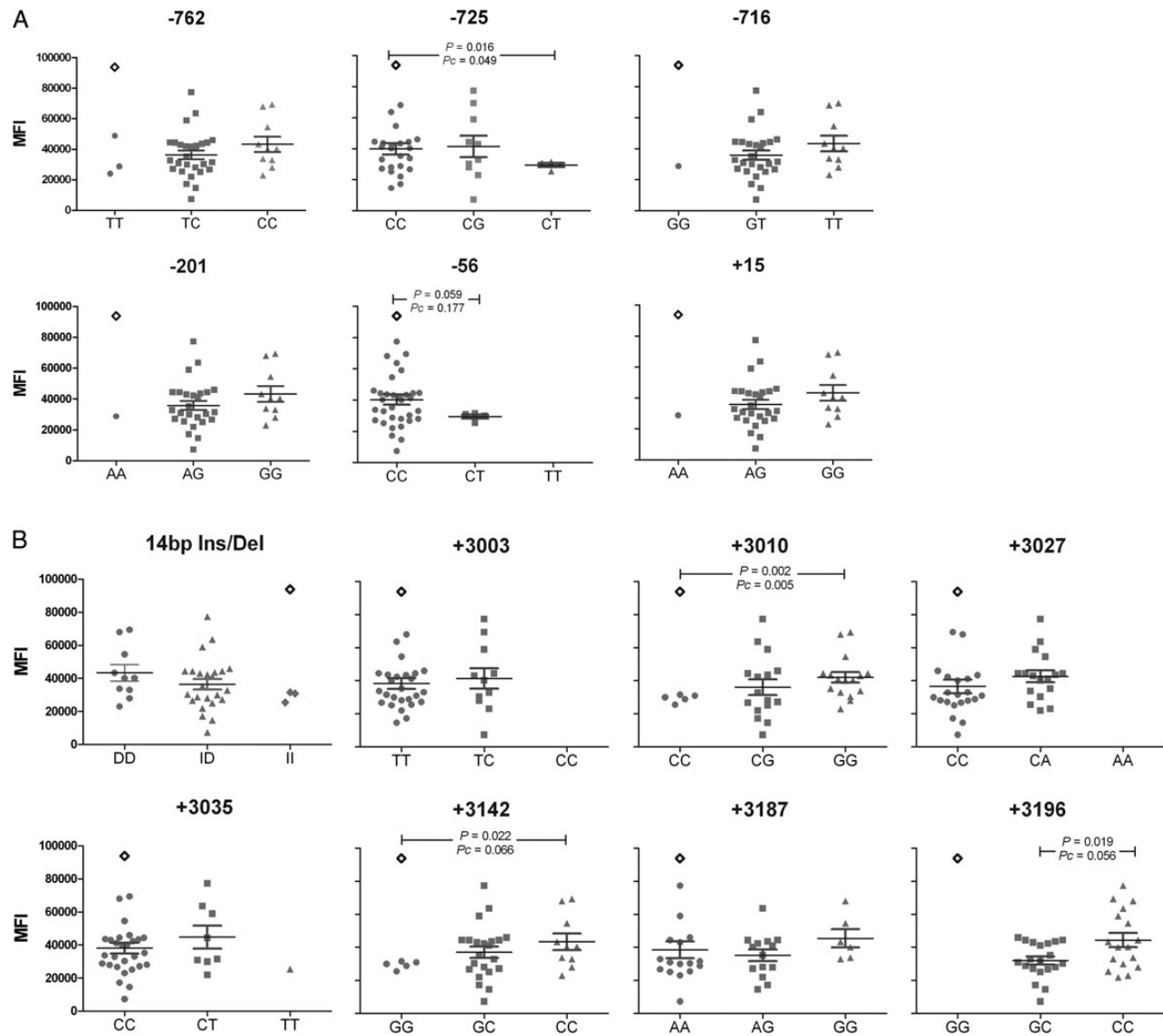
Figure 5 Association of selected SNPs in the 5′-URR and 3′-UTR of the *HLA-G* gene with membrane-bound HLA-G1 expression measured as the MFI. Samples that were heterozygous for the null allele were omitted from the analysis. (**A**) *HLA-G* promoter region/5′-URR polymorphisms. (**B**) *HLA-G* 3′-UTR polymorphisms. (**C**) Analysis according to the *HLA-G* 3′-UTR haplotype. One sample (Sample 9) was considered an outlier and not included in the analyses, although it is shown in the figures for reference. The four genotypes, −725, +3010, +3142 and +3187, with more than three samples in each genotype group were tested using the one-way (between groups) ANOVA, and the −725, +3010 and +3142 SNPs were significantly different after Welch's correction (not shown in graph). The statistical comparison was further based on an unpaired *t*-test with Welch's correction, when appropriate, comparing the two homozygous genotypes for each of the four SNPs. For the other SNPs, the two represented genotypes for each SNP were compared with the use of an unpaired *t*-test (with Welch's correction when appropriate). The *P*-values were adjusted by multiplying with the number of possible genotype outputs (*Pc* = corrected *P*-value). Both the adjusted and the non-adjusted *P*-values are shown, and the data are presented as mean ± SEM.

