Maternal NLRP7 and C6orf221 variants are not a common risk factor for androgenetic moles, triploidy and recurrent miscarriage

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ABSTRACT: Maternal effect genes control early events of embryogenesis. Maternal homozygous and compound mutations in two such genes, NLRP7 and c6orf221, have been detected in the majority of women experiencing recurrent biparental hydatidiform moles. It was suggested that other forms of reproductive wastage, including diploid androgenetic moles, partial moles, polyploidy, recurrent spontaneous abortions and stillbirths of uncertain etiology, may be caused by NLRP7 or c6orf221 mutations in the mother. To elucidate which subpopulations of women with adverse reproductive outcomes should be screened for NLRP7/C6orf221 variants, we sequenced coding sequence and exon/intron boundaries of NLRP7 and C6orf221 in a well-defined group of 17 women with recurrent miscarriage and additional triploidy or complete hydatidiform moles. The major findings for this group were non-synonymous variants of NLRP7, rather than clearly pathogenic mutations. To assess the role of these variants, we genotyped them in a larger group including women with primary recurrent miscarriage (n = 39), paternal triploid conceptions (n = 22) and women with proven fertility after age 37 and no prior history of miscarriage or pregnancy complications (n = 52). No associations between non-synonymous NLRP7 variants and primary recurrent miscarriage or partial hydatidiform molar pregnancies were detected. Our findings suggest that neither mutations nor variants in NLRP7 and C6orf221 are major factors contributing to the risk of these types of pregnancy complications. Further studies in larger groups of patients and controls are needed to specify the impact of NLRP7 rare non-synonymous variants on genetic susceptibility to recurrent reproductive wastage.

Key words: complete hydatidiform mole / recurrent miscarriage / triploidy / NLRP7 / C6orf221

Introduction

Hydatidiform mole is an abnormal human pregnancy characterized by impaired embryonic development and hyperplasia of the placental trophoblast, typically due to excess gene expression from the paternal genome (Kajii and Ohama, 1977). Complete hydatidiform moles (CHMs) are characterized by the absence of an embryo and mostly are diandric diploid (Lage et al., 1992). Partial hydatidiform moles (PHMs), which are almost always diandric triploid (McFadden and Langlois, 2000), show restricted embryonic and fetal development, largely as a secondary consequence of abnormal placental growth (McFadden and Robinson, 2006). CHM is regarded as a predominantly sporadic condition with about 1% recurrence risk (Berkowitz et al., 1998). A portion of the recurrent cases is familial, has diploid biparental origin (FBiHM) and displays abnormal expression of some maternally imprinted genes (Judson et al., 2002; El-Maarri et al., 2003). The development of recurrent biparental moles has been previously associated with maternal homozygous and compound mutations in NLRP7 or C6orf221 maternal effect genes (Murdoch et al., 2006; Parry et al., 2011). Maternal effect genes are those from maternal genome that are expressed in the oocyte during oogenesis and are needed prior to the onset of gene activation in the embryo, and thus their expression is essential for normal embryo development. It is suggested that NLRP7 and C6orf221 act during human oogenesis and/or early embryogenesis, controlling oocyte maturation, early embryo cleavage and the establishment of primary maternal epigenetic marks (Deveault et al., 2009; Zheng and Dean, 2009). There is a growing body of evidence that NLRP7 and C6orf221 may be involved in a diversity of pregnancy complications in addition to FBiHM (Deveault et al., 2009; Puechbert et al., 2009; Messaed...
et al., 2011b; Slim et al., 2011). Several studies have established the association between the degree to which NLRP7 variants affect gene function and the severity of placental phenotype. Specifically, biallelic protein-truncating mutations in NLRP7 and C6orf221 were found in some women with FBiHM, whereas monoallelic truncating or missense mutations were found in some women with sporadic androgenetic CHMs, partial moles, polyhydramnios and cases of miscarriages and stillbirths of uncertain etiology (Messaed et al., 2011b; Qian et al., 2011; Slim et al., 2011). It was speculated that rare non-synonymous NLRP7 variants (NSVs) might contribute to genetic susceptibility to recurrent reproductive wastage (Messaed et al., 2011b).

