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Genetic Study of Hydatidiform Moles by Restriction Fragment Length Polymorphisms(RFLPs) Analysis

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Twenty three hydatidiform moles (HMs) were studied using the techniques of "RFLPs" employing a minisatellite deoxyribonucleic acid probe. Among the 23 HMs, 17 were homozygous types resulting from a duplicated haploid sperm, and two were heterozygous types resulting from fertilization two independent sperms(dispermy). It was revealed that the four histopathologically diagnosed complete HMs (CHMs) were partial HMs (PHMs) with one maternal and 2 paternal chromosome contribution(diandry) or two maternal and 1 paternal alleles (digyny). The locus specific minisatellite probes were useful in classifying CHM into heterozygous and homozygous types as well as in diagnosing PHM. One heterozygous (50%) and 5 homozygous (29. 4%) CHMs, and one PHM (25%) progressed to persistent gestational trophoblastic disease (p>0.5).

Key Words: *hydatidiform mole, heterozygous CHM, homozygous CHM, partial mole, restriction fragment length polymorphisms, locus specific minisatellite probe*

INTRODUCTION

HM, typically characterized by hydropic swelling of the villi and hyperplasia of the trophoblast (Szulman & Surti, 1978), can progress to a trophoblastic tumor (Fisher et al.,1989). Vassilakos & Kajii (1976) classified HM into CHM and PHM based on histopathologic appearance. Recently, cytogenetic studies on HM revealed androgenesis playing an important role in the genesis of HM (Jacobs et al., 1980; Lurain et al., 1983; Coullins et al., 1985; Verjerslev et al., 1987; Azuma et al., 1991), and the techniques of molecular biology, especially those of RFLPs, have subsequently subdivided CHM into homozygous and heterozygous type.

Investigators have discovered that the majority of CHMs are homozygous type, which appear to originate from fertilization of an empty egg by a duplicated haploid sperm (Lawler et al., 1979 ; Jacobs, et al., 1980) with chromosome constitution of 46,XX. About 10% of CHMs are heterozygous type arising from fertilization of an enucleated egg by two different sperms (dispermy) (Ohama et al., 1981; Patillo et al., 1981; Surti et al., 1982; Fisher et al., 1984). This type of CHM, thus, has the karyotype of either 46, XX or 46, XY. PHMs, on the other hand, are predominantly triploid (Szulman & Surti, 1978) having one maternal and two paternal halploid genomes (Jacobs et al., 1982; Lawler et al., 1982) or two maternal and one paternal contributions (Szulman and Surti, 1984).

CHM is more likely to progress to trophoblastic tumor than PHM (Davis et al., 1984; Wake et al., 1984; Lawler et al., 1991). Among CHMs, an increased risk of malignant transformation has been reported in the heterozygous type compared with the homozygous one (Kajii, 1980; Ohama et al., 1981; Davis et al., 1984; Wake et al., 1984; Wake et al., 1987).

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The 46, XY heterozygous CHM can be distinguished from other types of CHM by using cytogenetic techniques such as karyotyping, Y-body staining (Davis et al., 1984) or by using DNA probes specific for Y chromosome sequences. The heterozygous CHM with 46, XX genotype can be identified possible by using multiple locus specific minisatellite probes (Wong et al., 1986) designed to detect RFLPs in individual DNA. Several other probes has been used for detection of similar RFLPs, but different results were obtained due to the heterozygosity of genomic structure for the probe (1989; Wong et al., 1987: Fisher et al).
This study was attempted to study HM genetically and relate its genetic type to the risk of malignant transformation. We analyzed the DNAs from various HMs by RFLPs analysis. Patients were followed up.

MATERIALS AND METHODS

Samples

Molar tissues were obtained from 23 patients by suction curettage. Blood samples (10ml) were collected from patients and their respective spouses and were heparinized. After washing to remove any maternal cells, the molar tissues were carefully separated and a portion was used for histopathologic examination and the remainder was frozen at -70°C for RFLPs analysis.

