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ORIGINAL ARTICLE

Comprehensive genotype–phenotype correlations between *NLRP7* mutations and the balance between embryonic tissue differentiation and trophoblastic proliferation

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ABSTRACT

Background Hydatidiform mole (HM) is a human pregnancy with excessive trophoblastic proliferation and abnormal embryonic development that may be sporadic or recurrent. In the sporadic form, the HM phenotype is driven by an abnormal ratio of paternal to maternal genomes, whereas in the recurrent form, the HM phenotype is caused by maternal-recessive mutations, mostly in *NLRP7*, despite the diploid biparental origin of the HM tissues. In this study, we characterised the expression of the imprinted, maternally expressed gene, *CDKN1C* ($p57^{KIP2}$), the genotype, and the histopathology of 36 products of conception (POC) from patients with two defective alleles in *NLRP7* and looked for potential correlations between the nature of the mutations in the patients and the various HM features.

Methods/results We found that all the 36 POCs are diploid biparental and have the same parental contribution to their genomes. However, some of them expressed variable levels of $p57^{KIP2}$ and this expression was strongly associated with the presence of embryonic tissues of inner cell mass origin and mild trophoblastic proliferation, which are features of triploid partial HMs, and were associated with missense mutations. Negative $p57^{KIP2}$ expression was associated with the absence of embryonic tissues and excessive trophoblastic proliferation, which are features of androgenetic complete HMs and were associated with protein-truncating mutations.

Conclusions Our data suggest that *NLRP7*, depending on the severity of its mutations, regulates the imprinted expression of $p57^{KIP2}$ and consequently the balance between tissue differentiation and proliferation during early human development. This role is novel and could not have been revealed by any other approach on somatic cells.

INTRODUCTION

Hydatidiform mole (HM) is an abnormal human pregnancy characterised by the absence of, or abnormal, embryonic development, excessive trophoblastic proliferation and hydropic degeneration of chorionic villi. Common moles are usually sporadic, not recurrent and affect 1 in 600 pregnancies in western countries[1] but have higher frequencies in developing countries.[2 3] Based on histopathological examination, HMs are divided into two categories, complete HMs (CHMs) and partial HMs (PHMs). CHMs display circumferential trophoblastic proliferation and do not contain extraembryonic membranes (chorion and amnion), fetal nucleated red blood cells (NRBC) or any other embryonic tissue of inner cell mass origin. PHMs have moderate focal trophoblastic proliferation and may contain extraembryonic membranes and/or embryonic tissues of inner cell mass origin.

Common sporadic CHMs are mostly diploid androgenetic with two copies of the paternal genome. Common sporadic PHMs are mostly triploid dispermic with two different copies of the paternal genome and one copy of the maternal genome. $p57^{KIP2}$ is the product of the paternally imprinted, maternally expressed gene, *CDKN1C*, which is expressed in the nuclei of cytotrophoblast and some stromal cells of triploid dispermic PHMs, but not in those of diploid androgenetic CHMs.[4] Consequently, $p57^{KIP2}$ immunohistochemistry is an ancillary marker that is commonly used by pathologists to indirectly identify the presence of the maternal genome and help them in dividing HMs into PHMs and CHMs, which may share some histopathological features, when evacuated at early gestational stages.[5] This differential expression of $p57^{KIP2}$ is believed to be due to the absence of the maternal genome in androgenetic CHMs. However, even in androgenetic CHMs, $p57^{KIP2}$ is expressed in the nuclei of extravillous trophoblast cells, which indicates that $p57^{KIP2}$ imprinting is not maintained on paternal alleles in all trophoblastic cells. Consequently, the exact mechanism underlying the imprinted expression of $p57^{KIP2}$ only in some cellular types of first trimester placenta is not known.[6 7] Ki-67 is a nuclear protein coded by *MKI67* (antigen identified by monoclonal antibody Ki-67) and a proliferation marker known to be expressed in all active phases of the cell cycle (G1, S, G2 and mitosis), but not in resting G_0 cells.[8] Ki-67 is expressed in normal first trimester human placenta; however, its expression level is higher in sporadic HMs. Among these, triploid PHMs express lower Ki-67 levels than androgenetic

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CHMs,[9 10] which reflects their milder trophoblastic proliferation. Recurrent HMs occur in 1%–6% of patients with a prior mole[11–17] and may occur in patients with no family history of HMs (singleton cases) or in related women from the same family (familial cases). By studying familial cases of recurrent HMs, two maternal effect genes, *NLRP7* and *KHDC3L*, responsible for recurrent HMs have been identified.[18 19] *NLRP7* is a major gene for recurrent HMs and is mutated in 48%–80% of patients, depending on patients' ascertainment criteria and populations.[20–23] *KHDC3L* is a minor gene for recurrent HMs and is mutated in only 10%–14% of patients with no *NLRP7* mutations.[19 23 24] To date, approximately 47 different mutations have been reported in patients with two *NLRP7*-defective alleles (http://fmf.igh.cnrs.fr/ISSAID/infevers/). The role of NLRP7 protein in the pathophysiology of moles is not fully understood, but we do know that NLRP7 downregulates intracellular inflammation and impairs interleukin-1β secretion in various cellular models,[25–27] including peripheral blood mononuclear cells from patients with two *NLRP7*-defective alleles.[27] Recently, a study by Mahadevan *et al* demonstrated that *NLRP7* knockdown in human embryonic stem cells accelerates trophoblast differentiation.[28]

At the genotypic level, the parental contribution to approximately 80 HMs from patients with two *NLRP7*-defective alleles has been analysed so far and were found all diploid biparental[21–23 29–39] with the exception of two moles that were found to be triploid digynic[23] and triploid diandric.[38] Despite their diploid biparental genome, HMs from patients with *NLRP7* or *KHDC3L* mutations lack maternal methylation marks on several imprinted, paternally expressed genes and display gain of methylation marks on some imprinted, maternally expressed genes.[21 40–42] Recently, altered DNA methylation in cells with *NLRP7* mutations or knockdown has been shown to extend beyond imprinted genes and affect many non-imprinted genes.[28 43 44] Using immunohistochemistry, four studies have investigated the imprinted expression of $p57^{KIP2}$ in diploid biparental CHMs from patients with two *NLRP7*-defective alleles. These studies demonstrated the absence of $p57^{KIP2}$ expression in the cytotrophoblast and villous stroma of these diploid biparental moles similar to the absence of $p57^{KIP2}$ expression in androgenetic CHMs.[23 37 39 45] To date, no studies have investigated Ki-67 expression in diploid biparental moles caused by *NLRP7* mutations.

To better understand the role of *NLRP7* mutations in HMs, we first characterised $p57^{KIP2}$ expression in 36 products of conception (POCs), mostly HMs, from patients with two *NLRP7*-defective alleles. We found that some of them express variable levels of $p57^{KIP2}$ in the cytotrophoblast and villous stroma, which was in contradiction with previously reported data in the field and suggested either the presence of aneuploidies, genotypic mosaicisms or incomplete inactivation of $p57^{KIP2}$. We next used three DNA-based approaches to comprehensively characterise these tissues and demonstrated their diploid biparental genome. We looked for potential correlation between $p57^{KIP2}$ expression, the nature of mutations, Ki-67 expression and morphological features of the HMs. We found that some missense mutations do not completely repress $p57^{KIP2}$ expression and are associated with the presence of embryonic tissues of inner cell mass origin, mild trophoblastic proliferation and low expression of Ki-67. However, protein-truncating mutations repress $p57^{KIP2}$ expression and are associated with the absence of embryonic tissues of inner cell mass origin and the presence of excessive trophoblastic proliferation.

