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Association Between p53 Expression and Bcl-2, P-glycoprotein, Topoisomerase II Alpha, Thymidylate Synthase and Thymidine Phosphorylase as Potential Therapeutic Targets in Colorectal Cancer Patients

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Abstract: We evaluated the expression of molecular markers in colorectal adenocarcinoma in relation to p53 protein expression. Tissue samples of 54 patients with colorectal adenocarcinoma were obtained at surgery at university hospitals in the years 2000-2003. These were analyzed by immunohistochemical techniques using primary antibodies for p53, Bcl-2, P-gp, topoisomerase II alpha and Thymidylate Synthase (TS), thymidine phosphorylase/PD- ECGF (TP) and LSAB detection kit. The highest prevalence of expression among six analyzed markers were P-gp and p53 with 77% expression and the lowest one was Topo II with 35% expression. No clinicopathological significance was recorded in colorectal cancer patients. Several immunophenotypes were observed between p53 and other molecular markers. Additionally the prevalence of lack of expression of Bcl-2, Topo II and TS was higher in $p53^+$ tumors than in p53-tumors. A significant association ($p = 0.021$) existed between p53/Bcl-2 coexpression and mean age of patients (63.5 [10.1]y vs. 52.3 [15.2] y) and between p53/TP coexpression and sex (66.7% male; ($p = 0.022$). Overexpression of mutated p53 seen in tumor samples may alter the expression pattern of other molecular markers that are predictors of tumor response to chemotherapy regimens. Age and sex of patients could also affect the p53 related proteins such as Bcl-2 and TP, which can affect therapeutic outcome and disease prognosis. These findings emphasize the importance of tumor immunophenotypes as valuable prognostic or predictive markers in clinical settings.

Key words: Colorectal adenocarcinoma, p53, Bcl-2, P-glycoprotein, Topoisomerase II alpha, Thymidylate synthase, thymidine phosphorylase, therapeutics targets

INTRODUCTION

Colorectal cancer results from stepwise progression through several genetic alterations, including in the p53 gene (Catalano *et al*., 2001). Mutation or deletion of the p53 gene is one of the most frequently detectable genetic changes in colorectal cancer (Hollstein *et al*., 1991). Mutation in p53 may also affect expression of other genes, though this issue remains controversial (Anwar *et al*., 2004; Allegra, 2003). Whether the expression of p53 predicts a worse response to treatment and can serve, as a prognostic factor remains controversial (Pomp, 1998). Response of malignant tumors to therapy is a complex process influenced by a variety of intrinsic and acquired factors (Nagy, 1994).

We assessed the value of p53, in relation to certain markers, either alone or in combination, in colorectal cancer patients. Our primary aim was to define the clinicopathological significance of p53 and the secondary aim was to assess the association between p53 expression and other protein alterations (p53) immunophenotypes in colorectal cancer patients.

MATERIALS AND METHODS

Patients: Tissue samples of patients with colorectal adenocarcinoma who had previously undergone surgery at one of the two university hospitals (Shohadaye Tajrish and Imam Khomeini) during the years 2000-2003 were studied. Twenty-five patients with prior chemotherapy or radiotherapy, familial history of colorectal cancer, history of coexisting diseases, alcoholism or substance abuse were excluded. Immunohistochemical studies were thus performed on 54 of the total 104 cases. All our patients

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were from different geographical locations within Iran. Data were collected about age at surgery, sex and pathological diagnosis. Histological data contained tumor anatomical location, pathological type and size, histological differentiation (malignancy grade), stage and lymphatic invasion.

This study was in accord with the ethical Committee on human experimentation of Tehran University of Medical Sciences.

Primary antibodies: Six primary mouse monoclonal antibodies with following specifications were used in the present study: anti-human p53 protein, Clone D)-7 with optimized dilution of 1:100; Bcl-2 oncoprotein, Clone 124 with optimized dilution of 1:80; anti-P-glycoprotein, Clone C494 with optimized dilution of 1:200; anti-human topoisomerase II α, Clone Ki-S1 with optimized dilution of 1:50 and thymidylate synthase (TS) Ab-1, Clone TS 106 with optimized dilution of 1:40 and thymidine phosphorylase/PD-ECGF Ab-1, Close P-GF.44C (both from Labvision, USA) with optimized dilution of 1:200.