RFLPs

Three different hypervariable minisatellite probes (MR 24/1-HVR, α-globin 3'-HVR and Mucin-HVR) and a Y-chromosome specific DNA probe were supplied from Amersham (U.K.) (Table 1). The probe MR 24/1-HVR contained the pseudoautosomal region at the telomeres of the X and Y chromosomes. The frequency of heterozygosity was above 90% and the allele band size was a 2.0-5.0 kb. The α-Globin 3'-HVR consisted of a tandem repeat of 17 base pairs, and was localized to 16p12-pter. The heterozygosity was above 90% and the probe size was smaller than 4.0kb. The probe Mucin-HVR was localized to 1q21 and the allele band size ranged from 3.0 to 7.0 kb. The heterozygosity frequency was above 85%.

The size of the probe for Y-chromosome was 2.45 kb. The probes were isolated by *Hinf* I (Boerhinger Mannheim, Germany) digestion of the plasmid and gel electrophoresis analysis. The probes were labelled with [γ-^{32}P]-dCTP (Amersham, PB 10205) by random primed DNA labelling kit and the specific activity of the labelled probes was 1.8 X 10^9cpm/g.

DNAs from the molar tissues and mononuclear cells were isolated by proteinase K digestion and phenol extraction (Sambrook et al. 1989). DNAs (10 μg) were digested with *Hinf* I, electrophoresed in 0.8% agarose gels and transferred to nylon membranes (Hybond-N$^+$, Amersham, U.K.) as described by Southern(1975). The filter was prehybridized for 4 hours at 68°C in hybridization solution without the probe and then hybridized overnight at 68°C in hybridization buffer containing 5X SSPE, 0.2% SDS, 5X Denhardt's solution, 10% dextran sulfate and 150 μg/ml denatured sonicated salmon sperm DNA, and again hybridized overnight at 68°C in the prehybridization buffer containing labelled probe. The filter was washed twice at room temperature for 5 minutes in 1XSSPE/0.1% SDS and then twice at 65°C for 20 minutes in 0.1X SSPE/0.1% SDS.

Autoradiography

The filter was exposed to AGFA Curix-XP film (Agfa, Germany) and an intensifying screen a -75°C for 3-5 days. Probes were removed by placing the filters in the boiling solution of 0.1% SDS, 1mM EDTA for 2 minutes, which was followed by rapid cooling at 20°C in the same solution . In the autoradiographs of RFLPs, PHM usually had two paternal and one maternal alleles. When two paternal alleles were seen without maternal contribution, it represented heterozygous CHM. With the karyotype of 46, XX, or 46, XY, Y chromosome was distinguished using Y chromosome specific probes in certain cases. Homozygous CHM (46,XX) had one paternal contribution without maternal alleles.

Patient follow-up

After molar evacuation, patients were monitored weekly until serum β-hCG has been normal for three consecutive weeks, i.e., each time less than 1.5mIU/ml, which with no clinical or radiologic disease evidence was the criteria of complete remission. The diagnosis of persistent gestational trophoblastic disease (GTD) after evacuation was based on three consecutive rising or plateauing of serum β-hCG levels, the demonstration of metastasis, and/or a histopathologic diagnosis of choriocarcinoma from examination of surgical specimens or spontaneously passed tissue (Lurain et al., 1983).

Table 1. Locus specific minisatellite probes

	MR24/1-HVR	α-globin 3'-HVR	Mucin-HVR
Chromosomal localization	telomeres of the X & Y chromosome	16p 12-pter	1q21
Allele length range(kb)	2.0-5.0	<4.0	3.0-7.0
Heterozygosity	>90%	>90%	>85%

p : short arm of chromosome
q : long arm of chromosome
t : translocation
r : ring chromosome

Table 2. Genetic classification of HM

HM types		No. of Patients
CHM		
	Heterozygous	2
	Homozygous	17
PHM		4

HM : hydatidiform mole
CHM : complete hydatidiform mole
PHM : partial hydatidiform mole

Statistical analysis

Fisher's exact test was used where appropriate.

