Expression of Pro-Apoptotic Bax and Anti-Apoptotic Bcl-2 Proteins in Hydatidiform Moles and Placentas With Hydropic Changes

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Abstract- Morphologic examination still forms the main diagnostic tool in the differential diagnosis of molar placentas. However the criteria are subjective and show considerable inter-observer variability among pathologists. The aim of the present study was to investigate the role of Bcl-2 and Bax immunostaining in the differential diagnosis of molar placentas. Bax and Bcl-2 immunohistochemical staining were performed in 19 molars (8 partial and 11 complete hydatidiform mole) and 10 non-molar (hydropic abortion) formalin-fixed, paraffin-embedded tissue samples. Ploidy analysis using flow cytometry had confirmed diploidy in hydropic abortions and complete hydatidiform moles and triploidy in partial hydatidiform moles. Bcl-2 expression was observed only in syncytiotrophoblasts, No immunoreactivity was detected in Cytotrophoblasts, and stromal cells, the total score averages of Bcl-2 immunoexpression in partial hydatidiform moles and hydropic abortions were significantly higher than in complete hydatidiform moles, whereas no significant difference was observed between partial hydatidiform moles and hydropic abortions. Bax immunoreactivity was observed in cytotrophoblasts, stromal cells and occasionally in syncytiotrophoblasts. No statistically significant difference in Bax immunoexpression total score was observed among various groups. Based on the results of this study, Bcl-2 immunostaining offers a potential adjunctive diagnostic tool to distinguish complete hydatidiform mole from partial hydatidiform mole and hydropic abortion, but not partial hydatidiform mole from hydropic abortion, Bax immunostaining cannot be helpful in this regard.

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Keywords: Hydatidiform mole; Abortion; Immunohistochemistry; Bcl-2; Bax

Introduction

Gestational trophoblastic disease (GTD) comprise a spectrum of disorders from the premalignant conditions of complete hydatidiform mole (CHM) and partial hydatidiform mole (PHM) through to the malignant invasive mole (IM), choriocarcinoma (CC) and very rare placental site trophoblastic tumour (PSTT) and epithelioid trophoblastic tumour (ETT) (1). Hydatidiform mole (HM) is the most common form of GTD; both the partial and complete forms of HMs are primarily benign but can develop into persistent trophoblastic diseases that require chemotherapy (2,3). HM is an abnormal pregnancy characterized by hydropic swelling of placental villi, and trophoblastic hyperplasia (4), Placentas characterized by hydropic swelling of chorionic villi occur in a spectrum of pathologic conditions including HA, PHM and CHM known as hydropic placentas. Accurate diagnostic classification of hydropic placentas is important as the risk of persistent GTD, or Gestational trophoblastic neoplasia (GTN) is different among the three entities (5). Whereas HA is completely benign, HMs have a significant risk for developing persistent GTD, with a higher incidence in patients with CHM (10-30%) than in patients with PHM (0.5-5%) (6). GTN is used to refer to a group of uncommon malignant gynecological tumors arising from trophoblastic cells, including IM, CC, PSTT, and ETT

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(7). Histologic examination forms the main tool in the diagnosis of molar pregnancies. However, there is considerable overlap in the histologic features between molar and nonmolar pregnancies and between CHMs and PHMs, resulting in significant inter-observer and intra-observer variability in the diagnosis (8-10); Especially that during early pregnancy the diagnostic criteria are subtly different from the classical pathological features (11). Recently, pathologists have relied on molecular techniques, such as DNA flow cytometry, chromosome in situ hybridization, and polymerase chain reaction-based genotyping or HLA typing, which by showing DNA content differences, help to correctly identify the hydropic placentas (12). However, the molecular methods are expensive and time-consuming, and cannot be routinely applied in all laboratories. Thus, a time- and cost-effective ancillary tool, available in most laboratories, would be helpful. Placental apoptosis plays an important role in normal morphogenesis and tumorigenesis of the trophoblastic tissue (13). Bcl-2 and Bax are two important regulator genes in the apoptotic pathway (14). The present study was carried out to evaluate the expression pattern of Bcl-2 and Bax in HAs, PHMs, and CHMs, and to assess the value of this markers in differential diagnosis of the three entities.

Materials and Methods

Formalin-fixed, paraffin-embedded gestational products from 29 patients, including 11 CHMs, 8 PHMs and 10 HAs diagnosed in the Emam Reza and Qhaem Departments of pathology, Mashhad University of Medical Sciences were gathered. Tissue sections of the specimens were stained with routine hematoxylin-eosin and histopathologically reviewed by the pathologist using published criteria (15), for the confirmation of diagnosis. Ploidy analysis using flow cytometry was performed and confirmed diploidy in spontaneous abortions and complete moles, and triploidy in partial moles (16).

