Human Reproduction, Vol.30, No.1 pp. 232–238, 2015
Advanced Access publication on November 5, 2014 doi:10.1093/humrep/deu296

human reproduction

ORIGINAL ARTICLE ***Reproductive genetics***

No evidence for mutations in *NLRP7*, *NLRP2* or *KHDC3L* in women with unexplained recurrent pregnancy loss or infertility

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Submitted on May 28, 2014; resubmitted on September 4, 2014; accepted on September 10, 2014

STUDY QUESTION: Are mutations in *NLRP2/7 (NACHT, LRR and PYD domains-containing protein 2/7)* or *KHDC3L (KH Domain Containing 3 Like)* associated with recurrent pregnancy loss (RPL) or infertility?

SUMMARY ANSWER: We found no evidence for mutations in *NLRP2/7* or *KHDC3L* in unexplained RPL or infertility.

WHAT IS KNOWN ALREADY: Mutations in *NLRP7* and *KHDC3L* are known to cause biparental hydatidiform moles (BiHMs), a rare form of pregnancy loss. *NLRP2*, while not associated with the BiHM pathology, is known to cause recurrent Beckwith Weidemann Syndrome (BWS).

STUDY DESIGN, SIZE, AND DURATION: Ninety-four patients with well characterized, unexplained infertility were recruited over a 9-year period from three IVF clinics in Sweden. Blood samples from 24 patients with 3 or more consecutive miscarriages of unknown etiology were provided by the Recurrent Miscarriage Clinic at St Mary's Hospital, London, UK.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Patients were recruited into both cohorts following extensive clinical studies. Genomic DNA was isolated from peripheral blood and subject to Sanger sequencing of *NLRP2*, *NLRP7* and *KHDC3L*. Sequence electropherograms were analyzed by Sequencher v5.0 software and variants compared with those observed in the 1000 Genomes, single nucleotide polymorphism database (dbSNP) and HapMap databases. Functional effects of non-synonymous variants were predicted using Polyphen-2 and sorting intolerant from tolerant (SIFT).

MAIN RESULTS AND THE ROLE OF CHANCE: No disease-causing mutations were identified in NLRP2, NLRP7 and KHDC3L in our cohorts of unexplained infertility and RPL.

LIMITATIONS, REASONS FOR CAUTION: Due to the limited patient size, it is difficult to conclude if the low frequency single nucleotide polymorphisms observed in the present study are causative of the phenotype. The design of the present study therefore is only capable of detecting highly penetrant mutations.

WIDER IMPLICATIONS OF THE FINDINGS: The present study supports the hypothesis that mutations in *NLRP7* and *KHDC3L* are specific for the BiHM phenotype and do not play a role in other adverse reproductive outcomes. Furthermore, to date, mutations in *NLRP2* have only been associated with the imprinting disorder BWS in offspring and there is no evidence for a role in molar pregnancies, RPL or unexplained infertility.

† The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors and are listed in alphabetical order.

STUDY FUNDING/COMPETING INTEREST(S): This study was funded by the following sources: Estonian Ministry of Education and Research (Grant SF0180044s09), Enterprise Estonia (Grant EU30020); Mentored Resident research project (Department of Obstetrics and Gynecology, Baylor College of Medicine); Imperial NIHR Biomedical Research Centre; Grant Number C06RR029965 from the National Center for Research Resources (NCCR; NIH). No competing interests declared.

Key words: recurrent pregnancy loss / unexplained infertility / *NLRP2* / *NLRP7* / *KHDC3L*

Introduction

Infertility and recurrent pregnancy loss (RPL) are important reproductive phenotypes of multifactorial origin that affect up to 30% and 5–15% of couples of reproductive age attempting a pregnancy, respectively (Rai and Regan, 2006; Boivin *et al.*, 2007; Smith *et al.*, 2011). Maternal comorbidities along with maternal and fetal genetic factors play a role in the etiology of these conditions, but despite extensive research, a cause cannot be identified in up to 13% (SART, 2010) of cases with unexplained infertility and 40–50% (Allison and Schust, 2009) with RPL. Previous human studies, as well as animal models with reproductive phenotypes suggest that numerous genes with high-impact mutations may contribute to a small number of cases, supporting the evaluation of plausible candidate genes in cohorts of affected patients (Carp *et al.*, 2001; Suzumori and Sugiura-Ogasawara, 2010; Su *et al.*, 2011).

