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Original Article

P63 and Ki-67 expression in trophoblastic disease and spontaneous abortion

Minoo Erfanian[a], Nourieh Sharifi[b], Abas Ali Omidi[c]*

Abstract

BACKGROUND: Despite well-described histopathologic criteria, the distinction of spontaneous abortion from hydatidiform mole and complete hydatidiform mole from partial hydatidiform mole remains a problem because of interobserver and intraobserver variability. The aim of this study was to evaluate the value of two immunohistochemical markers in the differential diagnosis of subgroups of lesions of villous trophoblasts and spontaneous abortions.

METHODS: Immunohistochemistry with the P63 and Ki-67 antibody was performed in formalin-fixed paraffin-embedded samples of non hydropic abortion (n = 14), partial hydatidiform mole (n = 12), complete hydatidiform mole (n = 12) and choriocarcinoma (n = 12). The Ki-67 and P63 labeling index (number of positive nuclei/total number of nuclei) for villous stromal cells, cytotrophoblasts and syncytiotrophoblasts were evaluated separately by counting 100 cells of each population. Statistical analysis was carried out by $\chi 2$ analysis, and the Mann-Whitney U test. Statistical significance was determined at $p < 0.05$ on the basis of 2-tailed tests.

RESULTS: None of nonhydropic spontaneous abortions analyzed exhibited positive cytotrotrophoblastic and syncytiotrophoblastic cells for P63. The syncytiotrophoblastic cells were negative for p63 in all of choriocarcinomas. All of choriocarcinomas analyzed exhibited severe expression of Ki-67 in cytotrotrophoblastic cells. None of abortions and partial moles was diffusely labeled with Ki-67.

CONCLUSIONS: Ki-67 labeling index in cytotrophoblastic cells is the best index to differentiate between abortion and subgroups of lesions of villous trophoblasts as well as between different subgroups of lesions of villous trophoblasts. Ki-67 is a better marker than P63 to attain this goal.

KEYWORDS: Partial Hydatidiform Mole, Complete Hydatidiform Mole, Choriocarcinoma, Abortion, P63, Ki-67, Immunohistochemistry.

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Gestational trophoblastic diseases are a group of interrelated diseases of trophoblastic tissue that include partial hydatidiform mole, complete hydatidiform mole, invasive mole, choriocarcinoma, and placental site trophoblastic tumor.

The risk of persistant gestational disease is higher in complete hydatidiform mole in comparison to partial hydatidiform mole. In addition, rare instances of choriocarcinoma have followed partial hydatidiform mole,[1-3] while choriocarcinoma follows complete hydatidiform mole in 2-5% of cases.[4] Choriocarcinomas are clearly malignant neoplastic lesions but hydatidiform moles are just abnormal placental tissues with a potential for malignant change.[5] Thus, clinicians require an accurate diagnosis of these entities for both prognosis and patient management and a diagnosis reflecting uncertainty such as "cannot rule out molar pregnancy" or "lesion suspicious for HM" is insufficient.

Despite well-described histopathologic criteria, the distinction of spontaneous abortion from hydatidiform mole and complete hydatidiform mole from partial hydatidiform

[a] Resident of Anatomoclinical Pathology, Mashhad Medical University, Mashhad, Iran.
[b] Associate Professor of Anatomoclinical Pathology, Mashhad Medical University, Mashhad, Iran.
[c] Professor of Anatomoclinical Pathology, Mashhad Medical University, Mashhad, Iran.
* Corresponding Author
E-mail: nourieh_sharifi@yahoo.com

mole remains a problem because of interobserver and intraobserver variability.[6,7] Especially, in early pregnancy in that the diagnostic criteria are subtly different from the classical pathological features.[8] Thus, development of new methods that allow to differentiate these pathologies in doubtful cases is important.

