IMMUNOHISTOCHEMICAL EXPRESSION OF P53 AND BCL-2 PROTEINS IN ADVANCED ESOPHAGEAL CANCER PATIENTS

S. Arbabi Bidgoli[1], B. Minaee[2], M.H. Ghahremani[1], S.N. Ostad[1], M. Djamali-Zavarei[3] and E. Azizi[1*]

1) Department of Pharmacology and Toxicology, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

2) Department of Anatomy and Embryology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

3) Department of Pathology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

Abstract- The current challenges in the management of esophageal cancer are to obtain a better understanding of underlying molecular alterations to provide new treatment options. We studied the p53 and Bcl-2 protein expression in esophageal carcinomas to correlate molecular alterations with clinicopathological findings. Tissue samples of 37 patients with advanced esophageal carcinoma were analyzed by immunohistochemical techniques. Positive immunostaining for p53 and Bcl-2 were observed in 67.6% and 43.6% of tumor samples, respectively. The prevalence of Bcl-2 overexpression was significantly greater in $p53^{+}$ tumors as compared with p53- tumors (P = 0.003). Unlike p53, positive Bcl-2 immunostaining correlated significantly with tumor type (P = 0.001) and histological differentiation (P = 0.007). Our data also showed that 35% of patients were positive for both proteins and 32.4% of patients were positive for p53 but negative for Bcl-2 expression. These results indicate two types of double gene alterations that obviously would affect tumor biology and response to chemotherapy. Therefore, it is advisable to determine expression profile of certain genes including p53 and Bcl-2 in tumor samples before selecting chemotherapy regimen.

Acta Medica Iranica, 43(5): 313-322; 2005

Keywords: Esophageal cancer, p53, Bcl-2, immunohistochemistry, molecular markers

INTRODUCTION

Esophageal cancer (predominantly squamous cell carcinoma) represents the third most common gastrointestinal malignancy and ranks among the 10 most frequent cancers in the world (over 300,000 new cases/year). It is also very common in developing countries with marked geographic variations in incidence and mortality (1). Iran is one of the known areas with a high incidence of esophageal cancer (2). Despite the high prevalence of esophageal carcinoma in different parts of Iran, the expression patterns of molecular markers are not clear among Iranian patients.

Various studies have revealed the importance of genetic alterations in the development of cancers, including esophageal cancer (3). The p53 tumor suppressor gene is one of the commonly mutated genes in pathogenesis of different types of cancers including esophageal cancer. The p53 protein is well known for its role in the control of cell proliferation, apoptosis and genetic stability (4). There are many reports indicating an inverse relationship between mutant p53 overexpression and apoptosis (5, 6). It is accepted that p53 mutation is associated with resistance to chemotherapeutic effects of many anti-cancer drugs including 5-FU and cisplatin (7, 8). However, the direct effect of specific p53 mutations

Received: 25 July 2004, Revised: 14 Feb. 2005, Accepted: 14 Feb 2005

*** Corresponding Author:**
E. Azizi, Department of Pharmacology and Toxicology, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran
Tel: +98 21 66959100
Fax: +98 21 66461178
E-mail: aziziebr@sina.tums.ac.ir

on esophageal tumor biology and chemotherapy outcome remains unknown. This may be due to the fact that p53 interacts with several key molecules including Bcl-2 (9, 10). Numerous preclinical and clinical studies have suggested that impact of p53 status on responses to chemotherapy or radiotherapy depends on status of other genes such as Bcl-2 (9, 10).

The Bcl-2 family has been reported to be involved in the resistance to different therapeutic regimens which ultimately will induce cell death through apoptosis (11). There are evidences referring to the development of a multi-drug resistance phenomenon as a result of over expression of Bcl-2 protein (12). Moreover, over expression of wild type Bcl-2 causes inhibition of apoptosis, which contributes to neoplastic transformation. On the other hand, the decreased expression of Bcl-2 achieved by antisense method increases the susceptibility of cancer cells to apoptosis induction by many chemotherapeutic drugs (13).

