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Influence of the c-erb B-2, nm23, bcl-2 and p53 protein markers on colorectal cancer

C-erb B-2, nm23, bcl-2 ve p53 protein belirteçlerinin kolorektal kanser üzerine olan etkileri

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Background/aims: *Under stringent intra-laboratory conditions we evaluated the relationship between the expression of four protein markers and clinicopathologic characteristics of colorectal tumors.* ***Methods:*** *124 patients with colorectal cancer from 1999 to 2002 were assessed.* ***Results:*** *The expression of c-erb B-2, nm23 and p53 was mostly determined in tumors located in the rectum. However, about 20% of the rectal lesions had bcl-2 expression. p53 and c-erb B-2 expression was significantly demonstrated in the lesions with vascular and lymph node involvement. However, the difference between the markers and staging was not statistically significant (p=0.388, p=0.301). C-erb B-2 and p53 were more frequently expressed in the patients with large tumors (more than 5 cm) with moderate and poor differentiation grade. About half of the tumors expressing c- erb B-2 and p53 had vascular invasion and more than 70% had N1 and N2 lymphatic invasion as well. In the patients with tumors expressing c-erb B-2 and p53, recurrences often occurred and both disease-free survival (DFS) and overall survival (OS) in the first two years after surgery were shorter than of the patients with tumors expressing nm23 and bcl-2.* ***Conclusion:*** *In this study, c-erb B-2 and p53 were frequently expressed in the Astler-Coller stage C large tumors located in the rectum and a high degree of vascular and lymphatic invasion was observed. In the patients with tumors expressing c-erb B-2 and p53, recurrences were determined more frequently and DFS and OS were shorter than in patients with tumors expressing nm23 and bcl-2. Thus, two different protein markers should be taken into consideration when evaluating the clinical outcome of patients with colorectal cancer.*

Key words: Colorectal cancer, carcinogenesis, oncoproteins, c-erb B family

Amaç: *Kolorektal kanserli hastalarda prognostik değeri olan belirli klinikopatolojik özellikler ile c-erb B-2, nm23, bcl-2 ve p53 protein belirteçleri uygun laboratuvar şartları altında karşılaştırıldı.* ***Yöntem:*** *1999-2002 tarihleri arasında 124 kolorektal kanserli hasta çalışmaya alındı.* ***Bulgular:*** *C-erb B-2, nm23, p53 varlığı gösteren tümörlerin sıklıkla rektumda yerleşim gösterenler olduğu görüldü. Ama rektal lezyonların ancak %20 sinde bcl-2 görülmüştü. p53 ve c-erb B-2, damarsal ve lenfatik nod yayılımı gösteren tümörlerde daha fazla bulundu. Bu protein belirteçler ile tumor evreleri arasında bulunan fark ise istatistiksel olarak anlamlı değildi (p=0.388, p=0.301). C-erb B2 ve p 53 sıklıkla hem 5 cm'den büyük hemde orta ve kötü diferansiyasyon derecesi gösteren tümörlerde gösterildi. C-erb b-2 ve p53 gösteren tümörlerin yaklaşık yarısı damarsal yayılım gösterirken bunların %70'inden fazlası ise lenfatik yayılım göstermekteydi. Aynı zamanda bu belirteçleri gösteren tümörlerde rekürrens sık olarak gözlenirken, 2 yıllık hastalıksız yaşam ve tüm yaşam süreleri oransal olarak daha kısa bulundu.* ***Sonuç:*** *Çalışmada c-erb B-2 ve p53 ün sıklıkla Astler-Coller evrelemesine göre C evresinde bulunan, rektumda yerleşmiş ve diferansiyasyon derecesi orta ve kötü olan büyük tümörlerde bulunmaktadır ve bu tümörler de belirgin damarsal ve lenfatik yayılım göstermektedir. Bu durum aynı özellikli tümörlerde, rekürrensin ilk iki yıl içerisinde istatistiki olarak belirgin olmasa da görece sık olarak karşımıza çıkmasına ve hastalıksız yaşamın daha kısa olmasına neden olan bir etmen olarak belirmektedir.*

Anahtar kelimeler: Kolo-rektal kanser, onkoproteinler, karsinogenez, c-erb B ailesi

INTRODUCTION

Colorectal cancer (CRC) is the sixth most common cancer and persists as a significant cause of cancer mortality in Turkey (1). Prognosis remains poor despite the considerable number of cancer researches and depends on the stage of cancer at the time of presentation. Colorectal carcinogenesis is complex and probably matched by dependent genetic alterations. In recent decades researchers have tried to identify biological markers in order to individualize chemotherapy and predict clinical outcome and tumor sensitivity (2).

