MHR Basic science of reproductive medicine

ORIGINAL RESEARCH

A genome-wide scan in affected sibling pairs with idiopathic recurrent miscarriage suggests genetic linkage

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ABSTRACT: Previously, siblings of patients with idiopathic recurrent miscarriage (IRM) have been shown to have a higher risk of miscarriage. This study comprises two parts: (i) an epidemiological part, in which we introduce data on the frequency of miscarriage among 268 siblings of 244 patients with IRM and (ii) a genetic part presenting data from a genome-wide linkage study of 38 affected sibling pairs with IRM. All IRM patients (probands) had experienced three or more miscarriages and affected siblings two or more miscarriages. The sibling pairs were genotyped by the Affymetrix GeneChip 50K XbaI platform and non-parametric linkage analysis was performed via the software package Merlin. We find that siblings of IRM patients exhibit a higher frequency of miscarriage than population controls regardless of age at the time of pregnancy. We identify chromosomal regions with LOD scores between 2.5 and 3.0 in subgroups of affected sibling pairs. Maximum LOD scores were identified in four occurrences: for rs10514716 (3p14.2) when analyzing sister-pairs only; for rs10511668 (9p22.1) and rs341048 (11q13.4) when only analyzing families where the probands have had four or more miscarriages; and for rs10485275 (6q16.3) when analyzing one sibling pair from each family only. We identify no founder mutations. Concluding, our results imply that IRM patients and their siblings share factors which increase the risk of miscarriage. In this first genome-wide linkage study of affected sibling pairs with IRM, we identify regions on chromosomes 3, 6, 9 and 11 which warrant further investigation in order to elucidate their putative roles in the genesis of IRM.

Key words: recurrent miscarriage / affected sibling pair analysis / risk of miscarriage / epidemiology / genome-wide linkage study

Introduction

Recurrent miscarriage (RM) is defined as three or more consecutive intrauterine miscarriages. RM affects 1–3% of couples who wish to reproduce. Uterine abnormalities, abnormal karyotypes in the couple, endocrine disorders and the presence of the lupus anticoagulant or antinuclear antibodies are relatively well-documented risk factors for miscarriage (Stephenson, 1996; Baek et al., 2007). However, in about 50% of couples suffering from RM none of these factors can be found, and the condition is thus termed idiopathic RM (IRM). It has been proposed that RM has a multifactorial etiology (Christiansen et al., 2008).

Further, the risk of miscarriage has earlier been reported to be enhanced for siblings of IRM patients (Christiansen et al., 1990). This led to our hypothesis that IRM has a partially genetic etiology.

Various maternal genotypes have been reported to be associated with RM. These include the thrombophilic genetic variants factor II

and factor V Leiden, which have been associated with both early and late RM (Rey et al., 2003). The discovery that sisters who are HLA identical by descent (IBD) with probands with RM exhibit a higher risk of miscarriage than those who are not HLA identical, suggests that genes in the HLA region are involved in RM (Christiansen et al., 1989). An association with HLA-DRB1*03 has been reported (Christiansen et al., 1994; Kruse et al., 2004), especially for patients with four or more miscarriages or secondary RM. Hviid et al., 2004 found that homozygosity for a 14-bp insertion in exon 8 of the HLA-G gene is correlated with an increased risk of RM. High cytokine-producing genotypes of IFN-γ (Daher et al., 2003) as well as a specific haplotype of IL10 promoter polymorphisms have been associated with RM (Zammiti et al., 2006), although there is no consensus on this (Babbage et al., 2001). Low levels of plasma mannose-binding lectin (MBL) in the plasma (Kilpatrick et al., 1995; Kruse et al., 2002) and carriage of MBL2 genotypes disposing to MBL deficiency have also been associated with RM (Christiansen et al., 2009b).

Recently, in a genome-wide association study, 430 microsatellites were investigated in 44 patients with two or more idiopathic miscarriages and 44 unrelated controls. The authors found significant associations between 6q27, 9q33.1 and Xp22.11 and IRM (Li *et al*., 2010).

In this paper we present results from a study with an epidemiological part confirming that siblings of IRM patients exhibit a higher risk of miscarriage. In the genetic part of the study we investigate a multicase cohort using affected sibling pair analysis (Risch 1990) and attempt to identify genetic markers shared among the affected siblings with IRM.