RESULTS

Results of autoradiographs

DNAs from various HM tissues, maternal and paternal mononuclear cells were digested with *Hinf* I and the polymorphism of each genome was tested with 3 different probes complementary to hypervariable regions (MR 24/1-HVR probe, α–globin-3'-HVR probe and Mucin-HVR probe) and a Y chromosome specific probe. In each figure, the numbers 1, 2 and 3 represent the allele of one case with the numbers 4, 5 and 6, and 7, 8, and 9 representing a second and a third case, respectively. The first lane of each case reflects maternal DNAs, while the second and the third lanes reflect molar and paternal DNAs, respectively.

Fig. 1 shows the autoradiograph from the hybridization using MR 24/1-HVR probe. Shown in molar DNAs (lane 2) are two maternal alleles(lane 1) and one paternal allele (lane 3) consistent with findings of PHM. Lane 5 (molar DNAs) showing two paternal bands (lane 6) without maternal contribution (lane 4) was comparable to that of a heterozygous CHM. The one case present with a paternal allele (lane 9) while lacking maternal alleles (lane 7) in lane 8 (molar DNAs) was that of a homozygous CHM.

The same membrane was hybridized using α–globin HVR probe after washing (Fig. 2). The first case with the molar DNAs (lane 2) showing two maternal (lane 1) and one paternal allele (lane 3) was a PHM. The second case showing the characteristics of heterozygous CHM revealed only two paternal alleles (lane 6). Any maternal alleles in lane 4 failed to be identified. The third molar DNAs (lane 8) expressed one paternal allele (lane 9) without any maternal alleles (lane 7), and were those of homozygous CHM. The apparent lack of maternal bands in the autoradiograph was probably due to the long size of-globin HVR probe.

The membrane using the mucin-HVR probe was seen in Fig. 3. The lane 2 (molar DNAs) showed two maternal (lane 1) and one paternal band (lane 3), believed to represent the PHM. Oddly enough, the heterozygous CHM (lane 5) showed one maternal (lane 4) and one paternal (lane 6) band. The molar DNAs of lane 8 expressed one maternal (lane 7) and one paternal (lane 9) contribution suggesting a homozygous CHM. The lower heterozygosity of mucin-HVR probe used in this study (Table 1) makes it necessary to use a probe of higher heterozygosity for detecting heterozygous CHM.

The Fig. 4 showed the film taken from the same membrane hybrydized by Y-chromosome specific probe. All paternal bands (lane 3, 6 and 9) and the DNA of 46,XY heterozygous CHM (lane 5) showed Y-chromosome. The molar DNAs in lanes 2 and 8 (PHM and homozygous CHM) did not show Y-chromosome.

Genetic classification of hydatidiform mole (Table 2)

Among the 23 HMs studied, all of which has

Table 3. Prognosis of HM based on genetic types

HM types	No. of SR[a](%)	No. of P-GTD[b](%)
CHM		
Heterozygous	1(50%)	1(50%)
Homozygous	12(70.6%)	5(29.4%)
PHM	3(75%)	1(25%)

