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Research Paper

Role of Apoptotic Regulators in Human Epithelial Ovarian Cancer

Rajarshi Kar[1]
Sudip Sen[1]
Archna Singh[1]
Himani Sharma[1]
Sunesh Kumar[2]
Siddhartha Dutta Gupta[3]
Neeta Singh[1,*]

Departments of [1]Biochemistry; [2]Obstetrics and Gynecology and [3]Pathology; All India Institute of Medical Sciences New Delhi, India

*Correspondence to: Neeta Singh; Room no. 3027A; Department of Biochemistry; All India Institute of Medical Sciences; New Delhi 110029, India; Tel.: +91.11.26594945; Fax: +91.11.26588663; Email: singh_neeta26@rediffmail.com

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KEY WORDS

ovarian cancer, apoptotic index, telomerase activity, ascitic cells, Bcl-2 family, BRCA1, Survivin

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ABSTRACT

We assessed molecular markers such as BRCA1, K-ras, p53, Bcl-2, Bcl-XL, Survivin and telomerase activity in untreated ovarian cancer tissue samples, ascitic cells and normal ovarian tissues and gathered insights into their correlation with each other and also with apoptotic index. The expression of these proteins was analyzed by Western blotting and immunohistochemistry. Apoptotic index was determined by TUNEL assay and telomerase activity was measured by PCR-ELISA kit. p53, Bcl-2, Bcl-XL, K-ras and Survivin were found to be over expressed in tumors and ascitic cells as compared to normal controls whereas there was no significant difference in expression of BRCA1. A significantly higher telomerase activity and lower apoptotic index in tumors as compared to controls was observed. p53 positively correlated with Bcl-2, Bcl-XL, K-ras and Survivin expression and also clinical stage of the disease. A positive correlation between Survivin and Bcl-2, Bcl-XL was seen. Apoptotic Index, telomerase activity and BRCA1 expression showed no correlation with any of the parameters. Our study confirms the fact that multiple gene interactions govern the pathogenesis of ovarian cancer, and analyzing ascitic cells of ovarian cancer patients may help to delineate molecular profile of the primary tumor.

INTRODUCTION

Ovarian cancer is the second most common gynecological malignancy and is associated with high mortality as most cases are diagnosed late.[1] Insignificant symptoms and lack of appropriate tumor markers prevents early detection. Only about 25% of patients are diagnosed when the disease is still confined to the ovary.[2] At advanced stages (stages III and IV), when the disease has spread beyond the ovary, treatment becomes increasingly ineffective.

The aberrant expression of various oncogenes and defective tumor suppressor genes has been implicated in the genesis of ovarian cancer. p53 gene has been reported to be mutated in 50–90% of advanced ovarian cancers.[3] The loss or dysfunction of activated p53 allows the unchecked replication of cells with genetic damage, which result in tumor development if they harbour other damaged proto-oncogenes or tumor suppressor genes. Germline mutations in BRCA1/2 is responsible for as much as 10% of all ovarian cancer cases and are present in most families containing either site-specific ovarian cancer cases or breast and ovarian cancers together.[4] Mutations in K-ras are also frequently seen in ovarian tumors and are present in the early stages of development of these tumors.[5]

The regulation of apoptosis is through a balance between the pro and antiapoptotic genes and a tilt of the balance towards antiapoptotic genes is an important factor in genesis of most cancers including ovarian cancer. Most of the anti-apoptotic genes, like Bcl-2, Bcl-xl and Survivin, have been shown to be over expressed in ovarian cancer.[6-8] Telomeres gradually shorten during cell proliferation and serve as an indicator for dividing cells to stop replicating. In rapidly proliferating cells like germ cells their length is maintained by telomerase, a ribonucleoprotein complex. Activity of this enzyme is negligible in somatic cells thus checking their uncontrolled proliferation. Reactivation of telomerase has been linked to the development of ovarian tumors.[9] However, there are very few studies which deal with interaction of all these genes in ovarian tumorigenesis.

