Correlation between bcl-2, c-myc and GSH in esophageal carcinoma

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Abstract
Esophageal carcinoma has a high incidence rate in India, but its etiology is largely unknown. It is believed to arise as a result of interaction of multiple genetic and environmental factors during multistep tumorigenesis process. In the present study, expressions of bcl-2, c-myc protein and reduced glutathione levels were evaluated in forty surgically resected tissue specimens from tumor, tissue adjoining to the tumor, and normal mucosa from a distant site in response to the neo-adjuvant therapy (NAT). Group A consisted of patients who had received preoperative chemotherapy followed by transthoracic esophagectomy. Group B consisted of surgically operated patients without any preoperative chemotherapy. Protein expression analysis and their localization was carried out using Western Blotting and by immunohistochemistry. Glutathione levels were biochemically analyzed. Expression of bcl-2 protein, in tumor and its adjoining tissue of patients was found to be significantly down-regulated than in the normal tissue of all the patients without NAT. The levels of this anti-apoptotic protein after NAT were significantly increased. On comparison between two treatment groups, no statistically significant effect of neo-adjuvant therapy was observed on levels of c-myc in tumor tissues. This study is reporting for the first time a significant inverse correlation between bcl-2 and c-myc protein expression in esophageal carcinoma. Levels of endogenous antioxidant, reduced glutathione (GSH) in tumor of patients on NAT were found to be significantly less than the levels in tumors of patients without NAT. Positive correlation was observed between bcl-2 and GSH levels in tumor tissue of patients receiving NAT. Whereas, inverse correlation between c-myc expression and GSH was found in tumor tissues of patients who had undergone NAT. This study for the first time has shown the relationships between oncoproteins bcl-2/c-myc and GSH in esophageal carcinoma which might be helpful in adjusting the apoptotic threshold in clinical setting.

Keywords: bcl-2, c-myc, reduced glutathione, neo-adjuvant therapy and esophageal carcinoma.

INTRODUCTION
The striking feature of esophageal carcinoma is its multifactorial origin and wide geographical variation in its incidence. Over the past two decades it has become increasingly clear that cancer is produced by errors in the normal genetic programs that guide the multiplication, specialization and death of cells. Our molecular understanding of apoptosis has advanced profoundly since its original description by Wyllie and colleagues (Kerr et al., 1972). Apoptosis is highly regulated at the molecular level, and bcl-2, a 26 KDa, protein is now emerging as the prototype of a rapidly growing family of interacting proteins which share its ability to modulate apoptosis. Bcl-2 family of proteins regulates late step in apoptotic pathway (Srinivas et al., 2000). The main role of bcl-2 in neoplasia is to facilitate clonal selection, and thus increase the lifetime of invasive cells (Strasser, 1995) which favors the acquisition and accumulation of genetic alterations (Yin and Schimke, 1996). Over expression of bcl-2 has been reported in a number of human cancers, although its correlation with tumor differentiation, and the clinical outcome are conflicting and depend on tumor type and site (Jordan et al., 1996). Bcl-2 protein is believed to be involved in imparting resistance to apoptosis induced by chemotherapeutic agents and radiation (Srinivas et al., 2000). Expression levels of the bcl-2 family of proteins are critical not only for tumor development but also for tumor progression and resistance to therapy (Bargou et al., 1995), and hence the treatment outcome.

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In human beings, several recent findings correlate alterations in the c-myc oncogene with malignant neoplasms. The proto-oncogene c-myc is at the center of transcription factor network that regulates cellular proliferation, differentiation and apoptosis (Oster et al., 2002). Despite numerous studies, the molecular mechanism responsible for these biological activities of c-myc is unclear. Studies on the interaction of bcl-2 and c-myc have demonstrated that bcl-2 prevented the apoptosis induced by c-myc, but did not interfere with its proliferative effect (Fanidi et al., 1992, Wagner et al., 1993). This provides a novel mechanism for oncogene cooperation in the process of carcinogenesis and drug resistance in tumors.