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Widespread genetic mutations in colorectal carcinogenesis exist on chromosomes 17, 18 and 5. As tumor-suppressor genes, p53, nm23 and proto-oncogene c-erb B-2 are located on these chromosomes (3). Rather few data exist for epithelium-derived tumors regarding bcl-2 protein. bcl-2 protein prolongs survival of a variety of cells by blocking apoptosis. Particularly in CRC, there have been few investigations regarding the role of bcl-2 (4, 5). p53 is a DNA binding cell cycle-regulating transcription factor and participates in the balance between cell survival and death. Losses, deletions, and mutations of p53 have been determined in the pathogenesis of a wide range of tumors including CRCs (4, 6). nm23 is a possible tumor metastasis suppressor gene. nm23-H1 and nm23-H2 are two different genes which encode nucleoside phosphate proteins, demonstrate approximately 90% amino acid sequence identity, and are involved in cell proliferation and differentiation, as well as basement membrane formation and growth arrest (7-9). C-erb B-2 proto-oncogene on chromosome 17q21 produces a transmembrane glycoprotein in the type I kinase receptor family which interferes in a number of cellular proliferations in normal circumstances (10, 11). Clinically, c-erb B-2 overexpression has been associated with poor prognosis in a number of breast, ovarian, gastric and CRCs (12-14), but it has not been recognized as a prognostic indicator in large studies.

In this study, we aimed to determine the frequency of overexpression of these four proteins and compared each protein as a marker in the clinicopathologic features in patients with stage B-C CRC.

MATERIALS AND METHODS

Archived paraffin-embedded samples were available for 124 of 132 patients in the database of our surgical clinic who underwent elective surgery for CRC from 1999 to 2002. Detailed pathologic data was retrospectively verified for each specimen embedded in paraffin block. Pathologic and clinical data [patient gender and age, site of primary tumor, degree of differentiation, size of tumor, stage according to Astler-Coller classification, and some clinical findings such as recurrence, disease-free survival (DFS) and overall survival (OS) in the follow-up period] were assessed (Table 1). Ninety-eight of 124 suitable patients were all alive with a median follow-up of 27 months (11-55 mos). Thirty-three patients (26.6%) had recurrence during the first two years of follow-up. Eight patients were lost to follow-up.

Table 1. Clinicopathologic characteristics of the patients enrolled

Patient characteristics		No. Pts.
Sex	Male	58
	Female	74
Age	<50	28
	51-60	49
	>61	55
Localization	Cecum & ascending	24
	Transverse colon	19
	Descending & sigmoid	53
	Rectum	36
Differentiation	Well	39
	Moderate	34
	Poor	59
Astler–Coller	B1	22
	B2	31
	C1	47
	C2	32

Histopathologic procedure and pathologic analysis entailed routine microscopic examination of the tumor, margins and lymph nodes (LNs). A single section was routinely performed. Slices were stained with hematoxylin-eosin (H&E). Slices from paraffin blocks were sectioned in multiple slices 4 microns thick and were also stained with H&E in the pathological evaluation. The formalin-fixed, paraffin-embedded specimens were examined immunohistochemically using a standard immunoperoxidase method using respective antibodies to c-erb B-2 (Clone CB11) (1:10 dilution; Biogenex Corporation, CA), nm23 (Clone 37.6) (1:50 dilution; Biogenex Corporation, CA), bcl-2 (Clone 100) (1:200 dilution; Biogenex Corporation, CA), and p53 (Clone Bp53-12-1) (1:40 dilution; Biogenex Corporation, CA). Positive controls were selected cases known to be positive for the primary antibody, such as breast carcinoma for nm23, c-erb B-2, and p53 and normal LN for bcl-2. Negative controls were stained with a nonspecific Ig G (normal rabbit Ig G) and Tris-buffered saline.

Quantitative Analysis

The degree of expression of these four protein markers was estimated by classifying according to two-grade scoring: 0=no staining, 1=focal and weak staining and 2=moderate and strong staining. Sections with grade 2 were considered positive (34). Sections with no or weak expression were considered negative for c-erb B-2 protein marker. For the other markers, defect in any cell structure was considered positive. Figures 1 through 4 demonstrate expression of the four markers in the cell.