IHC method: Tissues were deparaffinized and rehydrated as described previously (Arbabi Bidgoli *et al*., 2005). Following the blocking step, the slides were incubated with primary antibodies at optimized dilutions for 18 h at 4°C. The bound antibodies were detected using the streptavidin-biotin immunoperoxidase detection kit (LSAB2; Dakocytomation and DAB chromogen (Dakocytomation) based on the manufacturer's instruction with necessary modifications. Sections were also counterstained with Meyer's hematoxylin. In negative controls, incubation with the primary antibody was omitted. As positive control, an adenocarcinoma sample known to positively stain for each antibody was included in each staining run. Staining was considered negative only after careful examination of the entire tissue section. Quantitation of the intensity and number of positive tumor cells was done by two independent pathologists (BM and MD) blinded to the clinical outcome. In case of disagreement, the immunohistochemical scoring was repeated.

Scoring method: Tumor samples were classified into four categories based on the molecular expression of p53 and Topo II alpha and cytoplasmic expression of Bcl-2, P-glycoprotein, TS and TP. Tumor cells were scored as 3+ if they had strong staining (>50%), 2+ if they had moderate staining (25-50%), 1+ if they had mild staining (5-25%) and o if staining was <5%. According to the guidelines for pathological studies on colorectal carcinoma, histological grade and pathological stage of tumors were also determined (Rubin *et al*., 1998).

Statistical analyses: Values were expressed as frequency rates or as the mean±standard deviation (SD). The non-parametric Kruskall Wallis H (Woolson 2002) was used to define the association between the scores of all markers and p53 scores and also the association between p53 expression and binominal clinico-pathological parameters. Probability values of <0.05 were considered significant. Statistical analysis was performed with SPSS 10 statistical software

RESULTS

Patient's characteristics: Table 1 shows the clinicopathological features of these patients.

Results of staining with each antibody: Expression of markers was analyzed in all of the cases for p53 and Bcl-2, in 52 cases for P-gp and Topo II and in 51 cases for TS and TP expression (Fig. 1) p53 and Topo alpha showed nuclear and the others showed cytoplasmic expression (Table 2). The highest incidence of expression was with P-gp and p53 with 77% expression and the lowest with Topo II with 35% expression (Fig. 2).

Clinicopathological significance of p53 in colorectal carcinoma: Kruskall Wallis H test showed no significant association between p53 scores of expression and evaluated clinicopathological findings (Table 3).

Significant Immunophenotypes

P53 and Bcl-2: Tumors were classified to four immunophenotypes: a) p53+/Bcl-2+ (12/54; 22%), b) $p53^{+}$/$Bcl\text{-}2^{-}$ (29/54; 54%), c) $p53^{-}$/$Bcl\text{-}2^{+}$ (6/54; 11%) and d) $p53^{-}$/$Bcl\text{-}2^{-}$ (7/54; 13%). Among $p53^{+}$ tumors, the incidence of Bcl-2 negative expression (29/41, 71%) was

Table 1: Clinicopathological features of colorectal cancer patients (N = 54)

Age (Mean±SD)	54.8 year±14.94 (Range: 15-85)	Tumor size (Mean±SD)	5 cm±1.84 (Range: 0.5-9)
Sex		**Lymphatic invasion**	
M	24 (44.4%)	Positive	30 (55.6%)
F	30 (55.6%)	Negative	24 (44.4%)
TAL		**Pathological stage**	
Colon	35 (64.8%)	Stage A	3 (5.6%)
Rectum	19(35.2%)	Stage B1	3 (5.6%)
HG		Stage B2	24 (44.4%)
W	25 (46.3%)	Stage C1	2 (3.7%)
M	22 (40.7%)	Stage C2	10(18.5%)
P	4 (7.4%)	Stage D	12(22.2%)
U	3 (5.6%)		

