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Immunohistochemical analysis of c-erbB-2, Bcl-2, p53, p21$^{WAF1/Cip1}$, p63 and Ki-67 expression in hydatidiform moles

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ABSTRACT

Hydatidiform moles (HM) are characterized by an abnormal proliferating trophoblast with a potential for a malignant transformation. Similar to other human tumors, trophoblastic pathogenesis is likely a multistep process involving several molecular and genetic alterations. The study was performed to investigate the expression patterns of c-erbB-2 and Bcl-2 oncoproteins, p53, p21$^{WAF1/CIP1}$ and p63 tumor suppressor proteins and Ki-67 cell proliferation marker in HM.

We conducted a retrospective study of 220 gestational products, including 39 hydropic abortions (HA), 41 partial HM (PHM) and 140 complete HM (CHM). The expression of c-erbB-2, Bcl-2, p53, p21$^{WAF1/CIP1}$, p63 and Ki-67 was investigated by immunohistochemistry on archival tissues. c-erbB-2 expression was observed in three PHM and 10 CHM. Bcl-2 immunostaining was significantly higher in PHM (61%) and CHM (70.7%) compared with HA (7.7%, $p = 0.001$ and $p < 0.0001$, respectively). p53 expression was stronger in CHM (73.6%) compared with PHM (24.4%, $p < 0.0001$) and HA (12.8%, $p < 0.0001$). p21$^{WAF1/CIP1}$ staining was observed as well in molar and non-molar gestations ($p > 0.05$). p63 immunoexpression was significantly described in CHM (85.7%) and PHM (78%) compared with HA (10.2%, $p < 0.0001$ and $p = 0.0001$, respectively). Ki-67 was significantly expressed in CHM (72.1%) compared with HA (46.2%, $p = 0.005$).

Altered expression of Bcl-2, p53, p63 and Ki-67 reflects the HM pathological development. Immunohistochemical analysis is beneficial to recognize the HM molecular and pathogenic mechanisms. Furthermore, it could serve as a useful adjunct to conventional methods for refining HM diagnosis.

1. Introduction

Gestational trophoblastic disease (GTD) is a heterogeneous disease of placental trophoblasts with clinically distinct physiopathology, ranging from benign hydatidiform moles (HM) to choriocarcinomas, placental site trophoblastic tumors and epithelioid trophoblastic tumors [12,37]. Occurring more frequently in South-East Asia, HM is an aberrant human pregnancy characterized by significant hydropic enlargement and excessive trophoblastic proliferation, involving part of or all chorionic villi [13,57,61]. According to clinical features, histopathology and genetic differences, HM is sub-classified into two separate entities, including partial hydatidiform mole (PHM) and complete hydatidiform mole (CHM) [4,47]. Although the well-established histopathological criteria, the distinction between non-molar hydropic abortions (HA), PHM and CHM, especially in the first trimester, remains challenging in clinical practice [12,22,59]. Given that 10–30% of CHM and 0.5–5% of PHM cases progress to persistent GTD or even choriocarcinoma, clinical differentiation of these lesions has important therapeutic and prognostic implications [4,59]. Several ancillary techniques have been proposed to resolve the diagnostic dilemmas of HM, including immunohistochemistry, DNA ploidy, conventional cytogenetics, fluorescence *in situ* hybridization and genotyping analyses [5,18,35,44]. During the two last decades, the utility of the p57^{KIP2} immunohistochemistry has been well-established [3,26,34,38]. The

Abbreviations: CHM, complete hydatidiform moles; HA, hydropic abortions; HM, hydatidiform moles; GTD, gestational trophoblastic disease; GTN, gestational trophoblastic neoplasia; PHM, partial hydatidiform moles

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P57^{KIP2} gene is a strongly paternally imprinted gene and is expressed only by the maternal allele in most tissues. Thus, p57^{KIP2} staining is a highly useful marker in confirming CHM. In fact, p57^{KIP2} expression is absent in CHM and present in PHM, HA and normal trophoblasts. The DNA genotyping is the most recent additional technique using polymerase chain reaction (PCR) amplification of short tandem repeat (STR) loci and has emerged as a powerful diagnostic tool to accurately classify both CHM and PHM [3,6,17,26,38,50].

During the implantation, the trophoblast behavior is similar to that of tumor cells. In fact, human placental trophoblasts can be considered pseudomalignant tissue, with firmly controlled proliferation, apoptosis and invasiveness [60]. As other human cancers, GTD is a multistep process characterized by aberrant proliferative and apoptotic activities and dysregulation of cell signaling pathways, involving several genetic and cellular alterations and leading to inactivation of tumor suppressor genes and activation of oncogenes [9,10,36,43–45]. Although there is a growing understanding of the molecular biology of HM, the precise pathogenic mechanisms of HM development are still unclear and should be further explored.

c-erbB-2 proto-oncogene encodes a transmembrane protein with tyrosine kinase activity. It is mainly expressed in embryonic tissue and in certain normal adult tissues. c-erbB-2 protein plays a decisive role in the transduction of cell proliferation and differentiation signals. Overexpression of c-erbB-2 may cause abnormal cell proliferation resulting in the malignant cell transformation [67].

Bcl-2 is a proto-oncogene enhancing growth and survival of cells. Bcl-2 plays a critical role in normal placental growth and ageing. Inappropriate Bcl-2 regulation has been involved in various pregnancy disorders [58].

TP53 is an important tumor suppression gene involved in apoptosis, cell cycle, genomic stability and carcinogenesis as well as in embryogenesis [51]. p53 is involved in cellular differentiation by stimulating the expression of several p53-target genes in first-trimester trophoblasts [16].

p21$^{WAF1/Cip1}$ is a tumor suppressor by promoting cell cycle arrest in response to various stimuli and it acts as principal effector of various tumor suppressor pathways for promoting anti-proliferative activities that are p53-independent. Hence, p21$^{WAF1/Cip1}$ can promote cell proliferation and oncogenicity [1].

The transcription factor p63, localized in the long arm of the third chromosome (3q27-29], is a homolog of the *TP53* gene. p63 protein is expressed in the cytotrophoblasts of the normal placenta and contributes to the stem cell maintenance, as well as growth and development of various epithelial tissues [42].