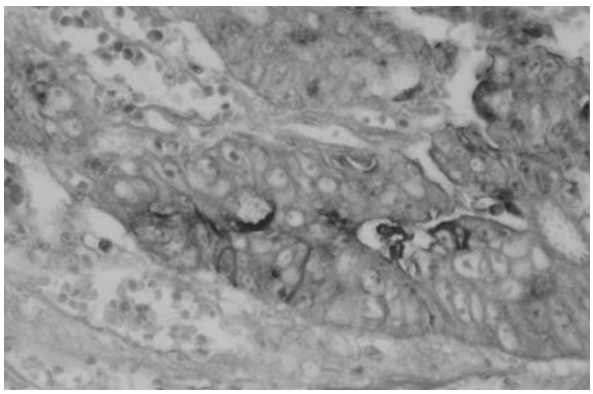

Figure 1. C-erb B-2 (membranous) expressed by intrahepatic cholangiocytes

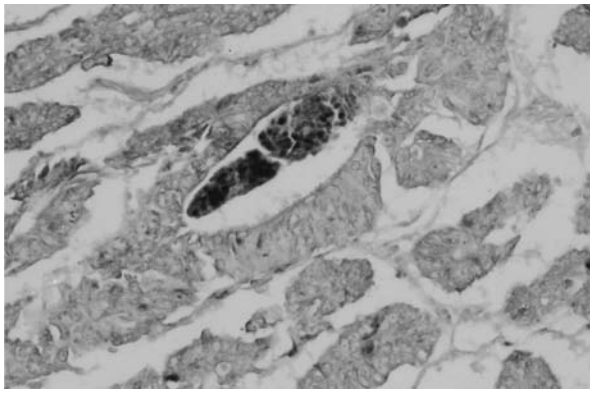

Figure 2. Cytoplasmic Bcl-2 expression following staining using intrahepatic cholangiocytes

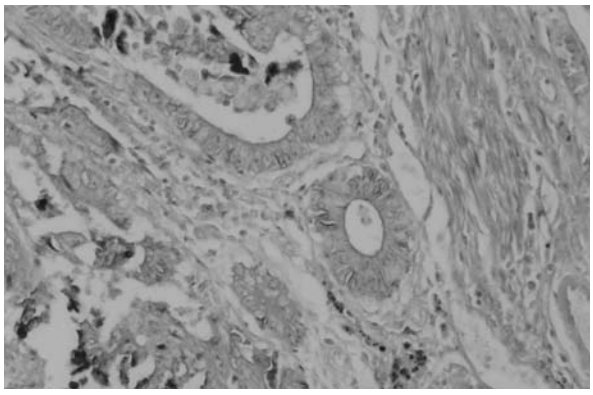

Figure 3. Nm-23 expressed in nucleus as demonstrated by staining with intrahepatic cholangiocytes

Statistical Analysis

Pearson's chi-square analysis was used for the variables. P values <0.05 were considered significant.