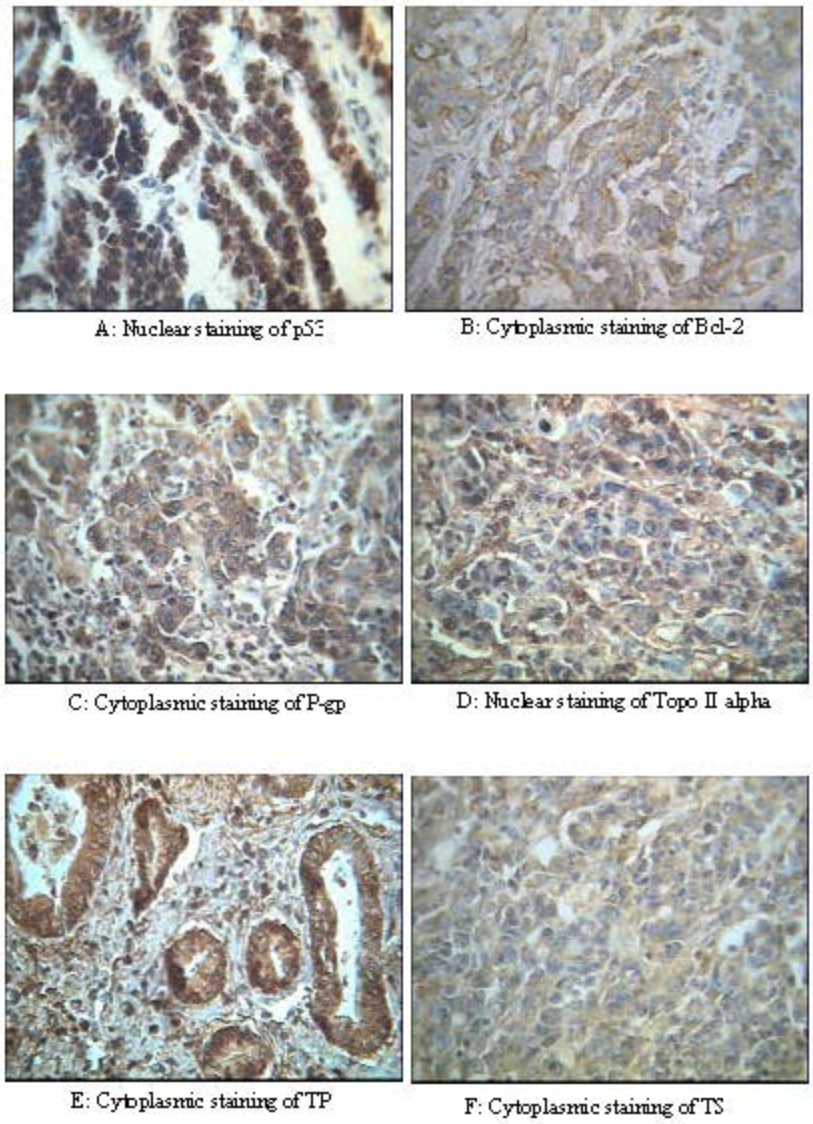

Fig. 1: Immunohistochemical staining of markers in tumor samples of patients with Colorectal Carcinoma. Tumor sections were stained using primary monoclonal antibodies as described in materials and methods (Chromogen DAB, magnification x 400)

Table 2: Status of marker expressions in Colorectal cancer

Markers	No. of cases	Positive	Negative	1+	2+	3+
P53	54	41 (75.9%)	13 (24.1%)	19 (35.2%)	16 (29.6%)	6 (11.1%)
Bcl-2	54	18 (33.3%)	36 (66.7%)	10 (18.5%)	6 (11.1%)	2 (3.7%)
P-gp	52	40 (76.9%)	12 (23.1%)	13 (25%)	15 (28.8%)	12 (23.1%)
Topo II	52	18 (34.6%)	34 (65.4%)	9 (17.3%)	6 (11.5%)	3 (5.8%)
TS	51	21 (41.2%)	30 (58.8%)	13 (25.5%)	6 (11.8%)	2 (3.9%)
TP	51	24 (47.1%)	27 (52.9%)	15 (29.4%)	8 (15.7%)	1 (2%)

significantly higher than Bcl-2 positive expression (29%). Kruskall Wallis H test showed a significant association between p53 and Bcl-2 scores of expression ($p = 0.021$) (Table 4).

P53 and P-gp: Tumors were classified to four immunophenotypes a) p53+/P-gp+ (30/51, 58.8%), b) p53+/P-gp- (9/51, 17.65%), c) p53-/P-gp+ (9/51, 17.65%) and d) p53 -/P-gp- (3/51,5.9%). More than half of the

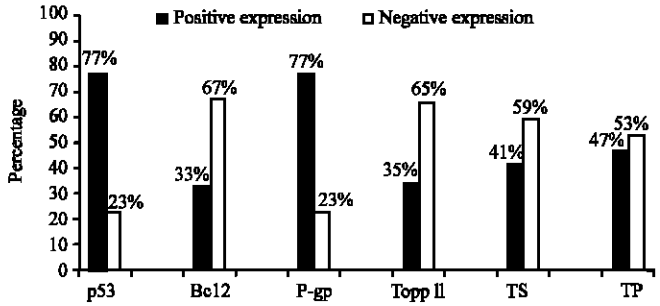

Fig. 2: Status of p53, Bcl-2, P-gp, Topo II, TS and TP expression in Colorectal carcinoma (n = 54)

Table 3: Lack of clinicopathological significance of p53 in colorectal carcinoma (N = 54)