A reliable and complementary method to the pathologic interpretation is a genetic study of the conceptus.[9] Assessment of ploidy used in situ hybridization or flow cytometry can distinguish diploid CM or hydropic abortion from triploid PM. However, ploidy studies cannot distinguish between CM and diploid nonmolar products of conception, molar and nonmolar triploids, monospermic and dispermic CM or androgenetic and biparental CM. To diagnose these entities, the parental origin of the nuclear chromosomes must be determined using DNA polymorphisms in conjunction with polymerase chain reaction (PCR), which may be carried out even on small amounts of fixed tissue from paraffin blocks.[10] However, these methods are time-consuming and require both maternal and paternal blood samples in addition to molar tissue.[11]

Another complementary method to the pathologic interpretation is immunohistochemistery. One of the advantages of these methods is the ability to apply them retrospectively to sections of routinely formalin-fixed and paraffin-embedded tissue. Another advantage is that there is no need for expensive or sophisticated equipments.

Among the immunohistochemical markers, proliferation markers such as Ki-67 have been established as a valuable reflection of the tissue proliferative compartment and thus could be of value in studying the biologic behavior of gestational tophoblastic diseases. The Ki-67 gene encodes a large nuclear protein with 2 isoforms in which their biologic functions remain unclear. Ki-67 immunoreactivity can be found in all phases of cell cycle except in the quiescent G0 phase.[12,13]

Other markers that have been investigated for this purpose are tumor suppressor genes such as P53 and P63 that is a P53 homologue.[14] However, P63 is not a classical tumor suppressor gene and P63 expression is associated with several malignancies.[15] These data indicate that P63 may act as an oncogene in the genesis of these tumors.[16] In normal placentas P63 is expressed in the cytotrophoblast cells [17,18] but The role of P63 in gestational trophoblastic diseases, however, merits further investigation.

The aim of this study therefore was to evaluate the expressions of a proliferation marker (Ki-67) and of P63 tumor protein in nonhydropic abortion, PHM, CHM and choriocarcinoma and also to assess the values of these markers in the differential diagnosis of subgroups of lesions of villous trophoblasts and spontaneous abortions.

Methods

Subjects

Twelve partial hydatidiform moles, 12 complete hydatidiform moles, 12 choriocarcinomas, and 14 first-trimester nonhydropic spontaneous abortions (control group) diagnosed previously in the Qhaem and Imam reza Department of Pathology, Mashhad University of Medical Sciences were included in the study after reevaluation of each case to confirm the diagnosis by two pathologists. In order to differentiate PHM from CHM the histological features of the specimens were assessed according to the diagnostic criteria of Genest.

Immunohistochemistry

Multiple 5-µm-thick sections of representative formalin-fixed, paraffin-embedded tissues were cut for immunohistochemical studies. A polymer-based technique was used for the detection of Ki-67 (Clone MIB-1, N1633, DakoCytomation, Glostrup, Denmark) and P63 (Clone 4A4, 1:100 Dilution, DakoCytomation, Glostrup, Denmark). Normal prostatic tissue was used as the positive control for P63 and reactive lymph node was used as the positive control for Ki-67.

Report

All immunostained sections were examined by the same two observers with a ×400 objective

under the light microscope (Olympus Bx50; Olympus Optical Co, Ltd, Tokyo, Japan) for evaluation of Ki-67, and P63 expressions. In the abortion and hydatidiform mole specimens Ki-67 and P63 expression for villous stromal cells, cytotrophoblasts, and syncytiotrophoblasts were evaluated separately by counting 100 cells of each population. P63 and Ki-67 expression was quantitatively assessed as 0 (no stained cells), + (less than 10% positive cells), ++ (10-50% positive cells), and +++ (more than 50% positive cells). The intensity was scored as 0 (absence), + (weak), ++ (moderate), or +++ (strong). In the choriocarcinoma specimens 100 cytotrophoblastic and 100 syncytiotrophoblastic cells were counted in each case, and Ki-67 and P63 expression and intensity were assessed by the same criteria noted above.

Statistical Analysis

The results obtained from each case groups were compared in pairs for all the parameters included in the study (P63 and Ki-67 labeling index, distribution of Ki-67 and P63 immunostaining, intensity of Ki-67 and P63 immunostaining) by means of the Student t test, $\chi 2$ analysis, and the Mann-Whitney U test. Statistical significance was determined at $p < 0.05$ on the basis of 2-tailed tests.