The Ki-67 is a cell proliferation marker expressed by the cell nucleus of all active phases of the cell cycle (G1, S, G2 and mitosis) except the resting cells (G0). Thus, Ki-67 expression presents an excellent marker of tissue proliferation compartment and could be of value in better understanding the biological behavior of HM [56].

To further explore the pathogenesis of HM compared with non-molar pregnancies, the study investigated the expression pattern of c-erbB-2 and Bcl-2 oncoproteins, p53, p21$^{WAF1/Cip1}$ and p63 tumor suppressor proteins, as well as Ki-67 cell proliferation marker in a large series of molar and non-molar gestational products in Tunisia.

2. Materials and methods

2.1. Tissue samples

We carried out a retrospective study of 220 specimens of the first trimester gestational products received in the Pathology Department, Farhet Hached University Hospital of Sousse, Tunisia, during 2005-2008. These cases originated from the Department of Gynecology and Obstetrics, Farhet Hached University Hospital of Sousse and Taher Sfar University Hospital of Mahdia (Tunisia). This study was approved by the local Human Ethics Committee at the Farhet Hached University Hospital of Sousse (Tunisia) and it conformed to the provisions of the Declaration of Helsinki.

Each case was categorized as HA, PHM, or CHM based on clinical, biochemical (BhCG) and histological criteria [48]. Histological diagnosis review of all cases was performed by two pathologists using Hematoxylin and eosin stained sections.

Prior to analysis, all specimens were already phenotyped by p57^{KIP2} immunohistochemistry as we previously reported [34]. Concordant results were obtained in 210 cases. p57^{KIP2} immunoexpression was detected in 41 PHM and 38 HA, whereas no p57^{KIP2} immunostaining was found in 131 CHM. Among the 10 discordant cases, 9 cases were diagnosed initially as eight PHM and one HA and showed no p57 staining, indicating the absence of maternal contribution. In these 9 cases, CHM diagnosis was retained. In The 10th discordant case, the histological diagnosis between PHM and HA was equivocal [34].

In all these discordant cases (n = 10) as well as in 20 p57^{KIP2}-negative CHM, microsatellite DNA genotyping analysis was performed using fluorescently labeled primers for a panel of 15 STR markers, including D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818 and FGA amelogenin sex marker according to the manufacturer protocol (AmpFlSTR™ Identifiler™ PCR Amplification Kit, Applied Biosystems) [34]. As, the DNA genotyping analysis showed a normal biparental origin of the embryo, the diagnosis of HA was retained in 10th discordant case [34].

Finally, combining the initial histopathological diagnosis and the results of p57^{KIP2} immunohistochemistry and nuclear DNA microsatellite polymorphism, the 220 abortuses samples were classified as 39 HA, 41 PHM and 140 CHM [34].

The patient' age at diagnosis ranged between 18 and 53 years with a median age of 33 years. Gestational age ranged from five to 14 weeks (median: nine weeks). All tissues had been routinely fixed in 10% buffered formalin and paraffin embedded. One or two tissue blocks were selected to confirm that diagnostic tissue as originally reported was adequately represented in remaining tissue blocks.

2.2. Immunohistochemistry

Immunohistochemistry was performed on sections of four microns thickness. After dewaxing and rehydration, the antigen unmasking was carried out in a citrate buffer at 95 °C for 40 min (Table 1). The endogenous peroxidase activity was blocked by 3% hydrogen peroxide. Slides were then incubated with the primary antibody at room temperature (20–25 °C) for 30 min (Table 1). The revelation was made by

Table 1
Immunohistochemistry conditions and evaluation.

Marker	Clone	Provenance	Dilution	Retrieval solution	Positive immunostaining
c-erbB-2	Polyclonal (A0485)	Dako	1/300	Citrate 0.01 M, pH 6.0	Membranous staining
Bcl2	124	Dako	1/50	Citrate 0.01 M, pH 6.0	Cytoplasmic staining
p53	Do-7	Dako	1/50	Citrate 0.01 M, pH 6.0	Nuclear staining
p21$^{WAF/Cip1}$	SX 118	Dako	1/50	Citrate 0.01 M, pH 9.0	Nuclear staining
p63	4A4	Dako	1/50	Citrate 0.01 M, pH 9.0	Nuclear staining
Ki-67	Mib1	Dako	1/50	Citrate 0.01 M, pH 6.0	Nuclear staining

the Envision + Dual Link System HRP kit (Dako, code K4063). Diaminobenzidine was used as the chromogen for the immunostaining. Finally, sections were counterstained with hematoxylin and mounted. Appropriate positive controls were performed according manufacture's instruction. Negative controls were obtained by substitution of the primary antibody by phosphate buffered saline. Images were captured by the microscopic digital camera Olympus system.

Immunohistochemistry evaluation was independently performed by two pathologists. The stained cell types were identified as villous cytotrophoblasts, villous intermediate trophoblasts, villous syncytiotrophoblasts, villous stromal cells or decidual cells. The immunoexpression patterns were scored semi-quantitatively by evaluating the percentage of positive cells and staining intensity as described elsewhere [20,21,31]. The immunoreactivity scores were calculated by multiplying the percentage of positive cells by the staining intensity. The percentage of positive cells was estimated by counting ~100 cells per slide (×400 magnification) and scored as follows: 0 (< 5% staining), 1 (5–25% staining), 2 (25–50% staining), 3 (50–75% staining), and 4 (> 75% staining). The staining intensity was scored as follows: 0, negative, 1, weakly positive, 2, moderately positive, and 3, strongly positive.

2.3. Statistical analysis

Statistical analysis was performed with Chi-square test, using the statistical Package for Social Science (SPSS) software Version 19.0 (IBM Corp., Armonk, NY, USA). Probability values (p) of 0.05 or less were considered statistically significant.

3. Results

The immunoexpression of c-erbB-2, Bcl2, p53, $p21^{WAF1/Cip1}$, p63 and Ki-67 in non-molar and molar pregnancies was summarized in Tables 2 and 3.