Infertility is defined as the inability to conceive within 1 year of unprotected regular intercourse whereas a diagnosis of unexplained infertility is made when no cause is found despite a complete workup with documented evidence of ovulation, adequate ovarian reserve, patent Fallopian tubes, normal semen analysis and no evidence of endometriosis or pelvic adhesions (Jerome and Strauss, 2009). The American College of Obstetrics and Gynecology (ACOG) defines RPL as two or more repetitive pregnancy losses prior to 20 weeks of gestation, while the Royal College of Obstetricians and Gynecologists defines RPL as the loss of three or more consecutive pregnancies prior to viability, i.e. 24 weeks of gestation. Similar to infertility, causes can be multiple, including maternal and placental factors as well as genetic defects in the mother or fetus. Accordingly, several genes have been causally associated with RPL (Daniely *et al.*, 2001; Suzumori and Sugiura-Ogasawara, 2010; Daher *et al.*, 2012).

One specific form of pregnancy loss, hydatidiform mole (HM) is an abnormal human pregnancy characterized by cystic degeneration of chorionic villi and trophoblastic hyperplasia. Complete HM are usually sporadic and androgenetic (AnCHM), but rare forms of CHM are biparentally inherited (BiHM) and can be a cause of RPL in affected women (Murdoch *et al.*, 2006; Kou *et al.*, 2008; Wang *et al.*, 2009). Most affected women with recurrent BiHM have been found to have mutations of *NLRP7 (NACHT, LRR and PYD domains-containing protein 7) or KHDC3L (KH Domain Containing 3 Like)*. The loss of methylation phenotype observed in BiHM tissues is suggestive of a role for NLRP7, and possibly KHDC3L, in reprogramming imprints in the maternal germline, believed to be relevant to the pathogenesis of BiHM (Judson *et al.*, 2002; Murdoch *et al.*, 2006; Parry *et al.*, 2011).

It has recently been reported that heterozygous mutations in *NLRP7* might be associated with other forms of reproductive failure, such as recurrent miscarriage and sporadic HM (Messaed *et al.*, 2011). However, given the specificity of the imprinting defects seen in BiHM tissues, the association of *NLRP7* with other forms of adverse reproductive outcomes is difficult to reconcile, and subsequent reports have thus far not been able to confirm these findings (Andreasen *et al.*, 2012, 2013; Dixon *et al.*, 2012; Mahadevan *et al.*, 2013; Manokhina *et al.*, 2013). In contrast, given the observation that NLRP7 and KHDC3L may be important for proper switching of imprinting marks in the maternal germline, these genes could be associated with unexplained apparent infertility, if the clinical presentation of preimplantation developmental arrest of embryos resulted from disruption of DNA methylation at imprinted or non-imprinted loci.

To address the hypothesis that mutations in *NLRP7* and *KHDC3L* may cause infertility, we sequenced *NLRP7* and *KHDC3L* in DNA from 94 women with extensively characterized unexplained infertility. To explore the current controversy about the association of mutations in these genes with RPL, we also performed mutational analysis in DNA of 24 women with RPL.

Finally, since *NLRP7* is believed to have evolved from *NLRP2 (NACHT, LRR and PYD domains-containing protein 2)* (Tian *et al.*, 2009a,b), a maternal effect gene associated with Beckwith Weidemann Syndrome (BWS) (Meyer *et al.*, 2009), and a recent report has suggested an association between *NLRP2* and recurrent miscarriages (Huang *et al.*, 2013) we have also sequenced *NLRP2* in both cohorts.