Results

None of nonhydropic spontaneous abortions analyzed exhibited positive cytotrotrophoblastic and syncytiotrophoblastic cells for P63. The syncytiotrophoblastic cells were negative for P63 in all of choriocarcinomas (Figure 1).

All of choriocarcinomas analyzed exhibited severe expression of Ki-67 in cytotrotrophoblastic cells. None of abortions and partial moles was diffusely labeled with Ki-67 (> 50%) (Figure 2).

In order to analyze these data, the four groups of complete and incomplete diagnosed hydatidiform moles, spontaneous abortions and choriocarcinomas were matched in pairs and evaluated according to the P63 and Ki-67 expression. The results of statistic analysis are summarized in tables 1-10.

Table 1. Ki-67 labeling index (% of positively stained nuclei/total number of nuclei counted) in abortion and lesions of villous trophoblasts

Type of lesion	Ki-67 labeling index in cytotrophoblasts	Ki-67 labeling index in syncytiotrophoblasts	Ki-67 labeling index in stromal cells
Abortion	7.35 ± 7.63	1.50 ± 2.62	2.00 ± 1.83
PHM	25.83 ± 10.62	3.66 ± 2.18	3.16 ± 8.49
CHM	52.08 ± 19.70	14.16 ± 19.28	5.83 ± 11.37
CC	80.41 ± 9.40	58.75 ± 18.10	0

All values are expressed as mean ± SD
PHM: partial hydatidiform mole; CHM: complete hydatidiform mole; CC: choriocarcinoma

Table 2. P63 labeling index (% of positively stained nuclei/total number of nuclei counted) in abortion and lesions of villous trophoblasts

Type of lesion	P63 labeling index in cytotrophoblasts	P63 labeling index in syncytiotrophoblasts	P63 labeling index in stromal cells
Abortion	0	0	0.64 ± 0.74
PHM	38.16 ± 18.09	5.41 ± 8.82	0.75 ± 0.86
CHM	63.75 ± 14.63	21.91 ± 17.50	3.16 ± 5.49
CC	56.66 ± 21.35	0	0

All values are expressed as mean ± SD
PHM: partial hydatidiform mole; CHM: complete hydatidiform mole; CC: choriocarcinoma

Table 3. Distribution of Ki-67 immunoreactivity in abortion and lesions of villous trophoblasts

Type of lesion	Distribution of Ki-67 immunoreactivity											
	Cytotrophoblast				Syncytiotrophoblast				Stromal			
	0	+	++	+++	0	+	++	+++	0	+	++	+++
Abortion	0	10	4	0	6	7	1	0	3	11	0	0
PHM	0	0	12	0	1	11	0	0	6	5	1	0
CHM	0	0	5	7	3	3	5	1	5	5	2	0
CC	0	0	0	12	0	0	4	8	12	0	0	0

PHM: partial hydatidiform mole; CHM: complete hydatidiform mole; CC: choriocarcinoma. 0 (no stained cells), + (less than 10% positive cells), ++ (10-50% positive cells), and +++ (more than 50% positive cells)

Table 4. Distribution of P63 immunoreactivity in abortion and lesions of villous trophoblasts

Type of lesion	Distribution of P63 immunoreactivity											
	Cytotrophoblast				Syncytiotrophoblast				Stromal			
	0	+	++	+++	0	+	++	+++	0	+	++	+++
Abortion	14	0	0	0	14	0	0	0	8	6	0	0
PHM	0	1	9	2	4	6	2	0	5	7	0	0
CHM	0	0	3	9	2	3	7	0	3	8	1	0
CC	0	0	3	9	12	0	0	0	12	0	0	0

PHM: partial hydatidiform mole; CHM: complete hydatidiform mole; CC: choriocarcinoma. 0 (no stained cells), + (less than 10% positive cells), ++ (10-50% positive cells), and +++ (more than 50% positive cells)