Many of the chemical and physical stimuli capable of inducing apoptosis are known to increase the steady state concentration of reactive oxygen species (ROS). These highly reactive compounds can act as initiators and/or promoters of carcinogenesis, cause DNA damage, activate procarcinogens, and also alter the cellular antioxidant defense system. GSH is an endogenous molecule for the effective detoxification mechanism. Changes in GSH homeostasis have also been implicated in the etiology and progression of a number of human diseases. Treatment with radiation and most chemotherapeutic agents results in a dramatic change in cellular gene expression (Fornace et al., 1992). The use of NAT prior to surgery in the treatment of esophageal carcinoma has increased in the recent years. However, not all the patients respond equally and therefore, the understanding of molecular factors responsible for response and/or resistance to chemotherapy is required to improve the treatment results. The present study, therefore, was carried out to find relationship between expression profiles of bcl-2, c-myc and levels of GSH in esophageal carcinoma in response to NAT. In the present study, for the first time, we are reporting correlations between bcl-2, c-myc and GSH in the tumor tissue, tissue adjoining to tumor and normal tissue of esophagus of patients suffering from esophageal carcinoma.

**MATERIALS AND METHODS**

**Patients and Tissue Samples**

Forty surgically resected tissue specimens from tumor, adjoining to tumor tissue and paired microscopically normal mucosa (3 cm or more away from tumor tissue) were obtained from Department of General Surgery, Postgraduate Institute of Medical Education and Research, Chandigarh, India, with written informed consent from the patients. Patients were divided into two main groups, A and B. Group A consisted of thirty patients who had received preoperative chemotherapy comprising of cisplatin and 5-FU (5-flourouracil). Group B consisted of ten patients without any preoperative chemotherapy. The patients in group A were administered cisplatin (30mg/mt²/ day) and 5-FU (750mg/mt²/day) daily for three days and were operated surgically by transhiatal esophagectomy (THE) after four weeks of neo-adjuvant therapy. Patients receiving steroids, harboring more than one malignancy and who were on antioxidant therapy were not included in the study. The mean age of patients was 52 years (range 30-75). The ratio of males and females was found to be 1: 0.66. Relevant information regarding age, sex, dysphagia, associated medical illness, site of lesion, type of histology, smoking, alcohol intake and lymph node metastasis was collected.

**Antibodies**

Primary bcl-2, c-myc, rabbit polyclonal IgG and secondary anti-rabbit IgG were procured from Santa Cruz Biotechnology, USA.

**Protein Extraction**

Approximately 500 mg of tissue sample was homogenized in 1.5 ml of lysis buffer (50 mM Tris pH 8.8, 150 mM NaCl, 10 µg/ml leupeptin, 20 µg/ml aprotinin and 1 mM (phenylmethylsulfonyl fluoride). The homogenate was centrifuged twice at 10,000g for 10 min at 4°C and supernatant was stored at −80°C. Protein concentration was determined by Lowry’s method (1951).

**Reduced Glutathione (GSH) Assay**

GSH was measured immediately in esophageal homogenate by the method of Moron et al. (1979). To 125 mg tissue equivalent homogenate, 100 µl of 25 % TCA was added and centrifuged at 3000 rpm for 10 minutes. The reaction was initiated by the addition of 2 ml of 0.6 mM 5,5'- dithio-bis, 2- nitrobenzoic acid (DTNB). Optical density of the yellow colored complex formed by GSH and DTNB (Ellman’s reagent) was recorded at 412 nm against a reference cuvette containing 0.1 or 0.2 ml of 5 % TCA. All assays were done in duplicates.

