Bcl-2 and p53 expressions in Indian women with complete hydatidiform mole

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ABSTRACT

Introduction: Hydatidiform moles have a high incidence rate in Asian countries like India. The molecular pathway leading to the pathogenesis and progression of hydatidiform moles is not yet understood. This study aimed to investigate the biological significance of Bcl-2 and p53 in complete hydatidiform moles (CHMs) as well as their influence on disease progression in the Indian population.

Methods: Archival tissues from 35 patients with CHMs and 35 age-matched controls were examined for Bcl-2 and p53 expressions by immunohistochemistry.

Results: Bcl-2 was found to be immunolocalised in the cytoplasm of the syncytiotrophoblast, whereas p53 was observed in both the nucleus and cytoplasm of the syncytiotrophoblast and cytotrophoblasts. In CHMs, Bcl-2 was detected in 23 percent of patients and p53 nuclear expression, in 66 percent. A significant decrease in Bcl-2 expression was observed in CHMs (p-value is 0.015), and the down-regulation of Bcl-2 significantly correlated with higher nuclear expression of p53 (p-value is 0.002), indicating an inverse association between the two proteins (p-value is 0.0001). However, no correlation was found between the clinical progress of patients with CHMs and p53 and those with Bcl-2 protein expression.

Conclusion: The current study demonstrated the significance of Bcl-2 and p53 in the pathogenesis of CHMs but did not reveal any association with disease progression.

Keywords: Bcl-2, complete hydatidiform moles, placenta, p53

INTRODUCTION

Apoptosis is an intrinsic cellular self-destruction mechanism that is essential for a variety of biological events such as developmental sculpturing, tissue homeostasis and removal of unwanted cells.[1] The molecular pathway leading to apoptotic cell death is highly preserved and regulated by several proteins that either support or oppose apoptosis. A variety of molecules are known to regulate the process of apoptosis.[1] Bcl-2 was the first of its family to be discovered, and serves as a powerful antidote to cell death by countermanding the effect of both caspase-dependent and caspase-independent modes of cell death through manifold independent functions.[2] It is believed to play a decisive role in normal placental growth and ageing, and its inappropriate regulation has been implicated in several pregnancy disorders.[3,4] Tumour suppressor protein p53 is a well-known transcription factor that is responsible for the direct activation and inhibition of numerous genes involved in apoptosis. It plays both transcription-dependent and transcription-independent roles during apoptosis.[5] It is involved in the development and progression of various tumour types.[6] Several studies have shown an association between Bcl-2 and p53 in a variety of cancers.[7]

Hydatidiform mole (HM), a rare placental disorder, has a high occurrence rate in Asian countries like India (one in 502 pregnancies).[8] It is an aberrant conceptus that is characterised by enlarged, oedematous and vesicular hydropic chorionic villi, absence of blood vessels and proliferation of trophoblasts to a varying degree. The aetiology of molar pregnancy remains elusive. Based on morphological and genetic criteria, HMs are classified as partial (PHMs) or complete (CHMs).[8] It has been observed that 8%–30% of cases develop persistent trophoblastic disease (PTD) requiring therapeutic interventions;[9] hence, the molecular mechanisms behind this disorder need to be explored. It has also been observed that CHMs are associated with a higher incidence of malignant transformation than PHMs. There have been no reports to date on the analysis of Bcl-2 and p53 expressions in

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Table I. Correlation of clinicopathological parameters with Bcl-2 and p53 expressions.

Clinicopathological parameter (n = 35)	Bcl-2 expression		p-value	p53 expression		p-value
	Negative (−; +)	Positive (++; +++)		Negative (−; +)	Positive (++; +++)	
Maternal age (yrs)						
≤ 30	23	8	0.55	10	21	0.59
> 30	4	0		2	2	
Gestational age (wks)						
8–12	10	1	0.39	3	14	0.71
13–20	17	7		9	9	
Parity						
Primigravida	13	3	0.42	6	11	0.72
Multigravida	14	5		9	9	
Abortions						
nil	17	7	0.39	9	15	0.71
≥ 1	10	1		3	8	
Bad obstetric history*						
No	24	5	0.12	9	20	0.39
Yes	3	3		3	3	
Disease progression (n = 17)						
Progressed	9	1	0.25	3	7	0.35
Regressed	4	3		4	3	

* Bad obstetric history includes history of tubal pregnancy, prenatal death, stillbirth, ovarian cyst or multiple fibroids.
(−): no staining; (+): mild staining; (++): moderate staining; (+++): intense staining

Indian women with molar pregnancy. The present study aimed to explore the expression profiles of apoptotic regulators Bcl-2 and p53 in CHMs and to elucidate their possible involvement in the pathogenesis and progression of disease in patients of Indian origin.

