European Journal of Obstetrics & Gynecology and Reproductive Biology 170 (2013) 188–192

Contents lists available at SciVerse ScienceDirect

European Journal of Obstetrics & Gynecology and Reproductive Biology

journal homepage: www.elsevier.com/locate/ejogrb

Novel *NLRP7* mutations in familial recurrent hydatidiform mole: are *NLRP7* mutations a risk for recurrent reproductive wastage?

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ARTICLE INFO

Article history:
Received 10 September 2012
Received in revised form 2 March 2013
Accepted 24 June 2013

Keywords:
Familial recurrent hydatidiform mole
NLRP7
KHDC3L
Recurrent reproductive wastage

ABSTRACT

Objective: Familial recurrent hydatidiform mole is an exceedingly rare clinical condition. Affected women are predisposed to molar pregnancies of diploid, biparental origin rather than androgenetic origin. At present, *NLRP7* and *KHDC3L* (*C6orf221*) are the only genes known to be associated with familial recurrent hydatidiform mole. This study investigated the genetic dispositions in two large Turkish families with recurring molar conceptuses.
Study design: Copy number variation analysis was performed followed by *NLRP7* gene sequencing. The finding of a mono-allelic condition in one family led to investigation of the adjacent *NLRP2* gene and recently associated *KHDC3L* gene. Sampled molar tissues were genotyped using microsatellite markers.
Results: In one family, a homozygous single nucleotide insertion that caused a frameshift leading to an early stop codon, c.2940_2941insC (p.Glu981ArgfsX13), was identified in the affected sisters. In the other family, a heterozygous 60-kb deletion eliminating substantial portions of the *NLRP2* and *NLRP7* genes on one allele was found. Screening of *NLRP2* and *KHDC3L* genes revealed no alterations that were considered to be pathological. Genotyping of six independent molar conceptions revealed that five were of diploid, biparental origin and one was of diandric, triploid origin.
Conclusions: Two novel protein-truncating mutations in the *NLRP7* gene were found to be associated with familial recurrent hydatidiform mole. Mutations in the *NLRP7* gene causing recurrent biparental hydatidiform mole may also be associated with other forms of recurrent reproductive wastage.
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1. Introduction

Hydatidiform mole (HM) is an aberrant human pregnancy in which there is abnormal embryonic development and proliferation of placental villi. These abnormal pregnancies are composed of two main types, complete hydatidiform moles (CHMs) and partial hydatidiform moles (PHMs), based on their histological features and the genetic origin of the molar tissue. Approximately 75% of clinically ascertained molar pregnancies are CHMs and mostly of diploid, androgenetic origin, and the remaining 25% are PHMs and mostly of diandric, triploid origin [1]. In both PHMs and CHMs, hydrops and excessive trophoblast proliferation are associated with two copies of the paternal genome and consequent over-expression of genes transcribed from the paternally inherited allele [2]. Following any molar pregnancy, the patient is at increased risk of further HMs in subsequent pregnancies. A second HM is, however, still infrequent (approximately 1%) and a third HM is extremely unlikely in subsequent pregnancies, except in the case of women with a rare autosomal-recessive condition, familial recurrent hydatidiform mole (FRHM), in which affected patients have an inherited predisposition to multiple CHMs [3,4]. Despite the fact that CHMs in this condition are frequently pathologically indistinguishable from typical androgenetic CHMs, they are diploid, biparental in origin, without an excess of paternally inherited genetic material [2].

A study of families with recurrent hydatidiform mole (RHM) revealed two defective genes, *NLRP7* and *KHDC3L*, but the precise pathophysiology underlining the development of molar pregnancies is unknown [5,6]. Although little is known about the function of the *KHDC3L* gene, data are emerging to indicate that the *NLRP7* gene is involved in innate immunity, leading to the hypothesis that abnormal immune responses in early pregnancy underlie molar development in these conceptions [2,7]. An alternative role for

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http://dx.doi.org/10.1016/j.ejogrb.2013.06.028

these genes in setting or maintaining the maternal imprint within the ovum has also been proposed [8,9]. Besides the risk of persistent gestational trophoblastic neoplasia, women with FRHM are unable to have normal pregnancies. To date, only two families have been reported to have normal live births in addition to recurrent CHMs [10,11]. This article reports two novel protein-truncating *NLRP7* mutations in two Turkish families with RHMs and reproductive wastage.