**Western Blotting**

Western blotting was performed by the method of Towbin et al. (1979). Samples were dissolved in sample buffer (0.125M Tris, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.1% bromophenol blue) pH 6.8 and heated at 100°C for 4-5 minutes. Briefly, 40 µg protein was subjected to 10 % SDS-PAGE followed by electroblotting onto nitrocellulose membrane at 40 mA. The membrane was incubated overnight with 4% skimmed milk at 4°C. Equal protein loading was verified by Ponceau red staining. Blot was incubated with primary bcl-2 antibody (1:1000) and c-myc antibody.
antibody (1:1000) for 1 hr at 37°C. Three washings with phosphate buffered saline (PBS) and 0.05 % Tween-20 were given. Blot was then incubated with secondary antibody bcl-2 (1 : 15000) and c-myc (1 : 7500) dilution for 1 hr at 37°C. Again three washings with PBS and 0.05% Tween-20 were given, and the final washing was given with PBS. Blot was incubated at 37°C with substrate BCIP/NBT (5-Bromo-4-Chloro-3-indolyl phosphate/ nitroblue tetrazolium) till bands appeared. For quantitative analysis, the intensity of the protein bands was evaluated by densitometer using Imaging Denstitometer software (Bio-rad model GS-670). The intensity of bands from the adjoining and tumor tissues were calculated with respect to the intensity of normal sample taken as 100 and hence a relative value of expression was obtained for tumor and adjoining tissues. For negative control, blot was incubated without primary antibody and proceeded in similar fashion with other blots. Specimens from patients who were very strongly expressing bcl-2/ c-myc were used as positive controls for the present study.

Immunohistochemical Staining

Briefly, tissue sections were kept in oven for 5 min at 80°C and deparaffinized in xylene rehydrated through graded alcohol (100%, 70%, 50% and 30%) and finally brought to water. The sections were incubated for 30 minutes at room temperature in endogenous block solution to quench the endogenous peroxidase activity. Antigen unmasking was carried out by incubating in citrate buffer for 10 minutes at high power in a microwave oven. Slides were allowed to cool to room temperature, washed twice in PBS-Triton-x and then in PBS alone. Sections were incubated with appropriately diluted primary antibody at room temperature for 2 hrs to overnight depending upon the results. After similar two washings with PBS-Triton-x and one washing with PBS alone, the sections were incubated with respective secondary antibody for 1 to 2 hours at room temperature and processed with avidin-biotin complex peroxidase method. Thereafter, sections were washed 3 times in PBS. Color was developed by adding chromogen DAB (3,3′- diaminobenzidine tetrahydrochloride) for 2-5 minutes. After washing with distilled water, the sections were counter stained with haematoxylin. Finally, sections were subjected to alcohol and xylene baths, air-dried and then mounted for examination under light microscope. A lymph node reactive case was used as a positive control. The negative controls did not show any evidence of non-specific staining. Protein expression was evaluated by semi-quantitative scoring by assessing the percentage of positive cells from total of 500 cells counted. Stainings were classified as negative (-), mild (+), moderate (+++) or intense (++++) expression based on a grading score pattern.

Statistical Analysis

Spearman rank order correlation was used to test correlation between bcl-2 and c-myc protein expression. Chi-Square test was used for testing association between protein expression and various clinicopathological parameters. Statistical evaluation between groups was carried out with One Way Analysis of Variance and Mann Whitney Test. The P <= 0.05 was considered to be statistically significant.

RESULTS

Western Blotting: bcl-2 and c-myc

Fig. 1a shows bcl-2 protein expression on a representatative immunoblot in normal (N), adjoining (A) and tumor (T) esophageal carcinoma (I), and their relative values (Mean ± SEM ) without (-ve NAT) and with (+ve NAT) neoadjuvant therapy (II);

* P <0.05;  **P <0.01;  ***P < 0.001, (N = 10-30). a) with respect to to normal; b) wrt to its adjoining; c) (+ve NAT) wrt (-ve NAT)

N Vs A (-ve NAT): Wilcoxon Signed Rank Test.
N Vs T (-ve NAT) : Paired- t-test;
A Vs T (-ve NAT) : Paired- t-test ;
T (+ve NAT) Vs T (-ve NAT) : Mann-Whitney Rank Sum Test;
A (+ve NAT) Vs A (-ve NAT) : Mann-Whitney Rank Sum Test

Figure 1: Bcl-2 protein expression on immunoblot in normal (N), adjoining (A) and tumor (T) esophageal carcinoma (I), and their relative values (Mean ± SEM ) without (-ve NAT) and with (+ve NAT) neoadjuvant therapy (II);

RESULTS

Western Blotting: bcl-2 and c-myc

Fig. 1a shows bcl-2 protein expression on a representative immunoblot in normal (N), adjoining (A) and tumor tissue (T) obtained from two esophageal cancer patients. Bcl-2 protein expression in tumor tissue was up-regulated in 16 patients and down-regulated in 14 patients in response to NAT. In group B patients treated without chemotherapy the down-regulation of bcl-2 was 100% both in tumor and its adjoining tissue with respect to the normal tissue. The values (mean ± SEM) for bcl-2 protein expressions in normal, adjoining and tumor tissue of patients with (+ve) and without (-ve) NAT are depicted in Fig. 1b.