a : spontaneous remission

b : persistent gestational trophoblastic disease

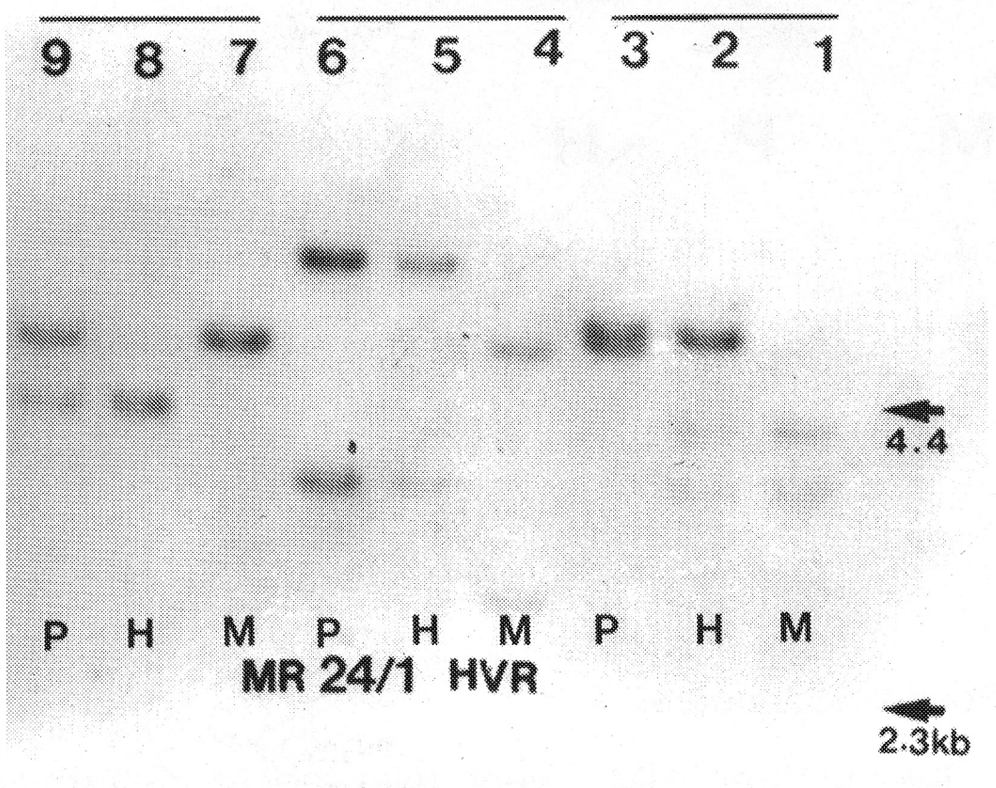

Fig. 1. Autoradiograph of RFLPs detected with locus-specific minisatellite MR 24/1-HVR probe in DNA 1, 2 & 3 lanes are PHMs, 4, 5 & 6 lanes are heterozygous CHM, 7, 8, & 9 lanes are homozygous CHM (M: patient, H: molar tissue, P: father).

been initially diagnosed as CHMs histopathologically, four were revealed as PHMs with the remainder, 19 revealed as CHMs by genetic analysis. The genetic analysis showed that CHMs were androgenetic in origin by excluding a maternal contribution to the genome.

PHM : PHM presents a triploid karyotype apparently resulted from two mechanisms of diandry, i.e., fertilization of a normal egg by two independent sperms(dispermy) and fertilization by a sperm carrying the total paternal load of 46, XY. A minority (20-25%) of triploids are of maternal origin and probably represent a state of digyny. Three of our 23 cases represented one maternal and two paternal alleles carried by diandry ; one of the them showing two maternal and one paternal allele (Fig.1-3, lanes 1-3) by digyny.

Heterozygous CHM : Heterozygous CHMs with 46, XY genotype can be identified by using cytogenetic techniques such as karyotyping, Y-body staining or by using Y chromosome specific DNA