Materials and Methods

Participants

Hospital records of all RM patients referred to the Danish Miscarriage Clinic between 1986 and 2004 (709 patients) were evaluated in 2004–2005. *A priori* the following patients were excluded: those with no living, full siblings in Denmark ($n = 70$); incomplete medical investigation ($n = 52$); less than three confirmed miscarriages ($n = 25$); abnormal parental karyotype ($n = 6$); abnormal uterine anatomy ($n = 14$); irregular menstrual cycle ($n = 8$) and lupus anticoagulant in the plasma ($n = 6$), and we also excluded non-Danish-speaking or non-Caucasian patients ($n = 12$); those who had emigrated ($n = 6$) or were deceased ($n = 4$). Thus, the eligible patients (probands) fulfilled the criteria for IRM, having had at least three consecutive miscarriages and no established risk factors.

Five hundred and six probands were contacted and asked for an update on their reproductive history and the names and addresses of their full siblings. Of these, 102 did not wish to participate and 160 patients did not reply despite a reminder, leaving 244 patients (48%) and 381 full siblings in the study group. From the 381 siblings we obtained reproductive data from 268 sisters and brothers' wives.

Twenty-three of the probands had one responding sibling who had had at least two miscarriages, and three had two responding siblings with two or more miscarriages.

The siblings' miscarriages were not necessarily consecutive. For both probands and siblings, second trimester miscarriages and biochemical pregnancies were included. In a few instances, karyotype data were available, but not in the majority of cases. Ectopic pregnancies were not counted as miscarriages for probands or siblings. From 2005 to 2008, an additional five multicase families were included, applying the protocol indicated above. In one of these families there were two affected siblings. In the remaining four there was one sibling with two or more miscarriages. From these 31 families, blood samples from the proband and the affected sibling(s) were collected for DNA chip analysis.

All probands and siblings in multicase families were also HLA typed as part of a larger cohort, the results of which are previously published (Kolte *et al*., 2010).

Laboratory work

The DNA was extracted using a salting-out method, as described elsewhere (Miller *et al*., 1988). For the genome-wide linkage study we used the Affymetrix® GeneChip® 50K *Xba*I (Affymetrix, Santa Clara, CA, USA) platform, according to the manufacturer's instructions. Briefly: First, the DNA was digested for 2 h at 37°C with *Xba*I (New England BioLabs, Ipswich, MA, USA). After ligation with a 31-bp adaptor (Affymetrix), the DNA was amplified by one-primer PCR using Platinum *Pfx* polymerase (Invitrogen, Carlsbad, CA, USA). The amplicons were then fractionated to less than 180 bp by DNaseI (Affymetrix). We increased fragmentation time from 35 to 40 min. After labeling via TUNEL assay, the amplicons were hybridized to GeneChips (Affymetrix). The hybridized amplicons were visualized with streptavidin R-phycoerythrin (SAPE) (Invitrogen), biotinylated anti-streptavidin (Vector Labs, Burlingame, CA, USA) and SAPE. Finally the chips were scanned in an Affymetrix® GeneChip® scanner.

Data processing and statistics

The prevalence of miscarriage among the probands' siblings was evaluated using the Mantel Haenzel χ^2 test for linear association at a 95% significance level, inputted in SPSS. Only pregnancies where the woman's age was known were considered.

The SNPs were annotated using the following database: genome version: ucsc: hg18, ncbi build 36.1; snp version: dbsnp 126; annotation version: netaffx build 27. For genotype calling, the CRLMM algorithm for base calling was used as implemented in the 'oligo' R package. All CELL files were called in the same batch. In total, 58 959 SNPs were called. Of these 336 did not have annotation for either physical or genetic mapping position. Two SNPs mapped to the same position and thus a total of 338 SNPs were removed. Using the method of moments estimator from the 'plink' software, IBD sharing between individuals was determined. Based on this, one sample was declared misspecified and the family containing this sample was removed from further analysis. Analysis for sex misspecification and inbreeding showed no problems. Further SNP cleaning: 5404 SNPs had a minor allele frequency lower than 1% and were removed; 3563 SNPs had more than 5% missing data and were removed; 31 275 SNPs were in LD with adjacent markers ($r^2 > 0.1$ and LOD score >2) and were removed. The method we use for the linkage analysis is based on the assumption that there is no LD in the analyzed data. Therefore r^2 is set at a particularly low level of 0.1. A total of 18 379 SNPs remained after cleaning. Complying with the MIAME standards, raw data are available through the ArrayExpress Archive: http://www.ebi.ac.uk/arrayexpress/, accession number E-MEXP-2942.

