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ORIGINAL ARTICLE

Immunohistochemical Analysis of E-Cadherin, p53 and Inhibin-α Expression in Hydatidiform Mole and Hydropic Abortion

Onur Erol[1] · Dinç Süren[2] · Birsel Tutuş[2] · Tayfun Toptaş[3] · Ahmet Arda Gökay[2] · Aysel Uysal Derbent[1] · Mustafa Kemal Özel[1] · Cem Sezer[2]

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Abstract The purpose of this study was to investigate the role of E-cadherin, p53, and inhibin-α immunostaining in the differential diagnosis of hydropic abortion (HA), partial hydatidiform mole (PHM), and complete hydatidiform mole (CHM). E-cadherin, p53, and inhibin-α protein expression patterns were investigated immunohistochemically using paraffin-embedded tissue sections from histologically diagnosed cases of HA ($n=23$), PHM ($n=24$), and CHM ($n=23$). Expression patterns of these markers were scored semi-quantitatively according to the staining intensity, percentage of positive cells, and immunoreactivity score. Classification of cases was established on histologic criteria and supported by the molecular genotyping. Immunostaining allowed the identification of specific cell types with E-cadherin, p53, and inhibin-α expression in all cases. E-cadherin expression was detected on the cell surface of villous cytotrophoblasts. We observed a marked decline in the expression of E-cadherin from HAs to PHMs to CHMs. The p53-positive reaction was restricted to the nucleus of villous cytotrophoblasts. Significantly increased p53 expression was observed in CHMs, compared with HAs and PHMs. The expression of inhibin-α was localised in the cytoplasm of villous syncytiotrophoblasts, and the expression of this marker was significantly higher in PHMs and CHMs than HAs. In conclusion, immunohistochemical analysis of E-cadherin, p53, and inhibin-α expression could serve as a useful adjunct to conventional methods in the differential diagnosis of HA, PHM, and CHM.

Keywords E-cadherin · p53 · Inhibin-α · Hydatidiform mole

✉ Onur Erol
dronurerol@hotmail.com

[1] Department of Obstetrics and Gynecology, Antalya Training and Research Hospital, Antalya, Turkey

[2] Department of Pathology, Antalya Training and Research Hospital, Antalya, Turkey

[3] Department of Gynecologic Oncology, Antalya Training and Research Hospital, Antalya, Turkey

Introduction

Gestational trophoblastic diseases (GTDs) encompass a heterogeneous set of diseases that arise from abnormal trophoblast tissue, including hydatidiform moles (HM), invasive HM, choriocarcinomas, placental site trophoblastic tumours, and epithelioid trophoblastic tumours [1]. HM is the most common form of GTD, which is an abnormal pregnancy, characterised by hydropic swelling of placental villi and trophoblastic hyperplasia. HMs are subclassified further into complete hydatidiform mole (CHM) and partial hydatidiform mole (PHM), based on morphological, genetic, and clinical features. CHMs are derived exclusively from the paternal genome (androgenetic diploidy), whereas PHMs contain one maternally derived and two paternally derived haploid genomes (diandric triploidy). Additionally, hydropic abortions (HAs) can mimic HMs morphologically. HAs are typically characterized by biparental diploidy (one maternal and one paternal chromosome complement). Despite well-described histopathological criteria, distinguishing HA from HM, and CHM from PHM, remains a problem in clinical practice [2]. Several ancillary techniques have been applied to resolve these diagnostic problems, including immunohistochemistry, conventional cytogenetics (karyotyping), flow cytometry, digital image analysis, fluorescence in situ hybridisation (FISH) and molecular genotyping. The value of an immunohistochemical analysis of p57 expression for improving the diagnosis of HMs has been well established [3]. The p57 gene is

paternally imprinted and expressed predominantly from the maternal allele in most tissues. As CHMs lack a maternal genomic component, p57 is a highly specific and sensitive marker for CHMs due to an absence of nuclear staining in villous stromal cells and cytotrophoblasts. In contrast, both PHMs and HAs contain a maternal chromosomal complement and express p57. The most recent ancillary technique, molecular genotyping using polymerase chain reaction amplification of short tandem repeat (STR) loci allows for determination of parental source of polymorphic alleles and their ratios. In particular, this analysis can distinguish androgenetic diploidy, diandric triploidy and biparental diploidy, which are characteristic of CMs, PHMs and HAs, respectively.