Despite the progress in chemotherapy in recent years, the benefits of chemotherapy in esophageal cancer remain modest. The most widely used combination in esophageal cancer, 5-FU and cisplatin, has shown objective responses in 20-40% of cases, but had no impact on overall survival (13). It has been suggested that tumors expressing both Bcl-2 and mutant p53 have the lowest rate of apoptosis (9).

Since genetic alterations of p53 are frequently observed in esophageal cancers, alteration in the expression of Bcl-2 in esophageal carcinomas may further worsen the prognosis. In addition, lack of enough information on significance of the alterations in the expression of both p53 and Bcl-2 makes it difficult to clearly define the outcome of cancer therapy and patients survival in cases with esophageal cancers.

Our aim in the present study was to evaluate the importance of two apoptosis regulators as prognostic markers in a clinical setting for the first time in Iranian patients. Using immunohistochemical (IHC) techniques, we determined the expression of p53 and Bcl2 proteins in patients with esophageal cancer and correlated the results with data collected on clinicopathological parameters.

MATERIALS AND METHODS

Patients

Tissue samples of 37 patients with squamous cell carcinoma and adenocarcinoma of the esophagus were randomly selected for this study. Samples of esophageal tumors were obtained from patients who underwent surgery at two different university hospitals (Shohadaye Tajrish and Imam Khomeini) during the year 2000-2003. The patients included were Iranians from different geographical locations within Iran. Based on the designed questionnaire, data were collected for age at surgery, sex and pathological diagnosis.

Histopathological data contained tumor anatomical location, tumor pathological type, tumor size, histological differentiation (malignancy grade), stage and lymphatic invasion.

Immunohistochemistry

As previously described (14), formaldehyde fixed and paraffin embedded sections (3-5 μm) were mounted on slides, deparaffinized in xylene and rehydrated in graded ethanol solutions.

Tissue sections were then subjected to antigen retrieval using microwave oven and boiling citrate buffer (pH = 6.0) for 9 min. After blocking endogenous peroxidase activity with 0.3% hydrogen peroxide in methanol for 30 min, sections were incubated at room temperature for 60 min in 5% BSA for blocking nonspecific bindings. Sections were then incubated overnight at 4°C in humid chamber with p53 mouse monoclonal antibody (DO-7; Dako Cytomation, Denmark) that recognizes an epitope in the N-terminal, between amino acids 19-26, of the human p53 protein. In case of Bcl-2, the mouse monoclonal antibody (124; Dako Cytomation, Denmark) that reacts with wild type Bcl-2 protein was used. The primary antibodies were used at dilution of 1:100 for p53 and 1:80 for Bcl-2. The LSAB-2 detection kit and Diaminobenzidine (DAB) chromogen (Dako Cytomation, Denmark) were used to visualize the results based on the manufacturer's instruction with necessary modifications. Sections were also counterstained with Meyer's hematoxylin. In each series, a section in which incubation with the primary antibody was

omitted was used as negative control. The areas of highest protein expression evident at low-power scanning were taken for analysis. Staining was considered negative only after careful examination of the entire tissue section. Quantitation of the intensity and number of positive tumor cells were performed by two independent pathologists blinded to the clinical outcome as explained previously (15). In cases in which the investigators disagreed, the immunohistochemical scoring was repeated to agree on same scoring by both observers. Tumor samples were then classified into four categories based on the nuclear (p53) or cytoplasmic (Bcl-2) staining of tumor cells as 3+ (strong staining, >50%), 2+ (moderate staining, 25-50%), 1+ (mild staining, 5-25%) and 0 (negative, <5% or unstained).

Statistical analysis

For the statistical analysis, descriptive data were expressed as the mean ± SD. In order to compare the immunostaining pattern of p53 and Bcl-2 in relation to continuous variables, the independent-sample *t* test and for categorical variables, matched paired and ranked prognostic variables, the McNemar's Chi square (λ^2) test were applied using SPSS ver.10 software (l6). The significant relationship between p53 and Bcl-2 expression and between each marker expression with tumor anatomical location, tumor pathological type, tumor size, histological differentiation, gender, age at surgery and lymph node involvement were statistically evaluated.

