MOLECULAR ASSOCIATIONS OF REPRODUCTIVE FAILURE

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Approximately one in four pregnant women experience one or more miscarriages, making spontaneous abortion the most common pregnancy complication, and of public health importance. Recurrent spontaneous abortion (RSA) can be defined as the loss of two or more pregnancies and affects 1% of couples. This prevalence is higher than expected by chance, suggesting some couples have an underlying systemic cause for RSA.

We have chosen to study two immunological aspects of pregnancy loss. The first involves maternal defense against infection in terms of predicted mannose binding lectin (MBL) plasma levels. The second approach is to analyze the human leukocyte antigen-G (HLA-G) gene, which is believed to play a role in maternal recognition of paternal antigens at the maternal-fetal interface.

The case population included women having two or more clinically recognized spontaneous abortions as well as having unknown etiology for RSA. Control subjects were selected from healthy primiparous women with no history of miscarriage. Cases and controls were genotyped for five MBL single nucleotide polymorphisms (SNPs). Both populations genotyped were in Hardy-Weinberg equilibrium, at all five sites. Fisher's exact test of cases and controls was not significant at each of the five sites, p-values > 0.05. No association was observed between MBL genotypes or predicted MBL plasma levels and risk of RSA, or presence of live birth and recurrent pregnancy loss, among women with unexplained RSA.

Using the same population, the HLA-G promoter region and 3' untranslated region (UTR) was sequenced in cases and controls. Twenty-three SNPs were observed with a minor allele frequency >0.02 in the promoter region. Linkage disequilibrium was detected throughout 1400 base pairs of the promoter region that were sequenced. While SNP data revealed allele

frequency differences between cases and controls, haplotype data proved even more beneficial; one haplotype potentially predicting increased risk of RSA, while the other potentially protecting against risk of RSA. Finally, cases had a higher frequency of individuals homozygous for the 14 base pair insertion in the 3' UTR.

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1.0 INTRODUCTION: SPONTANEOUS ABORTION

Reproductive failure is a broad term meaning the inability to conceive, known as infertility, or the inability to maintain a pregnancy, known as miscarriage (Wold and Arici 2005). A miscarriage is any loss before 20 gestational weeks (Devi Wold et al. 2005). Miscarriages affect 15% of women, primarily in the first trimester, recurrent pregnancy loss occurs in one to two percent of this population (Clark et al. 2001). Most recurrent abortions involve a number of consecutive losses, three or more. Recurrent spontaneous abortion (RSA) has also been defined as two or more spontaneous abortions (Lanasa and Hogge 2000). Patients with a single live birth followed by a series of miscarriages are considered to have similar conditions to those with only miscarriages (Clark et al. 2001). A genetic component may be involved when a woman experiences a mixture of live births and miscarriages (Clark et al. 2001).

Determining a cause for miscarriage has become the topic of many studies. In couples experiencing repetitive pregnancy loss, approximately two-thirds can establish a definite cause after thorough evaluation, i.e. genetic endocrinologic, anatomic, immunologic, thrombophilic and physician assessment (Stephenson 1996). Women with two losses have identifiable problems just as frequently as women with three or more losses (Devi Wold et al. 2005), therefore it is reasonable to group both populations together in research and clinical situations.

Lack of success of previous pregnancies is a risk factor affecting incidence of miscarriage in women; reproductive history is important for couples trying to have children. Women with no live births experiencing repeated pregnancy loss are known as primary recurrent spontaneous aborters, while women with previous live births experiencing spontaneous abortions are classified as secondary recurrent spontaneous aborters. Primary recurrent aborters have a rate of miscarriage as low as five percent that increases to 50% after three or more losses (Dhont 2003).

The risk of a fourth loss is 30% in secondary recurrent aborters with three or more losses (Dhont 2003).

Known causes of recurrent miscarriages include genetic factors, environmental influences, infection, metabolic and endocrine effects as well as anatomic defects. Approximately 20-50% of recurrent miscarriages however have unknown etiologies (Laird et al. 2003). Because of the multi-factorial nature, recurrent spontaneous abortion is difficult to diagnose and treat. Maternal and fetal immune response is an important but understudied area that has been suggested to provide answers for unknown spontaneous abortions.

It is extremely likely that more than one immune cause for recurrent miscarriage exists. There are two theories linking pregnancy loss to immune factors. The first idea involves maternal and fetal defense against infection. If the mother is exposed to pathogens, the body's method of resistance to microorganisms could also harm the pregnancy. Furthermore, because the fetus has an immature immune system, introduction of infection could directly harm the fetus, resulting in termination. The second theory involves maternal immune system recognition of paternal antigens on the feto-placental unit, followed by damage to fetal cells and abortion (Laird et al. 2003). The mechanisms that protect a pregnancy from the maternal immune system are not well understood therefore immunological assessment of normal pregnancy, let alone abnormal pregnancy, is challenging (Laird et al. 2003).

We plan to test both theories by looking at two different genes responsible for immune responses. Mannose binding lectin (MBL) may be responsible for opsonization of pathogens and activated by maternal response to infection while human leukocyte antigen-G (HLA-G) may be a factor in paternal antigen recognition by mother and/or the fetus.

2.0 RESEARCH

2.1 HYPOTHESIS

Multi-site haplotypes in the MBL gene and in the promoter region of HLA-G are associated with increased genetic risks of recurrent pregnancy loss.

2.2 OBJECTIVES

2.2.1 Specific aim one

Several infectious agents have been proposed as a cause for recurrent miscarriage and preterm labor. The first specific aim is to genotype mis-sense mutations related to reduced MBL, as well as MBL promoter region polymorphisms, associated with altered MBL expression. We will classify our cases and controls by high, intermediate and low MBL levels based on the mis-sense mutations and promoter polymorphisms to see if there is a relationship between certain MBL alleles and predicted quantitative MBL levels in case and control populations.

2.2.2 Specific aim two

The HLA-G gene is primarily expressed by trophoblast cells, which are in contact with mother and fetus. The second specific aim is to sequence 1400 base pairs in the HLA-G promoter region in order to study the polymorphisms and haplotypes. With an abundance of literature concerning the HLA-G gene and only a few groups investigating the promoter region, there is a large

amount to be learned concerning the gene's structure and function in respect to recurrent pregnancy loss.

3.0 BACKGROUND

3.1 MANNOSE BINDING LECTIN

3.1.1 Innate immune system

The innate immune system is the principal defense against infection if/when the adaptive immune response is either compromised or too immature to react. Mannose binding lectin (MBL), also known as mannan binding lectin, is produced by the liver and travels though blood. MBL is a calcium dependant lectin and a component of the innate immune system. More specifically, MBL is a collectin, which is a C-type lectin that plays a role in the immune response against invading organisms (Bernig et al. 2004; Holmskov et al. 2003). MBL is one of five collectins, the remaining four being the pulmonary surfactant proteins SP-A and SP-D, and the plasma lectins, conglutinin and CL-43.

Collectins have a collagen-like region and a lectin region, each with an identifiable purpose. The collagen portion interacts with the effector elements of the innate immune response, while the lectin, or protein, region binds to sugar molecules on the surface of the pathogen (Mohr 2006). MBL, as well as the other collectins, function by binding to carbohydrate structures on the surface of microorganisms and foreign particles including bacteria, fungi, viruses and parasites (Eisen and Minchinton 2003). The interaction of the C-type lectin domain and the microbial carbohydrates leads to activation of the lectin complement system (Bernig et al. 2004; Eisen and Minchinton 2003; Holmskov et al. 2003).

The complement system consists of three pathways: the classical pathway, the alternative pathway and the lectin pathway. Together they are responsible for fighting infection via recruiting inflammatory cells, opsonization and eradicating pathogens (Figure 1) (Janeway 2005).

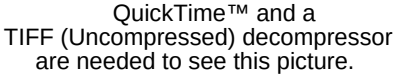

Figure 1: Complement system pathways

The lectin pathway is mediated by MBL (Kaiser 2006). A set of plasma proteins designed to respond to extracellular antigens circulate in the blood in inactive forms until they recognize foreign microorganisms, at which time they become active (Kaiser 2006). Because pathogens have different surface structures (i.e. mannose groups) they can be differentiated by MBL from mammalian cells. The lectin pathway is initiated by the binding of MBL to the mannose groups of microbial carbohydrates, resulting in chemotactical attraction of phagocytes, inflammation, B-lymphocyte activation, etc. (Kaiser 2006). This response is part of an individual's protection against disease.

3.1.2 Mannose binding lectin gene

Circulating levels of MBL and functional activity are partially regulated by genetic variation in the MBL2 gene, which encodes for MBL (Bernig et al. 2004). The MBL2 gene is located on chromosome 10q11.2-21 and consists of four exons. An MBL pseudo-gene, MBL1, has been located in the same chromosomal region (Garred et al. 2003). Three different variant alleles in MBL2 coding for structurally abnormal proteins have been identified in codons 52, 54 and 57 of exon 1, also known as B, C and D alleles. In the proximal promoter region there are two other single nucleotide polymorphisms (SNPs), located at –550 and –221 or L/H and X/Y. Together, these SNPs can be used to form haplotypes, which have been reported to partially explain changes in complement activation and decreased circulating levels of MBL (Bernig et al. 2004).

Individuals heterozygous for codon 52, 54 and 57 SNPs are susceptible to certain infections, due to low concentration levels of MBL, while concurrently protected against particular pathogens, specifically intracellular microorganism that depend on the MBL system for phagocytosis (Bernig et al. 2004). These structural variants have a significant reduction effect on serum MBL levels, more so than the promoter variants (Baxter et al. 2001). Ninety percent of individuals who are homozygous for the wild type structural gene have MBL concentratiosn >0.6 μg/ml, while the majority of heterozygotes have plasma MBL levels <0.6 μg/ml (Baxter et al. 2001; Madsen et al. 1994). Individuals who are homozygous or compound heterozygous for the less common alleles have MBL plasma concentrations of <1% of the wild type concentrations (Baxter et al. 2001). And heterozygotes have MBL plasma concentrations of about 10% of the wild type levels (Baxter et al. 2001).

Most individuals who are MBL deficient do not get infections. One theory suggests a second immune defect must occur for susceptibility of infection to become pronounced (Summerfield 2003). Diseases such as bacterial infections (i.e. meningococcal disease), parasitic infection (i.e. malaria), cystic fibrosis and systemic lupus erythematosus have been associated with certain haplotypes and low serum MBL levels (Summerfield 2003). Furthermore, infection due to immunodeficiency (i.e. common variable immunodeficiency and bone-marrow transplantation) and vascular disorders are more common in those with low plasma MBL levels

(Tsutsumi et al. 2005). However, in some situations low MBL production can protect against disease. An MBL deficiency decreases the "infectivity" of some intracellular pathogens by limiting opsonization and phagocytosis (Worthley et al. 2005). Individuals with a significantly higher MBL levels than controls show resistance to *Mycobacterium leprae* (leprosy) as well as tuberculosis meningitis (Turner and Hamvas 2000; Worthley et al. 2005).

3.1.3 Mannose binding lectin and pregnancy

Reduced MBL levels in the mother and/or the fetus can have a negative effect on pregnancy. MBL deficient fetuses are more susceptible to infection and or inflammatory events in utero (Kilpatrick et al. 1999). This is because the fetus is less reactive to infection and therefore unable to properly activate the complement cascade and phagocytosis. If an infection is not detected early and/or is too severe, the fetus will not be able to adequately manage the pathogen and abortion may occur.

Infection is also a concern for a pregnant mother with low MBL levels. A less sensitive innate immune response indicates the mother will be more susceptible to a prolonged and sustained inflammatory response. The consequence is a down-regulation of insulin sensitivity in response to the sustained release of inflammatory cytokines. This has the potential to contribute to gestational diabetes. Insufficient amounts of MBL have been linked to gestational diabetes mellitus, preterm birth and recurrent infection, all of which increase risk for RSA (Annells et al. 2004; Megia et al. 2004).

Furthermore if the fetus is exposed to more viruses, bacteria and disease as a result of the mother's poor ability to fight infection adequately, pregnancy complications become more likely. Low maternal MBL levels can cause excess inflammatory responses and can harm the fetus directly by altering the fetus's environment. The involvement of MBL deficiency with acute infantile infection suggests that a lack of sufficient MBL may lead to miscarriage (Baxter et al. 2001; Madsen et al. 1994).

3.1.4 Mannose binding lectin literature review

A 1999 study was performed with 146 Danish women and 46 Scottish women experiencing recurrent miscarriage. MBL levels were determined by serum or plasma assays, blood was drawn when patients were not pregnant. MBL levels were also investigated in 444 controls taken from males and females. Results showed a correlation between the frequency of MBL deficiency and number of miscarriages, $p < 0.01$ (Christiansen et al. 1999).

Another study found MBL concentrations less than or equal to 0.1 μg/ml were clinically significant to risk of miscarriage (Kilpatrick et al. 1999). By studying levels in 397 males and females experiencing RSA and 376 controls, it was confirmed that low MBL concentrations are over-represented in couples with RSA. The authors concluded the fetus's MBL genotype is a critical factor in successful pregnancy and women with MBL less than or equal to 0.01 μg/ml are twice as likely to experience miscarriage as women with median levels of MBL (Kilpatrick et al. 1999).

In 2000, Kilpatrick conducted a study of MBL concentration during normal pregnancy. Using 14 patients, measurements of MBL were taken before and during pregnancy. Three patients experienced significant increases in MBL concentrations, however "significant increase" was not well defined. Conclusions were that a modest but significant increase occurs as a result of pregnancy, however it was also noted that MBL levels rarely increase during the first trimester but increase later in pregnancy (Kilpatrick 2000).

In a comprehensive study by Baxter et al. 2001, the authors looked at the frequency of variants in MBL, tumour necrosis factor (TNF) and lymphotoxin alpha (LTA). Alleles in 76 Caucasian couples experiencing RSA were examined. MBL variant frequencies associated with susceptibility to infection showed similar haplotype frequencies in cases and controls. Haplotype variation in TNF and LTA, which are associated with severity of infection, were also similar in cases and controls (Baxter et al. 2001).

More data was generated when pregnancy success and MBL serum levels were compared in 217 women experiencing RSA and 111 of their husbands. Controls were 104 couples with no history of reproductive complications and 210 blood donors. Blood was taken from patients while they were not pregnant. Women experiencing RSA had insignificantly higher rates of MBL levels, less than or equal to 0.1 µg/ml (Kruse et al. 2002). Patients with MBL levels less than or equal to 0.1 µg/ml did show an increase in abortion rate than patients with normal MBL levels (Kruse et al. 2002).

Finally of interest, a study was conducted in order to determine whether MBL interacts with *Candida* spp., yeast cells that cause valvovaginal candidiasis (Pellis et al. 2005), one of the most frequent vaginal infections. MBL, along with C1q and C3, are the molecules of the complement system therefore they included all three candidates in the analysis. Cases included 47 women experiencing gynecological symptoms of infection and 23 healthy women with no clinical signs of inflammations. Findings showed when there is vaginal infection MBL and C3 are present in the vaginal cavity acting as recognition molecules for the infectious agents (Pellis et al. 2005). MBL, but not C1q, binds to bacteria and fungi in the vagina, suggesting a lectin pathway and a classical pathway play a role in immune defense (Pellis et al. 2005).

In summary, it has been confirmed that MBL levels are a factor in pregnancy. It is also apparent that MBL plays a role in the defense against vaginal infection. MBL is essential to successful pregnancy in regards to maternal defense against infection. Yet there is still some debate as to significant differences in maternal MBL levels between cases and controls associated with pregnancy loss. MBL is involved in maternal immune function and therefore affects the vaginal cavity's response to disease. Given that MBL has a role in maternal and fetal immune response and that disease potentially affects pregnancy, there is justification for further analysis of MBL and its association to RSA.

3.2 HUMAN LEUKOCYTE ANTIGEN-G

3.2.1 Maternal fetal interface

The placenta, a derivative of the trophoblast, makes up the maternal-fetal interface in all mammals (Agrawal and Pandey 2003). Trophoblst cells separate from the inner cell mass as the zygote, or fertilized egg, travel through the Fallopian tube prior to implantation in the uterine lining (Agrawal and Pandey 2003). These cells line the outside of the blastocyst, a hollow ball of cells approximately five to eight days old (Merck Manuals 2006). During implantation they bind to the endometrium and develop into two layers, an inner layer of mononuclear cytotrophoblasts and an outer layer of multinuclear cytoplasm (MedicalGlossary.org 2004). This outer layer forms the maternal-fetal interface, the placenta. The development and role of the placenta is important due to its physical connection of the genetically dissimilar embryo to the uterus.

By definition a syncytuim is a large region of cytoplasm that contains many nuclei (Wikipedia 2006). The multinuclear cytoplasmic layer is also known as syncytiotrophoblasts, a mass of cytoplasm not separated into individual cells. The syncytiotrophoblast is a membrane directly exposed to maternal blood and all of the immune system agents circulating (Agrawal and Pandey 2003). This membrane surrounds the fetus and functions as a "biological dialysis membrane" involved in cooperative exchange of molecules into and out of the fetal environment (Agrawal and Pandey 2003). It is believed that the syncytiotrophoblast cells are responsible for the fetus's immunological exemption from the mother's immune system.

Cytotrophoblasts, the inner layer of cells between the syncytiotrophoblast and chorionic villus capillaries, attach to the placenta and the maternal decidua (Wikipedia 2006). At the decidua, extravillous trophoblast (EVT) cells enter and natural killer (NK) cells become more proliferate (Moffett-King 2002). Extravillous trophoblast cells are the only trophoblast cells that express MHC class I antigens and interact with NK cells (Parham 2004). Human leukocyte antigen-G (HLA-G), a ligand for NK cell receptors, is expressed only by EVT and medullary epithelial cells (found in the thymus) whereas some of the other MHC class I molecules (i.e. HLA-C and HLA-E) are more common.

3.2.2 Natural killer cells

NK cells are large granular lymphocytes. Their activity is regulated by the interaction of cell surface receptors with NK cell ligands expressed on the surface of pathogens. In contrast, interaction of an inhibitory receptor, such as killer cell immunoglobulin receptors (KIRs), with its ligand, negatively controls NK cell activity. NK cells are not classified as T cells or B cells however they do express cell surface antigens CD16 and CD56. NK cell function includes direct elimination of virus infected cells and the production of cytokines, providing rapid but non-specific response to infection (Wold and Arici 2005). NK cells recognize and lyse target cells by two mechanisms, antibody dependant cell cytotoxicity and natural cytotoxicity (Yamada et al. 2005).

A distinctive feature of the uterine mucosa during pregnancy is the presence of a large population of NK cells known at uterine natural killer cells (uNK). Uterine natural killer cells can be differentiated by their high expression of CD56, a neural cell adhesion molecule and NK cell marker, in comparison to peripheral blood NK cells (Wold and Arici 2005). In early pregnancy uNK cells comprise approximately 70% of the total leukocyte population in the decidua (Wold and Arici 2005). It is not until after 20 weeks of gestation that the population of uNK cells decreases (Moffett-King 2002).

When pregnancy occurs, the uNK cells localize in the decidua coming into contact with the invading trophoblast (van der Meer et al. 2004). Their presence during implantation and the expression of HLA-G receptors suggest a critical role during implantation through interaction with HLA-G, which are expressed on the invading trophoblast (Figure 2) (Emmer et al. 2002; van der Meer et al. 2004).

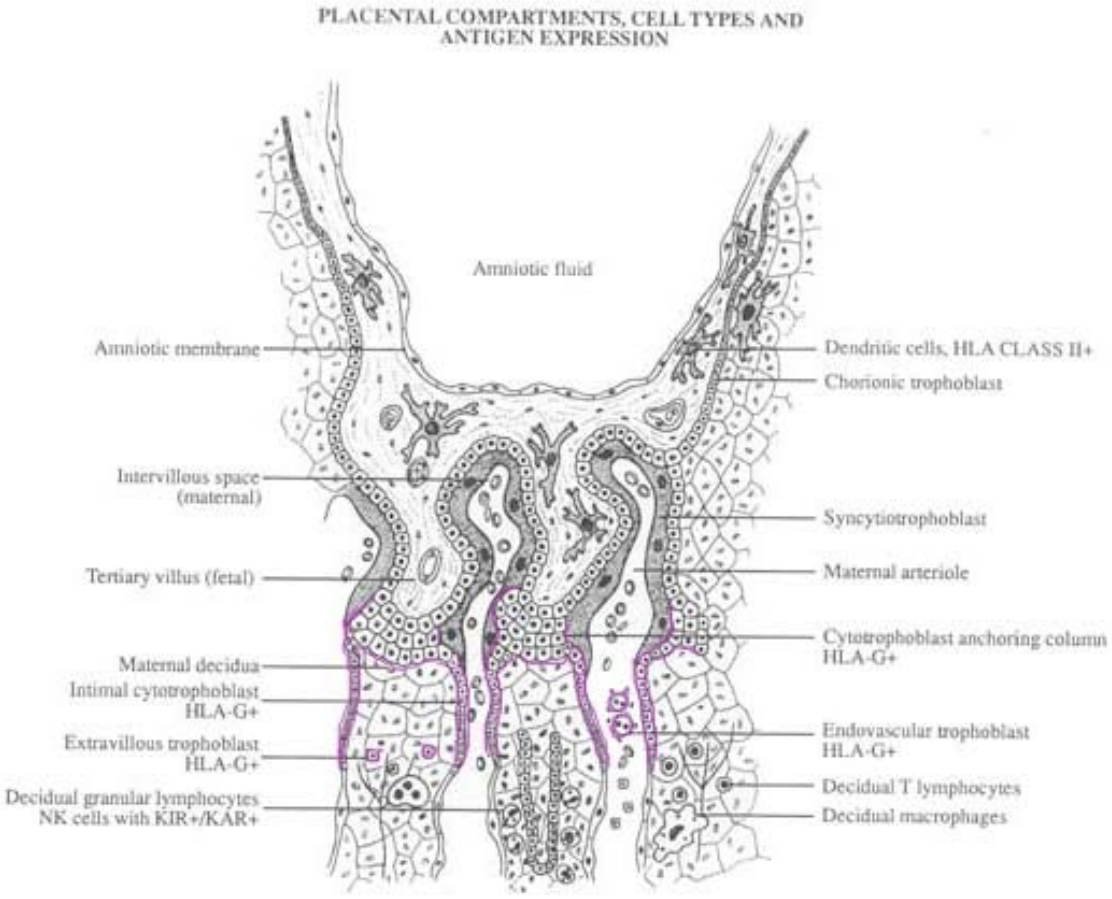

Figure 2: Location of HLA-G expression (Carter 2000)

The uNK cell resistance is the result of an interaction between HLA-G and KIRs (Khalil-Daher et al. 1999). KIRs obstruct cytolytic functions of killer cells, in this case uNK cells, by specific interaction with major histocompatability complex (MHC) class I molecules (Cho et al. 1999), i.e. HLA-G. Consequently, a deletion or mutation of the HLA-G gene could lead to loss of fetus in early pregnancy (Rouas-Freiss et al. 1997).