Cadherins comprise a family of calcium-dependent adhesion glycoproteins that mediate cell-cell binding to maintain differentiated tissue structure and morphogenesis. It is now established that the cadherins are implicated in diverse biological processes, such as cell adhesion, cell signalling, cell recognition, control of cell division, inhibition of apoptosis, migration, differentiation, morphogenesis, embryo implantation, tumour development and metastasis [4]. E-cadherin is arguably the prototypic member of the cadherin family and is expressed predominantly at the membrane of epithelial cells, and involved in maintaining epithelial architecture and cell polarity. Absent or altered expression of E-cadherin results in reduced cell-cell connections and enhanced cellular mobility, and correlates with the neoplastic transformation of epithelial cells [5].

The p53 protein is a critical component of cellular mechanisms, and known to be activated as a transcription factor in responses to genotoxic stresses such as hypoxia, hyperthermia, and DNA damage, to induce cell cycle arrest or apoptosis. Wild-type p53 protein has a short half-life (usually less than 30 min), and functions as a negative regulator of cell growth in tumour suppression. In contrast, mutant type p53 protein has a prolonged half-life, of several hours, promotes cell growth, and functions as an oncogene [6].

Inhibins belong to the transforming growth factor superfamily and are secreted by granulosa cells of the ovary and Sertoli cells of the testis. Inhibins consist of an α-subunit and one of two possible β-subunits (βA or βB), resulting in the formation of inhibin A (α-βA) or B (α-βB). In addition to their primary role in modulating follicle-stimulating hormone production, inhibins are expressed in a wide range of human tissues outside the reproductive axis (e.g., prostate, brain, adrenal, placenta) and are involved in the regulation of cell growth and differentiation [7]. Moreover, differential expression of the inhibin subunits suggests that they play an important role in malignant cell transformation [8]. It has been demonstrated that inhibin-α may act as a tumour suppressor, with its expression linked to tumour progression and poorer patient survival [9].

The present study was carried out to evaluate the expression patterns of E-cadherin, p53, and inhibin-α in HAs, PHMs, and CHMs, and to assess the value of these markers in the differential diagnosis of the three entities.

Material and Methods

In total, 70 formalin-fixed paraffin-embedded placental tissues were retrieved from the files of the Department of Pathology, Antalya Training and Research Hospital between January 2010 and June 2013 after institutional review board approval. The cases were selected to represent HA ($n=23$), PHM ($n=24$), and CHM ($n=23$) and the diagnosis of each case was obtained from the original pathology report. Haematoxylin and eosin-stained sections of the specimens were reviewed independently by two pathologists with no knowledge of the specimens' clinical information and molecular genotyping test results, and were classified according to the main morphological findings [10]. CHM is characterized by hydropic swelling of villi with central cisterns, circumferential trophoblastic hyperplasia with diffuse and marked atypia and trophoblastic inclusions. Morphologic features of PHM include focal trophoblastic hyperplasia, a dimorphic villous population with an admixture of hydropic and normal villi, scalloping and prominent stromal trophoblastic inclusions, and mild trophoblastic atypia. HA is characterized by villous edema without trophoblastic hyperplasia. The histological diagnosis of three entities was confirmed by cell ploidy analysis in all samples using STR genotyping. Patient demographic data were obtained through a chart review.

Tissue Preparation and Evaluation of Immunohistochemical Staining

Briefly, 4-μm-thick, representative sections from formalin-fixed, paraffin-embedded tissue blocks were obtained in each case, incubated for 120 min at 60 °C and then overnight at 37 °C. The tissue sections were deparaffinised in xylene and alcohol, rehydrated, and washed in a solution buffered with 10 % sodium citrate in a microwave oven (800 W). The slides were left to cool at room temperature for 20 min. Endogenous peroxidase activity was blocked using 0.3 % hydrogen peroxide, and the slides were washed in phosphate-buffered saline (PBS, 10 mM, pH 7.4). As the primary antibody, mouse monoclonal antibodies against p57 (clone 25B2), E-cadherin (clone 36B5), p53 (clone IMX25), and inhibin-α (clone AMY82) were incubated with the slides for 60 min at room temperature, according to the manufacturer's protocol (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK). The slides were washed in PBS, and the sections were incubated for 20 min with biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA). The chromogenic reaction was performed using 3,3'-diaminobenzidine. Then, the sections were

washed in distilled water, counterstained with haematoxylin, and mounted with Entellan (Merck, Darmstadt, Germany). Appropriate positive and negative controls were run for each case. The evaluation of protein expression was performed by two independent pathologists (DS and BT). The stained cell types were identified as villous cytotrophoblasts, villous intermediate trophoblasts, villous syncytiotrophoblasts, villous stromal cells or decidual cells. Four slides per case ($n = 280$ slides total) were evaluated for immunohistochemical analysis.

