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A single base-pair mutation in the 3′-untranslated region of HLA-G mRNA is associated with pre-eclampsia

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Human leukocyte antigen-G (HLA-G) is a non-classical class I HLA molecule that is expressed by extravillous cytotrophoblast cells. This protein may play a critical role in the protection of cytotrophoblasts from maternal immune response, allowing these semi-allogeneic cells to invade the uterus unimpeded. We have demonstrated that diminished placental HLA-G expression is associated with pre-eclampsia. In order to explore fundamental mechanisms underlying this reduced HLA-G expression in pre-eclampsia, we looked for, and found by sequences analysis, a single base-pair mutation in the HLA-G gene 3′-untranslated region (3′UTR) adjacent to an AUUUA motif. This mutation is significantly associated with pre-eclampsia, the severe form being more strongly associated with homozygosity for this mutation than the mild form. Since the null allele was discovered in the HLA-G mRNA 3′UTR adjacent to an AUUUA motif, we also examined the effect of this mutation on HLA-G mRNA stability, and found that half-lives of HLA-G mRNA with the mutation were significantly shorter than without the mutation. These data provide evidence that this mutation could be one of the fundamental mechanisms for lower levels of placental HLA-G protein expression in patients with pre-eclampsia.

Keywords: HLA-G; gene mutation; RNA stability; pre-eclampsia

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Introduction

Pre-eclampsia is a disease that affects ~5–10% of pregnant women and is one of the major causes of maternal perinatal morbidity and mortality (Sibai *et al*., 2005). Despite extensive study, the underlying etiology remains elusive. However, it is generally agreed that pre-eclampsia is associated with shallow or absent placental cytotrophoblast invasion into the uterus (Widschwendter *et al*., 1998). Many hypotheses have been put forward to explain the mechanisms for the development of pre-eclampsia; one hypothesis implicates a breakdown in the natural mechanism that protects the semi-allogeneic fetal allograft from rejection by the maternal immune system (Redman and Sargent, 2005).

Human leukocyte antigen-G (HLA-G) is a non-classical class I HLA molecule that is expressed by extravillous cytotrophoblast cells (McMaster *et al*., 1995). This protein may play a critical role in the protection of cytotrophoblasts from maternal immune response, allowing these semi-allogeneic cells to invade the uterus unimpeded (O'Brien *et al*., 2000). Therefore, it has been proposed that the reduced HLA-G gene transcription (Colbern *et al*., 1994; Hara *et al*., 1996; Lim *et al*., 1997) and translation (Hara *et al*., 1996; Lim *et al*., 1997; Goldman-Wohl *et al*., 2000; Yie *et al*., 2004, 2005) observed in women with pre-eclampsia may contribute to the pathogenesis of pre-eclampsia (O'Brien *et al*., 2000). Since pre-eclampsia, according to epidemiological studies, has a strong familial component, HLA-G may be an ideal candidate gene for mutations predisposing to this condition (O'Brien *et al*., 2000). A number of potential polymorphisms or mutations in the HLA-G gene have been screened for an association (O'Brien *et al*., 2000), but most were found not to be associated with pre-eclampsia. However, a 14 bp deletion/insertion polymorphism in the 3′-untranslated region (3′UTR) of exon 8 has been linked to differences in the levels of HLA-G expression and in HLA-G mRNA splicing (Hviid, 2004; Hviid *et al*., 2004). In a case–controlled study, an over-representation of the homozygous HLA genotype was detected in pre-eclampsia offspring compared with controls. In some cases, differences between these cases and controls were associated with transmission from the father of this 14 bp deletion/insertion polymorphism in exon 8 (Hylenius *et al*., 2004). Nevertheless, more recent studies (Vianna *et al*., 2007; Iversen *et al*., 2008) found that the fetal HLA-G 14 bp genotype is reflected in the decidual HLA-G mRNA splice form profile, but does not appear to be associated with increased risk for development of pre-eclampsia. The steady-state levels of a particular mRNA species depend not only on its rate of synthesis, but also on its rate of degradation. Adenylate/uridylate (AU)-rich element is a sequence consisting mostly of many uridines and some adenosines in the 3′UTR of mRNA and was first identified as a *cis*-acting degradation signal of the mRNAs of certain lymphokines, cytokines and proto-oncogenes. Using RNA binding assays, several groups have identified proteins that interact with AU-rich elements and many of these proteins have been implicated in the regulation of mRNA stability. The 3′UTR of HLA-G mRNA contains one AUUUA motif and one AUUAUUU repeat.