To date, it is unclear which subpopulations of women with adverse reproductive outcomes, other than FBiHM, should be screened for NLRP7/C6orf221 variants. Furthermore, the knowledge of the frequency of such variants in the general reproductive population is insufficient to evaluate any potential pathogenic effects. To further elucidate the incidence and relevance of such variants, we sequenced all coding regions and exon-intron boundaries of NLRP7 and C6orf221 in a clinically well-defined group of women with a pregnancy history of diandric CHM or triploidy and additional miscarriages. As the major findings for this group were non-synonymous variants of NLRP7, rather than clearly pathogenic mutations, we further genotyped these variants in a larger group of women with similar pregnancy pathology and in healthy controls.

Materials and Methods

Subjects

Samples utilized in this study were ascertained as part of previous studies of recurrent miscarriage (Beever et al., 2003; Hanna et al., 2010), origin of triploidy (McFadden and Robinson, 2006) or molar pregnancy (Bourque et al., 2011). To enhance for the presence of a genetic defect, only women with primary recurrent miscarriage (PRM), defined as three or more consecutive miscarriages 20 weeks before gestation with no prior live birth, were included in the present study (PRM, n = 39, see Table I and Supplementary data, Table SI for case details). Women with an abnormal karyotype were excluded. In the group of women with triploid conceptions, we evaluated only those of paternal origin (confirmed by microsatellite genotyping, TP, n = 22), as it was the presence of the PHM phenotype specifically that was hypothesized to be associated with NLRP7 mutations (Deveauaut et al., 2009). Detailed pregnancy history was not available on the TP group. The healthy control group (HC, n = 52) consisted of women with proven fertility (at least one live born after 37 years) and no history of miscarriage or major pregnancy complications (see Supplementary data, Table SI for control group details) and overlapped the controls used for our previous studies of recurrent miscarriage (e.g. Hanna et al., 2010). This study was approved by the University of British Columbia Clinical Ethics Review Board.

Post hoc power analysis has been performed to estimate measurable effect size for the taken study groups using the G*Power 3.1.5 software (Faul et al., 2009). The statistical power of 0.80 was taken as a standard of adequacy. The effect size was calculated as 0.325 (RSA versus HC) and 0.361 (TP versus HC), which would allow the detection of a medium effect size with the statistical significance <0.05; however, in the analysis of the variants independently, the use of Bonferroni correction for multiple comparisons reduces this effect size to 0.398 and 0.441, respectively, with the statistical significance of 0.008. Given the strict criteria for the inclusion, we would expect, if present, a stronger association.

Sequencing

A subgroup of 17 women was selected for sequencing of the NLRP7 and C6orf221 genes (Table I). These included 13 PRM cases that additionally had at least one confirmed tripliod or CHM miscarriage, 2 secondary recurrent miscarriage cases with confirmed partial mole (RSA148, RSA 253) and 2 additional cases (PL70, PM211) with a CHM only (molecularly confirmed to be monospermic due to homoyzogosity of a large series of microsatellite markers).

DNA was extracted from whole peripheral blood (except decidua for case PL70), using conventional methods. All coding exons (2–11) of NLRP7 and (1–3) C6orf221 genes, including the intron/exon boundaries, were amplified with conventional PCR, using primer sequences obtained from previous studies (Hayward et al., 2009; Parry et al., 2011). Sequencing was performed in both directions (where possible), using a 3130xl Genetic Analyzer (Applied Biosystems, Foster City CA, USA), and BigDye Terminator V3.1 for sequencing chemistry. Sequence data were analyzed using Chromas 2.33 (Technylisium Pty Ltd, Australia) and SeqDoc (Crowe, 2005) and annotated according to NM_001172755.1 (NLRP7) and NM_001017361.2 (C6orf221) reference sequences. In order to evaluate the potential impact of variants, protein prediction was performed using the PolyPhen-2 ver.2.2.2 online software (Adzhubei et al., 2010). Scores between 0 and 1 (interpreted as benign and potentially damaging, respectively) were generated for each variant. In this study we assess only non-synonymous variants, those that lead to amino acid change and may impact on protein function.

mRNA analysis

For PM211, total RNA was extracted from whole blood using the RiboPure Blood total RNA extraction kit (Ambion, Inc., Austin, USA). To prepare cDNA, 300 ng of RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Amplification of the cDNA fragments of interest was performed with conventional PCR with the primers designed with the Primer3 software (Supplementary data, Table SII). Amplicons were then sequenced and analyzed according to NM_001172755.1 as a reference mRNA sequence.