MATERIALS AND METHODS

Patients and mutation analysis

A total of 36 POCs from 17 patients were included in this study. Patients were referred to our laboratory from various national and international collaborators for DNA testing. All the patients have been screened for *NLRP7* mutations as previously described,[18] and the results of their mutation analysis were either previously reported[18 20 33 34 46] or generated during this study and are described in the 'Results' section and online supplementary table I.

Histopathological characterisation of the POCs

Morphological examination

For morphological examination and diagnosis, sections from the POCs were stained with H&E, examined and scored independently by two pathologists for the degree of trophoblastic proliferation, the degree of hydropic changes and the presence of extra-embryonic membranes, NRBC and/or other embryonic tissues according to previously reported guidelines.[47] The two pathologists were blinded to the data generated by the other methods, and we deliberately did not change the histopathological diagnosis based on the results of the other methods because our aim is to look for correlations between the parental contribution to the POC genomes, the histopathological features of the HMs and the mutations in the patients. The number of available blocks and/or slides from each POC is provided in table 1 and online supplementary table I.

$p57^{KIP2}$ immunohistochemistry

$p57^{KIP2}$ immunohistochemistry were performed on 4 µm sections from formalin-fixed paraffin-embedded tissues as previously described.[5] For all cases of $p57^{KIP2}$ immunohistochemistry, the presence or absence of nuclear staining was assessed in cells from the cytotrophoblast, villous stroma, extravillous trophoblast and maternal decidua, independent of histopathological and genotyping data. $p57^{KIP2}$ was considered 'conclusive' when maternal decidua and/or extravillous trophoblastic cells, serving as internal positive control, exhibited nuclear expression of $p57^{KIP2}$ in several areas of the analysed slides. Cases were considered 'inconclusive' when maternal decidua and/or extravillous trophoblastic cells did not express $p57^{KIP2}$ or when the staining of $p57^{KIP2}$ was not nuclear due to non-optimal quality of tissue preparation or immunohistochemistry.

Ki-67 immunohistochemistry

Ki-67 expression level was evaluated by scoring the percentage of positive cells in the cytotrophoblast in 10 different fields. Immunohistochemistry analysis was performed under light microscopy at 200× magnification.

Comprehensive characterisation of the parental contribution to POC

Microsatellite genotyping

Five serial 8 µm sections were prepared from paraffin blocks containing the largest amount of chorionic villi that are separated from maternal tissues. These sections were stained with H&E and areas containing chorionic villi were defined using a stereomicroscope or an inverted microscope. Pinpoint solution (Zymo Research, Orange, California, USA) was applied to the areas that only contain chorionic villi and was left to dry for 30–45 min at room temperature.[48] The tissues were removed with the Pinpoint gel and were used for DNA extraction using phenol-chloroform. Multiplex fluorescent microsatellite

Table 1 Recapitulation of p57^{KIP2} and Ki-67 expression by immunohistochemistry, presence of embryonic tissues of inner cell mass origin, histopathology and mutations of 32 products of conceptions (POCs) from patients with two *NLRP7*-defective alleles

Case ID-patient ID	Block ID (N)	GA in weeks	p57^{KIP2} expression		Inner cell mass derivatives	Ki-67 expression % of positive CT cells	Pathologists		*NLRP7* mutations or NSVs in the patients
			CT	VM			1	2	
MoLb1-4	2151 (1)	8	+++	+++	No	n.a	CHM	CHM	p.[**G118fs;** V319I];[**G118fs;** V319I]
Molb1-6	4199 (1)	11	+++	+++	No	70%	CHM	CHM	p.[**G118fs;** V319I];[**G118fs;** V319I]
MoUs99-655	6526 (6)	n.a	+++	+++	Membranes	51%	PHM	PHM	p.[**L750V**];[**L750V**]
MoUs99-657	238 (6)	17	+++	+++	Membranes	13%	PHM	PHM	p.[**L750V**];[**L750V**]
Mous167-712	3932 (1)	n.a	+++	+++	No	n.a	CHM	CHM	p.[V319I(;)**P716A**(;)**Cys931X**]
MoCa179-744	27404 (4)	8	+++	+++	No	69%	CHM	eCHM	p.[**E340QfsX10**];[**R693W**]
MoLb1-6	1524 (1)	n.a	+++	−−−	No	n.a	CHM	PHM	p.[**G118fs;** V319I];[**G118fs;** V319I]
MoLb1-6	6190 (1)	n.a	+++	−−−	No	n.a	CHM	PHM	p.[**G118fs;** V319I];[**G118fs;** V319I]
MoUs99-655	1554 (11)	9	+++	−−−	NRBC, membranes	31%	SA	SA	p.[**L750V**];[**L750V**]
MoFr101-662	M251 (1)	9	+++ (45%), −−−(55%)	−−−	Complete fetus with a mole	n.a	PHM	SA	p.[**L964P**];[**L964P**]
MoIn103-671	G1814 (1)	8	+++ (58%), −−−(42%)	−−−	No	99%	PHM	PHM	p.[**R693P**];[**R693P**]
MoUs99-655	7246 (3)	9	−−−(95%), ++(5%)	−−−	NRBC	81%	PHM	PHM	p.[**L750V**];[**L750V**]
MoUs99-655	2777 (10)	9	−−−(95%), ++(5%)	−−−	NRBC	58%	PHM	PHM	p.[**L750V**];[**L750V**]
MoLb1-4	5411 (2)	14	−−−	−−−	No	n.a	CHM	CHM	p.[**G118fs;** V319I];[**G118fs;** V319I]
MoIn69-480	G1071 (2)	10	−−−	−−−	No	52%	HM	CHM	p.[**N913S**];[**R693P**]
MoCh76-519	523 (1)	7	−−−	−−−	No	100%	CHM	CHM	p.[**E99X**; V319I];[**D657V**]
MoUs99-657	7814 (3)	n.a	−−−	−−−	No	99%	CHM	CHM	p.[**L750V**];[**L750V**]
MoUs99-657	1858 (1)	n.a	−−−	−−−	No	96%	PHM	CHM	p.[**L750V**];[**L750V**]
MoIn104-674	G574 (2)	10	−−−	−−−	No	n.a	PHM	PHM	p.[**R693P**];[**R693P**]
MoNz 170-725	7759 (1)	n.a	−−−	−−−	No	n.a	CHM	CHM	p.[**Q310Hfs**; A481T];[**R693W**]
MoNz 170-725	8508 (1)	n.a	−−−	−−−	No	100%	CHM	CHM	p.[**Q310Hfs**; A481T];[**R693W**]
MoUs171-733	15636 (5)	n.a	−−−	−−−	No	71%	CHM	CHM	p.[**L750V**];[**L750V**]
MoUs171-733	3005 (2)	n.a	−−−	−−−	No	100%	CHM	CHM	p.[**L750V**];[**L750V**]
MoCa179-744	21689 (1)	9	−−−	−−−	No	100%	CHM	PHM	p.[**E340Qfs**];[**R693W**]
MoMx341-1074	9449 (6)	8	−−−	−−−	No	96%	CHM	CHM	p.[**Tyr872X**];c.[**2810+2T>G**]
MoCa179-744	100090 (3)	n.a	−−−	−−−	No	n.a	CHM	eCHM	p.[**E340QfsX10**];[**R693W**]
MoCa179-744	10282 (3)	n.a	−−−	−−−	No	99%	CHM	eCHM	p.[**E340QfsX10**];[**R693W**]
MoUs420-1200	8454 (3)	11.5	−−−	−−−	No	94%	CHM	CHM	p.[**R693Q(;)c.2130-2A>G=A/G**]
MoUs420-1200	5644 (15)	8	−−−	−−−	No	87%	PHM	PHM	p.[**R693Q(;)c.2130-2A>G=A/G**]
MoCa408-2000	10509 (2)	10.5	−−−	−−−	No	95%	CHM	CHM	p.[G487E; **Glu508Aspfs*27**];[G487E; **Glu508Aspfs*27**]
MoCa408-2000	17467 (1)	n.a	−−−	−−−	No	n.a	CHM	CHM	p.[G487E; **Glu508Aspfs*27**];[G487E; **Glu508Aspfs*27**]
MoCa408-2000	3661 (1)	8	−−−	−−−	No	100%	CHM	CHM	p.[G487E; **Glu508Aspfs*27**];[G487E; **Glu508Aspfs*27**]

