

Variable Number Tandem Repeat (VNTR) Genotyping of Hydatidiform Mole in Iranian Patients

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Abstract

Background: Classification of molar gestation into Complete Hydatidiform Mole (CHM) and Partial Hydatidiform Mole (PHM) is done according to clinical, ultrasonographic, histologic and genetic criteria. However, making a distinction between CHM and PHM using histologic criteria alone may be difficult and several studies have shown that misclassifications are frequent, even for experienced pathologists. CHM is the most common precursor to choriocarcinoma and heterozygous moles carry an increased predisposition to transformation.

Methods: Formalin-fixed, paraffin-embedded tissue sections of patients as well as peripheral blood of patients and their partners' were collected in EDTA tubes. Tissue samples were obtained by curettage. Histological evaluation was performed on routine section stained with Hematoxylin and Eosin. Variable Number Tandem Repeats (VNTRs) genotyping was performed for 30 cases in two groups of CHM (n=21) and PHM (n=9), with Polymerase Chain Reaction (PCR) amplification of 2 different polymorphic loci, namely the Col2A1 and D1S80.

Results: The results of DNA analysis by VNTR genotyping showed that in 16 cases of CHM, amplification of the VNTR polymorphic loci showed androgenetic mono-spermic moles (homozygote) and in 5 cases of CHM androgenetic dispermic moles (heterozygote) in molar tissue. In cases of PHM, 6 samples were triploid dispermic and 3 samples were diploid biparental.

Conclusion: This study confirmed that VNTR genotyping can identify the parental source of polymorphic alleles in hydatidiform mole. Compared to STR genotyping, VNTR genotyping was performed by PCR amplification of several minisatellite markers of DNA. This method significantly requires less time and is cost-effective.

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Introduction

Hydatidiform Mole (HM) (OMIM 231090) is an abnormal gestation characterized by significant hydropic enlargement and variable trophoblastic proliferation involving part of or all chorionic villi. HMs are sub-classified into Complete HM (CHM) and Partial HM (PHM),

based on clinical features, histopathology and genetic differences¹⁻³.

HMs are relatively common and occur in approximately 1 in 1500 pregnancies in Europe and North America. This incidence varies between ethnic groups and is 2-10 times

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higher in some countries of Latin America, the Middle East, and the Far East with the highest frequencies being in Mexico, Iran and Indonesia⁴⁻⁷.

Karyotype and genotype analyses have shown that sporadic moles may have different genotypic types with the majority of the CHMs being diploid androgenetic and the majority of the PHMs being triploid diandric dispermic. Among androgenetic moles, the majority are mono-spermic (homozygous) and 10-20% are dispermic (heterozygous)⁸⁻¹². Studies have suggested that heterozygous mole may have a more malignant potential than its homozygous counterpart^{13,14}.

With the increased use of high resolution ultrasonography in early pregnancy, the diagnosis and sub-classification of hydatidiform moles have become more difficult, as CHMs are now commonly evacuated at an earlier stage and before the development of classical morphologic and histologic features. Recently, pathologists have relied on molecular techniques, such as DNA flow cytometry, chromosome *in situ* hybridization, polymerase chain reaction-based genotyping or HLA typing, showing DNA content differences for CHM and PHM¹⁵⁻¹⁷. Approximately 75% of clinically ascertained molar pregnancy are CHMs and mostly of diploid, androgenetic origin, and the remaining 25% are PHMs and mostly of diandric, triploid origin^{18,19}. Vanda Repiska *et al* suggested that PCR amplification of polymorphic VNTR regions will be a useful tool in helping to determine relative risk of choriocarcinoma in patients with complete hydatidiform moles. Closer clinical surveillance of patients with heterozygous moles may prove to be an important part of management in the future²⁰.

Persistent gestational trophoblastic disease develops after a CHM in 10 to 30% of cases and after a PHM in 0.5 to 5%. Choriocarcinoma arises in up to 3% of CHM and is rare but reported to be one of the consequences of PHM^{21,22}. Despite the difference in risk for persistent disease or metastases, the post evacuation surveillance recommendations are

identical with frequent monitoring of serum human chorionic gonadotropin levels until normalization and monthly monitoring for 6 month thereafter^{23,24}.

