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HIGH RISK OF GESTATIONAL TROPHOBLASTIC NEOPLASIA DEVELOPMENT IN RECURRENT HYDATIDIFORM MOLES WITH *NLRP7* PATHOGENIC VARIATIONS

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ABSTRACT

Objective: Pathogenic variations of the *NLRP7* and *KHDC3L* genes are responsible for familial recurrent hydatidiform moles, a rare autosomal recessive phenomenon that can lead to severe comorbidities. Little is known about the diversity of genetic defects or the natural course of disease progression among recurrent hydatidiform mole cases from distinct ethnicities. In this study, we aimed to investigate the mutation profile and pregnancy outcomes in patients with multiple molar pregnancies.

Material and Methods: Three unrelated cases with recurrent molar pregnancies are included in this study. None of the patients had a known family history of molar pregnancy. Clinical findings and follow-up results are documented. Sanger sequencing is used to reveal genetic defects in exons and exon-intron boundaries of *NLRP7* and *KHDC3L* genes.

Results: *NLRP7* pathogenic variants were found in all three cases. In two cases, homozygous, c.2471+1G>A canonical splice cite variant was identified and in one case a homozygous, c.2571dupC (p.Ile858HisfsTer11) frameshift variant was identified. No variant in the *KHDC3L* gene was found in any case. In all cases, the development of gestational trophoblastic neoplasia complicated the clinical course and the treatment plans.

Conclusions: We found that defects of the *NLRP7* gene are principally responsible for etiology in our region, and the mutation profile suggests a founder effect in the Turkish population. We suggest early genetic diagnosis and counseling in molar pregnancies and recommend close follow-up in terms of conversion to gestational trophoblastic neoplasia.

Key words: Genetic; *KHDC3L*; *NLRP7*; Recurrent hydatidiform mole

INTRODUCTION

Gestational trophoblastic disease is defined as the spectrum of aberrant cellular expansions originated from the placental villous trophoblast. Main forms of gestational trophoblastic disease include benign hydatidiform moles (HM) and four malignant gestational trophoblastic neoplasias (GTN), i.e. invasive mole, choriocarcinoma, placental site trophoblastic tumor and epithelioid trophoblastic tumors [1]. The incidence varies but the highest incidences are recorded in some regions of Asia. In Turkey, the average HM incidence is 1.87 per 1,000 deliveries and incidence for GTNs are around 0.38 per 1,000 deliveries [2, 3]. At the histopathological level, hydatidiform mole is classified as complete hydatidiform mole (CHM) or partial hydatidiform mole (PHM). CHMs are generally of diploid androgenetic origin and have a 15% risk of GTN, while PHMs are mostly of triploid dispermic origin with a lesser 5% risk. Early diagnosis and follow-up is crucial due to the risk of malignancy. Sporadic and recurrent cases are reported [4]. The presence of two or more molar pregnancies in the same case is defined as recurrent hydatidiform mole (RHM). A subgroup of RHMs are familial (FRHM), where an autosomal recessive genetic defect causes molar development in a diploid embryo with maternal and paternal genetic contribution, i.e., biparental mole (BiCHM). FRHMs are mostly BiCHMs, in contrast to androgenetic CHMs [5]. BiCHMs are caused by homozygous pathogenic variations in the maternal genotype and the paternal genotype is not involved in pathogenesis

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[6]. The two most frequently involved maternal gene loci are *NLRP7* and *KHDC3L*, causing 75-80% and 5-10% of FRHM cases, respectively [5]. These syndromes are termed HYDM1 (OMIM#231090) when caused by mutations in the *NLRP7* gene and HYDM2 (OMIM#614293) when caused by mutations in the *KHDC3L* gene [7]. For cases with FRHM, in vitro fertilization with oocyte donation may be offered, but even then, HM and subsequent failure of achieving normal pregnancy may occur [6].

In this report, we present three cases of recurrent hydatidiform mole and their genetic analysis results. All three patients were treated with single agent chemotherapy due to the development of GTN after evacuation of the retained products of conception. We discussed potential implications in light of the current literature.

