ORIGINAL ARTICLE

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

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1,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 *L 7*, despite the diploid biparental origin of the HM tissues. In this study, we characterised the expression of the imprinted, maternally expressed gene *CDK 1C* (p57

^{KIP2}), the genotype, and the histopathology of 36 products of conception (POC) from patients with two defective alleles in *L 7* 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 *L 7*, 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

despite the diploid biparental origin of[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

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, *7* and *C3* , responsible for recurrent HMs have been identified.[18 19] *7* is a major gene for recurrent HMs and is mutated in 48%–80% of patients, depending on patients' ascertainment criteria and populations.[20–23] *C3* is a minor gene for recurrent HMs and is mutated in only 10%–14% of patients with no *7* mutations.[19 23 24] To date, approximately 47 different mutations have been reported in patients with two *7*-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-1 secretion in various cellular models,[25–27] including peripheral blood mononuclear cells from patients with two *7*-defective alleles.[27] Recently, a study by Mahadevan demonstrated that *7* 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 *7*-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 *7* or *C3* 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 *7* 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 *7*-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 *7* mutations.

To better understand the role of *7* mutations in HMs, we first characterised $p57^{KIP2}$ expression in 36 products of conception (POCs), mostly HMs, from patients with two *7*-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 laboratorythe HMs.401.6(1to)7.1(o903.748

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 *P* -defective alleles

Case ID-patient ID	Block ID (N)	GA in weeks	p57^{KIP2} expression		Inner cell mass derivatives	Ki-67 expression	Pathologists		NLRP7 mutations or NSVs in the patients
			CT	VM		% of positive CT cells	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.[100% CHM CHA481TT141Tf23.3650Td()-9096.1

genotyping was performed with PowerPlex 16 HS System (Promega, Corporation, Fitchburg, Wisconsin, USA). The reac-

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 7.

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 7-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); thr20%3g68.f p57

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 *7* 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 *7* 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 stillbi6i53(inzygous)-a25T5c15796.9s1T

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

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 *7* 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 *7* 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 *7* 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 *7* mutations. Among the four studies that have investigated $p57^{KIP2}$ expression in diploid biparental HMs from patients with two *7*-defective alleles,[23 37 39 45] one major and important study included 34 HMs and demonstrated that all of them are $p57^{KIP2}$

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