Each human somatic cell contains 6.4 billion base pairs of DNA. Portions of this DNA encode over 30,000 genes, while the remainders are non-coding. The human genome contains hundreds of highly polymorphic segments, each of which is characterized by allelic variation and attributed to the difference in the copy number of the tandemly repeated DNA sequences, such as Variable Number of Tandem Repeats (VNTRs). There are two principal families of VNTRs: minisatellite and microsatellite. Minisatellites are composed of different repeated "core" DNA sequences arranged in tandem. The size of the core sequence can vary from 7 to 100 *bp* in different VNTRs. Due to their high polymorphic content, VNTR is a useful tool in linkage analysis, forensic identification, paternity testing and population genetic studies²⁵⁻²⁷. VNTRs or minisatellite loci are polymorphic and due to the variation in the number of copies of their repeat unit, VNTRs are multi-allelic, so they display variation in length of the repeats and don't vary from one generation to the next. They are easily amenable to Polymerase Chain Reaction (PCR). Regarding the above mentioned criteria, these markers would be useful and informative in studying genetic variation among human population²⁸. In a study performed in Iran, it was shown that both ApoB and D1S80 are very polymorphic in Iranian populations²⁶. The Col2A1 minisatellite as one of VNTR markers, located at 3' of last exon of Col2A1 gene has been shown to be highly variable and informative²⁹.

Therefore, the aim of this study was to show the value of VNTR amplification for genotyping of CHM and PHM.

Materials and Methods

Patients

Thirty cases were selected from gynecologic oncology service. Formalin-fixed, paraffin-

embedded tissue sections of the patients were collected from the routine gynecologic pathology service files of the Mirza Koochak Khan Hospital, a referral gynecologic hospital in Tehran, Iran. Before sample collection, the study was reviewed and approved by the Tarbiat Modares University Ethics Committee.

The mean age of patients with complete mole was 26.4 years (range, 15-41 years) and in patients with partial mole, it was 30 years (range, 24-37 years). Peripheral blood of patients and their partners' were collected in EDTA tubes. Tissue samples were obtained by curettage. Histological evaluation was performed on routine section stained with Hematoxylin and Eosin and diagnoses were made by gynecologic pathologist.

For each specimen, the maternal deciduas and molar villi on paraffin block were separated using scalpel and a consecutive section stained with Hematoxyline and Eosin (Figure 1). Then three, 20 μm thick sections from FFPE tissue blocks were collected in 1.5 *micro* tubes for each case.

DNA isolation

DNA was isolated from FFPE tissue as well as from peripheral blood of patients and their partners. DNA extraction from peripheral blood was performed using modified salting out method. For each extraction, 500 μl blood (EDTA, whole blood) was used. Then, 1000 μl cell lysis buffer was added into the sterile 1.5 *ml* tube and centrifuged at 7000 RPM for 4 *min* and the supernatant was discarded. This step was repeated once more. To

the remaining white pellet, 300 μl nuclei lysis buffer was added and incubated at RT for 30 *min* until the pellet was re-suspended. Then, 100 μl NaCl (Merck, Germany) 6M and 600 μl chloroform (Merck, Germany) were added and centrifuged at 7000 RPM for 4 *min*. The supernatant (upper layer) was transferred to a new 1.5 tube and 1000 μl cold Ethanol (Merck, Germany) 100% (-20°C) was added. The tube was inverted 5-6 times gently until Hank of DNA was obtained, then centrifuged at 1000 RPM for 2 *min*. The supernatant was discarded and cold Ethanol (Merck, Germany) 70% (-20°C) was added, again tube was centrifuged. The supernatant was discarded and 100 μl distilled water was added to resuspended DNA.