MATERIALS AND METHODS

Patient Selection

RHM patients who were referred to the Department of Gynecologic Oncology (Tepecik Training and Research Hospital) and to the Center for Genetic Diagnosis (Dokuz Eylul University) between the years 2018 and 2020 were retrospectively reviewed. All 3 cases were included in the study. In addition to the gynecologic work up, Sanger Sequencing of the *NLRP7, KHDC3L* coding regions and exon-intron boundaries were performed. This study was in conformity with the Declaration of Helsinki on ethical principles for medical research. All individuals provided written informed consent for molecular analysis and also for the publication of this paper.

Sample Collection and DNA Extraction

Total genomic DNA was extracted from 4 ml peripheral blood from all patients via magnetic bead purification method. MagPurix Blood DNA Extraction Kit (Zinexts, Taiwan) was used with the MagPurix Automated Extraction System (Zinexts, Taiwan) according to the manufacturer's protocol. DNA quality and concentration measurements were performed by NanoDrop ND1000® Spectrophotometer (Thermo Fisher Scientific, USA). After proper quality (50-100 ng/µl concentration and A260/A280: 1.8-2.0 purity) of DNA was ensured, the DNA was stored at -20°C until further use.

Sanger Sequencing

Direct amplification and sequencing of the *KHDC3L* and *NLRP7* genes were performed primarily using the primer sequences shown on Table 1. Exons and splice-site junctions were amplified using a standard PCR procedure

Table 1. Amplification and sequencing primers of *NLRP7* and *KHDC3L* genes

Sequence of the primers (5'→3') used for the mutation analysis of *KHDC3L*		
Exon	**5' Primer (Forward)**	**3' Primer (Reverse)**
1	GTTCCTCCTACCGGGTGCG	CGATCCTCACCAGTAGCCAAT
2	GGCTTCTTTCTGCCACCCATA	TCTCCGGTGGAGGTGCAG
3	GCTGGGAATAGGGCTACCTG	GTGGCGAGGAAGGATGATGT
Sequence of the primers (5'→3') used for the mutation analysis of *NLRP7*		
Exon	**5' Primer (Forward)**	**3' Primer (Reverse)**
1	UTR – not amplified	UTR – not amplified
2	TCTTGGCACACAGGAAACTG	TGTAAGGCTGGAGTGCAGTG
3	CATGCCTGGCTGACACTTTA	TCTGCTCATTGCAACCTCTG
4 - part 1	CTCAAGTGATCCACCCACCT	AGGAAGATGTTACCCAGGGC
4 - part 2	GGCCTTGATGAGCTGAAAGT	CCTCAGCTTCCAGCAGTTTC
4 - part 3	CTGTTCCTGGACGGAGACAT	TGTCAGAATTTCCCTCTGGC
5	TTGTGGTTTTTGCCATTGAA	AGGAAGACCCTGAACGATGA
6	CCCGGCCAAGAACTTCTAAT	GTAACCACTCCAGATGCCGT
7	AGGCTGCAGTGAGGTGAGAT	AACACCTGACTTACTGCGCC
8	GATGAACAGGAAGGGCTGAA	GCACATGAATTCAAGCAGGA
9	GCAAGCCCACCTGGAAGTAT	AGTGTTTGGAAATCTGGAAATCC
10	CTCCCGAAGTGTTGGGATTA	ACCTCTGCCTCTCAGGTTCA
11	GGCATCCTGGGTAGTTGAGA	TTTTTGGGAGATTCTGCACG

that utilized the AmpliTaq Gold™ 360 DNA Polymerase (Applied Biosystems - Thermo Fisher Scientific, USA). Amplifications were performed using Eppendorf 5332 Mastercycler (Eppendorf AG, Germany). PCR products were verified by 2% agarose gel electrophoresis and ethidium bromide staining. Sequencing reactions were performed with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems - Thermo Fisher Scientific, USA), and electrophoresed on an ABI 3130 Capillary Electrophoresis System (Applied Biosystems - Thermo Fisher Scientific, USA). Sequence alignment and evaluation were performed using CLC Genomics Workbench v3.6.5 (Qiagen, Germany). GRCh38.p13 human genome reference in the "Ensembl" database, with a ENST00000370367 transcript of the *KHDC3L* gene and a ENST00000592784 transcript of the *NLRP7* gene as reference. Detected variants were classified according to the current guidelines [8].

