# Semiquantitative Evaluation of HLA-G mRNA in Placental Tissue of Preeclamptic Patients

Thesis

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By

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# Contents

# LIST OF ABBREVIATION

| | |
|---|---|
| APC | Antigen-Presenting Cells |
| AT1 | Angiotensin Subtype 1 |
| BAbs | Blocking Antibodies |
| CD | Cluster of Differentiation |
| CTL | Cytotoxic T Lymphocytes |
| DCs | Dendritic Cells |
| DN | Double Negative T Cells |
| dNK | Decidual Natural Killer Cell |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| EVT | Extravillous Trophoblast |
| EVCT | Extravillous Cytotrophoblasts |
| Fc_R | Receptor for Fc fragment of immunoglobulins |
| HLA | Human Leukocyte Antigen |
| HDL | High Density Lipoprotein |
| HO | Heme Oxygenase |
| ICAM-1 | Inter-Cellular Adhesion Molecule-1 |
| IDO | Indoleamine 2,3-Dioxygenase |
| IFN-γ | Interferon-gamma |
| IgG | Immunoglubulin G |
| IL | Interleukin |
| ILT | Immunoglobulin-Like Transcript |
| IUGR | Intrauterine Growth Retardarion |
| KIR | Killing Inhibitory Receptor |
| LDL | Low Density Lipoprotein |
| LIR | Leukocyte Immunoglobulin-like Receptor-1 |
| MHC | Major Histocompatibility Complex |
| mRNA | messenger Ribonucleic Acid |
| NADPH | reduced Nicotinamide Adenine Dinucleotide Phosphate |
| NK | Natural Killer |
| NKAR | Natural Killer Activating Receptor |
| NKARL | Natural Killer Activating Receptor Ligand |
| NO | nitric oxide |
| PBMC | Peripheral Blood Mononuclear cells |
| PE | Pre-eclampsia |
| PlGF | Placental Growth Factor |
| RA | Recurrent Abortions |
| ROC curve | Reciever Operating Characteristics |
| RT-PCR | Reverse transcription-Polymerase Chain Reaction |
| sHLA-G | Soluble Human Leukocytic Antigen-G |
| SNP | Single Nucleotide Polymorphism |
| TCR | T Cell Receptor |
| TGF-β | Transforming Growth Factor-Beta |
| TNF-α | Tumor Necrosis Factor-alpha |
| UMC | Uterine Mononuclear cells |
| VEGF | Vascular endothelial growth factor |
| VCAM-1 | Vascular-Cellular Adhesion Molecule-1 |

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# INTRODUCTION

Successful mammalian pregnancy depends upon tolerance of a genetically incompatible fetus by the maternal immune system. The mammalian fetus may be perceived, paradoxically, as a successful allograft, a successful tumor, and a successful parasite (**Arck et al, 1999**).

Several specialized mechanisms have evolved to protect the fetus from maternal immunological rejection. These involve hormonal changes, specific functions of immune cells and cytokines at the uteroplacental interface, and the expression of unique MHC and non-MHC cell surface molecules (**Aluvihare et al, 2004).**

Specialized fetal tissue (trophoblast cells) that invades maternal uterine tissue at the site of implantation strictly regulates their expression of HLA genes and the production of their proteins. It is these proteins that, if recognized as foreign by maternal immune cells, would stimulate maternal anti-fetal cytotoxic T lymphocytes (CTL) capable of destroying HLA-expressing fetal cells. Instead, the antigens expressed in trophoblast cells program maternal leukocytes into pathways consistent with tolerance. Human trophoblast cells express one class Ia molecule (HLA-C) and all three class Ib molecules (HLA-E, -F, and –G). The HLA-C gene is moderately polymorphic, and could stimulate maternal anti-fetal acquired immunity if paternal alleles differed from maternal. Yet allelic disparity at the HLA-C locus does not seem to be a causal factor in infertility or termination of pregnancy. The other HLA class Ib are distinguished by low numbers of alleles that differ at the protein level (**Hunt et al, 2005; Hviid, 2006).**

HLA-G is a specialized antigen expressed only by trophoblast cells and exhibits greatly decreased polymorphism. HLA-G specifically binds to inhibitory receptors expressed by decidual leukocytes (T and B lymphocytes, NK cells and mononuclear phagocytes) and abrogate activating signals received by these cells (**LeMaoult et al, 2004)**.