The expression of c-erbB-2 oncogene was observed in 13 M samples, including three PHM (7.3%) and 10 CHM (7.1%). These cases exhibited membranous staining of cytotrophoblastic cells (Fig. 1A), whereas no c-erbB-2 immunoexpression was observed in all HA cases. No significant difference of c-erbB-2 expression pattern was reported between PHM and CHM cases ($p > 0.05$).

Bcl-2 expression was found in only three cases of HA. By contrast, cytoplasmic Bcl-2 immunostaining of the syncytiotrophoblastic cells was reported in 25 PHM (61%) and 99 CHM (70.7%, Fig. 1B-C). Compared with HA, Bcl-2 expression was statistically higher in PHM ($p = 0.001$) and CHM ($p < 0.0001$).

Only five HA and 10 PHM exhibited p53 nuclear immunostaining in villous cytotrophoblasts (Fig. 2A). By contrast, p53 expression was observed in 103 CHM (73.6%). There was a significant difference compared with PHM ($p < 0.0001$) and HA ($p < 0.0001$).

Nuclear expression of $p21^{WAF1/Cip1}$ was observed in 64.1%, 82.9% and 85.7% of HA, PHM and CHM cases, respectively (Fig. 2B). The expression was found in villous syncytiotrophoblasts. There was no significant difference between molar and non-molar gestations ($p > 0.05$ for all).

Among molar specimens, p63 immunoexpression was detected in the cytotrophoblasts nucleus of 32 PHM and 120 CHM (Fig. 2C) and it was significantly higher compared with HA (only 10.2%, $p = 0.001$ and $p < 0.0001$, respectively).

Nuclear Ki-67 immunostaining was described in cytotrophoblastic cells of 46.2%, 65.8%, 72.1% of HA, PHM and CHM, respectively (Fig. 2D). Ki-67 expression was significantly detected in CHM compared with HA ($p = 0.005$).

Herein, three CHM progressed to persistent GTD and exhibited c-erbB-2, Bcl2, p53, $p21^{WAF1/Cip1}$, p63 and Ki-67 expression.

Table 3
Statistical analysis of the immunohistochemistry results in molar and non-molar pregnancies.

Expression	HA/PHM	HA/CHM	PHM/CHM
c-erbB-2	$p > 0.05$	$p > 0.05$	$p > 0.05$
Bcl-2	$p = 0.001$	$p < 0.0001$	$p > 0.05$
p53	$p > 0.05$	$p < 0.0001$	$p < 0.0001$
$p21^{WAF1/Cip1}$	$p > 0.05$	$p > 0.05$	$p > 0.05$
p63	$p = 0.001$	$p = 0.0001$	$p > 0.05$
Ki-67	$p > 0.05$	$p = 0.005$	$p > 0.05$

Note: CHM, complete hydatidiform moles; HA, hydropic abortions; PHM, partial hydatidiform moles.

4. Discussion

In this study, we extensively analyzed the immunohistochemical expression of c-erbB-2 and Bcl-2 oncoproteins, p53, $p21^{WAF1/Cip1}$ and p63 tumor suppressor proteins and Ki-67 cell proliferation marker in a large series of molar and non-molar pregnancies in Tunisian women. When compared with non-molar abortions, we found a significant expression of Bcl-2 and p63 in PHM cases as well as a higher immunoexpression of Bcl-2, p53, p63 and Ki-67 in CHM samples. However, only p53 expression was significantly different between PHM and CHM.

The c-erbB-2 oncogene is overexpressed in several malignant tumors, including breast, colorectal, gastric and gallbladder tumors. In persistent GTD, Cameron et al. [7] neglected any significance of c-erbB-2 expression since only one case showed positive staining. However, later studies have reported the involvement of c-erbB-2 oncogene in HM pathogenesis [21,24,29,68,69]. Yang et al. [68] considered that the altered c-erbB-2 expression could be important in the pathogenic mechanisms of CHM as well as a strong predictor for the malignant transformation of this disease. Using quantitative and semi-quantitative analysis of c-erbB-2 immunostaining, Yazaki-Sun et al. [69] confirmed these findings and advocated the c-erbB-2 usefulness in the establishment of therapeutic protocol. More recently, Erol et al. [21] described a significantly higher positive c-erbB-2 staining in CHM compared with PHM and HA. Interestingly, Hasanzadeh et al. [29] reported that c-erbB-2 overexpression is associated with malignant progression of molar pregnancies and could predict GTN during the early stages. Herein, although our results were not statistically significant, a higher c-erbB-2 expression was found in CHM and a progression to persistent GTD was reported in three c-erbB-2-positive CHM. Hence, we suggest that c-erbB-2 expression could be a useful in prediction of HM malignant progression. Indeed, this hypothesis should be confirmed in a large series of HM giving rise to GTN.

Dysregulation of Bcl-2 has been involved in various pregnancy

Table 2
Expression of c-erbB-2, Bcl2, p53, $p21^{WAF1/Cip1}$, p63 and Ki-67 in 220 M and non-molar gestations.

Histological Diagnosis	c-erbB-2	Bcl-2	p53	$p21^{WAF1/Cip1}$	p63	Ki-67
HA (n = 39)	0	3 (7.7%)	5 (12.8%)	25 (64.1%)	4 (10.2%)	18 (46.2%)
PHM (n = 41)	3 (7.3%)	25 (61%)	10 (24.4%)	34 (82.9%)	32 (78%)	27 (65.8%)
CHM (n = 140)	10 (7.1%)	99 (70.7%)	103 (73.6%)	120 (85.7%)	120 (85.7%)	101 (72.1%)

Note: CHM, complete hydatidiform moles; HA, hydropic abortions; PHM, partial hydatidiform moles.