For Immunohistochemical staining, 5μm thick sections were cut and incubated for 60 min at 60º C, then the sections deparaffinized in xylene and rehydrated in a descending ethanol series. Endogenous peroxidase activity was blocked by a 20-minute treatment with three percent hydrogen peroxidase in phosphate-buffered saline (PBS). The slides were then washed twice in PBS, pH 7.4 and subsequently transferred to retrieval buffer (10-Mm sodium citrate buffer, pH 6.0) and heated in a microwave oven (at a power of 700 W). The slides were left to cool at room temperature, then were incubated with mouse monoclonal antibody (Bcl-2: prediluted (ready to use), Clone 124, Dako, Glostrup, Denmark) and rabbit polyclonal antibody (Bax: dilution: 1/20, Clone Ab-1 Dako, Glostrup, Denmark) for 30 min at room temperature. Later the sections were rinsed in PBS and incubated with polymer-based Envision (Dako Cytomation, Glostrup, Denmark). The chromogenic reaction was performed by 3, 3-diaminobenzidine (DAB), (Dako Cytomation, Glostrup, Denmark). The sections were then counterstained with Mayer's hematoxylin. The sections of lymph nodes with hyperplasia were used as a positive control for Bcl-2 and normal breast tissues for Bax, negative controls were stained by skipping primary antibody incubation. Evaluation of protein expression was carried out. All immunostained sections were independently examined by the same two observers with a ×200 objective under the light microscope (Olympus BX-51, Olympus, Tokyo, Japan), while they did not know about the slide diagnosis, therefore the analysis was double-blind (17,18). Immunostainig of Bcl-2 and Bax were analyzed semiquantitatively based on the score value of staining intensity which was: 0 if there were no stained cells; 1 for weak staining; 2 for moderate staining and 3 for strong staining. Percentages of positive cells were estimated and graded as follows: 0, no stained cells; 1, ≤ 25%; 2, 26-50%; and 3, >50%. The total score of Immunoreactivity for each case was the sum of the values for the two parameters, which ranged from 0 to 6 (14). Collected data were analyzed by means of the Kruskal-Wallis test followed by Mann-Whitney tests. The differences were considered statistically significant at a P less than 0.05.

Results

Bax and Bcl-2 immunoreactivity were detected in all cases. Bcl-2 expression was observed only in syncytiotrophoblasts with varying degrees of intensity. Cytotrophoblasts and stromal cells were clearly negative in all cases (Figure 1a-c).

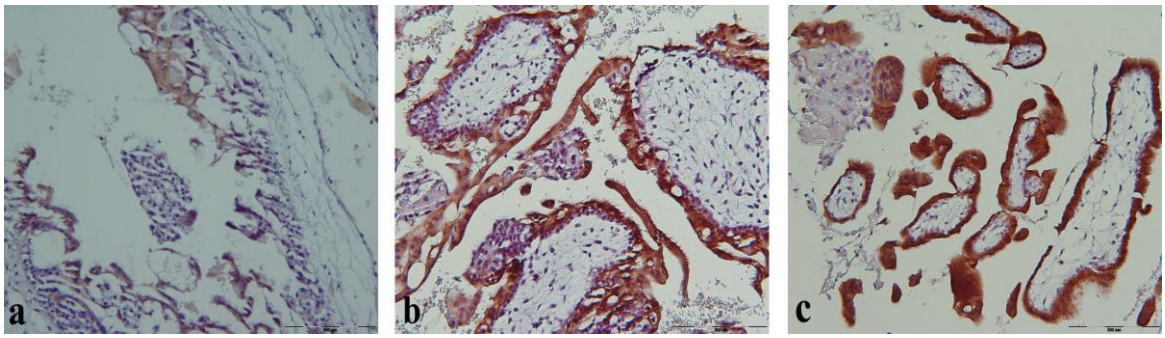

Figure 1. Immunoreactivity with Bcl-2 in CHM (a), PHM (b) and HA (c) was found exclusively in syncytiotrophoblasts, whereas cytotrophoblasts and stromal cells were negative.

Distribution and intensity of immunoreactive cells in each category are tabulated in tables 1 and 2 based on the calculation of immunoexpression total score, the total score averages of Bcl-2 immunoexpression in PHMs (5.12) and HAs (5.60) were significantly higher than in CHMs (3.63), whereas no significant difference was observed between PHMs and HAs (Table 5).

Bax immunoreactivity was observed in cytotrophoblasts, stromal cells and occasionally in syncytiotrophoblasts (Figure 2a-c). Distribution and intensity of immunoreactive cells in each category were tabulated in (Table 3,4). No statistically significant difference in Bax immunoexpression total score was observed among various groups (Table 5).