RESULTS

Case 1

The first case was a gravida 3, para 0 (one early pregnancy loss and two consecutive HMs) 25-year-old woman. Her main complaint was amenorrhea. β-hCG level was measured as 271,793 mIU/mL. Ultrasonography (USG) revealed a 50 mm empty gestational sac with an irregular contour and there was no yolk sac. Several hypoechoic areas in the endometrial cavity were seen. Suction evacuation was performed without complications. Pathologic investigation revealed HM. Monitoring of β-hCG levels was initiated. Eighteen days after the evacuation, β-hCG levels lowered to 365 mIU/mL, but 7 days later it rose to 586 mIU/mL. The increase of β-hCG is considered as a sign of GTN development. The diagnosis was stage 1, low risk GTN with a WHO score of 1. Single agent chemotherapy protocol consisting of four doses of methotrexate and four doses of folinic acid was started. After 3 cycles of chemotherapy, β-hCG levels returned to the normal range after 40 days, and the treatment was concluded with one additional cycle of chemotherapy.

One and a half years after the initial management of the case, the patient was admitted to our clinic again, due to the suspicion of another pregnancy. The β-hCG level was 258,164 mIU/mL. A hyperechogenic lesion (50x32 mm) containing multiple millimetric cystic areas was seen in USG evaluation. Evacuation procedure was performed without complications and the β-hCG level was normal after two months. The material derived from evacuation was reported as HM by the pathology laboratory.

Because the case had four consecutive HM without any live births, genetic consultation was needed. Family history revealed that the patient had 9 siblings. Only one of her siblings was married and although she had 7 children, she had no history of molar pregnancy.

Sanger sequencing of the patient's DNA identified homozygous, c.2471+1G>A (rs104895505) pathogenic variation (Guideline criteria: PVS1, PM2, PP3) [8] in 7th intron of the *NLRP7* gene (Figure 1).

Case 2

The second patient was a gravida 3, para 0 (three consecutive HMs) 19-year-old woman. The first admission of the patient was on suspicion of pregnancy. Her β-hCG level was 440,873 mIU/mL. During USG, there was a HM in nodular form (81x47x84 mm) which included numerous millimetric cystic degeneration areas. It was located at corpus and fundus filling the endometrial cavity, with deep myometrial infiltration close to the serosa. Thyroid function related hormones were measured out of the normal range due to an increase in the β-hCG level: free T3 was 7.19 pg/mL, free T4 was 2.17 ng/dL, TSH was 0.02 mIU/mL. Two months after the evacuation of the products of conception, her β-hCG level was 9.63 mIU/mL. The pathology report was consistent with HM.

Eight months later, she had another admission because of amenorrhea. The USG revealed a 66x52 mm

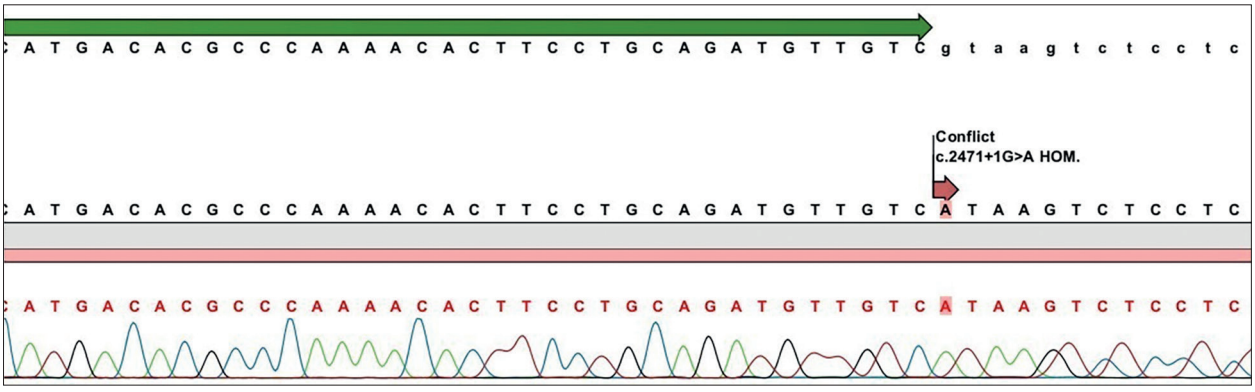

Figure 1. Sequence chromatogram of homozygous NLRP7: c.2471+1G>A (rs104895505) pathogenic variation.

lesion in the endometrial cavity, compatible with HM. The β-hCG level was 197,328 mIU/mL and thyroid function tests were also impaired: free T3 was 4.53 pg/mL, free T4 was 1.86 ng/dL and TSH was 0.02 mIU/mL. One and a half months after the evacuation procedure, her hormone profile was normal. Pathology confirmed HM.