On the basis of the staining pattern reported in the literature, the specimens were interpreted as "positive" for p57 staining when distinct nuclear staining (>50 %) of villous stromal cells and cytotrophoblasts was observed. The p57 stain was interpreted as "negative" when there was no distinct staining or limited nuclear staining (<10 %) of villous stromal cells and cytotrophoblasts but intermediate trophoblasts and/or maternal decidua exhibited nuclear expression of p57 (which served as the positive internal control for all cases). Nuclear expression in villous stromal cells and cytotrophoblasts in the focally positive range (10–50 %) was considered an equivocal result [11]. Syncytiotrophoblastic cells were used as negative controls.

The expression patterns of E-cadherin, p53 and inhibin-α were scored semi-quantitatively by evaluating the percentage of positive cells, staining intensity, and immunoreactivity score (IRS), as described elsewhere [12]. The IRS was calculated by multiplying the percentage of positive cells by the staining intensity. After all relevant slides had been examined, 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 %, 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. Membranous, nuclear, and cytoplasmic staining were the criteria for positive E-cadherin, p53, and inhibin-α reactions, respectively. As positive controls, slides with histological sections containing invasive ductal breast carcinoma for E-cadherin, colonic adenocarcinoma for p53, and adrenal cortical carcinoma for inhibin-α were used. Analyses were performed using a digital microscope and software (Nikon DS-Fi1 digital microscope camera and Nikon Digital Sight DS-L2 monitor; Nikon, Tokyo, Japan). Representative case examples are illustrated in Fig. 1.

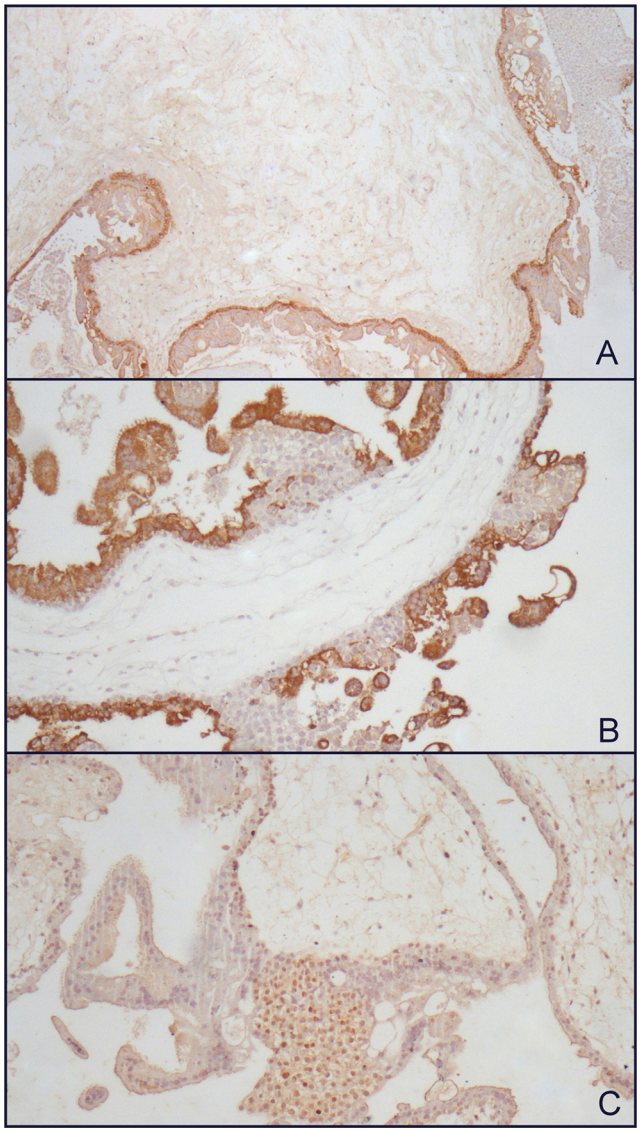

Fig 1 **a** E-cadherin expression on the surface of villous cytotrophoblasts in complete hydatidiform mole (magnification, ×100); **b)** the expression of inhibin-α in the cytoplasm of villous syncytiotrophoblasts in partial hydatidiform mole (magnification, ×100); **c)** the expression of p53 protein in the nuclei of villous cytotrophoblasts in complete hydatidiform mole (magnification, ×100)