Fig. 2 depicts c-myc protein expression on a representative immunoblot in normal (N), adjoining (A)
and tumor (T) tissues obtained from two esophageal cancer patients. In all the patients who were not administered with chemotherapeutic agents, there was significant up regulation of the c-myc protein expression in tumor and its adjoining tissue as compared to normal mucosa. However, levels of c-myc were decreased in tumor and adjoining tissue after NAT (Fig. 2II).

Figure 2: c-Myc protein expression on immunoblot in normal (N), adjoining (A) and tumor (T) esophageal carcinoma (I) and their relative values (Mean ± SEM) without (-ve NAT) and with (+ve NAT) neoadjuvant therapy (II);

* P <0.05;  **P <0.01;  ***P < 0.001, (N = 10-30). a) with respect to normal; b) wrt to its adjoining; c) (+ve NAT) w.r.t (-ve NAT)

N Vs A (-ve NAT) : One Way Analysis of Variance (Dunn’s Method);
N Vs T (-ve NAT) : One Way Analysis of Variance (Dunn’s Method);
N Vs A (+ve NAT): One Way Analysis of Variance (Dunn’s Method);
A Vs T (+ve NAT) : Wilcoxon Signed Rank Test;
A (-ve NAT) Vs A (+ve NAT) : Student’s-t-test

Immunohistochemical Staining

Immunohistochemical results of bcl-2 expression showed 31% patients with negative staining and 69% patients with positive staining. Cytoplasmic localization of bcl-2 with mild, moderate and strong immunoreactivity was present in 26%, 19% and 55% of immunohistochemically positive tumors respectively. The photomicrograph of bcl-2 staining shows islands of tumor cells with differential cytoplasmic staining as indicated by brown color (Fig. 3I).

Results of c-myc staining showed 23% negative and 77% positive immunostaining. Further, distribution analysis of positive c-myc staining showed 61% mild, 8% moderate and 8% strong positivity. Both moderate and strong c-myc immunoreactivity was found mainly in cytoplasm of esophageal tumors. Mild c-myc positivity displayed heterogeneous staining pattern with both nuclear and cytoplasmic staining in esophageal carcinoma patients as indicated by brown color in Fig. 3II. The levels of protein staining in tissue by immunostaining were found to be positively correlated with levels of protein expression as analyzed by western-blot.

Figure 3I: The photomicrograph of bcl-2 staining shows islands of tumor cells with differential cytoplasmic staining.

Figure 3II: The photomicrograph of c-myc staining showing both nuclear and cytoplasmic staining in esophageal carcinoma patients.

Clinicopathological parameters

Clinico-pathological parameters and their relationship with bcl-2 and c-myc protein levels are shown in Table 1 [Supplementary data]. No significant association was
found between any of the parameters and bcl-2/c-myc protein expression levels by Chi-Square test.

**Reduced Glutathione**

Levels of GSH did not statistically differ in the tumor, its adjoining tissue and normal tissue of patients who did not receive NAT. However, GSH levels in the tumor and adjoining tissues decreased significantly in comparison to the normal tissue after NAT as depicted in Fig. 4. On comparison between treatment arms (NAT +ve Vs NAT –ve), GSH levels were found to be significantly decreased in the tumor of patient’s with NAT as compared to patients without NAT.

**Relationship between bcl-2 and c-myc**

An inverse correlation between c-myc and bcl-2 protein levels in esophageal tumors was observed in patients without NAT as determined by Spearman Rank Order Correlation (r = -0.67; P = 0.029). The negative relationship remained significant in patients who received NAT (r = -0.31; P = 0.091) (Fig. 5A).