The presence or absence of p57^{KIP2} expression in villous cytotrophoblast (CT) and mesenchyme (VM) are indicated by '+' or '−' ; +++, indicates 100% of cells are positive; ++, 20%–50% of cells are positive; ---, all cells are negative. Different populations of chorionic villi with discordant p57^{KIP2} expression are separated by a comma. The percentage of chorionic villi in each population compared with the total number of chorionic villi on the analysed slide is indicated between parentheses. Family ID is provided in the first column followed by the patient ID. Histopathological diagnosis of products of conception was made independently by two pathologists.
Mutations are in bold; rare or low-frequency non-synonymous variants (NSV) are underlined; common NSVs are in standard font.
HM, hydatidiform mole (HM is used when the pathologist did not distinguish between partial and complete HM); CHM, complete HM; PHM, partial HM; N, number of available blocks for each POC; NRBC, nucleated red blood cells; SA, spontaneous abortion.

genotyping was performed with PowerPlex 16 HS System (Promega, Corporation, Fitchburg, Wisconsin, USA). The reaction consisted of short tandem repeat multiplex PCR assays that amplify DNA at 15 different short tandem repeat loci and a fragment from the Amelogenin gene that distinguishes the X and Y chromosomes. Amplified PCR products were resolved by capillary electrophoresis using an ABI3130 Genetic Analyzer and the genotypes of the molar tissues were compared with those of the patients and their available partners in order to determine the parental origin of the alleles. The average number of amplified loci was 12.

Flow cytometry

Ten sections of 20 μm containing a substantial amount of chorionic villi from each available POC were used to assess the ploidy of the tissues by flow cytometry using propidium iodide according to standard methods.[49]

FISH

Fluorescent in situ hybridisation (FISH) was performed on 4 μm sections. Slides were hybridised with probes from the centromeres of three chromosomes, X, Y and 18, as previously described.[50] In addition, probes from other chromosomes were also used on some tissues to solve some genotypic discrepancies and to investigate whether additional peaks detected occasionally with microsatellite markers are due to trisomies. For each POC, more than 100 cells from different microscopic fields were scored with each probe.

Statistics

The significance of the association between the tissues with $p57^{KIP2}$ expression and the presence of embryonic tissues of inner cell mass origin (fetal membranes, NRBC or fetus) was determined by Fisher's exact test. Similar statistical test was used to compare between $p57^{KIP2}$ expression and the severity of the mutations, between the presence of embryonic tissues and the severity of the mutations. The significance of association between Ki-67 and $p57^{KIP2}$ expression was determined by two-tailed unpaired t test. All statistical tests were done using GraphPad Prism software; p values <0.05 were considered statistically significant.

RESULTS

Identification of three novel protein-truncating mutations in *NLRP7*

In this study, we analysed 36 POCs from a total of 17 patients, each with two *NLRP7*-defective alleles. *NLRP7* mutations found in 13 out of the 17 patients were previously reported.[18 33 34 46]