Trophoblast cells in normal, healthy placentas are unlikely to elicit a CTL cell response. This is due to the fact that polymorphic HLA class I antigens are absent on syncytiotrophoblast and are scarce on other subpopulations. Moreover, membrane-associated and/or soluble HLA-G eliminate alloreactive (antipaternal) T cells both by inducing $CD8^+$ T cell to die by apoptosis and by down-regulating the expression of $CD8\alpha$ mRNA and protein (**Contini** et al, 2003).

Maternal tolerance to HLA-G in terms of antibody production has been demonstrated in 91%. Yet tolerance is not absolute: ~9% of women who had undergone at least one pregnancy generated anti-HLA-G antibodies that were readily identified in maternal sera by ELISA and immunoblotting. Not unexpectedly, maternal anti-HLA-G antibodies have no deleterious effect; all the women who developed these antibodies had multiple successful pregnancies. There is no relationship between exposure to a foreign (paternal) HLA-G protein on placental cells and maternal production of anti-HLA-G. How tolerance is achieved remains to be elucidated, but data accumulated to date strongly support the idea that inhibition is specific to HLA-G. The deficiency of anti-HLA-G antibodies is not due to maternal, pregnancy-associated reduction in B cell function. Mothers produce high levels of other types of antibodies in order to provide defense against pathogens

during pregnancy and assure transfer of protective antibodies to the fetus (**Hunt et al, 2005**).

Pre-eclampsia affects 2–7% of all pregnancies with varying severity and is a leading cause of maternal and fetal mortality and morbidity. The aetiology involves almost certainly a combination of genetic predisposition with maternal and fetal contributions and environmental factors. Epidemiological studies have shown that it occurs more often in nulliparous than multiparous women, and multiparous women changing partner also have a higher risk in a subsequent pregnancy. Furthermore, a long period of cohabitation preceeding pregnancy protects against developing pre-eclampsia, and there seems to be some evidence that the use of barrier contraceptive methods increases the risk of developing pre-eclampsia. The risk is elevated if other women in the family (mother, grandmother, sister) have had pre-eclampsia. These and other observations have led to speculations regarding an immunological basis or component in pre-eclampsia because previous exposure to foreign or paternal antigens appears to reduce the risk of pre-eclampsia (***Hylenius*** et al, 2004).

## AIM OF THE WORK

Since low or aberrant expression of membrane-bound and soluble HLA-G may have implications for maternal immune cell interactions and cytokine profiles during pregnancy, the aim of this study is to assess the expression of HLA-G mRNA in the placental tissue of preeclamptic versus normal pregnant women. The results will be correlated with the different parameters defining pregnancy outcome.

# IMMUNOLOGICAL ADAPTATION DURING PREGNANCY

The maternal tolerance to the semiallogeneic fetus is still a central theme in reproductive immunology. During placentation, fetally-derived, genetically dissimilar tissue and cells come into close contact with maternal tissue and cells, thus forming the so-called ***feto-maternal interface***. The most extensive contact between fetally-derived and maternal blood cells is formed by the villous trophoblastic barrier, where the syncytiotrophoblast surface permanently floats in maternal blood. Further contact is made by some extravillous cytotrophoblast cells, either located at villous tips, in so-called cell islands, or the endovascular trophoblast population within the uteroplacental spiral arteries. The third contact zone is the so-called ***junctional zone*** within the decidua where the invading extravillous trophoblast cells encounter all maternal tissue leukocytes, which are mainly natural killer (NK) cells, macrophages and T cells; this junctional zone extends at the edge of the placenta to the amnio-chorionic membranes where the chorionic laeve trophoblast has intimate contact with decidua tissue. It is worth mentioning that evidence has shown that even in healthy pregnancies fetal and maternal lymphoid cells are able to transgress the trophoblastic barrier, which, anatomically, seems completely impermeable (Douglas and King, 1990, Cross et al 1994, Arck et al,1999).