Based on these SNPs, genome-wide non-parametric linkage analysis was performed using the S_{ALL} statistic (Whittemore and Halpern, 1994) as implemented in Merlin (Abecasis *et al*., 2002).

This was done both with all sibling pairs and with three different subsets of these: (i) sister pairs only (not including brothers), (ii) pairs containing probands with four or more miscarriages only and (iii) one sibling pair from each family only (randomly removing one of the siblings in all families with two affected siblings).

We also performed a relatedness analysis on the same data using the software package Relate (Albrechtsen *et al*., 2009) in order to look for founder mutations. Relate was run on all pairs of individuals between families assuming none of the individuals share more than one chromosome IBD and assuming that the rate of IBD state change along the genome is a function of the IBD sharing.

For both the linkage analyses and the relatedness analysis, we used genetic distances inferred from the Kong map (Kong *et al*., 2002).

Results

Epidemiological data

In 2005, former patients in the Danish Recurrent Miscarriage Clinic and their siblings were contacted for evaluation of reproductive history. Data were obtained from 244 patients with IRM (probands) and 268 siblings.

Frequency of miscarriage among the probands' siblings is 25.3 versus 13.1% in the general population (Nybo Andersen *et al*., 2000). Figure 1 illustrates that the increased risk among patients'
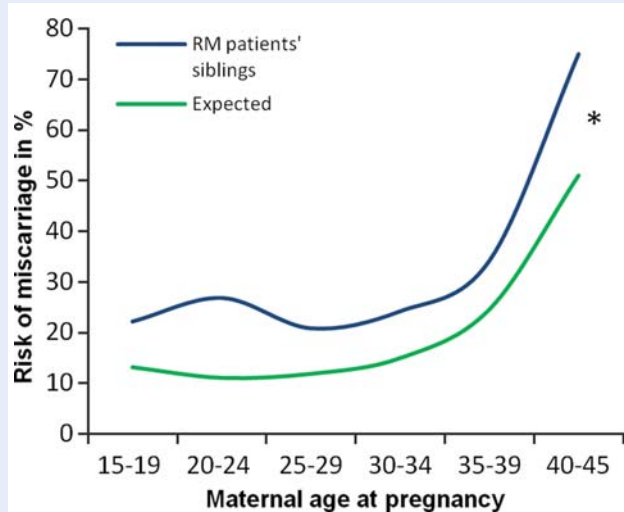
Figure 1 Risk of miscarriage among IRM patients' sisters and brothers' wives compared with the expected rate of miscarriage in the Danish population (34). *OR = 2.04, CI 1.4–3.0; P-value <0.001.

siblings is regardless of age at the time of pregnancy (OR = 2.04, confidence interval: 1.4–3.0; P-value <0.001).

Genome-wide linkage study

The families where a sister or brother's partner had had two or more miscarriages were included in the genetic part of the study. Thirty probands, 25 sisters and 9 brothers fulfilled the criteria, shown in Table I.

In a genome-wide linkage study using an SNP platform, the cut-off value for significance is usually set at LOD score 3.0 to compensate for multiple testing.

Applying a significance level of LOD score ≥3.0, no significantly linked markers were identified. However, in subanalyses, we did identify markers with LOD score >2.5 but <3.0, which suggests linkage. When studying sister pairs (28 pairs) exclusively, a LOD score of 2.9 was calculated for the marker rs10514716 (3p14.2). In sibling pairs where the proband had had four or more miscarriages (18 pairs), two peaks were found. Maximum LOD scores were found for rs10511668 (9p22.1) and rs341048 (11q13.4). When looking only at one sibling pair in each family (30 pairs), the highest LOD score was for rs10485275 (6q16.3) (see Table II for a description of the identified peaks in the subanalyses and Fig. 2 for an illustration

Table I The distribution of affected siblings and probands in the 30 families.