2. Materials and methods

2.1. Families

The complete pedigree structures of the two unrelated families, HM-01 and HM-02, are shown in Figs. 1A and 2A, respectively.

HM-01 is a large consanguineous family reporting no history of molar pregnancy in previous generations. The proband, IV:4, was a 26-year-old woman diagnosed with PHM who was referred to the authors' hospital for genetic counselling. Her history included three molar pregnancies and two miscarriages in early gestation. Two of her sisters, IV:1 and IV:3, also reported a history of reproductive failures. The elder sister, IV:1, married to a second cousin, had three CHMs and two miscarriages of unknown origin. Her husband had had three viable pregnancies with his second wife. Cytogenetic analysis revealed a normal karyotype for IV:1 and IV:2. The proband's younger sister, IV:3, married to a first cousin, had experienced five pregnancies in 7 years, resulting in three CHMs and two miscarriages. The medical history of the consanguineous parents, III:1 and III:2, included recurrent reproductive wastages with four stillbirths, one unremarkable early neonatal death within the first month of life and one miscarriage. The proband's father, III:2, reported no reproductive failure with his first wife.

The proband of family HM-02, III:3, was a 29-year-old woman in a consanguineous marriage. She had experienced six pregnancy losses and no normal pregnancies over a 9-year period. Her third, fourth and sixth pregnancies were diagnosed as CHMs by ultrasonographic examination and confirmed histopathologically. After her third molar pregnancy, III:3 developed a low-risk gestational trophoblastic neoplasm and was treated with chemotherapy. Cytogenetic analysis revealed a normal karyotype in the couple. Her elder sister, III:1, had a history of three consecutive CHMs in a 4-year period, followed by one uneventful pregnancy leading to a healthy male child. This was succeeded by another CHM, treated with chemotherapy due to the development of a low-risk gestational trophoblastic neoplasm. Her sixth conception proceeded normally and led to a healthy male child. Sister III:6 was married to a first cousin. She had experienced two CHMs and one PHM evacuated in early gestation. The patients' mother, II:2 (gravida 7, para 7), had an uneventful obstetric history except for one child who died at 2 years of age.

2.2. Genetic investigations

This genetic study was approved by the institutional review board of Kanuni Sultan Süleyman Training and Research Hospital. Informed consent was obtained from all individuals for DNA isolation and genetic analysis of their samples and molar tissues.

In family HM-01, histopathological examination of two molar tissues, V:1 and V:2, confirmed the diagnosis of PHM. Cytogenetic analysis was performed on fresh molar tissue, V:2. DNA samples were isolated from paraffin-embedded molar tissues of V:1 and V:2 from family HM-01; and paraffin-embedded molar tissues of IV:1 and IV:2, and fresh molar tissues of IV:3 and IV:4 from family HM-02. DNA was isolated from peripheral blood samples taken from six members of family HM-01 (III:1, III:2, IV:1, IV:3, IV:4 and IV:5) and 10 members of family HM-02 (II:1, II: 2, III:1, III:2, III:3, III:4, III:5, III:6, III:7 and III:8).

DNA samples from IV:1 of family HM-01, and from III:3 of family HM-02 were analyzed by Affymetrix SNP 6.0 Array (Santa Clara, California, USA). The data were reviewed at ≥200 kb for duplications and at ≥50 kb for deletions with ≥20 markers.

All the coding, deep exon-intron boundaries and total untranslated regions (UTRs) of *NLRP7* gene (NM_139176.3) were sequenced for IV:4 of family HM-01 and for III:3 of family HM-02. In III:2 of family HM-02, *KHDC3L* (NM_001017361.2) and *NLRP2* (NM_017852.3) genes were also sequenced. DNA mutation numbering was based on the recommendations of the Human Genome Variation Society. Ascribed variations were sequenced in each sampled individual to determine segregation. Genotyping was revealed using informative microsatellite markers for V:1 and V:2 from family HM-01, and IV:1, IV:2, IV:3 and IV:4 from family HM-02 with parental DNA samples.