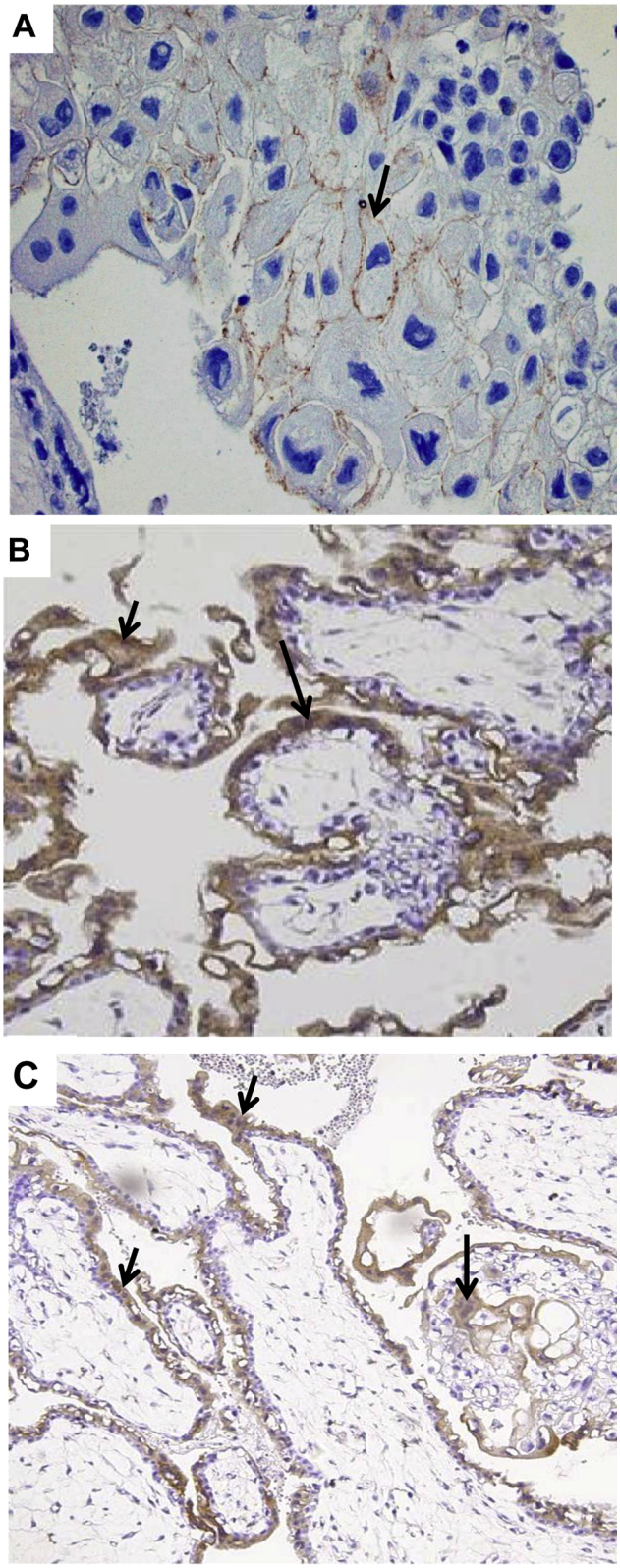

Fig. 1. Expression pattern of c-erbB-2 and Bcl-2 oncoproteins in HM samples. (A) Membrane expression of c-erbB-2 in cytotrophoblastic cells of CHM (Mx400). (B–C) Cytoplasmic expression of Bcl-2 in syncytiotrophoblastic cells of PHM (Mx200) and CHM (Mx100). Expression localization was pointed with black arrows.

disorders. In GTD, almost a dozen studies have investigated the Bcl-2 expression with conflicting results [2,8,21,24,31,52,53,64,65]. Fulop et al. [24] found a significantly stronger Bcl-2 expression in the syncytiotrophoblast of CHM and choriocarcinoma compared with both normal placenta and PHM. In molar chorionic villi, Hussein [31] reported a moderate expression of Bcl-2 suggesting its involvement in the development of HM. In our study, analyzing a larger series of HM samples, we reported a significantly higher Bcl-2 expression in villous syncytiotrophoblasts of CHM as well as PHM cases compared with HA. However, other studies described a significant decrease in Bcl-2 expression in CHM cases as compared with the control group [21,52,54,65,66]. In the study of Candelier et al. [8], Bcl-2 expression was reported in syncytiotrophoblasts of the normal placenta and HM and increased gradually during the normal differentiation. Recently, a significantly increased Bcl-2 expression pattern was observed in HA compared with PHM and CHM by Erol et al. [21]. The decrease of Bcl-2 expression was associated with the excessive proliferation of trophoblastic cells in HM [65].

In contrast to malignant tumors, p53 immunoexpression in the trophoblast is due to upregulation of the *TP53* tumor suppressor gene that could be essential for controlling excessive trophoblast proliferation and considered a defensive mechanism against the abnormal cell development during the normal placentation [27,40]. Nevertheless, several reports investigated the possible role of p53 immunoexpression in the HM pathogenesis with controversial findings [2,11,15,20,27,31,33,36,39,52,55,64,68]. Some previous analyses showed p53 overexpression in PHM and even in HA [11,27,64]. Chen et al. [11] found significantly higher p53 immunostaining in PHM than HA concerning the percentage of stained cells. Herein, however, no significant difference was observed between HA and PHM consistent with some earlier reports [23,55]. Considering only staining in the villous intermediate trophoblasts, Al-Bozom [2] identified an increased p53 expression in CHM compared with PHM with no expression in HA. Interestingly, in our study, significantly increased p53 expression was reported in CHM as compared with HA and PHM as previously shown [20,23,33,49]. Taken together, these results proved the higher cell activity and apoptosis in the trophoblasts of CHM that could be associated with HM malignant transformation [62,69]. Nevertheless, using a highly sensitive pyrosequencing approach, a very recent study of Chan et al. [10] identified specific nonsense and missense mutations in exons 6, 7 and 8 of the *TP53* tumor suppressor gene in CHM and even in PHM, suggesting the presence of p53 mutants in a small population of HM trophoblasts.

According to the literature, there were only few earlier studies investigating $p21^{WAF1/Cip1}$ expression in GTD pathogenesis [14,23,36]. A significantly stronger expression of $p21^{WAF1/Cip1}$ was reported in CHM and choriocarcinoma compared with normal placentas and PHM, advocating an important role in the pathogenic development of both CHM and choriocarcinoma [14,23,36]. By contrast, in our study, no statistical difference of $p21^{WAF1/Cip1}$ immunostaining was observed between molar and non-molar samples although CHM cases exhibited more frequently $p21^{WAF1/Cip1}$ expression.