DNA Analysis by STR Genotyping

DNA extraction was performed on formalin-fixed, paraffin embedded tissue following a standard procedure using an automated system (Magna Pure LC, Roche Diagnostics). Quantitative fluorescent polymerase chain reaction (QF-PCR) methodology was used to determine the diploidy status of the extracted DNA. Short-tandem repeat loci were evaluated in each sample using the ChromoQuant® QF-PCR kit (CyberGene AB, Solna, Sweden), which allows for DNA amplification and fluorescence analysis of 22 loci from different chromosomes and the amelogenin locus simultaneously. The amplified microsatellite fragment size data were analysed using ChromoQuant Visualizer STaR ver. 4.03 analysis software. QF-PCR amplification and capillary electrophoresis were performed according to the manufacturer's instructions. Capillary electrophoresis data from villous tissues were analysed to identify alleles at each locus. For each locus from which two alleles were identified, the allelic ratio was calculated by dividing the peak height of the longer allele by the

peak height of the shorter allele. Allelic ratios of 0.8–1.4 were considered consistent with diploidy. Allelic ratios between 0.3 and 0.6 or 1.6 and 2.0 were considered consistent with triploidy. Allelic ratios that fell between the normal and abnormal ranges were classed as inconclusive. In addition, loci with three and two alleles identified were consistent with triploidy and diploidy, respectively (Fig 2). At least two informative loci were required for the final interpretation.

Statistical Analysis

The semi-quantitative data are expressed as means ± SD. Numerical comparisons among the three groups were performed using the Kruskal-Wallis test, and *post hoc* comparisons were conducted using the Mann-Whitney *U*-test with Bonferroni correction. Categorical data were compared using Pearson's chi-square test. All tests were two-sided at a significance level of $p < 0.05$. All data were analysed using the SPSS software (ver. 18.0; SPSS Inc., Chicago, IL, USA).

Results

The ages of the 70 patients ranged from 17 to 45 (median, 30) years. Patients with PHM (median, 25 years) were younger than those with CHM (median, 30 years) and HA (median, 32 years). The median gestational age at the time of diagnosis