METHODS

A total of 70 women who attended the Department of Obstetrics and Gynaecology at Vardhman Mahavir Medical College and Safdarjung Hospital between September 2007 and March 2009 were enrolled in the study. Evacuated tissue samples from the archive with reconfirmed CHMs (n = 35) were taken as the study group. Placental specimens from medically terminated normal pregnancies at 8–20 weeks, with no concurrent medical illness, history of abortions or smoking/alcohol habits were included as age-matched controls (n = 35). Patient data included gestational age, gravidity, parity as well as abortion and obstetrics history (Table I). All patients inducted in the study had CHMs. The gestational age of the CHMs, based on the last menstrual period, was 8–20 (median 14) weeks. The study was approved by the institutional ethics committee involving human material, and written informed consent was obtained from each subject. The median age of the patients in the CHM group was 24 (range 19–35) years. Follow-up could be obtained in 17 of the 35 patients with a diagnosis of CHM

in this study. Among the 17 patients, seven underwent spontaneous regression and ten developed PTD.

Immediately after vaginal delivery, the desired portion of the tissue specimen, consisting of villi, was fixed in 10% buffered formalin for 24 hours. Following fixation, the samples were routinely processed for paraffin embedding. Later, 5-μm thick sections were cut and mounted on poly-L-lysine-coated slides, air-dried and processed for immunohistochemical analysis. The sections were deparaffinised, rehydrated and immunostained with Bcl-2 and p53 antibodies, respectively. In order to attain adequate visualisation of proteins with minimal background, the antibodies were tested at different dilutions, and the antigen retrieval conditions (buffer, heating intensity and time) were standardised. For Bcl-2, the optimisation of immunohistochemical staining was achieved by retrieval in Tris-EDTA buffer (pH 9.0), whereas p53 retrieval was performed in citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked with 0.03% H_2O_2 in methanol. The sections were then incubated at 4°C overnight with mouse anti-human Bcl-2 monoclonal antibody (Dako Cytomation, Glostrup, Denmark) at 1:200 dilution or p53 monoclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) at 1:100 dilution. Later, the sections were rinsed in phosphate buffered saline and incubated with polymer-based Envision™ (Dako Cytomation, Glostrup,
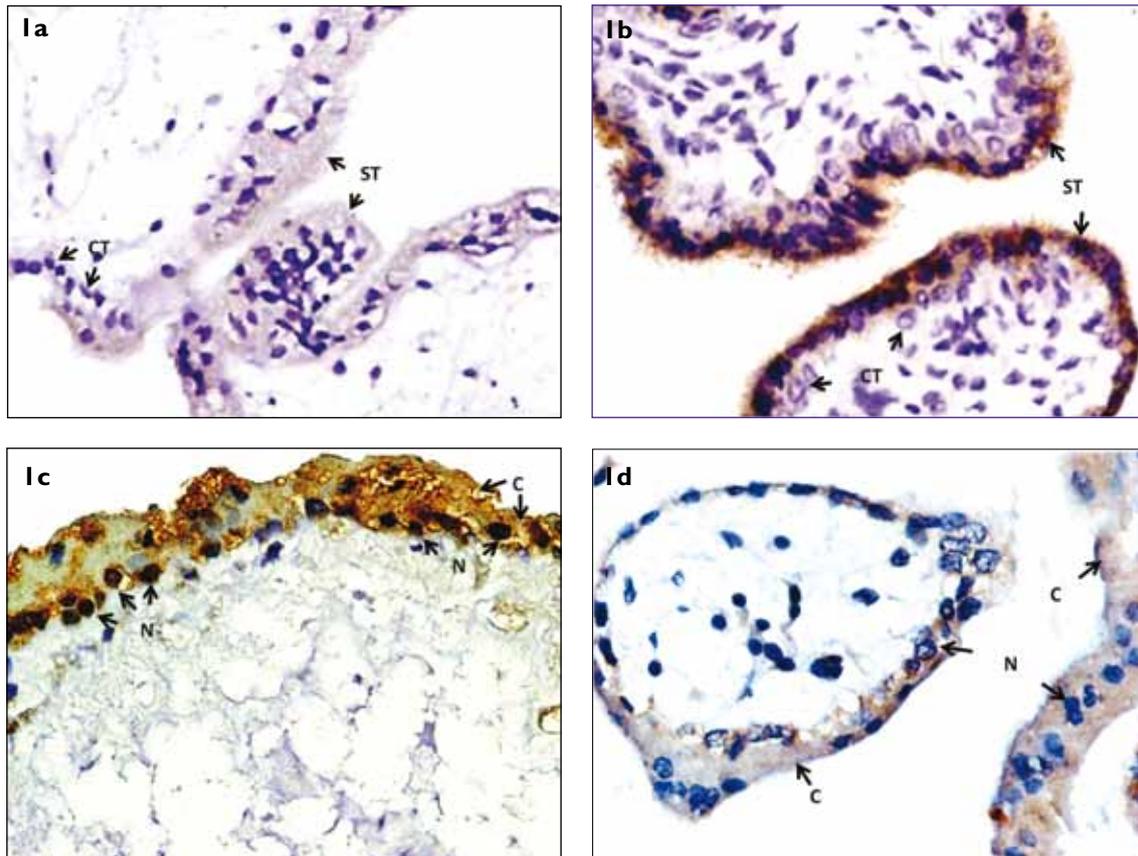
Fig. I Immunohistochemical localisation of Bcl-2 and p53 in complete hydatidiform moles (CHMs). Photomicrographs show (a) CHM (16 wks) with remarkable loss in the expression of Bcl-2; and (b) in comparison, age-matched normal placenta depicting intense immunostaining within the cytoplasm of the syncytiotrophoblasts [ST]. Negative immunostaining was observed in the cytotrophoblasts [CT]. Photomicrographs show (c) molar placental tissue (12 wks) with distinct nuclear [N] and moderate cytoplasmic [C] staining for p53; and (d) control placenta demonstrating mild cytoplasmic [C] and no nuclear [N] expressions for p53 (IHC, x 400 for all).