NLRP7 genotyping

All samples were assessed for six non-synonymous NLRP7 variants (c.929A>G, c.930G>T, c.931C>A, c.955G>A, c.1441G>A and c.2301G>T) using pyrosequencing-based genotyping assays for PyroMark Q24 (Qiagen, Inc., Toronto, ON). Genotyping assays were designed with the PSQ Assay Design software (Biotage, Uppsala, Sweden); primer sequences are represented in Supplementary data, Table SII. Common and rare allele genotypes were validated by sequencing for quality assurance.

Population stratification

Study participants represented a western Canadian population, with self-reported ethnicity being predominantly Caucasian. To exclude population stratification between the PRM and control groups, 21 ancestry informative SNPs were genotyped (Hanna et al., 2010). Population stratification is unlikely to be a confounding factor in this study, as genotype distribution was not significantly different between recurrent miscarriage and control women for these 21 ancestral informative SNPs (Supplementary data, Table SIV).

Statistical analysis

Hardy–Weinberg equilibrium was tested for each of the non-synonymous NLRP7 variants in controls (Supplementary data, Table SIV). Fisher’s exact
Table I  Detailed pregnancy history and sequencing data for 17 cases

<table>
<thead>
<tr>
<th>Case</th>
<th>Pregnancy history</th>
<th>Genetic and histopathological evaluation</th>
<th>NSVs in NLRP7</th>
<th>Predicted change</th>
<th>PolyPhen2 score</th>
<th>NSVs in c6orf221</th>
<th>Predicted change</th>
<th>PolyPhen2 score</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSA 1</td>
<td>G5SA5</td>
<td>SA3: 47,XY+7; pat. meiotic, SA4: 48,XX+15, +16, SA5:69,XXY</td>
<td>c.955G&gt;A+ [=]</td>
<td>p.Val319Ile</td>
<td>0.003</td>
<td>c.602C&gt;G+ [=]</td>
<td>Ala21Gly</td>
<td>0.997</td>
</tr>
<tr>
<td>RSA 9</td>
<td>GSTA1SA4</td>
<td>SA2: monospermic androgenetic CHM, SA3: 46,XY, SA4: 46,XY</td>
<td>c.1441G&gt;A</td>
<td>p.Ala481Thr</td>
<td>0</td>
<td>c.602C&gt;G+ [=]</td>
<td>Ala21Gly</td>
<td>0.997</td>
</tr>
<tr>
<td>RSA 27</td>
<td>G4SA4</td>
<td>SA3: 70,XXY, +7, SA4: 46,XY/47,XY, +15</td>
<td></td>
<td></td>
<td></td>
<td>c.602C&gt;G+ [=]</td>
<td>Ala21Gly</td>
<td>0.997</td>
</tr>
<tr>
<td>RSA 31</td>
<td>G7SA7</td>
<td>SA7: 69,XXY</td>
<td>c.930G&gt;T+ [=]</td>
<td>p.Leu311His</td>
<td>0.884</td>
<td>c.289G&gt;C+ [=]</td>
<td>Glu97Gln</td>
<td>0.015</td>
</tr>
<tr>
<td>RSA 36</td>
<td>G5SA5</td>
<td>SA4: molar pregnancy (?)</td>
<td>c.289G&gt;C</td>
<td>p.Ala481Thr</td>
<td>0</td>
<td>c.602C&gt;G+ [=]</td>
<td>Ala21Gly</td>
<td>0.997</td>
</tr>
<tr>
<td>RSA 68</td>
<td>G5SA5</td>
<td>SA5: 69,XXY, dispermic</td>
<td>c.289G&gt;C</td>
<td>p.Ala481Thr</td>
<td>0</td>
<td>c.602C&gt;G+ [=]</td>
<td>Ala21Gly</td>
<td>0.997</td>
</tr>
<tr>
<td>RSA 75</td>
<td>G6SA4T2</td>
<td>SA2: PHM, SA4: 47,XX, +17</td>
<td>c.289G&gt;C</td>