Number of families	Probands ≥3 miscarriages	Sisters ≥2 miscarriages	Brothers' spouses: ≥2 miscarriages	Sibling pairs
18	1	1	0	18
3	1	2	0	9
8	1	0	1	8
1	1	1	1	3

In total, 30 probands, 25 affected sisters and 9 brothers affected through their spouses were included, which gave 38 affected sibling pairs.

Table II Chromosomal areas with LOD score ≥2.5.

| Chromosomal position	Marker[a]	Physical position[b]	LOD score	P-value	Gene containing the SNP	Borders of linkage peak	Width[d]
Only sister pairs							
3p14.2	rs10514716	59 456 628	2.9	0.00012	FHIT[c]	Chr3: 58 166 473–60 246 217	208
Sibling pairs with proband ≥4 miscarriages							
9p22.1	rs10511668	19 012 470	2.6	0.0003	Hypothetical protein LOC 158297, part of FAM154A	Chr9:18 244 023–19 583 084	1339
11q13.4	rs341048	71 975 928	2.9	0.00013	PDE2A	Chr11:68,435,538–74 753 514	6317
One sibling pair per family							
6q16.3	rs10485275	102 272 746	2.5	0.0004	GRIK2	Chr6:102 022 335–104 005 966	1983

In the analysis of all 38 sibling pairs, no markers had an LOD score ≥2.5.
[a]The SNP with the highest LOD score.
[b]In base pairs.
[c]SNP located 254 kb centromeric to AUG in FHIT.
[d]In kilobase.

Figure 2 The four chromosomes in which peaks with LOD score >2.5 was identified in subgroups.
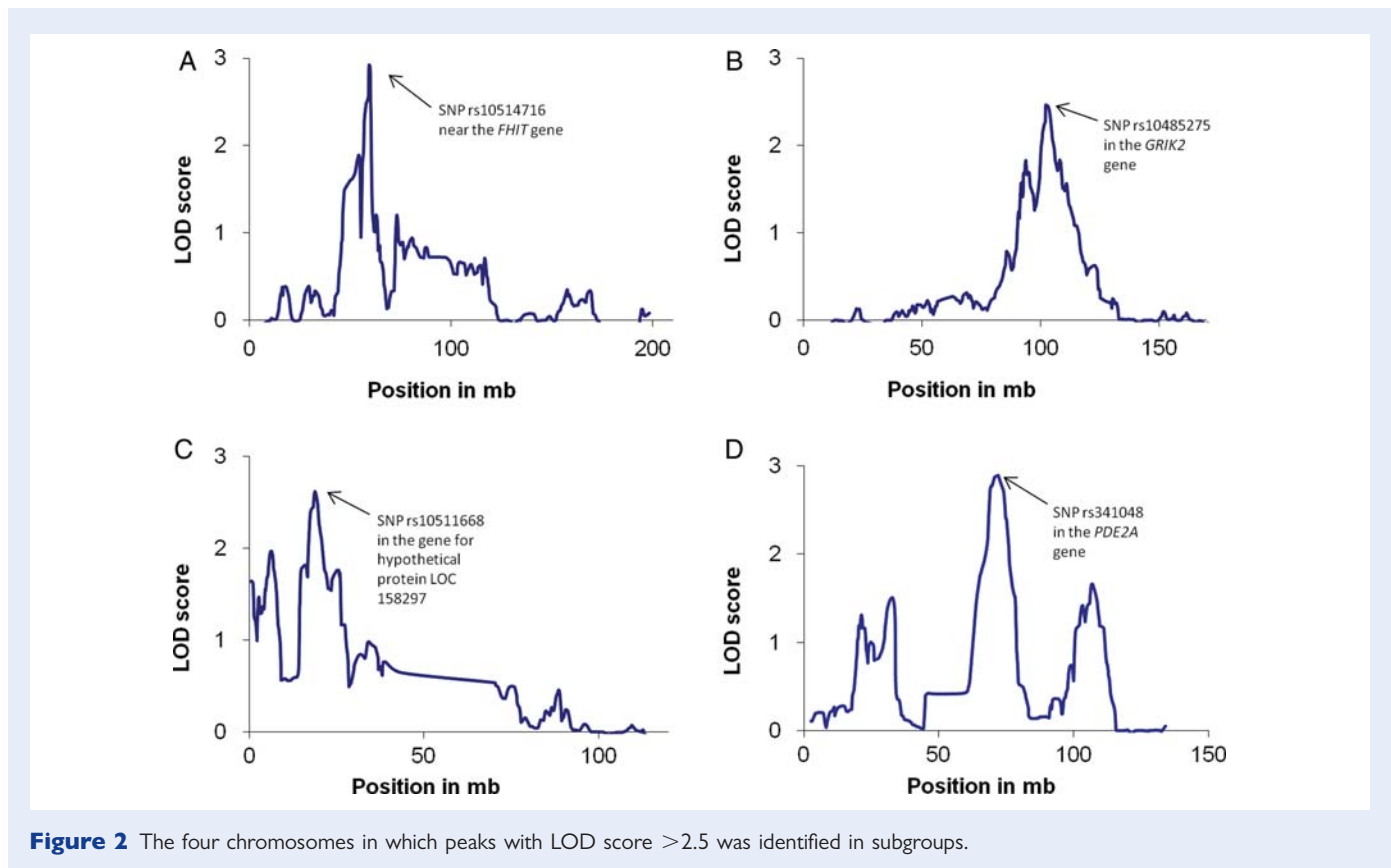
of the chromosomes in which we identified markers with LOD score >2.5). A full list of genes in the chromosomal areas which had an LOD score >2.5 in our analyses can be found via the UCSC genome browser at http://genome.ucsc.edu/cgi-bin/hgGateway.