In late stages ovarian cancer is often associated with development of ascites. Presence of malignant cells in ascitic fluid is an indicator of bad prognosis.[2] Cytology alone is not a sensitive or specific diagnostic tool.[2] Because the presence of malignant cells in the peritoneal fluid is an important prognostic indicator, there is a critical need for new diagnostic methods which will be able to improve our ability to detect microscopic disease

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in the peritoneal cavity. Immuno-cytochemistry using primary antibodies and immunophenotyping using flow cytometry do offer the possibility to detect cancer cells in cytological negative specimens. There is need to develop tumor markers which can augment detection of malignant cells in ascites.

In this study we compared the expression of p53, BRCA1, K-ras, Bcl-2, Bcl-xl and Survivin in primary, untreated epithelial ovarian cancer and normal ovarian tissue and gathered insights into their correlation with each other and also with apoptotic index and telomerase activity. We also focused on examining if the expression of these proteins in the ascitic cells is able to reflect the molecular profile of the corresponding primary tumor.

MATERIALS AND METHODS

Sample collection and processing. Twenty five primary, untreated epithelial ovarian cancer samples and 10 normal ovarian tissue samples for normal controls were obtained from the Department of Obstetrics and Gynecology, AIIMS, New Delhi, India. The samples were snap frozen and stored at -70°C, till further use. Concurrent sections of 8 μm were cut in a cryostat. Ascitic fluid was collected from seven of the untreated patients of epithelial ovarian cancer concurrently with their primary tumors. Cells isolated from the ascitic fluid by differential centrifugation were stored at -70°C till further processing.[10] The histopathological classification of the ovarian tissue samples was done according to WHO criteria and the staging using FIGO (International Federation of Gynecology and Obstetrics) guidelines.

Immunohistochemical analysis. Protein expression of anti-apoptotic genes was done by immunohistochemistry using appropriate antibodies as described earlier.[10] Tonsil tissue for Bcl-2 and Bcl-XL, placental tissue for survivin, breast tissue for p53 and BRCA1 and skin tissue for K-ras were used as positive controls. The negative controls lacked primary antibody.

Western blot analysis. Tumor tissue sections/ascitic cells were analyzed by Western blot using rabbit Bcl-2, Bcl-XL, Survivin, K-ras and mouse p53 primary antibodies (Santacruz biotechnology, USA) as described earlier.[10] However BRCA1 was analyzed by immunohistochemistry only as we were unable to standardize the Western Blot for it due to the large size of the protein (220 kD). β-actin was used for normalization.

TUNEL assay. Apoptotic cells were identified by the Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) technique using the Dead End Colorimetric Cell Death Detection kit (Promega Inc., USA) as previously described.[11]

Telomerase assay. Telomerase assay was done on 25 tumors and 10 normal controls as described previously, using the PCR ELISA kit from Roche Diagnostics, GmbH, Germany and following the manufacturer's guidelines.[11]

Statistical analysis. Statistical analysis of the samples was done using SPSS (version 10.0) software. Wilcoxon's signed rank test was used for comparison within groups i.e., ascites and tumors. Mann Whitney test was used to compare between tumors and controls. Correlation was determined using Spearman's correlation coefficient.

RESULTS

p53, Bcl-2, Bcl-xl, K-ras and survivin was overexpressed in ovarian tumors. The percentage of tumors and controls positive for p53, Bcl-2, Bcl-xl, Survivin, K-ras and BRCA1 and their mean expression are shown in Table 1. Representative Western blots are shown in Figure 1A. The expression of p53, Bcl-2, Bcl-xl, K-ras and Survivin was significantly higher in tumors as compared to normal controls (Fig. 1B). Immunohistochemistry results were corroborative (Fig. 2).