between HLA-G protein expression on trophoblast cells. The −725 tri-allelic SNP in the 5′-URR ($P = 0.030$, one-way ANOVA, Welch), the +3010 SNP ($P = 0.004$; one-way ANOVA, Welch) and the +3142 SNP ($P = 0.014$; one-way ANOVA, Welch) in the 3′-UTR (Fig. 5A and B, only *P*-values from the post *t*-tests are depicted in the figure). Furthermore, in the 3′-UTR, the +3196 C/C genotype was significantly associated with a higher HLA-G surface expression ($P = 0.019$, $Pc = 0.056$, unpaired *t*-test). The HLA-G surface expression on

trophoblast cells was higher for the UTR-1 haplotype compared with the UTR-2 haplotype, although not statistically significant, most likely due to sample size (Fig. 5C). A single sample (Sample 9) represented the H10/H10 extended haplotype, which includes the UTR-2/UTR-2 3′-UTR haplotype. Of all the samples, this one sample exhibited the highest surface expression and was, due to inconsistency in the genotype/surface expression association assessment, considered an outlier and excluded from the SNP/14 bp analysis (Fig. 5).
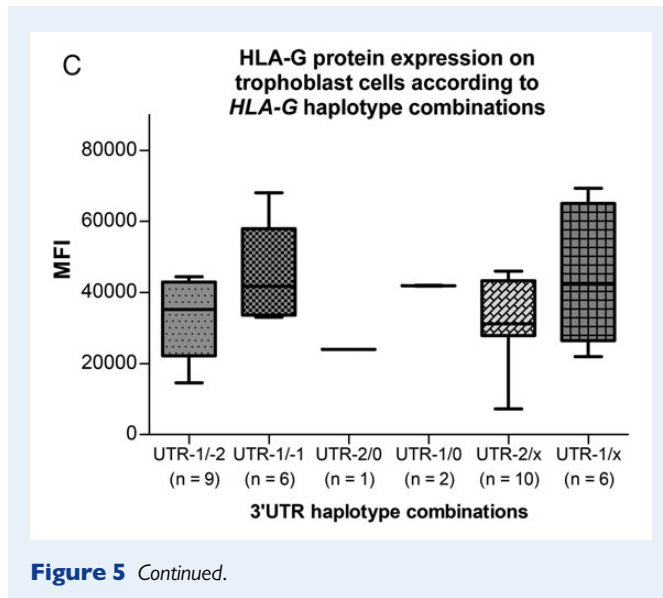
Figure 5 *Continued*.

Discussion

The main finding of the present study is that the *HLA-G* 14 bp ins/del polymorphism exhibits allelic imbalance, also described as differential mRNA expression of the two alleles. Allelic imbalance was shown to have functional consequences, leading to a significantly lower surface expression on primary trophoblast cells, while allelic balance was associated with significantly higher surface expression. Accumulating evidence emphasizes the importance of HLA-G expression on extravillous trophoblast cells at the feto-maternal contact zone during first trimester of pregnancy, and possibly already at the time of implantation (Liu *et al.*, 2013; Lombardelli *et al.*, 2013; Dahl *et al.*, 2014; Lynge Nilsson *et al.*, 2014). In essence, maternal acceptance of the semi-allogenic fetus is attributed to the effects of HLA-G on long-term immune modulation or tolerance (LeMaoult *et al.*, 2005; Hviid, 2006). Although studies show that HLA-G is not genetically imprinted and that bi-parental alleles are co-dominantly expressed (Hashimoto *et al.*, 1997; Hviid *et al.*, 1998), other factors may regulate gene expression levels so that the allelic frequency is not always 1:1, a phenomenon described as allelic imbalance. *Cis*-acting polymorphisms may alter regulation for one allele through a change to promoter/enhancer regions or through 3′-UTR polymorphisms that affect mRNA stability or microRNA binding, and this may increase or decrease the expression of one allele to a lesser or higher degree, resulting in imbalance. Allelic imbalance might have functional and thus clinical significance, as observed in studies with cancer (Shen *et al.*, 2011; Antczak *et al.*, 2013). In accordance, the significantly lower HLA-G protein expression associated with allelic imbalance in the 14 bp ins/del polymorphism presented in the current study may influence the tolerance-inducing function of HLA-G. In heterozygous sample pairs, allelic imbalance was observed as specific expression skewed toward either the 14 bp insertion or the deletion allele. Differential expression has been indicated in previous studies, which have shown that the 14 bp deletion allele is present at higher concentrations in cells (Hviid *et al.*, 2003; Rousseau *et al.*, 2003), although these studies failed to distinguish that an actual balanced group also existed, and importantly, that allelic imbalance versus allelic balance has consequences for the levels of HLA-G surface expression.