Table 5. Intensity of P63 immunoreactivity in abortion and lesions of villous trophoblasts

Type of lesion	Intensity of P63 immunoreactivity											
	Cytotrophoblast				Syncytiotrophoblast				Stromal			
	N	W	M	S	N	W	M	S	N	W	M	S
Abortion	14	0	0	0	14	0	0	0	8	5	1	0
PHM	0	1	4	7	4	2	4	2	5	0	4	3
CHM	0	0	1	11	2	0	6	4	3	1	3	5
CC	0	1	7	4	12	0	0	0	12	0	0	0

PHM: partial hydatidiform mole; CHM: complete hydatidiform mole; CC: choriocarcinoma
N: negative; W: weak; M: moderate; S: severe

Table 6. Intensity of Ki-67 immunoreactivity in abortion and lesions of villous trophoblasts

Type of lesion	Intensity of Ki-67 immunoreactivity											
	Cytotrophoblast				Syncytiotrophoblast				Stromal			
	N	W	M	S	N	W	M	S	N	W	M	S
Abortion	0	0	8	6	6	0	6	2	3	0	10	1
PHM	0	1	4	7	1	1	4	6	6	0	3	3
CHM	0	0	1	11	3	0	6	3	5	1	3	3
CC	0	0	7	5	0	0	6	6	12	0	0	0

PHM: partial hydatidiform mole; CHM: complete hydatidiform mole; CC: choriocarcinoma
N: negative; W: weak ; M: moderate; S: severe

Table 7. Results of statistic analysis to compare P63 expression between choriocarcinoma and abortion, partial hydatidiform mole and complete hydatidiform mole

Type of lesions to be separated	P63 labeling index		Distribution of P63 expression		Intensity of P63 expression	
	Cyto	Syncytio	Cyto	Syncytio	Cyto	Syncytio
Abortion, CC	(p < 0.001) *	(p = 1.000) **	(p < 0.001) *	n	(p < 0.001) *	n
PHM, CC	(p = 0.039) *	(p < 0.001) *	(p = 0.015) *	(p = 0.002) *	(p = 0.441) **	(p = 0.007) *
CHM, CC	(p = 0.443) **	(p < 0.001) *	(p = 1.000) **	(p < 0.001) *	(p = 0.012) *	(p < 0.001) *

PHM: partial hydatidiform mole; CHM: complete hydatidiform mole; CC: choriocarcinoma; cyto: cytotrophoblast; syncytio: syncytiotrophoblast. All of values are p values.
* Statistical significance. ** Absence of statistical significance. n: no statistics are computed

Table 8. Results of statistic analysis to compare Ki-67 expression between choriocarcinoma and abortion, partial hydatidiform mole and complete hydatidiform mole

Type of lesions to be separated	Ki-67 labeling index		Distribution of Ki-67 expression		Intensity of Ki-67 expression	
	Cyto	Syncytio	Cyto	Syncytio	Cyto	Syncytio
Abortion, CC	(p < 0.001)*	(p < 0.001) *	(p < 0.001) *	(p < 0.001) *	(p = 0.951) **	(p = 0.019) *
PHM, CC	(p < 0.001)*	(p < 0.001)*	(p < 0.001) *	(p < 0.001) *	(p = 0.341) **	(p = 0.494) **
CHM, CC	(p < 0.001)*	(p < 0.001) *	(p = 0.012) *	(p = 0.009) *	(p = 0.009) *	(p = 0.135) **

PHM: partial hydatidiform mole; CHM: complete hydatidiform mole; CC: choriocarcinoma; cyto: cytotrophoblast; syncytio: syncytiotrophoblast. All of values are p values.
* Statistical significance. ** Absence of statistical significance. n: no statistics are computed

Table 9. Results of statistic analysis to compare Ki-67 expression between abortion and partial hydatidiform mole, abortion and complete hydatidiform mole, and partial hydatidiform mole and complete hydatidiform mole