The role of p63 expression in molar pregnancies' pathogenesis has been analyzed and remains unclear [11,19,30,41,53]. No significant difference of p63 immunoexpression between PHM and HA was reported by Chen et al. [11]. Although the nuclear immunoreactivity of p63 was higher in molar than non-molar gestations and in PHM than CHM, Heidarpour and Khanahmadi [30] considered that using this marker alone is an inaccurate diagnostic tool in view of its low sensitivity and specificity. Currently, a significant difference of p63 expression was observed between molar and non-molar abortions, but not between PHM and CHM supporting its involvement in molar pathogenesis. Nevertheless, p63 staining has been described differently between PHM and CHM [53] and has been advocated even in routine clinical practice to improve HM diagnosis mainly in case of limited resource settings [41].

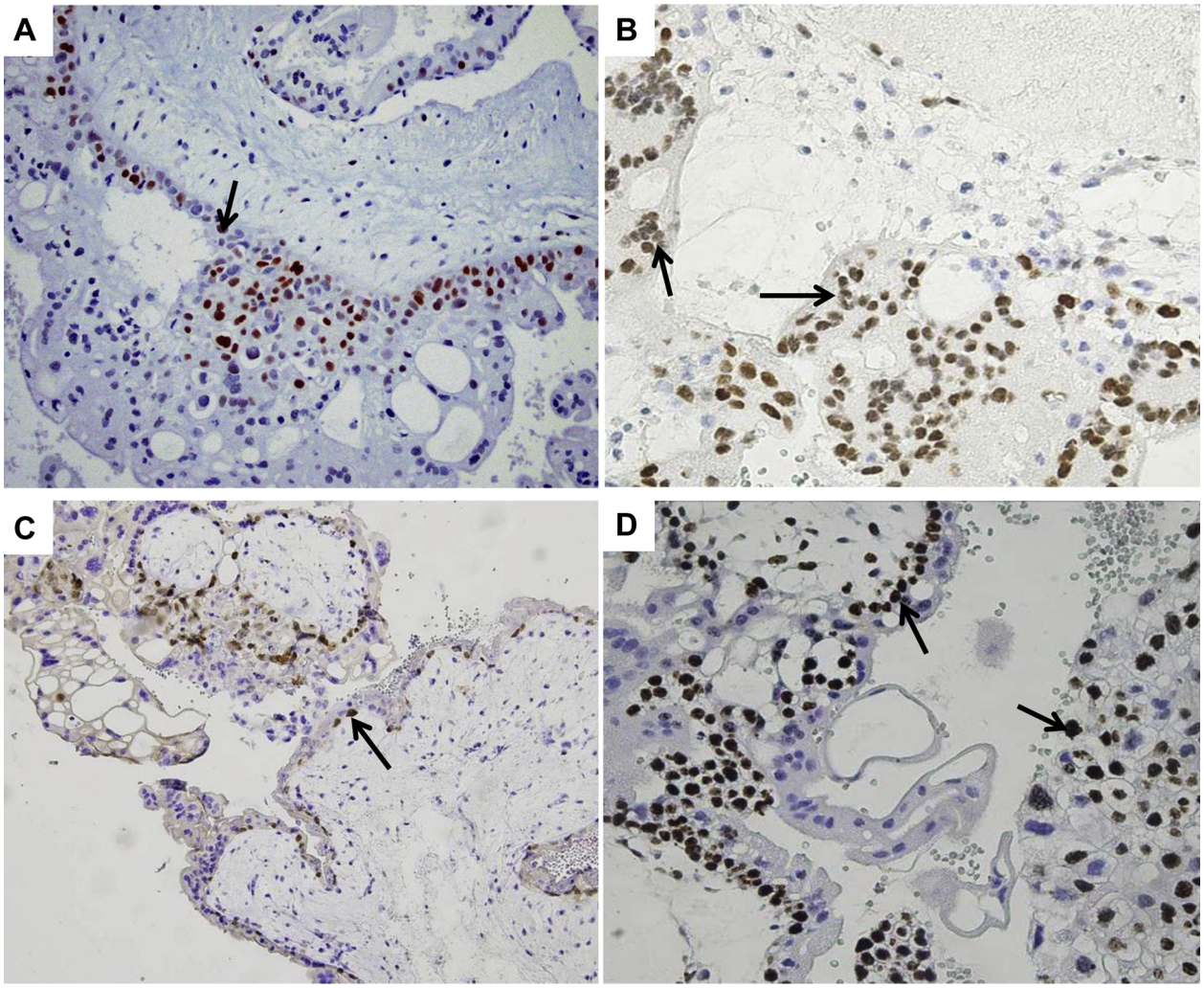

Fig. 2. Expression pattern of p53, $p21^{WAF/Cip1}$ and p63 tumor suppressor proteins and Ki-67 cell proliferation marker in HM samples.
(A) Nuclear p53 expression in cytotrophoblastic cells of CHM (B, Mx200). (B) Nuclear $p21^{WAF/Cip1}$ expression in syncytiotrophoblastic cells of CHM (Mx200). (C) Nuclear p63 expression in cytotrophoblastic cells of PHM (Mx100). (D) Strong nuclear Ki-67 expression in cytotrophoblastic cells of CHM (Mx200). Expression localization was pointed with black arrows.

Since Ki-67 is involved in the trophoblast differentiation and is mainly expressed by cytotrophoblastic cells, it seems an excellent marker for the tissue proliferation compartment, determining the GTD biological behavior [56]. In HM, the usefulness of Ki-67 immunostaining is still conflicting [11,13,19,25,28,32,33,39,44,46,55,63,70]. Earlier reports found no significant difference of Ki-67 expression between molar and normal placentas as well as between HM which progressed to persistent GTD and those that did not [13,25,32,46,55]. More recently, Sundara et al. [63] showed that only the GTN was found to be significantly different from the normal placenta. Hence, the Ki-67 staining of placental trophoblastic cells in patients with a molar placenta could present a high predictive value for the detection of GTN progression [28,39,63]. Herein, we showed that Ki-67 expression was significantly different between CHM and HA, confirming its role in CHM development. In addition, a progression to persistent GTD was reported in three Ki-67-positive CHM cases, supporting the predictive value of this marker. Despite the low number of analyzing samples, Erfanian et al. [19] considered that Ki-67 immunostaining in cytotrophoblastic cells is the best factor to differentiate between HA and HM, as well as between CHM and PHM. Later reports confirmed the usefulness of Ki-67 proliferation marker in the differential diagnosis of non-molar and molar gestations [11,39,44,70], mainly in distinguishing CHM from PHM [70] and even PHM from HA if combined with E-cadherin expression [44].