Our previous study in HLA-G1-transfected cells indicated that the 14 bp deletion transcript is the least stable (Svendsen *et al.*, 2013). In line with this finding, the higher abundance of the deletion transcript may compensate for the seemingly lower mRNA transcript stability of this allele. A lower expression of insertion transcripts found in samples with allelic imbalance is equivalently observed as lower expression of the −92 bp transcript variant in our study. Apart from the 14 bp ins/del, no extended haplotype or single polymorphism was associated with allelic imbalance. Interestingly, allelic balance, in contrast, was associated with three genotypes: +188 C/C, +748 A/A and +3027 C/C. All represented in the G*01:01:03 allele, which also represents the 3′-UTR haplotype, 7-UTR, discussed below. The G*01:01:03 allele has been associated with reduced sHLA-G plasma levels (Rebmann *et al.*, 2001), which could indicate a different effect of this allele on cell surface expression versus circulating levels. Despite the fact that the coding region of G*01:01:03 represents synonymous nucleotide variations, commonly perceived as neutral, two out of three SNPs that differed between the allelic balanced and imbalanced groups, reside in intron 1 and exon 3 of the coding region. Indeed, recent data show that synonymous nucleotide variations can induce significant changes in the mRNA folding, thereby affecting higher-order structure, and may in fact not be neutral at all (Shabalina *et al.*, 2013).

Studies on *HLA-G* polymorphisms are mainly focused on the 14 bp ins/del due to a well-documented association with pregnancy complications, autoimmune disease, transplantation and cancer (LeMaoult *et al.*, 2005; Dahl *et al.*, 2014; Sabbagh *et al.*, 2014). However, several recent studies have shown that other polymorphic sites, primarily residing in the 3′-UTR of the *HLA-G* gene, are associated with differential HLA-G solubility levels in non-pregnant donors (Hviid, 2004; Chen *et al.*, 2008; Di Cristofaro *et al.*, 2013; Martelli-Palomino *et al.*, 2013) and variable levels of membrane-bound HLA-G on myeloid antigen-presenting cells (Locafaro *et al.*, 2014). Although these associations have been demonstrated for 3′-UTR polymorphisms, we wanted to explore the significance of 3′-UTR, coding and 5′-UTR nucleotide variations on HLA-G surface expression on trophoblast cells. To this end, the full HLA-G gene was DNA sequenced for each placental sample. We found similar trends as a recent study investigating associations between HLA-G genotypes and sHLA-G in plasma (Martelli-Palomino *et al.*, 2013), more specifically in relation to the 14 bp ins/del, +3003, +3010, +3142 and +3187 SNPs, but not for the +3027 and +3035 SNPs. In the Martelli-Palomino *et al.* (2013) study, no association was found between sHLA-G and the +3196 SNP otherwise speculated to be involved in mRNA stability. In our study, the differences between SNP genotypes were statistically significant for +3010, +3142 and +3196, and interestingly, the +3010 C and +3142 G alleles, which presented with the lowest HLA-G surface expression in the current study, were recently linked to systemic lupus erythematosus (SLE) susceptibility (Lucena-Silva *et al.*, 2013).

The transcription rate of HLA-G is regulated by the 5′-URR, but only the tri-allelic −725 C/G/T SNP has been associated with differences in HLA-G expression, where the presence of the G variant significantly increases the HLA-G transcription rate in a JEG-3 cell line (Ober *et al.*, 2006). We found no significant difference in the HLA-G cell surface expression on trophoblast cells between the −725 C/C genotype and the −725 C/G genotype, possibly due to high surface expression variation in the −725 C/G group. However, we did find a significant higher HLA-G surface expression for the −725 C/C compared with the −725 C/T

genotype. The −56 C/T genotype in the 5′-URR showed a trend for a lower HLA-G cell surface expression compared with the −56 C/C genotype. Interestingly, the −56 SNP is located in a Ras Response Element previously described as a binding site for the repressor factor RREB1 (Ras Responsive Element Binding 1) implicated in transcriptional repression of HLA-G expression (Flajollet et al., 2009).