3.2.3 Human leukocyte antigen-G gene

There are two groups of MHC HLA genes: classical HLA class Ia (HLA-A, -B and -C) and II (HLA-DR, -DQ and -DP) and non-classical HLA class Ib (HLA-E, -F and -G). Less variation has been reported in HLA Ib genes in contrast to the highly polymorphic HLA Ia genes (Hviid

2006). Both the HLA Ia and Ib genes are located in the same region on the short arm of chromosome 6. In particular, HLA-G is located on chromosome 6p22.1; it is 3363 base pairs long, of which 1842 base pairs make up its six exons. The promoter region and 3' untranslated region (UTR) surrounding the gene are polymorphic and of interest in many spontaneous abortion studies. HLA-G is expressed in all EVT populations including the cytotrophoblast cells, interstitial trophoblasts, endovascular trophobalsts and placental cells (Agrawal and Pandey 2003). Because of HLA-G's proximity to fetal tissue, a genetically foreign entity, and its role in the maternal and fetal immune defense, it is likely that HLA-G expression and subsequent interaction with uNK cells and KIRs is a factor in all, successful and unsuccessful, pregnancy.

3.2.4 Human leukocyte antigen-G literature review

3.2.4.1 HLA-G exon 2 and exon 3

In 2001 Pfeiffer et al. studied 78 Caucasian couples experiencing three or more RSA. The control population consisted of 52 Caucasian women with at least one successful pregnancy and no history of infertility. After sequencing exon 2 and exon 3, 14 different alleles were found in the cases and controls. Two HLA-G alleles, *01013 and *0105N, were more frequent in patients with RSA compared with fertile controls. These alleles are also associated with lower levels of circulating soluble HLA-G (Pfeiffer et al. 2001). The conclusion was HLA-G genotype may be a contributing risk factor in RSA.

A different study suggested that HLA-G alleles *0104 and *0105N are associated with couples experiencing RSA (Aldrich et al. 2001). One hundred thirteen couples with unexplained RSA and no more than one live birth were genotyped for seven polymorphism defining twelve HLA-G alleles. Ethnicities included Caucasians, African-Americans, Hispanics, one East Asian and one Asian Indian. This study also supported the association between HLA-G*0105N Pfeiffer et al. reported. However, HLA-G allele *01013 was not associated with pregnancy loss in this study.

Most recently, a study of 120 Indian women experiencing three or more consecutive miscarriages compared exon 2 and exon 3 polymorphisms to those in 120 fertile controls. Eleven

alleles were found and data supported their hypothesis that HLA-G polymorphism may contribute to recurrent fetal loss. In this study HLA-G alleles *010103, 010105 and 010108 were more frequent in women experiencing RSA (Abbas et al. 2004).

3.2.4.2 HLA-G 3' untranslated region

The 3' UTR of HLA-G, frequently referred to as exon 8 in the literature, was first investigated in 1995 by K.E. Humphrey. Within the 3'UTR is a 14 base pair insertion/deletion that is associated with a splice variant resulting in a 92 base pair removal in the 3' UTR upstream of the 3'UTR insertion/deletion. The 92 bp deletion is located at the beginning of "exon 8" and extends to the 3' UTR to a potential acceptor AG site (Rousseau et al. 2003).

With this information, pedigrees and the 14 base pair deletion were analyzed via linkage analysis and genotyping (Humphrey et al. 1995). One hundred ninety-six individuals including pre-eclamptic/eclamptic (PE/E) pregnant women, individuals from PE/E pregnancies, relatives, husbands and controls were genotyped. Looking only at the 3'UTR they stated genotypic and gene frequencies were not significantly different in the groups studied and linkage between maternal expression of HLA-G and PE/E is unlikely (Humphrey et al. 1995).

In 2003 placentas from normal and mild pre-eclampsia (PE) pregnancies were used to examine HLA-G's possible role in pregnancy. Rousseau et al. analyzed the effect of the 14 base pair deletion on the stability of HLA-G mRNAs by performing actinomycin D treatments on JEG-3 choriocarcinoma cell lines and M8 melanoma cell lines transfected with HLA-G*010102 allele. They found HLA-G mRNAs with the 92 base pair deletion are more stable than complete mRNA. Conclusions suggested this region to be involved in mechanisms for controlling post-transcriptional regulation of HLA-G molecules associated with allelic variants (Rousseau et al. 2003).

This was further studied in a group of 90 women consisting of in vitro fertilization (IVF) patients and those experiencing RSA, as well as 93 controls. It was concluded that the homozygous individuals with the 14 base pair insertion in the 3' UTR were significantly

associated with reduced fertility in respect to unsuccessful IVF treatments and increased risk of recurrent miscarriage (Hviid et al. 2004a). Also in 2004 Hviid published a paper looking at healthy and PE pregnancies in a Danish population. Associations between the 14 base pair deletion in the 3' UTR were compared with birth weight, placental weight and placental ratio (Hviid 2004). A presence of the deletion was associated with increased birth weight in relationship to gestational age and with placental weight at birth. There was also a slightly higher placental ratio in the offspring homozygous for the 14 base pair insertion genotype (Hviid 2004).

One of the largest studies of the insertion/deletion in the 3' UTR included 120 women with no children, 110 had 3 or more RSA and 120 healthy women with three or more live births. Hardy-Weinberg equilibrium (HWE) showed no difference between homozygotes for the insertion/deletion in normal fertile women and RSA women. They did find a difference between heterozygotes in normal women and women with RSA. Frequency of heterozygotes was significantly increased in RSA women compared to normal fertile women (X^2 =6.014, p <0.01) (Tripathi et al. 2004). The frequency of the 14 base pair deletion was 52% in fertile women and 51.6% among women experiencing recurrent fetal losses with no known cause (Tripathi et al. 2004). These results are contradictory to Hviid et al. 2002, whose group reported observing more heterozygotes in controls as compared to RSA cases.

3.2.4.3 HLA-G exons and 3' untranslated region

A comprehensive HLA-G study was conducted in 1996 by Ober et al. Eighty individuals were randomly selected from a group of several hundred Hutterites, a population known to have naturally high fertility rates (Ober et al. 1996). Six HLA-G alleles were defined by exon 2, exon 3 and the 3' UTR variation. Low levels of variation in exon 2 and exon 3 were reported. In general, this study did not have a fertility or infertility focus however they showed Hutterites with polymorphisms did not experience deleterious affects on pregnancy outcomes (Ober et al. 1996).

Examining exons 2, 3, 4 and the 3'UTR, in 61 Danish couples experiencing 3 or more spontaneous abortions and 47 control couples, Hviid et al. reported a greater number of heterozygotes for the 14 base pair insertion/deletion polymorphism in controls than in cases.

HLA-G histo-incompatibility between the fetus and placental unit and the mother was not supported (Hviid et al. 2002). This was followed up in 2003 with a study of trophoplast biopsies from 12 Caucasian first trimester placentas from elective termination pregnancies. Primers in exon 2 and the 3' UTR were used to generate products of HLA-G transcripts from a heterozygous sample (Hviid et al. 2003). A relationship between HLA-G polymorphisms and mRNA levels of different alternatively spliced HLA-G isoforms in first trimester trophoblast cell populations were reported (Hviid et al. 2003). However when the same group looked at placentas from 20 uncomplicated and PE pregnancies, no association between HLA-G protein expression and HLA-G genotype in placentas was found (Hviid et al. 2004b).

Using a definition of RSA as three or more consecutive fetal deaths prior to 28 weeks gestation, Hviid and Christiansen looked at polymorphisms again in exons 2, 3, 4 and the 3' UTR. The case population included 57 Danish women and 47 of their husbands, while the control population consisted of 44 couples. They reported strong linkage disequilibrium existing between HLA-GR3 (an HLA class II gene) locus and HLA-G* 010102 in RSA patients (Hviid and Christiansen 2005).

3.2.4.4 HLA-G promoter region

In 1999, Hviid et al. performed the first investigation of the HLA-G promoter region. Their goal was to detect any possible sequence variation that may affect transcription factor binding, possibly influencing the level or developmental regulation of HLA-G expression (Hviid et al. 1999). Their study included ten DNA samples, consisting of two randomly selected samples each to represent the five HLA-G alleles currently known in 1999. They analyzed sequence from -1437 to -793 and from -383 to 81. After sequencing, they reported seven SNPs (-1413, -1306, -1179, -1140, -1121, -964, -201) and one deletion (-1233) in the promoter region (Hviid et al. 1999).

Variation in the HLA-G promoter region was next examined in relation to miscarriage. Forty-two Hutterite women and men were genotyped for the 14 base pair insertion/deletion in the 3' UTR and five of the seven promoter region SNPs reported by Hviid (Ober et al. 2003). The women experienced multiple conceptions including both miscarriages and successful

pregnancies. These 42 individuals were specifically chosen to represent all eight HLA-G alleles, and not on the basis of reproductive history. Eighteen SNPs (-1306, -1179, -1155, -1140, -1138, -1121, -964, -762, -725, -716, -689, -666, -633, -486, -477, -369, -201, -56) were found in the 1300 bp region upstream of exon 1. One polymorphism, -725 was reported to be associated with fetal loss when both parents carried a G allele at this site. It was further shown that a G allele created a CpG dinucleotide (Ober et al. 2003).

A study of 85 Danish individuals (43 women and 42 men) enrolled in IVF treatment supported Ober et al.'s suggestion of -725 being a factor in pregnancy outcomes. Of the 12 SNPs reported (-964, -762, -725, -716, -689, -666, -633, -486, -477, -369, -201, -56) SNP -725 was observed in samples with homozygous insertions for the 14 base pair marker in the 3' UTR. These individuals did not have detectable soluble HLA-G (sHLA-G1) and HLA-G5 in serum screens, p =0.03 (Hviid et al. 2004c). Based on these findings, Hviid et al. concluded that variation in HLA-G gene's promoter region leads to decreased levels of sHLA-G and may be of possible importance in preventing pregnancy complications (Hviid et al. 2004c).

In order to determine the effect of nucleotide change at -725 and -1121 on haplotypes, a luciferase reporter assay was preformed on five selected HLA-G promoter haplotypes (Ober et al. 2006). The goal was to determine whether variation in the promoter region influenced transcription. Findings showed differences in expression levels between promoters, the haplotypes with a G allele at -725 had significantly higher expression levels compared to a C allele or T allele (Ober et al. 2006).

In 2006 SNP -477 was highlighted to have a possible functional significance due to high levels of interleukin-10 (IL-10) (Hviid et al. 2006). Their population of 61 male and female Caucasian donors was genotyped for the 3' UTR 14 base pair insertion/deletion and sequencing was analyzed between -762 an -400 from start site of transcription. Eleven SNPs (-762, -725, -716, -689, -666, -646, -633, -509, -486, -477, -400), one insertion (-540) and one deletion (-533) were detected. Using PHASE 2.1 and the thirteen variants for analysis they reported eighteen haplotypes along with possible implications for IL-10 secretion from peripheral blood

mononuclear cells, instrumental in immune responses and immune escape or tolerance (Hviid et al. 2006).

Most recent SNP counts were reported from DNA collected in African Americans, European Americans and Chinese individuals participating in an asthma study. Twenty-seven SNPs were found in the promoter region of HLA-G (Tan et al. 2005). These SNPs include the eighteen reported by Ober et al. in 2003 (see above) as well as variation at -990, -922, -810, -646, -509, -483, -443, -400 and -391. With these 27 SNPs, thirteen different haplotypes were reported (Tan et al. 2005).

The previous articles are the most informative when it comes to background studies of HLA-G. For additional articles of interest, see Appendix A. In summary, information concerning HLA-G and spontaneous abortion has been in the literature since 1996. Exon 2 and exon 3 are mostly used to identify the different HLA-G alleles when comparing samples in a study. The 14 base pair insertion/deletion found in the 3' UTR has received a substantial amount of attention in spontaneous abortions studies. And while the promoter region appears to be related to pregnancy loss, previous studies have had small populations and contradictory conclusions.

3.3 MATERIALS AND METHODS

Brief summaries of the materials and methods are discussed below; methods are described in more detail in the manuscripts that follow.

3.3.1 Population

Patient samples (cases) were collected from women who experienced two or more recurrent pregnancy losses. The cases were drawn from the general obstetrical population served by the University of Pittsburgh, Magee Women's Hospital and the University of Minnesota, Center for Reproductive Medicine. All protocols were approved by the Institutional Review Boards of the

University of Pittsburgh and the University of Minnesota and each participant gave written informed consent to participate. Details of the recruitment have been reported by Lanasa et al. 2001. Briefly, idiopathic recurrent spontaneous abortion was defined by 1) having two or more clinically recognized spontaneous abortions and 2) having recurrent spontaneous abortion of unknown cause (Lanasa et al. 2001). Women were excluded from enrollment if they were known to have abnormal hysterosalpinogram, antiphosophlipid antibodies, connective tissue disorder, if either parent had an abnormal karyotype (i.e. balanced translocation) and if the mother had skewed X-inactivation. Ages of participants ranged from 25-52 years, mean of 33.3 years, median of 33 years and mode of 34 years.

Control subjects were selected from healthy primiparous women aged 14-44 years who were seeking prenatal care prior to 20 weeks' gestation at Magee-Womens Hospital in Pittsburgh, Pennsylvania. Of these women screened for possible participation in the study, factors such as planned delivery at another hospital or not being pregnant were deemed ineligible and removed from the study population. After enrollment, those women who experienced spontaneous abortion, termination of pregnancy, molar pregnancy or ectopic pregnancy were determined ineligible and removed from the study population.

3.3.2 Genotyping

High molecular weight DNA was isolated from EDTA anticoagulated whole blood using the PureGene kit (Gentra Systems). The MBL study consisted of 219 cases and 235 controls, while the HLA-G study included 238 cases and 233 controls. Differences in "n" (numbers) can be accounted for due to variation in genotyping and sequencing success across the studies. All populations consisted of Caucasian samples only.

Mannose binding lectin genotyping included three mis-sense variants in exon 1 (R52C, G54D and G57E), each of which is associated with reduced levels of circulating MBL (Kilpatrick 2002), and two promoter variants which are known to influence the expression of the MBL gene (Juliger et al. 2000). The SNPs were analyzed individually by sequence specific priming-polymerase chain reaction (SSP-PCR), restriction enzyme digest or pyrosequencing.

HLA-G promoter region alleles and haplotypes were defined by sequencing cases and controls. Two primer pairs were used due to the 1400 base pairs of HLA-G promoter region we were interested in. Selecting primers to sequence the promoter region of HLA-G was accomplished using the NCBI BLAST website http://www.ncbi.nlm.nih.gov/blast/Blast.cgi. It was important to verify that the primers chosen were unique to the promoter region of HLA-G. For this reason, after choosing the sets of primers, the sequences were reentered into BLAST and checked for homology with the HLA-G gene. SNPs at the HLA-G locus were identified by sequencing the coding and regulatory regions using the BigDye 3.1 terminator ready reaction kit, and running them on the Prism ABI 3730 fluorescent sequencer. We analyzed the aligned sequences using SEQUENCHER 4.5 (Gene Codes). The SNPs in exon 2 were identified using methods similar to the promoter region sequencing, while the 14 base pair insertion/deletion in the 3' UTR was detected by PCR and gel electrophoresis on a 3% gel. All HLA-G sequence and variation was compared to the current sequence reported in SNPper (Appendix B).

3.3.3 Data analysis

Allele frequencies in both the MBL and HLA-G projects were estimated by gene counting and compared to the expectations of HWE by chi-square and Fisher's exact test analysis. Gene counting was carried out using R Consol (R) (RDevelopmentCoreTeam 2005) to ensure accuracy. Odds ratio and power analysis were calculated using an interactive website (Statistics 2004). We compared allele and haplotype frequencies in cases and controls to identify alleles/haplotypes that were non-randomly associated with recurrent pregnancy loss. R was used to calculate Fisher's exact tests and to find odds ratios (RDevelopmentCoreTeam 2005). Chi-square results were calculated using an interactive website (Preacher 2001). MATCH version 1.0 was used to analyze TFBS (Quandt et al. 1995). All common and minor alleles were used in the TFBS searches in order to reveal binding sites that are both created and destroyed due to polymorphisms in the promoter region. Three consensus sequences were used to locate TFBS (Appendix C). Linkage disequilibrium was analyzed using Haploview (Barrett et al. 2005) and haplotypes were constructed using PHASE version 2.1.1 (Stephens 2006).

3.4 RESULTS

3.4.1 Mannose binding lectin

Case and control populations were genotyped for five sites that have been suggested to influence plasma MBL levels: -550, -221, codon 52, codon 54 and codon 57. All five sites were in Hardy-Weinberg equilibrium (HWE). Cases and controls were grouped and predictions of high, intermediate and low plasma MBL levels were assigned; no significant differences were found between cases and controls, X^2 =0.4098 p =0.8147. Cases were stratified by number of RSA to examine frequency of RSA with genotype. No relationship between plasma MBL levels and number of spontaneous abortions was found. Power analysis was performed and we detected 80% power at p=0.05 to observe an odds ratio $\geq$ 1.63 when comparing the high MBL plasma level group to medium and low plasma level groups combined. Therefore, this study was large enough to have shown an effect of RSA due to MBL haplotypes, if a relationship was present.

3.4.2 Human leukocyte antigen-G

The HLA-G study identified a total of 32 SNPs spanning the 1400 bp region downstream of exon 1, 22 of which have minor allele frequencies greater than two percent. Two SNPs, -477 and -369, in the case population deviate significantly from Hardy-Weinberg expectations, p =0.0039 and p =0.0049 respectively. These same two SNPs were also found to be in strong LD with one another (LOD >2 D' =1). Further analysis involved comparing counts at each SNP between case and control groups to determine if variation could be protective of, or account for, RSA. Three sites showed significant differences between case and control SNP frequencies, -486, -477 and -369. Odds ratio calculations for these SNPs indicate an approximate 1.456 fold increase (CI =1.1077-1.9137) for risk of RSA.

Results of the 3' UTR insertion/deletion variation were in HWE and there was no significant difference between cases and controls, X^2 =5.71, p =0.0576. Exon 2 was sequenced in order to provide HLA-G allele nomenclature. Of the six sites, four were in HWE and two sites had frequency of variations too low for accurate HWE testing.

Forty-six TFBS were found in the HLA-G promoter regions. Of these 46, seven have a 5' to 3' core match that is altered by a presence or absence of an allele. Transcription factors affiliated include: Elk-1, Foxd3, Comp1, v-Myb, Hnf-4 and Cdp Cr1.

Haplotype frequencies in case and control populations for exon 2 and the 3' UTR polymorphism as well as for the promoter region were calculated (Appendix G and Appendix F). Many HLA-G papers in the literature define individuals by HLA-G nomenclature based on specific variation in exon 2, exon 3 and the 3' UTR. In our samples, exon 2 and the 3' UTR 14 bp polymorphism were analyzed in order to identify HLA-G allele nomenclature and compare it to the alleles found by other investigators. Six sites, originally identified by others, in exon 2 were genotyped: 188, 294, 374, 410, 484 and 487. We were able to distinguish 16 haplotypes in our case and control population (Appendix D). Allele frequency differences between case and control populations for the 16 haplotypes were not significant, $p = 0.48$. While we found six sites of variation in Exon 2, three sites are usually discussed and include 294, 374 and 410. Therefore we also performed analysis after removing SNPs 188, 484 and 487 in order to provide nomenclature comparable to that which is in the literature (Appendix E). Promoter region haplotypes were constructed; a total of 55 different haplotypes were found in the case and control population (Appendix F). Haplotype frequencies in cases and control groups did not differ significantly, $p = 0.06$, although a trend towards significance is observed.