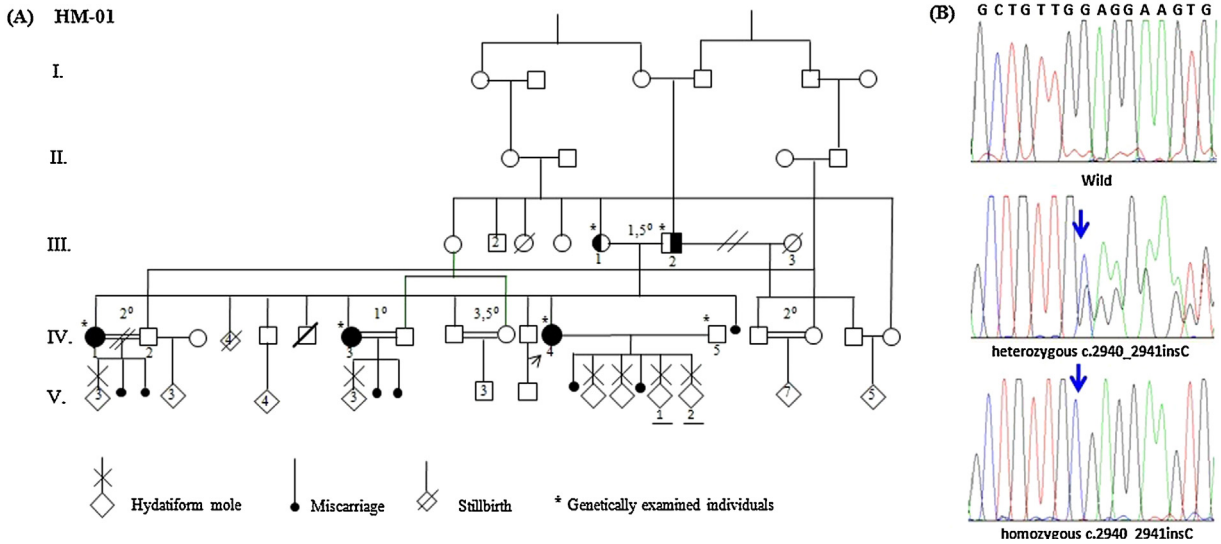

Fig. 1. (A) Pedigree structure of family HM-01. Asterisks identify individuals sampled for karyotyping and/or DNA isolation. Underlined symbols identify subjects evaluated cytogenetically. (B) Sequence chromatograms of the mutation region of the *NLRP7* gene: wild, heterozygous and homozygous types.