**Relationship analysis between protein markers and GSH, an anti-oxidant enzyme**

Fig. 5B shows a positive correlation between bcl-2 and GSH levels in tumor tissue of patients receiving NAT (r = 0.75, P = 0.04). However, the association between c-myc and GSH in tumor tissue of patients who had undergone NAT prior to surgery was found to be negative (r = 0.71; P = 0.04) (Fig. 5C).

**DISCUSSION**

In oncogenesis, the bcl-2 protein acts in cellular immortalization and/or transformation. The growing family of bcl-2 can register diverse forms of intracellular damage, gauge whether other cells have provided positive or negative stimulus, and integrate these competing signals to determine whether the cell is “to be or not to be” (Jerry and Suzanne 1998). Bcl-2, an anti-apoptotic protein, is expressed in a variety of tumors and its expression modulates the sensitivity of tumor cells to wide spectrum of chemotherapeutic agents (Simonian et al., 1997). In the present study, patients of esophageal cancer who had undergone NAT showed an increase in the bcl-2 protein expression in 16 patients and decrease in the remaining 14 out of 30 patients. Similar bcl-2 expression pattern was observed in the adjoining tissue. This observation suggests that bcl-2 protein levels fail to predict a definite pattern in patients on NAT and varies from patient to patient. Whereas, in all the patients without any treatment prior to surgery showed decreased bcl-2 levels in malignant tissue. Increase of anti-apoptotic bcl-2 protein expression upon NAT administration suggests that it may play a role in inducing chemotherapy resistance or tumor relapse. A number of studies have correlated bcl-2 increased expression with poor clinical outcome in various malignancies (Reed 1999). Recently, Galante GM et al., (2008), have documented that overexpression of bcl-2 may confer resistance to anoikis (a special type of programmed cell death) in pancreatic cancer cell lines. Earlier, the findings in mutant p53 cell lines (AL27 and PANC3) have shown that after 5-FU exposure there was a significant increase in bcl-2 protein which was significantly correlated with 5-fluorouracil (5-FU) resistance (Mirjolet et al., 2000). Studies on advanced gastric cancer have also shown that 5-FU-induced apoptosis was suppressed by the over expression of bcl-2 (Inada et al., 1997). Chemotherapeutic agents such as taxol and 5-FU have also been shown to induce post translational modification of bcl-2 through phosphorylation (Halder et al., 1994), which in turn modulates its anti-apoptotic activity (Halder et al., 1995). Over-expression of bcl-2 can attenuate excision-repair activity, which would enhance mutagenesis and might have an effect on the genes responsible for apoptosis (Liu et al., 1997).

Mutations which disrupt regulation or expression levels of the c-myc gene, a pro-apoptotic gene, are among the most common found in human and animal cancers.
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(Cole and McMahon 1999). De-regulated c-myc expression has been observed in variety of tumors, and its enforced expression in animals lead to cellular transformation and tumorigenesis (Marcu et al., 1992). In most of the patients without any treatment prior to surgery, there was increase in c-myc protein expression in both tumor and adjoining tissue. We found that in patients, who were given NAT, there was decrease in c-myc levels in tumor and adjoining tissue as compared to the normal tissue. This beside bcl-2 may be another contributory factor for inhibition of apoptotic pathways. In this context, the present study for the first time has shown significant inverse correlation between bcl-2 and c-myc irrespective of the treatment arm followed. It has been reported that acquisition of high bcl-2 expression mitigates the apoptotic effects of deregulated c-myc expression without affecting its ability to promote continuous cell growth, so providing a mechanistic basis for the oncogene synergy between these two proto-oncogene (Fanidi et al., 1992). We feel that high levels of bcl-2 with low levels of c-myc in tumor cells would create a conducive environment for tumor cell proliferation leading to metastasis and thus poor prognosis. Whereas, in patients with down-regulated bcl-2 and up-regulated c-myc there may be a good prognosis. Hence, it seems that the c-myc expression is carefully controlled by many mechanisms, and its response to various treatment modalities are modulated by interactions with other regulatory proteins and hence, requires further investigations.