To define LD across the promoter region we used Haploview. SNP data was analyzed for cases and controls together, (Appendix I) as well for the two individual groups (Appendix J and Appendix K). LD between SNPs and across LD blocks was summarized. We are able to conclude that there is LD across the 1400 bp of promoter region upstream of exon 1.

4.0 MANNOSE BINDING LECTIN MANUSCRIPT

The following manuscript was submitted to the American Journal of Obstetrics and Gynecology

Mannose binding lectin genotypes are not associated with recurrent spontaneous abortion

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This study found no evidence to support a relationship between mannose binding lectin genotypes, or phenotypes, and the risk of recurrent spontaneous abortion.

Mannose binding lectin genotypes are not associated with recurrent spontaneous abortion

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Objective: Infectious agents are known to cause pregnancy loss; an infectious etiology for recurrent spontaneous abortion (RSA) has been proposed. Low mannose binding lectin (MBL) plasma levels, a factor in innate immunity, is an explanation for severe infantile infection and has been proposed as a cause of RSA.

Study Design: To test the hypothesis that MBL genotypes, leading to reduced levels of circulating MBL, are significantly more frequent among women experiencing idiopathic RSA, we conducted a case (n=219)/control (n=235) study of RSA. Subjects were genotyped for promoter variations and missense mutations in exon 1.

Results: Populations genotyped are in Hardy-Weinberg equilibrium. Fisher's exact test is not significant, p-values all >0.05. Number of miscarriages and a presence of live births are unaffected by predicted MBL plasma level.

Conclusion: No association is observed between MBL genotypes or predicted MBL plasma levels and risk of recurrent pregnancy loss among women with unexplained RSA.

Key Words: Mannose binding lectin, Recurrent spontaneous abortion, Genotyping

INTRODUCTION

Recurrent spontaneous abortion (RSA) is the most common pregnancy complication with the majority of cases having unknown causes. RSA was defined by Lanasa et al. 2001 as two or more clinically recognized spontaneous abortions. Because the prevalence of RSA is higher than expected by chance, it has been suggested that some couples have an underlying systemic cause for repeated pregnancy loss [1]. A number of factors have been identified for RSA and include: 1) anatomic abnormalities of the uterus or cervix, 2) cytogenetic disorders such as recurrent aneuploidy or parental balanced translocations, 3) endocrine factors such as inadequate luteal phase, thyroid dysfunction, or diabetes, 4) reproductive tract infections, 5) immunologic causes such as anticardiolipin syndrome [2, 3] and 6) female carriers of X linked recessive male-lethal traits[4]. These etiologies, however, explain causes of recurrent pregnancy loss in only 20 to 50% of cases, depending on the indication for referral [5-8].

Several bacterial and viral infectious agents have been proposed as a cause for miscarriage [9]. It is known that the presence of pathogenic organisms in the maternal genital tract or placenta result in poor pregnancy outcomes [1, 10, 11]. This suggests pregnancy outcome may be determined by maternal or fetal-host immune response. Mannose binding lectin (MBL), one of many elements of the innate immune system, has been indicated as an important component in successful pregnancy. Low MBL concentration has been proposed to be a risk factor for spontaneous abortion (SA) [12]. It has been confirmed that plasma MBL concentrations are determined by a combination of genotypes and polymorphisms in both MBL coding and promoter sequence [13, 14]. Three different variant alleles coding for structurally abnormal proteins have been identified in codons 52, 54 and 57 of exon 1 in the MBL2 gene located on chromosome 10q11.2-q21 [15]. Two single nucleotide polymorphisms (SNPs) lie in the proximal promoter at positions -550 and -221 of the flanking 5‘ region of the MBL2 gene [16], which also influences protein coding. Ninety percent of individuals who are homozygous for the wild type structural gene have MBL concentrations >0.6 ug/ml, while the majority of heterozygotes have plasma MBL levels <0.06 ug/ml [14, 17]. Individuals who were homozygous or compound heterozygous for the less common alleles had MBL plasma concentrations of <1% of the wild type concentrations.

Plasma levels of MBL are largely genetically determined and can be predicted by examining SNPs in the MBL gene and surrounding sequence [15]. We therefore tested the hypothesis that MBL genotypes leading to reduced levels of circulating MBL are significantly more frequent among women who have experienced idiopathic recurrent pregnancy loss.

MATERIALS AND METHODS

The cases (n=219) are Caucasian women who experienced two or more recurrent pregnancy losses. They were drawn from the general obstetrical population served by the University of Pittsburgh, Magee-Womens Hospital and the University of Minnesota, Center for Reproductive Medicine. The Institutional Review Boards of the University of Pittsburgh and the University of Minnesota approved all protocols, and each participant gave written informed consent. Details of the recruitment have been reported by Lanasa et al. [4]. Briefly, idiopathic RSA was defined by: 1) having two or more clinically recognized spontaneous abortions and 2) having RSA of unknown cause. Women were excluded if they were known to have abnormal hysterosalpinogram, antiphosophlipid antibodies, connective tissue disorder, skewed X-inactivation, or if either parent had an abnormal karyotype (i.e. balanced translocation). Control subjects (n=236) were selected from healthy Caucasian women aged 14-44 years, who were seeking prenatal care at Magee-Womens Hospital in Pittsburgh, Pennsylvania and known to have a normal pregnancy outcome.

High molecular weight DNA was isolated from EDTA anticoagulated whole blood using the PureGene kit (Gentra Systems). Mannose binding lectin genotyping included three missense variants in exon 1 (R52C, G54D and G57E) and two functional promoter variants (-550 C>G and -221 G>C). To detect polymorphisms at -550, DNA was subjected to sequence specific priming-polymerase chain reaction (SSP-PCR) [14]. Briefly, this method uses twin-pair primers to detect allelic nucleotides differentially[18]. Methods are described in detail by Matsushita et al. 1998. The -221 site was investigated by the methods from Roelofs et al. 2003. PCR was carried out, the amplification product was digested by BasJI, and the fragments were separated by

electrophoresis through agarose gels [19]. Exon 1 SNPs were identified by pyrosequencing using amplification [20]. Primers were: forward 5'-CGTCTTACTCAGAAACTGTGACCTGTGAGG-3' and reverse 5'-TTCCTCTGGAAGGTAAAGAATTGCAG-3' with an internal sequencing primer: 5'-GGTTCCCCCTTTTCT-3'.

Genotype frequencies were estimated by gene counting and compared to the expectations of Hardy-Weinberg equilibrium by chi square analysis and Fisher's exact test. Dipolotypes were estimated and classified based on methodology as described in Baxter et al. [1]. Allele and genotype frequencies were compared in cases and controls using chi square contingency table analysis.

RESULTS

A total of 219 cases and 236 controls were genotyped for the 5 polymorphisms, Table 1. Overall the number of miscarriages in the case population ranges from 2 SA to 10 SA per patient (mean = 3.575, median = 3, Figure 1). In patients with more than 4 SA the mean number of losses per patient is 6.636.

The case and control populations genotyped are in Hardy-Weinberg equilibrium, at all five sites. Fisher's exact test of cases and controls is not significant at each of the 5 sites, p-values for -550, -221, codon 52, codon 54 and codon 57 are 0.7803, 0.3867, 0.2933, 0.8051 and 0.6046 respectively (Table I).

Plasma MBL concentration is determined by a combination of genotypes and is influenced by mutations in the structural gene as well as by two dimorphic loci in the promoter region [13, 14]. Therefore we classified our cases and controls as high, intermediate and low in predicted MBL plasma concentrations.

Using the classification methods described by Baxter et al. [1], it is possible to define the collection of genotypes as phenotypes (Figure 2). There is no significant association in cases and

controls between number of spontaneous abortions and predicted plasma levels of MBL (2 degrees of freedom, X^2= 0.4098, p= 0.81472) nor is there an association between lack of live births and MBL levels (2 degrees of freedom, X^2=0.6620, p=0.7182). There is no association between a history of RSA and multi-SNP genotypes at the MBL locus. There is no significant difference in risk of RSA and plasma MBL levels (high, intermediate, low) with number of SA in cases and controls.

COMMENT

In a recent study of 217 women with three or more unexplained SA, Kruse et al. [21] reported women with low maternal serum MBL levels had a higher abortion rate than patients with normal MBL levels [21]. A similar study of 146 Danish women and 49 Scottish women with three or more unexplained SA reported MBL deficiency was found with increased frequency among women with RSA in both populations, reaching statistical significance in the Scottish population [22]. These and other studies suggest that MBL levels could account for previously unexplained RSA. However, Baxter et al. in 2001 published contradictory results of recurrent miscarriage and MBL, stating no association between recurrent miscarriage and variant alleles in the MBL gene. Our study too has shown recurrent spontaneous abortion is not influenced by MBL genotypes, supporting Baxter et al. [1].

Traditionally RSA has been defined as three or more spontaneous, consecutive pregnancy losses. It has been reported that patients with a single live born followed by a series of miscarriages have similar disorders to those patients with only miscarriages [23]. Also, because risk of recurrent miscarriage increases with each prior miscarriage it has been argued that evaluation can begin after two losses instead of waiting until three [24]. The RSA criteria of two or more miscarriages were required for eligibility in this study, as opposed to the RSA definition of three or more recurrent pregnancy losses. It is our belief that the population used gives a broader range of pregnancy loss cases and results in a more comprehensive genotyping study. Due to differences in the definition of RSA, it was therefore important to determine if women with 2 SA would have similar phenotypes to those women experiencing 3 or more SA. It is clear from Figure 2 that had we examined women experiencing 3 or more SA, leaving out the population

experiencing 2 SA, the results would not have changed. Our case population does not produce significantly different levels of MBL than our control population.

Investigating MBL plasma levels in pregnant mothers implies we are studying a maternal condition or phenotype; the influence of a genotype. The mechanism we propose involves women with low MBL plasma levels being at an increased risk for recurrent miscarriage because of their diminished ability to detect and fight infection. This would result in potentially every pregnancy being lost to miscarriage if exposed to maternal genital tract infection. Eighty of our cases experienced two or more miscarriages with no live births and were observed to have similar predicted MBL plasma levels to our control population, p=0.7182, Figure 2. Having no live births does not impact predicted MBL plasma levels in women suffering from RSA. One suggestion to further strengthen these findings would be future analysis of infection rates and rates of exposure to infections in the populations studied.

The diplotypes analyzed in this study were estimated based on previous findings [1]. Given that we did not measure plasma levels in each case and control, our findings rely on the reported diplotype frequencies and relationships previously reported. Given the consensus in the literature, we are confident that our methods of estimating MBL concentrations were true and informative.

Given our sample size of 219 cases and 235 controls, we had 80% power (at p=0.05) to detect an odds ratio $\geq$1.63 when comparing the proportion of individuals with genetically determined high MBL levels versus individuals with genetically determined intermediate or low MBL levels between the two groups. If the true odds ratio, between individuals with high MBL levels compared to individuals with intermediate and low MBL, is less than 1.63 we would not have adequate power to detect significant differences.

Despite prior smaller studies [13, 21, 22] of a correlation between reduced plasma MBL levels and RSA, we find no evidence to support a relationship between MBL genotypes or phenotypes and risk of RSA among Caucasian women. Our conclusion is to reject the hypothesis that

genetically determined levels of plasma MBL are related to the risk or number of idiopathic spontaneous abortions in Caucasian women.

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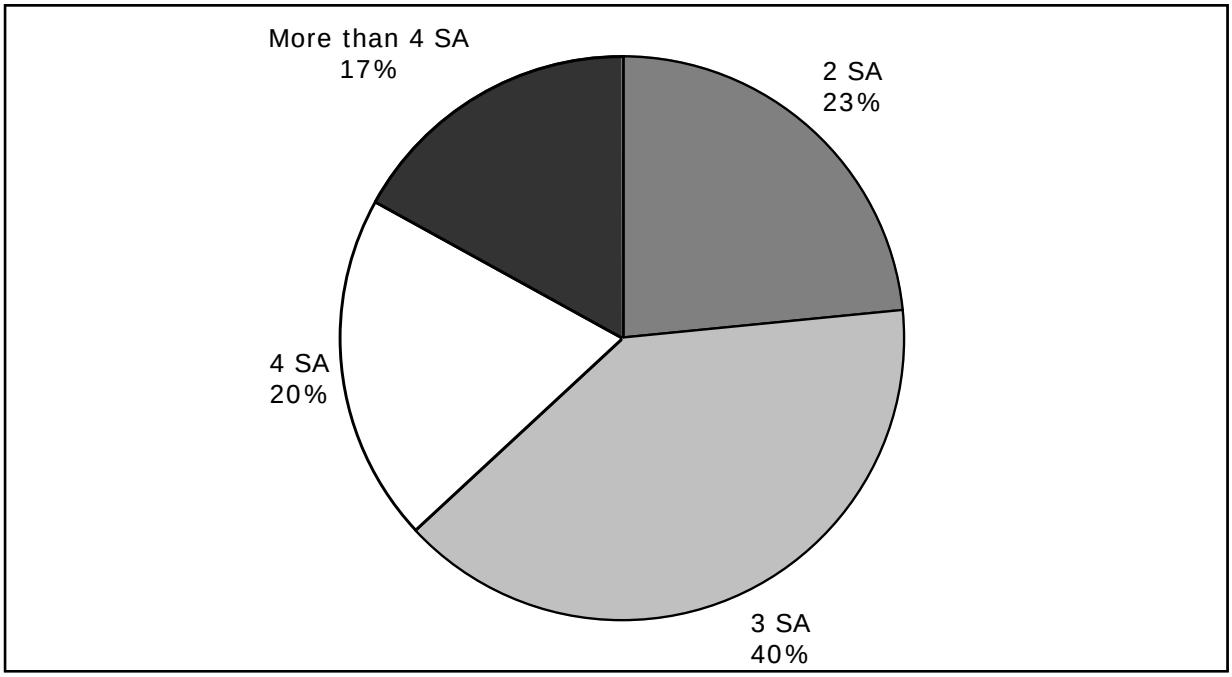

Figure 1: Categorization of spontaneous abortion cases (n=219) according to number of miscarriages experienced. 2 SA, 3 SA, 4 SA and 4 or more SA are depicted, n=51, 87, 44 and 37 respectively.

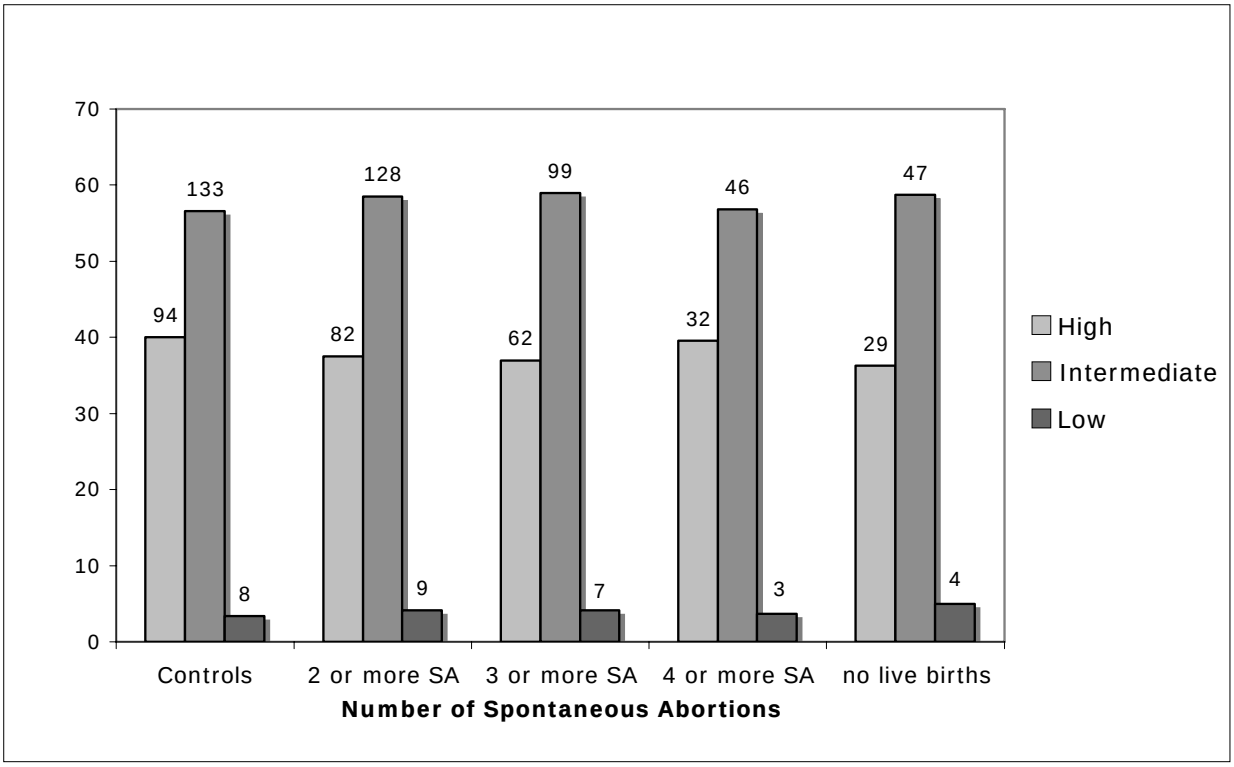

Figure 2: Percent of classified MBL production in comparison with the number of spontaneous abortions. Each individual, in cases and controls, was classified by predicted MBL plasma level concentration and separated by number of miscarriages.

Table 1: Genotype counts and minor allele frequency (q) of MBL alleles affecting mannose binding lectin concentrations.

	-550	-221	Codon 52	Codon 54	Codon 57
Cases					
	CC: 92	GG: 127	CC: 199	GG: 159	GG: 213
	CG: 96	GC: 84	CT: 20	GA: 56	GA: 6
	GG: 31	CC: 8		AA: 4	
q	0.3607	0.2283	0.0457	0.1461	0.0137
Controls					
	CC: 97	GG: 150	CC: 207	GG: 178	GG: 226
	CG: 110	GC: 76	CT: 29	GA: 54	GA: 9
	GG: 29	CC: 10		AA: 4	
q	0.3559	0.3649	0.0614	0.1314	0.0191

5.0 HUMAN LEUKOCYTE ANTIGEN-G MANUSCRIPT

A comprehensive analysis of HLA-G's promoter region and the implications for recurrent spontaneous abortion

ABSTRACT

Background: Miscarriage is one of the most common pregnancy complications. Recurrent spontaneous abortion (RSA) is defined as two or more pregnancy losses. Human leukocyte antigen-G (HLA-G) is a ligand for natural killer (NK) cell receptors and has the ability to block NK cell activity, which if not blocked can potentially harm a fetus. Consequently a deletion or mutation of the HLA-G gene could lead to miscarriage.

Study design: Cases (n=238) include Caucasian women experiencing two or more spontaneous abortions (SA) and controls (n=233) include women with at least one live birth and no history of SA. Sequencing was performed for 1400 base pairs (bp) of HLA-G's promoter region, genotyping for the 14 bp insertion/deletion was carried out and haplotypes and diplotypes were constructed using information from sequencing.

Results: Twenty-three single nucleotide polymorphism (SNPs) were observed in the promoter region with a minor allele frequency >0.02. Twelve SNPs differed significantly in frequency between cases and controls. Two haplotypes incorporating these 12 SNPs differed significantly in frequency between cases and control populations. Cases also had a higher frequency of individuals homozygous for the 14 bp insertion. Linkage disequilibrium (LD) was detected throughout the 1400 bp promoter region.

Conclusion: Cases and controls differed significantly in frequency of both individual SNPs and resulting haplotypes. We therefore conclude that RSA is associated with promoter region polymorphisms in the HLA-G gene.

INTRODUCTION

Approximately one in four pregnant women experiences at least one pregnancy loss, making spontaneous abortion the most common pregnancy complication (Wilcox et al. 1988). This prevalence is higher than expected by chance and suggests that some couples have an underlying systemic cause for repeated pregnancy loss (Baxter et al. 2001). Recurrent spontaneous abortion (RSA) can be defined as two or more clinically recognized spontaneous abortions (Lanasa et al. 2001).

RSA may be a result of maternal immune system recognition of paternal antigens on the feto-placental unit, followed by damage to fetal cells and abortion (Laird et al. 2003). The mechanisms that protect a pregnancy from the maternal immune system are not well understood. Therefore immunological assessment of normal pregnancy, let alone abnormal pregnancy, is challenging (Laird et al. 2003). Human leukocyte antigen-G (HLA-G) is an HLA-class I gene located in the major histocompatibility complex (MHC) on chromosome 6, and is expressed at the maternal-fetal interface. Uterine natural killer (uNK) cells are present in and around trophoblast cells and can cause fetal damage if not properly regulated. HLA-G is expressed on fetal extravillous trophoblasts (EVT), placental macrophages and mesenchymal chorionic villi. Trophoblast cells are known to be immunologically inert, lacking MHC I or MHC II molecules. Without MHC molecules the cells would be destroyed. However HLA-G is believed to inhibit trophoblast damage caused by natural killer (NK) cells during pregnancy, potentially protecting the fetus's genetically dissimilar cells from the mother's immune system. Because of HLA-G's proximity to fetal and maternal tissue, which are genetically different from each other, and its role in immune defense, it is likely that HLA-G expression and subsequent interaction with uNK cells and killing inhibitory receptors, is a potential factor in all pregnancies, whether successful or unsuccessful. Consequently, a deletion or mutation in the HLA-G gene could lead to loss of the fetus in early pregnancy (Rouas-Freiss et al. 1997).