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This study was initiated to determine whether a DNA polymorphism exists in the HLA-G mRNA 3′UTR, at or near the AUUUA motif, and test whether this polymorphism is associated with pre-eclampsia and/or HLA-G mRNA stability.

Materials and Methods

Patients

This study was approved and monitored by the Research Ethics Committee of Women's College Hospital. Using informed consent, we recruited 29 pre-eclamptic patients and 15 normal control women for this study. All the subjects were seen in the labour and delivery suite of the Women's College Hospital, University of Toronto, Toronto, Canada from 1996 to 1997. Pre-eclampsia was diagnosed, and sub-classified as mild versus severe, according to the guideline published by the American College of Obstetricians and Gynecologists (ACOG Committee on Obstetric Practice, 2002). Briefly, nulliparous parturient who were not known to be hypertensive before pregnancy were diagnosed with pre-eclampsia if they had: an increase in systolic pressure of 30 mm Hg or diastolic pressure of 15 mm Hg compared with blood pressures (BP) obtained before 20 gestational weeks; proteinuria ≥1–2+ on dipstick testing; and return to normal BP and clearance of proteinuria by 12 weeks' post-partum. Moreover, all pre-eclamptic subjects manifested BP at term that exceeded a systolic of 140 mm Hg or a diastolic of 90 mm Hg. Patients were sub-classified as severe pre-eclamptic if: systolic BP was found to be 160 mm Hg or greater and diastolic >110 mm Hg on at least two occasions; proteinuria 3–4+ on at least two occasions; and other features including oliguria (<500 ml/24 h), cerebral or visual disturbances, pulmonary edema, epigastric or right upper quadrant pain, impaired liver function, thrombocytopenia and intrauterine growth restriction. Finally, the assignment to the pre-eclampsia versus control groups was adjudicated *post hoc* by a panel of five obstetricians who reviewed the complete clinical and biochemical course of each pregnancy. Subjects with evidence of illicit drug or cigarette use, chronic hypertension, or other medical illnesses, and patients who had a multiple gestation or chorioamnionitis were excluded from the study. According to the protocol, the next patient who delivered, having had an uncomplicated pregnancy and delivery, was approached to participate. Of these, 15 of 29 control women agreed to participate. A summary of the clinical characteristics of the study groups is shown in Table I.

DNA extraction and HLA-G gene sequence

DNA was extracted from placenta tissues and blood cells using a phenol/chloroform (Bell *et al.*, 1981). DNA concentration and purity were measured by UV spectrophotometry at 260/280 nm. The dried DNA pellets were dissolved in 10–20 μl TE buffer, pH 8.0. HLA-G exon 8, containing the HLA-G mRNA 3′UTR sequence, was amplified by polymerase chain reaction (PCR): 200 ng of DNA was made up to a final volume of 50 μl with primers (sense: 5′-TGTGGGACTGAGTGGCAAGT-3′and anti-sense: 5′-TTTGTCT CTAAATTTCAGGAATC-3′) at initial denaturation of 94°C for 5 min, 30 cycles of 94°C for 1 min, 51°C for 1 min and 72°C for 2 min and the final extension step at 72°C for 10 min.

Each sample PCR product was purified from a 2% low-melting point agarose gel by using QIAquick Gel-Extraction Kit (Qiagen, Hilden, Germany). Approximately 50 ng of the purified products were then sequenced in both directions by using ABI PRIME Big-Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) on an ABI DNA analyzer (Applied Biosystems).

In vitro mutagenesis

To generate *in vitro* mutagenesis during reverse transcription, total RNA from cultured JEG-3 cells was extracted using Trizon® Reagents (Gibco, Burlington, ON, Canada) according to the manufacturer's manual. Reverse transcription reactions were performed using MMLV reverse transcriptase (Epicentre Technologies, Madison, WI, USA) with HLA-G gene specific primers HLAGΔG: 5′-TAAACTTTTTCATTTAAAT*G*-3′ (normal sequence) and HLAGΔA: 5′-TAAACTTTTTCATTTAAAT*A*-3′ (mutation sequence). The primers correspond to HLA-G mRNA 3′UTR nucleotides +1754 to +1773 (Fig. 1), differing in one base-pair (Δt−Δc at +1754). The cDNAs were amplified by PCR with primers (GF: 5′-CACCACCCTGTCTTTGACTA-3′ and GB: 3′-ATCTTGGAACAGGGTGGTCC-5′) denatured at 94°C for 5 min, and then 35 cycles at 94°C for 1 min, at 50°C for 1 min and at 72°C for 2 min. The PCR product was checked on a 1.2% argarose gel stained with ethidium bromide and cloned into a cDNA™ 3.1 directional TOPO expression vector (Invitrogen Corporation, Carlsbad, CA, USA). Cloned plasmids were transfected into the SP/02 myeloma cell line, which does not express HLA-G, by using Lipofectamine 2000 (Invitrogen), according to the manufacturer's manual.