DNA was prepared from tissue using modified Phenol-chloroform method. In each case, three, 20 μm -thick sections from FFPE tissue blocks were collected in 1.5 *micro* tubes. Briefly, the tissue sections were deparaffinized with Xylene (Merck, Germany), washed with 100%, 96% and 70% Ethanol (Merck, Germany) and dried in thermo block. The dried tissue was then incubated in 200 μl tissue lysis buffer (Genetbio, Korea) and 50 μl proteinase K (Genetbio, Korea). After overnight incubation at 46°C, the tube was incubated at 75°C for 1 *hr* until complete digestion of tissue. Then, 250 μl Phenol-Chloroform-Isoamyl alcohol (Sinaclon, Iran) was added and centrifuged. The upper layer was transferred to a new 1.5 tube and equal volume of Isopropanol alcohol (Merck, Germany) was added and then incubated at 4°C overnight. The next day, the tube was inverted several times and then centrifuged at 13000 RPM for 30 *min*. The supernatant was discarded and 50 μl distilled water was added to eluted DNA.

PCR amplification

The DNA samples were subjected to polymerase chain reaction (PCR) amplification of 2 VNTRs (variable number tandem repeats) polymorphic loci, namely the Col2A and D1S80. The primer sequences for 2VNTR locus²⁹⁻³¹ are given in table 1.

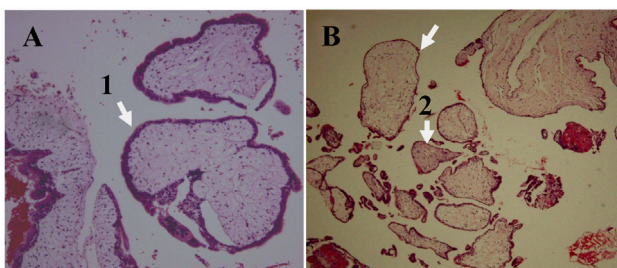


Figure 1. H&E staining of hydatidiform mole. A) CHM characterized by, 1: hydropic villi with circumferential trophoblastic hyperplasia $\times 100$ B) PHM characterized by, 1: hydropic and 2: fibrotic villi with focal mild trophoblastic hyperplasia $\times 100$

Table 1. Minisatellite primers

Locus	Location	Repeat	Primer sequence
D1S80	1P	16 bp	5'-GTC TTG TTG GAG ATG CAC GTG CCC CTT GC-3' 5'-GAA ACT GGC CTC CAA ACA CTG CCC GCC GCC G-3'
Col2A1	12q13	26 bp	5'-GCT TCC TCC AGG TTA AGG TTG ACA G-3' 5'-ACC TGC TCT CCT CCG ACC C-3'

All the DNA samples were subjected to PCR with slight modification in a thermocycler (CreaCon- Netherlands). To determine the genetic origin of each HM, 1 μ l DNA from the patient and her partner and 1.5 μ l DNA from each of the HM were amplified using two pairs of primers (BioNEER, South Korea) in a final reaction volume of 25 μ l containing DNA, 2 $\times$ Taq Master mix (Amplicon, Denmark) 12.5 μ l and 50 pmols of each primer.

The cycle conditions were as follows: for Col2A1, 30 cycles of denaturation at 94°C for 30 s; annealing at 60°C for 50 s; extension at 72°C for 50 s, for D1S80, 30 cycles of denaturation at 94°C for 30 s; annealing at 70°C for 50 s; extension at 72°C for 50 s. The initial denaturation and final extension were carried out at 94°C for 5 min and 72°C for 10 min for Col2A1 and D1S80.

Following amplification, 8 μ l of each PCR reaction product was analyzed by electrophoresis in a 2% agarose gel with a 100 bp molecular weight marker, stained with ethidium bromide and documented in a gel documentation system. In the interpretation of genotyping results, the allele sizes of minisatellite polymorphisms found in the molar villi were

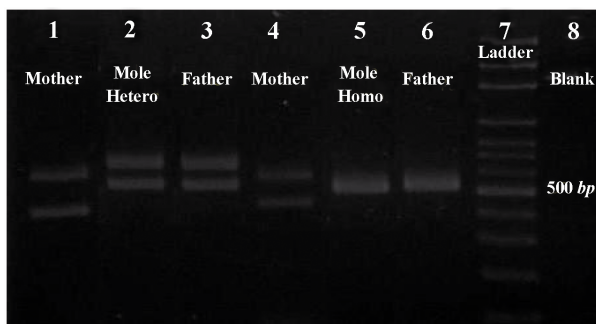


Figure 2. Results of the PCR amplification of D1S80 VNTR locus, line 1: mother; line 2: CHM of heterozygous; line 3: father; line 4: mother; line 5: CHM of homozygous; line 6: father; line 7: ladder; line 8: blank

then compared with the corresponding allele sizes observed in the maternal and paternal samples as shown in figures 2-4.

