Mannose binding lectin genotypes are not associated with increased risk of unexplained recurrent pregnancy loss

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Abstract

Purpose Immune response to infections has been associated with recurrent pregnancy loss (RPL). Low plasma mannose binding lectin (MBL) levels, an innate immunity factor in infections, has been related to RPL. In this study, we tested the hypothesis that MBL genotypes that are known to cause reduced plasma MBL levels are significantly more frequent among women experiencing unexplained RPL.

Methods This study included 219 Caucasian women diagnosed with unexplained RPL and 236 control women. All participants were genotyped for two promoter (−550 C > G and −221 G > C) and three missense (R52C, G54D and G57E) mutations in exon 1. These mutations are known to be associated with variations in plasma MBL levels. Genotype frequencies were estimated by gene counting and were compared to the expectation of Hardy-Weinberg equilibrium

by chi-squared (χ^2) analysis and Fisher's exact test. Allele and genotype frequencies were compared in cases and controls using χ^2 contingency table analysis.

Results There was no difference in demographics between cases and controls. The number of miscarriages in the participants with RPL ranged from 2 to 10 spontaneous abortions (SAB's) per participant. Populations genotyped were in Hardy-Weinberg equilibrium. There was no association between a history of RPL and multi-SNP genotypes at the MBL locus. In unexplained RPL, the number of SAB's and live birth rates were unaffected by MBL genotype. There was no association between MBL genotype and the risk of unexplained RPL. The occurrence of live birth was not associated with MBL genotype. **Conclusion** Genotypes known to cause low MBL plasma levels are not associated with an increased risk of unexplained RPL.

Capsule Population variation in the mannose binding lectin gene is not a risk factor for recurrent pregnancy loss.

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Background

Recurrent pregnancy loss (RPL) is one of the most common pregnancy complications with the majority of cases having unknown causes [1]. Because the prevalence of RPL is higher than expected by chance, it has been suggested that some couples have an underlying systemic cause for RPL [2]. A number of factors have been identified in the etiology of RPL including 1) anatomic müllerian abnormalities of the uterus or cervix, 2) cytogenetic disorders such as aneuploidy or parental balanced translocations, 3) endocrine disorders such as thyroid dysfunction and diabetes, 4) reproductive tract infections such as mycoplasma, 5) immunologic causes such as antiphospholipid syndrome, and 6) skewed X-inactivation

[2–9]. These etiologies however explain causes of RPL in only 20 to 50 % of cases.

Several bacterial and viral infectious agents have been proposed to be a cause for miscarriage [10]. It is known that the presence of pathogenic organisms in the maternal genital tract or placenta result in poor pregnancy outcomes [11–15]. This suggests that pregnancy outcome may be determined by maternal or fetal-host immune response. Mannose binding lectin (MBL), a calcium dependent plasma lectin, is one of many elements of the innate immune system that has been indicated as an important component in successful pregnancy [16]. Low MBL concentration has been proposed to be a risk factor for spontaneous abortion (SAB) [17, 18]. It has already been documented that plasma MBL concentrations are determined by a combination of genotypes and polymorphisms in both MBL coding and promoter sequence [19–22]. Three variant alleles coding for missense mutations leading to loss of function of MBL have been identified in codons 52 (rs5030737), 54 (rs1800451) and 57 (rs1800451) of exon 1 in the Mannose Binding Lectin 2 gene (MBL2) located on 10q11.2–q21 [23]. Two single nucleotide polymorphisms (SNPs) lie in the proximal promoter at positions –550 (rs11003125) and –221 (rs7096206) of the flanking 5' region of the MBL2 gene, which also influences plasma levels of MBL [23]. 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 [19, 20]. Individuals who are homozygous or compound heterozygous for the less common alleles had MBL plasma concentrations of <1 % of the wild type concentrations [19, 20]. Although MBL serum concentrations increase during pregnancy, the effect of genotype is maintained with heterozygotes having significantly reduced MBL levels compared to controls [24].

Plasma levels of MBL are largely genetically determined and can be predicted by examining the three missense mutations in exon 1 and two promoter polymorphisms [19, 20]. Several studies have investigated the relationship between MBL gene polymorphisms and its associated sequence variation with obstetric outcomes [25–27]. We therefore tested the hypothesis that MBL genotypes that are known to lead to reduced levels of circulating MBL are significantly more frequent among women who have experienced unexplained RPL than women who did not experience RPL.