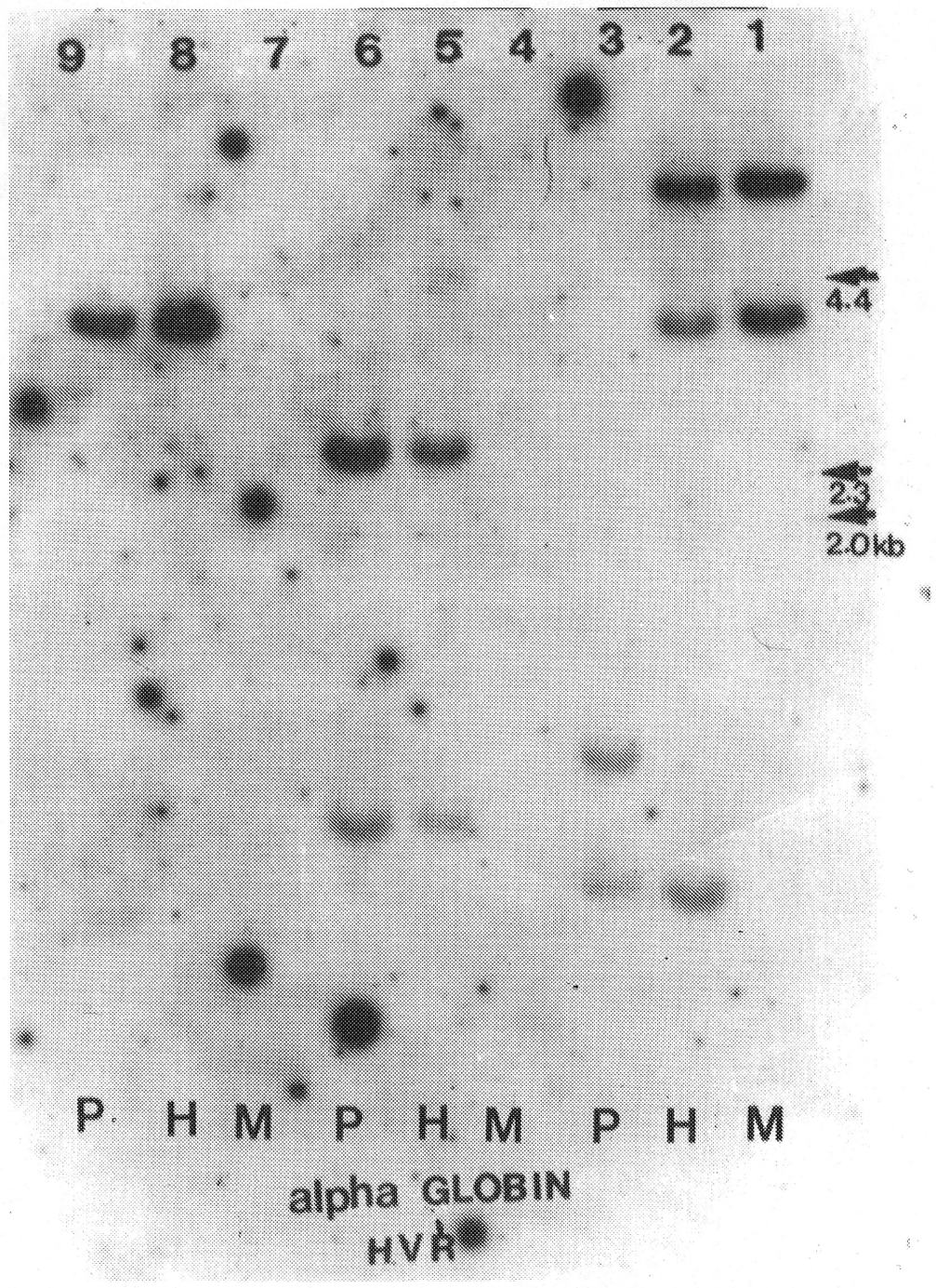

Fig. 2. Autoradiograph of RFLPs detected with α-globin-3'-HVR probe using same membrane.

probes. Two of our 19 CHM cases had two paternal alleles without maternal contribution (Fig.1-3, lanes 4-6) and represented Y chromosome identified by using DNA probes specific for Y chromosome sequences (Fig. 4, lane 5), which likely arose by dispermy. Their karyotype was 46, XY. Identifying a 46,XX CHM (heterozygous type) is difficult but possible using multiple locus specific minisatellite probes. However in this study, we found no 46, XX heterozygous CHMs.