We estimated the pairwise relatedness between families, but our results showed no sign of relatedness between families. Thus, no founder mutations were identified.

Discussion

The present study is the largest in which an investigation of reproductive outcome in siblings of patients with IRM has been carried out and furthermore it is the first-ever study that has carried out linkage analysis in affected sibling pairs with IRM using a genome-wide scan.

We confirm that siblings of IRM patients experience significantly higher numbers of miscarriages than the general population, and we demonstrate that this occurrence is irrespective of maternal age at the time of pregnancy. We hypothesized that some part may be genetic, and we conducted a genome-wide linkage study among 38 sibling pairs. This resulted in our identification of four genetic areas with suggestive linkage to IRM in subgroups of affected sibling pairs.

Interpretation of our results is limited by three factors.

First, there may have been a biased response rate in favor of families with affected siblings, which may tend to increase the reported miscarriage rate among siblings.

However, this would not affect the genetic part of the study as the comparisons of genetic markers were made within each family. By looking into our records obtained at the patients' first consultation (where the patient gave information about their siblings' reproductive history), we can confidently establish that the included multicase families can be considered representative for the whole IRM population, as the vast majority of multicase families have been included in the genetic part of the study.

Second, ascertainment bias may play a role, because families with two or more siblings with three or more miscarriages may preferentially be referred to our clinic. Thirteen of the 34 siblings in the genetic part of the study had had three or more miscarriages.

Finally, the present study has an inherent selection bias. If RM is indeed a condition for which genetic factors play a role it is likely that the patients' mothers also had reduced reproductive fitness. Women or couples carrying genetic variants that most severely affect reproductive fitness would therefore be expected to produce a limited number of children (one child or perhaps none). Such families would not be ascertained in the present study focusing on affected sibling pairs. This selection bias will thus result in an underestimation of the contribution of genetic variants to IRM. One could argue that miscarriage has positive effects on reproductive fitness as it prevents the birth of abnormal children and increases spacing between children, allowing the parents to improve physical and socioeconomic status. However, data from our group suggest decreased reproductive fitness due to repeated miscarriage, decreased fertility as a consequence of increasing age, salpingitis after surgical evacuation of miscarriage, abstinence from new pregnancy due to fear of miscarriage and dissolution of partnership as a consequence of stress and depression (unpublished data).

Our study is corroborated by a number of strengths. The study group is unique; the probands have been seen in the Danish RM clinic for periods of more than 20 years and as such represent the largest cohort of IRM patients worldwide to our knowledge. Furthermore, all probands had had three or more miscarriages and all probands were thoroughly investigated for known causes of RM, limiting our study group by the most rigorous criteria for IRM. Whenever possible, miscarriages reported by the participants were confirmed by hospital records. Finally, the Affymetrix GeneChip platform has been used in numerous large-scale association (Welcome Trust Case Control Consortium, 2007) and linkage studies (Romanos et al., 2008) can thus be considered as validated.