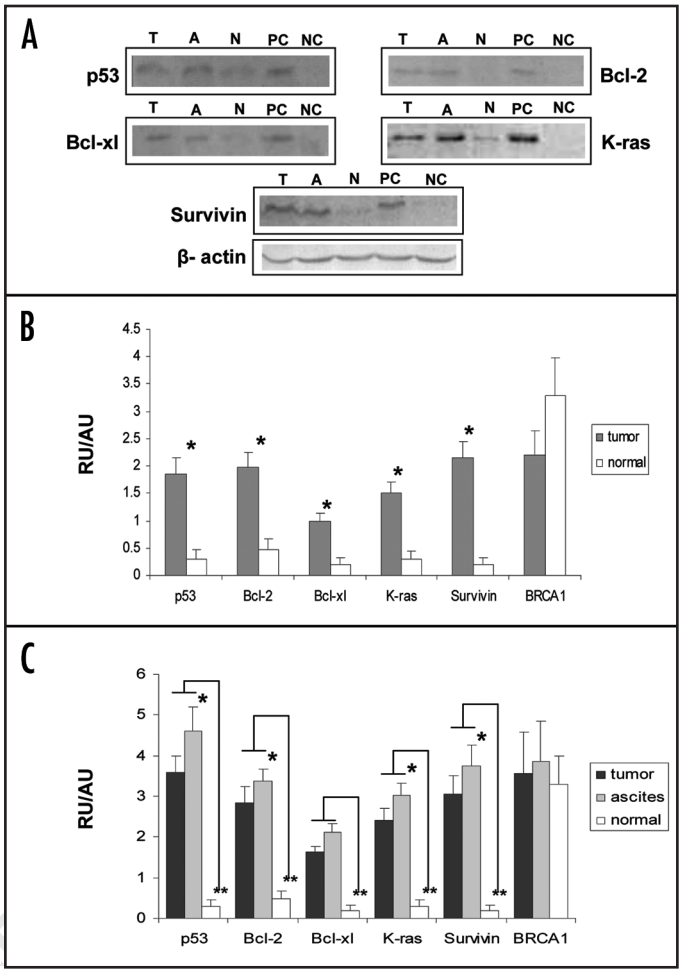

Figure 1. (A) Representative western blots for p53, Bcl-2, Bcl-xl, K-ras and Survivin (T, tumors; A, ascitic cells; N, controls; PC, positive control; NC, negative control). (B) Graphical representation of mean expression of p53, Bcl-2, Bcl-xl, K-ras, Survivin and BRCA1 in tumors (n = 25) and controls (n = 10). *p < 0.01. (C) Graphical representation of mean expression of p53, Bcl-2, Bcl-xl, K-ras, Survivin and BRCA1 in ascitic cells (n = 7), corresponding tumors (n = 7) and controls (n = 10). (AU, arbitrary units as by immunohistochemistry for BRCA1; RU, relative units as by western blotting for p53, Bcl-2, Bcl-xl, K-ras and survivin). *p < 0.05, **p < 0.01.

Ascitic cells reflected the molecular profile of the corresponding tumor. The mean expression of these proteins in ascitic cells were compared with their corresponding tumors (Table 2). Expression of p53, Bcl-2, Survivin and K-ras was higher in ascitic cells than their corresponding tumors whereas the expression of Bcl-xl and BRCA1 were similar in tumors and ascitic cells (Fig. 1C). There was increase in expression of p53, Bcl-2, Bcl-xl, K-ras and Survivin and no difference in BRCA1 protein expression, in both ascitic cells and their corresponding primary tumors, when compared with controls (Fig. 1C). So, ascitic cells could reflect the molecular changes in the corresponding tumors.