Table 1. Distribution of Bcl-2 immunoreactivity in various groups (%)

Type of lesion	Cytotrophoblasts				Syncytiotrophoblasts				Stromal Cells			
	0	**1**	**2**	**3**	**0**	**1**	**2**	**3**	**0**	**1**	**2**	**3**
CHM	0	0	0	0	0	0	63.64	36.36	0	0	0	0
PHM	0	0	0	0	0	0	12.5	87.5	0	0	0	0
HA	0	0	0	0	0	0	0	100	0	0	0	0

0 (no stained cells), 1 (≤ 25 % positive cells), 2 (26-50 % positive cells) and 3 (>50 % positive cells)

Table 2. The intensity of Bcl-2 immunoreactivity in various groups (%)

Type of lesion	Cytotrophoblasts				Syncytiotrophoblasts				Stromal Cells			
	0	**1**	**2**	**3**	**0**	**1**	**2**	**3**	**0**	**1**	**2**	**3**
CHM	0	0	0	0	0	72.73	27.27	0	0	0	0	0
PHM	0	0	0	0	0	12.5	50	37.5	0	0	0	0
HA	0	0	0	0	0	10	20	70	0	0	0	0

0 (no stained cells), 1 (weak staining), 2 (moderate staining) and 3 (strong staining)

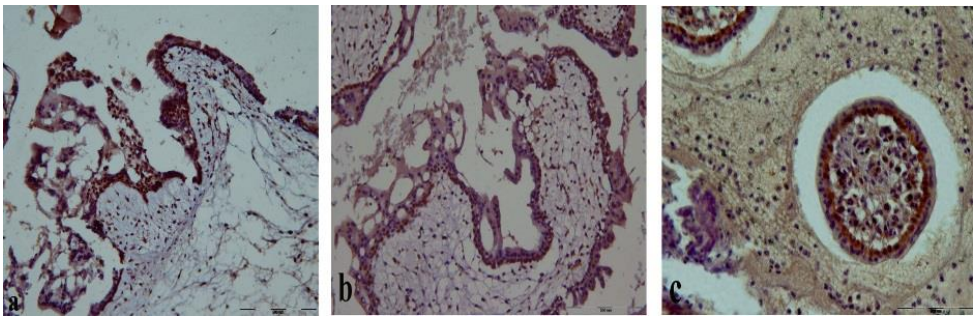

Figure 2. Immunoreactivity with Bax in CHM (a), PHM (b) and HA (c), which was demonstrated in cytotrophoblasts, stromal cells and occasionally in syncytiotrophoblasts. Scale bar = 500µm

Table 3. Distribution of Bax immunoreactivity in various groups (%)

Type of lesion	Cytotrophoblasts				Syncytiotrophoblasts				Stromal Cells			
	0	**1**	**2**	**3**	**0**	**1**	**2**	**3**	**0**	**1**	**2**	**3**
CHM	0	0	0	100	27.27	54.55	18.18	0	0	27.27	54.55	18.18
PHM	0	0	0	100	25	50	25	0	0	50	37.5	12.5
HA	0	0	0	100	40	50	10	0	0	20	60	20

0 (no stained cells), 1 (≤ 25 % positive cells), 2 (26-50 % positive cells) and 3 (>50 % positive cells)

Table 4. The intensity of Bax immunoreactivity in various groups (%)

Type of lesion	Cytotrophoblasts				Syncytiotrophoblasts				Stromal Cells			
	0	**1**	**2**	**3**	**0**	**1**	**2**	**3**	**0**	**1**	**2**	**3**
CHM	0	18.18	45.46	36.36	27.27	45.46	18.18	9.09	0	27.27	54.55	18.18
PHM	0	12.5	62.5	25	25	50	25	0	0	25	62.5	12.5
HA	0	30	60	30	0	40	20	0	0	50	30	20

0 (no stained cells), 1 (weak staining), 2 (moderate staining) and 3 (strong staining)

Table 5. Results of statistical analysis to compare Bcl-2 and Bax Total Score Averages between groups

Types of lesions	Bcl-2			Bax		
	Cy	**Syn**	**Str**	**Cy**	**Syn**	**Str**
CHM, PHM	n	(*P*= 0.004)	n	(*P*= 0.820)	(*P*= 0.368)	(*P*= 0.536)
CHM, HA	n	(*P*< 0.001)	n	(*P*= 0.217)	(*P*= 0.500)	(*P*= 0.711)
PHM, HA	n	(*P*= 0.105)	n	(*P*= 0.284)	(*P*=0.394)	(*P*= 0.818)

All of the values are *P*, n: no statistics are computed. Cy:Cytotrophoblast, Syn: Syncytiotrophoblast, Str: Stromal Cells