Epidemiology

The discovery that siblings of IRM patients have an increased frequency of miscarriage irrespective of age is important as it implies that the patient and her siblings share factors that increase susceptibility to pregnancy loss. Restricting the analysis to sisters only does not yield a significantly different result (data not shown). However, the number of reported pregnancies where the woman's age is unknown is higher in the group of brothers' wives, so caution should be taken when interpreting the results for this group. We compare the estimated risk of miscarriage among IRM patients' siblings with data from the Nybo Andersen study, which is based on information from the national discharge registry and the medical birth registry. Here, diagnoses at the time of discharge from a hospital are recorded. The data comprise information on maternal age and outcome for all pregnancies intended to be carried to birth in the period 1978–1992. Therefore, only miscarriages leading to hospital contact are registered here (Nybo Andersen et al., 2000). A possible bias is that we may overestimate risk of miscarriage among our patients as they may not have been admitted to a hospital following an early miscarriage. However, we estimate that only a minor number of the reported miscarriages in the group of probands' siblings have not led to a hospital contact as we have hospital documentation for the large majority. Furthermore, The Danish Recurrent Miscarriage Clinic is free of charge and women are referred to us from all over Denmark. Therefore, we have no reason to believe that our patients constitute a different socioeconomic group than the general population. Neither ultrasonic diagnosis of pregnancy nor sensitivity of urine or serum hCG has changed significantly between 1992 and 2008. Thus, we find the comparison between the estimated risk of miscarriage among our patients' siblings and the general population valid.

The hypothesis was that at least some of the factors leading to a familial clustering of miscarriages may be genetic. However, the impact of factors such as intrauterine environment, imprinting and epigenetic phenomena shared by siblings of the same mother as well as comparable early life circumstances have not, to our knowledge, been investigated in affected sibling pairs with IRM.

Genome-wide linkage study

In the linkage study based on the genome-wide scan of all affected sibling pairs, an LOD score >2.5 could not be identified in any region. Genetic variants connected with risk of miscarriage may only play a role when found in the maternal genome, e.g. genes of importance for maternal immune tolerance to the trophoblast or genes affecting maternal progesterone production. A linkage analysis restricted to affected women is therefore highly relevant. In the sub-analysis of females only (probands and sisters), the peak in 3p14.2 is not located within a gene. The nearest known gene is the FHIT gene located 254 kb telomeric from the SNP with maximum LOD score. The FHIT gene (Fragile Histidine Triad gene), a member of the histidine triad gene family, encodes a diadenosine 5′,5′′′-P1,P3-triphosphate hydrolase involved in purine metabolism. As the genomic region in which the FHIT gene located is unstable, we have checked for copy number variations and find no abnormalities; hence the peak is not due to technical difficulties. Aberrant transcripts of the FHIT gene have been found in about one-half of all gastro-intestinal cancers (Hassan et al., 2010). As FHIT has functions inducing apoptosis, one could speculate that aberrant expression may have an influence on for instance, trophoblast invasion and therefore risk of miscarriage, but as far as we know, this has not been investigated.

IRM patients with four or more miscarriages have a markedly increased risk of miscarrying in subsequent pregnancies and may constitute a separate disease entity (carrying more harmful genetic variants) compared with patients with fewer miscarriages (Christiansen et al., 1989). We identified two peaks when analyzing these probands and their affected siblings. First, in 9p22.1 the highest LOD score was observed for a SNP in the gene for hypothetical protein LOC 158297, a member of the FAM154A protein family. To our knowledge, this protein family has not been associated with specific functions or diseases. Second, linkage to an SNP in the PDE2A gene (11q13.4) was suggested by an LOD score of 2.9. The PDE2A (phosphodiesterase 2A, cGMP-stimulated isoform 1) protein hydrolyzes cAMP and cGMP. The gene is expressed in brain, heart, placenta, lung, skeletal muscle, kidney and pancreas.