Transfected SP/02 cell line was cultured for 48 h in RPMI 1640, supplemented with 10% fetal calf serum in the presence of 50 IU/ml penicillin, 50 μg/ml streptomycin and amphotericin B 50 μg/ml at 37°C, 5% CO_2.

Actinomycin D study

During the experiments, the cells were washed three times with serum-free medium (RPMI 1640). The cells were maintained in the serum-free medium and actinomycin D (Sigma-Aldrich Corp. St. Louis, MO, USA) was added to the cell culture for 0, 0.5, 1, 2 and 4 h, at a final concentration of 5 μg/ml. The cells were then collected by centrifugation at 4°C, 800 rpm for 5 min and cell pellets were stored at −80°C until assay. Empty plasmids of cDNA™ 3.1 directional TOPO expression vectors were also used to detect non-specific background.

RNA extraction

Total RNA of the cell pellets at each time point was extracted with Trizon® reagents, as described earlier. HLA-G stability was measured by either a RT–PCR-ELISA or an RNAase protection assay (RPA) as follows:

RT–PCR– ELISA

An HLA-G fragment prepared from JEG-3 cells by RT–PCR using primers G256 and G1225 (Kirszenbaum *et al.*, 1994), corresponding to nucleotides +256 to +1225, was used as a probe. 1 μg/ml of the fragment was denatured at 95°C for 5 min, put on ice for 3 min and coated on a 96-well microtiter plate (Dynatec, Chantilly, VA, USA) in 50 μl per well of 0.1 M PBS/1 M NaCl coating buffer (pH = 7.2) at 4°C overnight. The plate was then washed twice, and dried and stored at −20°C until use.

The total RNA of each time point was amplified by RT–PCR during 30 cycles (94°C 1′, 58°C 1′, 72°C 2′) in the presence of biotin-labelled primer sets (G256 and G1225). Five microlitre of each PCR product in triplicate was denatured using 1 N NaOH and hybridized to plate a coated probe at 50°C for 2 h. Fifty microlitre per well of 1:1000 streptoavidin–horseradish peroxidase (HRP) conjugate (Sigma) diluted in sample buffer (0.01 M PBS/150 mM NaCl, 0.5% block solution, 5 mM EDTA and 1% Tween-20) was added following four washes with 0.01 M of PBS washing solution containing 0.05% Tween-20. After 1 h incubation at room temperature, the plate was

Table I. Clinical characteristics of patients.

Characteristics	Pre-eclampsia cases ($n = 29$)	Normal controls ($n = 15$)
Age (years)	30.3 ± 4.8 (range 24–40)	32.1 ± 5.3 (range 25–41)
Primiparas*	16/20 (80%)	9/14 (64%)
Gestational age (weeks)*	35.3 ± 2.4 (range 31–39)	39.4 ± 1.8 (range 38–42)
Birthweight (grams)*	2319 ± 708 (range 1215–3595)	3324 ± 482 (range 2580–4264)
IUGR*	5/20 (25%)	0/14 (0%)
Disease severity	mild – 50% severe – 50%	n/a

*$P < 0.05$. IUGR, intrauterine growth restriction (<10th percentile for gestational age); n/a, not applicable.

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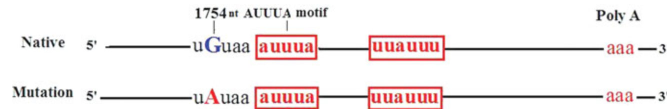

Figure 1: Shows the sequence of HLA-G mRNA 3′-untranslated region. Initial sequences analysis shows a correlation with a mutation (G→A) in the 3′-untranslated region adjacent to an AUUUA motif.

washed four times again with the wash solution and 100 μl of TMB (Sigma) was added. After a 15 min incubation, colour reactions were stopped by adding 50 μl per well of 1 M HCl and read at 450/630 nm at a microplate reader (Awareness Technology, Palm City, FL, USA). The cloned plasmids were used as a standard and amplified together with sample cDNAs. HLA-G mRNA levels at each time point were determined by comparison to the standard.