Type of lesions to be separated	Ki-67 labeling index			Distribution of Ki-67 expression			Intensity of Ki-67 expression		
	Cyto	Syncytio	Stromal	Cyto	Syncytio	Stromal	Cyto	Syncytio	Stromal
Abortion , PHM	(p < 0.001) *	(p = 0.009) *	(p = 0.193) **	(p < 0.001) *	(p = 0.069) **	(p = 0.127) **	(p = 0.321) **	(p = 0.077) **	(p = 0.059) **
Abortion, CHM	(p < 0.001) *	(p = 0.013) *	(p = 0.527) **	(p < 0.001) *	(p = 0.105) *	(p = 0.099) **	(p = 0.009) *	(p = 0.591) **	(p = 0.104) **
PHM, CHM	(p = 0.002) *	(p = 0.143) **	(p = 0.671) **	(p = 0.002) *	(p = 0.009) *	(p = 0.809) **	(p = 0.158) **	(p = 0.334) **	(p = 0.779) **

PHM: partial hydatidiform mole; CHM: complete hydatidiform mole; CC: choriocarcinoma; cyto: cytotrophoblast; syncytio: syncytiotrophoblast. All of values are p values.
* Statistical significance.** Absence of statistical significance. n: no statistics are computed

Table 10. Results of statistic analysis to compare P63 expression between abortion and partial hydatidiform mole, abortion and complete hydatidiform mole, and partial hydatidiform mole and complete hydatidiform mole

Type of lesions to be separated	P63 labeling index			Distribution of P63 expression			Intensity of P63 expression		
	Cyto	Syncytio	Stromal	Cyto	Syncytio	Stromal	Cyto	Syncytio	Stromal
Abortion, PHM	(p < 0.001) *	(p = 0.003) *	(p = 0.820) **	(p < 0.001) *	(p < 0.001) *	(p = 0.671) **	(p < 0.001) *	(p = 0.004) *	(p = 0.013) *
Abortion, CHM	(p < 0.001) *	(p < 0.001) *	(p = 0.041) *	(p < 0.001) *	(p < 0.001) *	(p = 0.283) **	(p < 0.001) *	(p < 0.001) *	(p = 0.013) *
PH M, CHM	(p = 0.001) *	(p = 0.012) *	(p = 0.078) **	(p = 0.015) *	(p = 0.108) **	(p = 0.457) **	(p = 0.158) **	(p = 0.292) **	(p = 0.543) **

PHM: partial hydatidiform mole; CHM: complete hydatidiform mole; CC: choriocarcinoma; cyto: cytotrophoblast; syncytio: syncytiotrophoblast. All of values are p values.
* Statistical significance.** Absence of statistical significance. n: no statistics are computed