RESULTS

Patients' characteristics

Our study included 37 post operative esophageal cancer patients, 21 (56.8%) male and 16 (43.2%) females. The mean age of these patients was 61 yr ± 12.57 (range: 34-80). Our patients had received no chemotherapy or radiotherapy before surgery. No one had familial history of esophageal cancer, history of background diseases, and/or history of alcohol and abuse-substances addiction.

Clinicopathological features

The distribution of tumor anatomical level was 2 (5.4%) in the upper third, 8 (21.6%) in the middle third and 27 (73%) in the lower third of esophagus. The mean macroscopic size of the resected tumors was 4.9 cm (range: 1.5- 8.5). Tumor pathological types were squamous cell carcinoma (SCC) in 31 (83.8%) and adenocarcinoma (AC) in 6 (16.2%) patients. The carcinomas were well differentiated in 16 (43.3%), moderately differentiated in 12 (32.4%), poorly differentiated in 8 (21.6%) and undifferentiated in 1 (2.7%). More than half of the samples, 23 (62.2%), were also lymph node positive and 14 (37.8%) of these samples had negative lymphatic invasion (Table 1). All of the tumors were in advanced stage of esophageal carcinoma due to observed perineural, vascular and adventitia invasion according to the guidelines for clinical and pathological studies on carcinoma of the esophagus (17).

Out of 37 esophageal tumor samples, 25 (67.67%) showed nuclear p53 expression. The distribution of positive nuclear staining for p53 (Fig.1a) was 7 (18.9%) with strong staining (3+), 8 (21.6%) with moderate staining (2+) and 10 (27%) with mild staining (1+).

Table 1. Clinicopathological features of esophageal cancer patients (n= 37)*

Age (yr)†	61 ± 12.57(range: 34-80)
Tumor size (cm)†	4.9 ± 2.07(range: 1.5-8.5)
Tumor type	
Squamous cell carcinoma	31(83.8%)
Adenocarcinoma	6 (16.2%)
Lymphatic invasion	
Positive	23 (62.2%)
Negative	14 (37.8%)
Sex	
Male	21 (56.8%)
Female	16 (43.2%)
Tumor anatomical level	
Upper third	2 (5.4%)
Middle third	8 (21.6%)
Lower third	27 (73%)
Histological grade	
Well differentiated	16 (43.2%)
Moderately differentiated	12 (32.4%)
Poorly differentiate	8 (21.6%)
Undifferentiated	1 (2.7%)

* Data are given as number (percent) unless specified otherwise.
† Mean ± SD.