<td>p.Ala481Thr</td>
<td>0</td>
<td>c.602C&gt;G+ [=]</td>
<td>Ala21Gly</td>
<td>0.997</td>
</tr>
<tr>
<td>RSA 148</td>
<td>G4T1SA3</td>
<td>SA3: PHM</td>
<td>c.289G&gt;C</td>
<td>p.Ala481Thr</td>
<td>0</td>
<td>c.602C&gt;G+ [=]</td>
<td>Ala21Gly</td>
<td>0.997</td>
</tr>
<tr>
<td>RSA 165</td>
<td>G3SA3</td>
<td>SA3: 69,XXY</td>
<td>c.289G&gt;C</td>
<td>p.Ala481Thr</td>
<td>0</td>
<td>c.602C&gt;G+ [=]</td>
<td>Ala21Gly</td>
<td>0.997</td>
</tr>
<tr>
<td>RSA 220</td>
<td>G4SA3T1</td>
<td>SA3: PHM (tetraploid)</td>
<td>c.289G&gt;C</td>
<td>p.Ala481Thr</td>
<td>0</td>
<td>c.602C&gt;G+ [=]</td>
<td>Ala21Gly</td>
<td>0.997</td>
</tr>
<tr>
<td>RSA 240</td>
<td>G8TA2SA6</td>
<td>SA7: PHM</td>
<td>c.1441G&gt;A+</td>
<td>p.Ala481Thr</td>
<td>0</td>
<td>c.289G&gt;C+ [=]</td>
<td>Glu97Gln</td>
<td>0.015</td>
</tr>
<tr>
<td>RSA 253</td>
<td>G5TA1T1SA3</td>
<td>SA3: PHM</td>
<td>c.1441G&gt;A+</td>
<td>p.Ala481Thr</td>
<td>0</td>
<td>c.289G&gt;C+ [=]</td>
<td>Glu97Gln</td>
<td>0.015</td>
</tr>
<tr>
<td>RSA 290</td>
<td>G4SA4</td>
<td>SA4: 69,XX (mat. MII)</td>
<td>c.289G&gt;C+</td>
<td>p.Ala481Thr</td>
<td>0</td>
<td>c.289G&gt;C+ [=]</td>
<td>Glu97Gln</td>
<td>0.015</td>
</tr>
<tr>
<td>RSA 300</td>
<td>G18SA18</td>
<td>SA5: 69,XX, SA11: 69,XXY, SA16: 69,XX, SA17: 69,XX</td>
<td>c.289G&gt;C+</td>
<td>p.Ala481Thr</td>
<td>0</td>
<td>c.289G&gt;C+ [=]</td>
<td>Glu97Gln</td>
<td>0.015</td>
</tr>
</tbody>
</table>

PolyPhen2 scores predicting possibly damaging mutations are in bold. Self-reported ethnicity was Caucasian for RSA 36, 68, 75, 148, 165, 220, 253, 300 and 301; Asian for PM211 and unknown in all other cases.

G, gestations; SA, spontaneous abortions; TA, therapeutic abortion; T, term delivery; PT, preterm delivery; CHM, complete hydatidiform mole; DCDA, dichorionic - diamniotic; PHM, partial hydatidiform mole; RM, recurrent miscarriage.
probability and its Freeman–Halton extension were used for comparisons of allele and genotype frequencies between PRM versus HC and TP versus HC groups. The Benjamini–Hochberg False Discovery Rate model was used to correct for multiple analyses of the ancestry marker genotypes (Hochberg and Benjamini, 1990).

**Results**

**Mutation screening of NLRP7 and C6orf221**

The search for mutations in 17 patients revealed 1 novel (c.2301G>T) and 5 previously described non-synonymous variants in a coding region of NLRP7: c.929A>G (1 allele), c.930G>T (1 allele), c.931C>A (1 allele), c.955G>A (5 alleles), c.1441G>A (6 alleles). The novel variant, c.2301G>T, was within the first coding nucleotide of exon 7, in a heterozygous state in a patient with an androgenetic CHM co-existing with normal pregnancy (PM211). mRNA analysis did not reveal a splice site breakage, suggesting that the variant results in a missense mutation p.Arg767Ser. PolyPhen-2 software predicted this variant to be benign (score 0.015).