Denmark). The chromogenic reaction was performed by 3,3-diaminobenzidine (DAB). (Dako Cytomation, Gilostrup, Denmark). Specimens were then counterstained with Mayer's haematoxylin and examined under a light microscope (Olympus BX-51, Olympus, Tokyo, Japan). For negative control, primary antibody was replaced by isotype-specific IgG.

Evaluation of protein expression was carried out in two arbitrarily chosen slides of each case, and every slide was studied by two observers who were blinded to the gestation and experimental group. An average of ten microscopic fields were examined (each slide) at 100 times and 400 times magnification under a light microscope. Bcl-2 expression was scored according to the subjectively evaluated intensity of staining and the proportion of stained villous cells. Cytoplasmic stain was the criteria for a positive Bcl-2 reaction.[10] Each observer used a simple intensity scoring system, where (−) indicated no staining, (+) weak staining, (++) moderate staining and (+++) intense staining. Specimens were considered to be immunopositive if > 10% of villous cells showed immunoreactivity. In

the same subset of HMs and age-matched controls, p53 immunohistochemistry was also carried out and further correlated with Bcl-2 expression. For p53, only nuclear staining was considered positive.[10] Here, the percentages of immunoreactive nuclei were assessed and the results scored on a scale with grades, where (−) represented < 10%; (+) 10%–25%; (++) 25%–50%; and (+++) > 50% positive nuclei. The agreement between observers was 80%, and the cases that scored within this range were considered for the final semi-quantitative evaluation. Data obtained from the study and control groups were compared for Bcl-2 and p53 expressions by means of Fisher's exact test (two-sided) and chi-square analysis. The data was analysed using the Statistical Package for the Social Sciences version 10.0 (SPSS Inc, Chicago, IL, USA). The minimum significance level was set at $p < 0.05$.