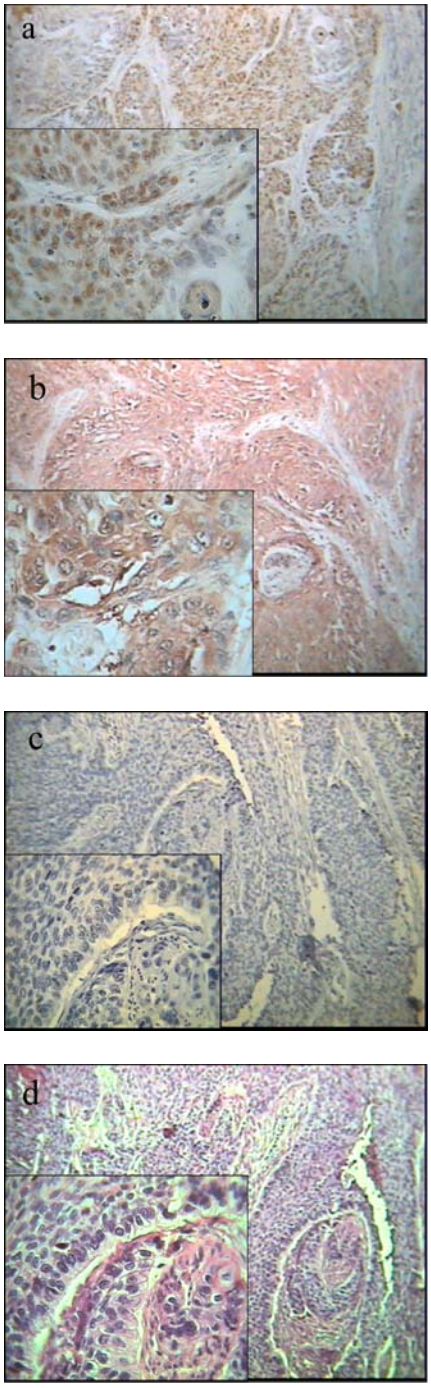

Fig. 1. Immunohistochemical staining in well differentiated squamous cell esophageal tumor samples. Tumor sections were stained using primary monoclonal antibodies as described in materials and methods. a) strong nuclear staining of p53 mouse monoclonal antibody (DO-7; Dako Cytomation, Denmark), dilution 1:100, x 100; b) strong cytoplasmic staining of Bcl-2, the mouse monoclonal antibody (124; Dako Cytomation, Denmark), dilution 1:80, x 100; c) negative control x 100; d) H&E staining x 100 (inset x 400).

For Bcl-2, its staining with cytoplasmic localization (Figure 1b) was found in 15 (40.5%) tumor samples. The distribution of positive staining was 3 (8.1%) with strong staining (3+), 6 (16.2%) with moderate staining (2+) and 6 (16.2%) with mild staining (1+).

Relationship between p53 and Bcl-2 expression

The prevalence of Bcl-2 over expression was significantly (P = 0.03) greater in p53+ tumors (35.14%) compared with p53- tumors (5.41%). Tumors were classified to four immunophenotypes (Fig. 2): 1) p53-/Bcl-2- (10/37; 27.02%), 2) p53 +/Bcl-2- (12/37; 32.43%), 3) p53- /Bcl-2+ (2/37; 5.40%) and 4) p53+/ Bcl-2+ (13/37; 35.17%).

Relationship between p53 and Bcl-2 expression and clinicopathological findings

Our results indicate no significant difference between positive and negative groups of markers with respect to age and gender of patients as well as the tumor size. Unlike p53, Bcl-2 immunostaining was significantly related to pathological tumor type (P = 0.001), histological differentiation (P = 0.007), and relatively to lymphatic invasion (P = 0.077) as shown in figure 3.

Relationship of p53 and Bcl-2 coexpression and clinicopathological findings

Our results showed significant relationship between p53 and Bcl-2 coexpression (P = 0.003).

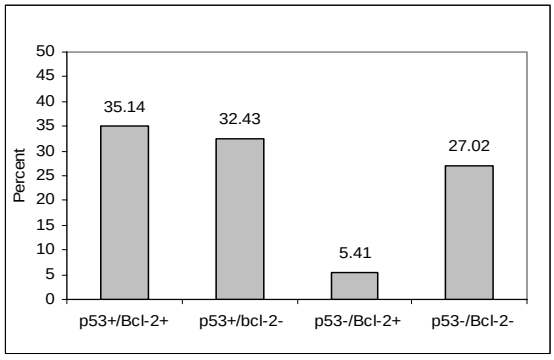

Fig. 2. Relationship between p53 and Bcl-2 expression in esophageal cancer. Expression of p53 and Bcl-2 was determined in tumor samples using immunohistochemical technique as indicated in the methods.

The relationship between coexpression patterns of markers with clinicopathological data were compared with other mentioned immunophenotypes (Table 2).

Positive p53 and Bcl-2 immunophenotype differed significantly with respect to tumor pathological type ($P = 0.0001$), tumor differentiation ($P = 0.007$) and lymph node involvement ($P = 0.021$) but not with respect to age and gender of patients when compared with other immunophenotypes.

Relationship of p53+/Bcl-2- immunophenotype and clinicopathological findings

Our data showed a high frequency of p53+/ Bcl-2+. The clinicopathological features of p53 positive and Bcl-2 negative cases were compared in table 3. This combination of expression pattern showed significant relationship with tumor type ($P = 0.0001$), histological differentiation ($P = 0.001$), tumor location ($P = 0.001$) and lymphatic invasion ($P = 0.013$).