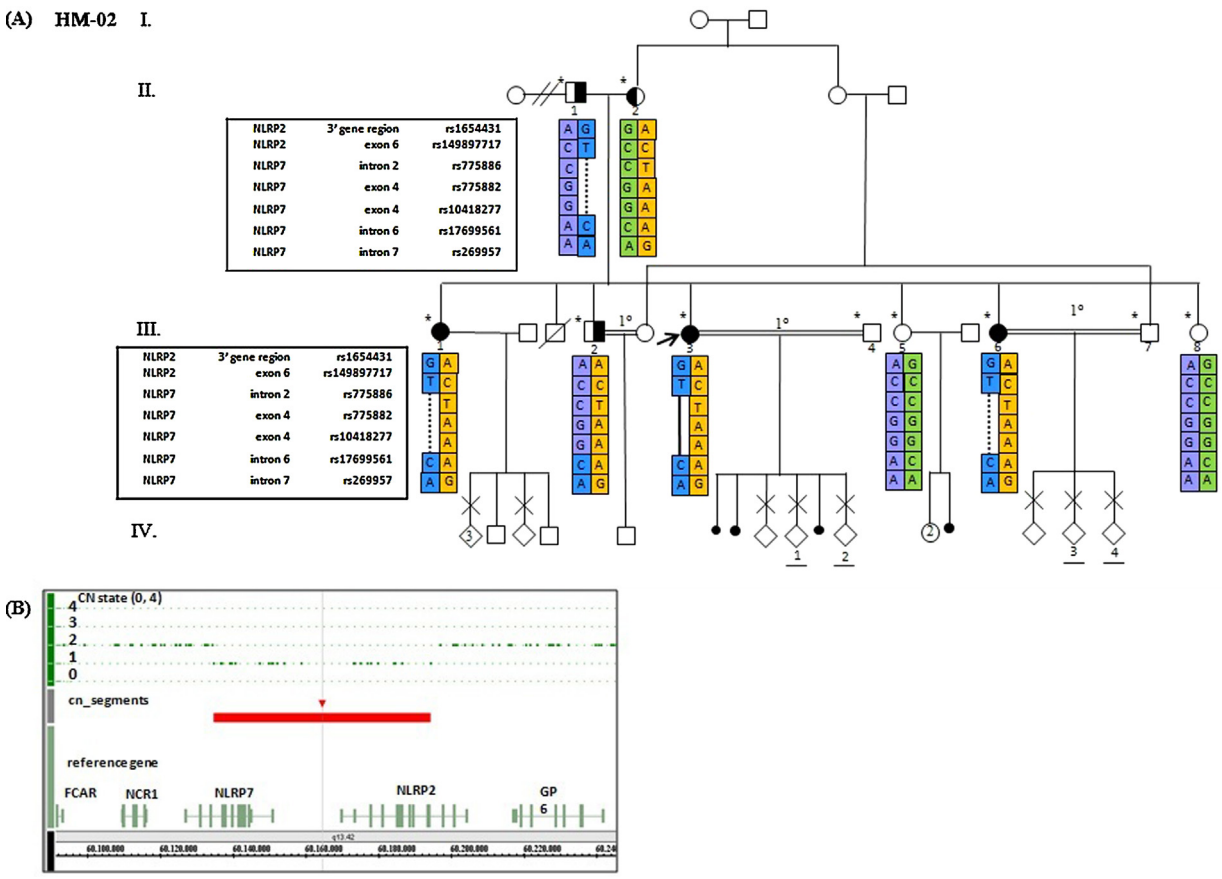

Fig. 2. (A) Pedigree structure of family HM-02. Asterisks identify individuals sampled for DNA isolation. Underlined symbols identify subjects evaluated cytogenetically. Haplotypes built from single-nucleotide polymorphism (SNP) results are presented in columns. Deletion from array result is shown with a black line for III:3, and a dotted line for indirectly ascertained individuals II:1, III:1 and III:6. (B) Affymetrix SNP 6.0 array for III:3, presenting the single dose deletion of the 60-kb region (red area) at chromosome 19 q13.42 on Chromosome Analysis Suite, extending from intron 8 of the *NLRP7* gene to intron 11 of the *NLRP2* gene. Bright green dot identifies copy number probes in the region. (For interpretation of the references to color in the figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Genotyping of molar tissues

In family HM-01, two informative microsatellite markers showed that mole V:1 was disomic, consistent with a diploid, biparental origin. Mole V:2, of the same parents, had a trisomic genotype with two paternal and one maternal allele, and cytogenetic analysis revealed a 69, XXY karyotype in 17 metaphases obtained from long-term cell culture. In family HM-02, biparental contribution was demonstrated at three informative loci in moles IV:1 and IV:2, and at two informative loci in moles IV:3 and IV:4, by the presence of one allele from each parental genome (Table 1).

Table 1
Informative microsatellite markers identified in DNA from the patients, their partners and hydatidiform mole tissues in families HM-01 and HM-02.

Polymorphic microsatellite	Patient	Molar tissue		Partner
Family HM-01	(IV:4)	HM (V:1)	HM (V:2)	(IV:5)
D4S2983	1,2	1,3	2,3,4	3,4
D15S131	1,2	1,3	2,3,4	3,4
Family HM-02	(III:3)	HM (IV: 1)	HM (IV:2)	(III:4)
D5S2949	3,4	1,4	1,4	1,2
D10S1730	1,4	1,3	2,4	2,3
D15S131	1,3	3,4	3,4	2,4
	(III:6)	HM (IV:3)	HM (IV:4)	(III:7)
D2S1384	1,3	3,4	1,2	2,4
D15S131	1,3	2,3	1,4	2,4

3.2. Mutation screening of the NLRP7 gene

In family HM-01, a novel c.2940_2941insC change, resulting in a frameshift and protein truncation (p.Glu981ArgfsX13), was identified in a homozygous state in three sisters (IV:1, IV:3 and IV:4) (Fig. 1B). The parents, III:1 and III:2, were heterozygous, confirming the autosomal-recessive mode of inheritance. Affymetrix SNP 6.0 analysis of the proband did not reveal any damaging gross deletion/duplication in the genome or on the *NLRP7* gene.