RNase protection analysis

RPAs were performed by using the SuperSignal®RPAIII™ kit (Ambion, Austin, TX, USA) according to the manufacturer's manual. Firstly, a 728 antisense nucleotide HLA-G probe was generated from the plasmid by *in vitro* transcription using T7 RNA polymerase. Transcribed riboprobe was then digested with 10 U DNase at 37°C for 30 min and purified using a QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. The probe was labelled by *in vitro* transcription with a UTP-biotin kit (Roche Diagnostics, Montreal, QC, Canada) according to the manufacturer's instructions.

Secondly, the probe was hybridized to total RNA and treated with RNAase at 37°C for 2 h. Non-hybridized RNA and free probes were digested by incubation with RNase A and T1 for 45 min at 30°C. Enzyme activity was stopped by addition of proteinase K.

Thirdly, the protected fragments of HLA-G mRNA were determined by 6% TBE gel electrophoresis in denaturing conditions, and then transferred to a positively charged nylon membrane in 0.5× TBE transfer buffer at 30 V for 1 h at room temperature. Following incubation with a 1:1000 dilution of streptoavidin– HRP conjugate (Sigma), the visualization of the biotin-labelled probe was developed with a BrightStar BioDetect Kit (Ambion). Unprotected HLA-G mRNA and biotin-labelled probe were loaded as size markers and positive controls. Identity of the bands protected from RNase degradation was established by comparing their size with the size of the bands of the positive control samples. For negative control, the transfected cell RNA was substituted with yeast RNA.

Finally, the density of the bands representing the protected fragments was measured using AlphaImager 1220 Analysis and Documentation System. Semi-quantification was achieved by normalizing the optical densities of the specific bands to the optical densities of β-actin gene. The optical density of the specific protected bands was expressed as relative values.

Statistical analysis

HLA-G genotype frequencies were compared with Hardy–Weinberg expectations using χ^2 tests. The frequency of the 1754ΔA allele was compared between pre-eclampsic patients and controls, as well as severe and mild subgroups of pre-eclampsia, with a χ^2 test. Comparisons of HLA-G mRNA half-life between normal controls and the mutation were carried out by using a Student's *t*-test.

Results

Definition of detected HLA-G alleles and comparison of the allele frequencies between pre-eclampsia patients and healthy controls

We identified a polymorphism, ΔA (mutant) or ΔG (native), located at +1754 of the HLA-G gene exon 8 in the HLA-G mRNA 3′UTR that is adjacent to the solitary AUUUA motif in this region (Fig. 1). This polymorphism was evident when we studied previously reported HLA-G sequences.

By DNA sequence analysis, the observed A allele frequency in the control group was 0.27 (4/15), and 0.74 (21.5/29) in the pre-eclampsia group. The difference between the two groups was statistically significant (Fisher's exact test, $P = 0.009$). The observed A allele frequency in the severe pre-eclampsia group was 0.86 (12/14), and 0.63 (9.5/15) in the mild pre-eclampsia group. No statistical significance was found between these two groups (Fisher's exact test, $P = 0.215$). However, when HLA-G genotype frequencies were compared with Hardy–Weinberg expectations by using χ^2 tests, the following placental HLA-G alleles with respect to this polymorphism were observed in the pre-eclampsia group: ΔG/ΔG = 2, ΔG/ΔA = 11 and ΔA/ΔA = 16; while in normal pregnant women: ΔG/ΔG = 11, ΔG/ΔA = 0 and ΔA/ΔA = 4 ($\chi^2 = 15.6$; df = 1; $P < 0.0001$). Thus, a greater number of 1754ΔA alleles were found in the placentas of women with pre-eclampsia compared with those of healthy pregnant women (Table II). Furthermore, 12/14 placentas were found to be homozygous ΔA/ΔA in patients with severe pre-eclampsia, while only 4/15 were found among those with mild disease (Table III). Statistical analysis indicated that the homozygous ΔA/ΔA allele genotype was significantly higher in association with severe disease than mild case ($\chi^2 = 9.19$; df = 1; $P = 0.001$).

Effect of the mutation on the stability of HLA-G mRNA

Since steady-state mRNA levels can be reduced by either inhibiting transcription or by decreasing mRNA stability, and the mutant allele

Table II. Patient group genotypes for the ΔA[b] mutation[a].