RESULTS

The correlation of clinicopathological parameters with Bcl-2 and p53 expressions is summarised in Table I. Both Bcl-2 and p53 expressions did not

Table II. Immunohistochemical analysis of Bcl-2 in the CHM group vs. control group (8–20 wks).

Group	Bcl-2 immunostaining				p-value; OR; CI
	(−)	(+)	(++)	(+++)	
CHM (n = 35)	17	10	8	0	0.015; 0.5; 0.361–0.746
Control (n = 35)	0	13	15	7	

CHM: hydatidiform mole; OR: odds ratio; CI: confidence interval; (−): no staining; (+): mild staining; (++): moderate staining; (+++): intense staining

Table III. Immunohistochemical analysis of p53 in the CHM group vs. control group (8–20 wks).

Group	p53 immunostaining		p-value; OR; CI
	Negative (−; +)	Positive (++; +++)	
CHM (n = 35)	12	23	< 0.002; 0.5; 0.362–0.941
Control (n = 35)	30	5	

CHM: hydatidiform mole; OR: odds ratio; CI: confidence interval; (−): no staining; (+): mild staining; (++): moderate staining; (+++): intense staining

Table IV. Relationship between Bcl-2 and P53 in CHMs.

Case (n = 35)		Bcl-2 immunostaining		p-value; OR; CI
		Negative (−; +)	Positive (++; +++)	
p53 immunostaining	Negative	6	6	0.015; 0.5; 0.361–0.746
	Positive	21	2	

CHM: hydatidiform mole; OR: odds ratio; CI: confidence interval; (−): no staining; (+): mild staining; (++): moderate staining; (+++): intense staining

reveal any correlation with the clinicopathological parameters analysed, including the disease progression (Table I). Bcl-2 was expressed in the cytoplasm of the syncytiotrophoblast in both the CHM and control groups. The cytotrophoblasts, mesenchymal cells of the villous core and the extravillous trophoblast showed negative immunostaining (Figs. 1a & b). The results of the immunohistochemical analysis of Bcl-2 are summarised in Table II. Of the 35 CHMs examined, 27 (77%) displayed reduced levels of Bcl-2 expression (17 showed Bcl-2 loss and ten displayed mild expression) (Fig. 1a). In comparison group, 63% (22/35) of the age-matched controls demonstrated higher Bcl-2 immunostaining (15 moderate and seven intense) (Fig. 1b). The statistical analysis demonstrated that the expression of Bcl-2 was significantly down-regulated in CHMs (p = 0.015; OR 0.5; confidence interval [CI] 0.361–0.746).

Strong p53 nuclear staining was detectable in 66% (23/35) of cases with CHM (Fig. 1c), while 86% (30/35) of the controls showed no nuclear expression for p53 (Fig. 1d & Table III). This difference between the CHM and control groups was statistically significant (p = 0.002; OR 0.5; CI 0.362–0.941), where the former expressed higher nuclear positivity for p53. Out of the 35 CHMs examined, 23 (66%) showed distinct nuclear localisation for p53. Of these 23 CHMs, 21 (91%) demonstrated a loss of Bcl-2 expression. Statistical analysis showed a significant inverse association between Bcl-2 protein expression and nuclear p53 (p < 0.0001; OR 0.3; CI 0.150–0.742) (Table IV).

DISCUSSION

Apoptosis has been reported in the human placenta under both normal and pathological conditions.[3] However, the reports presented by different authors regarding apoptotic events in placental development at different gestations and under different pathologic conditions remain controversial.[10-15] Several molecules are associated with the induction and prevention of apoptosis. Bcl-2 is one such molecule whose expression is considered as an anti-apoptotic factor that is responsible for inhibiting apoptosis.[11]