Homozygous CHM : The majority of CHMs are homozygous type, which appear to originate from fertilization of an empty egg by a duplicated hap-

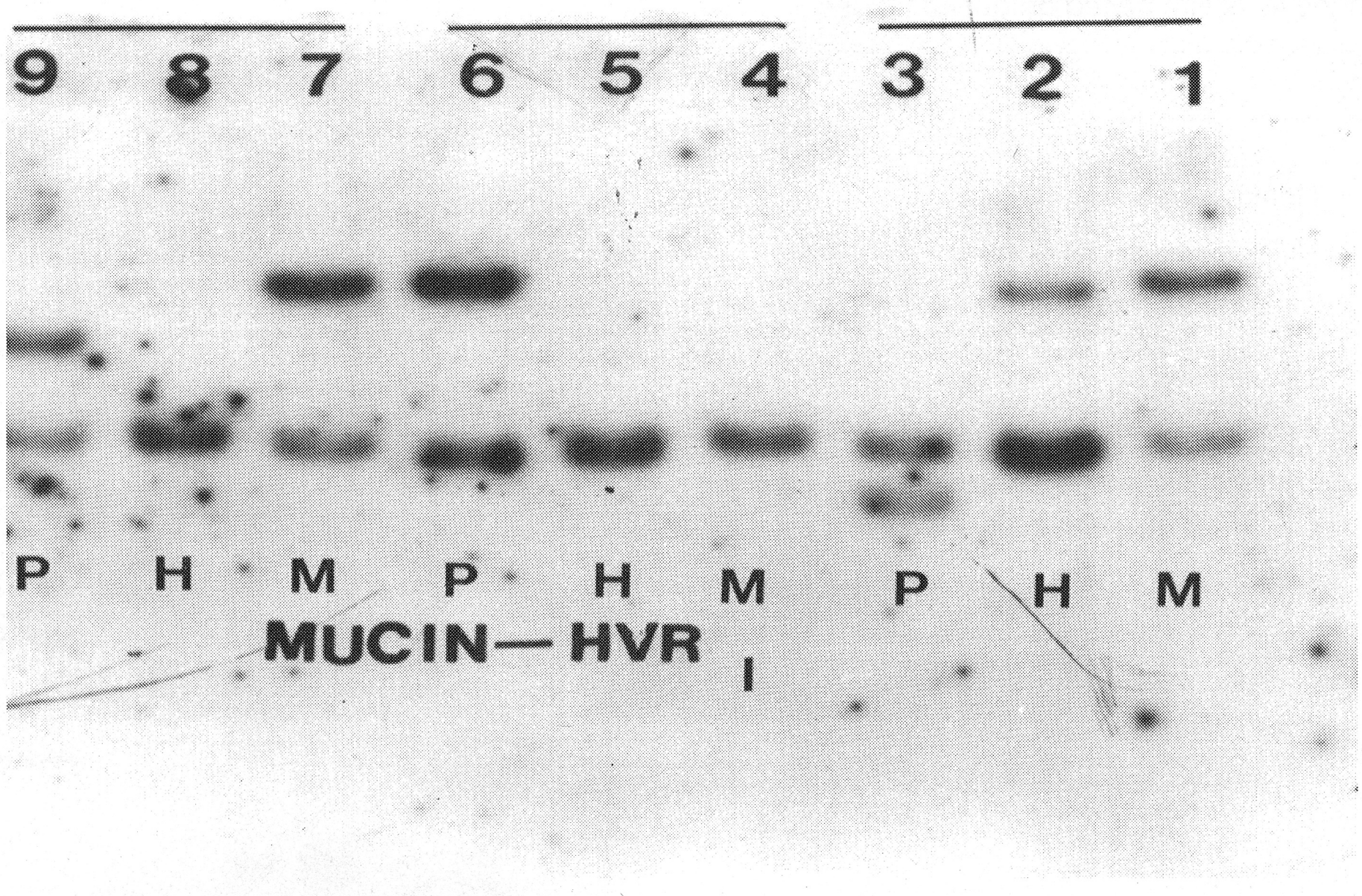

Fig. 3. Autoradiograph of RFLPs detected with Mucin-HVR probe using same membrane.