Results

The results of DNA analysis by VNTR genotyping as well as other related information about patients including clinical pa-

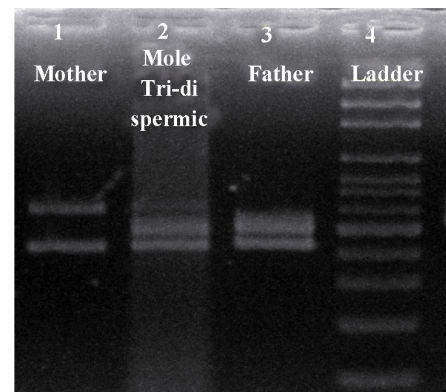


Figure 3. Results of the PCR amplification of D1S80 VNTR locus, line 1: mother; line 2: PHM of triploid dispermic; line 3: father; line 4: ladder

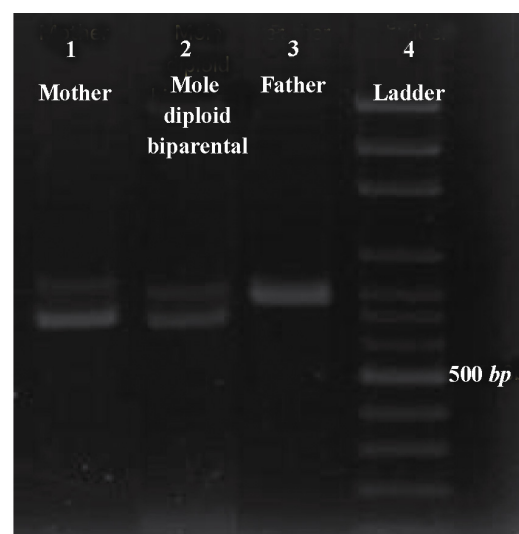


Figure 4. Results of the PCR amplification of Col2A1 VNTR locus, line 1: mother; line 2: PHM of diploid biparental; line 3: father; line 4: ladder

Table 2. Clinical parameter, histological diagnosis and genotyping findings of patients examined

Patient no.	Patient age(yrs)	Weeks of gestation	Pathologic diagnosis	B-hCG (mIU/ml)	Genotyping
1	29	6W	CHM	200000	A.mono S
2	24	6W+6d	CHM	120000	A.mono S
3	30	12W+3d	CHM	140000	A.mono S
4	27	7W+4d	CHM	100000	A.mono S
5	27	7W	CHM	98000	A.di S
6	20	9W	CHM	20700	A.di S
7	20	10W	CHM	40000	A.mono S
8	41	11W+5d	CHM	140000	A.mono S
9	29	9W+4d	CHM	14800	A.mono S
10	26	8W+5d	CHM	150000	A.mono S
11	27	10W+5d	CHM	180000	A.mono S
12	29	8W+1d	CHM	110000	A.mono S
13	22	11W+1d	CHM	150000	A.di S
14	23	11W+4d	CHM	140000	A.di S
15	38	10W+2d	CHM	120000	A.mono S
16	20	9W+6d	CHM	200000	A.mono S
17	22	11w+3d	CHM	20400	A.mono S
18	28	11W	CHM	300000	A.di S
19	28	9W+5d	CHM	273000	A.mono S
20	31	11W+6d	CHM	30000	A.mono S
21	15	12W	CHM	180000	A.mono S
22	33	7W+4d	PHM	225	Diploid biparental
23	30	9W+2d	PHM	1600	Triploid di S
24	31	8w+5d	PHM	1608	Diploid biparental
25	37	10W+3d	PHM	2000	Triploid di S
26	25	8W	PHM	1624	Triploid di S
27	35	11W+2d	PHM	1200	Triploid di S
28	30	9W	PHM	1524	Diploid biparental
29	31	10W+3d	PHM	17000	Triploid di S
30	24	11W	PHM	2650	Triploid di S

parameter and histologic diagnosis are shown in table 2. In 16 cases of CHM, amplification of the VNTR polymorphic loci showed androgenetic mono-spermic moles (homozygote) in molar tissue (76.2%). In 5 cases of CHM, androgenetic dispermic moles (heterozygote) were seen in molar tissue (23.8%). In cases of PHM, 6 samples were triploid dispermic (66.7%) and 3 samples were diploid biparental (33.3%).