We performed a third subgroup analysis in which we examined only one pair of affected siblings per family, removing a third affected sibling randomly to ensure independent observations. A peak was identified within the GRIK2 gene, suggesting linkage to 6q16.2. GRIK2 encodes the glutamate receptor, ionotropic, kainate 2. The protein functions as a ligand-activated ion channel. Mutations in the gene have been associated with autosomal recessive mental retardation (Motazacker et al., 2007). Affected sibling pair analysis has also linked GRIK2 with autism (Jamain et al., 2002). Variations in the gene have been associated with variations in age-of-onset of Huntington's disease (MacDonald et al., 1999) and the expression has been found to be down-regulated in patients with schizophrenia (Choi et al., 2009).

None of the above-mentioned genes and their products has previously been linked with IRM or other pregnancy complications, and none of the aforementioned SNPs generated an LOD score ≥3.0. Thus these loci are only suggestive, but may be candidate loci in further research.

We could not confirm the loci associated with IRM in the Li et al., 2010 study. There may be several reasons for this. The research population differs in several aspects; the cohort in the Li et al. study is of Han Chinese origin and the patients had had two or more miscarriages (no information on how many had three or more miscarriages). Additionally, their study is a case–control study and they used a technique of pooled DNA and 430 microsatellites (Li et al., 2010). Thus,

the Li *et al.* study differs from the present in multiple instances. Additionally, lack of confirmation of markers associated with a condition is a well-known problem to genome-wide linkage/association studies, e.g. large differences in genetic variants associated with type 2 diabetes even in the same ethnic groups have been reported (Christiansen *et al.*, 2009a).

We also did not find linkage to the *MBL2* gene on chromosome 10q11.2–q21 or the HLA region on chromosome 6p21.3, which have been linked to RM in previous studies (Christiansen *et al.*, 2009b; Kolte *et al.*, 2010). However, our previous studies have suggested that the impact on RM risk of proposed susceptibility HLA alleles is complex. HLA class II alleles restricting immunity against male-specific minor histocompatibility (HY) antigens only impact pregnancy outcome negatively in RM patients with a firstborn boy (Nielsen *et al.*, 2009). There is also some evidence that a 14 base-pair insertion in exon 8 of the HLA-G gene may only confer susceptibility to RM when inherited from the patient's mother (Kolte *et al.*, 2010). Furthermore, the HLA class II alleles and HLA-G variants suggested to confer susceptibility to RM are frequent and are found in >50% of the population. This complexity obviously makes it more difficult to establish significant linkage to the HLA region in a sibling pair study of limited size.

The lack of significantly linked markers can be explained by several mechanisms. First, the number of included families may be too small to generate sufficient statistical power. Second, IRM may have a polygenic inheritance pattern with relatively low size of effect of each causal variant. In a study like ours, susceptibility genes with low penetrance would not be identified. If this is the case, large, population-based studies of IRM patients and unrelated controls would be necessary, similar to what we have seen for type 2 diabetes in recent years. However, as IRM is a relatively rare condition, it does not seem feasible except in a large collaborative setting, where discrepancies in marker prevalence across different ethnicities would limit interpretation.

Third, the genetic contribution to IRM may be monogenic, but with a strong genetic heterogeneity. In this case, IRM is monogenic in individual families, but the susceptibility genes are different from family to family. It cannot be ruled out with our study design. Conversely, analysis of large pedigrees, including several generations and more distant relations, could contribute to the investigation of such a hypothesis.

Conclusions

This is the first genome-wide linkage study of affected sibling pairs with IRM. We have demonstrated that siblings of IRM patients have a higher risk of miscarriage and that this susceptibility is regardless of age at pregnancy. We did not observe LOD scores >3.0, which may be due to polygenic inheritance, genetic heterogeneity, insufficient power or lack of genetic contribution to IRM etiology. However, we identified SNPs with LOD scores very close to 3.0, thus pinpointing genetic areas which warrant further research in order to confirm or refute linkage between IRM and these genetic regions. We warmly welcome replication studies and envision a repetition of this study including more families in the future. Should linkage be confirmed, fine mapping of these chromosomal areas might provide further insight in the pathogenesis of IRM.

Ethics

The Regional Ethics Committee for Frederiksberg and Copenhagen approved the study in accordance with Danish legislation (approval number KF-01-104/04). Oral and written consent was obtained from all participants.

Authors' roles

Funding

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