Her third admission was made 2 years later, due to a suspected pregnancy. The β-hCG value was 272,283 mIU/mL. Free T3 was 5.19 pg/mL, free T4 was 1.48 ng/dL and TSH was 0.02 mIU/mL. The lesion measured 66x40 mm and it was consistent with HM again. Evacuation of the products of conception was performed, and β-hCG monitoring was initiated. The β-hCG level was 2.98 at week 3 and increased to 3.66 at week 4. It increased again to 9.35 at week 5. The lesion was considered as post-molar stage 1, low risk GTN and single agent chemotherapy (methotrexate) was planned. After two cycles of chemotherapy, β-hCG was normal and two more cycles of chemotherapy were given. After treatment, free T3 was measured as 3.97 pg/mL, free T4 was 0.83 ng/dL and TSH was 0.37 mIU/mL.

The patient had homozygous c.2471+1G>A (rs104895505) pathogenic variation in the *NLRP7* gene. This variation was identical to the variation found in case 1. Although the patients were specifically questioned, no blood relation was found between them.

Case 3

The third patient was a gravida 2, para 0 (two consecutive HMs) 33-year-old woman. She was admitted to the hospital because of a suspected pregnancy. Her β-hCG level was 573,347 mIU/mL. USG revealed 70x80 mm HM with cystic areas and without any fetus. One week after the evacuation procedure the β-hCG level measured 11,758 mIU/mL. The patient chose not to be followed-up after the procedure. The pathology report confirmed HM. One and half months later, the patient was readmitted to another hospital where curettage was performed. Monitored β-hCG showed a decrease to 137 mIU/mL, followed by a plateau and consequent increase to 166 mIU/mL. The case was considered a Stage 1 Low Risk GTN, and single agent chemotherapy was started. A total of two cycles of methotrexate were given. After one year of a contraception period, the patient decided to try another pregnancy and folic acid prophylaxis was given. Following conception, the β-hCG level was 276,400 mIU/mL and USG revealed HM with cystic areas and a diameter of 50 mm. Following evacuation, the β-hCG values were normal after 1.5 months. Pathology confirmed HM. Genetic evaluation by Sanger sequencing identified a homozygous c.2571dupC, p.Ile858HisfsTer11 (rs766731093) pathogenic (Guideline criteria: PVS1, PM1, PM2) frameshift variant in 8th exon of the *NLRP7* gene (Figure 2).

DISCUSSION

Familial cases of HM were first reported in the early 1980s [9, 10]. Recurrent HMs affect 1.5-9.3% of women with a history of HM and may be associated with autosomal recessive inheritance when encountered in more than one family member [11]. While interspersed normal pregnancies can be seen in recurrent PHM cases, which is generally sporadic, consecutive molar pregnancies are more common in recurrent CHM cases, of which a subgroup is familial [4]. The underlying mechanism has been identified to be the development of varying degrees of 'erroneous' paternal imprint markers on maternal chromosomes [12]. Similar loss of imprinting has also been previously demonstrated in choriocarcinomas [13].

To evaluate this recent data in the literature, we investigated the *NLRP7* and *KHDC3L* genes in 3 cases of RHM. A homozygous c.2471+1G>A (rs104895505) pathogenic variation in the *NLRP7* gene was detected in two cases. The third case had homozygous c.2571dupC (p.Ile858HisfsTer11, rs766731093) in the 8th exon of the *NLRP7* gene. Both variants have been reported previ-