Materials and Methods

Study subjects: unexplained infertility

This study group included 94 women with a diagnosis of primary unexplained infertility. All women were Swedish or Finnish in origin. Blood samples were collected following written informed consent in three IVF clinics in the Department of Obstetrics and Gynecology, Karolinska University Hospital Huddinge; Fertility Center Scandinavia, Stockholm and Department of Women's and Children's Health, Uppsala University Hospital, Sweden between 2000 and 2009. Unexplained infertility was diagnosed after confirmed ovulation, normal ovarian function and normal serum levels of FSH, prolactin, thyroid-stimulating hormone and thyroid hormone. At least two analyses of the partner's semen were shown to be normal according to the World Health Organization diagnostic criteria (World Health Organisation, 1999). Furthermore, all infertile women showed patent tubes on hysterosonosalpingogram, and no endometriosis was identified by clinical evaluation, ultrasonography or diagnostic laparoscopy. The mean age was 33.0 + 3.5 years, BMI was 22.9 + 3.6 kg/m^2. All subjects had regular menstrual cycles with mean cycle length of 28.5 + 2.1 days and duration of menstrual flow of 4.7 + 0.8 days. None of the patients has been pregnant before, and had not used any hormonal medication at least 3 months prior to sampling.

The Ethical Review Boards at Karolinska Institutet, Uppsala University and Baylor College of Medicine approved the study. Peripheral blood samples were collected in EDTA tubes. Genomic DNA was extracted from blood using QIAamp DNA Blood Maxi kits according to manufacturer's instructions (Qiagen, Venlo, The Netherlands).

Study subjects: RPL

This study group included 24 women with a mean of 4.7 (range 3–10) miscarriages of unknown etiology referred to the Recurrent Miscarriage Clinic at St Mary's Hospital, London. Twenty-two women were of Caucasian origin and two Asian. Blood samples were collected after informed consent. Peripheral blood samples were collected in EDTA tubes. Genomic DNA was extracted from blood using QIAamp DNA Blood Mini kits according to manufacturer's instructions (Qiagen, UK). This study was approved by the local research ethics committee of St Mary's Hospital NHS Trust, London, UK.

Sequencing of *NLRP2*, *NLRP7* and *KHDC3L*

Primers used to sequence the coding exons of the genes *NLRP7* and *KHDC3L* were those that have been used previously (Wang *et al.*, 2009; Mahadevan *et al.*, 2013). Primers used to sequence *NLRP2* are provided in Table I. PCR was performed on 20–50 ng of genomic DNA under standard conditions. Purified PCR products were sequenced in the forward orientation at Beckman Coulter Genomics (Danver, MA, USA and UK). Confirmation of mutations was carried out on repeated PCR reactions in both directions. Sequence electropherograms were analyzed using Sequencher v5.0 software. Sequence variations were compared with those in the 1000 Genomes, single nucleotide polymorphism database (dbSNP) and HapMap public databases. The predicted functional effects of novel non-synonymous single nucleotide polymorphisms (SNPs) were evaluated using PolyPhen-2 and sorting intolerant from tolerant (SIFT). Minor allele frequencies (MAFs) were calculated for non-synonymous variants in our cohorts and compared with those reported in dbSNP. MAFs are expressed as frequencies with which SNPs are observed, where a heterozygous SNP was counted once and a homozygous SNP was counted twice as per MAF nomenclature.

Results

Sequence analysis of the coding exons and exon–intron boundaries of *NLRP2, NLRP7* and *KHDC3L* revealed no putative disease-causing mutations in either patient cohort.

Unexplained infertility cohort

Sequencing of *NLRP2* revealed several SNPs, most of which were present in dbSNP and had MAF scores available. One SNP in exon 4 seen in a heterozygous state in a single patient causing a c.361G>A variation changed amino acid 121 from Aspartic acid to Alanine (p.Asp121Ala). This SNP was not previously reported in dbSNP and was predicted by PolyPhen-2 to be benign and by SIFT to be tolerated. Seven other SNPs (rs142463014, rs150097185, rs148817929, rs142528551, rs189403101, rs61735082, rs61735083) were present in dbSNP but did not have MAF scores associated with them. However, given the low frequency with which we observed these SNPs in our study group, we speculate that they are unlikely to make a major contribution to infertility (Table II). More studies of ethnically matched controls will be necessary to establish or rule out causality.