Immunohistochemical results show bcl-2 positivity mainly localized to cytoplasm of neoplastic cells with positive staining in 69% of tumors examined, which is very close to findings quoted in the literature (Patel et al., 1997). Interestingly in our study, apart from nuclear c-myc staining, majority of cases showed both nuclear and cytoplasmatic staining. It has been suggested that aberrant cytoplasmic localization may be due to alterations in the C terminus of the protein, reducing the efficiency of nuclear targeting (Royds, 1992). It seems that alteration in c-myc localization and deregulated expression may play a role in cancer development and it may be used as predictive biomarker in esophageal squamous cell carcinoma.

Oxyradicals are involved in multiple mutational events, and can contribute to the conversion of healthy cells to malignant cells (Levy et al., 1999). Since, there are hardly any reports available in literature on GSH (endogenous antioxidant) changes in response to NAT in esophageal carcinoma patients, this aspect was also evaluated in the present study. ROS induced apoptosis in cellular system is mainly based on the excessive formation of ROS as well as on depletion of cellular antioxidants. Intracellular ROS formation play a physiological role as second messengers through regulation of gene expression and cell proliferation (Schulze-Osthoff et al., 1995). Thus, it seems that depending upon the extent of ROS-mediated oxidative stress cell may proliferate or undergo apoptosis (Maellaro et al., 1996, Zhong-Ying et al., 2003). The oxidant-antioxidant balance is thought to be important in the initiation, promotion, and therapy-resistance of cancer. It has been shown that GSH plays a central role in this delicate balance by maintaining the redox status of the cell for protecting it from ROS, and other toxic chemicals. A very important finding of the present study was significant decline in GSH levels in patients after NAT. It is believed that such a situation would enhance the drug sensitivity by promoting apoptotic cascade and tumor killing in esophageal tumor tissue. GSH depletion almost invariably predisposes cells to apoptosis by other pro-apoptotic stimuli. When GSH levels are lowered, this leads to an increase in cell stress, increase in ROS formation, and increase in sphingomyelin metabolites, all of which are important intracellular signaling molecules in apoptosis. Conversely, replenishing GSH generally prevents apoptosis (Chau et al., 1998). Further, bcl-2 and GSH showed a significant positive association in the tumor tissue of patients in response to NAT. Our results indicate that the over expression of bcl-2 may shift the cellular redox potential to a more reduced state. Studies on the redox aspects of bcl-2 function has led to a hypothesis that bcl-2-expressing cells have enhanced antioxidant capacities that suppresses apoptotic pathways initiated in the malignant cells. Biochemical studies also have implicated bcl-2 in the regulation of the redox potential of the cell, as ectopic expression of bcl-2 suppresses cell death induced by oxidizing agents (Zhong et al., 1993). It has also been shown that bcl-2 expressing cells have higher intracellular and intra nuclear levels of GSH. The higher nuclear levels of GSH may block nuclear alterations associated with apoptosis (Voehringer et al., 1998). This is further supported by the observation that depletion of GSH by culturing cells in tissue culture medium lacking the amino acids cysteine and methionine or treating cells with diethylmaleate to directly deplete GSH caused over expressing cells bcl-2 to become sensitized to apoptotic induction (Voehringer et al., 1998). The positive correlation between bcl-2 and GSH in esophageal carcinoma indicates that further reduction in tumor GSH may be helpful for better prognosis of patients having low levels of bcl-2.

FUTURE STUDIES

RNA interference (RNAi) as a mechanism to selectively degrade mRNA (mRNA) expression has emerged as a powerful method for specific gene silencing which may also lead to promising novel therapeutic strategies. Given the ability to knock down essentially any gene of interest, RNA interference via siRNAs and short hairpin RNA (shRNA) is able to achieve a therapeutic effect. Silencing a specific
oncogene in tumor cells brings about cell death both in vitro and in vivo. There are an increasing number of large-scale RNAi screens that are designed to identify the important genes in various biological pathways. In future studies, it will be interesting to use RNAi to find genes of interest in esophageal cancer progression and also to silence one gene of interest which could help establish the effect on the other gene for example on bcl-2/ c-myc in the present study.

References