Although the biology of HLA-G suggests a possible involvement in successful pregnancy, the exact function of HLA-G is unknown; thus, its role in pregnancy is beneficial to investigate. The majority of HLA-G studies have focused on three single nucleotide

polymorphisms (SNPs) in exon 2, three SNPs in exon 3 as well as a 14 base pair (bp) insertion/deletion in the 3' untranslated region (UTR). Exon polymorphisms revealed HLA-G allele *01014, *010102, *0105N, *010108 and possibly *01013 (studies are contradictory) to be associated with RSA (Abbas et al. 2004; Aldrich et al. 2001; Hviid and Christiansen 2005; Pfeiffer et al. 2001). The 3' UTR has conflicting findings concerning the 14 bp insertion/deletion in RSA as well. One report suggests homozygosity for the insertion is associated with increased risk of RSA and heterozygotes are less frequent in RSA groups (Hviid et al. 2004a). In contrast, others find that heterozygotes are more frequent in RSA populations (Tripathi et al. 2004) compared to another study reporting heterozygotes are less frequent in RSA case (Hviid et al. 2002).

Eighteen SNPs were initially identified in the HLA-G promoter region in Hutterite couples who experienced both fetal loss as well as successful pregnancy (Ober et al. 2003). More recently this group has updated their findings to include 27 SNPs (Tan et al. 2005). Single nucleotide polymorphisms defining eight HLA-G promoter haplotypes have been identified and differentially associated with risk of miscarriage in the Hutterites (Ober et al. 2003).

Although the most frequently studied, SNPs are not the only factors in a gene to influence function and protein synthesis. The promoter region is the sequence before exon 1 that initiates and regulates the transcription of a gene. Protein binding sites in a promoter are known as transcription factor binding sites (TFBS). These are short sequences, made up of core and matrix sequence, totaling approximately 10-20 base pairs, which enable specific recognition by a corresponding transcription factor (TF). Transcription initiation influences whether a gene is expressed and consequently how much protein is produced (Cartharius et al. 2005). The ability to predict potentially functional TFBS is important to promoter analysis (Cartharius et al. 2005).

If functional HLA-G is important in successful pregnancy, then molecular methods such as screening for variation in HLA-G may be beneficial in identifying individuals at risk of recurrent pregnancy loss. In order to assess association between RSA and genotype we studied case and control populations for HLA-G polymorphisms in the promoter region. We predicted

there would be a relationship between the presence of certain HLA-G alleles and an increased risk for recurrent pregnancy loss.

METHODS

The cases (n=238) were Caucasian women who experienced two or more recurrent pregnancy losses. The Institutional Review Boards of the University of Pittsburgh and the University of Minnesota approved all protocols, and each participant gave written informed consent. Details of the recruitment have been reported by Lanasa et al. 2001. Briefly, idiopathic RSA was defined by: 1) having two or more clinically recognized spontaneous abortions and 2) having RSA of unknown cause. Women were excluded if they were known to have abnormal hysterosalpinogram, antiphospholipid antibodies, connective tissue disorder, if either parent had an abnormal karyotype (i.e. balanced translocation) and if the mother had skewed X-inactivation. Mean, median and mode for the patient population were 33.46 years, 33 years and 34 years respectively. Control subjects (n=233) were selected from healthy Caucasian women aged 14-44 years, who were seeking prenatal care prior to 20 weeks gestation at Magee-Womens Hospital in Pittsburgh, Pennsylvania.

High molecular weight DNA was isolated from EDTA anticoagulated whole blood using the PureGene kit (Gentra Systems, Minneapolis, MN). We were interested in the 1400 bp region of the promoter adjacent to the coding region of the HLA-G gene. Primer pairs (PF2 and PR2; PF4 and PR4) were designed with overlapping amplification products of ~75 bp so that the entire region of interest was amplified (Table I). DNA was amplified by polymerase chain reaction (PCR) on a Bio-Rad MJ Thermal Cycler (Richmond, CA) with an initial denaturing step of 95 degrees Celsius (C) for 2 minutes, followed by 35 cycles each of 94 degrees C for 30 seconds, 58 degrees C annealing for 30 seconds and 72 degrees C for 30 seconds, with a final extension step of 72 degrees C for 1 minute. An EXO Sap step was performed on the PCR products generated from the primer sets in order to remove excess primers and deoxyribonucleotide triphosphates (dNTPs). EXO Sap products were cycle sequenced using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions, and then electrophoresed through an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City,

CA) at the University of Pittsburgh's Genomics and Proteomics Core Sequencing Facility. Sequence analysis was completed using Sequencher v4.5 RC2 (Gene Codes Corporation). In order to compare results with previous studies we sequenced exon 2 as well. Exon 2 analysis was similar to promoter region analysis; primers used were Exon2F with Exon2R (Table I).

The 14 bp insertion/deletion in the 3' UTR was investigated via PCR and gel electrophoresis, using primers IDF and IDR (Table I). Samples were amplified on a Bio-Rad MJ Thermal Cycler (Richmond, CA) at 95 degrees C for 5 minutes, followed by 45 cycles of 95 degrees C for 30 seconds, 52 degrees C for 30 seconds and 72 degrees C for 30 seconds, with a final extension of 72 degrees C for 5 minutes. Samples were electrophoresed through a 3% agarose gel. Insertions and deletions were interpreted by two independent investigators.

R Consol was used to count SNPs and to calculate Fisher's exact tests and odds ratios (RDevelopmentCoreTeam 2005). Chi-squared results were calculated using an interactive website (Preacher 2001). MATCH version 1.0 was used to analyze TFBS (Quandt et al. 1995). All common and minor alleles were used in the TFBS searches in order to reveal binding sites that are both created and destroyed due to polymorphisms in the promoter region. Linkage disequilibrium was analyzed using Haploview (Barrett et al. 2005).

Haplotypes and diplotypes were constructed using Phase v2.1.1 (Stephens 2006). A diplotype consists of haplotype pairs from one individual, in this study the mother. One haplotype is passed to an offspring for each parent and Phase software is able to analyze both haplotypes and diplotypes allowing for analysis of possible maternal diplotypes and possible maternal haplotypes, one of which is inherited by the fetus.

RESULTS

Promoter region sequence

Sequencing of the cases' and controls' DNA inclusively between -1305 and -201 bp identified 23 SNPs with minor allele frequencies greater than two percent (Table II). All SNPs were in Hardy-Weinberg equilibrium (HWE) in the control samples. Two SNPs in the cases deviated

significantly from Hardy-Weinberg expectations, -477 and -369 p-values 0.0039 and 0.0049 respectively. SNP -725, which has three alleles, is not included in Table II. Frequencies for C, G and T alleles at this locus in controls were 0.8201, 0.1495, and 0.0304 respectively whereas those for cases were 0.8146, 0.1313, and 0.0542 respectively.

Twelve SNPs differed significantly in allele frequency between cases and controls (Table III). Odds ratios for these 12 SNPs (Table III) indicated there is approximately a 1.456 fold increase in the presence of one allele over another, confidence interval (C.I.) 1.0705-1.8285.

Fourteen base pair insertion/deletion

The 3' UTR of HLA-G has a 14 base pair insertion/deletion. Cases and controls were in HWE and were not significantly different from each other, X^2= 5.71 p=0.058 (Table II). Five of the samples in the case population were not typed for the insertion/deletions analysis because of poor amplification.

Transcription factor binding sites

We identified 46 potential TFBS matches within the HLA-G promoter region. Of these, seven have a 5' to 3' core match that is changed by the presence or absence of an allele. These seven affected SNPs include -1179, -1155, -762, -689, -486 and two at -201. The factors involved are Elk-1, Foxd 3, Comp 1, v-Myb, Hnf-4 and Cdp Cr1. The percentage core match for each TFBS is ≥76.5%.

Haplotype and diplotype data

We first constructed haplotypes using all 23 variants present in case and controls. We identified a total of 55 haplotypes in our population. Phase calculations indicate that neither promoter region nor exon 2 haplotypes differed significantly in frequency between cases and controls, p=0.06 and p=0.48 respectively. We kept exon 2 haplotypes separate from promoter region haplotypes because an excess of haplotypes were created when all the SNP data were combined; separate analysis was more useful in case and control comparison. Eight promoter-region haplotypes for cases and controls had frequencies >1% (supplemental Table VIII) and accounted for

approximately 90% of the haplotypes in our samples. Diplotype testing showed no significant haplotype pairs at $p \leq 0.01$.

We repeated the haplotype analysis using the twelve SNPs which differed significantly in frequency between cases and controls (Table III). This time, 28 haplotypes were identified. Two haplotypes (AGATGGTACGAA [haplotype 13] and GAGCTAGGACCG [haplotype 19]) were significantly more frequent in one population than the other (Table IV). Again diplotype analysis did not reveal significant findings.

Linkage disequilibrium (LD) for the entire population, as well as for cases and controls separately, was analyzed using Haploview (Figure 1). The average D' value of SNPs across the 1400 bp regions was >0.95, indicating strong LD. Interestingly, the Haploview images of LD for case populations and control populations appear to be different (Figure 1B and 1C). For example in the controls, site -1138 has LOD<2, D'<1 and -486, -391 and -369 have LOD<2 D'=1 which indicates weaker LD. In contrast, the cases exhibit strong LD at these same locations, LOD≥2, D'=1. These results suggest a higher frequency of common haplotypes in the cases than in the controls. However, the controls also have an increase in rare variants that are not present in the case population, which may cause a gap at certain locations in the LD image.

DISCUSSION

Traditionally RSA has been defined as three or more spontaneous, consecutive pregnancy losses. It has been reported that patients with a single live birth followed by a series of miscarriages have similar disorders to those patients with only miscarriages (Clark et al. 2001). Also because risk of recurrent miscarriage increases with each prior miscarriage it has been argued that evaluation should begin after two losses instead of waiting until three (ACOG 2001). The RSA criteria of two or more miscarriages were required for eligibility in this study, as opposed to the RSA definition of three or more recurrent pregnancy losses. It is our belief that the population used gives a broader range of pregnancy loss cases and results in a more comprehensive genotyping study. RSA, consecutive or not, allows for the possibility of a genetic mutation which if recessive, would occur in 25% of conceptions; live births could be interspersed with

miscarriage(s). The case population includes these individuals, as well as those experiencing two or more SA, whereas a study requiring three or more consecutive miscarriages would have eliminated their participation. This becomes important because HLA-G is expressed on trophoblast cells and is of fetal origin, therefore we are considering a fetus's maternally inherited risk and each pregnancy will be dependant on the fetus's HLA-G expression. An individual who experiences two or more non-consecutive spontaneous abortions is more likely to have miscarried due to genetic factors, and should be included in this analysis, as opposed to experiencing loss due to maternal causes such as medical illness, hormonal issues or anatomic causes (Clark et al. 2001).

Insertion/deletion data for the 3' UTR were similar for cases and controls in our study. The frequency of the 14 bp deletion was 56.438% in cases and 62.876% in controls. The number of heterozygotes was lower in cases, n=97, than in controls, n=107, which contrasts reports by a previous study that showed significantly more heterozygotes in the case population, X^2=6.014, p<0.01 (Tripathi et al. 2004). Our results could vary due to ethnic differences; our study was conducted in the United States and the other was conducted in India. Furthermore we were able to exclude cases with abnormal chromosomes, anatomic irregularity and endocrine disorders. Our data are more consistent with research by Hviid et al. 2002 who reported a significantly greater number, according to HWE, of heterozygotes for the 14 bp polymorphism in controls. Homozygotes for the insertion were more frequent in cases than controls (Hviid et al. 2002), which corresponds with our findings.

Factor Hnf-4 has a TFBS interrupted by SNP -486, which is of interest because this SNP differs significantly in allele frequency between cases and controls. However, Hnf-4 is currently not known to play a role in decidua formation, pregnancy or miscarriage. Of the five other TFs associated with potential HLA-G promoter binding site identified in this study, Elk-1, Foxd3, Comp1, v-Myb and Cdp Cr1, only one (Foxd3) has a connection to pregnancy. It has been demonstrated that Foxd3 is required in the trophoblast progenitor cell line in murine embryos (Tompers et al. 2005). The binding site for Foxd3 is created by a G allele at SNP -1155. However, both the cases and controls had high frequencies of G alleles at -1155, and the allele frequencies were not significantly different between the two groups. Nevertheless, we did

identify two cases who were homozygous for the minor alleles and one control individual homozygous for the minor allele at this site. It is possible that the groups did not have significantly different allele frequencies at -1155 because selection has already taken place to eliminate variation in cases and controls.

Consistent with previous reports, we have determined that there is variation in the promoter region of HLA-G. Other investigations have reported that the odds ratio for pregnancy loss among couples with the G allele at -725 is 2.76, confidence interval (CI) 1.08 - 7.09 (Ober et al. 2003; Ober et al. 2006) . These authors proposed that promoters with a G allele at -725 allele and perhaps a T allele at -1121 would have increased HLA-G expression which would negatively affect pregnancy outcome (Ober et al. 2006). However, our analysis did not reveal SNP -725 or -1121 to be significant when comparing frequencies of the C, G or T alleles in cases and controls. Previously, a SNP at -477 was associated with IL-10 concentrations, but only before statistical correction (Hviid et al. 2006). The authors initially believed -477 to be influential due to its proximity to a heat shock element (Hviid et al. 2006). Because heat shock elements are important for transcription, a variant in the sequence could be significant. Our investigations support this study as we found SNP -477 allele frequencies to be one of 12 SNPs that differed significantly in allele frequency between cases and controls.

Sequencing revealed haplotype variation could be a risk factor for RSA and it is likely that a combination of SNPs play a role in RSA. Twelve SNPs were identified to differ significantly in allele frequency between cases and controls, p-values < 0.05 (Table III). These 12 SNPs have similar odds ratios suggesting one SNP is no more important than another. Haplotype 19 is significantly more common in RSA controls than cases, $p=0.0004$, whereas haplotype 13 is significantly more common in cases, $p=0.00782$ (Table IV). Given this information we conclude haplotype 13 to be a risk factor for RSA whereas haplotype 19 is likely to be protective against RSA. Furthermore, by examining the LD in the cases, Figure 1B, we show the presence of shared haplotypes, which further strengthens the theory of variants being linked. Given this information we then went on to test diplotypes. Both diplotype and haplotype information are important because of the fetal genotype's implications. We did not see significant findings in diplotype analysis. One explanation is that HLA-G is expressed on

trophoblast cells which are of fetal origin yet we were constructing maternal diplotypes. Had we analyzed fetal DNA, we would have been more likely to see diplotype associations.

Novel findings include three SNPs in the promoter region, -486, -477 and -369 whose allele frequencies differed significantly between cases and controls, p-values <0.005 (Table III). Sites -477 and -369 were in strong LD, LOD=197.39 D'=1.0. Our findings suggest that having an A allele at -486 could be protective against RSA. Also the likelihood of a G allele at -477, which corresponds to having an A allele at -369, could indicate susceptibility to RSA. Although these three SNPs do suggest a relationship to RSA, we strongly believe there are haplotype factors, not individual SNP variations, associated with RSA because of the strong LD across the 1400 bp promoter region sequenced.

HLA-G is expressed in all EVT populations including the cytotrophoblast cells, interstitial trophoblasts, endovascular trophobalsts and placental cells (Agrawal and Pandey 2003). When considering HLA-G's influence on pregnancy, it is important to take into account that HLA-G is found on trophoblasts at the maternal-fetal interface, and that they are considered to be the primary expresser of HLA-G. These trophoblast cells however are of fetal origin, not maternal origin; meaning that HLA-G nucleotide structure is based on genetic information from both the mother and father. For this reason it is questionable as to whether examining maternal HLA-G variation and diplotypes is the most effective method of studying HLA-G in women experiencing RSA. HLA-G has frequently been reported to play a role in immunity as well as in pregnancy loss, however too often the focus has been on maternal HLA-G status as opposed to fetal. We have attempted to compensate for this potential weakness by looking at haplotypes. One maternal haplotype is inherited by the fetus and therefore affects trophoblast HLA-G expression or lack of expression. To test our hypothesis further, sequencing of fetal DNA would be useful. However obtaining a sample of fetal DNA would be challenging. In the future we could sequence the paternal HLA-G promoter region in order to predict the fetal diplotype and attempt to compare miscarriages to fetal genotype.

Based on our study, we believe that polymorphism in the promoter of HLA-G influences RSA, but because of strong LD across the region, haplotype differences, rather than individual

nucleotide variation are more important. By grouping SNPs into haplotypes we were able to condense and utilize all promoter region allele information simultaneously. Furthermore, TFBSs located in the promoter region may be created or destroyed depending on an individual's haplotype, therefore affecting the likelihood of successful pregnancy.

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Table I: Primer names, direction and sequences used for RSA case and control analysis.

Primer name	Sequence (5'-3')
PF2	5'-CTGAACACTTACAACTGTGAGG-3'
PR2	5'-CCGACACAGGTTAGGAGAAGG-3'
PF4	5'-GATACGCACACTAGTTAC-3'
PR4	5'-CCAGGACATCTCCTCAAG-3'
Exon2F	5'-GAGGGTCGGGCGGGTCTCAAC-3'
Exon2R	5'-GCATGGAGGTGGGGGTCGTGA-3'
IDF	5'-GTGATGGGCTGTTTAAAGTGTCACC-3'
IDR	5'-GGAAGGAATGCAGTTCAGCATGA-3'

Table II: Allele frequencies for SNPs with minor frequencies (q) greater than two percent.

	Cases				Controls			
Location	Alleles	p	q	HWE p value	Alleles	p	q	HWE p value
Promoter								
-1305	A=G	0.5	0.5	0.2	G→A	0.588	0.412	0.92
-1179	G→A	0.556	0.444	0.07	A→G	0.54	0.46	0.9
-1155	G→A	0.884	0.116	0.78	G→A	0.914	0.086	0.88
-1140	A→T	0.622	0.378	0.42	A→T	0.667	0.333	0.92
-1138	A→G	0.949	0.051	0.15	A→G	0.972	0.028	0.91
-1121	C→T	0.917	0.083	0.39	C→T	0.915	0.085	0.9
-964	G→A	0.505	0.496	0.15	G→A	0.591	0.409	1
-762	C→T	0.502	0.498	0.17	C→T	0.583	0.417	0.62
-716	T→G	0.513	0.487	0.17	T→G	0.581	0.419	0.42
-689	A→G	0.511	0.489	0.15	A→G	0.58	0.42	0.32
-666	G→T	0.511	0.49	0.12	G→T	0.598	0.402	0.73
-646	A→G	0.983	0.017	0.97	A→G	0.984	0.016	0.97
-633	G→A	0.513	0.487	0.15	G→A	0.588	0.412	0.72
-543	del→in	0.973	0.028	0.91	del→in	0.986	0.014	0.98
-536	in→del	0.977	0.023	0.94	in→del	0.972	0.028	0.92
-509	C→G	0.964	0.036	0.43	C→G	0.975	0.026	0.93
-486	A=C	0.5	0.5	0.09	A→C	0.582	0.418	0.5
-477	C→G	0.449	0.551	0.0039	C→G	0.547	0.453	0.63
-400	G→A	0.949	0.051	0.17	G→A	0.968	0.032	0.89
-391	G→A	0.946	0.054	0.27	G→A	0.968	0.032	0.89
-369	C→A	0.447	0.553	0.0049	C→A	0.544	0.457	0.68
-201	G→A	0.509	0.492	0.1	G→A	0.572	0.428	0.51
3'UTR								
	del→in	0.565	0.435	0.09	del→in	0.629	0.371	0.97

Major alleles (p) are listed to the left of the arrows while minor alleles are to the right of the arrows. HWE= Hardy Weinberg Equilibrium

Table III: SNP summary

SNP	Reference SNP No. or Surrounding Seq	Calculation	p-value	Allele	Odds Ratio	CI
-1305	CCACA*TTCAG	X^2=8.823	0.012	A	1.429	1.095-1.866
-1179	GCAGC*GAACT	X^2=8.863	0.012	G	1.466	1.124-1.912
-1155	ACATT*TTTTA	Fisher's exact test	0.372			
-1140	AGATT*TTA*T	X^2=2.157	0.34			
-1138	T*CATCCATC	Fisher's exact test	0.2			
-1121	AGAGC*TCGCT	Fisher's exact test	0.795			
-964	rs1632947	X^2=7.453	0.024	A	1.42	1.079-1.870
-762	rs1632946	X^2=9.529	0.009	T	1.388	1.066-1.809
-725	rs1233334	Fisher's exact test	0.478			
-716	rs2249863	X^2=9.063	0.011	G	1.321	1.016-1.717
-689	rs2735022	X^2=10.108	0.006	G	1.325	1.020-1.722
-666	TTACC*ATTAG	X^2=10.367	0.006	T	1.424	1.095-1.854
-646	CATTC*GGGGT	Fisher's exact test	1			
-633	rs1632944	X^2=8.389	0.015	A	1.355	1.041-1.765
-543	AGG*GGAAAA	X^2=2.088	0.149			
-536	GAAA*AAAAC	X^2=0.187	0.665			
-509	AATCC*AGGGC	Fisher's exact test	0.687			
-486	rs1736933	X^2=11.245	0.004	C	1.409	1.073-1.851
-477	rs1736932	X^2=16.646	0.0002	G	1.483	1.129-1.948
-400	GACAG*GATTC	Fisher's exact test	0.315			
-391	TCCGG*ATGAA	Fisher's exact test	0.228			
-369	rs1632943	X^2=15.647	0.0004	A	1.475	1.121-1.942
-201	rs1631950	X^2=8.553	0.014	G	1.291	0.988-1.686

Reference SNP number or location, and results of chi-squared analysis or Fisher's exact test for each SNP as well as odds ratios and confidence intervals for SNPs with p-values < 0.05.