Sequencing of *NLRP7* also revealed several SNPs which have been reported previously in other studies across various ethnicities. We found one novel SNP, p.Met427Leu, which was predicted by SIFT to be damaging (score 0.02) but by Polyphen-2 to be benign (score 0.007). We observed this SNP in only one patient and in the heterozygous state. Four SNPs (rs149175257, rs1654634, rs61745087, rs104895545) were present in dbSNP without associated MAF scores (Table III). Similar to the SNPs observed in *NLRP2*, since we observed these SNPs at a very low frequency in our study group, we speculated that they are unlikely to be predictive or causative of infertility.

Sequencing of *KHDC3L* revealed three known SNPs, all of which were at frequencies comparable with those reported in dbSNP except for rs144291287, which had no associated MAF score (Table IV).

RPL cohort

Sequencing of *NLRP2* in this cohort also yielded known SNPs comparable with reported frequencies (Table II). Two SNPs did not have documented MAF scores. rs149735961 (c.1010G>A; p.Arg337Gln) was predicted by both PolyPhen-2 and SIFT to be damaging (SIFT score 0.04 and PolyPhen-2 score 0.989) and this SNP was seen in a heterozygous state in a single patient. rs61735082 (c.1480A>G; p.Thr494Ala) and rs61735083 (c.1499A>G; p.Glu500Gly) were both predicted by PolyPhen-2 and SIFT to be benign changes.

No novel mutations were identified in the RPL cohort in either of the two genes implicated in familial HM, *NLRP7* or *KHDC3L*. Sequencing of these genes identified a number of previously described polymorphisms in both genes, at frequencies comparable with published databases (Tables III and IV).

Table I Primer sequences for *NLRP2 (NACHT, LRR and PYD domains-containing protein 2)*.

Exon	Forward primer 5′–3′	Reverse primer 5′–3′	Tm	Product (bp)
2	agcaggaactggcatttgag	gcctggccttctgaatttct	60°C	451
3	tgaaggcaataaaatcttgagc	tgctaagtccggcatttctt	60°C	223
4	gactcaggggtccaacttga	ttggagagagatggggttctt	60°C	250
5	atcagcctgcctccttttct	tgctttgtgttacataggaaagtt	60°C	244
6	ggccaacacaaggcattt	tgggatgaggctaaagatgg	60°C	1704
7	tgatgcttcttgggtgttga	ctctcaattccctgtgtctcg	60°C	463
8	gcccctggtttccatttaag	tcctcacgtatggttgtcca	60°C	441
9, 10	ctgcatccaacattagagtcag	gcatcatgcttggaactttt	60°C	1387
11	aactcacaggttcgggtttg	aaccatcctagtaaatgctcgat	60°C	360
12	cagatccccaacacacgag	agaaattggctgggtgtga	60°C	400
13	ccctgtgcctccttaacaga	cagaggcagaatcatggcta	60°C	383

Table II Non-synonymous variants identified in *NLRP2* in cases of primary infertility or RPL.