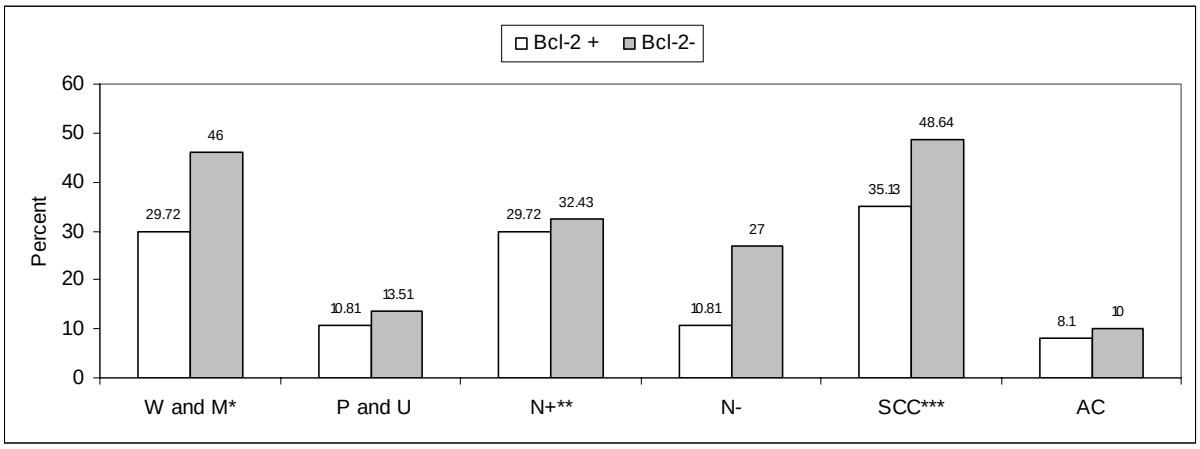

Fig. 3. Relationships between Bcl-2 overexpression and clinicopathological feature of patients in esophageal cancer. Expression of Bcl-2 was determined in tumor samples using immunohistochemical technique as indicated in the methods. * Histological grade: W, well differentiated; M, moderately differentiated; P, poorly differentiated; U, undifferentiated. ** Lymphatic invasion: N+, with lymphatic invasion; N-, without lymphatic invasion. *** Tumor type: SCC, squamous cell carcinoma; AC, adenocarcinoma.

Table 2. Clinicopathological feature of $p53^+$/ $Bcl\text{-}2^+$ cases versus other immunophenotypes of p53 and Bcl-2 in esophageal cancer*

Variables	No of patients	$P53^+/Bcl\text{-}2^+$ (n= 13)	Other immunophenotypes	*P* values
Tumor type				
Squamous cell carcinoma	31 (83.7%)	10 (27%)	21 (56.75%)	0.0001
Adenocarcinoma	6 (16.2%)	3 (8.1%)	3 (8.1%)	
Tumor size				
Over 4.9 cm	20 (54%)	7 (18.9%)	13 (35.13%)	0.167
Under 4.9 cm	17 (46%)	6 (16.2%)	11 (29.7%)	
Histological grade				
Well and moderately differentiated	28 (75.67%)	10 (27%)	18 (48.64%)	0.001
Poorly differentiated and undifferentiated	9 (24.32%)	3 (8.1%)	6 (8.1%)	
Lymph node involvement				
Positive	23 (62.16%)	10 (27%)	13 (35.13%)	0.021
Negative	14 (37.83%)	3 (8.18%)	11 (29.72%)	
Tumor anatomical level				
Lower third	27 (73%)	8 (21.62%)	19 (51.35%)	0.007
Upper and middle third	10 (27%)	5 (13.51%)	5 (13.51%)	

* Data are given as number (percent).