Variable	No. of patients	P53(+) n = 41 (75.9%)	P53(-) n = 13 (24.1%)	p-value
Age				
Over 55	28 (51.85%)	21(38.8%)	7 (12.96%)	0.721
Under 55	26 (48.14%)	20 (37.07%)	6 (11.1%)	
Tumor location				
Colon	35 (64.8%)	25 (46.3%)	10 (18.51%)	0.610
Rectum	19 (35.2%)	16 (29.6%)	3 (5.55%)	
Tumor size				
Over 5 cm	31 (57.4%)	23 (42.6%)	8 (14.8%)	0.877
Under 5 cm	23 (42.59%)	18 (33.3%)	5 (9.25%)	
Histological grade				
WandM[1]	47 (87%)	37 (68.51%)	10 (18.51%)	0.245
PandU[2]	7 (13%)	4 (7.4%)	3 (5.55%)	
Lymph node involvement				
Positive	30 (55.6%)	18	12	0.5
Negative	24 (44.4%)	23	1	
Metastasis				
Positive	12(22.2%)	9 (16.7%)	3 (5.5%)	0.790
Negative	42 (77.8%)	32 (59.2%)	10 (18.6%)	
Tumor pathological stage				
A	3 (5.6%)	3	0	0.894
B1	3 (5.6%)	2	1	
B2	24 (44.4%)	18	6	
C1	2 (3.7%)	1	2	
C2	10 (18.5%)	8	3	
D	12 (22.2%)	9	13	

Table 4: Relationships between p53 and other markers in colorectal carcinoma patients (N = 54)

Tumor markers	P53(+) n = 41	P53(-) n =13	p-value
Bcl-2(+) (n =18)	12/54,22.2%	6/54,11.1%	0.021*
Bcl-2(-) (n = 36)	29/54,53.7%	7/54,18.91%	
P-gp(+) (n = 39)	30/51,58.8%	9/51,17.65%	0.459
P-gp(-) (n = 12)	9/51,17.65%	3/51,5.9%%	
Topo II(+) (n = 39)	13/51,25.5%	7/51,13.7%	0.375
Topo II(-) (n = 12)	26/51,51%	5/51,9.8%	
TS(+) (n = 41)	16/53,30.2%	7/53,13.2%	0.715
TS(-) (n = 12)	25/53,47.17%	5/53,9.43%	
TP(+) (n = 24)	18/51,35.3%	6/51,11.75%	0.873
TP(-) (n = 27)	21/51,41.2%	6/51,11.75%	

patients had coexpression of p53 and P-gp but no statistical significance was recorded between them (Table 4).

P53 and Topo isomerase II alpha: Tumors were classified to four immunophenotypes a) p53+/Topo II+ (13/52, 25%), b) p53+/Topo- (26/52, 50%), c) p53-/Topo II+ (5/52, 10%) and d) p53 -/Topo II-(8/52, 15%). Among $p53^+$ tumors, the incidence of Topo II negative expression (26/39, 66.7%) was significantly higher than Topo II positive expression (13/39, 33.3%) but no statistical significance was recorded between them.

P53 and Thymidylate Synthase (TS): Tumors were classified to four immunophenotypes a) p53+/TS+ (14/51, 27%), b) p53+/TS- (25/51, 49%), c) p53-/TS+ (7/51, 14%) and d) p53 -/TS- (5/51, 10%). Among $p53^+$ tumors, the prevalence of TS negative expression (25/39, 64%) was higher than TS positive expression (14/39, 36%) but no statistical significancy was recorded between them (Table 4).

P53 and Thymidine Phosphorylase (TP): Tumors were classified to four immunophenotypes a) p53+/TP+ (18/51, 35%), b) p53+/TP- (21/51, 41%), c) p53-/TP+ (6/51, 12%) and d) p53 -/TP- (6/51, 12%). Among TP- tumors, the incidence of p53 positive expression (21/27, 78%) was higher than p53 negative expression (6/27,22%) but no statistical significance was recorded between them (Table 4).

Clinicopathological significance of immunophenotypes: Primarily the clinicopathological significance of significant immunophenotype and then other immunophenotypes in each group were evaluated.

P53 and Bcl-2: There was a significant correlation between p53/Bcl-2 coexpression and age (p = 0.021).That means the mean age of patients with coexpression of p53 and Bcl-2 was 63.5±10.1041 which is comparable with other groups with mean age of 52.3±15.257. Other immunophenotypes were also evaluated without any correlation with clinicopathological data.

P53 and P-gp: Neither p53+/P-gp+ expression as the most prevalent immunophenotype nor other immunophenotypes were associated with clinicopathological findings.

P53 and Topo isomerase II alpha: Neither p53+/Topo II- expression as the main immunophenotype nor other immunophenotypes were associated with clinicopathological findings.