Methods

Samples

Two hundred and nineteen Caucasian women (age range 16–43) who experienced unexplained RPL (defined as two

or more SAB's) were included in this study. Participants were recruited 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 a written informed consent. Inclusion criteria included the following: 1) having had two or more clinically recognized SAB's and 2) having RPL of unknown etiology. An SAB was defined by a positive urine or serum beta human chorionic gonadotropin (β HCG), followed by either vaginal bleeding consistent with loss of the products of conception or an ultrasonographic diagnosis of fetal demise with subsequent reduction in serum β HCG [2, 7, 28–30]. The two losses did not need to be consecutive. Ectopic and molar pregnancies were excluded. Losses must have occurred before 20 weeks of gestation. Cases were ascertained solely through the RPL phenotype; participants were not enrolled on the basis of family history of pregnancy loss, excess female offspring or X chromosome-linked disease [2, 7, 28–30].

The diagnosis of unexplained RPL was based on a standard set of diagnostic criteria that included an evaluation of potential anatomic, cytogenetic, infectious, immunologic, hormonal and toxic causes [31]. Women were excluded from enrollment if they were found to have diabetes, thyroid disease, abnormal uterine anatomy, antiphospholipid syndrome, connective tissue disorder, exposure to abnormally excessive environmental toxins, or if either parent had an abnormal karyotype (i.e. balanced translocation) [2, 7, 28–30].

Control subjects included 236 gravida 1, para 1 women with no history of pregnancy loss and having had healthy uncomplicated pregnancies. The control participants were healthy Caucasian women (age range 14–44 years) who were seeking prenatal care prior to 20 weeks gestation at University of Pittsburgh, Magee-Womens Hospital and the University of Minnesota, Center for Reproductive Medicine.

Genotyping

High molecular weight DNA was isolated from ethylenediaminetetraacetic acid (EDTA) anticoagulated whole blood using the PureGene kit (Gentra Systems, Minneapolis MN). 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) as previously described [20]. Briefly, this method uses twin-pair primers to detect allelic nucleotides differentially [32]. The –221 site was investigated by the method from Roelofs et al. [33]. Polymerase chain reaction was carried out, the amplification product was digested by *BasJI*, and the fragments were separated by

electrophoresis through agarose gels, as previously described [33]. Exon 1 SNPs were identified by pyrosequencing using amplification [34]. Primers included forward 5'-CGTCTTACTCAGAACTGTGACCTGTGAGG-3' and reverse 5'-TTCCTCTGGAAGGTAAAGAATTGCAG-3' with an internal sequencing primer: 5'-GGTCCCCCTTTTCT-3'.

Analysis

Genotype frequencies were estimated by gene counting and compared to the expectations of Hardy-Weinberg equilibrium by chi-squared (χ^2) analysis and Fisher's exact test. Diplootypes were estimated and classified based on methodology described by Baxter et al. [11]. Allele and genotype frequencies were compared in cases and controls using χ^2 contingency table analysis. With a sample size of 219 cases and 236 controls, we had 80 % power (at $p=0.05$) to detect an odds ratio ≥ 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.

Results

A total of 219 cases and 236 controls were genotyped for 5 polymorphisms (Table 1). There was no difference in demographics between cases and controls (these data were published in our previous report) [2, 7, 29]. Overall the number of miscarriages in the participants with RPL ranged from 2 to 10 SAB's per patient (mean = 3.6 SAB's per patient) (Fig. 1). In patients with more than 4 SAB's, the average number of losses per patient was 6.6 SAB's.

The case and control populations genotyped were in Hardy-Weinberg equilibrium, at all five polymorphism sites. Fisher's exact test of cases and controls was not significant at any of the

5 sites: p -values for -550, -221, codon 52, codon 54 and codon 57 were 0.78, 0.38, 0.29, 0.80 and 0.60 respectively (Table 1).

We then classified our cases and controls as high, intermediate and low in predicted MBL plasma concentrations; as analyzed by Baxter et al. [11] (Fig. 2). There was no significant association in cases and controls between the number of SAB's and the predicted plasma MBL level based on genotype ($p=0.81$), nor was there an association between women who had no live births and predicted MBL levels ($p=0.71$). There was no association between a history of RPL and multi-SNP genotypes at the MBL locus.

Discussion

In this study, we examined the genotype for two promoter (-550 C > G and -221 G > C) and three missense (R52C, G54D and G57E) mutations in the MBL2 gene in 219 Caucasian participants and found no association between MBL genotype and the risk of idiopathic RPL. The advantages of our study are 1) it included larger sample size than the previously published studies to date pertaining to the relationship between MBL genotype and unexplained RPL 2) it had adequate power analysis, and 3) unlike all the previous data [18, 35, 36], our definition for RPL was based on the recent definition of this diagnosis by the American Society of Reproductive Medicine [37] i.e., the occurrence of 2 or more, rather than 3 or more, SAB's.