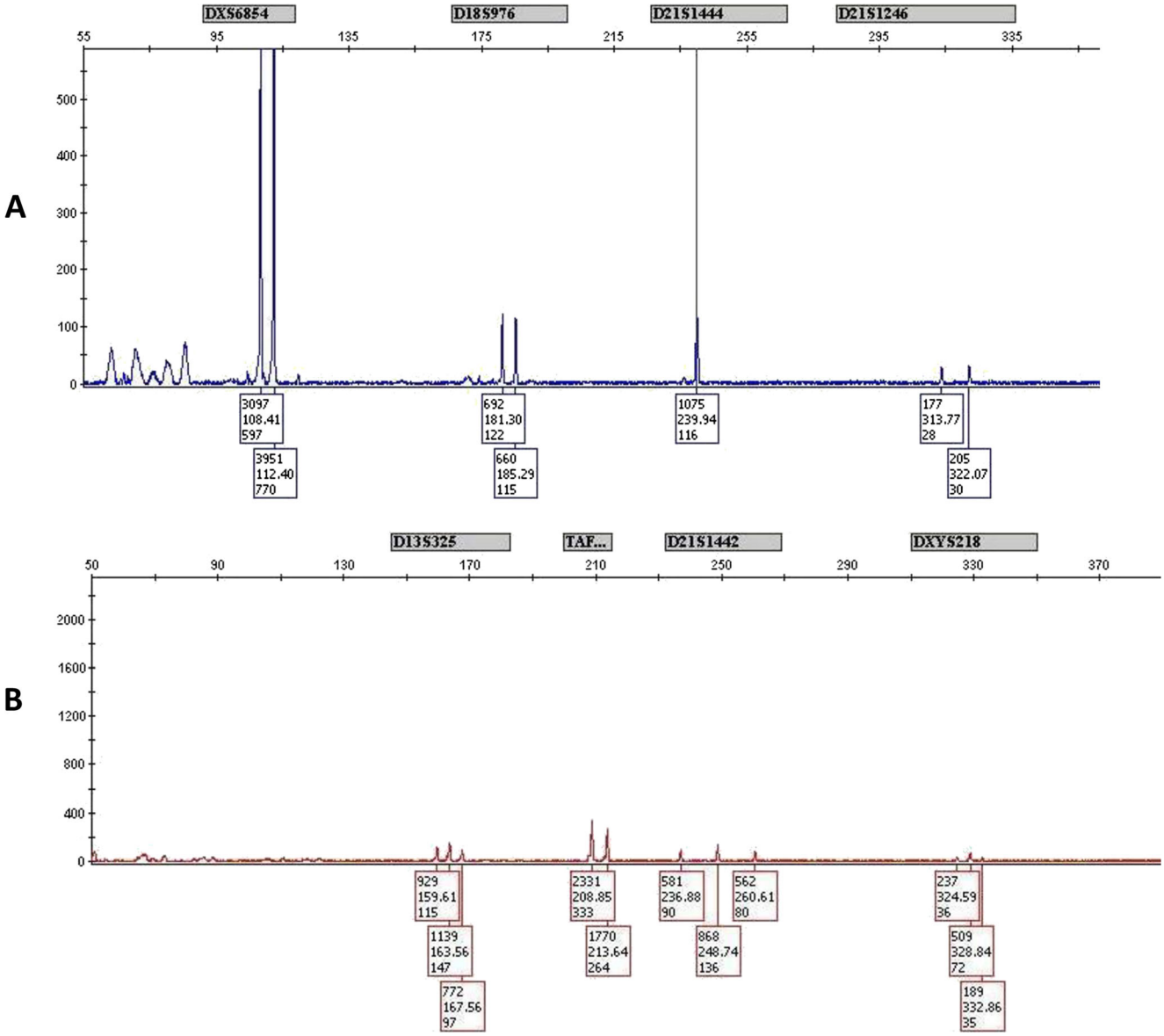

Fig 2 Representative examples of diploid and triploid histograms produced by short-tandem repeat amplification. **a** Three loci (DXS6854, D18S976 and D21S1246) each demonstrate two alleles, consistent with diploidy (two peaks with approximate 1:1 ratios); **b** Three loci (D13S325, D21S1442 and DXYS218) each demonstrate three alleles, consistent with triploidy

was 8 weeks for the HA cases, 10 weeks for PHM cases, and 9 weeks for CHM cases. All 23 cases that had been morphologically diagnosed as CHM exhibited a striking lack of p57 positive staining in villous cytotrophoblasts and stromal cells. Although the percentage of positive p57 staining tended to be higher in PHMs (75 %, $n=18$) than in HAs (69.6 %, $n=16$), the difference was not significant ($p=0.677$).

All HA, PHM and CHM cases with informative morphologic results demonstrated biparental diploidy, diandric triploidy and androgenetic diploidy, respectively. Immunostaining allowed the identification of specific cell types positive for E-cadherin, p53, and inhibin-α expression in all cases. As shown in Table 1, the expression patterns (percentage of positive cells, staining intensity, IRS) of the three proteins varied among HA, PHM, and CHM.

E-cadherin expression was observed on the cell membrane of villous cytotrophoblasts. Villous intermediate trophoblasts and villous syncytiotrophoblasts showed negative immunostaining. The expression pattern of E-cadherin was significantly higher in HAs compared with PHMs and CHMs, and in PHMs compared with CHMs (adjusted $p<0.017$ for all).

Immunostaining for p53 was found in the nucleus of villous cytotrophoblasts, whereas villous intermediate trophoblasts and villous syncytiotrophoblasts showed negative immunostaining. A significantly increased expression pattern of p53 was observed in CHMs compared with HAs and PHMs (adjusted $p<0.001$ for all), but there was no significant difference between HAs and PHMs (adjusted $p>0.017$).