Ovarian Tumors had lower apoptotic index than normal ovarian tissues. The apoptotic index (AI) varied from 0-0.048(0–4.8%) in tumors (n = 25) and from 0.02-0.12(1–12%) in controls (n = 10). The mean apoptosis in tumors was 2.11% (±0.34) while that in

Table 1 **Percentage of tumors and controls positive for p53, Bcl-2, Bcl-xl, Survivin, K-ras and BRCA1 and their mean expression (± standard deviation)**

	Tumors		Controls	
	% Positive	Mean Expression	% Positive	Mean Expression
p53	76	1.85 ± 0.3 RU	30	0.3 ± 0.16 RU
Bcl-2	80	1.99 ± 0.25 RU	40	0.48 ± 0.18 RU
Bcl-xl	72	0.99 ± 0.14 RU	20	0.19 ± 0.12 RU
K-ras	84	1.52 ± 0.18 RU	30	0.30 ± 0.15 RU
Survivin	80	2.14 ± 0.31 RU	20	0.20 ± 0.13 RU
BRCA1	56	2.20 ± 0.44 AU	80	3.30 ± 0.67 AU

AU, arbitrary units for immunohistochemistry; RU, relative units for western blotting.

Table 2 **Mean expression (± standard deviation) of p53, Bcl-2, Bcl-xl, survivin, K-ras and BRCA1 in ascitic cells and corresponding tumors**

	Ascites	Corresponding Tumors
p53	4.6 ± 0.59 RU	3.63 ± 0.43 RU
Bcl-2	3.37 ± 0.30 RU	2.85 ± 0.37 RU
Bcl-xl	2.12 ± 0.2 RU	1.64 ± 0.13 RU
K-ras	3.02 ± 0.30 RU	2.4 ± 0.29 RU
Survivin	3.76 ± 0.47 RU	3.06 ± 0.44 RU
BRCA1	3.86 ± 1.0 AU	3.57 ± 0.97 AU

AU, arbitrary units for immunohistochemistry; RU, relative units for western blotting.