Exon	Nucleotide change	Protein change	dbSNP		MAF Score (Minor Allele)	
			ID	MAF (Minor Allele)	Unexplained infertility	RPL
2	c.11C>T	p.S4L	rs142463014	Unavailable	0.005(T)	0.0208(T)
2	c.15G>A	p.A5A	rs269912	0.122(A)	0.191(A)	0.1875(A)
2	c.175C>T	p.L59F	rs150097185	Unavailable	0.005(T)	—
3	c.312G>A	p.K104K	rs2217659	0.214(A)	0.154(A)	0.1875(A)
4	c.361G>A	p.D121A	Novel	Unavailable	0.005(A)	—
6	c.519C>T	p.F172F	rs10403648	0.145(T)	0.042(T)	0.0417(T)
6	c.596C>T	p.T198M	rs17699678	0.061(T)	0.313(T)	0.1041(T)
6	c.715C>T	p.L239L	rs56073572	0.047(T)	0.138(T)	0.1041(T)
6	c.979G>C	p.E327Q	rs148817929	Unavailable	0.010(C)	—
6	c.982G>C	p.E328Q	rs142528551	Unavailable	0.010(C)	—
6	c.1010G>A	p.R337Q	rs149735961	Unavailable	0.0157(A)	0.0208(A)
6	c.1009T>C	p.F337L	rs62124644	0.005(C)	0.005(C)	0.0208(C)
6	c.1056C>T	p.D352D	rs3826883	0.144(T)	0.207(C)	0.2291(C)
6	c.1072C>T	p.R358C	rs189403101	Unavailable	0.005(T)	—
6	c.1480A>G	p.T494A	rs61735082	Unavailable	0.015(G)	0.0208(G)
6	c.1500A>G	p.E500E	rs3745905	0.149(G)	0.047(G)	0.0208(G)
6	c.1499A>G	p.E500G	rs61735083	Unavailable	0.015	0.0625
6	c.1506T>C	p.D502D	rs61735084	0.009(C)	0.015(C)	0.0208(C)
6	c.1519A>G	p.T507A	rs34804158	0.160(G)	0.212(G)	0.25(G)
6	c.1608T>C	p.F536F	rs10412915	0.306(C)	0.313(C)	0.3125(C)
6	c.1749C>G	p.L583L	rs11672113	0.293(G)	0.382(G)	0.375(G)
7	c.2044C>T	p.L704L	rs61733928	0.018(T)	0.026(T)	—
13	c.2994C>A	p.I1020I	rs12768	0.442(C)	0.558(C)	0.5208(C)
13	c.3089C>A	p.A1052E	rs1043673	0.359(A)	0.367(A)	0.3958(A)

SNP not found in the study group.
MAF, minor allele frequency.

Discussion

We analyzed women with unexplained infertility and women with RPL for mutations in *NLRP2*, *NLRP7* and *KHDC3L* and found no disease-causing mutations in these genes in our study cohorts. This observation is in agreement with our hypothesis that mutations in *NLRP2*, *NLRP7* and *KHDC3L* are unlikely to be causative of RPL. These studies also show that, at least in this cohort of 94 patients, they are not associated with unexplained infertility. These data thus indicate that mutations in *NLRP7* and *KHDC3L* are primarily associated with the recurrent, biparentally inherited HMs. In addition, our current results, as well as those of other studies (Andreasen *et al*., 2012) have thus far not revealed an association of *NLRP2* with any of these conditions.

The mutational spectrum of *NLRP7* in the context of HM has been tested in various ethnic groups (Kou *et al*., 2008; Messaed *et al*., 2011; Muhlstein *et al*., 2011; Qian *et al*., 2011; Dixon *et al*., 2012; Landolsi *et al*., 2012; Estrada *et al*., 2013). There have been several reports suggesting an immunological role for *NLRP7* in the etiology of adverse reproductive outcomes such as miscarriage, AnCHM, spontaneous abortion, etc. (Qian *et al*., 2007, 2011; Slim *et al*., 2011; Reddy *et al*., 2012). These attempts were rationalized by the knowledge that NLRP7 is a member of the NLRP family of proteins which have been found to have a role in innate immunity and apoptosis (Kufer and Sansonetti, 2011; Radian *et al*., 2013). This has been used to support the hypothesis that mutations in *NLRP7* cause BiHM because its loss of function creates a sub-optimal uterine environment that fails to reject the abnormal pregnancy. However, given the spatio-temporal patterns of expression of NLRP7 (Zhang *et al*., 2008) in the female germ line and early embryo and the fact that imprint reprogramming occurs in the germ line and early embryo, our alternate hypothesis proposes a more direct role for NLRP7 in the process of imprint acquisition and/or maintenance. More recent research in an *in vitro* model provides the first concrete evidence that loss of NLRP7 affects trophoblast differentiation and genome-wide DNA methylation (Mahadevan *et al*., 2014). Furthermore, although the function of KHDC3L has not yet been defined, there is no evidence for a potential role in immune defense and it also belongs to a family of proteins with documented preferential expression and function in the female germ line and early embryo (Pierre *et al*., 2007; Tian *et al*., 2009a,b; Tashiro *et al*., 2010). We therefore hypothesize that NLRP7 and KHDC3L perform similar, potentially overlapping functions in reprogramming of maternal imprinting marks.