Expression of inhibin-α was localised in the cytoplasm of villous syncytiotrophoblasts. We did not identify inhibin-α expression in villous intermediate trophoblasts or villous cytotrophoblasts. The expression pattern of inhibin-α was significantly higher in PHMs and CHMs than in HAs (adjusted $p<0.017$ for all), whereas no significant difference was observed between PHMs and CHMs (adjusted $p>0.017$).

Discussion

As the risk of persistent GTD differs for HA, PHM, and CHM, accurate diagnosis of the three entities has clinical management and prognostic implications. Whereas HA is completely benign, HMs have a significant risk of developing persistent GTD, with a higher incidence in patients with CHM (10-30 %) than in those with PHM (0.5–5 %) [13]. A careful microscopic evaluation of the morphological features on

Table 1 The expression pattern of E-cadherin, p53 and inhibin-α in hydropic abortions, partial and complete hydatidiform moles

Proteins	HA ($n=23$)	PHM ($n=24$)	CHM ($n=23$)	p	p^a	p^b	p^c
E-cadherin							
Stained cell type	cytotrophoblast	cytotrophoblast	cytotrophoblast				
Localization	cell membrane	cell membrane	cell membrane				
Staining intensity	2.7 ± 0.47	1.91 ± 0.67	1.53 ± 0.51	<0.001*	0.012*	0.009*	<0.001*
Percentage of positive cells	3.15 ± 0.83	2.61 ± 0.85	2.18 ± 0.88	0.023*	0.008*	0.012 *	<0.001*
Immunoreactivity scores	8.1 ± 3.04	6.26 ± 3.23	3.65 ± 2.23	<0.001*	<0.001*	0.002*	<0.001*
P53							
Stained cell type	cytotrophoblast	cytotrophoblast	cytotrophoblast				
Localization	nucleus	nucleus	nucleus				
Staining intensity	0.12 ± 0.33	0.22 ± 0.42	1.95 ± 0.39	<0.001*	<0.001*	0.418	<0.001*
Percentage of positive cells	0.12 ± 0.33	0.35 ± 0.78	2.6 ± 0.59	<0.001*	<0.001*	0.508	<0.001*
Immunoreactivity scores	0.12 ± 0.33	0.35 ± 0.78	5.1 ± 2.03	<0.001*	<0.001*	0.508	<0.001*
Inhibin-α							
Stained cell type	syncytiotrophoblast	syncytiotrophoblast	syncytiotrophoblast				
Localization	cytoplasm	cytoplasm	cytoplasm				
Staining intensity	2.12 ± 0.49	2.7 ± 0.57	2.78 ± 0.42	<0.001*	0.74	0.008*	0.006 *
Percentage of positive cells	2.88 ± 0.69	3.3 ± 0.73	3.61 ± 0.58	0.006*	0.145	0.001*	<0.001*
Immunoreactivity scores	6.35 ± 2.93	9.1 ± 2.94	10.17 ± 2.59	0.001*	0.172	<0.001*	<0.001*

Values are given as mean ± SD (standart deviation)

HA hydropic abortion, *PHM* partial hydatidiform mole, *CHM* complete hydatidiform mole, p between three groups

p^a between partial and complete hydatidiform mole, p^b between partial hydatidiform mole and hydropic abortion, p^c between complete hydatidiform mole and hydropic abortion.

Adjusted significance level for p^a, p^b and $p^c = 0.017$.

* Significant difference

haematoxylin and eosin-stained slides remains the cornerstone of diagnosis for these three entities. Immunohistochemical markers that allow differentiation of these pathologies are important in clinical practice. Although immunohistochemical analysis of p57 can be helpful in distinguishing CHM from its mimics, it cannot discern a PHM from a HA. Thus, we evaluated the combined expression patterns of three embryogenesis-related proteins—E-cadherin, p53, and inhibin-α—by immunohistochemistry in HAs, PHMs, CHMs, and assessed their clinical application in the differential diagnosis of the three entities.