Patients	*n*	ΔG/ΔG[b]	ΔA[c]/ΔG[b]	ΔA/ΔA[c]	ΔA[c]Frequency	*P*-value
Control	15	11	0	4	0.071	
Pre-eclampsia	29	2	11 genotype frequencies	16	0.549	<0.0001

[a]HLA-G were calculated according to Hardy–Weinberg Equilibrium equation: $p^2 + 2pq + q^2 = 1$, in which p^2 was denoted for the ΔA/ΔA homozygotes, q^2 for the ΔG/ΔG homozygotes and $2pq$ for the ΔA/ΔG heterozygotes. Then, the HLA-G genotype frequencies were compared with Hardy–Weinberg expectations using χ^2 tests; [b]ΔG = Native allele; [c]ΔA = Mutant allele.

Table III. Comparison of genotype frequency between mild and severe pre-eclampsia[a].

Samples	*n*	ΔG/ΔG[b]	ΔA/ΔG[c, b]	ΔA/ΔA[c]	1754ΔA[c] frequency	*P*-value
Severe	14	2	0	12	0.735	
Mild	15	0	11	4	0.399	0.001

[a]Calculation and comparison of HLA-G genotype frequencies were performed as the same as in Table II; [b]ΔG = Native allele; [c]ΔA = Mutant allele.

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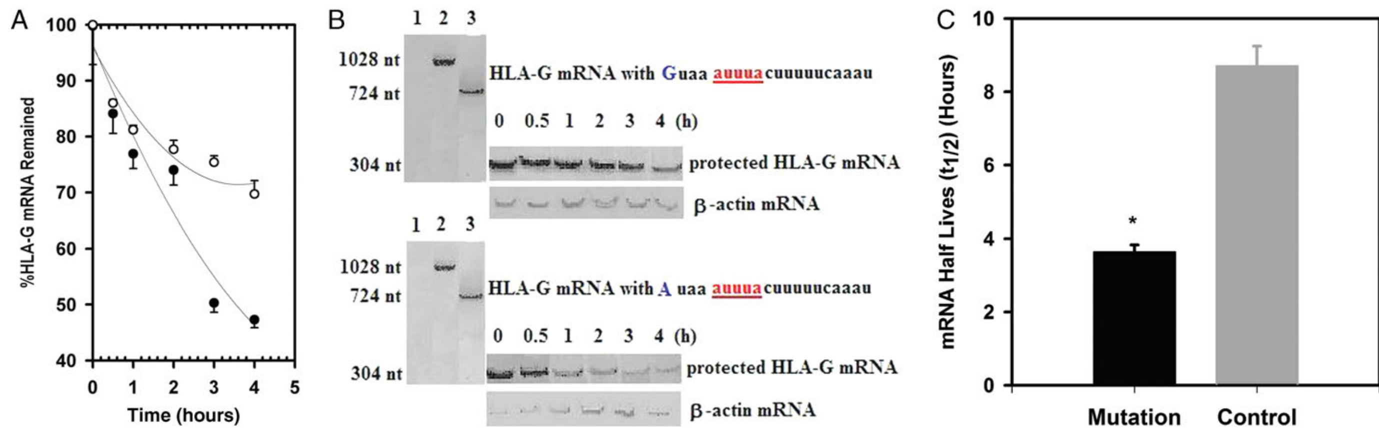

Figure 2: Evaluation of HLA-G mRNA stability.
Transfected SP/02 cells were treated with actinomycin D for various time periods. In (**A**), HLA-G mRNA expression was determined by RT–PCR-ELISA in which the untreated cells were taken as baseline (100%) and HLA-G mRNA concentrations in various time periods in normal (↓) and mutation groups (↑) were calculated as percentage of the baseline, and defined as remained HLA-G mRNA (%).(**B**) shows a representative RPA of HLA-G mRNA and the housekeeping gene β-actin in which (1) = negative control, unprotected HLA-G mRNA (2) and biotin labeled probe (3) were used as positive controls. The results shown are one of three representative independent experiments. The relative optical density values of the mRNA bands were normalized to the relative optical density of the housekeeping gene β-actin. Comparison of half-lives between HLA-G mRNA with ΔA allele and with ΔG was shown in (**C**). There was a significant difference between two genotypes (3.63 ± 0.203 versus 8.70 ± 0.550 h, $P = 0.0001$).