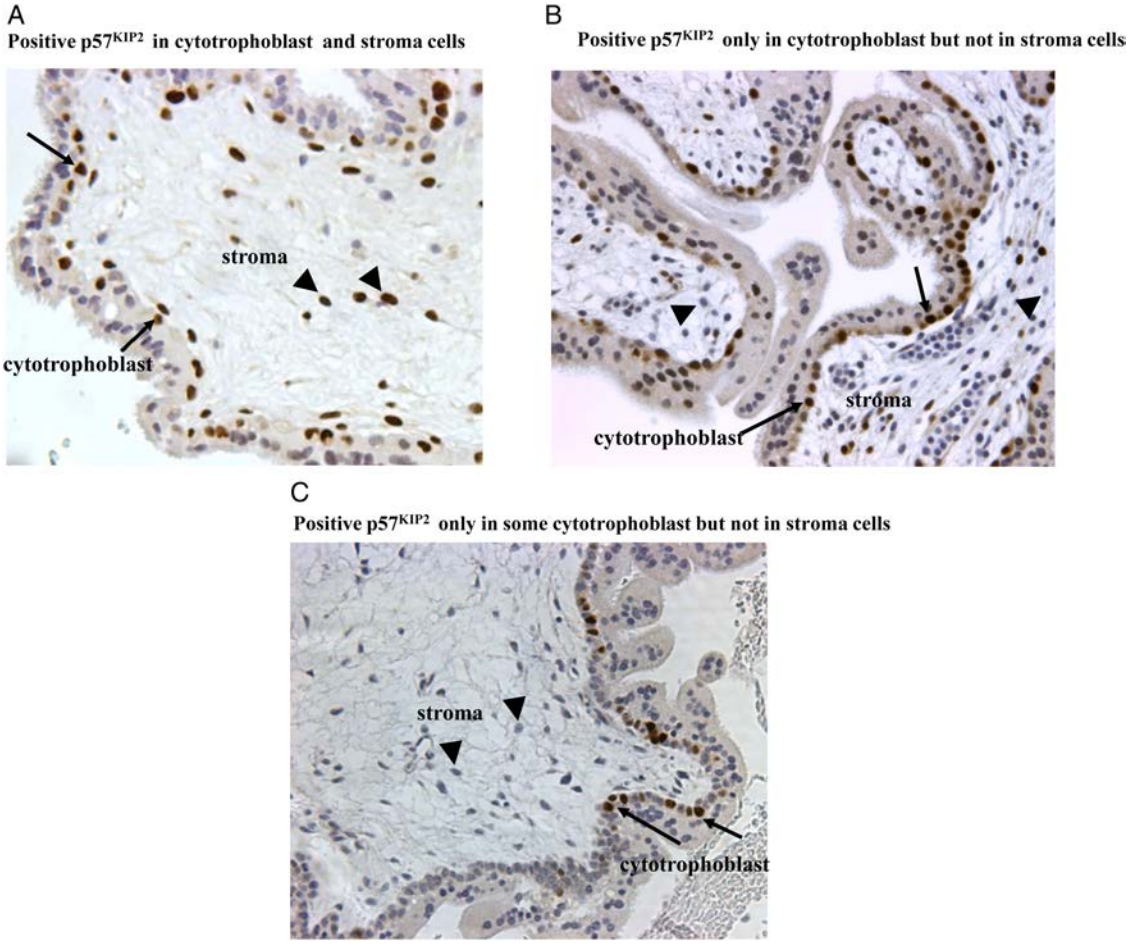


Figure 1 Examples of the variations in the expression of $p57^{KIP2}$ in diploid biparental hydatidiform moles from patients with two *NLRP7*-defective alleles. (A) In products of conception (POC) 6526 from patient 655, $p57^{KIP2}$ is expressed strongly (brown) in all nuclei of cytotrophoblast (CT) (arrow) and villous stroma cells (arrowhead) (magnification 200×). (B) In POC 1554 from patient 655, $p57^{KIP2}$ is expressed in all nuclei of CT (arrow) but not in villous stroma cells (arrowhead) (200×). (C) In POC 2777 from patient 655, $p57^{KIP2}$ is expressed in 20%–50% of CT (arrow) but not in villous stroma cells (arrowhead) (200×).

Mutation analysis in the remaining four new patients, 1074, 1142, 1200 and 2000, whose POCs are included in this study, was performed during this study as previously described.[18] This analysis identified three novel protein-truncating mutations, a stop codon, c.2616C>A, p. Tyr872Stop in exon 8; a splice mutation, c.2130-2A>G affecting the invariant acceptor site at the junction of intron 5 and exon 6; and an insertion of 22-bp, c.1517_1518ins22, p.Glu508Aspfs*27 in exon 4 (see online supplementary table I). In some new or previously reported patients, in which more than one mutation was found, the phase was established either by testing the parents for the identified DNA changes or by amplifying a PCR fragment containing both mutations, cloning and sequencing. The results of this analysis are summarised in online supplementary table I and are annotated according to the Human Genome Variation Society guidelines (http://www.hgvs.org/) for haplotype annotations. In conclusion, all the patients whose POCs are included in this study had two defective alleles in *NLRP7*.

Some HMs from patients with two *NLRP7*-defective alleles express $p57^{KIP2}$

Using immunohistochemistry, we first analysed the expression of $p57^{KIP2}$ in 36 POCs from 17 patients with two *NLRP7*-defective alleles. Of the analysed tissues, 32 were conclusive. Of these, 19 (59%) did not express $p57^{KIP2}$ in the cytotrophoblast or the villous stroma and were therefore $p57^{KIP2}$ negative and 13 (41%) displayed variable levels of $p57^{KIP2}$ positive cells ranging from 20% to 100% (table 1 and figure 1A–C). Among the 13 POCs with some $p57^{KIP2}$ expression, six expressed $p57^{KIP2}$ strongly in all cytotrophoblast and villous stroma cells (figure 1A); three expressed $p57^{KIP2}$ only in the cytotrophoblast, but not in the villous stroma, and this pattern was observed in all chorionic villi (figure 1B); and four expressed $p57^{KIP2}$ in 20%–50% of cytotrophoblast cells, but not in villous stroma cells, and this pattern was observed only in 5% of chorionic villi (table 1 and figure 1C). These data demonstrate that $p57^{KIP2}$ silencing does not occur in all diploid biparental moles and that some diploid biparental HMs from patients with two *NLRP7*-defective alleles do express $p57^{KIP2}$ in cytotrophoblast and villous stroma cells. The expression of $p57^{KIP2}$ in diploid biparental moles from patients with two *NLRP7*-defective alleles is novel and has not been previously reported.

Comprehensive characterisation of the parental contribution to HMs from patients with recessive *NLRP7* mutations

The presence of HMs with variable levels of positive $p57^{KIP2}$ expression raised the possibility that these tissues may be aneuploid, for instance triploid, in mosaic or non-mosaic state. We, therefore, undertook a comprehensive genotypic characterisation of the parental contribution to these 36 POCs using three

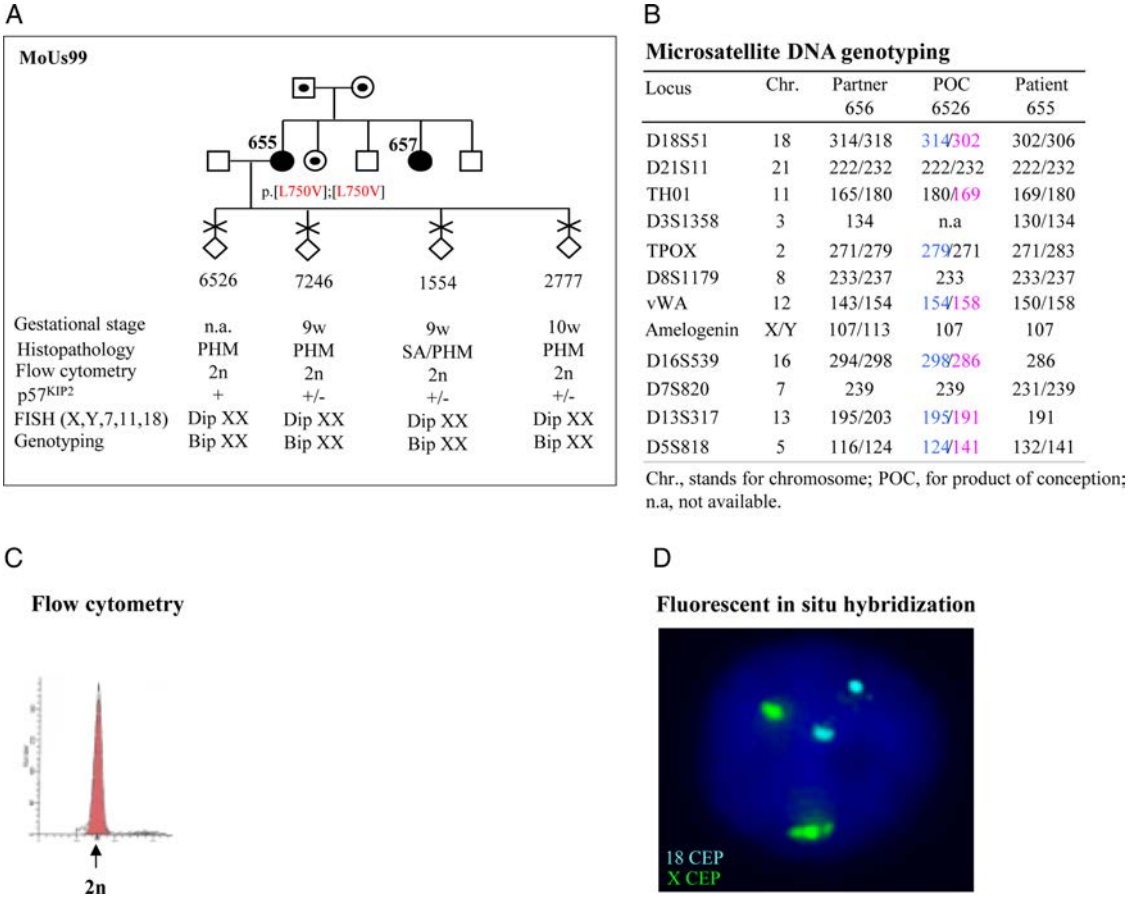


Figure 2 Representative example of our comprehensive analysis of products of conceptions (POCs) from patients with two defective alleles in *NLRP7*. (A) Pedigree structure, reproductive outcomes and recapitulation of the results of the characterisation of four POCs from patient 655 using three DNA-based approaches. (B) DNA genotyping demonstrating the biparental contribution to POC 6526. Maternal alleles are in pink and paternal alleles in blue. (C) Flow cytometry results demonstrating the presence of a single diploid DNA peak. (D) FISH with centromeric probes from chromosomes X, Y and 18 confirming diploidy and the presence of two X chromosomes in the POC. HM, hydatidiform moles; Dip, diploid; Bip, biparental; w, week; n.a., not available; PHM, partial hydatidiform mole; SA, spontaneous abortion.