Discussion

The histologic separation of spontaneous abortions especially those with hydropic changes from partial moles and of partial moles from complete moles may be difficult. All authors agree on the risk of molar disease to developing persistent gestational trophoblastic tumors, and most of them have emphasized the importance of some ancillary techniques as cytometry, molecular genotyping, histochemistry, and immunohistochemistry to improve diagnosis (19-23). The value of immunohistochemical analysis of paternally imprinted maternally expressed the p57 gene for improving the diagnosis of HMs has been demonstrated in a number of recent studies, (24,25). However, p57 immunohistochemistry can identify CHMs (androgenetic diploidy) by the lack of p57 expression but cannot distinguish PHMs (diandric monogynic triploidy) from non-molar (biparental diploidy) specimens (25). Apoptosis plays important roles in the normal placental morphogenesis and in the pathogenesis of gestational trophoblastic diseases (13). Bcl-2 and Bax are two important regulator genes in the apoptotic pathway (14). The present study was carried out to evaluate the expression pattern of Bcl-2 and Bax in HAs, PHMs, and CHMs, and to assess the value of this marker in differential diagnosis of the three entities. In this study, Bcl-2 expression was observed only in syncytiotrophoblasts with varying degrees of intensity. Cytotrophoblasts and stromal cells were clearly negative in all cases. This is consistent with previous studies performed by Wong *et al*., (14), Qiao *et al*., (18) and chandelier *et al.,* (26) on the other hand, the studies done by Ratts *et al*., (27) and Ishihara *et al.,* (28) confirmed this pattern of immunoexpression for Bcl-2 in normal placentas.

Bcl-2 gene is a major regulator of apoptosis. It inhibits apoptotic cell death (29). The syncytiotrophoblast is a terminally differentiated non-proliferating fetal epithelium which constitutes permanent continuous surface barrier directly in contact with maternal cells. It was postulated that Bcl-2 might be a type of proliferation or maturation-related marker of trophoblasts, which shows decreased expression along with terminal differentiation and maturation (30).

The expression of Bcl-2 in the syncytiotrophoblast could be a fundamental mechanism by which this important epithelial component of the placenta is preserved (31).

On the other hand, Toki *et al*. speculated that the major role of Bcl-2 in syncytiotrophoblast might be to inhibit the spread of apoptosis to the other nuclei sharing the same cytoplasm, rather than to immortalize the syncytiotrophoblast. If the syncytiotrophoblast, as a

multinuclear giant cell in which the nuclei all share the same cytoplasm, tends to undergo spreading nuclear DNA fragmentation, such apoptotic changes would need to be controlled by certain suppressors (32).

Based on the calculation of immunoexpression total score, the total score averages of Bcl-2 immunoexpression in PHMs and hAs were significantly higher than in CHMs.

This finding is in line with the publications of Wong *et al.*, (14), Qiao *et al*., (18) and Wargasetia *et al*., (33). Bcl-2 is antiapoptosis, so the decrease in the expression of Bcl-2 could lead to an increase in apoptosis. This is consistent with some studies which showed higher levels of apoptosis observed in CHMs compared with PHMs and spontaneous abortions (14,18,34).

Conversely, Al-Bozom showed strong and diffuse positivity of Bcl-2 immunoreactivity in the syncytiotrophoblasts of most molar and non-molar placentas (35), and a study by Fulop *et al.*, demonstrated a significantly stronger expression of Bcl-2 protein in CHM and CC as compared with the normal placenta and PHM (36). In this opinion, these discrepancies in the results can be attributed to the use of different antibody clones and retrieval methods (13). Furthermore, in our study ploidy analysis using flow cytometry, was performed as an efficient adjunct diagnostic tool to the differential diagnosis of samples.

In this study, Bax immunoreactivity was observed in cytotrophoblasts, stromal cells and occasionally in syncytiotrophoblasts. We found a few studies on the Bax immunoexpression properties in molar pregnancies and spontaneous abortions.

It has been reported that Bax immunopositivity was found in cytotrophoblasts of normal placenta and CHM cases, but not in syncytiotrophoblast and cells within the mesenchymal cores (18). In accordance with this study, Wong *et al*. reported Bax immunoreactivity in cytotrophoblasts, syncytiotrophoblasts and occasionally in villous stromal cells (14). No statistically significant difference in Bax immunoexpression total scores was observed among various groups. This is consistent with previous studies done by Wong *et al.*, (14) and Qiao *et al.*, (18). It was reported that Bcl-2 expression is probably regulating apoptosis in normal placenta and gestational trophoblastic disease, whereas Bax expression is not (14). Based on the results of this study, Bcl-2 immunostaining offers a potential adjunctive diagnostic tool to distinguish CHM from PHM and HA, but not PHM from HA, Bax immunostaining cannot be helpful in this regard.

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