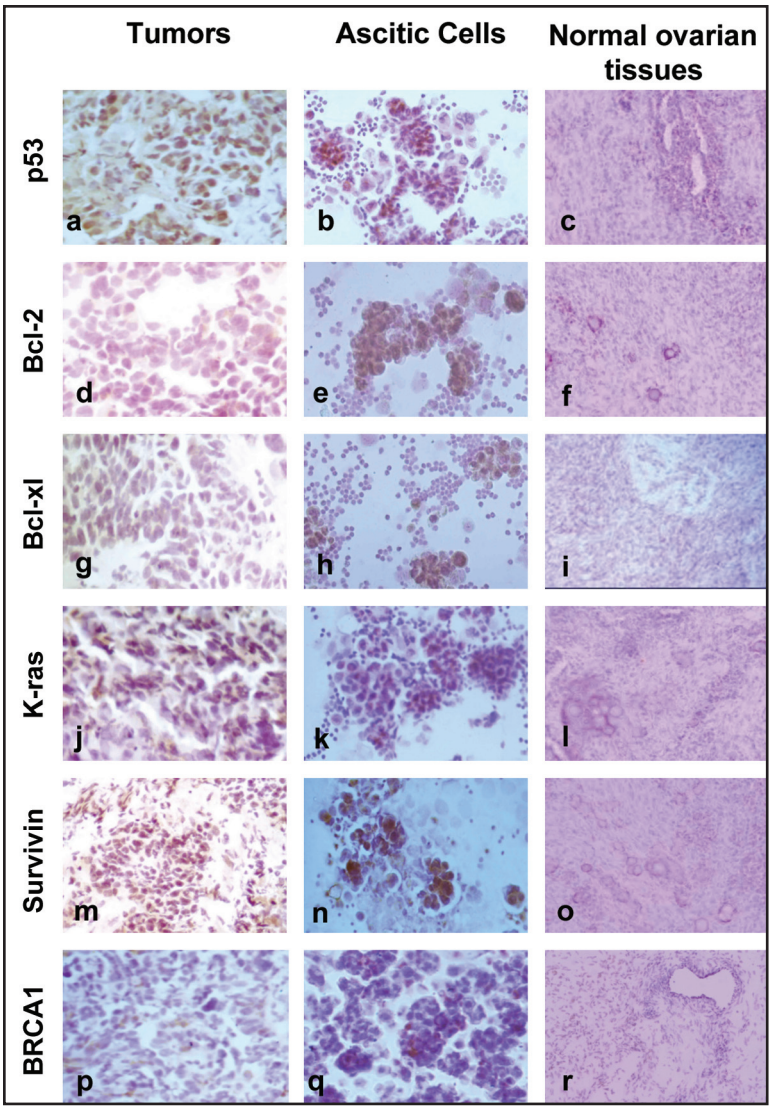

Figure 2. Representative micrographs of immunohistochemstry and immunocytochemistry of tumors, ascitic cells and controls for p53 (a–c), Bcl-2 (d–f), Bcl-xl (g–i), K-ras (j–l), Survivin (m–o) and BRCA1(p–r). Magnification 200X.

controls was 4.74% (±1.03) (Fig. 3). The difference in apoptotic index between tumors and controls was significant (p = 0.01). Apoptotic index was also calculated for the 7 ascitic fluid samples and was compared with the corresponding primary tumors. The mean apoptosis in ascitic cells and their primary tumors were 3.37% (±0.87) and 3.17% (±.71), respectively. The difference was not statistically significant.

High telomerase activity was seen in ovarian tumors. The mean telomerase activity in tumors was 0.694 (±0.11) whereas in controls it was 0.296 (±0.14). The difference in telomerase activity in tumors and controls was statistically significant (p = 0.022).

Correlation between apoptosis related proteins. p53 positively correlated with Bcl-2, Bcl-xl, K-ras and Survivin expression and also clinical stage of the disease. Survivin positively correlated with Bcl-2 and Bcl-xl. Apoptotic index, telomerase activity and BRCA1 expression showed no correlation with any of the parameters.

DISCUSSION

We studied a panel of seven molecular markers and apoptotic index in ovarian tumors, their corresponding ascitic cells, and normal ovarian tissue and evaluated their diagnostic potential. We also examined how interactions between these molecules play a role in the pathogenesis of ovarian tumors.

p53 is a tumor suppressor gene, mutations of which are associated with accumulation or over expression of this protein.[12] p53 mutations and/or protein accumulations have been described in 50% to 90% of ovarian tumors, depending upon the tumor type and methodology employed.[3] In our study we detected mutated p53 in 76% of tumors. The expression of p53 in tumors was significantly higher than normal controls.

BRCA genes are tumor suppressor genes which inhibit the growth of cancer cells through their role in the maintenance of genomic integrity, DNA repair, cell cycle control and apoptosis. Though mutations of BRCA genes are rare in sporadic ovarian tumors, decreased expression due to loss of heterozygosity, hypermethylation of the BRCA promoter or other mechanisms is a frequent event in sporadic ovarian tumors.[4] In our study there was a decrease in expression of wild type BRCA1 protein in tumors as compared to normal ovaries.

Ras proteins are GTPases which when activated influence

growth and differentiation, cell cycle regulation, cell survival and angiogenesis.[13] K-ras expression is more commonly associated with mucinous ovarian tumors than serous tumors.[14] However, in our study which consisted mostly of serous tumors we detected expression of K-ras in 84% of tumors. Our findings are consistent with another study which reports that activating mutations of K-ras are not associated with the differentiation of the epithelial cells.[5]

Bcl-2 and Bcl-xl proteins are antiapoptotic and down stream targets for p53.[15] Previous studies report Bcl-2 and Bcl-xl expression in 27% to 57% and 60% to 70% of ovarian tumors, respectively.[6,7] We report Bcl-2 and Bcl-xl expression in 80% and 72% of ovarian tumors, respectively. Keeping with previous reports their expression was significantly higher in ovarian tumors as compared to normal controls, suggesting that the antiapoptotic members of Bcl-2 family play a major role in the pathogenesis of ovarian cancer.