During pregnancy, the maternal immune system is clearly active, and under certain conditions may contribute to fetal damage/death. Well-defined pathological processes include destruction of fetal erythrocytes (Rh antigen, erythroblastosis) and platelets (alloimmune thrombo-cytopenia) by maternal antibodies and infections of

pregnancy, where activated macrophages secreting high levels of Th1-type cytokines alter the delicate cytokine balance at the maternal-fetal interface (Marzi et al, 1996).

Although anti-paternal HLA antibodies are common in pregnant women, they do no damage. Even novel HLA antigens expressed in the fetal membranes are tolerogenic rather than immunogenic (Hunt et al, 2003). Within the uterus, a dramatic change in endometrial leukocyte subpopulations occurs as a consequence of implantation. After a brief inflammatory reaction caused by blastocyst breaching of the uterine epithelium, a reaction best documented in rodents, the altered endometrium (now termed decidua) settles into a pattern where local protection is provided by the innate immune system. From this point onward, the major players in acquired immunity, T and B lymphocytes, are identified mainly in the myometrium distal to fetal tissues whereas members of the innate immune system, natural killer (NK) cells and macrophages, predominate in the decidua (Givan et al, 1997). An exception is the $T_{reg}$ subset of $CD4^+/CD25^+$ cells, which produce interleukin-10 (IL-10) and transforming growth factor-β (TGF-β), and are believed to be critical to maintenance of tolerance (Sakaguchi, 2000; Read and Powrie, 2001). These cells, whose proliferation is stimulated by estrogen (Polanczyk and Carson, 2004) comprise ~14% of $CD4^+$ cells in early decidua (Heikkinen et al, 2004). Similar reports have appeared in human pregnancy (Hunt, 2005).

The trophoblast cells circumvent antibody-mediated damage by exhibiting high levels of the complement regulatory proteins (Hsi et al, 1991), and reduce cell-mediated immunity by expressing inhibitory members of the B7 family (Petroff et al, 2003), and apoptosis-inducing members of the tumor necrosis factor (TNF) family of

ligands (Chen et al,1991). As with cells in the decidua, fetal cells produce immunosuppressive cytokines, chemokines, and prostaglandins that dampen T lymphocyte proliferation and export high levels of immune suppressive hormones such as progesterone (Hunt et al, 2005).