Alleles from the 3′-UTR polymorphic sites are associated with HLA-G production and are in strong LD with each other, illustrating a scenario in which their influence may not be mutually exclusive (Castelli et al., 2010). Indeed, the 14 bp ins/del and the 7 3′-UTR SNPs earlier described exist in different combinations generating 8 different 3′-UTR haplotypes, UTR-1 to UTR-8 (Castelli et al., 2010). Individuals carrying the UTR-1/UTR-1 genotype have significantly higher sHLA-G levels than the UTR-2/UTR-2 haplotype (Di Cristofaro et al., 2013). We observed the same tendency for membrane-bound HLA-G: UTR-1 exhibited higher surface expression than UTR-2. This applied for UTR-1 homozygous samples, UTR-1 and UTR-2 heterozygous samples and UTR-1 and UTR-2 samples heterozygous for the null allele. Interestingly, our allelic imbalance study showed that UTR-7 was significantly more represented in the allelic balanced samples, and thus, UTR-7 was associated with the highest HLA-G cell surface expression level. The UTR-7 haplotype is characterized by the presence of G at position +3142 and, conversely, has been described as a low-producer haplotype by others, but only for sHLA-G in plasma (Martelli-Palomino et al., 2013). The same study indicated UTR-5 as a low producer haplotype associated with low sHLA-G levels in blood plasma compared with other 3-′UTR haplotypes (Martelli-Palomino et al., 2013), which is in direct conflict with another study, where UTR-5 exhibited the highest sHLA-G in donor blood serum, even despite individuals being heterozygous (Di Cristofaro et al., 2013). In support of the latter findings, the UTR-5 haplotype is characterized by the −56 T and −725 T alleles in the 5′-URR, and high-soluble expression levels have previously been reported for these alleles (Jassem et al., 2012). Although further studies are needed, it should be stressed that single polymorphisms in the 3′-UTR and the combined 3′-UTR haplotypes could have different effects on soluble versus cell surface expression of HLA-G.

In the placenta, the primary transcript encoding the full-length HLA-G1 comprises the majority of all seven alternatively spliced HLA-G transcripts known, while the G5 transcript encoding the soluble form is the least abundant (Blaschitz et al., 2005). These previously reported findings are supported in the present study. It is still not clear, however, exactly, which of the isoforms of HLA-G are responsible for its recognized immunomodulatory functions. In a study with single embryos, the predominant forms were G3 and G4, while the other alternatively spliced transcripts were more varied in their expression (Yao et al., 2005). We did not find a pattern supporting that a specific isoform distribution is associated with allelic balance/imbalance but we did, however, note that the G3 isoform is significantly more abundant for the del/del 14 bp genotype; this observation has been previously reported (Hviid et al., 2003). The significance of higher abundance of G3 transcripts is unclear, although isoform-specific functions are likely to exist. A recent study re-evaluated the specificity of the antibody MEM-G/9, which is commonly used to detect the native membrane-bound G1 and soluble G5 protein isoform, and showed that it also detects G3 on the cell surface (Zhao et al., 2012). Thus, a fraction of the HLA-G cell surface expression detected in the current study could represent the G3 protein isoform. Transcriptional regulation is likely to have an impact on the overall distribution of HLA-G mRNA and protein isoforms, and future studies are warranted to elucidate whether isoform distribution is associated with specific HLA-G alleles/genotypes, a shift in isoform repertoire—as observed in embryos—during the course of pregnancy, and, importantly, potential isoform-specific functions.

It is generally accepted that 5′-URR and 3′-UTR polymorphisms might influence gene expression. It is not clear, however, whether the allelic imbalance observed in the current study reflects regulation at the transcriptional or post-transcriptional level. The extended HLA-G promoter haplotypes would have the most prominent effect in the former case, while the 3′-UTR haplotypes would have more impact in the latter. Most likely, HLA-G expression is tightly regulated at both the transcriptional level and at the level of mRNA. Nonetheless, this study shows that HLA-G1 (and possibly HLA-G3) is highly expressed on the surface of trophoblast cells that are allelic balanced for the 14 bp ins/del, and with expression levels that even exceed those of the del/del genotype, often associated with highest expression. If previous findings hold true that sHLA-G is immune-modulating in a dose-dependent manner (Kapasi et al., 2000), the significantly lower surface expression of HLA-G we observed in samples exhibiting evident allelic imbalance could have implications for genetic studies in relation to disease, especially pre-eclampsia and recurrent spontaneous abortions, as well as cancer immunology. Our findings could, at least in part, explain some of the discrepancies in other studies investigating the 14 bp ins/del polymorphism and other HLA-G SNPs in relation to expression levels. Furthermore, the high membrane-bound HLA-G protein expression in cases of allelic balance may favor some embryos, or fetuses, that are heterozygous for HLA haplotypes and fits into the concept of balancing selection working at the HLA-G locus (Tan et al., 2005; Sabbagh et al., 2014). Likely, conventional biological interpretations of the genotype−phenotype relationship should be reconsidered and allelic expression of the 14 bp ins/del polymorphism, and a range of 3′-UTR haplotypes, should be further studied in future experiments in order to fully understand and exploit the suppressive activity of HLA-G in a wide range of associated diseases, and especially in relation to reproduction.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