Extensive research is also being performed toward identification of biomarkers and therapeutic targets for unexplained infertility, with

Table III Non-synonymous variants identified in *NLRP7* in cases of primary infertility or RPL—SNP not found in the study group.

Exon	Nucleotide change	Protein change	dbSNP		MAF Score (Minor Allele)	
			ID	MAF (Minor Allele)	Unexplained infertility	RPL
2	c.66A>G	p.L22L	rs149175257	Unavailable	0.005(G)	—
4	c.390G>A	p. Q130Q	rs775883	0.373(A)	0.297(A)	0.33(A)
4	c.1137G>C/T	p.K379K	rs10418277	0.191(T)	0.196(C)	0.187(C)
4	c.1725G>T	p.L575L	rs73055288	0.019(A)	0.047(A)	—
4	c.467G>A	p.R156Q	rs61746625	0.001(A)	0.010(A)	—
4	c.1279A>T	p.M427L	Novel	Unavailable	0.005(T)	—
4	c.1288T>C	p.F430L	rs1654634	Unavailable	0.005(C)	—
4	c.1290C>T	p.F430F	rs61745087	Unavailable	0.005(T)	—
4	c.1302C>T	p.D434D	rs104895545	Unavailable	0.005(T)	—
4	c.1441G>A	p.A481T	rs61747414	0.101(A)	0.090(A)	0.1041(A)
4	c.1104T>C	p.I368I	rs1654636	0.027(C)	0.005(C)	—
4	c.574A>C	p.M192L	rs104895529	0.000(C)	0.005(C)	—
4	c.929A>G	p.Q310R	rs77812009	0.009(G)	—	0.0208(G)
4	c.931C>A	p.L311I	rs79513034	0.009(A)	0.015(A)	0.0208(A)
4	c.955G>A	p.V319I	rs775882	0.278(A)	0.19(A)	0.2083(A)
4	c.1104T>C	p.I368I	rs1654636	0.028(C)	—	0.0208(C)
4	c.1460G>A	p.G487E	rs775881	0.115(A)	0.02(A)	0.0416(A)
4	c.1491C>T	p.I497I	rs775880	0.083(T)	0.026(T)	0.0416(T)
4	c.1532A>G	p.K511R	rs61743949	0.025(G)	0.005(G)	0.0208(G)
4	c.1725G>T	p.L575L	rs73055288	0.025(T)	0.04(T)	0.0208(T)
6	c.2156C>T	p. A719V	rs104895526	<0.001(T)	0.005(T)	0.0208(T)
7	c.2444G>A	p.R815H	rs150034626	0.001(A)	0.005(A)	—
9	c.2682T>C	p. Y894Y	rs269951	0.425(T)	0.468(T)	0.4583(T)
9	c.2775A>G	p.A925A	rs269950	0.425(A)	0.468(G)	0.4583(G)

Table IV Non-synonymous variants identified in *KHDC3L (KH Domain Containing 3 Like)* in cases of primary infertility or RPL—SNP not found in the study group.

Exon	Nucleotide change	Protein change	dbSNP		MAF Score (Minor Allele)
			ID	MAF (Minor Allele)	Unexplained infertility
RPL					
1	c.14G>A 0.0106(A)	p.R5K —		rs144291287	Unavailable