different DNA-based approaches, microsatellite DNA genotyping, flow cytometry and FISH. Each of the used methods has its advantages and limitations. Of the three methods used in our genotypic evaluation, microsatellite DNA genotyping is the only one that allows determining the parental origin of DNA present in the POCs. A distortion in the heights of the maternal and paternal alleles is indicative of an imbalance in the ratio between the parental genomes. Flow cytometry is an easy and fast method to determine ploidy (2n or 3n). FISH is another method to determine ploidy and the only method to identify mosaicisms, but only in cases of mixed cellular populations with different ploidies, different gender or high frequency of aneuploidy cells. An example of our comprehensive genotyping approach is provided in figure 2.

The detailed results of the three approaches are summarised in online supplementary table I and demonstrated that all the POCs are diploid biparental. Among these 36 tissues, 35 had a single cellular population and 1 was found mosaic with two cellular populations. In this mosaic molar tissue, the main cellular population was diploid XY and was found in all cytotrophoblast cells and in 90% of villous stroma cells. The second minor cellular population was diploid XX and was found only in 10% of cells from the villous stroma (figure 3; see online supplementary figure S1). It is important to note that the mosaicism in this POC was not detected by the multiplex DNA genotyping due to the low amount of cells from the minor cellular population, which prevented us from determining its parental origin. However, we had noticed an imbalance in the heights of the X and Y allele peaks at the Amelogenin gene marker, which was not due to contamination with maternal DNA as judged by the profiles of other informative microsatellite markers (figure 3). It is, therefore, possible that this POC may have originated from a dispermic fertilisation followed by postzygotic diploidisation at the first cellular division leading to a diploid biparental XY cell and another diploid XX cell.[51] Such mosaicism would be in agreement with the mosaic $p57^{KIP2}$ pattern observed with this POC, but does not explain the positive expression of $p57^{KIP2}$ in the major cell line of this POC, which is diploid biparental. Therefore, our genotyping data on the 36 POCs demonstrate that molar tissues from patients with two *NLRP7* defective alleles are mostly diploid biparental with a single cellular population, but failed to identify aneuploidies that could underlie their positive $p57^{KIP2}$ expression.

$p57^{KIP2}$ expression correlates with the presence of embryonic tissues of inner cell mass origin

We next used histopathological examination to characterise the 36 POCs from patients with two *NLRP7* defective alleles. H&E slides were reviewed independently by two pathologists who were blinded to the genotyping results and $p57^{KIP2}$ staining. Of

A

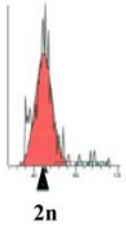
Multiplex microsatellite genotyping

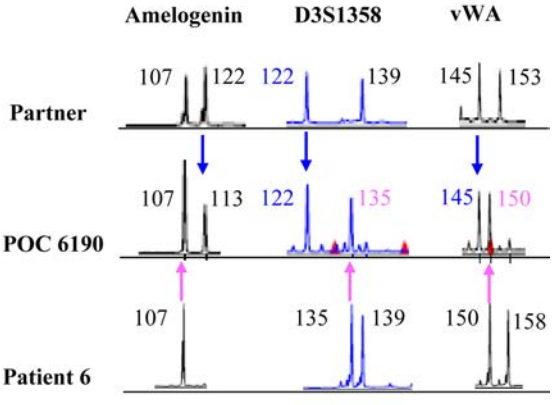
Loci	Chr	Partner	POC 6190	Patient 6
D21S11	21	222/232	222	222
TH01	11	166/178	178 /170	170/181
D3S1358	3	122/139	122 /135	135/139
vWA	12	145/153	145 / 150	150/158
Amelogenin	X/Y	107/113	107/113	107
D16S539	16	281	281/294	281/294
D7S820	7	227/231	227 /235	227/235
D13S317	13	191/199	191 /199	199
D5S818	5	133/137	133/137	133/137

Chr., stands for chromosome; POC, product of conception. The X allele with a higher peak at the Amelogenin marker is underlined.

B

Flow cytometry





C

Fluorescent in situ hybridization

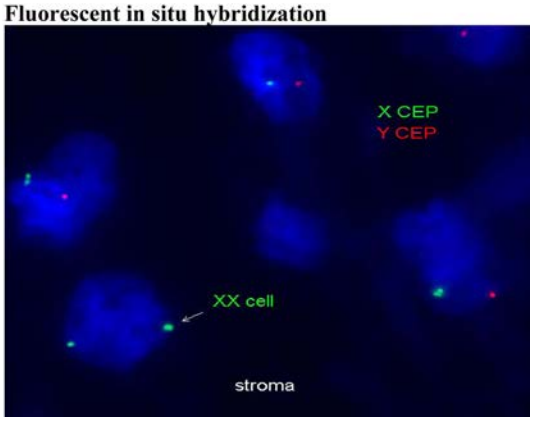


Figure 3 Mosaic mole from a patient with two *NLRP7*-defective alleles. (A) DNA genotyping demonstrating the biparental contribution to this hydatidiform mole. Maternal alleles are in pink and paternal alleles in blue. (B) Flow cytometry results demonstrating the presence of a single diploid DNA peak. (C) FISH with centromeric probes from chromosomes X and Y showed two cellular populations, diploid XY in the cytotrophoblast and villous stroma and diploid XX in approximately 10% of cells from the villous stroma. A photo of the whole villous showing the presence of the XX cells in the villous stroma is provided in online supplementary figure S1.

the analysed 36 POCs, there was an agreement between the two pathologists on the diagnosis of 81% of the cases, which is in line with previously reported data in the field.[52–54] Among the 32 POCs that were conclusive for $p57^{KIP2}$ staining, 13 expressed $p57^{KIP2}$ in the cytotrophoblast and/or the villous stroma and 6 (46%) of them had embryonic tissues of inner cell mass origin, namely, extraembryonic membranes and NRBC inside the chorionic villi (table 1) (figure 4, upper panel). These six POCs had mild trophoblastic proliferation and consequently were diagnosed as PHMs or non-molar spontaneous abortions (SAs). However, none of the 19 POCs that did not express $p57^{KIP2}$ had extra-embryonic membranes or NRBC (figure 4, lower panel). The association between positive expression of $p57^{KIP2}$ and the presence of embryonic tissues was highly significant (p=0.00189) (table 2). In addition, among the 32 analysed tissues, 12 were from patients with at least one protein-truncating mutation in the coding region (E99X, Q310Hfs, E340Qfs, Y872X, E508Dfs, C931X) and all these POCs did not have embryonic tissues of inner cell mass origin (p=0.04277) (table 2) and had strong trophoblastic proliferation. Moreover, 10 of these 12 tissues did not express $p57^{KIP2}$ at all (p=0.03191) (table 2).