Authors' roles

S.D. designed the study, performed the experiments, performed data analysis and data presentation, and wrote the first draft of the manuscript. T.V.F.H. designed the study, performed data analysis, and critically reviewed and edited the manuscript. S.T. and C.K.T. were responsible for clinical sample collection and critically reviewed the manuscript. O.B.C. was involved in discussions related to the study and critically reviewed the manuscript.

Funding

Conflict of interest

None declared.

References

Antczak A, Migdalska-Sek M, Pastuszak-Lewandoska D, Czarnecka K, Nawrot E, Domanska D, Kordiak J, Gorski P, Brzezianska E. Significant frequency of allelic imbalance in 3p region covering RARbeta and MLH1 loci seems to be essential in molecular non-small cell lung cancer diagnosis. *Med Oncol* 2013;**30**:532.

Blaschitz A, Juch H, Volz A, Hutter H, Daxboeck C, Desoye G, Dohr G. The soluble pool of HLA-G produced by human trophoblasts does not include detectable levels of the intron 4-containing HLA-G5 and HLA-G6 isoforms. *Mol Hum Reprod* 2005;**11**:699–710.

Bortolotti D, Gentili V, Melchiorri L, Rotola A, Rizzo R. An accurate and reliable real time SNP genotyping assay for the HLA-G +3142 bp C>G polymorphism. *Tissue Antigens* 2012;**80**:259–262.

Carosella ED. The tolerogenic molecule HLA-G. *Immunol Lett* 2011; **138**:22–24.

Castelli EC, Mendes-Junior CT, Deghaide NH, de Albuquerque RS, Muniz YC, Simoes RT, Carosella ED, Moreau P, Donadi EA. The genetic structure of 3′untranslated region of the HLA-G gene: polymorphisms and haplotypes. *Genes Immun* 2010;**11**:134–141.

Castelli EC, Mendes-Junior CT, Veiga-Castelli LC, Roger M, Moreau P, Donadi EA. A comprehensive study of polymorphic sites along the HLA-G gene: implication for gene regulation and evolution. *Mol Biol Evol* 2011;**28**:3069–3086.

Chen XY, Yan WH, Lin A, Xu HH, Zhang JG, Wang XX. The 14 bp deletion polymorphisms in HLA-G gene play an important role in the expression of soluble HLA-G in plasma. *Tissue Antigens* 2008;**72**:335–341.

Colbern GT, Chiang MH, Main EK. Expression of the nonclassic histocompatibility antigen HLA-G by preeclamptic placenta. *Am J Obstet Gynecol* 1994;**170**:1244–1250.

Dahl M, Djurisic S, Hviid TV. The many faces of human leukocyte antigen-G: relevance to the fate of pregnancy. *J Immunol Res* 2014;**2014**:591489.

Darmochwal-Kolarz D, Kolarz B, Rolinski J, Leszczynska-Gorzelak B, Oleszczuk J. The concentrations of soluble HLA-G protein are elevated during mid-gestation and decreased in pre-eclampsia. *Folia Histochem Cytobiol* 2012;**50**:286–291.

Di Cristofaro J, El Moujally D, Agnel A, Mazieres S, Cortey M, Basire A, Chiaroni J, Picard C. HLA-G haplotype structure shows good conservation between different populations and good correlation with high, normal and low soluble HLA-G expression. *Hum Immunol* 2013;**74**:203–206.

Djurisic S, Sorensen AE, Hviid TV. A fast and easy real-time PCR genotyping method for the HLA-G 14-bp insertion/deletion polymorphism in the 3′ untranslated region. *Tissue Antigens* 2012;**79**:186–189.

Donadi EA, Castelli EC, Arnaiz-Villena A, Roger M, Rey D, Moreau P. Implications of the polymorphism of HLA-G on its function, regulation, evolution and disease association. *Cell Mol Life Sci* 2011;**68**:369–395.