To our knowledge, our study constitutes the first report investigating simultaneously the expression of six different genes on a large series of molar and non-molar gestations with already confirmed diagnosis by $p57^{KIP2}$ immunohistochemistry and DNA genotyping analysis. Based on our findings, altered expression of Bcl-2, p53, p63 and Ki-67 reflects the pathological development of HM. The immunohistochemical study of molar and non-molar pregnancies is of great help in better understanding the molecular and pathogenic mechanisms of HM. Therefore, it could be a useful adjunct to conventional methods for refining HM diagnosis. Further analyses of more PHM and HA cases are required to confirm these results.

Conflict of interest

None of the authors have any conflict of interest to disclose.

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Disclaimer

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References

[1] T. Abbas, A. Dutta, p21 in cancer: intricate networks and multiple activities, Nat. Rev. Cancer 9 (2009) 400–414.
[2] I.A. Al-Bozom, p53 and Bcl-2 oncoprotein expression in placentas with hydropic changes and partial and complete moles, APMIS 108 (2000) 756–760.
[3] N. Banet, C. DeScipio, K.M. Murphy, et al., Characteristics of hydatidiform moles: analysis of a prospective series with p57 immunohistochemistry and molecular genotyping, Mod. Pathol. 27 (2014) 238–254.
[4] R.S. Berkowitz, D.P. Goldstein, Current management of gestational trophoblastic diseases, Gynecol. Oncol. 112 (2009) 654–662.
[5] N. Buza, P. Hui, Immunohistochemistry and other ancillary techniques in the diagnosis of gestational trophoblastic diseases, Semin. Diagn. Pathol. 31 (2014) 223–232.
[6] J. Bynum, K.M. Murphy, C. DeScipio, et al., Invasive complete hydatidiform moles: analysis of a case series with genotyping, Int. J. Gynecol. Pathol. 35 (2016) 134–141.
[7] B. Cameron, A.M. Gown, H.K. Tamimi, Expression of c-erbB-2 oncogene product in persistent gestational trophoblastic disease, Am. J. Obstet. Gynecol. 170 (1994) 1616–1621.
[8] J.J. Candelier, L. Frappart, T. Yadaden, et al., Altered p16 and Bcl-2 expression reflects pathologic development in hydatidiform moles and choriocarcinoma, Pathol. Oncol. Res. 19 (2013) 217–227.
[9] L. Carey, B.M. Nash, D.C. Wright, Molecular genetic studies of complete hydatidiform moles, Transl. Pediatr. 4 (2015) 181–188.
[10] K.K. Chan, E.S. Wong, O.G. Wong, H.Y. Ngan, A.N. Cheung, Identification of non-synonymous TP53 mutations in hydatidiform moles, Mutat. Res. 89 (2018) 20–23.
[11] Y. Chen, D. Shen, Y. Gu, P. Zhong, J. Xie, Q. Song, The diagnostic value of Ki-67, P53 and P63 in distinguishing partial hydatidiform mole from hydropic abortion, Wien. Klin. Wochenschr. 124 (2012) 184–187.
[12] A.N. Cheung, Pathology of gestational trophoblastic diseases, Best Pract. Res. Clin. Obstet. Gynaecol. 17 (2003) 849–868.
[13] A.N. Cheung, H.Y. Ngan, R.J. Collins, Y.L. Wong, Assessment of cell proliferation in hydatidiform mole using monoclonal antibody MIB1 to Ki-67 antigen, J. Clin. Pathol. 47 (1994) 601–604.
[14] A.N. Cheung, D.H. Shen, U.S. Khoo, L.C. Wong, H.Y. Ngan, p21WAF1/CIP1 expression in gestational trophoblastic disease: correlation with clinicopathological parameters, and Ki67 and p53 gene expression, J. Clin. Pathol. 51 (1998) 159–162.
[15] A.N. Cheung, G. Srivastava, L.P. Chung, et al., Expression of the p53 gene in trophoblastic cells in hydatidiform moles and normal human placentas, J. Reprod. Med. 39 (1994) 223–227.
[16] M. Cohen, A. Meisser, L. Haenggeli, I. Irminger-Finger, P. Bischof, Status of p53 in first-trimester cytotrophoblastic cells, Mol. Hum. Reprod. 13 (2007) 111–116.
[17] T.J. Colgan, M.C. Chang, S. Nanji, E. Kolomietz, DNA genotyping of suspected partial hydatidiform moles detects clinically significant aneuploidy, Int. J. Gynecol. Pathol. 36 (2017) 217–221.
[18] H. Crisp, J.L. Burton, R. Stewart, M. Wells, Refining the diagnosis of hydatidiform mole: image ploidy analysis and p57KIP2 immunohistochemistry, Histopathology 43 (2003) 363–373.
[19] M. Erfanian, N. Sharifi, A.A. Omidi, P63 and Ki-67 expression in trophoblastic disease and spontaneous abortion, J. Res. Med. Sci. 14 (2009) 375–384.
[20] O. Erol, D. Suren, B. Tutus, et al., Immunohistochemical analysis of E-cadherin, p53 and inhibin-α expression in hydatidiform mole and hydropic abortion, Pathol. Oncol. Res. 22 (2016) 515–521.
[21] O. Erol, D. Suren, B. Tutus, et al., Comparison of p57, c-erbB-2, CD117, and bcl-2 expression in the differential diagnosis of hydatidiform mole and hydropic abortion, Eur. J. Gynaecol. Oncol. 37 (2016) 522–529.
[22] M. Fukunaga, H. Katabuchi, T. Nagasaka, Y. Mikami, S. Minamiguchi, J.M. Lage, Interobserver and intraobserver variability in the diagnosis of hydatidiform mole, Am. J. Surg. Pathol. 29 (2005) 942–947.
[23] V. Fulop, S.C. Mok, D.R. Genest, I. Gati, J. Doszpod, R.S. Berkowitz, p53, p21, Rb and mdm2 oncoproteins. Expression in normal placenta, partial and complete mole, and choriocarcinoma, J. Reprod. Med. 43 (1998) 119–127.
[24] V. Fulop, S.C. Mok, D.R. Genest, I. Szigetvari, I. Cseh, R.S. Berkowitz, c-myc, c-erbB-2, c-fms and bcl-2 oncoproteins. Expression in normal placenta, partial and complete mole, and choriocarcinoma, J. Reprod. Med. 43 (1998) 101–110.
[25] J. Gerdes, H. Lemke, H. Baisch, H.H. Wacker, U. Schwab, H. Stein, Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67, J. Immunol. 133 (1984) 1710–1715.
[26] M. Gupta, R. Vang, A.V. Yemelyanova, et al., Diagnostic reproducibility of hydatidiform moles: ancillary techniques (p57 immunohistochemistry and molecular genotyping) improve morphologic diagnosis for both recently trained and experienced gynecologic pathologists, Am. J. Surg. Pathol. 36 (2012) 1747–1760.