These data demonstrate a significant association between missense *NRLP7* mutations (presumably with some residual activity), positive $p57^{KIP2}$ expression, the presence of embryonic tissues of inner cell mass origin and mild trophoblastic proliferation. On the contrary, truncating *NRLP7* mutations (presumed to completely abolish the function) correlated with negative $p57^{KIP2}$ expression and absence of embryonic tissues of inner cell mass origin. We note that some patients with invariant splice mutations had more variability in their reproductive outcomes than patients with protein-truncating mutations in the coding region. The best example of these is the case of family MoLb1, in which three patients are homozygous for an invariant splice mutation, c.352+1G>A, p.Gly118fs, and had the full spectrum of reproductive loss ranging from moles to early neonatal death and including SAs and stillbirths.[18] In addition, two patients from this family had, each, one live birth of a healthy baby and now adults. In this family, MoLb1, four moles were found $p57^{KIP2}$ positive (table 1).

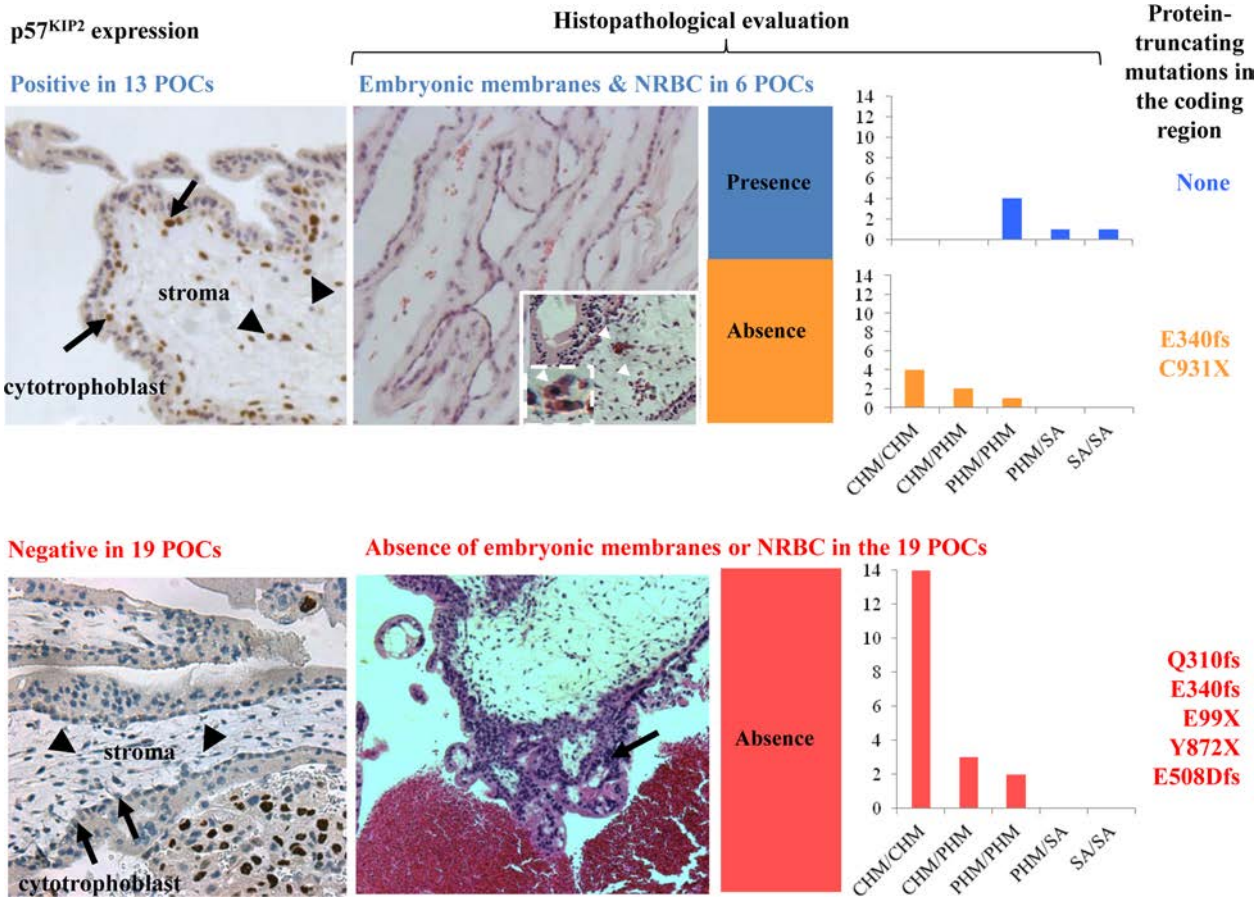


Figure 4 Recapitulation of $p57^{KIP2}$ expression, histopathological features of the hydatidiform moles and *NLRP7* mutations in the patients. In the upper panel, from left to right, a representative view from one product of conception (POC) demonstrating positive $p57^{KIP2}$ staining (brown colour) in both cytotrophoblast (arrow) and villous stroma (arrowhead) (200×); H&E staining of the same POC showing the presence of embryonic membranes (100×) and of another POC showing nucleated red blood cells inside a chorionic villous (200×); and final diagnoses made independently by two pathologists. Among the 13 POCs with some positive $p57^{KIP2}$ staining, six (blue) had embryonic tissues of inner cell mass origin (embryonic membranes and/or nucleated red blood cells) and seven did not have (orange). In the lower panel, from left to right, a representative view from one POC demonstrating negative $p57^{KIP2}$ staining in both cytotrophoblast (arrow) and villous stroma (arrowhead) (200×); H&E staining of the same POC showing circumferential trophoblastic proliferation (arrows) (100×); final diagnoses of the 19 POCs by two pathologists (red); and protein-truncating mutations in the coding region found in the patients who had these 19 $p57^{KIP2}$-negative POCs. Diagnoses by the two pathologists are separated by '/'. POC, product of conception; CHM, complete hydatidiform mole; PHM, partial hydatidiform mole; SA, spontaneous abortion; NRBC, nucleated red blood cells.

Table 2 Correlations between the severity of the mutations, $p57^{KIP2}$ expression and the hydatidiform mole (HM) features

	Embryonic development		$p57^{KIP2}$ expression	
	Present	Absent	Positive	Negative
$P57^{KIP2}$ expression				
Positive	6	7		
Negative	0	19		
	p=0.00189			
Mutation severity				
Missense	6	14	11	9
Truncating	0	12	2	10
	p=0.04277		p=0.03191	

Negative correlation between $p57^{KIP2}$ and Ki-67 expression

In the histopathological analysis, the degree of trophoblastic proliferation was evaluated by the two pathologists based on microscopic examination, which is a descriptive analysis known to be subject to interobserver and intraobserver variabilities.[52–54] To have a more accurate evaluation of the trophoblastic proliferation of the POCs from patients with two defective *NLRP7* alleles, we analysed 22 of the 32 POCs that were analysed with $p57^{KIP2}$ and from which sufficient materials were available for Ki-67 expression by immunohistochemistry. This analysis showed that the eight POCs that expressed $p57^{KIP2}$ had significantly lower levels of Ki-67 immunoreactivity in the cytotrophoblast than the 14 POCs that did not express $p57^{KIP2}$ (p=0.0012) (figure 5A–C).