Flajollet S, Poras I, Carosella ED, Moreau P. RREB-1 is a transcriptional repressor of HLA-G. *J Immunol* 2009;**183**:6948–6959.

Fujii T, Ishitani A, Geraghty DE. A soluble form of the HLA-G antigen is encoded by a messenger ribonucleic acid containing intron 4. *J Immunol* 1994;**153**:5516–5524.

Fuzzi B, Rizzo R, Criscuoli L, Noci I, Melchiorri L, Scarselli B, Bencini E, Menicucci A, Baricordi OR. HLA-G expression in early embryos is a fundamental prerequisite for the obtainment of pregnancy. *Eur J Immunol* 2002;**32**:311–315.

Hackmon R, Koifman A, Hyodo H, Glickman H, Sheiner E, Geraghty DE. Reduced third-trimester levels of soluble human leukocyte antigen G protein in severe preeclampsia. *Am J Obstet Gynecol* 2007;**197**:255.e1–255.e5.

Hara N, Fujii T, Yamashita T, Kozuma S, Okai T, Taketani Y. Altered expression of human leukocyte antigen G (HLA-G) on extravillous trophoblasts in preeclampsia: immunohistological demonstration with anti-HLA-G specific antibody '87G' and anti-cytokeratin antibody 'CAM5.2'. *Am J Reprod Immunol* 1996;**36**:349–358.

Harrison GA, Humphrey KE, Jakobsen IB, Cooper DW. A 14 bp deletion polymorphism in the HLA-G gene. *Hum Mol Genet* 1993;**2**:2200.

Hashimoto K, Azuma C, Koyama M, Nobunaga T, Kimura T, Shimoya K, Kubota Y, Saji F, Murata Y. Biparental alleles of HLA-G are co-dominantly expressed in the placenta. *Jpn J Hum Genet* 1997;**42**:181–186.

Huggett JF, Foy CA, Benes V, Emslie K, Garson JA, Haynes R, Hellemans J, Kubista M, Mueller RD, Nolan T et al. The digital MIQE guidelines: minimum information for publication of quantitative digital PCR experiments. *Clin Chem* 2013;**59**:892–902.

Hviid TV. HLA-G genotype is associated with fetoplacental growth. *Hum Immunol* 2004;**65**:586–593.

Hviid TV. HLA-G in human reproduction: aspects of genetics, function and pregnancy complications. *Hum Reprod Update* 2006;**12**:209–232.

Hviid TV, Moller C, Sorensen S, Morling N. Co-dominant expression of the HLA-G gene and various forms of alternatively spliced HLA-G mRNA in human first trimester trophoblast. *Hum Immunol* 1998;**59**:87–98.

Hviid TV, Sorensen S, Morling N. Polymorphism in the regulatory region located more than 1.1 kilobases 5′ to the start site of transcription, the promoter region, and exon 1 of the HLA-G gene. *Hum Immunol* 1999; **60**:1237–1244.

Hviid TV, Hylenius S, Rorbye C, Nielsen LG. HLA-G allelic variants are associated with differences in the HLA-G mRNA isoform profile and HLA-G mRNA levels. *Immunogenetics* 2003;**55**:63–79.

Hviid TV, Rizzo R, Melchiorri L, Stignani M, Baricordi OR. Polymorphism in the 5′ upstream regulatory and 3′ untranslated regions of the HLA-G gene in relation to soluble HLA-G and IL-10 expression. *Hum Immunol* 2006;**67**:53–62.

Hylenius S, Andersen AM, Melbye M, Hviid TV. Association between HLA-G genotype and risk of pre-eclampsia: a case–control study using family triads. *Mol Hum Reprod* 2004;**10**:237–246.

Ishitani A, Geraghty DE. Alternative splicing of HLA-G transcripts yields proteins with primary structures resembling both class I and class II antigens. *Proc Natl Acad Sci USA* 1992;**89**:3947–3951.

Ishitani A, Sageshima N, Lee N, Dorofeeva N, Hatake K, Marquardt H, Geraghty DE. Protein expression and peptide binding suggest unique and interacting functional roles for HLA-E, F, and G in maternal–placental immune recognition. *J Immunol* 2003;**171**:1376–1384.

Iversen AC, Nguyen OT, Tommerdal LF, Eide IP, Landsem VM, Acar N, Myhre R, Klungland H, Austgulen R. The HLA-G 14bp gene polymorphism and decidual HLA-G 14bp gene expression in pre-eclamptic and normal pregnancies. *J Reprod Immunol* 2008;**78**:158–165.