Table IV: Haplotype 19 and Haplotype 13 case and control comparisons of haplotype counts

Haplotype 19: chi-square 15.641, p=0.0004			
GAGCTAGGACCG			
	19,19	19,X	X,X
Cases	52	97	89
Controls	58	126	49

Haplotype 13: chi-square 9.701, p=0.00782			
AGATGGTACGAA			
	13,13	13,X	X,X
Cases	61	100	77
Controls	33	114	86

X represents any haplotype other than the haplotype of interest.

QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

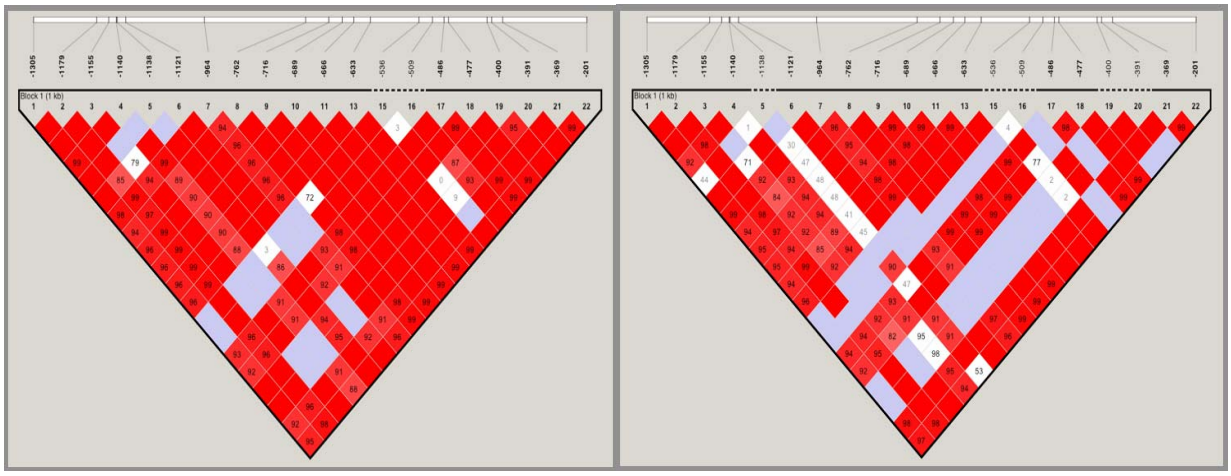

Figure 1: Haploview results

Linkage disequilibrium (LD) between pairs of SNPs in the HLA-G promoter region for (a) cases and controls combined, (b) cases and (c) controls alone. Images were generated in Haploview, using only SNPs with minor allele frequencies >2%. Areas in shades of pink-dark red indicate strong LD (LOD≥2, D'=1), blue indicates weak LD (LOD<2, D'=1) and white indicates no LD (LOD<2, D'<1).

SUPPLIMENTAL DATA

We observed individuals with SNP frequencies below two percent, Table V. While they are rare, they are included for completeness.

Table V: Counts of observed variants below two percent

Location	**-1410**	**-1255**	**-922**	**-810**	**-779**
SNP	CT	GT	CA	CT	CA
No. samples	1	1	5	4	1
Case/Cont	1/0	1/0	2/3	4/0	0/1
Location	**-755**	**-483**	**-399**	**-361**	**-250**
SNP	GA	AG	GA	CT	GA
No. samples	2	6	2	1	1
Case/Cont	0/2	3/3	1/1	1/0	1/0

We genotyped three SNPs in exon 2 and one insertion/deletion polymorphism in the 3'UTR in order to determine HLA-G alleles present in population. Four HLA-G alleles, *01041, *01013, *01012 and *01011, were found to be represented in our study.

Table VI: Haplotypes, counts and HLA-G allele results from exon 1 and insertion/deletion genotyping. Haplotypes consist of the following locations in exon 2: 294, 374, 410 as well as the 14 bp insertion or deletion in the 3' UTR.

Haplotype	Counts	HLA-G Allele
AGCD	467	Other
AACI	325	01041
AACD	93	01013 and 01012
AGCI	33	01011
TGCI	22	Other
TACI	2	Other

We wanted to examine haplotypes constructed using all 23 promoter region SNPs found in the 1400 bp promoter region.

Table VII: Haplotypes with more than one percent frequency in cases and controls. Numbers 1, 2 and 3 correspond to SNP -725 which has three alleles: 1=C, 2=G, 3=T.

Haplotype	Frequency
AGGTACAT1GGTAADICCGGGAA	0.3230
GAGAACGC1TAGAGDICACGGCG	0.3007
AGAAACAT1GGTAADICCGGGAA	0.0945
GAGAATGC2TAGAGDICACGGCG	0.0727
GAGAACGC2TAGAGDICACGGCG	0.0620
GAGAACGC1TAGAGDDCACGGCG	0.0234
GGGAGCGC3TAGGGDIGAGAAAG	0.0141
AGGTACAC1TAGAGDICACGGCG	0.0110

Table VIII: Haplotype number, haplotypes observed and counts in cases and controls combined using 12 SNPs in the promoter region.

Haplotype No.	Haplotype	Count
1	AGGTGGTACGAA	3
2	AGACTAGGAGAG	2
3	AGACTAGGACCG	11
4	AGACTAGGCCCG	2
5	AGACTATGCCCG	1
6	AGACTATACCCG	1
7	AGACGGGGACCG	1
8	AGATGGGGCGAG	1
9	AGATGGGAAGAA	1
10	AGATGGGACGAA	3
11	AGATGGTGCGAA	2
12	AGATGGTACGAG	1
13	AGATGGTACGAA	402
14	GGGCTAGGAGAG	38
15	GGGCTAGGACCG	4
16	GGGTGGTACGAA	1
17	GGATGGTACGAA	7
18	GAGCTAGGAGCG	1
19	GAGCTAGGACCG	443
20	GAGCTAGGACCA	3
21	GAGCTAGGCGAG	3
22	GAGCTGTACGAA	1
23	GAGCGAGGCGAG	1
24	GAGTTAGGACCG	2
25	GAGTGGGGAGAG	1
26	GAGTGGGGCGAG	4
27	GAGTGGTACGAA	1
28	GAACTAGGACCG	1

We examined the full TFBSs, core and matrix sequences, with the SNPs that interrupt the binding site matrix.

Table IX: Transcription factors and their binding site recognition sequences, which are interrupted by SNPs -1179, -1155, -762, -689, -486 and -201. Lowercase letters indicate matrix match, uppercase letters indicate core match. Bold letters signify location of SNP.

Position	Transcription factor binding site	Factor name	Factor prese
-1179	agcagC**G**GAActct	Elk-1	If G, no
-1155	caTT**G**TTttata	Foxd3	If G, no
-762	cagataCA**T**TGtctgggaaagtga	Comp1	If T, no
-689	accAAC**G**Ggc	v-Myb	If G, no
-486	tctggca**C**CAAGctccctg	Hnf-4	If C, no
-201	tgtatgG**A**TTGgggaggccccgcg	Comp1	If A, no
-201	tATGG**A**ttgg	Cdp Cr1	If A, no

6.0 CONCLUSION

It is reasonable to assume that there is more than one immunological cause of recurrent miscarriage. Using a select population of RSA cases and fertile controls, we were able to successfully investigating two aspects of immunology affecting pregnancy. Both studies provided us with data and rational conclusions, however during our efforts questions did arise that we were not able to answer. The MBL research we conducted was the largest MBL and RSA study to date. We were able to show that MBL genotypes, regardless of the number of miscarriages, did not affect pregnancy outcomes. What we were usable to establish however, was a direct link between MBL levels, infection and pregnancy loss. In order to confirm that low MBL plasma levels increase maternal and/or fetal infection, resulting in the miscarriages, we would have needed maternal exposure and infection rates. For future studies this information could possibly be gathered from socioeconomic background information, physician visits or self-reported by the patient. The HLA-G project, while also successful, could be improved upon by further tests and analysis. We based our investigation on maternal samples and data. Careful examination of the literature however suggests that HLA-G is expressed on trophoblast cells, which are derived by the fetus. This being true, maternal SNP data is only partially responsible for HLA-G expression therefore adding paternal, or even fetal, HLA-G sequence analysis would be extremely beneficial to our study design in future experiments.

During our twofold gene approach we did not find an association between molecularly deduced levels of plasma MBL in women and risk or number of RSA. Therefore, we do not consider maternal susceptibility to infection due to decreased plasma MBL levels to be a cause of RSA. We did generate 55 different HLA-G promoter region haplotypes, eight of which could account for more than 90% of the haplotypes found in cases and controls. Two of these, haplotype 13 and haplotype 19 were particularly interesting due differences seen in cases and

controls. Haploytpe 19 was significantly more frequent in controls while haplotype 13 was just the opposite, significantly more frequent in cases. The promoter region was discovered to be in LD across the 1400 bp region upstream of exon 1, and we were able to located 6 TFBS that are altered as a result of variation in the promoter region. Using the transcription factor data we discovered, we were able to incorporate it with data already known to create a map of the HLA-G promoter region (Figure 3). As research in the area continues, we hope that this map will be useful to other investigations.

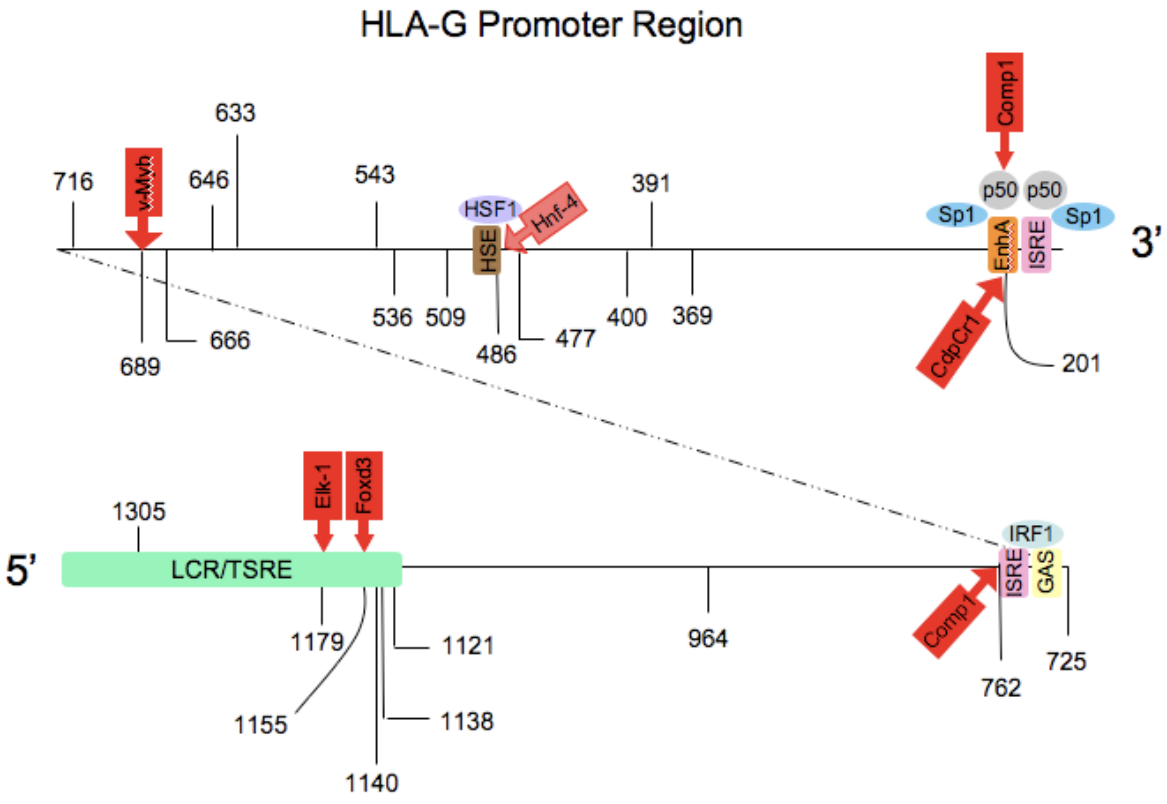

Figure 2: HLA-G promoter region

SNP and haplotype variation has proved to be a promising area of research in regards to MBL and HLA-G. Continuing research will be advantageous due to the abundance of variation in genes associated with immunology as well as the likelihood that genetic variation has a role in maternal immune response to a fetus, and the fetal environment.

APPENDIX A

ADDITIONAL ARTICLES OF INTEREST

A class I antigen, HLA-G, expressed in human trophoblasts

Population: First trimester villous cytotrophoblasts from four pooled individuals and four first trimester placental villi
Methods: Immuno precipitation of methionine-labeled HLA-G+LCL.221 transferent cells with MAb W6/32 was run on a gel to identify an autoradiographic spot array, characteristic of the HLA-G alpha chain and distinct from the spot arrays of HLA-A, -B, -C, -E, -F
Conclusion: Data shows a nonpolymprphic class I molecule, HLA-G, is expressed by cytotrophoblasts and suggests a function for these molecules in protecting the placenta from rejection. (Kovats et al. 1990)

Characterization of a new HLA-G allele encoding a nonconservative amino acid substitution in the alpha3 domain (exon 4) and its relevance to certain complications in pregnancy

Population: 22 fertile women, 22 fertile men, 106 healthy individuals, 31 women with PE, 19 women who had experienced three or more spontaneous abortions and their partners
Methods: Genotyped for polymorphism in exon 4
Conclusion: Distribution of genotypes for all three groups was in accordance with HWE. No association with the HLA-G allele was found in pre-eclamptic, abortion or healthy populations. (Hviid et al. 2001)

HLA-G unique promoter region: functional implications

Population: Transgenic HLA-G mouse models under the control of HLA-G promoter
Methods: pHLA-G-il-EGFP transgene was microinjected into 39 viable embryos, 4 mice were backcrossed. Placental cDNA from embryos was amplified.
Conclusion: PT-PCR demonstrated differential activity of HLA-G promoter in mouse transgenic placenta according to gestational stage. Once the placenta is fully formed, the functions of HLA-G might not be crucial. (Solier et al. 2001)

Altered phenotype of HLA-G expressing trophoblast and decidual natural killer cells in pathological pregnancies

Population: 9 women with recurrent miscarriage, 5 women with ectopic tubal pregnancy and 11 controls
Methods: Designed to reveal HLA-G expression and NK cell distribution in tissues
Conclusion: HLA-G protein is expressed at the cell surface of EVT cells in tissue from both recurrent miscarriage and ectopic pregnancies. In normal pregnancy there is a decrease and a morphological change in CD56+ NK cells upon interaction with HLA-G expressing trophoblasts. (Emmer et al. 2002)

HLA-G expression in early embryos is a fundamental prerequisite for the obtainment of pregnancy

Population: 285 supernatants corresponding to 101 IVF procedures
Methods: Soluable HLA-G levels were measured via ELISA
Conclusion: Expression of HLA-G products in embryo cells is a mandatory, but not sufficient, prerequisite for the development of pregnancy. (Fuzzi et al. 2002)

Expression of membrane-bound HLA-G at the maternal-fetal interface is not associated with pregnancy maintenance among patients with idiopathic recurrent pregnancy loss

Population: Trophoblast tissue from 47 women having dilation and curettage (D&C). Of these women, 30 had at least three first trimester pregnancy losses, 17 were elective terminations, 16 had normal chromosomes and 14 presented with fetal trisomy 16.
Methods: HLA-G protein expression at the maternal-fetal interface was studied. They studied immune-mediated RSA by using RSA patients with normal fetal chromosomes and patients with known chromosomal abnormalities.
Conclusion: No significant differences were found in levels of HLA-G between groups of RSA patients. HLA-G expression is not a major immunological determinant of pregnancy maintenance among patients with idiopathic RSA. (Patel et al. 2003)

Transcriptional status of HLA-G at the maternal-fetal interface in recurrent spontaneous abortion

Population: 17 women with RSA, 25 controls
Methods: HLA-G isoforms were expressed in terms of HLA-G transcript/ beta actin densitometric ratios
Conclusion: Suggests that a lower relative transcription of HLA-G isoforms may contribute to the physiology of RSA. (Abbas et al. 2006)