DISCUSSION

To date, approximately 80 molar tissues from patients with two *NLRP7* mutations have been genotyped, but only with one method, and found mostly diploid biparental.[21–23 29–39] Of these, 37 were characterised for $p57^{KIP2}$ expression and were all found to be negative.[23 37 39 45] In this study, we performed $p57^{KIP2}$ immunohistochemistry on 36 POCs, from patients with two *NLRP7*-defective alleles and found that six of them are $p57^{KIP2}$ positive in all chorionic villi and seven express variable levels of $p57^{KIP2}$ protein in some chorionic villi. This raised the question about possible genomic aneuploidies, for instance, triploidy or mosaicism that could explain positive or mosaic $p57^{KIP2}$ expression.

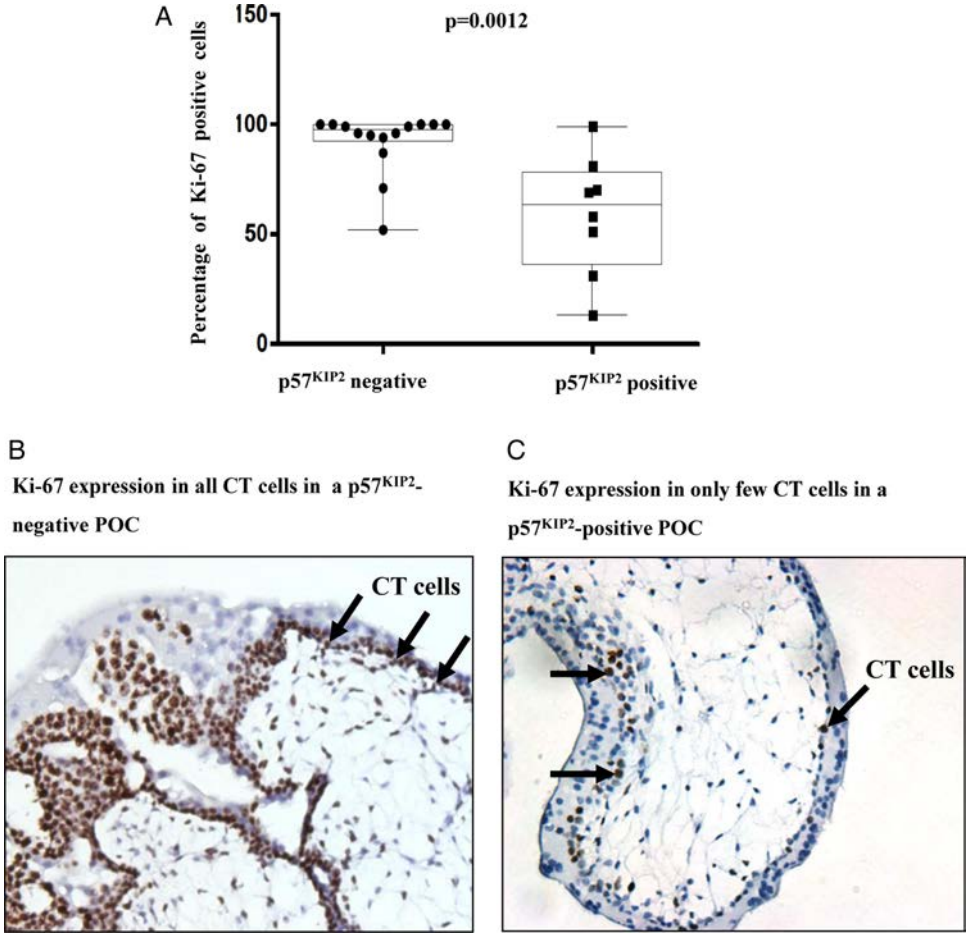


Figure 5 Ki-67 expression in 22 products of conceptions (POCs) from patients with two *NLRP7*-defective alleles. (A) Box plot comparing the expression of Ki-67 in moles that did not express $p57^{KIP2}$ and moles that expressed $p57^{KIP2}$. Fourteen moles that were negative for $p57^{KIP2}$ expression had significantly higher levels of Ki-67 expression in the nuclei of cytotrophoblast cells than eight moles with positive $p57^{KIP2}$ expression (p=0.0012). (B) Examples of different levels of Ki-67 staining. (Left panel) POC 8508 (from patient 725) demonstrating positive Ki-67 staining (brown) in all nuclei of cytotrophoblast (CT) (arrow). This POC did not express $p57^{KIP2}$ and is from a patient with one protein-truncating mutation in the coding region. (Right panel) POC 1554 (from patient 655) demonstrating positive Ki-67 staining in few CT cells (arrow). This POC expressed $p57^{KIP2}$, had embryonic membranes, mild trophoblastic proliferation and is from a patient with a missense mutation.

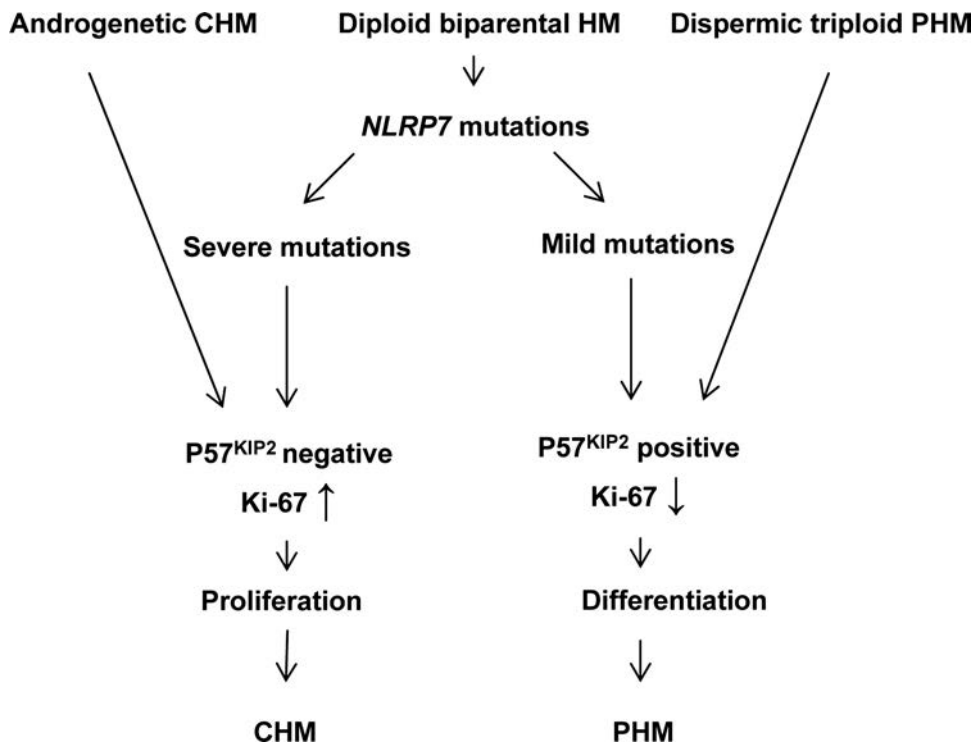


Figure 6 A suggested model of *NLRP7* action upstream of $p57^{KIP2}$ and Ki-67. Despite their different genotypes, both sporadic triploid dipsermic moles and some diploid biparental moles caused by mild *NLRP7* mutations acquire $p57^{KIP2}$ expression (positive) have low levels of Ki-67 expression, and are therefore diagnosed as partial hydatidiform moles. However, sporadic androgenetic moles and most diploid biparental moles caused by *NLRP7* protein-truncating mutations do not acquire $p57^{KIP2}$ expression (negative), have higher levels of Ki-67 expression and are therefore diagnosed as complete hydatidiform moles. CHM, complete hydatidiform mole; PHM, partial hydatidiform mole.