P53 and Thymidylate Synthase (TS): Neither p53+/TS- expression as the main immunophenotype nor other immunophenotypes were associated with clinicopathological findings.

P53 and Thymidine Phosphorylase (TP): There was a significant correlation between p53/TP coexpression and sex ($p = 0.022$). That means, out of 18 (35.3%) patients with coexpression of p53 and TP, 12/18 (66.7%) were males. As we previously reported (Rubin and Farber, 1998), TP immunostaining were also correlated with sex of patients ($p = 0.002$). The incidence of TP negative immunostaining was also significantly higher in females than males (74% versus 26%, respectively) and the incidence of TP positive staining was significantly higher in males than females (66.6% versus 33.3%, respectively).

DISCUSSION

We used immunohistochemistry as a powerful method (Jin *et al*., 2004) to detect the tissue expression of p53 and to analyze the clinicopathological significance of p53 as well as its association with other markers. Overexpression of altered p53 has been detected in 50-80% of colorectal carcinomas and has been suggested to be associated with a more rapid disease progression (Jessup *et al*., 1998). We included tumor samples from 54 colorectal cancer patients and showed p53 expression in 77% of patients. In other studies, including the largest one that included 995 patients with Duke's stage B or C colorectal cancer who were not receiving adjuvant chemotherapy, p53 mutation or overexpression of p53 was not diagnosed as a strong prognostic factor (Soong *et al*., 2000). The present data confirm this, for the first time among Iranian patients. Although p53 could not be considered as a prognostic factor in colorectal by itself but it affects many different pathways (Buglioni, 1999; Saleh, 2000; Manne, 1997) and expression of certain other genes (Anwar, 2004; Pomp, 1998). Tumors that expressed both Bcl-2 and mutant p53 had the lowest rate apoptosis overall (Jin, 2004) and worse prognosis (Soong, 2000). This phenomenon of $p53^+/Bcl\text{-}2^+$ immunophenotype was seen in 12/54 cases in this study. On the other side it has been suggested[15] that loss of Bcl-2 could be associated with an increased risk of death. Among our $p53^+$ tumors ($n = 41$), the incidence of Bcl-2 negative expression (71%) was significantly higher than Bcl-2 positive expression (29%).

Although this dominant immunophenotype was not associated with poor prognosis, it seems that the role of Bcl-2 as a prognostic factor is unclear and its evaluation with chemotherapy outcome and survival needs further analysis. Although more than half of the patients had coexpression of p53 and P-gp it was not considered as a significant immunophenotype among other related immunophenotypes but the clinical value of this immunophenotype requires further studies. Topo-mediated resistance could be caused by altered or reduced topoisomerase enzyme activity (Matsumoto, 2001) and reduced protein expression can be one of the mechanisms leading to reduced Topo II activity. Topoisomerase II-inhibiting antineoplastic agents are among the most effective antineoplastic drugs currently available. Among $p53^+$ tumors, the incidence of Topo II negative expression (66.7%) was higher than Topo II positive expression (33.3%). It seems that the p53 mutation could affect Topo II negative expression and may significantly affect successful chemotherapy in such patients. The same effect was observed in TS and p53 association. Among $p53^+$ tumors, the incidence of TS negative expression (64%) was higher than TS positive expression (36%). TS has been of interest as a target for 5-flurouracil, capecitabine and several new antifolate agents that are under clinical development (Aschele 2000). As p53 overexpression could affect the expression pattern of TS, the indirect role of p53 in chemotherapy outcome with this group of chemotherapy drugs is suggested. As we reported previously, male hormones can induce TP expression in colorectal carcinoma with larger size and male sex (Arbabi Bidgoli, 2005). The incidence of p53 positive expression (78%) was significantly higher than p53 negative expression (22%) among TP negative tumors; therefore a possible role of angiogenesis and sex on the expression patterns of p53 could be suggested. The present results suggest that p53 protein, which is over expressed in colorectal adenocarcinomas, could affect the expression of other critical proteins in chemotherapy regimens. P53 expression is an important step in tumor progression and metastasis. Different subgroups of patients and several immunophenotypes may show us the critical role of p53 mutation as an early clinical event. The pretreatment information given by molecular markers may prove useful in the design of multi disciplinary treatment strategies for colorectal cancer. Ours was a relatively small study. Larger studies with clear outcome, chemotherapy response and disease-free survival are needed to investigate the exact role of the p53 tumor suppressor gene in the expression of other molecular markers and to evaluate its role as a predictive or prognostic marker in colorectal adenocarcinoma.

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