APPENDIX B

SNPPER CONSENSUS SEQUENCE

SNPper Key: exons | CODING | Introns | **SNP** | 5'/3'

```
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APPENDIX C

CONSENSUS SEQUENCE FOR TRANSFAC

C.1 REFERENCE SEQUENCE: CASES, COMMON ALLELES

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C.2 REFERENCE SEQUENCE: CASES, MINOR ALLELES

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C.3 REFERENCE SEQUENCE: CASES, MINOR ALLELES AND -725 T

GTAGCAGGACCACTATAGAGAGAACACTCATGTAGCAGGTCATGGAACAGTGCTAG
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GGGATGAGAGGGACGGAGGGAAGGGCTGGAGGAGCAGGAGGTGAGGAAAAGGAG
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CTACAATCCCAGGGCGAGCTCACTCTCTGGCACCAAGCTCCCTGGGGTGATTTTTCT
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GTTTCTCCCTGGTTTCTCAGACAGCTCCTGGGCCAAGACTCAGGGAGACACTGAGAC
AGAACGCTTGGCACAAGAGTAGCGGGGTCAGGGCGAAGTCCCAGGGCCTCAAGCGT
GGCTCTCAGGGTCTCAGGCCCCACAGGCGGTGTATGGATTGGGGAGGCCCCGCGTT
GGGGATTCT

APPENDIX D

EXON 2 AND 3' UNTRANSLATED REGION HAPLOTYPES

Index	Haplotype	Count
1	CAGCTGD	456
2	TAACCGI	284
3	TAACCGD	88
4	CAACCGI	40
5	CAGCCGI	24
6	CTGCCTI	21
7	TAGCTGD	6
8	CAACTGD	5
9	CAGCCGD	4
10	TAGCTGI	4
11	CAGCTGI	3
12	CTATTI	2
13	TAGCGI	2
14	CTGCGI	1
15	TAGTTD	1
16	TAATGI	1

APPENDIX E

EXON 2 AND 3' UNTRANSLATED REGION HAPLOTYPES, SPECIFIC TO PUBLISHED NOMENCLATURE

Haplotype	Counts	HLA-G Allele
AGCD	467	Other
AACI	325	01041
AACD	93	01013 and 01012
AGCI	33	01011
TGCI	22	Other
TACI	2	Other

APPENDIX F

PROMOTER REGION HAPLOTYPES

Index	Haplotype	Count
1	AGGTACAT1GGTAADICCGGGAA	314
2	GAGAACGC1TAGAGDICACGGCG	290
3	AGAAACAT1GGTAADICCGGGAA	84
4	GAGAATGC2TAGAGDICACGGCG	71
5	GAGAACGC2TAGAGDICACGGCG	52
6	GAGAACGC1TAGAGDDCACGGCG	21
7	GGGAGCGC3TAGGGDIGAGAAAG	13
8	AGGTACAC1TAGAGDICACGGCG	10
9	GGGAGCGC3TAGAGIICAGAAAG	8
10	GGGAGCGC3TAGAGIIGAGAAAG	8
11	GGGTACAT1GGTAADICCGGGAA	7
12	GAGAATGC1TAGAGDICACGGCG	5
13	GGGAGCGC3TAGAGDIGAGAAAG	4
14	GAGAACGT1GGGAGDICCGGGAG	4
15	AGGTACAT1GGGAADICCGGGAA	3
16	GAGAACGC1TAGAGDICCGGGAG	3
17	AGGTACAC3TAGAGIIGAGAAAG	2
18	AGGTACAT1GGTAGDICCGGGAA	2
19	AGAAACGT1GGTAADICCGGGAA	2
20	GGGAACGC1TAGAGDICACGGCG	2
21	GAGAACGC1TAGAGDICACGGCA	2
22	GAGAACGT1GGTAADICCGGGAA	2
23	AGGAACGC2TAGAGDICACGGCG	1
24	AGGAACAC1TAGAADICCCGGCG	1

Index	Haplotype	Count
25	AGGAACAT1GGGAGDICCGGGAA	1
26	AGGAACAT1GGTAADICCGGGAA	1
27	AGGTACGT1GGTAADICCGGGAA	1
28	AGGTACAC1TAGAGDICCCGGCG	1
29	AGGTACAC2TAGAGDICACGGCG	1
30	AGGTACAT1GGTAADIGCGGGAG	1
31	AGGTATAT1GGTAADICCGGGAA	1
32	AGGTGCAT1GGTAADICCGGGAA	1
33	AGAAACAC1TATAGDICCCGGCG	1
34	AGAAACAC1GGGAGDICCCGGCG	1
35	AGAAACAT1GGGAADICAGGGAA	1
36	AAGAACGC2TAGAGDICACGGCG	1
37	GGGAACGC3TAGGGDIGAGAAAG	1
38	GGGAATGC1TAGAGDICACGGCG	1
39	GGGAGCGC2TAGAGIICAGAAAG	1
40	GGGAGCGC3TAGAGDICAGAAAG	1
41	GGGAGCGC3TAGAGDIGAGGAAG	1
42	GGGAGCGC3TAGGGIIGAGAAAG	1
43	GGGAGCGT1GGTAADICCGGGAA	1
44	GGAAGCGC3TAGAGDIGAGAAAG	1
45	GAGAACGC1TAGAGDICACAACG	1
46	GAGAACGC1TAGAGDDCAGGGCG	1
47	GAGAACGC1TGTAADICCGGGAA	1
48	GAGAACGC1GAGAGDICCGGGAG	1
49	GAGAACGT1TAGAGDICACGGCG	1
50	GAGAACGT1TAGAGDDCACGGCG	1
51	GAGAACGT1GGGAGDICAGGGAA	1
52	GAGAACAC1TAGAGDICACGGCG	1
53	GAGAATGC2TAGAGDICACAGCG	1
54	GAGAATGC2TAGAGDICACAACG	1
55	GAGTATGC2TAGAGDICACGGCG	1

APPENDIX G

EXON 2 AND 3' UNTRANSLATED REGION HAPLOTYPE FREQUENCIES

Index	Haplotype	E(freq)	S.E	Control Freq	S.E.(Control)	Case Freq	S.E.(Case)
1	AGCD	0.000021	0.000146	0	0	0.000041	0.000289
2	TACD	0.000021	0.000149	0	0	0.000042	0.000295
3	TACI	0.000032	0.000181	0	0.000009	0.000063	0.000359
4	AACI	0.000064	0.000310	0.000021	0.000213	0.000105	0.000542
5	AACD	0.000085	0.000313	0.000065	0.000367	0.000105	0.000518
6	AGCI	0.000085	0.000308	0.000085	0.00042	0.000084	0.00042
7	TACD	0.000106	0.000318	0	0	0.00021	0.00063
8	TACI	0.000127	0.000345	0	0	0.000252	0.000683
9	TGCD	0.000202	0.000443	0.000386	0.000825	0.000021	0.000209
10	TGCI	0.000435	0.000937	0.000558	0.000989	0.000315	0.001125
11	AACI	0.000456	0.000918	0.000708	0.00127	0.00021	0.000879
12	AACI	0.001166	0.001028	0.001585	0.001074	0.000756	0.001476
13	AACD	0.001178	0.002012	0.000558	0.001245	0.001784	0.003095
14	TGCI	0.001242	0.000945	0.00176	0.000878	0.000735	0.001495
15	AGCD	0.001534	0.000671	0.002167	0.000213	0.000914	0.00133
16	TACI	0.001667	0.001021	0.00161	0.000929	0.001722	0.001202
17	AGCI	0.003924	0.003844	0.0003	0.000815	0.007471	0.007271
18	AGCI	0.004809	0.004591	0.000816	0.001454	0.008719	0.008286
19	AGCD	0.005414	0.001490	0.004786	0.001017	0.00603	0.002593
20	AACD	0.005498	0.001832	0.002809	0.001158	0.008131	0.003256
21	AGCD	0.006370	0.002070	0.006502	0.001105	0.00624	0.003706
22	AGCI	0.008429	0.003213	0.007942	0.002596	0.008902	0.005002
23	AGCI	0.023009	0.002952	0.025136	0.002651	0.020929	0.005273
24	TGCI	0.023022	0.001433	0.021782	0.001065	0.024237	0.002586
25	AACI	0.043331	0.001979	0.044314	0.002405	0.042369	0.003172
26	AACD	0.100185	0.002844	0.090128	0.00234	0.110028	0.005064
27	AACI	0.292546	0.002849	0.264586	0.002654	0.319922	0.00537
28	AGCD	0.474957	0.003585	0.521332	0.00289	0.429558	0.006147

APPENDIX H

PROMOTER REGION HAPLOTYPE FREQUENCIES

Index		E(freq)	SE	Control Freq	SE(Control)	Case Freq	SE(Case)
1	AGGAACGC1TAGAGDICACGGCG	0.00017	0.000458	0.000064	0.000365	0.000273	0.000707
2	AGGAACGC2TAGAGDICACGGCG	0.000464	0.000693	0.000193	0.000614	0.000729	0.001
3	AGGAACGT1GGTAADICCGGGAA	0.000011	0.000106	0	0	0.000021	0.000209
4	AGGAACAT1GGTAADICCGGGAA	0.001146	0.000336	0.000107	0.000467	0.002164	0.000465
5	AGGAGCGC3TAGAGDIGAGGAAG	0.000064	0.000252	0	0	0.000126	0.000498
6	AGGAGCGC3TAGGGDIGAGAAAG	0.000021	0.000148	0	0	0.000042	0.000294
7	AGGTACGC1TAGAGDDCACGGCG	0.000032	0.000181	0.000021	0.000213	0.000042	0.000294
8	AGGTACGC3TAGAGDIGAGGAAG	0.000021	0.000148	0	0	0.000042	0.000294
9	AGGTACGT1GGTAADICCGGGAA	0.001168	0.00038	0.000129	0.000588	0.002185	0.000411
10	AGGTACAC1TAGAGDICACGGCG	0.010951	0.001448	0.010924	0.002179	0.010976	0.001418
11	AGGTACAC1TAGAGDICACGGCA	0.000021	0.000148	0.000043	0.0003	0	0.000002
12	AGGTACAC1TAGAGDICCCGGCG	0.001253	0.00042	0.000064	0.000365	0.002416	0.000751
13	AGGTACAC1TAGAGDDCACGGCG	0.000027	0.000174	0.000032	0.000261	0.000023	0.000218
14	AGGTACAC1TAGAADICACGGCG	0.000042	0.000208	0	0.000008	0.000084	0.000411
15	AGGTACAC1TAGAADICCCGGCG	0.000075	0.000271	0	0	0.000148	0.000538
16	AGGTACAC1GGGAGDICCCGGCG	0.000053	0.000261	0.000086	0.000421	0.000021	0.000209
17	AGGTACAC1GGGAADICCCGGAA	0.000426	0.000649	0.000711	0.00101	0.000147	0.000536
18	AGGTACAC2TAGAGDICACGGCG	0.001658	0.000681	0.001164	0.001326	0.002143	0.000294
19	AGGTACAC2TAGAGDICACAGCG	0.000032	0.000181	0	0	0.000063	0.000358
20	AGGTACAC3TAGAGDIGAGGAAG	0.000104	0.000315	0	0	0.000204	0.000622
21	AGGTACAC3TAGAGIICAGAAAG	0.000074	0.000292	0.000021	0.000213	0.000126	0.000545
22	AGGTACAC3TAGAGIIGAGAAAG	0.001964	0.000571	0.000064	0.000365	0.003824	0.00106
23	AGGTACAC3TAGGGDIGAGAAAG	0.000064	0.000293	0.000064	0.000365	0.000063	0.000358
24	AGGTACAT1TAGAGDICACGGCG	0.000021	0.000148	0.000043	0.0003	0	0
25	AGGTACAT1TAGAADICCCGGCG	0.000021	0.000148	0	0	0.000042	0.000294
26	AGGTACAT1TGTAADICCCGGAA	0.000032	0.000181	0	0	0.000063	0.000358
27	AGGTACAT1TGTAADICCCGGCG	0.000042	0.000256	0.000021	0.000213	0.000063	0.000358
28	AGGTACAT1GGGAGDICAGGGAA	0.000032	0.000229	0.000021	0.000213	0.000042	0.000294
29	AGGTACAT1GGGAGDICACGGCG	0.000032	0.000181	0.000043	0.0003	0.000021	0.000209
30	AGGTACAT1GGGAADICCGGGAA	0.003673	0.000737	0.00663	0.000994	0.000777	0.001018
31	AGGTACAT1GGTAGDICCGGGAA	0.00193	0.000658	0.00015	0.000547	0.003672	0.001186

32	AGGTACAT1GGTAADICACGGCG	0.000032	0.000181	0.000064	0.000365	0	0
33	AGGTACAT1GGTAADICCGGGAG	0.000053	0.000231	0.000064	0.000365	0.000042	0.000294
34	AGGTACAT1GGTAADICCGGGAA	0.32301	0.003352	0.299263	0.004601	0.346266	0.004207
35	AGGTACAT1GGTAADICCGGGCG	0.000032	0.000223	0.000043	0.000395	0.000021	0.000209
36	AGGTACAT1GGTAADICCGAGAA	0.00035	0.000499	0	0	0.000694	0.000988
37	AGGTACAT1GGTAADIGCGGGAG	0.001125	0.000264	0.000128	0.000533	0.002101	0.000002
38	AGGTACAT1GGTAAIICCGGGAA	0.00035	0.000499	0	0	0.000693	0.000988
39	AGGTACAT1GGTGADICCGGGAA	0.00017	0.000437	0.000043	0.0003	0.000295	0.000729
40	AGGTATAT1GGTAADICCGGGAA	0.002675	0.001668	0.001249	0.001486	0.004071	0.002493
41	AGGTGCAT1GGTAADICCGGGAA	0.001115	0.000262	0.002189	0.0003	0.000063	0.000358
42	AGAAACGT1GGTAADICCGGGAA	0.002261	0.000381	0.00236	0.000696	0.002164	0.000358
43	AGAAACAC1TAGAGDICACGGAA	0.000021	0.000149	0.000043	0.000301	0	0
44	AGAAACAC1TAGAGDICACGGCG	0.000848	0.00115	0.001414	0.001904	0.000294	0.000776
45	AGAAACAC1TAGAGDICCCGGCG	0.000437	0.000579	0.000818	0.001042	0.000063	0.000359
46	AGAAACAC1TAGAADICACGGCG	0.000021	0.000149	0.000043	0.000301	0	0
47	AGAAACAC1TAGAADICCCGGCG	0.000032	0.000181	0	0	0.000063	0.000359
48	AGAAACAC1TATAGDICACGGCG	0.000032	0.000181	0.000065	0.000367	0	0
49	AGAAACAC1TATAGDICCCGGCG	0.00054	0.000707	0.000941	0.001148	0.000146	0.000535
50	AGAAACAC1TATAADICCCGGCG	0.000011	0.000106	0	0	0.000022	0.000212
51	AGAAACAC1GGGAGDICCCGGAG	0.000032	0.000181	0.000064	0.000366	0	0
52	AGAAACAC1GGGAGDICCCGGCG	0.000446	0.000647	0.000816	0.001126	0.000084	0.000412
53	AGAAACAC1GGGAADICCCGGAA	0.000189	0.000407	0.000383	0.000822	0	0.000018
54	AGAAACAC1GGGAADICCCGGCG	0.000053	0.000231	0.000107	0.000468	0	0
55	AGAAACAC3TAGGGDIGAGAAAG	0.000021	0.000148	0.000021	0.000213	0.000021	0.000209
56	AGAAACAT1GGGAGDICAGGGAA	0.000011	0.000106	0.000021	0.000214	0	0
57	AGAAACAT1GGGAADICAGGGAA	0.001138	0.0005	0.002172	0.000863	0.000126	0.0005
58	AGAAACAT1GGTAGDICCGGGAA	0.000021	0.000149	0	0	0.000042	0.000294
59	AGAAACAT1GGTAADICCGGGAA	0.094545	0.002313	0.078391	0.003383	0.110359	0.003001
60	AGAAACAT1GGTAADICCGAGAA	0.000127	0.000376	0.000021	0.000213	0.000231	0.000657
61	AGAAGCGC3TAGAGDICAGAAAG	0.000042	0.000208	0	0	0.000084	0.000411
62	AGAAGCGC3TAGAGDIGAGGAAG	0.000329	0.000744	0	0	0.000651	0.001473
63	AGAAGCGC3TAGAGDIGAGAAAG	0.000021	0.000148	0	0	0.000042	0.000294
64	AGAAGCGC3TAGAGIICAGAAAG	0.000042	0.000208	0	0	0.000084	0.000411
65	AGAAGCGC3TAGAGIIGAGAAAG	0.000073	0.000269	0	0	0.000145	0.000532
66	AGAAGCGC3TAGGGDIGAGAAAG	0.000106	0.000352	0	0	0.000209	0.000696
67	AGAAGCGT1GGTAADICCGGGAA	0.000042	0.000208	0	0	0.000084	0.000412
68	AAGAACGC1TAGAGDICACGGCG	0.000363	0.000557	0.000043	0.0003	0.000677	0.001004
69	AAGAACGC1TAGAGDDCACGGCG	0.000021	0.000148	0.000043	0.0003	0	0.000001
70	AAGAACGC2TAGAGDICACGGCG	0.000298	0.00063	0.000022	0.000214	0.000569	0.001167
71	GGGAACGC1TAGAGDICACGGCG	0.002293	0.000491	0.002403	0.000911	0.002185	0.000412
72	GGGAACGC1TAGAGIICACGGCG	0.000265	0.00046	0.000536	0.000929	0	0
73	GGGAACGC2TAGAGDICACGGCG	0.000284	0.000549	0.000574	0.00111	0	0
74	GGGAACGC2TAGAGIICAGAAAG	0.000021	0.000148	0.000043	0.0003	0	0
75	GGGAACGC2TAGAGIICACGGCG	0.000106	0.000318	0.000215	0.000644	0	0
76	GGGAACGC3TAGGGDIGAGAAAG	0.001178	0.000468	0.002254	0.000732	0.000126	0.000499
77	GGGAACGC3TAGGGDDGAGAAAG	0.000032	0.000235	0.000043	0.0003	0.000021	0.000209
78	GGGAATGC1TAGAGDICACGGCG	0.001146	0.00031	0.000171	0.000628	0.002101	0.000007
79	GGGAATGC2TAGAGDICACGGCG	0.000202	0.000443	0.000408	0.000895	0	0
80	GGGAGCGC1TAGAGIICAGAAAG	0.000064	0.000252	0.000129	0.00051	0	0

81	GGGAGCGC1TAGAGIIGAGAAAG	0.000034	0.000186	0.000068	0.000376	0	0
82	GGGAGCGC2TAGAGDICACGGCG	0.000085	0.000325	0.000172	0.000657	0	0
83	GGGAGCGC2TAGAGIICAGAAAG	0.000295	0.000499	0.000598	0.001008	0	0
84	GGGAGCGC2TAGAGIICACGGCG	0.000053	0.000231	0.000107	0.000467	0	0
85	GGGAGCGC2TAGAGIIGAGAAAG	0.000212	0.000425	0.00043	0.000859	0	0
86	GGGAGCGC3TAGAGDICAGAAAG	0.001221	0.000417	0.000021	0.000213	0.002395	0.000806
87	GGGAGCGC3TAGAGDIGAGGAAG	0.000682	0.000653	0.000064	0.000366	0.001288	0.001183
88	GGGAGCGC3TAGAGDIGAGAAAG	0.003185	0.000684	0.002082	0.000564	0.004265	0.001178
89	GGGAGCGC3TAGAGIICAGAAAG	0.009101	0.000872	0.004963	0.001009	0.013151	0.001358
90	GGGAGCGC3TAGAGIIGAGAAAG	0.008206	0.000823	0.006867	0.000873	0.009517	0.001399
91	GGGAGCGC3TAGGGDIGAGAAAG	0.014062	0.000981	0.013136	0.001313	0.01497	0.001383
92	GGGAGCGC3TAGGGIIGAGAAAG	0.000616	0.000622	0.000021	0.000214	0.001197	0.001198
93	GGGAGCGT1GGTAADICCGGGAA	0.00121	0.000443	0.000279	0.000823	0.002122	0.000363
94	GGGAGTGC3TAGAGDIGAGAAAG	0.000138	0.000357	0	0	0.000273	0.000707
95	GGGTACAC3TAGAGIIGAGAAAG	0.00018	0.00057	0.000021	0.000213	0.000336	0.001032
96	GGGTACAT1GGTAADICCGGGAA	0.007565	0.001042	0.012631	0.001824	0.002605	0.001036
97	GGAAGCGC3TAGAGDICAGAAAG	0.000011	0.000106	0	0	0.000021	0.000209
98	GGAAGCGC3TAGAGDIGAGGAAG	0.000021	0.000205	0	0	0.000042	0.000404
99	GGAAGCGC3TAGAGDIGAGAAAG	0.000021	0.000149	0	0	0.000042	0.000295
100	GGAAGCGC3TAGAGIICAGAAAG	0.000106	0.000352	0	0	0.000211	0.000698
101	GGAAGCGC3TAGAGIIGAGAAAG	0.00017	0.000417	0	0	0.000336	0.000825
102	GGAAGCGC3TAGGGDIGAGAAAG	0.000277	0.00051	0.000064	0.000366	0.000486	0.000907
103	GAGAACGC1TAGAGDICAGGGAA	0.000021	0.000187	0.000043	0.000373	0	0
104	GAGAACGC1TAGAGDICACGGAG	0.000032	0.000186	0.000021	0.000213	0.000042	0.000294
105	GAGAACGC1TAGAGDICACGGAA	0.000064	0.000292	0.000128	0.00059	0	0
106	GAGAACGC1TAGAGDICACGGCG	0.300681	0.003328	0.334082	0.005133	0.267989	0.003732
107	GAGAACGC1TAGAGDICACGGCA	0.002399	0.000599	0.00266	0.001177	0.002143	0.000294
108	GAGAACGC1TAGAGDICACAACG	0.001093	0.000181	0.00221	0.000366	0	0
109	GAGAACGC1TAGAGDICCGGGAG	0.003641	0.000638	0.000772	0.001135	0.006449	0.000535
110	GAGAACGC1TAGAGDICCGGGAA	0.000032	0.000181	0.000064	0.000365	0	0
111	GAGAACGC1TAGAGDICCCGGCG	0.000106	0.000329	0.000065	0.000367	0.000147	0.000535