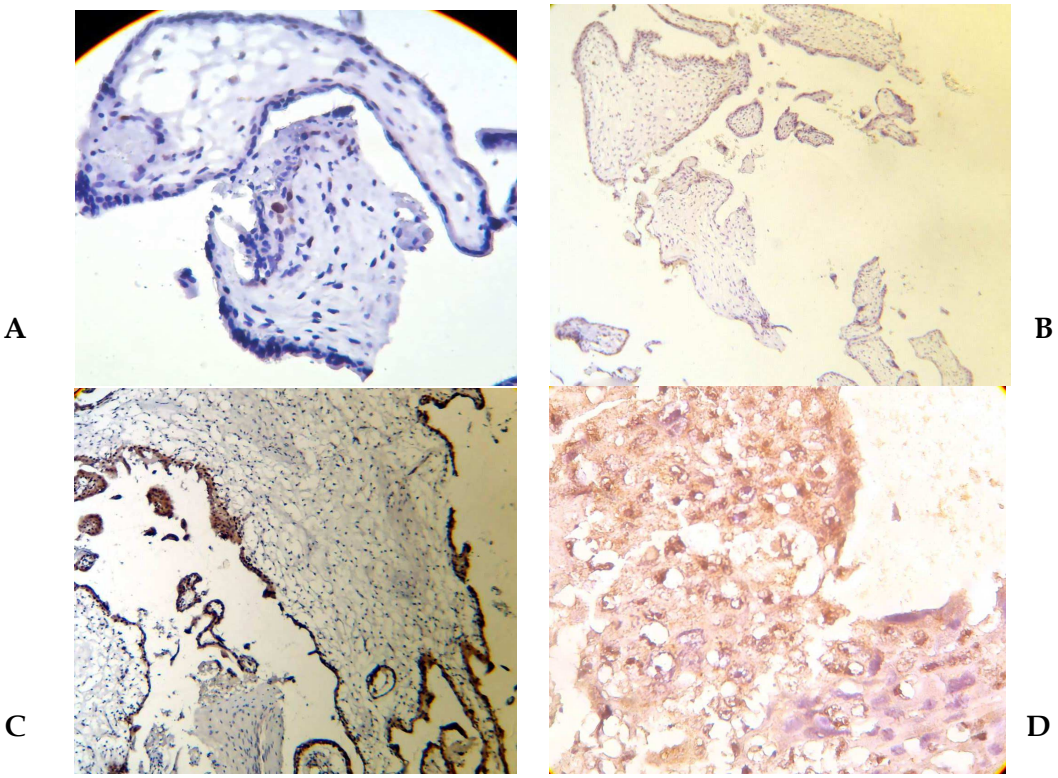

Figure 1. Immunohistochemical staining with P63 antibody in spontaneous abortion (A), partial (B) and complete(C) hydatidiform moles, and choriocarcinoma (D).

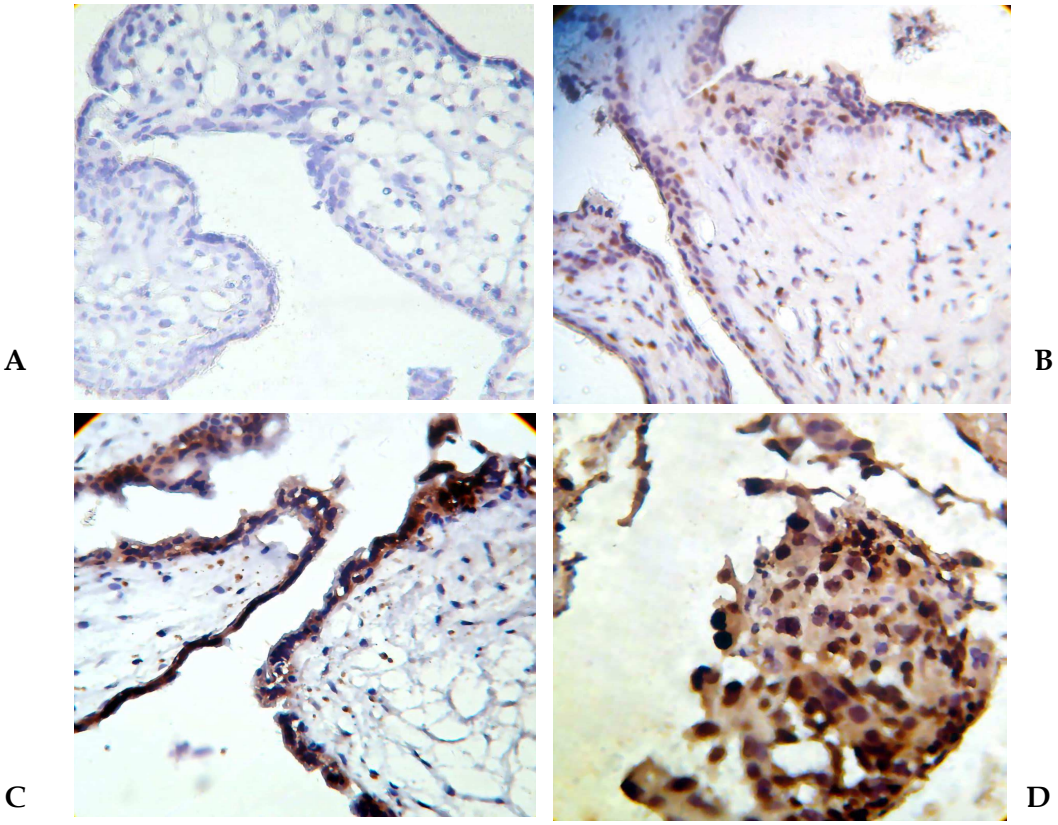

Figure 2. Immunohistochemical staining with Ki-67 antibody in spontaneous abortion (A), partial (B) and complete(C) hydatidiform moles, and choriocarcinoma (D).