E-cadherin is important for the maintenance of tissue architecture in the adult, as it is in the embryo. During embryogenesis, E-cadherin mediates a strong intercellular interaction between adjacent trophoblast cells. Additionally, E-cadherin is believed to be involved in trophoblast-endometrium interactions and its expression may be necessary in regulating the process of implantation and human placental development [14]. The dynamic expression of E-cadherin is relevant to the morphology of trophoblasts at the various stages of placental development. In the first and second trimesters, E-cadherin is localised along the lateral and apical surfaces of the cytotrophoblast columns. In the third trimester, E-cadherin is localised at the basal surface of syncytiotrophoblasts at sites where cytotrophoblasts are absent. Expression of E-cadherin is reduced from the first to the third trimester in the normal-term placenta [15]. This change may be attributable to the downregulation of E-cadherin gene expression during the differentiation of cytotrophoblasts to syncytiotrophoblasts as gestation advances. The downregulation of E-cadherin expression is associated with the invasiveness of trophoblasts [16, 17]. In this respect, a recent study found that expression of E-cadherin decreased gradually from normal villous trophoblasts in early pregnancy to benign HM to invasive HM [18]. However, no subclassification of benign HM was made and the stained trophoblast types were not identified. Xue et al. [19] reported a significant reduction of E-cadherin expression in CHM compared with that in normal first-trimester placenta; however, this finding was not corroborated by Balaram et al. [20]. In the present study, we observed a marked decline in the expression pattern of E-cadherin from HAs to PHMs to CHMs. This suggests that disturbance of chorionic integrity with uncontrolled proliferation and invasiveness of trophoblastic cells in HMs may, in part, be attributable to the loss of their adhesive properties, mediated by E-cadherin.

The expression of p53 has been described previously in first-trimester trophoblasts, where it contributes to cellular differentiation by stimulating the expression of various p53 target genes [21]. It has been suggested that immunohistochemically detectable expression of p53 in the trophoblast is due not to mutation of the gene, as in malignant tumours, but rather to upregulation of the p53 tumour suppressor gene that could be essential for controlling excessive trophoblast proliferation [22]. During normal placentation, continuous proliferation of trophoblasts leads to genomic instability in those cells. Thus, the trophoblasts either arrest the cell cycle and repairing genomic instability or undergo apoptosis. It is also thought that the overexpression of p53 is associated with extensive p53-dependent apoptosis in trophoblasts and that this is a defensive mechanism against the development of abnormal cells [23]. In addition to its pivotal roles in embryogenesis, several studies have revealed that overexpression of p53 is involved in the pathogenesis of GTD [24, 25]. Furthermore, immunohistochemical analysis of p53 expression has been proposed as a potential diagnostic tool to discriminate HMs and HAs. Al-Bozom [26] identified increased expression of p53 in CHMs compared with PHMs with absence of expression in HAs; however, only staining in the villous intermediate trophoblasts was considered for evaluation. A recent study reported significantly higher p53 expression in PHMs than HAs, but that study compared the two entities with regard to the percentage of stained cells [27]. Our findings demonstrated enhanced expression of p53 in CHMs compared with the PHMs and HAs, supporting the higher cell activity and apoptosis in the trophoblasts of CHM. We used a monoclonal antibody that recognises both wild-type and mutant p53, as did the above-mentioned studies; thus the discrepancies may be attributable to the use of different immunohistochemical evaluation methods.

The corpus luteum and placenta are the main sources of circulating inhibin during pregnancy. Inhibin has been shown to modulate the secretion of other placental hormones and the maternal immune response to invading trophoblastic cells [28]. The presence of inhibin subunits has also been reported in the endometrium, suggesting that these molecules could regulate decidualisation and implantation as paracrine modulators of early pregnancy [29, 30]. It has been shown that alteration of inhibin subunit expression may lead to abnormal placentation [31]. Immunohistochemical studies revealed that inhibin subunits are also localised in GTDs [32, 33]. Mylonas et al. [34] compared the expression patterns of all inhibin subunits in CHMs and PHMs. According to these authors, inhibin-βA and inhibin-βB markers may be useful in the differential diagnosis of the two entities. The present study is the first reported attempt to assess the value of inhibin-α expression for distinguishing HMs from HAs. Our results demonstrated a significant increase in inhibin-α expression in both CHMs and PMS, compared with HAs. Although the precise role of inhibin-α in HMs remains unclear, we suggest that increased expression of inhibin-α in trophoblastic cells from HMs is a compensatory mechanism to overcome impaired placentation as a modulator of early embryonic development.

In conclusion, the present study demonstrates that immunohistochemical evaluation of E-cadherin, p53, and inhibin-α expression could serve as a useful adjunct to conventional methods in the differential diagnosis of HA, PHM, and CHM.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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