112	GAGAACGC1TAGAGDIGACGGCG	0.000021	0.000154	0.000043	0.00031	0	0
113	GAGAACGC1TAGAGDDCAGGGCG	0.001125	0.000252	0.002231	0.00042	0.000042	0.000295
114	GAGAACGC1TAGAGDDCACGGCG	0.023354	0.001292	0.025344	0.002164	0.021405	0.001164
115	GAGAACGC1TAGAADICACGGCG	0.000361	0.000525	0.000043	0.0003	0.000672	0.00098
116	GAGAACGC1TAGAADICCCGGAA	0.000128	0.000377	0.000021	0.000213	0.000232	0.000658
117	GAGAACGC1TAGAADICCCGGCG	0.000106	0.000318	0	0	0.000209	0.000629
118	GAGAACGC1TAGAADDCACGGCG	0.000021	0.000149	0	0	0.000042	0.000294
119	GAGAACGC1TATAGDICACGGCG	0.000405	0.000516	0.000818	0.001042	0	0.000005
120	GAGAACGC1TATAGDICCCGGAG	0.000021	0.000149	0.000043	0.0003	0	0
121	GAGAACGC1TATAGDICCCGGCG	0.00017	0.000417	0.000322	0.000766	0.000021	0.00021
122	GAGAACGC1TATAADICCCGGAA	0.000033	0.000183	0	0	0.000064	0.000362
123	GAGAACGC1TATAADICCCGGCG	0.000032	0.000181	0	0	0.000063	0.000358
124	GAGAACGC1TGGAGDICAGGGAA	0.000403	0.000577	0.000771	0.001073	0.000042	0.000294
125	GAGAACGC1TGGAGDICAGGGCA	0.000127	0.000376	0.000257	0.00076	0	0
126	GAGAACGC1TGTAADICCGGGAA	0.000577	0.000626	0.001166	0.001265	0	0
127	GAGAACGC1TGTAADICCCGGAA	0.000094	0.000302	0	0	0.000187	0.000598
128	GAGAACGC1GAGAGDICACGGCG	0.000021	0.000149	0.000021	0.000213	0.000021	0.000209
129	GAGAACGC1GAGAGDICCGGGAG	0.00111	0.000513	0.002039	0.000767	0.0002	0.000617

130	GAGAACGC1GAGAGDICCGGGAA	0.000021	0.000149	0.000043	0.0003	0	0
131	GAGAACGC1GAGAGDICCCGGCG	0.000095	0.000383	0.000171	0.000656	0.000021	0.000209
132	GAGAACGC1GGGAGDICAGGGAA	0.000095	0.000427	0.000129	0.000593	0.000063	0.000358
133	GAGAACGC1GGGAGDICCGGGAG	0.000021	0.000149	0.000043	0.0003	0	0
134	GAGAACGC2TAGAGDICACGGCG	0.06202	0.002413	0.070509	0.00417	0.05371	0.002445
135	GAGAACGC2TAGAGDICACAGCG	0.000287	0.000528	0.000064	0.000365	0.000504	0.000897
136	GAGAACGC3TAGAGDIGAGGAAG	0.000085	0.000288	0	0	0.000167	0.000569
137	GAGAACGC3TAGGGDIGAGAAAG	0.000032	0.000228	0.000021	0.000213	0.000042	0.000294
138	GAGAACGT1TAGAGDICACGGCG	0.001116	0.000234	0.000088	0.000426	0.002122	0.000209
139	GAGAACGT1TAGAGDDCACGGCG	0.001093	0.000181	0	0.000007	0.002164	0.000358
140	GAGAACGT1GGGAGDICAGGGAA	0.000637	0.000728	0.001204	0.001334	0.000084	0.000412
141	GAGAACGT1GGGAGDICCGGGAG	0.003647	0.000814	0.006825	0.001311	0.000536	0.000939
142	GAGAACGT1GGGAADICAGGGAA	0.000061	0.000302	0.000102	0.000548	0.000021	0.000209
143	GAGAACGT1GGGAADICCGGGAA	0.000032	0.000221	0.000043	0.000389	0.000021	0.000209
144	GAGAACGT1GGTAGDICCGGGAA	0.000331	0.000766	0.000236	0.000723	0.000425	0.001064
145	GAGAACGT1GGTAADICCGGGAA	0.0027	0.000754	0.000888	0.001311	0.004474	0.000817
146	GAGAACAC1TAGAGDICACGGCG	0.001136	0.000483	0.000193	0.000647	0.002059	0.000662
147	GAGAACAT1GGTAGDICCGGGAA	0.000021	0.000148	0	0	0.000042	0.000294
148	GAGAACAT1GGTAADICCGGGAA	0.000103	0.000359	0.000037	0.00028	0.000168	0.000572
149	GAGAATGC1TAGAGDICACGGCG	0.004097	0.001804	0.005406	0.002372	0.002816	0.002198
150	GAGAATGC1TGTAADICCGGGAA	0.000612	0.000661	0.001236	0.001336	0	0
151	GAGAATGC1TGTAADICCCGGAA	0.000011	0.000107	0	0.000001	0.000022	0.000213
152	GAGAATGC2TAGAGDICACGGCG	0.072669	0.001866	0.076903	0.003338	0.068525	0.001872
153	GAGAATGC2TAGAGDICACAGCG	0.000255	0.000478	0.000021	0.000213	0.000484	0.000884
154	GAGAATGC2TAGAGDICACAACG	0.001093	0.000181	0.000064	0.000366	0.002101	0.000017
155	GAGAGCGC3TAGAGDIGAGGAAG	0.000021	0.000149	0	0	0.000042	0.000294
156	GAGAGCGC3TAGAGDIGAGAAAG	0.000042	0.000208	0.000085	0.00042	0	0
157	GAGTACGC2TAGAGDICACGGCG	0.000457	0.000547	0.000923	0.001105	0	0
158	GAGTATGC2TAGAGDICACGGCG	0.000647	0.000579	0.001287	0.001135	0.000021	0.000209

APPENDIX I

HAPLOTYPE DATA: CASES AND CONTROLS

I.1

SNP Position	ObsHET	PredHET	HWpval	%Geno	Minor AF
-1305	0.471	0.497	0.314	94.2	0.459
-1179	0.454	0.5	0.068	93.3	0.49
-1155	0.186	0.18	0.743	94.2	0.1
-1140	0.436	0.461	0.304	93.6	0.36
-1138	0.069	0.075	0.251	93.6	0.039
-1121	0.16	0.151	0.394	94	0.083
-964	0.462	0.497	0.194	88.7	0.459
-762	0.477	0.496	0.46	98	0.458
-716	0.481	0.496	0.57	99.1	0.455
-689	0.483	0.496	0.636	99.6	0.455
-666	0.469	0.494	0.302	98.9	0.447
-646	0.033	0.033	1	99.8	0.017
-633	0.468	0.495	0.284	98	0.451
-543	0.04	0.04	1	98.9	0.02
-536	0.052	0.05	1	98.9	0.026
-509	0.056	0.059	0.668	98.7	0.03
-486	0.473	0.497	0.349	98.4	0.459
-477	0.458	0.5	0.091	97.8	0.497
-400	0.074	0.08	0.336	98.9	0.041
-391	0.078	0.083	0.397	99.1	0.044
-369	0.455	0.5	0.071	96	0.495
-201	0.476	0.497	0.411	96	0.462

I.2

L1	L2	D'	LOD	r^2	CI low	Chi	Dist
-1305	-1179	1	137.77	0.81	0.98	1	126
-1305	-1155	1	17.7	0.13	0.9	1	150
-1305	-1140	0.95	89.52	0.61	0.91	0.98	165
-1305	-1138	0.85	2.41	0.03	0.4	0.96	167
-1305	-1121	1	10.17	0.08	0.83	1	184
-1305	-964	0.98	165.16	0.96	0.96	1	341
-1305	-762	0.94	140.13	0.87	0.9	0.96	543
-1305	-716	0.94	145.15	0.88	0.91	0.97	589
-1305	-689	0.93	143.26	0.87	0.9	0.96	616
-1305	-666	0.95	142.98	0.87	0.92	0.98	639
-1305	-633	0.96	148.08	0.89	0.92	0.98	672
-1305	-536	1	3.97	0.02	0.62	1	769
-1305	-509	1	2.96	0.03	0.51	1	796
-1305	-486	0.93	138.73	0.85	0.89	0.96	819
-1305	-477	0.92	107.79	0.72	0.87	0.95	828
-1305	-400	1	3.11	0.04	0.53	1	905
-1305	-391	1	3.4	0.04	0.56	1	914
-1305	-369	0.94	115.47	0.76	0.9	0.97	936
-1305	-201	0.94	141.05	0.88	0.91	0.97	1104
-1179	-1155	1	13.74	0.11	0.88	1	24
-1179	-1140	0.99	84.26	0.55	0.95	1	39
-1179	-1138	1	6.69	0.04	0.75	1	41
-1179	-1121	0.9	8.82	0.08	0.71	0.97	58
-1179	-964	0.99	131.34	0.82	0.96	1	215
-1179	-762	0.97	117.68	0.76	0.93	0.99	417
-1179	-716	0.98	121.61	0.77	0.94	1	463
-1179	-689	0.97	119.03	0.76	0.93	0.99	490
-1179	-666	0.99	122.54	0.76	0.96	1	513
-1179	-633	0.99	126.47	0.78	0.96	1	546
-1179	-536	1	4.22	0.03	0.63	1	643
-1179	-509	1	4.14	0.03	0.63	1	670
-1179	-486	0.95	114.76	0.74	0.91	0.98	693
-1179	-477	0.95	144.41	0.87	0.92	0.98	702
-1179	-400	1	6.33	0.04	0.74	1	779
-1179	-391	1	6.31	0.04	0.74	1	788
-1179	-369	0.98	155.75	0.92	0.95	1	810
-1179	-201	0.98	119.64	0.77	0.94	1	978
-1155	-1140	1	7.51	0.06	0.78	1	15
-1155	-1138	1	1.26	0	0.21	1	17
-1155	-1121	0.75	0.43	0.01	0.08	0.97	34
-1155	-964	0.93	13.64	0.12	0.79	0.98	191
-1155	-762	0.93	13.6	0.12	0.79	0.98	393
-1155	-716	0.97	15.28	0.13	0.84	1	439
-1155	-689	0.97	15.25	0.12	0.84	1	466
-1155	-666	0.94	14.57	0.12	0.8	0.98	489

-1155	-633	0.97	15.58	0.13	0.84	1	522
-1155	-536	1	0.38	0	0.07	0.98	619
-1155	-509	1	0.88	0	0.13	0.99	646
-1155	-486	0.97	14.81	0.12	0.83	1	669
-1155	-477	0.92	10.68	0.09	0.75	0.98	678
-1155	-400	1	1.3	0	0.22	1	755
-1155	-391	1	1.35	0	0.23	1	764
-1155	-369	1	13.4	0.11	0.87	1	786
-1155	-201	1	16.4	0.13	0.89	1	954
-1140	-1138	0.65	0.72	0.01	0.11	0.9	2
-1140	-1121	1	6.72	0.05	0.76	1	19
-1140	-964	0.99	95.7	0.65	0.96	1	176
-1140	-762	0.91	77.37	0.56	0.86	0.95	378
-1140	-716	0.92	80.57	0.58	0.87	0.96	424
-1140	-689	0.92	80.09	0.57	0.87	0.96	451
-1140	-666	0.89	77.2	0.56	0.84	0.94	474
-1140	-633	0.91	80.11	0.58	0.86	0.95	507
-1140	-536	1	3.06	0.01	0.54	1	604
-1140	-509	1	1.36	0.02	0.23	1	631
-1140	-486	0.92	78.74	0.56	0.86	0.96	654
-1140	-477	0.91	65.54	0.48	0.85	0.95	663
-1140	-400	1	0.8	0.02	0.13	0.98	740
-1140	-391	1	1.01	0.02	0.17	0.99	749
-1140	-369	0.93	69.41	0.5	0.87	0.97	771
-1140	-201	0.91	76.5	0.57	0.86	0.95	939
-1138	-1121	1	0.67	0	0.1	0.99	17
-1138	-964	0.84	2.18	0.02	0.37	0.96	174
-1138	-762	0.82	1.92	0.02	0.33	0.95	376
-1138	-716	0.83	2.09	0.02	0.36	0.95	422
-1138	-689	0.83	2.09	0.02	0.36	0.95	449
-1138	-666	0.82	1.9	0.02	0.33	0.95	472
-1138	-633	0.83	2	0.02	0.34	0.95	505
-1138	-536	0.7	0.04	0	0.04	0.96	602
-1138	-509	0.87	25.88	0.59	0.73	0.95	629
-1138	-486	0.84	2.17	0.02	0.37	0.95	652
-1138	-477	1	6.82	0.04	0.76	1	661
-1138	-400	0.93	37.84	0.79	0.83	0.98	738
-1138	-391	0.93	38.88	0.8	0.83	0.98	747
-1138	-369	1	6.79	0.04	0.76	1	769
-1138	-201	0.83	2	0.02	0.35	0.95	937
-1121	-964	1	9.35	0.08	0.82	1	157
-1121	-762	1	9.03	0.08	0.82	1	359
-1121	-716	1	8.82	0.08	0.81	1	405
-1121	-689	1	8.53	0.08	0.81	1	432
-1121	-666	1	8.02	0.07	0.8	1	455
-1121	-633	1	8.3	0.07	0.8	1	488
-1121	-536	1	0.51	0	0.08	0.98	585
-1121	-509	1	0.66	0	0.1	0.99	612
-1121	-486	1	8.8	0.08	0.81	1	635

-1121	-477	0.95	9.68	0.08	0.77	0.99	644
-1121	-400	1	0.71	0	0.11	0.99	721
-1121	-391	1	0.75	0	0.11	0.99	730
-1121	-369	0.95	9.75	0.08	0.77	0.99	752
-1121	-201	1	8.38	0.08	0.8	1	920
-964	-762	0.95	134.38	0.87	0.92	0.98	202
-964	-716	0.96	139.48	0.89	0.92	0.98	248
-964	-689	0.95	137.33	0.88	0.91	0.97	275
-964	-666	0.97	137.79	0.88	0.93	0.99	298
-964	-633	0.97	142.93	0.9	0.94	0.99	331
-964	-536	1	3.72	0.02	0.6	1	428
-964	-509	1	2.69	0.03	0.48	1	455
-964	-486	0.93	132.51	0.86	0.9	0.96	478
-964	-477	0.91	102.72	0.73	0.87	0.95	487
-964	-400	1	2.83	0.04	0.5	1	564
-964	-391	1	3.12	0.04	0.53	1	573
-964	-369	0.94	110.39	0.77	0.9	0.97	595
-964	-201	0.96	136.68	0.89	0.93	0.99	763
-762	-716	0.99	190.14	0.98	0.97	1	46
-762	-689	0.99	189.84	0.98	0.97	1	73
-762	-666	0.99	179.29	0.95	0.97	1	96
-762	-633	1	190.77	0.99	0.98	1	129
-762	-536	0.87	2.88	0.02	0.46	0.96	226
-762	-509	1	3.55	0.03	0.58	1	253
-762	-486	0.99	175.75	0.95	0.96	1	276
-762	-477	0.99	140.88	0.82	0.96	1	285
-762	-400	1	3.92	0.04	0.61	1	362
-762	-391	1	4.17	0.04	0.63	1	371
-762	-369	0.99	141.86	0.83	0.96	1	393
-762	-201	0.99	180.59	0.97	0.97	1	561
-716	-689	1	196.68	0.99	0.98	1	27
-716	-666	0.99	181.13	0.96	0.97	1	50
-716	-633	1	195.16	0.99	0.98	1	83
-716	-536	1	4.43	0.02	0.65	1	180
-716	-509	1	3.48	0.03	0.57	1	207
-716	-486	1	185.31	0.97	0.97	1	230
-716	-477	0.99	144.85	0.83	0.97	1	239
-716	-400	1	3.86	0.04	0.61	1	316
-716	-391	1	4.35	0.04	0.65	1	325
-716	-369	1	148.3	0.84	0.98	1	347
-716	-201	0.99	186.52	0.98	0.97	1	515
-689	-666	1	183.85	0.96	0.97	1	23
-689	-633	1	194	0.99	0.98	1	56
-689	-536	1	4.43	0.02	0.65	1	153
-689	-509	1	3.48	0.03	0.57	1	180
-689	-486	0.99	183.41	0.96	0.97	1	203
-689	-477	0.99	146.15	0.83	0.97	1	212
-689	-400	1	3.86	0.04	0.61	1	289
-689	-391	1	4.36	0.04	0.65	1	298

-689	-369	1	148.3	0.84	0.98	1	320
-689	-201	0.99	186.52	0.98	0.97	1	488
-666	-633	0.99	187.77	0.98	0.97	1	33
-666	-536	1	4.3	0.02	0.64	1	130
-666	-509	1	3.32	0.03	0.56	1	157
-666	-486	1	182.33	0.96	0.98	1	180
-666	-477	0.99	140.81	0.81	0.97	1	189
-666	-400	1	3.64	0.04	0.59	1	266
-666	-391	1	4.13	0.04	0.63	1	275
-666	-369	1	145.9	0.83	0.98	1	297
-666	-201	1	181.21	0.97	0.97	1	465
-633	-536	1	4.36	0.02	0.65	1	97
-633	-509	1	3.39	0.03	0.56	1	124
-633	-486	1	180.99	0.96	0.97	1	147
-633	-477	1	145.48	0.83	0.98	1	156
-633	-400	1	3.74	0.04	0.6	1	233
-633	-391	1	4.27	0.04	0.64	1	242
-633	-369	1	146.98	0.84	0.98	1	264
-633	-201	1	184.32	0.98	0.97	1	432
-536	-509	0.03	0.07	0	-0.01	0.2	27
-536	-486	1	4.53	0.02	0.66	1	50
-536	-477	0.87	3.09	0.02	0.48	0.97	59
-536	-400	0.01	0	0	-0.01	0.2	136
-536	-391	0.05	0.12	0	0	0.23	145
-536	-369	1	4.44	0.03	0.65	1	167
-536	-201	1	4.52	0.02	0.66	1	335
-509	-486	1	3.57	0.03	0.58	1	23
-509	-477	1	4.62	0.03	0.66	1	32
-509	-400	0.92	30.14	0.63	0.79	0.98	109
-509	-391	0.96	32.44	0.67	0.84	1	118
-509	-369	1	4.55	0.03	0.66	1	140
-509	-201	1	3.89	0.03	0.61	1	308
-486	-477	0.98	142.52	0.83	0.95	1	9
-486	-400	1	3.96	0.04	0.62	1	86
-486	-391	1	4.46	0.04	0.65	1	95
-486	-369	0.99	147.59	0.85	0.97	1	117
-486	-201	0.98	176.32	0.96	0.96	1	285
-477	-400	1	6.86	0.04	0.76	1	77
-477	-391	1	7.16	0.04	0.77	1	86
-477	-369	1	197.39	1	0.99	1	108
-477	-201	0.99	139.56	0.82	0.96	1	276
-400	-391	0.97	51.84	0.94	0.9	1	9
-400	-369	1	6.8	0.04	0.76	1	31
-400	-201	1	4.03	0.04	0.62	1	199
-391	-369	1	7.11	0.04	0.77	1	22
-391	-201	1	4.28	0.04	0.64	1	190
-369	-201	0.99	141.34	0.83	0.96	1	168

APPENDIX J

HAPLOVIEW DATA: CASES

J.1

SNP Position	ObsHET	PredHET	HWpval	%Geno	Minor AF
-1305	0.471	0.497	0.314	94.2	0.459
-1179	0.454	0.5	0.068	93.3	0.49
-1155	0.186	0.18	0.743	94.2	0.1
-1140	0.436	0.461	0.304	93.6	0.36
-1138	0.069	0.075	0.251	93.6	0.039
-1121	0.16	0.151	0.394	94	0.083
-964	0.462	0.497	0.194	88.7	0.459
-762	0.477	0.496	0.46	98	0.458
-716	0.481	0.496	0.57	99.1	0.455
-689	0.483	0.496	0.636	99.6	0.455
-666	0.469	0.494	0.302	98.9	0.447
-646	0.033	0.033	1	99.8	0.017
-633	0.468	0.495	0.284	98	0.451
-543	0.04	0.04	1	98.9	0.02
-536	0.052	0.05	1	98.9	0.026
-509	0.056	0.059	0.668	98.7	0.03
-486	0.473	0.497	0.349	98.4	0.459
-477	0.458	0.5	0.091	97.8	0.497
-400	0.074	0.08	0.336	98.9	0.041
-391	0.078	0.083	0.397	99.1	0.044
-369	0.455	0.5	0.071	96	0.495
-201	0.476	0.497	0.411	96	0.462

J.2

L1	L2	D'	LOD	r^2	CI low	Chi	Distance
-1305	-1179	1	71.68	0.8	0.97	1	126
-1305	-1155	1	9.48	0.13	0.82	1	150
-1305	-1140	0.99	50.21	0.6	0.93	1	165
-1305	-1138	1	3.28	0.05	0.55	1	167
-1305	-1121	1	6.54	0.09	0.75	1	184
-1305	-964	0.98	91.9	0.96	0.95	1	341
-1305	-762	0.94	75.67	0.87	0.9	0.98	543
-1305	-716	0.96	79.47	0.88	0.92	0.99	589
-1305	-689	0.96	79.19	0.88	0.92	0.99	616
-1305	-666	0.96	79.19	0.88	0.92	0.99	639
-1305	-633	0.96	78.14	0.87	0.92	0.99	672
-1305	-536	1	1.9	0.02	0.34	1	769
-1305	-509	1	1.98	0.04	0.36	1	796
-1305	-486	0.93	76.77	0.87	0.89	0.97	819
-1305	-477	0.92	57.87	0.72	0.85	0.96	828
-1305	-400	1	2.44	0.05	0.44	1	905
-1305	-391	1	2.73	0.05	0.49	1	914
-1305	-369	0.92	57.87	0.72	0.85	0.96	936
-1305	-201	0.95	76.22	0.87	0.9	0.98	1104
-1179	-1155	1	7.18	0.11	0.77	1	24
-1179	-1140	1	42.94	0.51	0.95	1	39
-1179	-1138	1	3.71	0.04	0.6	1	41
-1179	-1121	0.85	4.95	0.08	0.57	0.95	58
-1179	-964	0.99	67.54	0.78	0.94	1	215
-1179	-762	0.97	59.94	0.73	0.91	0.99	417
-1179	-716	0.99	63.54	0.74	0.94	1	463
-1179	-689	0.99	63.3	0.74	0.94	1	490
-1179	-666	0.99	63.3	0.74	0.94	1	513
-1179	-633	0.99	62.75	0.73	0.94	1	546
-1179	-536	1	2.04	0.03	0.37	1	643
-1179	-509	1	2.39	0.03	0.43	1	670