Jassem RM, Shani WS, Loisel DA, Sharief M, Billstrand C, Ober C. HLA-G polymorphisms and soluble HLA-G protein levels in women with recurrent pregnancy loss from Basrah province in Iraq. *Hum Immunol* 2012;**73**:811–817.

Kapasi K, Albert SE, Yie S, Zavazava N, Librach CL. HLA-G has a concentration-dependent effect on the generation of an allo-CTL response. *Immunology* 2000;**101**:191–200.

Kirszenbaum M, Moreau P, Gluckman E, Dausset J, Carosella E. An alternatively spliced form of HLA-G mRNA in human trophoblasts and evidence for the presence of HLA-G transcript in adult lymphocytes. *Proc Natl Acad Sci USA* 1994;**91**:4209–4213.

Kotze D, Kruger TF. HLA-G as a marker for embryo selection in assisted reproductive technology. *Fertil Steril* 2013;**100**:e44.

Kovats S, Main EK, Librach C, Stubblebine M, Fisher SJ, DeMars R. A class I antigen, HLA-G, expressed in human trophoblasts. *Science* 1990; **248**:220–223.

Kuersten S, Goodwin EB. The power of the 3′ UTR: translational control and development. *Nat Rev Genet* 2003;**4**:626–637.

Larsen MH, Hylenius S, Andersen AM, Hviid TV. The 3′-untranslated region of the HLA-G gene in relation to pre-eclampsia: revisited. *Tissue Antigens* 2010;**75**:253–261.

LeMaoult J, Rouas-Freiss N, Carosella ED. Immuno-tolerogenic functions of HLA-G: relevance in transplantation and oncology. *Autoimmun Rev* 2005;**4**:503–509.

Liu X, Gu W, Li X. HLA-G regulates the invasive properties of JEG-3 choriocarcinoma cells by controlling STAT3 activation. *Placenta* 2013;**34**:1044–1052.

Locafaro G, Amodio G, Tomasoni D, Tresoldi C, Ciceri F, Gregori S. HLA-G expression on blasts and tolerogenic cells in patients affected by acute myeloid leukemia. *J Immunol Res* 2014;**2014**:636292.

Lombardelli L, Aguerre-Girr M, Logiodice F, Kullolli O, Casart Y, Polgar B, Berrebi A, Romagnani S, Maggi E, Le Bouteiller P *et al.* HLA-G5 induces IL-4 secretion critical for successful pregnancy through differential expression of ILT2 receptor on decidual CD4(+) T cells and macrophages. *J Immunol* 2013;**191**:3651–3662.

Lucena-Silva N, de Souza VS, Gomes RG, Fantinatti A, Muniz YC, de Albuquerque RS, Monteiro AL, Diniz GT, Coelho MR, Mendes-Junior CT *et al.* HLA-G 3′ untranslated region polymorphisms are associated with systemic lupus erythematosus in 2 Brazilian populations. *J Rheumatol* 2013;**40**:1104–1113.

Lynge Nilsson L, Djurisic S, Hviid TV. Controlling the immunological crosstalk during conception and pregnancy: HLA-G in reproduction. *Front Immunol* 2014;**5**:198.

Martelli-Palomino G, Pancotto JA, Muniz YC, Mendes-Junior CT, Castelli EC, Massaro JD, Krawice-Radanne I, Poras I, Rebmann V, Carosella ED *et al.* Polymorphic sites at the 3′ untranslated region of the HLA-G gene are associated with differential hla-g soluble levels in the Brazilian and French population. *PLOS One* 2013;**8**:e71742.

McIntire RH, Morales PJ, Petroff MG, Colonna M, Hunt JS. Recombinant HLA-G5 and -G6 drive U937 myelomonocytic cell production of TGF-beta1. *J Leukoc Biol* 2004;**76**:1220–1228.

Menier C, Riteau B, Carosella ED, Rouas-Freiss N. MICA triggering signal for NK cell tumor lysis is counteracted by HLA-G1-mediated inhibitory signal. *Int J Cancer* 2002;**100**:63–70.

Ober C, Aldrich CL, Chervoneva I, Billstrand C, Rahimov F, Gray HL, Hyslop T. Variation in the HLA-G promoter region influences miscarriage rates. *Am J Hum Genet* 2003;**72**:1425–1435.

Ober C, Billstrand C, Kuldanek S, Tan Z. The miscarriage-associated HLA-G -725G allele influences transcription rates in JEG-3 cells. *Hum Reprod* 2006;**21**:1743–1748.

O'Brien M, McCarthy T, Jenkins D, Paul P, Dausset J, Carosella ED, Moreau P. Altered HLA-G transcription in pre-eclampsia is associated with allele specific inheritance: possible role of the HLA-G gene in susceptibility to the disease. *Cell Mol Life Sci* 2001;**58**:1943–1949.