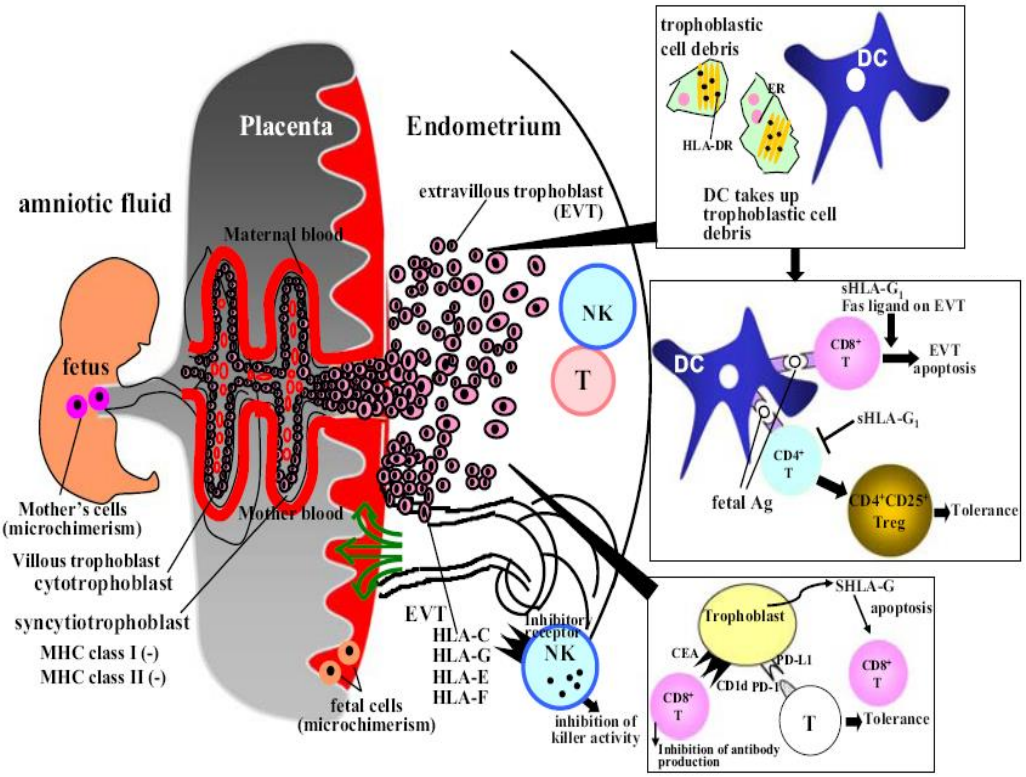


**Figure 1: Induction of maternal MHC antigen-specific tolerance during pregnancy in human.** Villous trophoblasts do not express MHC class I antigens or MHC class II antigens, but MHC class II antigens such as HLA-DR, and DQ are expressed on the endoplasmic reticulum (ER) in trophoblastic cell debris. These antigens may be presented by maternal dendritic cells, and activated maternal CD8+ T cells may be deleted by sHLA-G1 or the Fas/Fas ligand system. On the other hand, fetal antigen-recognized maternal CD4+ T cells may differentiate to CD4+CD25+ Treg cells. Furthermore, CEA associated with CD1d on trophoblasts may induce regulatory CD8+ T cells, and PD-L1 on trophoblasts may induce tolerance by the PD-L1/PD-1 system (**Wilczynski JR, 2007)**

# HLA CLASS I PROTEIN EXPRESSION IN THE HUMAN PLACENTA

Trophoblast cells strictly regulate their expression of HLA genes and the production of their proteins. Using immunohistochemistry, Blaschitz et al, (2001) investigated the expression of classical and non-classical HLA class I proteins in human placenta using various monoclonal antibodies. They reported that classical HLA class I proteins are expressed in all non-trophoblastic cells including the fetal and maternal cells. Comparison of HLA-A and HLA-B staining intensities within the villous stroma indicates that during first trimester of pregnancy the fetal HLA-B proteins are expressed before HLA-A appears. Among the trophoblast populations, the syncytiotrophoblast does not show any HLA class I staining, but the extravillous cells express high amounts of HLA-G together with HLA-C. With the antibodies used in their study, the authers did not detect HLA-E in any trophoblasts.

Shobu et al, (2006) showed that HLA-G was expressed strongly in the cytoplasm and on the cell surface during all stages of pregnancy. In extravillous trophoblasts (EVTs), HLA-F was expressed only in the cytoplasm weakly during the first trimester, after which expression increased and moved to the cell surface with the progression of pregnancy from the second trimester, which was confirmed by the results of double-labeled immunofluorescence staining with anti-HLA-F and anti-HLA-G antibodies, and by flow cytometry using trophoblasts isolated from the decidua. HLA-E showed similar expression as HLA-F, though it was expressed on the cell surface from the first trimester.