Discussion

Gestational trophoblastic disease is defined as a spectrum of abnormal gestations and neoplasm arising from villous or extravillous trophoblast that are associated with pregnancy. It takes several forms, each with its own risk of mortality and responsiveness to chemotherapy. Differential diagnosis of these diseases by routine histopathologic examination can be challenging. Studies have recently shown that immunohistochemistry for various markers is useful for confirming the diagnosis. In a study done by Cheville JC et al growth fraction (number of positive cells/total number of cells) of Ki-67 in cytotrophblastic cells was useful in separating complete mole from partial moles but not partial moles from hydropic abortion.[19] In this study growth fraction on stromal cells did not differ among these three entities. These findings correlate with present results that growth fraction of Ki-67 in syncytiotrophoblastic cells is useful in separating partial moles from hydropic abortion but not complete mole from partial moles and the distribution of Ki-67 in cytotrophblastic cells was also useful in separating these three entities. According to present results, intensity of Ki-67 immunostaining may be useful in separating hydropic abortion from complete mole (but neither hydropic abortion from partial moles nor partial mole from complete mole) in only cytotrophblastic cells group. In summary, it seems that among these three groups of cells (cytotrophblasts, syncytiotrophoblasts, stromal cells) and among these three indexes (Ki-67 labeling index, Ki-67 distibution, Ki-67 intensity of immunostaining) Ki-67 labeling index and Ki-67 disribution of immunostaining in cytotrophblastic cells are the best indexes in separating these three entities.

In other studies Ki-67 was not expressed in syncytiotrophoblastic cells. However, in this study Ki-67 expression was observed in syncytiotrophoblastic cells in abortion, partial hydatidiform mole, complete hydatidiform mole and choriocarcinoma. The highest Ki-67 expression was observed in choriocarcinoma. In addition, Ki-67 labeling index was useful in separating choriocarcinomas from nonhydropic abortion, partial mole and complete mole.[20,21]

Kale et al showed the expression of Ki-67 was significantly higher in hydatidiform moles (complete, partial and invasive) than in the control group (abortion) [22] which means that these findings are parallel to present results.

A study done by Shih et al showed all of choriocarcinomas were diffusely labeled with Ki-67 (> 50%).[18] These findings correlate with present results. In addition, according to present results, none of abortions and partial moles were diffusely labeled with Ki-67 (> 50%). Present results suggest that the distribution of Ki-67 may be useful in separating choriocarcinomas from nonhydropic abortion, partial mole and complete mole.

In one study Ramalho et al did not find any P63-positive cells in choriocarcinomas.[23] They concluded that P63 might be useful to differentiate a choriocarcinoma from other gestational trophoblastic diseases and might have a role in malignant transformation of gestational trophoblastic diseases but Shih et al showed that six of eight choriocarcinomas (63%) analyzed by them were positive for P63. In present study, all of choriocarcinomas were positive for P63. According to literature data, choriocarcinomas express several proteins that antagonize apoptosis [24,25] and P63 is one of these proteins.