Pfeiffer KA, Rebmann V, Passler M, van der Ven K, van der Ven H, Krebs D, Grosse-Wilde H. Soluble HLA levels in early pregnancy after in vitro fertilization. *Hum Immunol* 2000;**61**:559–564.

Ponte M, Cantoni C, Biassoni R, Tradori-Cappai A, Bentivoglio G, Vitale C, Bertone S, Moretta A, Moretta L, Mingari MC. Inhibitory receptors sensing HLA-G1 molecules in pregnancy: decidua-associated natural killer cells express LIR-1 and CD94/NKG2A and acquire p49, an HLA-G1-specific receptor. *Proc Natl Acad Sci USA* 1999;**96**:5674–5679.

Rajagopalan S, Long EO. A human histocompatibility leukocyte antigen (HLA)-G-specific receptor expressed on all natural killer cells. *J Exp Med* 1999;**189**:1093–1100.

Rebmann V, van der Ven K, Passler M, Pfeiffer K, Krebs D, Grosse-Wilde H. Association of soluble HLA-G plasma levels with HLA-G alleles. *Tissue Antigens* 2001;**57**:15–21.

Riteau B, Rouas-Freiss N, Menier C, Paul P, Dausset J, Carosella ED. HLA-G2, -G3, and -G4 isoforms expressed as nonmature cell surface glycoproteins inhibit NK and antigen-specific CTL cytolysis. *J Immunol* 2001;**166**:5018–5026.

Rizzo R, Andersen AS, Lassen MR, Sorensen HC, Bergholt T, Larsen MH, Melchiorri L, Stignani M, Baricordi OR, Hviid TV. Soluble human leukocyte antigen-G isoforms in maternal plasma in early and late pregnancy. *Am J Reprod Immunol* 2009;**62**:320–338.

Rousseau P, Le Discorde M, Mouillot G, Marcou C, Carosella ED, Moreau P. The 14 bp deletion–insertion polymorphism in the 3′ UT region of the HLA-G gene influences HLA-G mRNA stability. *Hum Immunol* 2003;**64**:1005–1010.

Sabbagh A, Luisi P, Castelli EC, Gineau L, Courtin D, Milet J, Massaro JD, Laayouni H, Moreau P, Donadi EA *et al.* Worldwide genetic variation at the 3′ untranslated region of the HLA-G gene: balancing selection influencing genetic diversity. *Genes Immun* 2014;**15**:95–106.

Shabalina SA, Spiridonov NA, Kashina A. Sounds of silence: synonymous nucleotides as a key to biological regulation and complexity. *Nucleic Acids Res* 2013;**41**:2073–2094.

Shen J, Medico L, Zhao H. Allelic imbalance in BRCA1 and BRCA2 gene expression and familial ovarian cancer. *Cancer Epidemiol Biomarkers Prev* 2011;**20**:50–56.

Steinborn A, Varkonyi T, Scharf A, Bahlmann F, Klee A, Sohn C. Early detection of decreased soluble HLA-G levels in the maternal circulation predicts the occurrence of preeclampsia and intrauterine growth retardation during further course of pregnancy. *Am J Reprod Immunol* 2007;**57**:277–286.

Svendsen SG, Hantash BM, Zhao L, Faber C, Bzorek M, Nissen MH, Hviid TV. The expression and functional activity of membrane-bound human leukocyte antigen-G1 are influenced by the 3′-untranslated region. *Hum Immunol* 2013;**74**:818–827.

Tan Z, Shon AM, Ober C. Evidence of balancing selection at the HLA-G promoter region. *Hum Mol Genet* 2005;**14**:3619–3628.

Yao YQ, Barlow DH, Sargent IL. Differential expression of alternatively spliced transcripts of HLA-G in human preimplantation embryos and inner cell masses. *J Immunol* 2005;**175**:8379–8385.

Yie SM, Taylor RN, Librach C. Low plasma HLA-G protein concentrations in early gestation indicate the development of preeclampsia later in pregnancy. *Am J Obstet Gynecol* 2005;**193**:204–208.

Zhao L, Teklemariam T, Hantash BM. Reassessment of HLA-G isoform specificity of MEM-G/9 and 4H84 monoclonal antibodies. *Tissue Antigens* 2012;**80**:231–238.

Zhu X, Han T, Yin G, Wang X, Yao Y. Expression of human leukocyte antigen-G during normal placentation and in preeclamptic pregnancies. *Hypertens Pregnancy* 2012;**31**:252–260.