Although the genomic structure of *HLA-G* is similar to other class I genes, it is unique in most other respects. The *HLA-G* gene has eight exons encoding a signal peptide (exon 1), the $\alpha$1, $\alpha$2, and $\alpha$3 domains (exons 2, 3, and 4, respectively), the transmembrane domain (exon 5), and the intracellular domain (exons 6 and 7), similar to other class I genes. However, a premature stop codon in exon 6 results in a truncated cytoplasmic tail that reveals a cryptic retrieval motif (Park et al, 2001). This results in the slower turnover and prolonged expression of HLA-G at the cell surface, and possibly the inefficient presentation of exogenous peptides. Park and colleagues interpret this as evidence that the primary function of HLA-G is not antigen presentation but as an inhibitory ligand for NK cells (Park et al, 2001).

A second unique feature of *HLA-G* is that it encodes multiple isoforms as a result of alternative splicing. The full-length isoform HLA-G1 is structurally similar to other class I genes, except for the truncated cytoplasmic tail. The G2 isoform results from the removal of exon 3 and homodimerizes to form an HLA class II-like structure (Ishitani and Geraghty, 1992; Morales et al, 2003). These two isoforms are expressed as soluble proteins (HLA-G5 and -G6, respectively) due to the inclusion of intron 4 sequences in the mature mRNA, resulting in secreted proteins with an additional 21 amino acids (encoded by intron 4 sequences) following the $\alpha$3 domain (Fujii et al, 1994). HLA-G3 results from the removal of exons 3 and 4. HLA-G4 and -G7 mRNAs are not abundant in placentas. Exon 4 (encoding the $\alpha$3 domain) is spliced out of the HLA-G4 transcript; the HLA-G7 transcript includes exon 2 and part of intron 2 and is predicted to encode a small soluble isoform (Hunt et al, 2005).