In family HM-02, Affymetrix SNP 6.0 analysis of the proband III:3 revealed 60-kb deletion presented by 27 copy number markers on 19q13.4 covering a significant portion of the *NLRP2* and *NLRP7* genes (Fig. 2B). Sequencing of the complete *NLRP7* gene did not reveal any other pathological variations and few clinically unrelated polymorphisms. Haplotyping of the SNP markers (rs1654431, rs149897717, rs775886, rs775882, rs10418277, rs17699561 and rs269957) presented a hemizygous region on rs775886, rs775882 and rs10418277 for III:1, III:3 and III:6 (Fig. 2A). As this region coincides with the deletion presented by the array analysis of III:3, the haplotyping suggested that III:1 and III:6 also carry this deletion, presumably inherited from II:1.

3.3. Mutation screening of the KHDC3L and NLRP2 genes

Sequencing of the *KHDC3L* gene in III:3 of family HM-02 revealed a single common mis-sense polymorphism in a homozygous state (rs561930) and no pathological alterations. Sequencing the *NLRP2* gene in the same individual revealed a rare mis-sense polymorphism in a heterozygous state, c.1736C>T (rs149897717),

altering proline to leucine (NP_060322.1) at codon 579 (p.Pro579-Leu). Screening of this alteration in other sampled individuals of family HM-02 showed that it is in a *-cis* position with the deletion allele (Fig. 2A).

4. Comments

The majority of females with FRHM studied to date have been found to be homozygote or compound heterozygote mutation carriers of the *NLRP7* and *KHDC3L* genes, confirming that these genes are the major cause of maternal effect recessive recurrent biparental molar pregnancies. The *NLRP7* gene is transcribed in various human tissues, including unfertilized oocytes at the germinal vesicle and metaphase II stages, and endometrium [12]. The *NLRP7* gene is a negative regulator of caspase-1-dependent interleukin (IL)-1β secretion, a pleiotropic and pro-inflammatory cytokine that activates a number of inflammatory and immunological pathways. IL-1β is normally expressed at high levels in the uterine milieu around the peri-implantation period, where it facilitates implantation of the blastocyst, regulates the protease network and controls the extent to which the trophoblast may invade the maternal endometrium [5]. It is therefore hypothesized that abnormal stimulation of the inflammatory process causes molar pregnancies. An alternative role for the *NLRP7* and *KHDC3L* genes in setting or maintaining the maternal imprint within the ovum has also been proposed. A number of genes that normally carry a maternal methylation imprint have been shown to assume a paternal epigenetic pattern on the maternal allele in biparental CHMs, suggesting that the phenotypic abnormalities observed in biparental CHMs, such as androgenetic CHMs, are associated with increased expression of paternally transcribed genes [2,8,9].

A full review of previously described families with RHM showed similar clinical heterogeneity in most of the reported families, with the pregnancies of affected women generally described as CHMs, PHMs, stillbirths or miscarriages [10,13,14]. Approximately 6% of pregnancies with FRHM are reported as PHM but when these PHM have been genotyped, they have been found to be diploid and biparental rather than diandric triploids [2]. In the minority of described pedigrees, some normal pregnancies have been interspersed with recurrent molar pregnancies [10,11]. Fisher and Hodges reviewed 152 pregnancies that had been reported by a number of groups. They looked at the clinical outcomes of patients who had FRHM and reported seven (5%) normal pregnancies [15]. In the present study, only one woman, III:1 of family HM-02, had any live-born offspring. Despite the fact that III-1 has the same deletion as her sisters, it is not certain that all affected individuals in family HM-02 have exactly the same genotype given that a second mutation was not identified. In addition, it was not possible to identify whether the RHMs of III:1 were of diploid, biparental origin due to unavailable molar tissue. On the other hand, both sisters in family HM-02, III:3 and III:6, have had RHMs consistent with a diploid, biparental origin. Accordingly, the presence of the same mutation and RHM, together with diploid, biparental molar conceptions in these two sisters, suggests that III:1 with live-born offspring could not simply be a carrier for poor reproductive history. In the two families, of the six patients with RHM, four had also experienced at least two miscarriages but tissue was not available for histopathological diagnosis and genetic investigation. These data are in line with a report by Messaed et al. documenting the association of *NLRP7* mutations with RHM and additional instances of reproductive wastage [7].