Table 3. Clinicopathological feature of $p53^+$/ $Bcl\text{-}2^-$ cases versus other immunophenotypes of p53 and Bcl-2 in esophageal cancer*

variables	No of patients	$P53^+/Bcl\text{-}2^+$ (n= 12)	Other immunophenotypes	*P* values
Tumor type				
Squamous cell carcinoma	31 (83.78%)	11 (29.7%)	20 (54.05%)	0.0001
Adenocarcinoma	6 (16.2%)	1 (2.7%)	5 (13.5%)	
Tumor size				
> 4.9 cm	20 (54.05%)	8 (21.62%)	12 (32.43%)	0.077
< 4.9 cm	17 (45.94%)	4 (10.8%)	13 (35.13%)	
Histological grade				
Well and moderately differentiated	28 (75.6%)	9 (24.32%)	19 (51.35%)	0.001
Poorly differentiated and undifferentiated	9 (24.32%)	3 (8.1%)	6 (16.21%)	
Lymph node involvement				
Positive	23 (62.16%)	9 (24.32%)	14 (37.83%)	0.013
Negative	14 (37.83%)	3 (8.1%)	11 (29.7%)	
Tumor anatomical level				
Lower third	27 (72.97%)	10 (27%)	17 (45.9%)	0.001
Upper and middle third	10 (27.02%)	2 (5.4%)	8 (21.6%)	

* Data are given as number (percent).

DISCUSSION

Various studies have shown that complex alterations of gene expression underlie the development of different malignant phenotypes of esophageal cancer cells (18). However, because of the lack of standardization and small number of cases over the years, the clinical evaluation of prognostic and predictive markers for esophageal cancer has been difficult to assess (19). As the roles of specific markers and their correlations become defined, the necessity for looking at multiple markers and their interactions with clinicopathological parameters and clinical outcome becomes clearer.

Most studies have correlated expression of molecular markers in the pretreatment biopsy with the response to treatment in the resected specimen and/ or the outcome following the treatment. These markers have usually been identified by immunohistochemical staining techniques (20). Accumulation of mutant p53 protein has been demonstrated in a number of human malignancies and shown to be associated with a poor prognosis in patients with breast, gastric, and colorectal carcinomas (21-23). But conflicting results have been reported regarding the expression of p53 and prognosis for esophageal cancer (24-26). Kitamura *et al.* findings support the role of p53 as a predictive marker in esophageal cancer (27). They assayed p53 protein expression in biopsy specimens of 95 advanced esophageal cancers immunohistochemically. All patients received one course of either chemoradiotherapy or hyperthermo-chemoradiotherapy preoperatively. Forty-one per cent of specimens were positive for p53 protein staining and treatment was histopathologically effective in 72%, the efficiency rate was 59% in p53 negative patients.

Shimada *et al.* correlated p53 expression in pretreatment biopsy samples with drug sensitivity in 59 patients with esophageal squamous cell carcinoma receiving cisplatin chemotherapy followed by esophagectomy (28). They reported that p53 expression in the pretreatment biopsy samples is correlated with resistance to cisplatin. One multivariable analysis demonstrated a significant relationship between cancer-specific death and the high-level expression of p53 (P = 0.04) in esophageal cancer (26). On the other hand, other studies have showed no correlation between p53 and (25, 29). In our study, nuclear p53 expression in the neoplastic epithelial cells of esophagus was observed in 67.6% of tumor samples. This percentage is in the

upper range of the previously published data (42%-64%) by others (29-33). Difference in the antibodies and dilutions used in our study and also advanced pathological stage and lymph node involvement in our tumor samples could be the reason for observed high percentage of p53 positive staining compared to other studies (29-33). Our data similar to the other published findings in esophageal cancer (34-37) did not demonstrate a significant correlation between p53 expression and studied clinicopathological parameters.