-1179	-486	0.96	59.44	0.72	0.9	0.99	693
-1179	-477	0.96	78.59	0.88	0.92	0.99	702
-1179	-400	1	3.7	0.04	0.59	1	779
-1179	-391	1	3.68	0.04	0.59	1	788
-1179	-369	0.96	78.59	0.88	0.92	0.99	810
-1179	-201	0.98	61.29	0.73	0.93	1	978
-1155	-1140	1	4.74	0.08	0.67	1	15
-1155	-1138	1	0.85	0.01	0.13	0.99	17
-1155	-1121	0.79	0.28	0.01	0.06	0.97	34
-1155	-964	0.94	7.56	0.12	0.72	0.99	191
-1155	-762	1	9.51	0.13	0.82	1	393
-1155	-716	1	9.85	0.14	0.83	1	439
-1155	-689	1	9.86	0.14	0.83	1	466
-1155	-666	1	9.86	0.14	0.83	1	489

-1155	-633	1	9.98	0.14	0.83	1	522
-1155	-536	1	0.24	0	0.05	0.97	619
-1155	-509	1	0.52	0	0.08	0.98	646
-1155	-486	1	9.4	0.13	0.82	1	669
-1155	-477	1	7.4	0.11	0.78	1	678
-1155	-400	1	0.86	0.01	0.13	0.99	755
-1155	-391	1	0.91	0.01	0.14	0.99	764
-1155	-369	1	7.4	0.11	0.78	1	786
-1155	-201	1	9.76	0.14	0.83	1	954
-1140	-1138	1	1.54	0.03	0.27	1	2
-1140	-1121	1	4.32	0.06	0.64	1	19
-1140	-964	0.99	50.03	0.61	0.93	1	176
-1140	-762	0.89	37.83	0.52	0.81	0.95	378
-1140	-716	0.9	39.36	0.53	0.82	0.95	424
-1140	-689	0.9	39.17	0.53	0.82	0.95	451
-1140	-666	0.9	39.17	0.53	0.82	0.95	474
-1140	-633	0.88	38.22	0.52	0.8	0.94	507
-1140	-536	1	1.34	0.01	0.22	1	604
-1140	-509	1	0.83	0.02	0.13	0.99	631
-1140	-486	0.91	39.64	0.52	0.83	0.96	654
-1140	-477	0.91	33.16	0.44	0.82	0.96	663
-1140	-400	1	0.7	0.03	0.11	0.99	740
-1140	-391	1	0.89	0.03	0.14	0.99	749
-1140	-369	0.91	33.16	0.44	0.82	0.96	771
-1140	-201	0.88	37.57	0.51	0.8	0.94	939
-1138	-1121	1	0.22	0	0.05	0.97	17
-1138	-964	1	3.24	0.05	0.55	1	174
-1138	-762	1	2.53	0.05	0.46	1	376
-1138	-716	1	2.72	0.05	0.48	1	422
-1138	-689	1	2.72	0.05	0.48	1	449
-1138	-666	1	2.72	0.05	0.48	1	472
-1138	-633	1	2.68	0.05	0.48	1	505
-1138	-536	0.03	0.01	0	0	0.34	602
-1138	-509	0.86	14.23	0.55	0.66	0.95	629
-1138	-486	1	2.9	0.05	0.51	1	652
-1138	-477	1	3.94	0.04	0.62	1	661
-1138	-400	0.94	22.92	0.8	0.8	0.99	738
-1138	-391	0.95	23.92	0.81	0.81	0.99	747
-1138	-369	1	3.94	0.04	0.62	1	769
-1138	-201	1	2.45	0.05	0.44	1	937
-1121	-964	1	6.17	0.09	0.74	1	157
-1121	-762	1	5.7	0.09	0.72	1	359
-1121	-716	1	5.46	0.09	0.71	1	405
-1121	-689	1	5.46	0.09	0.71	1	432
-1121	-666	1	5.46	0.09	0.71	1	455
-1121	-633	1	5.38	0.09	0.71	1	488
-1121	-536	1	0.39	0	0.06	0.98	585
-1121	-509	1	0.28	0	0.06	0.98	612
-1121	-486	1	5.78	0.09	0.73	1	635

-1121	-477	0.92	5.9	0.09	0.66	0.98	644
-1121	-400	1	0.22	0	0.05	0.97	721
-1121	-391	1	0.26	0	0.06	0.97	730
-1121	-369	0.92	5.9	0.09	0.66	0.98	752
-1121	-201	1	5.54	0.09	0.72	1	920
-964	-762	0.94	72.92	0.85	0.89	0.97	202
-964	-716	0.96	76.68	0.87	0.92	0.99	248
-964	-689	0.96	76.41	0.87	0.92	0.99	275
-964	-666	0.96	76.41	0.87	0.92	0.99	298
-964	-633	0.96	75.4	0.86	0.91	0.99	331
-964	-536	1	1.89	0.02	0.34	1	428
-964	-509	1	1.96	0.04	0.35	1	455
-964	-486	0.93	73.97	0.85	0.88	0.97	478
-964	-477	0.91	56.31	0.71	0.85	0.96	487
-964	-400	1	2.43	0.05	0.44	1	564
-964	-391	1	2.71	0.05	0.49	1	573
-964	-369	0.91	56.31	0.71	0.85	0.96	595
-964	-201	0.96	74.84	0.86	0.91	0.99	763
-762	-716	1	103.96	0.98	0.98	1	46
-762	-689	1	103.66	0.98	0.98	1	73
-762	-666	1	103.66	0.98	0.98	1	96
-762	-633	1	101.98	0.97	0.97	1	129
-762	-536	0.72	0.88	0.01	0.14	0.92	226
-762	-509	1	2.26	0.04	0.41	1	253
-762	-486	0.98	95.82	0.95	0.95	1	276
-762	-477	0.98	72.05	0.78	0.93	1	285
-762	-400	1	2.97	0.05	0.52	1	362
-762	-391	1	3.25	0.05	0.55	1	371
-762	-369	0.98	73.14	0.8	0.93	1	393
-762	-201	0.99	99.5	0.97	0.96	1	561
-716	-689	1	109.23	1	0.98	1	27
-716	-666	1	109.23	1	0.98	1	50
-716	-633	1	106.72	0.99	0.98	1	83
-716	-536	1	2.1	0.02	0.38	1	180
-716	-509	1	2.17	0.04	0.39	1	207
-716	-486	1	101.36	0.97	0.97	1	230
-716	-477	1	76.89	0.8	0.97	1	239
-716	-400	1	2.88	0.05	0.51	1	316
-716	-391	1	3.42	0.06	0.57	1	325
-716	-369	1	77.56	0.81	0.97	1	347
-716	-201	0.99	103.65	0.98	0.96	1	515
-689	-666	1	109.23	1	0.98	1	23
-689	-633	1	106.42	0.99	0.98	1	56
-689	-536	1	2.1	0.02	0.38	1	153
-689	-509	1	2.17	0.04	0.39	1	180
-689	-486	1	101.07	0.97	0.97	1	203
-689	-477	1	76.89	0.8	0.97	1	212
-689	-400	1	2.88	0.05	0.51	1	289
-689	-391	1	3.42	0.06	0.57	1	298

-689	-369	1	77.31	0.81	0.97	1	320
-689	-201	0.99	103.35	0.98	0.96	1	488
-666	-633	1	106.42	0.99	0.98	1	33
-666	-536	1	2.1	0.02	0.38	1	130
-666	-509	1	2.17	0.04	0.39	1	157
-666	-486	1	101.07	0.97	0.97	1	180
-666	-477	1	76.89	0.8	0.97	1	189
-666	-400	1	2.88	0.05	0.51	1	266
-666	-391	1	3.42	0.06	0.57	1	275
-666	-369	1	77.31	0.81	0.97	1	297
-666	-201	0.99	103.35	0.98	0.96	1	465
-633	-536	1	2.08	0.02	0.38	1	97
-633	-509	1	2.14	0.04	0.39	1	124
-633	-486	1	99.65	0.96	0.97	1	147
-633	-477	1	75.98	0.8	0.97	1	156
-633	-400	1	2.84	0.05	0.5	1	233
-633	-391	1	3.38	0.05	0.57	1	242
-633	-369	1	76.63	0.8	0.97	1	264
-633	-201	0.99	101.75	0.97	0.96	1	432
-536	-509	0.03	0.03	0	0	0.32	27
-536	-486	1	2.19	0.02	0.4	1	50
-536	-477	1	2.02	0.03	0.36	1	59
-536	-400	0	0	0	0.03	0.96	136
-536	-391	0.09	0.14	0	0	0.37	145
-536	-369	1	1.97	0.02	0.35	1	167
-536	-201	1	2.1	0.02	0.38	1	335
-509	-486	1	2.28	0.03	0.41	1	23
-509	-477	1	2.75	0.03	0.49	1	32
-509	-400	0.87	15.03	0.54	0.67	0.96	109
-509	-391	0.93	17.03	0.6	0.75	0.99	118
-509	-369	1	2.81	0.03	0.5	1	140
-509	-201	1	2.5	0.04	0.45	1	308
-486	-477	0.99	76.87	0.81	0.95	1	9
-486	-400	1	3.07	0.05	0.53	1	86
-486	-391	1	3.62	0.06	0.59	1	95
-486	-369	0.99	77.58	0.82	0.95	1	117
-486	-201	0.99	98.01	0.96	0.96	1	285
-477	-400	1	4.08	0.04	0.63	1	77
-477	-391	1	4.38	0.04	0.65	1	86
-477	-369	1	109.16	1	0.98	1	108
-477	-201	0.99	73.57	0.79	0.95	1	276
-400	-391	0.95	29.38	0.91	0.85	0.99	9
-400	-369	1	4.12	0.04	0.63	1	31
-400	-201	1	2.88	0.05	0.51	1	199
-391	-369	1	4.4	0.04	0.65	1	22
-391	-201	1	3.15	0.05	0.54	1	190
-369	-201	0.99	74.63	0.8	0.95	1	168

APPENDIX K

HAPLOVIEW DATA: CONTROLS

K.1

SNP Position	ObsHET	PredHET	HWpval	%Geno	Minor AF
-1305	0.505	0.485	0.677	94.5	0.413
-1179	0.493	0.496	0.996	93.1	0.458
-1155	0.157	0.153	1	93.6	0.083
-1140	0.443	0.446	1	93.1	0.335
-1138	0.059	0.058	1	92.7	0.03
-1121	0.153	0.149	1	93.1	0.081
-964	0.495	0.484	0.914	84.4	0.41
-762	0.519	0.486	0.42	96.3	0.417
-716	0.528	0.487	0.284	98.2	0.418
-689	0.535	0.487	0.205	99.5	0.419
-666	0.505	0.481	0.582	98.2	0.402
-646	0.032	0.032	1	99.5	0.016
-633	0.51	0.484	0.568	96.3	0.412
-543	0.028	0.028	1	98.6	0.014
-536	0.056	0.054	1	98.6	0.028
-509	0.051	0.05	1	98.6	0.026
-486	0.524	0.486	0.348	97.2	0.417
-477	0.528	0.496	0.43	97.2	0.453
-400	0.065	0.063	1	99.1	0.032
-391	0.065	0.063	1	98.6	0.033
-369	0.525	0.496	0.512	93.6	0.453
-201	0.53	0.49	0.325	92.7	0.428

K.2

L1	L2	D'	LOD	r^2	CI low	Chi	Dist
-1305	-1179	1	64.63	0.83	0.96	1	126
-1305	-1155	1	7.78	0.13	0.79	1	150
-1305	-1140	0.92	39.7	0.62	0.84	0.96	165
-1305	-1138	0.44	0.17	0	0.04	0.86	167
-1305	-1121	1	3.8	0.06	0.6	1	184
-1305	-964	0.99	71.92	0.96	0.95	1	341
-1305	-762	0.94	63.42	0.87	0.89	0.97	543
-1305	-716	0.95	65.14	0.87	0.9	0.98	589
-1305	-689	0.95	63.78	0.85	0.9	0.98	616
-1305	-666	0.94	62.59	0.85	0.88	0.97	639
-1305	-633	0.96	69.28	0.91	0.91	0.99	672
-1305	-536	1	2	0.02	0.36	1	769
-1305	-509	1	1.17	0.02	0.19	0.99	796
-1305	-486	0.94	60.94	0.84	0.88	0.97	819
-1305	-477	0.92	48.65	0.72	0.85	0.96	828
-1305	-400	1	0.98	0.02	0.15	0.99	905
-1305	-391	1	1	0.02	0.16	0.99	914
-1305	-369	0.98	57.19	0.81	0.92	1	936
-1305	-201	0.97	64.75	0.89	0.92	0.99	1104
-1179	-1155	1	6.11	0.11	0.74	1	24
-1179	-1140	0.98	41.21	0.59	0.92	1	39
-1179	-1138	1	2.67	0.03	0.47	1	41
-1179	-1121	1	4.42	0.08	0.65	1	58
-1179	-964	1	62.88	0.87	0.96	1	215
-1179	-762	0.98	56.84	0.79	0.92	1	417
-1179	-716	0.97	57.58	0.79	0.91	0.99	463
-1179	-689	0.94	55.61	0.77	0.89	0.98	490
-1179	-666	0.99	57.96	0.78	0.94	1	513
-1179	-633	1	63.36	0.83	0.96	1	546
-1179	-536	1	2.1	0.02	0.38	1	643
-1179	-509	1	1.58	0.03	0.27	1	670
-1179	-486	0.94	54.34	0.77	0.88	0.98	693
-1179	-477	0.95	64.39	0.86	0.9	0.98	702
-1179	-400	1	2.36	0.04	0.42	1	779
-1179	-391	1	2.34	0.04	0.42	1	788
-1179	-369	1	77.14	0.96	0.97	1	810
-1179	-201	0.98	58.19	0.83	0.92	1	978
-1155	-1140	1	3.07	0.05	0.53	1	15
-1155	-1138	1	0.47	0	0.07	0.98	17
-1155	-1121	0.71	0.15	0	0.05	0.97	34
-1155	-964	0.92	5.75	0.12	0.66	0.98	191
-1155	-762	0.84	4.45	0.09	0.55	0.95	393
-1155	-716	0.92	5.45	0.11	0.65	0.98	439
-1155	-689	0.92	5.4	0.11	0.64	0.98	466
-1155	-666	0.85	4.97	0.1	0.57	0.95	489

-1155	-633	0.92	5.59	0.11	0.65	0.98	522
-1155	-536	1	0.13	0	0.05	0.97	619
-1155	-509	1	0.39	0	0.06	0.98	646
-1155	-486	0.92	5.37	0.11	0.64	0.98	669
-1155	-477	0.82	3.51	0.07	0.48	0.94	678
-1155	-400	1	0.5	0	0.08	0.98	755
-1155	-391	1	0.51	0	0.08	0.98	764
-1155	-369	1	5.51	0.1	0.71	1	786
-1155	-201	1	6.31	0.11	0.74	1	954
-1140	-1138	0.01	0	0	0.02	0.8	2
-1140	-1121	1	2.46	0.04	0.44	1	19
-1140	-964	1	45.34	0.69	0.95	1	176
-1140	-762	0.93	39.47	0.62	0.85	0.97	378
-1140	-716	0.94	41.23	0.63	0.87	0.98	424
-1140	-689	0.94	40.86	0.62	0.87	0.98	451
-1140	-666	0.89	37.7	0.6	0.81	0.94	474
-1140	-633	0.94	42.16	0.65	0.87	0.98	507
-1140	-536	1	1.68	0.01	0.3	1	604
-1140	-509	1	0.61	0.01	0.1	0.98	631
-1140	-486	0.93	38.87	0.61	0.85	0.97	654
-1140	-477	0.91	32.17	0.52	0.82	0.96	663
-1140	-400	0.95	0.25	0.02	0.06	0.97	740
-1140	-391	0.98	0.27	0.02	0.06	0.97	749
-1140	-369	0.95	36.63	0.58	0.88	0.99	771
-1140	-201	0.94	39.38	0.64	0.87	0.98	939
-1138	-1121	1	0.45	0	0.07	0.98	17
-1138	-964	0.3	0.06	0	0.03	0.84	174
-1138	-762	0.47	0.2	0.01	0.04	0.87	376
-1138	-716	0.48	0.21	0.01	0.04	0.87	422
-1138	-689	0.48	0.21	0.01	0.04	0.87	449
-1138	-666	0.41	0.13	0	0.04	0.86	472
-1138	-633	0.45	0.18	0	0.04	0.86	505
-1138	-536	1	0.15	0	0.04	0.97	602
-1138	-509	0.9	11.65	0.67	0.66	0.98	629
-1138	-486	0.47	0.19	0	0.04	0.87	652
-1138	-477	1	2.71	0.04	0.48	1	661
-1138	-400	0.91	14.68	0.77	0.72	0.98	738
-1138	-391	0.91	14.65	0.77	0.72	0.98	747
-1138	-369	1	2.7	0.04	0.48	1	769
-1138	-201	0.53	0.27	0.01	0.05	0.88	937
-1121	-964	1	3.37	0.06	0.56	1	157
-1121	-762	1	3.41	0.07	0.57	1	359
-1121	-716	1	3.45	0.07	0.57	1	405
-1121	-689	1	3.17	0.06	0.54	1	432
-1121	-666	1	2.68	0.06	0.48	1	455
-1121	-633	1	3.06	0.06	0.53	1	488
-1121	-536	1	0.12	0	0.04	0.97	585
-1121	-509	1	0.38	0	0.06	0.98	612
-1121	-486	1	3.13	0.07	0.54	1	635

-1121	-477	1	4.11	0.08	0.63	1	644
-1121	-400	1	0.49	0	0.08	0.98	721
-1121	-391	1	0.49	0	0.08	0.98	730
-1121	-369	1	4.15	0.08	0.63	1	752
-1121	-201	1	2.95	0.07	0.52	1	920
-964	-762	0.96	60.47	0.9	0.91	0.99	202
-964	-716	0.95	61.91	0.9	0.9	0.98	248
-964	-689	0.94	60.15	0.88	0.89	0.98	275
-964	-666	0.98	60.06	0.88	0.93	1	298
-964	-633	0.99	67.11	0.94	0.95	1	331
-964	-536	1	1.81	0.02	0.33	1	428
-964	-509	1	0.92	0.02	0.14	0.99	455
-964	-486	0.93	57.31	0.86	0.88	0.96	478
-964	-477	0.91	45.17	0.74	0.84	0.96	487
-964	-400	1	0.72	0.02	0.11	0.99	564
-964	-391	1	0.74	0.02	0.11	0.99	573
-964	-369	0.97	53.85	0.84	0.92	1	595
-964	-201	0.96	61.68	0.93	0.92	0.99	763
-762	-716	1	86.32	0.98	0.97	1	46
-762	-689	1	86.32	0.98	0.97	1	73
-762	-666	0.98	75.58	0.92	0.94	1	96
-762	-633	1	88.57	1	0.98	1	129
-762	-536	1	2.28	0.02	0.42	1	226
-762	-509	1	1.45	0.02	0.25	1	253
-762	-486	0.99	78.88	0.95	0.95	1	276
-762	-477	1	68.42	0.86	0.97	1	285
-762	-400	1	1.26	0.03	0.21	1	362
-762	-391	1	1.26	0.03	0.21	1	371
-762	-369	1	68.3	0.88	0.97	1	393
-762	-201	0.99	80.54	0.98	0.96	1	561
-716	-689	0.99	87.11	0.98	0.96	1	27
-716	-666	0.98	73.97	0.91	0.94	1	50
-716	-633	1	87.46	0.99	0.98	1	83
-716	-536	1	2.29	0.02	0.42	1	180
-716	-509	1	1.46	0.02	0.25	1	207
-716	-486	0.99	83.34	0.97	0.96	1	230
-716	-477	0.99	67.29	0.85	0.94	1	239
-716	-400	1	1.28	0.02	0.21	1	316
-716	-391	1	1.28	0.02	0.21	1	325
-716	-369	1	69.81	0.88	0.97	1	347
-716	-201	0.99	82.12	0.98	0.96	1	515
-689	-666	0.99	76.31	0.92	0.95	1	23
-689	-633	1	86.63	0.98	0.97	1	56
-689	-536	1	2.3	0.02	0.42	1	153
-689	-509	1	1.47	0.02	0.25	1	180
-689	-486	0.98	82.04	0.96	0.95	1	203
-689	-477	0.99	68.76	0.86	0.95	1	212
-689	-400	1	1.29	0.02	0.21	1	289
-689	-391	1	1.29	0.02	0.21	1	298

-689	-369	1	70.09	0.88	0.97	1	320
-689	-201	0.99	82.43	0.98	0.96	1	488
-666	-633	0.99	81.2	0.96	0.95	1	33
-666	-536	1	2.16	0.02	0.4	1	130
-666	-509	1	1.34	0.02	0.23	1	157
-666	-486	1	80.13	0.94	0.97	1	180
-666	-477	0.99	62.78	0.81	0.94	1	189
-666	-400	1	1.13	0.02	0.18	0.99	266
-666	-391	1	1.13	0.02	0.18	0.99	275
-666	-369	1	67.38	0.86	0.96	1	297
-666	-201	1	77.98	0.95	0.97	1	465
-633	-536	1	2.24	0.02	0.41	1	97
-633	-509	1	1.41	0.02	0.24	1	124
-633	-486	0.99	80.59	0.96	0.95	1	147
-633	-477	1	68.62	0.86	0.97	1	156
-633	-400	1	1.22	0.02	0.2	1	233
-633	-391	1	1.24	0.02	0.2	1	242
-633	-369	1	69.53	0.88	0.97	1	264
-633	-201	1	82.1	0.98	0.97	1	432
-536	-509	0.04	0.05	0	0	0.29	27
-536	-486	1	2.31	0.02	0.42	1	50
-536	-477	0.77	1.28	0.01	0.22	0.94	59
-536	-400	0.02	0.01	0	-0.01	0.26	136
-536	-391	0.02	0.01	0	-0.01	0.26	145
-536	-369	1	2.33	0.03	0.42	1	167
-536	-201	1	2.37	0.02	0.43	1	335
-509	-486	1	1.44	0.02	0.25	1	23
-509	-477	1	1.75	0.03	0.31	1	32
-509	-400	1	15.76	0.78	0.81	1	109
-509	-391	1	15.74	0.78	0.81	1	118
-509	-369	1	1.73	0.03	0.31	1	140
-509	-201	1	1.52	0.02	0.26	1	308
-486	-477	0.98	64.29	0.83	0.93	1	9
-486	-400	1	1.25	0.02	0.21	1	86
-486	-391	1	1.25	0.02	0.21	1	95
-486	-369	1	68.97	0.88	0.97	1	117
-486	-201	0.99	77.96	0.96	0.95	1	285
-477	-400	1	2.53	0.04	0.45	1	77
-477	-391	1	2.53	0.04	0.45	1	86
-477	-369	1	86.91	0.99	0.98	1	108
-477	-201	0.99	65.37	0.86	0.94	1	276
-400	-391	1	22.59	1	0.89	1	9
-400	-369	1	2.53	0.04	0.45	1	31
-400	-201	1	1.42	0.02	0.24	1	199
-391	-369	1	2.53	0.04	0.45	1	22
-391	-201	1	1.42	0.02	0.24	1	190
-369	-201	0.99	66.05	0.87	0.94	1	168

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