We also detected two common variants in C6orf221 (see Table I).

**Genotyping of non-synonymous NLRP7 variants**

To further assess the role of NLRP7 variants more generally, allele and genotype distributions for the six variants, c.929A>G, c.930G>T, c.931C>A, c.955G>A, c.1441G>A and c.2301G>T, were compared between women with PRM, those with androgenetic triploid conceptions (TP) and control women with proven fertility and no history of miscarriage or pregnancy complications (HC). Genotype and allele frequencies are presented in Table II, including the novel minor allele c.2301G>T. Genotyping analysis demonstrated collocation of alleles for two SNPs (c.929A>G, c.931C>A), indicating strong linkage disequilibrium. In these cases, genotype/allelic frequencies were calculated as if they were a single locus. For these rare variants, there was a non-significant trend toward an association with PRM (4 carriers of 39) when compared with the HC group (1 carrier of 52) (P = 0.160). No associations between non-synonymous NLRP7 variants and PRM or TP were detected (calculated for each variant separately, any detected variant, presence of more than one variant and any detected variant combinations).

Collocation of minor alleles c.929A>G/c.931C>A and c.1441G>A was detected in three PRM and one HC samples; c.929A>G/c.931C>A, c.955G>A, c.1441G>A in 1 PRM case; c.930G>T and c.955G>A in one PRM and two HC samples; c.955G>A, c.1441G>A in two PRM and one HC case. Pregnancy histories for PRM and HC groups along with genotyping data are presented in Supplementary data, Tables SI and SII.

**Discussion**

It was speculated that the reproductive outcomes of women with NLRP7 mutations are not limited to recurrent biparental moles, but may include a spectrum of related reproductive pathologies associated with disruption of early embryonic development (Murdoch et al., 2006; Messaed et al., 2011b; Qian et al., 2011; Slim et al., 2011). It was suggested that NLRP7 mutations could lead to complete androgenetic and partial mole formation by a two-hit mechanism: (i) the mutation first causes an increased rate of stochastic and mosaic aneuploidy leading to the formation of haploid, diploid and polyploid blastomeres during early embryo cleavage (ii) it alters the maternal immune system and its tolerance to abnormal conceptions, so the resulting embryo evades this selective elimination (Deveault et al., 2009; Messaed et al., 2011a). In support of this hypothesis, early cleavage abnormalities were detected during in vitro and in vivo development of embryos from patients with NLRP7 mutations (Deveault et al., 2009); though it should be noted that early cleavage abnormalities are generally common and may not be limited to mutation carriers (Mantzouratou and Delhanty, 2011).

Another maternal effect gene, C6orf221, has been recently described as a homolog of ECAT1, which is specifically expressed in ovaries of eutherian mammals (Pierre et al., 2007). In humans, biallelic mutations of C6orf221 were detected in three cases of FBrHM, which indirectly supports its having a similar role in human early embryogenesis (Party et al., 2011). Both genes are specific to eutherian animals and belong to rapidly evolving gene families, which complicates the interpretation of animal model data and, thus, determination of the regulation pathways involved (Zheng and Dean, 2009).

Here, we report NLRP7 and C6orf221 sequencing data of 13 women, who had at least one molar pregnancy (not recurrent biparental mole) or at least one triploid miscarriage and a history of PRM, 2 women with PHM and secondary recurrent miscarriage and 2 women with mole and co-existing normal pregnancy. No novel or previously reported truncating mutations within the coding exons or intron/exon boundaries were identified. One novel variant p.Arg767Ser was detected in the leucine-rich region of the protein, where the majority of NLRP7 missense mutations have been detected previously (Wang et al., 2009; Messaed et al., 2011b). While Protein Prediction Software estimated this substitution to be benign, the results from use of such software should be interpreted with caution, as there are limitations to the prediction algorithms. For example, while the damaging potential of Ala201Gly variant in C6orf221 was scored as high (PolyPhen-2 score = 0.997), this is clearly a benign polymorphism as the minor allele frequency was 0.48 (based on genotyping data of 8375 alleles, source: dbSNP build 137). The high score can be considered a false-positive prediction (PFP of the algorithm is 5%) likely based on overestimation of multiple alignment data. A further limitation is that we cannot exclude that undetected mutations may be present in intronic or untranslated regions, although the impact of these would be more difficult to evaluate. Furthermore, rare genetic variants may have been missed in the larger cohort due to the use of the targeted analysis; sequencing would provide a comprehensive assessment of the genetic variation of these genes and may be beneficial in future studies.