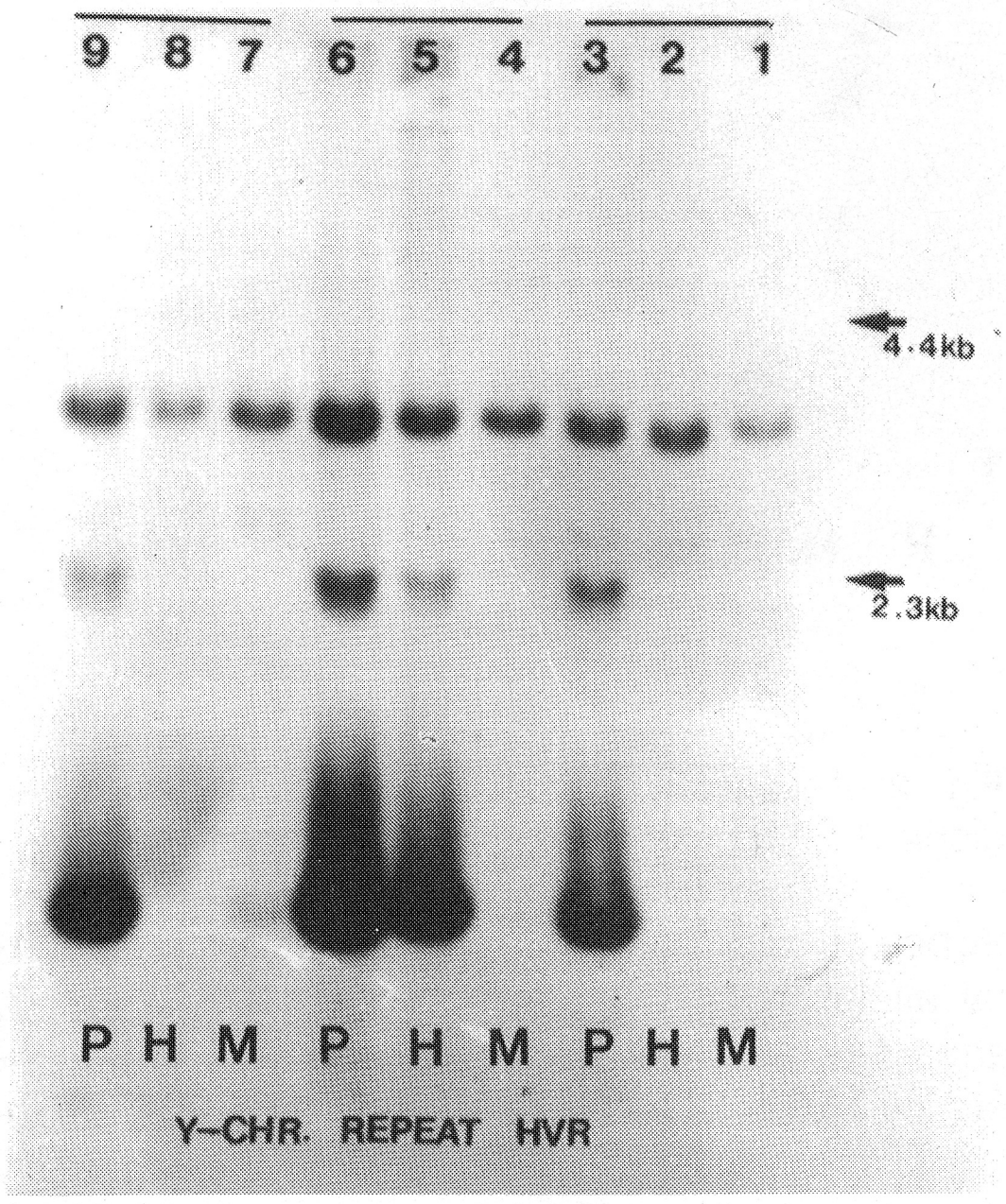

Fig. 4. Autoradiograph of RFLPs detected Y chromosome specific probe using same membrane.

loid sperm with chromosome constitution of 46,XX. Seventeen cases were homozygous CHMs having one paternal chromosome contribution without maternal alleles (Fig. 1-3, lanes 7-8).