is adjacent to an AUUUA motif in the HLA-G mRNA 3′UTR, we deduced that reduced HLA-G protein levels in pre-eclampsia may be caused by an increased rate of HLA-G mRNA degradation. To valuate the HLA-G mRNA stability in association with these alleles, HLA-G mRNA levels were measured at timed intervals after addition of actinomycin D by two methods: (i) an RT–PCR-ELISA and (ii) an RPA, as described in the Materials and Methods section. As shown in Fig. 2A, levels of HLA-G mRNA with the ΔA allele had decreased to $49.7 \pm 1.69\%$ of baseline (mean ± SE, $n = 7$) by 3 h after the addition of actinomycin D, whereas the ΔG containing HLA-G mRNA only decreased to $75.4 \pm 1.14\%$ (mean ± SE, $n = 7$) of baseline, as determined by the RT–PCR-ELISA. By RPA, HLA-G mRNA levels in cells translated with the ΔA HLA-G plasmid decayed more rapidly compared with that with the ΔG sequence (Fig. 2B). Statistical analyses showed that the HLA-G mRNA half-life in ΔA translated cells was significantly shorter than that with the ΔG sequence (3.63 ± 0.203 versus 8.70 ± 0.550 h, $P = 0.0001$; Fig. 2C).

Discussion

The results of this study indicate that frequency of the ΔA allele (1754ΔA) in HLA-G mRNA 3′UTR is significantly higher in placental tissue samples from patients with pre-eclampsia than in those of healthy controls (0.549 versus 0.071, $P < 0.0001$; Table II). The results also indicate that homozygosity for the ΔA allele is significantly associated with severity of the disease (0.735 versus 0.399, $P = 0.001$; Table III).

Since pre-eclampsia appears to be associated with poor placentation, it has long been considered that this disease may be a form of maternal immune rejection of the genetically foreign fetus (Redman and Sargent, 2005). However, cytotrophoblasts do not express the highly immunogenic transplantation antigens, HLA-A, -B and -D (Guleria and Sayegh, 2007). In fact, these invasive cytotrophoblasts that infiltrate maternal tissues during placentation express a unique combination of HLAs, namely HLA-C, -E and -G (Guleria and Sayegh, 2007). Of these, only HLA-C is highly polymorphic. In the mother's decidua, there are abundant natural killer (NK) cells that express the various KIR (killer immunoglobulin-like receptor) phenotypes. One study suggests that when a woman is homozygous for a group of KIR receptors, while the fetus is homozygous for the HLA-C2, pre-eclampsia may be more prevalent (Hiby *et al*., 2004).

Unlike HLA-C, HLA-G shows a limited polymorphism (Crew, 2007) and a restricted tissue distribution (McMaster *et al*., 1995). However, a large number of studies have demonstrated that HLA-G may play an important role in maternal–fetal immunotolerance by inhibiting activation of maternal T and NK cells resident in the deciduas (Le Bouteiller and Mallet, 1997). Therefore, a lower expression of HLA-G by invasive trophoblasts may diminish this protective effect. Indeed, a number of studies, including ours, show that both HLA-G gene transcription (Colbern *et al*., 1994; Hara *et al*., 1996; Lim *et al*., 1997) and translation (Hara *et al*., 1996; Lim *et al*., 1997; Goldman-Wohl *et al*., 2000; Yie *et al*., 2004, 2005) are reduced in women with pre-eclampsia.

In this study, the mutant allele was discovered in the HLA-G mRNA 3′UTR adjacent to an AUUUA motif but not the motif itself. Although AUUUA has been recognized as a key AU-rich motif sequence that mediates mRNA degradation, a number of reports have been suggested there were marked structural and functional heterogeneity in various human genes (Bakheet *et al*., 2003; Frevel *et al*., 2003; Tebo *et al*., 2003; Sully *et al*., 2004; Winzen *et al*., 2004). Since the HLA-G mRNA 3′UTR contains more than 58% AU nucleotides, we hypothesized that the mutation might also have an effect on HLA-G mRNA degradation. In order to test the hypothesis, we carried out *in vitro* mutagenesis. We have demonstrated that the mutant allele has a significant effect on HLA-G mRNA stability. This may help explain why HLA-G mRNA and resultant protein levels are reduced in pre-eclampsia (Fig. 2).

In summary, we have found a single base-pair point mutation in the HLA-G gene 3′UTR region that appears to be associated with pre-eclampsia and with decreased RNA stability *in vitro*. Therefore, the presence of this ΔA mutation could be an important factor to account for the lower placental HLA-G expression in association with pre-eclampsia.

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