In a previous study, an increased frequency of rare NLRP7 NSVs was detected among a group of women with either (i) three or more miscarriages or (ii) one CHM and at least one other pregnancy loss (Messaed et al., 2011b). A statistically significant association with deleterious outcomes was detected for the presence of any rare allele, as well as specifically for the c.1441G>A variant that was also associated with decreased cytokine secretion by mononuclear cells. Rare alleles are traditionally defined by relative population frequencies of <0.01, whereas Messaed et al. defined rare non-synonymous NLRP7 variants as those with minor allele frequency <0.064, which allowed inclusion...
of the c.1441G>A NSV and a number of other substitutions that may indirectly influence the prolongation of the abnormal pregnancy (Messaeed et al., 2011b). However, we did not find statistically significant associations between pathological phenotypes and any of these variants individually or in combination. We also did not include c.1441G>A in the ‘rare’ group as its minor allele frequency in the healthy controls was 11%. Furthermore, we detected the rare variants c.929A>G, c.931C>A and c.930G>T, with a similar location and with a stronger damaging potential (0.002/0.68 and 0.884, respectively) in controls with only normal reproductive outcomes.

A possible explanation for the absence of clinically significant alterations of NLRP7/C6orf221 in our study is that our criteria for patient selection may not have been consistent with the phenotype of such variant carriers. However, our PRM group was well characterized in terms of the detection and genotyping of NLRP7/C6orf221 variants in miscarriage and enhanced for cases less likely to be due to just chance. Even though some triploid miscarriages in the sequencing group may have been of a digynic origin, dispermic diandry occurs in over half of early triploid miscarriages (McFadden and Langlois, 2000; McFadden et al., 2002). The absence of any clear pathologic variants suggests that disruption of NLRP7/C6orf221 pathways is not a major mechanism for this spectrum of pathology. Partial hydatidiform mole (androgenetic triploid) cases were chosen for genotyping as they were previously suggested as a possible phenotype in pregnancies of NLRP7 mutation carriers (Deveault et al., 2009; Slim et al., 2011). However, the frequencies of all minor alleles were similar to the control group. Similarly, the recent study of Dixon et al. did not reveal NLRP7/C6orf221 mutations in nine cases of recurrent complete androgenetic molar pregnancies nor did it find any associations between rare NLRP7 variants in case versus control populations (Dixon et al., 2012). Interestingly, Andreasen et al. did not find maternal NLRP7/C6orf221 mutations in isolated moles with biparental contribution to the molar genome, including mosaic and non-mosaic cases (Andreasen et al., 2012). These results suggest the existence of alternative major mechanisms for mole formation.

Our findings suggest that NLRP7 and C6orf221 mutations or variants are not a major factor contributing to the risk of recurrent miscarriage in the general population, even if complete mole or triploidy has been detected. Further studies are needed to clarify whether deleterious reproductive outcomes, aside from recurrent biparental hydatidiform moles, are associated with NLRP7 and C6orf221 carriers. It is possible that the detected non-synonymous variants may have affected the development of observed phenotypes, but the sample size was too limited to reach significance. As our goal was to determine whether there is clinical indication to screen such women for these mutations, the low frequency of variants in the patient population and presence of the same variants in controls makes this unlikely to be clinically indicated as yet.

Supplementary data
Supplementary data are available at http://molehr.oxfordjournals.org/.

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Authors’ roles
I.M. participated in study design, performed the experiments, analyzed the data, wrote and revised the manuscript. C.W.H. corrected and revised the manuscript, participated in study design and data analysis. M.D.S and D.E.M. undertook patient recruitment/data collection and participated in the manuscript preparation/revision. W.P.R. supervised the study, participated in study design and corrected/revised the manuscript.
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