Follow-up studies

Twenty-three HMs were followed to determine whether HM genotypes were related with their prognosis. According to the criteria discussed, one heterozygous CHM (1/2; 50%) , five homozygous CHMs (5/17; 29.4%), and one PHM (1/4; 25%) progressed to persistent GTD (Table 2). These values were, however, not significantly different ($p>0.5$).

DISCUSSION

Cytogenetic techniques such as karyotyping and RFLPs analysis made it possible to classify CHM into "heterozygous" and "homozygous" types (Wallace et al., 1982; Fisher et al., 1989). Prior to the advent of this new method of HM classification, HM used to be classified largely based on histopathologic findings alone unanswering how to clearly differentiate one type of HM, CHM or PHM, from the other(Kajii et al., 1984).

The RFLPs method has been used to study DNA polymorphisms that occur about once in 1000 nucleotides in a gene pool of 3 billion nucleotides inherited to each individual (Lander, 1989). This method detects the DNA polymorphism when the restriction enzyme digestion of a DNA gives rise to a fragment of an altered length compared with that of the other DNA. Restriction enzymes are site

specific endonucleases capable of recognizing six or four base sequences and cleaving the DNA at specific points. The length alteration of restriction fragments is measured by the mobility change on gel electrophoresis. When the blotting technique (Southern, 1975) is combined, gain or loss of a restriction site in the DNA can be easily revealed. In our study, 4 histologically diagnosed CHMs revealed themselves to be PHMs when tested by RFLPs method (Table 2).

Heterozygous CHM results from dispermy. Many previous reports indicate more prone nature of heterozygous CHM to become malignant than the homozygous counterpart (Kajii, 1980; Ohama et al., 1981; Davis et al., 1984; Wake et al., 1984; Wake et a., 1987). This view, however, is not shared by all (Fisher & Lawler, 1984; Lawler & Fisher, 1987; Lawler et al.,1991). One of the 2 patients in the current series diagnosed as heterozygous CHM showed high serum β-hCG level (1, 960,100 mIU/ml) and was treated with etoposide, methotrexate and actinomycin D according to the protocol described by Jones (Jones, 1990), while the other with a low level of β-hCG (55,200 mIU/ml) had the β-hCG level completly regressing spontaneously in 8 weeks without chemotherapy. In the mean time, only five of the 17 homozygous CHM patients in this study showed plateaued or rising serum β-hCG levels and were treated with either chemotherapy or adjuvant hysterectomy. One homozygous case was an invasive mole, revealed after hysterectomy.

The majority of PHM shows diandry : fertilization of a normal egg by two different sperms or fertilization by a spermatozoon carrying the father's total complement of 46, XY(meiosis-I error), which has two paternal and one maternal allele. However, 20 to 25 percent of PHM cases are maternal origin and present a state of digyny, apparently a result of failure of reduction division and contains the total diploid maternal complement of 46, XX chromosomes (meiosis-I error). It is fertilized by a normal haploid spermatozoon (Szulman and Surti, 1984), as was the case in one PHM in this series. PHM reportedly carries better prognosis than CHM (Szulman & Surti, 1984). Lawler et al. (1991) reported no progression to GTD from their PHM cases. In our series, one of the four PHM cases progressed to persistent gestational trophoblastic disease and subsequently received 6 cycles of Dactinomycin chemotherapy. It was the case where the initial β-hCG level was high (662,000 mIU/ml), and was present with intercurrent hepatitis.

This study failed to predict patient prognosis according to the genetic types of HM. The trend was worth noting, although it fell short of showing statistical significance, of CHMs showing unfavorable prognosis compared with that of PHMs. This issue may be addressed further employing a larger patient sample in a future endeavor. Prophylactic treatment for patients at risk for malignant transformation would then substantially decrease the frequency and the mortality associated with postmolar trophoblastic disease. In conclusion, the genetic analysis of HM provides new insights into the genesis of HM and is a powerful tool for understanding its clinical biology.

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