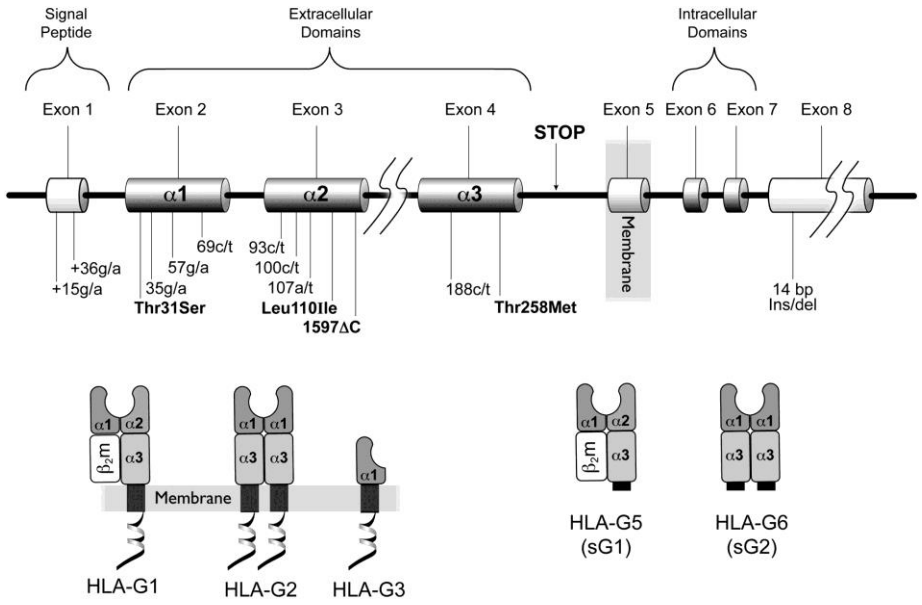


**Figure 2: Multiple HLA-G proteins result from alternative mRNA splicing.** Upper: The HLA gene is composed of 8 exons arranged in the same sequence as other HLA class I genes. The gene is alternatively spliced to yield 7 transcripts. In two of these, a stop sequence in intron 4 results in soluble isoforms. Alleles encoded by the polymorphisms and their amino acid substitutions or deletion are shown: *0103 (Thr31Ser), *0104 (Leu110ILe), *0105 (1597deltaC), *0106 (Thr258Met). A 14 bp insertion/deletion is present in exon 8 in the 3' UTR. α1, α2, α3 extracellular domains. Lower: Three messages encode membrane isoforms (HLA-G1, -G2, -G3) and two encode soluble isoforms (HLA-G5 and -G6, also known as sG1 and sG2, respectively). HLA-G1 and -G5 associate with light chain, β2m; the other three do not. Isoforms HLA-G4 and -G7 remain poorly defined and are not illustrated (Hunt et al, 2005).

Compared with the classical class I genes, the most polymorphic genes in the human genome, *HLA-G* has relatively little polymorphism in its coding region. HLA-G1 and/or -G5 proteins do indeed play an important role in the maintenance of pregnancy (Carosella et al, 2003).

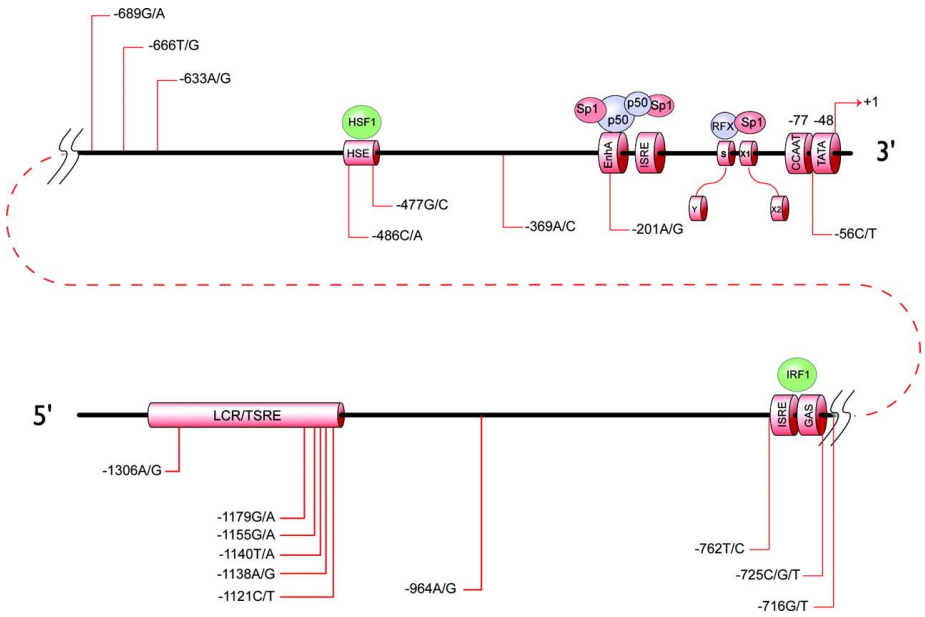


**Figure 3: Variation in the 5'-upstream regulatory region of HLA-G.** Eight unique haplotypes are defined by the polymorphisms. Polymorphisms are frequently associated with transcription factor binding sites and could affect the efficiency of transcription of HLA-G (Hunt et al, 2005).

New isoform-specific antibodies show that HLA-G5 is present throughout the placenta and within the chorion membrane, decidua, and maternal blood (Morales et al, 2003).

An antibody recognizing HLA-G2 and -G6 shows that one or another of these isoforms is prominent in/on extravillous cytotrophoblast cells distal to the placental villi, cytotrophoblast cells infiltrating the decidua, and some chorion membrane cytotrophoblast cells (Morales et al, 2003) HLA-G2/G6 is located in the same cells as HLA-G1 (Lim et al, 1997).

Blaschitz et al, (2005) investigated HLA-G isoform distribution in human first trimester and term placentas *in situ* and *in vitro*. The results obtained by applying