[27] R. Halperin, S. Peller, J. Sandbank, I. Bukovsky, D. Schneider, Expression of the p53 Gene and apoptosis in gestational trophoblastic disease, Placenta 21 (2000) 58–62.
[28] M. Hasanzadeh, N. Sharifi, H. Esmaieli, M.S. Daloee, A. Tabari, Immunohistochemical expression of the proliferative marker Ki67 in hydatidiform moles and its diagnostic value in the progression to gestational trophoblastic neoplasia, J. Obstet. Gynaecol. Res. 39 (2013) 572–577.
[29] M. Hasanzadeh, N. Sharifi, M. Farazestanian, S.S. Nazemian, F. Madani Sani, Immunohistochemistry study of P53 and C-erbB-2 expression in trophoblastic tissue and their predictive values in diagnosing malignant progression of simple molar pregnancy, Iran. J. Cancer Prev. 9 (2016) e4115.
[30] M. Heidarpour, M. Khanahmadi, Diagnostic value of P63 in differentiating normal gestation from molar pregnancy, J. Res. Med. Sci. 18 (2013) 462–466.
[31] M.R. Hussein, Analysis of p53, BCL-2 and epidermal growth factor receptor protein expression in the partial and complete hydatidiform moles, Exp. Mol. Pathol. 87 (2009) 63–69.
[32] M.D. Jeffers, J.A. Richmond, R. Smith, Trophoblastic proliferation does not predict progression to persistent gestational trophoblastic disease in complete hydatidiform mole, Int. J. Gynecol. Pathol. 15 (1996) 34–38.
[33] A. Kale, F. Söylemez, A. Ensari, Expressions of proliferation markers (Ki-67, proliferating cell nuclear antigen, and silverstaining nucleolar organizer regions) and of p53 tumor protein in gestational trophoblastic disease, Am. J. Obstet. Gynecol. 184 (2001) 567–574.
[34] H. Landolsi, N. Missaoui, S. Brahem, S. Hmissa, M. Gribaa, M.T. Yacoubi, The usefulness of p57(KIP2) immunohistochemical staining and genotyping test in the diagnosis of the hydatidiform mole, Pathol. Res. Pract. 207 (2011) 498–504.
[35] H. Landolsi, N. Missaoui, M.T. Yacoubi, et al., Assessment of the role of histopathology and DNA image analysis in the diagnosis of molar and non-molar abortion: a study of 89 cases in the center of Tunisia, Pathol. Res. Pract. 205 (2009) 789–796.
[36] H.W. Li, S.W. Tsao, A.N. Cheung, Current understandings of the molecular genetics of gestational trophoblastic diseases, Placenta 23 (2002) 20–31.
[37] J.R. Lurain, Gestational trophoblastic disease I: epidemiology, pathology, clinical presentation and diagnosis of gestational trophoblastic disease, and management of hydatidiform mole, Am. J. Obstet. Gynecol. 203 (2010) 531–539.
[38] J.M. Madi, A. Braga, M.P. Paganella, I.E. Litvin, E.M. Wendland, Accuracy of p57(KIP)(2) compared with genotyping to diagnose complete hydatidiform mole: a systematic review and meta-analysis, BJOG 125 (2018) 1226–1233.
[39] J. Makovitzky, A. Radtke, N. Shabani, K. Friese, B. Gerber, I. Mylonas, Invasive hydatidiform mole: immunohistochemical labelling of inhibin/activin subunits, Ki67, p53 and glycodelin A in A rare case, Acta Histochem. 111 (2009) 360–365.
[40] K. Marzusch, P. Ruck, H.P. Horny, J. Dietl, E. Kaiserling, Expression of the p53 tumour suppressor gene in human placenta: an immunohistochemical study, Placenta 16 (1995) 101–104.
[41] S. Masood, S.I. Kehar, S. Shawana, I. Aamir, Differential expression of p63 in hydropic and molar gestations, J. Coll. Physicians Surg. Pak. 25 (2015) 198–201.
[42] U.M. Moll, The role of p63 and p73 in tumor formation and progression: coming of age toward clinical usefulness, Clin. Cancer Res. 9 (2003) 5437–5441.
[43] G. Moser, K. Windsperger, J. Pollheimer, S.C. de Sousa Lopes, B. Huppertz, Human trophoblast invasion: new and unexpected routes and functions, Histochem. Cell Biol. 150 (2018) 361–370.
[44] R.A. Moussa, A.N. Eesa, Z.F. Abdallah, A. Abdelmeged, A. Mahran, H. Bahaa, Diagnostic utility of Twist1, Ki-67, and E-cadherin in diagnosing molar gestations and hydropic abortions, Am. J. Clin. Pathol. 149 (2018) 442–455.
[45] N.M.P. Nguyen, Y. Khawajkie, N. Mechtouf, The genetics of recurrent hydatidiform moles: new insights and lessons from a comprehensive analysis of 113 patients, Mod. Pathol. 31 (2018) 1116–1130.
[46] M. Olvera, S. Harris, C.A. Amezcua, et al., Immunohistochemical expression of cell cycle proteins E2F-1, Cdk-2, Cyclin E, p27(kip1), and Ki-67 in normal placenta and gestational trophoblastic disease, Mod. Pathol. 14 (2001) 1036–1042.
[47] F.J. Paradinas, P. Browne, R.A. Fisher, M. Foskett, K.D. Bagshawe, E. Newlands, A clinical, histopathological and flow cytometric study of 149 complete moles, 146 partial moles and 107 non-molar hydropic abortions, Histopathology 28 (1996) 101–110.
[48] F.J. Paradinas, C.W. Elston, H. Fox, M. Wells (Eds.), Gestational Trophoblastic Disease, Churchill Livingstone, Edinburgh, 2003, pp. 1359–1430.
[49] P. Petignat, R. Laurini, F. Goffin, I. Bruchim, P. Bischof, Expression of matrix metalloproteinase-2 and mutant p53 is increased in hydatidiform mole as compared with normal placenta, Int. J. Gynecol. Cancer 16 (2006) 1679–1684.
[50] P. Petignat, M.H. Billieux, J.L. Blouin, S. Dahoun, P. Vassilakos, Is genetic analysis useful in the routine management of hydatidiform mole? Hum. Reprod. 18 (2003) 243–249.
[51] A. Petitjean, M.I. Achatz, A.L. Borresen-Dale, P. Hainaut, M. Olivier, TP53 mutations in human cancers: functional selection and impact on cancer prognosis and outcomes, Oncogene 26 (2007) 2157–2165.
[52] S. Qiao, T. Nagasaka, T. Harada, N. Nakashima, p53, Bax and Bcl-2 expression, and apoptosis in gestational trophoblast of complete hydatidiform mole, Placenta 19 (1998) 361–369.
[53] L.N. Ramalho, M.S. Maggiori, A. Ribeiro-Silva, L.C. Peres, P63 expression in hydropic abortion and gestational trophoblastic diseases, Placenta 27 (2006) 740–743.
[54] G. Rath, S. Soni, C.P. Prasad, S. Salhan, A.K. Jain, S. Saxena, Bcl-2 and p53 expressions in Indian women with complete hydatidiform mole, Singapore Med. J. 52 (2011) 502–507.
[55] D.P. Schammel, T. Bocklage, p53, PCNA and Ki-67 in hydropic molar and non molar