Discussion

CHMs are generally diploid and androgenetic in origin, all 46 chromosomes being derived from the father. They may be mono-

spermic, arising by fertilization of an enucleate egg by a single spermatozoon which then doubles to provide a diploid chromosome complement, or dispermic arising from fertilization of an enucleate egg by two spermatozoa. PHMs are usually triploid, the extra haploid set of chromosome can have either a maternal origin (digynic triploidy) or a paternal one (diandric triploidy)^{14,15}.

Our results showed that 76.2% cases of CHM were homozygous and 23.8% heterozygous. Also, in cases of PHM, 66.7% were triploid dispermic and 33.3% diploid biparental. These results are consistent with reports in other investigations^{8,11}.

Persistent gestational trophoblastic disease develops after a CHM in 10 to 30% of cases and after a PHM in 0.5 to 5%^{21,22}. Homozygous and heterozygous CHM are two genetically distinct entities which can only be distinguished on the basis of genetic analysis and heterozygous mole may have a more malignant potential than its homozygous counterpart^{16,17}. In this study, five of 21 CHM cases, analyzed by genotyping, were found to be heterozygous and the remaining cases were homozygous.

Although many cases of hydropic abortion can be diagnosed on the basis of histology alone, it is difficult to estimate the proportion of cases evacuated at an early gestational age^{1,2,15}. Thus, accurate diagnosis of abnormal product of conception specimens and subsequent classification as complete or partial mole is mandatory to ascertain the actual risk of persistent GTD and to determine the appropriate nature and duration of clinical follow-up care. More advanced molecular genetics techniques have subsequently been applied to resolve this differential diagnosis problem¹⁵⁻¹⁷.

The PCR amplification of polymorphic VNTR region will be a useful tool in helping to determine relative risk of choriocarcinoma in patients with complete hydatidiform moles²⁰. VNTRs are the most informative markers for genetic studies. Although each VNTR locus is usually associated with a large number of different alleles in any population, each individual carries two alleles, one on each of the two homologous chromosomes. The major methodological advantage of VNTR is that all alleles are stable across generation, they are inherited in a Mendelian fashion and do not vary from parents to offspring^{25,27}.

Other techniques that can be used for genetic evaluation of molar pregnancy include Fluorescence In Situ Hybridization (FISH), flow cytometry and fluorescent microsatellite genotyping. However, comparison of VNTR genotyping with other methods showed that VNTR method is very simple, thus does not

require special device and also it is less time-consuming and more cost-effective.

In this study, it was shown that VNTR genotyping can identify the parental source of polymorphic alleles and thus discern androgenetic diploidy (homozygous and heterozygous), diandric triploidy, and biparental diploidy, which allows for specific diagnosis of CHMs, PHMs, and NMs, respectively. In a similar study, choriocarcinoma analyzed by PCR amplification of polymorphic VNTR in 9 out of 10 cases was identified as heterozygous²⁰.

The reliability of the diagnosis is crucial for appropriate counseling and to determine if a patient falls in to a "short-term" or "long-term" follow-up. In some instances, for example in women having a "lesion suspicious for HM", the diagnosis could be confirmed or ruled out, thus avoiding an unnecessary follow-up. This may be of particular importance in "older patients" having difficulties in conceiving and for whom a one-year wait may be extremely distressing^{9,13,16,24,32}.

Conclusion

In conclusion, the majority of cases of hydropic abortion can be diagnosed based on histological feature alone, but molecular biological techniques will be necessary to evaluate the proportion of cases evacuated at an early gestational age. Our results indicate that VNTR genotyping, a relatively simple, reliable, cost-efficient and rapid procedure, can be used successfully for diagnosis of HM and distinguishing CHM from its mimics, which is clinically important because the majority of persistent trophoblastic diseases and choriocarcinomas arise from CHM.

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