We, therefore, undertook a comprehensive characterisation of the 36 POCs using three DNA-based approaches to determine their parental contribution. We found that all the analysed POCs are diploid biparental with a single cellular population with the exception of only one that was found mosaic. Therefore, our data confirm previous reports[21–23 29–39] and demonstrate that HMs from patients with two mutated copies of *NLRP7* are mostly diploid biparental and exclude the presence of aneuploidies at the origin of positive $p57^{KIP2}$ expression in some of these tissues.

We next evaluated these tissues independently by two pathologists and found that missense mutations in *NLRP7* were associated with positive $p57^{KIP2}$ expression, the presence of embryonic tissues of inner cell mass origin and mild trophoblastic proliferation. However, protein-truncating mutations in the coding region of *NLRP7* were associated with negative $p57^{KIP2}$expression, absence of embryonic tissues of inner cell mass origin and severe trophoblastic proliferation. Interestingly, in all the analysed tissues, the trophoblastic proliferation was inversely correlated with that of $p57^{KIP2}$ expression, which indicates that these two functions, proliferation and differentiation, are tightly linked and regulated by the severity of *NLRP7* mutations. Among the four studies that have investigated $p57^{KIP2}$ expression in diploid biparental HMs from patients with two *NLRP7*-defective alleles,[23 37 39 45] one major and important study included 34 HMs and demonstrated that all of them are $p57^{KIP2}$ negative.[37] We explain the difference between $p57^{KIP2}$ expression in our study and that of Sebire *et al* by mainly three factors. First, in our analysis, we deliberately did not revise the histopathological diagnosis of the different POCs after the analysis of $p57^{KIP2}$ staining while Sebire *et al*, by analogy to the diagnosis of common sporadic androgenetic and triploid HMs, revised the final diagnosis of the conceptions based on $p57^{KIP2}$ expression. Second, in our study, we analysed all available POCs from the patients while Sebire *et al* analysed only the POCs that were diagnosed as HMs. Third, our analysis included more POCs from patients with missense mutations than that of Sebire *et al*. Therefore, our data are not in contradiction with those of Sebire *et al*, but simply, the two studies are not comparable because of their different inclusion criteria and design. Indeed, our study was designed to comprehensively characterise all the conceptions of patients with two *NLRP7*-defective alleles to better understand the effects of the various mutations and the origin of the variability in their reproductive outcomes.

CDKN1C is an imprinted, maternally expressed, gene in several mouse and human tissues. In humans, maternal loss-of-function mutations in *CDKN1C* are responsible for Beckwith–Wiedemann syndrome,[55] a paediatric overgrowth disorder in which the placenta share some histopathological features with PHMs.[56 57] In addition, a homozygous frameshift mutation in *NLRP2*, the closest *NLRP* gene to *NLRP7*, in the mother has been shown to be responsible for Beckwith–Wiedemann syndrome in her two offspring.[58] In mice, maternal $p57^{KIP2}$ null mutations lead to perinatal lethality due to altered cellular proliferation and differentiation in several tissues.[59–61] During mouse embryogenesis, positive $p57^{KIP2}$ expression is associated with terminally differentiated cells in several tissues.[62] Our data on molar tissues with the same parental contribution, diploid biparental and caused by recessive mutations in the same gene indicate that *NLRP7* plays, directly or indirectly, a role in the decision to switch between cellular differentiation and proliferation at a critical time during early development. Severe *NLRP7* mutations may prevent cytotrophoblast cells from exiting the cell cycle to terminally differentiate

and acquire $p57^{KIP2}$ expression and consequently, these cells continue to proliferate. However, mild mutations may allow some cytotrophoblast cells to exit the cell cycle to terminally differentiate, acquire $p57^{KIP2}$ expression and consequently, these cells stop to proliferate. Similar inverse correlations between $p57^{KIP2}$ and Ki-67 expression, tissue differentiation and proliferation have been observed in other cellular types, such as muscles, neurons, hepatocellular and pancreatic cancers.[63–66]

The most striking findings of our analyses are the similarities between partial diploid biparental moles, caused by mild *NLRP7*-defective alleles, and common partial triploid dispermic moles. The same similarities are observed between complete diploid biparental moles caused by severe *NLRP7*-defective alleles and common complete androgenetic moles (figure 6). Despite their different genotypes, both sporadic triploid dispermic moles and some diploid biparental moles caused by mild *NLRP7* mutations are $p57^{KIP2}$ positive, express low levels of Ki-67, have mild trophoblastic proliferation and may have some embryonic tissues. However, sporadic androgenetic moles and most diploid biparental moles caused by *NLRP7* protein-truncating mutations are $p57^{KIP2}$ negative, express high levels of Ki-67, have important trophoblastic proliferation and do not have embryonic tissues (figure 6). These data indicate that all mechanisms leading to HMs, including *NLRP7* mutations, act upstream of $p57^{KIP2}$ and Ki-67, and regulate the balance between tissue differentiation and proliferation.

The time at which *NLRP7* affects $p57^{KIP2}$ expression cannot be determined from our study, but the two genes are expressed in all oocytes and preimplantation stages.[18 67 68] Furthermore, previous studies have shown that mouse trophoblast stem cells express $p57^{KIP2}$ upon differentiation into trophoblast giant cells,[69] which are the equivalents of human extravillous trophoblast. The fact that $p57^{KIP2}$ expression was normal in the extravillous trophoblast of all molar tissues from patients with *NLRP7* mutations, but abnormal in the cytotrophoblast and the villous stroma indicates that *NLRP7* defects start to manifest after the terminal differentiation of the extravillous trophoblast and before the terminal differentiation of the cytotrophoblast and villous stroma cells. Our proposed role of *NLRP7* in regulating the balance between tissue differentiation and proliferation is in line with a recent interesting study demonstrating that reduced *NLRP7* expression in human embryonic stem cells alters trophoblast lineage differentiation.[28]

In conclusion, we report the most comprehensive and thorough analysis of 36 POCs from patients with two *NLRP7* mutations. Our data suggest that *NLRP7* mutations shift the cellular machinery from differentiation to proliferation at a very critical time during early development, a role that could not have been revealed by other approaches on somatic cells.

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Contributors NMPN performed DNA preparation and genotyping, $p57^{KIP2}$ screening and contributed to the writing of the manuscript. LZ, CD and RR contributed to genotyping experiments and *NLRP7* mutation screening. JA and AC examined and scored all POCs for histopathological diagnosis. LH and US performed the FISH analysis. MS, RB and PC referred some patients. GZ and RS provided some POC tissues. AA supervised ABI 3130 Analyzer work. RS supervised the work, provided financial support and wrote the manuscript.

Competing interests None.

Ethics approval McGill University IRB.

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