Women who have experienced biparental RHM, with or without a familial history, have limited reproductive options. Assisted reproductive techniques, including in vitro fertilization and intracytoplasmic sperm injection, combined with pre-implantation genetic diagnosis have been tested, but have not produced satisfactory outcomes to date [16]. Williams et al. suggested that counselling should include a discussion regarding the possibility of oocyte donation, as this may be the family's only viable option for a healthy pregnancy [1]. Even if a healthy embryo is achieved by this method, it remains unclear whether there is increased risk of miscarriage due to the inflammatory response expressed by the *NLRP7* gene or other genes that cause this phenotype. In a recent study, Fisher et al. reported a successful pregnancy through oocyte donation in a female with three biparental RHMs. Although this has only been attempted in a limited number of cases, this report established that oocyte donation can enable women with FRHM due to *NLRP7* mutations to achieve a normal pregnancy [17].

This study reported novel *NLRP7* mutations in two families with recurrent biparental CHM. In the first family (HM-01), three affected sisters were homozygous for a nonsense mutation, while in the second family (HM-02), three affected sisters were heterozygous for a 60-kb deletion eliminating substantial portions of the *NLRP2* and *NLRP7* genes. In family HM-02, despite having biparental CHMs, no other pathological variants were found on the opposite allele. Further testing of the adjacent *NLRP2* gene and recently associated *KHDC3L* gene revealed no other pathological alterations. All the previous reports on recurrent biparental CHM associated with *NLRP7* and *KHDC3L* genes disclosed either homozygous or compound heterozygous mutations, suggesting that heterozygosity for these genes may not be sufficient to cause recurrent biparental CHM [18]. This is supported by the observation that relatives of affected individuals, who are carriers, have reproductive outcomes similar to those of the normal population [19,20]. Nevertheless, gross deletion could result in a dominant negative effect on the healthy allele to cause insufficient NLRP7 protein in females. As FRHM is caused by defective alleles in females, not males, the heterozygous male individual in family HM-02, II:1, is not expected to be affected, unlike his carrier daughters. However, a second mutation somewhere in the *NLRP7* gene, not screened in this investigation (deep in intron activating criptical splice site), or another causative gene are also possibilities.

Since 2006, more than 50 disease-causing mutations have been identified in the *NLRP7* and *KHDC3L* genes. This study identified a 60-kb gross deletion, the largest reported probable disease-causing deletion in the *NLRP7* gene associated with FRHM. Previously, 1218-bp and 77-bp deletions associated with RHM have been reported [14,18]. Copy number variation (CNV) array platforms enable the identification of deletions/duplications as low as 1 kb in the genome, but another method of analysis is needed to support this finding. Real-time polymerase-chain reaction is one possibility. Haplotyping of the family members may serve as an alternative choice if informative SNP markers are available, as they were in family HM-02.

In the database of genomic variants, two different copy number variations occurring on the *NLRP7* gene are described. One is a single dose deletion of ~860 kb, removing the 5′untranslated region of exon 1 (variation_32274) that was identified in a single Caucasian male (NA07029) from 30 unrelated humans [21]. The other is a ~798-kb deletion/duplication covering the *NLRP7* gene completely (variation_4088) identified in 35 individuals drawn from a pool of 270 apparently healthy subjects representing four different populations with various ancestry; their genders as well as their life-outcome consequences, however, were not disclosed [22].

In conclusion, this study presented two novel protein-truncating mutations with additional evidence of the association between FRHM and the maternal gene, *NLRP7*. The results are in agreement with previous observations that *NLRP7* mutations are associated with recurrent biparental molar pregnancies and reproductive wastage.

Conflict of interest

None declared.

Acknowledgements

The authors wish to thank the families for participating in this study, and Prof. Dr. Seher Basaran for her critical comments on the manuscript.

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