Also in a study that was done by Ramalho et al there statistical difference was observed in distribution of P63 positive cytotrophoblastic cells between hydropic abortion and choriocarcinoma, partial hydatidiform mole and choriocarcinoma and complete hydatidiform mole and choriocarcinoma. However, according to present results, P63 labeling index and distribution of P63 immunostaining are useful in separating choriocarcinoma from hydropic abortion and partial hydatidiform mole but not from complete hydatidiform mole. In the study mentioned above none of moles (complete, partial) expressed P63 in syncytiotrophoblastic cells. However, according to present results P63 may be expressed in syncytiotrophoblastic

cells in this type of lesion. In addition, P63 labeling index and distribution of P63 immunostaining may be useful in differentiation of choriocarcinoma from partial hydatidiform mole and complete hydatidiform mole. However, according to present results no difference was found between partial hydatidiform mole and choriocarcinoma. Briefly it seems that among these two groups of cells (cytotrophblasts, syncytiotrophoblasts) and among these three indexes (P63 labeling index, P63 distibution, P63 intensity of immunostaining) none of indexes is able to differentiate between hydropic abortion and choriocarcinoma, as well as between partial hydatidiform mole and choriocarcinoma and complete hydatidiform mole and choriocarcinoma. By reviewing the above mentioned results it seems that Ki-67 is a better marker than P63 to attain this goal.

According to present results, P63 is negative in cytotrophoblastic and syncytotrophoblastic cells in all of abortions. All of partial moles and complete moles are positive for P63. Thus, P63 may be an ideal marker in separating abortion from mole in doubtful cases. In a study done by Ramalho et al no statistical difference was found in distribution of P63 positive cytotrophoblastic cells between hydropic abortion and partial hydatidiform mole, hydropic abortion and complete hydatidiform mole, and partial hydatidiform mole and complete hydatidiform mole. However, in present study distribution of P63 in cytotrophoblastic cells is useful in separating all of these three entities. In the study mentioned above, a difference was observed in the intensity of P63 immunostaining between hydropic abortion and partial hydatidiform mole, hydropic abortion and complete hydatidiform mole but not between partial hydatidiform mole and complete hydatidiform mole. Present results correlate with these findings.

According to present results, P63 labeling index in syncytiotrophoblastic cells is also useful in separating partial moles from hydropic abortion, complete mole from partial moles and hydropic abortion from complete mole. According to present results, distribution and intensity of P63 in syncytiotrophoblastic cells are also useful in separating partial moles from hydropic abortion and hydropic abortion from complete mole but not complete mole from partial mole.

Present results show that P63 labeling index in stromal cells is useful in separating hydropic abortion from complete mole but not partial moles from hydropic abortion and complete mole from partial moles and distribution of P63 immunostaining was not useful in separating these three entities. Intensity of P63 in stromal cells was useful in separating partial moles from hydropic abortion and hydropic abortion from complete mole but not complete mole from partial mole. In summary, it seems that among these three groups of cells (cytotrophblasts, syncytiotrophoblasts, stromal cells) and among these three indexes (P63 labeling index, P63 distibution, P63 intensity of immunostaining) P63 labeling index in cytotrophblastic and syncytiotrophoblastic cells are the best indexes in separating these three entities.

Conclusions

In summary Ki-67 labeling index in cytotrophoblastic cells is the best index to differentiate between abortion and subgroups of lesions of villous trophoblasts as well as between different subgroups of lesions of villous trophoblasts. Ki-67 is a better marker than P63 to attain this goal. The results of this study show that the evaluation of expressions of P63 in the cytotrophoblastic cells contributes to a reliable discrimination between spontaneous abortions and hydatidiform mole.

P63 is negative in cytotrophoblastic cells in all of abortions. Because of anti-apoptotic role of this protein, reduced expression of this protein might have a pathogenic role in abortion. The significance of these alterations in the pathogenesis of abortion, however, merits further investigation.

To the best of our knowledge this is the second study to investigate the expressions of P63 for the diagnosis of spontaneous abortions and hydatidiform moles. As mentioned above there

was some discrepancy between present results and results of previous study. So it is wise to investigate the expression of P63 in these lesions in another study with greater number of cases.

Conflict of Interests

Authors have no conflict of interests.

Authors' Contributions

ME carried out the design and coordinated the study, participated in most of the experiments and prepared the manuscript. NS provided assistance in the design of the study, coordinated and carried out all the experiments and participated in manuscript preparation. AAO provided assistance for all experiments. All authors have read and approved the content of the manuscript.

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