In a study of 217 women with 3 or more unexplained SAB's, Kruse et al. [36] reported women with low maternal serum MBL levels had a higher miscarriage risk than patients with normal MBL levels [36]. A similar study of 146 Danish women and 49 Scottish women with 3 or more unexplained SAB's reported that MBL deficiency was found with increased frequency among women with RPL in both populations, reaching statistical significance in the Scottish

Table 1 Mannose binding lectin (MBL) promoter region and exon sequencing results. Genotype counts and minor allele frequency (q) of MBL alleles affecting MBL 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		
<i>p</i>	0.64		0.77		0.95		0.85		0.99	
<i>q</i>	0.36		0.23		0.05		0.15		0.01	
Controls										
	CC:	97	GG:	150	CC:	207	GG:	178	GG:	227
	CG:	110	GC:	76	CT:	29	GA:	54	GA:	9
	GG:	29	CC:	10			AA:	4		
<i>p</i>	0.64		0.80		0.94		0.87		0.98	
<i>q</i>	0.36		0.20		0.06		0.13		0.02

Fig. 1 Categorization of spontaneous abortions (SAB) in cases, $n=219$, according to the number of miscarriages experienced. 2 SAB, 3 SAB, 4 SAB and 4 or more SAB are depicted, $n=51$, 87, 44 and 37 respectively

population [35]. These studies suggest that MBL levels could account for previously unexplained RPL. On the other hand, Baxter et al. [11], who also used 3 or more SAB's to define RPL, reported no significant association between RPL and variant alleles in the MBL gene. Similar to our study which uses the newest definition of RPL, their study also showed that unexplained RPL is not associated with MBL genotypes known to result in low MBL plasma levels.

Traditionally RPL has been defined as 3 or more spontaneous, consecutive pregnancy losses, but it has been shown that patients with a single live born followed by a series of miscarriages have similar disorders to those patients with only miscarriages and no live births [38]. Because the risk of recurrent miscarriage increases with each prior miscarriage, it has been argued that evaluation can begin after 2 losses instead of waiting until 3 SAB's [37, 39]. In our study, we used 2 or more SAB's for eligibility in the study. Due to differences in the definition of RPL, it was therefore important to determine if women with 2 SAB's are similar to those women experiencing 3 or more SAB's with no live births, making this category of cases consistent with the standard

definition of 3 or more consecutive miscarriages. It is clear from Fig. 2 that examining women experiencing 3 or more SAB's, which by definition excludes women experiencing 2 SAB's, have the same MBL genotype results compared to including women experiencing 2 SAB's. Furthermore, our case population, when analyzed together or by number of SAB's, does not show significantly different MBL genotypes than our control population.

Investigating MBL plasma levels in pregnant mothers implies studying a maternal condition or phenotype, the influence of a genotype. The mechanism we propose involves women with MBL genotype mutations being at an increased risk for RPL possibly because of their diminished ability to detect and respond to infection. This would result in potentially every pregnancy being lost to miscarriage if exposed to maternal genital tract infection. Seventy-five of our cases experienced 3 or more SAB's with no live births, and were observed to have similar MBL genotypes to our control population ($p=0.87$) (Fig. 2). Having no live births did not impact predicted MBL plasma levels in women suffering from unexplained RPL.

The diplotypes analyzed in this study were estimated based on previous findings [11]. MBL plasma levels were predicted using a combination of genotypes; MBL levels have been shown to be influenced by the mutations in the structural gene and by the two dimorphic loci in the promoter region. Baxter et al. [11] have shown that it is possible to define the collection of genotypes as plasma level phenotypes. Given that we did not measure plasma levels, our findings rely on the reported diplotype frequencies and relationships previously reported. Given the consensus in the literature, our methods of estimating MBL concentrations are reliable reflections of plasma MBL levels.

Despite prior smaller studies [17, 35, 36] of a correlation between reduced plasma MBL levels and RPL, we found in a large sample size population using the latest RPL definition that there is no evidence to support a relationship between MBL genotypes or phenotypes and the risk of idiopathic RPL among

Fig. 2 Percent of predicted mannose binding lectin (MBL) production in comparison with the number of spontaneous abortions (SAB). Each individual, in cases and controls, was classified by predicted MBL plasma level concentration and separated by number of miscarriages

Caucasian women. We postulate that genotypes known to cause low MBL plasma levels are not associated with unexplained RPL.

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