placentas: an immunohistochemical study, Int. J. Gynecol. Pathol. 15 (1996) 158–166.
[56] T. Scholzen, J. Gerdes, The Ki-67 protein: from the known and the unknown, J. Cell. Physiol. 182 (2000) 311–322.
[57] M.J. Seckl, N.J. Sebire, R.S. Berkowitz, Gestational trophoblastic disease, Lancet 376 (2010) 717–729.
[58] F. Sgarbosa, L.F. Barbisan, M.A. Brasil, et al., Changes in apoptosis and Bcl-2 expression in human hyperglycemic, term placental trophoblast, Diabetes Res. Clin. Pract. 73 (2006) 143–149.
[59] Ie M. Shih, Gestational trophoblastic neoplasia–pathogenesis and potential therapeutic targets, Lancet Oncol. 8 (2007) 642–650.
[60] E. Staun-Ram, E. Shalev, Human trophoblast function during the implantation process, Reprod. Biol. Endocrinol. 3 (2005) 56.
[61] S.J. Steigrad, Epidemiology of gestational trophoblastic diseases, Best Pract. Res. Clin. Obstet. Gynaecol. 17 (2003) 837–847.
[62] P. Sun, Q. Wu, G. Ruan, et al., Expression patterns of maspin and mutant p53 are associated with the development of gestational trophoblastic neoplasia, Oncol. Lett. 12 (2016) 3135–3142.
[63] Y.T. Sundara, E.S. Jordanova, B.S. Hernowo, S. Gandamihardja, G.J. Fleuren, Decidual infiltration of FoxP3$^+$ regulatory T cells, CD3$^+$ T cells, CD56$^+$ decidual natural killer cells and Ki-67 trophoblast cells in hydatidiform mole compared to normal and ectopic pregnancies, Mol. Med. Rep. 5 (2012) 275–281.
[64] A.K. Uzunlar, F. Yilmaz, G. Bayhan, Z. Akkuş, Expressions of p53, proliferating cell nuclear antigen and Ki-67 in gestational trophoblastic diseases, Eur. J. Gynaecol. Oncol. 23 (2002) 79–83.
[65] T.L. Wargasetia, N. Shahib, D. Martaadisoebrata, D. Dhianawaty, B. Hernowo, Characterization of apoptosis and autophagy through Bcl-2 and Beclin-1 immunoexpression in gestational trophoblastic disease, Iran. J. Reprod. Med. 13 (2015) 413–420.
[66] S.Y. Wong, H.Y. Ngan, C.C. Chan, A.N. Cheung, Apoptosis in gestational trophoblastic disease is correlated with clinical outcome and Bcl-2 expression but not Bax expression, Mod. Pathol. 12 (1999) 1025–1033.
[67] Z. Xu, B.D. Ford, Upregulation of erbB receptors in rat brain after middle cerebral arterial occlusion, Neurosci. Lett. 375 (2005) 181–186.
[68] X. Yang, Z. Zhang, C. Jia, J. Li, L. Yin, S. Jiang, The relationship between expression of c-ras, c-erbB-2, nm23, and p53 gene products and development of trophoblastic tumor and their predictive significance for the malignant transformation of complete hydatidiform mole, Gynecol. Oncol. 85 (2002) 438–444.
[69] S. Yazaki-Sun, S. Daher, M.M. de Souza Ishigai, M.T. Alves, T.M. Mantovani, R. Mattar, Correlation of c-erbB-2 oncogene and p53 tumor suppressor gene with malignant transformation of hydatidiform mole, J. Obstet. Gynaecol. Res. 32 (2006) 265–272.
[70] Y. Zhao, G.W. Xiong, X.W. Zhang, B.O. Hang, Is Ki-67 of diagnostic value in distinguishing between partial and complete hydatidiform moles?, A systematic review and meta-analysis, Anticancer Res. 38 (2018) 1105–1110.