Continued

different studies focusing on ovary and oocyte quality, or on endometrial receptivity and the implantation process (Horcajadas *et al.*, 2007; Aghajanova *et al.*, 2008; Altmae *et al.*, 2010). Several groups have been working on identifying genetic polymorphisms in candidate genes in women with unexplained infertility. It has been suggested that polymorphisms in the genes encoding aromatase (*CYP19A1*), hyaluronan-binding protein 2 and tissue factor pathway inhibitor could influence their levels of expression in receptive endometrium (Altmae *et al.*, 2009, 2011a,b). Associations of polymorphisms in genes encoding anti-Müllerian hormone and its receptor, estrogen receptor alpha and leukemia inhibitory factor with unexplained infertility have also been reported (Steck *et al.*, 2004; Rigon *et al.*, 2010; Du *et al.*, 2011). Many association studies have been performed in a search for molecular markers for recurrent miscarriage. Although there is some inconsistency in the results, polymorphisms in genes involved in inflammation, thrombosis and the cardiovascular system, the detoxification system, immune response, hormonal regulation and placental function have been shown to associate with a risk for RPL (Su *et al.*, 2011; Rull *et al.*, 2012).

In sequencing *NLRP2* in the unexplained infertility cohort, we observed eight SNPs, seven of which were reported in dbSNP but did not have associated MAF scores. Given the low frequency with which we observed these SNPs and the fact that they were all noted in the heterozygous state, we speculated that they are unlikely to be causal or predictive of the infertility of unknown etiology. Similarly in the RPL cohort, two of all the observed SNPs, did not have an associated MAF score and p.Arg337Gln was the only SNP observed in this cohort which was predicted to be possibly damaging to the function of the protein, as predicted by both Polyphen-2 and SIFT.

Sequencing of *NLRP7* in the unexplained infertility cohort revealed 1 novel SNP, p.Met427Leu which has not been described in dbSNP. The potential effect of this SNP on the function of *NLRP7* had discordant predictions from two commonly used algorithms. Polyphen-2 predicted that the SNP would be benign while SIFT predicted that the SNP would be damaging to the function of the protein. Additionally, four SNPs present in dbSNP but without associated MAF scores were observed, and all four of these were found in a single patient and in a heterozygous state. Similar to the speculations made for the SNPs seen in NLRP2, we concluded that the SNPs observed in NLRP7 are also unlikely to be causal or predictive of the phenotype. We observed a single patient each with a heterozygous p.Ala719Val change in both the infertility and RPL cohorts. This change has been noted in previous studies where it was suggested to be causative of the pathology (Qian *et al.*, 2011; Slim *et al.*, 2011). Whether this change could be causative of the phenotypes described in the present study is difficult to determine due to the size of the study. Our data and that of others only exclude the involvement of highly penetrant mutations.

Sequencing of *KHDC3L* in the unexplained infertility cohort did not reveal any novel SNPs or any changes that did not have comparable MAF scores to the control population, based on the scores provided by dbSNP. Identical results, with a lack of any potential disease-causing variants were found in the RPL cohort.

In conclusion, we did not find mutations in *NLRP2*, *NLRP7* and *KHDC3L* in women with RPL or unexplained infertility. This supports the hypothesis that mutations in *NLRP7* and *KHDC3L* are specific for the BiHM phenotype and do not play a role in other adverse reproductive outcomes. Furthermore, to date, mutations in *NLRP2* have only been associated with the imprinting disorder BWS in offspring and there is no evidence for a role in molar pregnancies, RPL or unexplained infertility.

Authors' roles

S.A. and A.S.-E. were involved in collection of samples for the Infertility cohort. L.R., N.S., R.A.F. and P.D. were involved in collection of samples for the Recurrent Miscarriage cohort and performing sequencing of *NLRP7* and *KHDC3L* in the same. L.A. and S.M. were involved in the sequencing of *NLRP7*, *NLRP2* and *KHDC3L* in the Infertility and *NLRP2* in the Recurrent Miscarriage cohorts. All authors contributed to drafting and editing the manuscript. I.B.V.V. was responsible for project design, oversight of all experiments, interpretation and final manuscript preparation.

Funding

This study was funded by the following sources: Estonian Ministry of Education and Research (Grant SF0180044s09), Enterprise Estonia (Grant EU30020); Mentored Resident research project (Department of Obstetrics and Gynecology, Baylor College of Medicine); Imperial NIHR Biomedical Research Centre; Grant Number C06RR029965 from the National Center for Research Resources (NCCR; NIH).

Conflict of interest

None to declare.

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