In the present study, we investigated the expression of Bcl-2 in CHMs, and our results demonstrated a significant decrease in Bcl-2 expression in CHMs (p = 0.015) compared with its age-matched controls, inferring an increased apoptotic profile in molar pregnancy. The present finding is consistent with that reported in previous studies conducted on the role of Bcl-2 in molar pregnancy.[10,12] Conversely, Al-Bozom showed strong and diffuse positivity of Bcl-2 in the syncytiotrophoblasts of most molar and non-molar placentas.[13] A study by Fulop et al demonstrated a significantly stronger expression of Bcl-2 protein in CHMs and choriocarcinoma as compared to both normal placentas and PHMs.[14] Furthermore, in a recent report, Hussein showed a relatively moderate expression of Bcl-2 in CHMs, and suggested that prominent expression of Bcl-2 may prevent the apoptotic cell death of these atypical trophoblastic cells, thus allowing them to acquire a more malignant potential. In his opinion, these discrepancies

in the results can be attributed to the use of different antibody clones and retrieval methods.[15]

As Bcl-2 is known for its oncogenic properties, its high expression in CHMs would have supported the view that CHMs are premalignant forms that are prone to malignant transformation. However, its loss, as observed in the current study, does not preclude such a possibility, since there have been reports demonstrating Bcl-2 loss in other malignant disorders like oral squamous cell, oesophageal and thyroid carcinomas.[16-18] In addition to its role in apoptosis, Bcl-2 is also known to have an antiproliferative effect, as it delays the progression of cell cycle to S-phase from quiescence.[16] Therefore, the loss of these suppressive effects of Bcl-2 on rapidly proliferating trophoblasts may be advantageous for potentially malignant HMs. The suppressive effect of Bcl-2 on proliferation has been observed in oral carcinoma, with increased proliferative activity reported in cases that had Bcl-2 loss.[19] Similarly, it can be assumed that the loss of Bcl-2 in CHMs may be associated with progression of disease, as it causes the trophoblasts to become more responsive to mitotic stimuli.

A wide variety of molecules can regulate Bcl-2 protein/mRNA, including lymphokines, transforming growth factor-beta, p53, retinoids and phorbol esters.[16] Among these, the tumour suppressor gene *p53* is known to play a central role in protecting against the propagation of DNA damage, uncontrolled proliferation and neoplastic transformation, primarily by inducing cell cycle arrest or apoptosis.[20] Bcl-2 may play a role in interacting with p53 in the regulation of these pathways. Hence, the expression profile of p53 was also analysed in the same CHM cohorts in order to elucidate its relationship with Bcl-2 in trophoblastic tissue. Unlike Bcl-2, the accumulation of p53 was significantly upregulated in CHMs compared to its gestational-matched controls (p = 0.002). Similar increases in p53 expression were reported in most of the previous studies, and this overexpression was presumed to be due to an upregulation of *p53wt* rather than the *mutp53* type.[21-25] Mutation of *p53* is the most frequent genetic alteration detected in human cancer, which inactivates its growth regulatory function and causes a loss of tumour-suppressive activity.[26] Hence, the loss of underlying *p53* mutation in CHMs, as observed in earlier studies, suggests the restoration of p53 tumour suppressor function.[26]

Furthermore, the current results revealed a significant inverse association between Bcl-2 and p53 expressions in CHMs (p < 0.0001). Although several investigators have studied the expression profile of these two proteins together,[10,13,15] only Hussein revealed a correlation

between the two proteins in CHMs.[15] This finding confirms the speculated association between Bcl-2 and p53, and suggests that the increase in p53 expression is likely an attempt to prevent excessive trophoblastic proliferation in CHMs, partly through modulation of regulators like Bcl-2. However, no correlation could be found between the clinical progress of patients with CHMs and the p53 (p = 0.35) or Bcl-2 protein (p = 0.25) expressions, probably due to the inadequate number of accessible patient follow-ups. Hence, it can be inferred that although the differential expression of p53 and Bcl-2 in CHMs suggests that they contribute to the pathogenesis of the disease, neither of the proteins was of independent prognostic significance. Despite the limitations, this is the first study to explore the significance of apoptosis regulators Bcl-2 and p53 in the pathogenesis of CHMs in women of Indian origin. Further larger-scale studies involving other apoptotic regulators, with a larger number of cases and follow-ups, are required to validate the current assumption and to elucidate the precise mechanism implicated in the progression of CHMs.

ACKNOWLEDGEMENTS

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