Bcl-2 is a mitochondrial protein with anti-apoptotic activities. Over expression of Bcl-2 causes a decrease in growth rate and sensitivity to cytotoxic drugs (38). High levels of Bcl-2 expression have been demonstrated in a variety of tumor types which is due to deregulation of Bcl-2 expression by several mechanisms (39). In particular, a negative regulation of Bcl-2 transcription by the p53 tumor suppressor gene has been reported (10). Since loss of p53 function following mutation is a very common alteration in human tumors, the expression of Bcl-2 and its anti-apoptotic function, has been implicated in drug resistance (39). Protection by Bcl-2 and related anti-apoptotic proteins against cell death induced by a variety of anti- tumor agents has been proposed as a novel mechanism of multi drug resistance (38). On the other hand, down-regulation of Bcl-2 by chemopreventive agents, suggest that induction of apoptosis by these drugs may be a mechanism by which they can intervene in esophageal carcinogenesis and indicate their potential benefit in chemoprevention of esophageal cancers (40).

Sarbia *et al.* assayed pretreatment tumor biopsies immunohistochemically for expression of p53 and Bcl-2 (41). They observed no correlation between the expression of Bc1-2 and the response to chemotherapy. But patients treated by radiochemotherapy and surgery with $p53^-$ tumors had a significantly better outcome than patients with $p53^+$ tumors with mean survival of 31.1 months vs 11.3 months. In our study, 43.6% of esophageal tumor samples showed cytoplasmic Bcl-2 expression in the neoplastic epithelial cells which is comparable to previous reports (14%-57.9%) by others (42-45). Although p53 expression as a single tumor marker did not correlate with our data on clinicopathological parameters, we observed a novel and significant relationship between Bcl-2 expression as a single marker and clinicopathological factors. The prevalence of Bcl-2 over expression was significantly (P = 0.007) greater in well and moderately differentiated tumors (27.72%) as compared with poorly and undifferentiated tumors (10.81%). The incidence of Bcl-2 overexpression was also relatively significant (P = 0.077) in lymph node positive tumors (29.72%) as compared with lymph node negative tumors (10.81%). We also found significant difference in Bcl-2 expression in squamous cell carcinoma compared to adenocarcinoma of the esophagus. These findings support the pathological importance of BcI-2 over expression in esophageal tumor progression and therefore, its potential to be considered as a valuable prognostic marker in esophageal cancer.

In addition, our data showed a significant relationship between p53 and Bcl-2 coexpression p= 0.03). Sarela *et al.* have also reported that p53 and Bcl-2 were significantly co-expressed in colorectal tumor samples (46). Co-expression of p53 and Bcl-2 implies that functional alterations in p53 protein may affect transcription regulation of Bcl-2 and consequently the over expression of BcI-2 protein (47). We observed over expression of mutated p53 protein and wild type Bcl-2 in esophageal tumor samples with lower histological differentiation. Our data also showed that lymph node positive cases had higher frequency of co-expression of p53 and Bcl-2 proteins. The prevalence of p53/Bcl-2 co-expression was also significantly (P = 0.021) greater in lymph node positive (27% as compared with lymph node negative samples (8.1 %). Our results also showed that 32.4% of tumors were p53 positive and Bcl-2 negative. This immunophenotype indicate another type of double gene alterations with significant relationship to pathological tumor type (P = 0.0001), tumor location (P = 0.001), malignancy grade (P = 0.001) and lymph node involvement (P = 0.013). These results suggest that alterations in both p53 and Bcl-2 gene expression may be correlated with tumor progression in esophageal cancer and further emphasize on their importance as prognostic markers.

In conclusion, our data on p53 and Bcl-2 overexpression, commonly observed in two tumor types of esophageal cancer would suggest the possibility of high drug resistance with low survival rate in these patients. Our results also showed the importance of Bcl-2 as valuable prognostic marker and its correlation with p53 with respect to clinicopathological findings. Therefore, it is advisable to analyze tumor samples of patients for determining the expression patterns of important molecular markers such as p53 and Bcl-2 proteins to identify patients who will most likely benefit from adjuvant therapy after esophagectomy.

Acknowledgment

Authors are thankful to the office of Vice - Chancellor for Research of Tehran University of Medical Sciences (TUMS) for their financial support of this research project. We also would like to thank Dr. I. Jahanzad and Dr. M. R. Khoshayand for their advices on IHC scoring and statistical analysis. We appreciate sincere cooperation of the staff of Shohadaye-Tajrish and Imam Khomeini University Hospitals on providing tumor samples.

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