Survivin, a member of the IAP family, is over expressed in ovarian tumors and interferes with both the cell-death machinery as well as mechanisms of cell-cycle progression and microtubule stability.[16] The percentage of Survivin positive cases varies from 51–86% in ovarian cancers.[8] In our study expression of survivin was seen in 80% of tumors and was significantly higher than normal controls. Apoptosis inhibition by Survivin, alone or in conjunction with p53, Bcl-2 and Bcl-xl may be responsible for onset and progression of ovarian carcinoma as suggested by the positive correlation seen between them. Our findings are in agreement with those reported in earlier studies.[8,17]

Wild type p53 has been shown to repress transcription of a number of genes, including Bcl-2 family and Survivin.[8,15,18] p53 mutations prevent DNA binding and compromise this transcriptional repression.[15] Since p53 protein overexpressed in most of the tumors is mutated, it is unable to repress the transcription of the antiapoptotic proteins. This explains the positive correlation seen between the expression of these proteins and mutated p53. Alternatively, mutated p53 allows the unchecked replication of cells which overexpress antiapoptotic genes or harbor damaged proto-oncogenes or tumor suppressor genes.[19] The correlation observed between stage of the tumor and p53 expression, as reported in previous studies,[20] also indicates its involvement in ovarian carcinogenesis.

Though we found a significantly lower mean apoptotic index in tumors as compared to normal ovaries, keeping with previous reports, many of the tumor samples showed high apoptotic index.[21,22] Only when the proliferative activity of the malignant tumors exceeds apoptotic cell death, an accumulation of tumor cells results. As many proteins affect apoptosis and the apoptotic index is the outcome of their collective influence, it is perhaps not possible to correlate apoptotic index with any single antiapoptotic protein, a fact highlighted by our results.

Studies on cervical cancer in our lab have revealed that measuring telomerase activity by PCR-ELISA technique has potential diagnostic and prognostic value.[11] Telomerase has been described as a potential marker for the detection of circulating ovarian tumor cells.[23] In our study higher telomerase activity was seen in ovarian tumors than normal controls as reported in a recent study.[9] Telomerase activity however did not correlate with any of the parameters studied.

From a purely mechanical stand point, it is plausible that carcinoma cells from primary tumors can be directly shed from the surface of the ovary, and these cells are expected to show little difference from the cells in the primary tumor from which they originated. However, our results show that ovarian cancer cells in primary tumors and those in effusions differ in their expression of p53, Bcl-2, Survivin and K-ras. Davidson et al[2] have also shown difference in expression of carbohydrate antigens and antiangiogenic molecules between malignant ovarian cells of ascites and primary tumor.

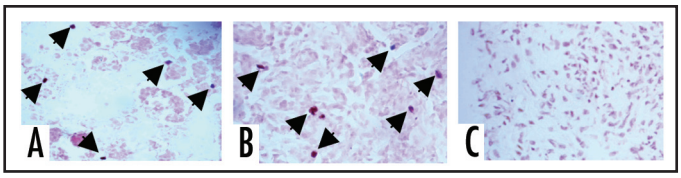

Figure 3. Representative micrographs of TUNEL assay in (A) tumors, (B) ascitic cells and (C) controls. Magnification 200X. Black arrows indicate TUNEL positive cells.

Several factors may contribute to the differences observed between cancer cells in primary tumors and effusions. Technical factors like better penetrance of the primary antibody in isolated cells as compared to cells in solid tissue and contribution of peritumoral stromal cells, endothelial cells and inflammatory cells to the total protein estimation must be also kept in mind. As the ascitic cells are derived from the clonal selection of specific populations that were able to metastasize from the primary tumor[2] and have higher malignant potential, they expressed increased level of these antiapoptotic proteins. Despite the differences, ascitic cells could still reflect the molecular profile of the primary tumor, when compared with normal controls.

Therefore studying ascitic cells of ovarian cancer patients may help to delineate the molecular profile of the primary tumor before initiation of therapy and also holds immense potential to be used as a diagnostic tool. Our study also confirms the fact that multiple gene interactions govern the pathogenesis of ovarian cancer and so single gene targeting approach may not be beneficial in ovarian cancer.

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