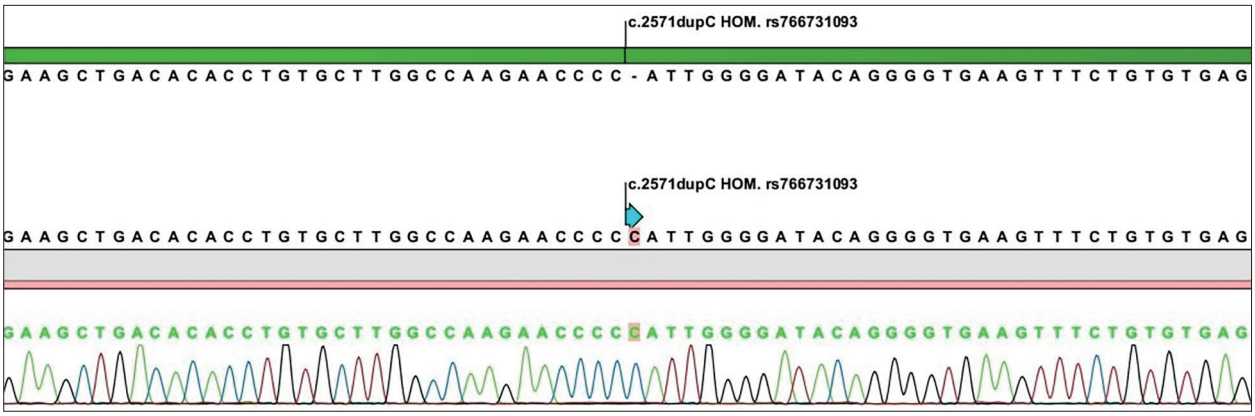

Figure 2. Sequence chromatogram of homozygous NLRP7: c.2571dupC, p.Ile858HisfsTer11 (rs766731093) pathogenic variation.

ously [11, 14]. All cases had a history of more than two HMs before their genetic testing. However, due to socioeconomic conditions, lack of family history and possibly due to frequent change of specialists during follow-up, HYDM1/HYDM2 investigation was initiated after at least the fourth molar pregnancy. This has led to an increased risk of GTN development, due to repeated attempts of new pregnancies, an increased risk of comorbidities due to repeated curettage, and considerable psychological damage. Therefore, we believe that early genetic counseling should be recommended on a case-by-case basis after the first or first repeated HM, considering criteria such as HM pathological classification, genetic constitution, and family history. During patient evaluation, family history would provide an important indicator in determining the indication for genetic testing. However, another common feature of our cases was the absence of a known family history. The sister of the first case had successful pregnancies. The other cases did not have a sister in the fertile period or any other known family history. Therefore, we think that although family history is important in determining the indication for genetic analysis, it should not be used as the only criterion in RHMs.

Post-molar GTN development was encountered in all cases. In the first and third cases, this complication was encountered in the 3rd molar pregnancy and in the second case in the 6th molar pregnancy. Apart from the first pregnancy loss of the first patient, interspersed non-molar pregnancies were not noticed. Because of the risk of GTNs and the failure to achieve normal pregnancies, we suggest a discussion of in vitro fertilization with oocyte donation in molecularly confirmed cases of HYDM1/2.

According to the literature, most of the cases have different genetic variations and frequent mutations that can be called a hotspot have not been identified [15]. To date, 275 variants have been identified in the *NLRP7* gene, the majority of which consist of benign, likely benign, silent or unclassified deep intronic variants [16]. Two of the three cases in our series have the same variant and there was no consanguinity between them. None of the patients had any pathogenic *KHDC3L* variant. As far as we know, only 8 families have been reported from Turkey so far [11, 15, 17, 18]. The variant seen in case three was previously reported in two Turkish families. Therefore, including the 3 families in this study, 3 out of 11 families (27%) carry the c.2571dupC variant, and 2 out of 11 (18%) carry the c.2471+1G>A variant. The lack of any established mutation hotspots and the fact that studies reporting the same mutations coming from different regions of the country suggest a founder effect in the Turkish population. Four or more RHMs were observed in these cases, suggesting that these variants lead to a clinically severe course. Research on a larger scale on this subject among the Turkish population is needed to prove whether there is a founder effect or not. None of the previously reported patients had any *KHDC3L* variant, which is in accordance with our results.

CONCLUSION

In conclusion, we contribute to the scientific literature with the clinical course and genetic results of 3 Turkish FRHM cases caused by *NLRP7* variants. We recommend closer follow-up of RHM cases, since GTN development is more frequent in these patients. In our opinion, genetic testing can be proposed to patients whose first pregnancy resulted in a mole, thus preventing possible delays and complications. In addition, we suggest that all RHM cases in our region should be principally investigated for pathogenic *NLRP7* variants, and that genetic counseling involving a discussion about in vitro fertilization with oocyte donation should be provided at the earliest opportunity to families with pathogenic *NLRP7* variants.

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None.

Conflict of Interest Statement.
The authors report no conflicts of interest.

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