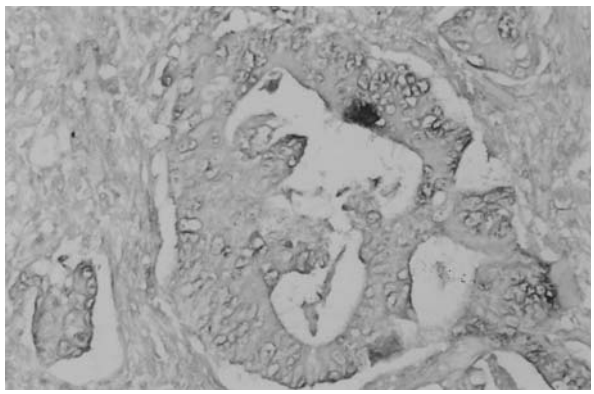

Figure 4. Demonstration of nuclear p53 expression

RESULTS

The relation of each protein marker to tumor size and localization, differentiation, staging, and vascular and lymphatic invasion is summarized in Table 2. In patients with CRC, c-erb B-2, nm23, bcl-2, and p53 were expressed at rates of 44.3%, 41.1%, 20.2% and 58.9%, respectively. In this series the size of tumors classified in three sequential subgroups were compared with the expression of c-erb B-2 and p53 (X^2=5.190, X^2=8.731, in general p<0.001). The majority of the patients had the tumor size of 5 cm or more. The expression of c-erb B-2, nm23 and p53 was mostly determined in the patients with tumor located in the rectum; only 20% of the tumors located in the rectum had bcl-2 expression. The relationship between the expression of protein markers and tumor localization was statistically significant (X^2=5.301, p=0.023). p53 and c-erb B-2 protein marker expression was significantly observed in the lesions located in the rectum with vascular and LN involvement (about 53-56% and 72-74%, respectively). Tumors with positive expression of c-erb B-2 and p53 protein markers were demonstrated more frequently in patients with Astler-Coller Stage C1 and C2 lesions, but the difference between the markers and staging was not statistically significant (X^2=1.591, p=0.207). In Table 2, it can be seen that c-erb B-2 and p53 protein markers were expressed in the patients with tumors with moderate and poor differentiation. The relation between the expression of these two protein markers and tumor differentiation was statistically different (X^2=16.697, p<0.001). As shown in Table 2, more than 50% of patients with tumors expressing c-erb B-2 and p53 had vascular invasion (Pearson X^2=6.200, p=0.013). Lymphatic invasion was frequently de-

Table 2. Comparison of the variables between the four protein markers and patient characteristics

		No. Pt. (n=124)	Tumor size (cm)			Location		Diff.			Stage (Astler-Coller) (n=41)				Vascular Invasion (n=62)		Lymphatic Invasion		p value M vs D M vs VI M vs S
			<1	1.1-5	>5	C	R	W	M	P	B1	B2	C1	C2	+	-	N1 (n=36)	N2 (n=11)	
C erb B2	+	55					31				4	5	27	19	22	6	32	11	<0.001
		44.3%	8	15	32	24	56.4%	5	19	31					53.5%		72.6%		=0.021
	-	69													8	5	p=0.388		X^2=5.190
Nm23	+	51					32				14	19	11	7	15	5	13	4	
		41.1%	19	23	9	19	62.7%	19	21	11					36.6%		27.4%		
	-	73													14	7			X^2=14.574
Bcl 2	+	25					5				16	7	2	1	5	17	4	2	
		20.2%	11	9	5	20	20%	17	6	2					12.2%		9.6%		
	-	99													8	11			X^2=8.569
P 53	+	73					47				6	13	21	33	23	3	35	11	<0.001 <0.001
		58.9%	7	24	42	26	64.4%	11	29	33					56.1%		74.2%		X^2=8.731
	-	51													13	2	p=0.301		X^2=37.063 <0.001

C: Entire colon except rectum, R: Rectum, Tumor differentiation, W: Well, M: Moderate, P: Poor, M vs D (Markers vs Differentiation), M vs VI (Markers vs. Vascular Invasion; M vs S (Markers vs Size)

monstrated, in 62 specimens. It was shown that in the tumors expressing c-erb B-2 and p53, N1 and N2 lymphatic invasion was observed in about 70%. Thirty-six of 47 patients had N1 and 11 had N2 lymphatic invasion according to the specimen reports. Moreover, tumors invading the vascular and lymphatic basin had no trace of expression of bcl-2 protein marker. Nevertheless, the variables were not measured as statistically significant (p=0.388, p=0.301). When recurrences were evaluated, 33 relapses (26.6%) were determined in the follow-up. Twenty of 33 recurrences occurred in patients with CRC expressing c-erb B-2 and p53. DFS in the first two years and OS during the follow-up period are shown in Table 3. The difference between the four tumor markers and DFS and OS was not statistically significant (X^2=3.526, p=0.060). We also made a comparison between postoperative complications and marker expression, as also seen in Table 3. In this series, early postoperative complication ratio was 13.7%.

DISCUSSION

Our aim was to review the significance of the combined expression of four protein markers believed to play a vital role in the carcinogenesis of CRC. When considering the four markers separately, both c-erb B-2 and p53 protein marker expression was positively related to vascular invasion and tumor size in general. In other words, patients with tumors expressing nm23 and bcl-2 had an estimated low probability of having adjacent tissue invasion. In this study, nm23-H1 was expressed in 41.1% of patients with CRC stage B and C. Moreover, in the patients with stage B and C, tumors more than 5 cm in size expressing both c-erb B-2 and p53 were mostly observed (p<0.001). But we were unable to determine a positive relationship between those protein markers and lymphatic invasion. Campo and Tannapfel in their studies (16,17) stated that nm23-H1 protein was expressed at rates from 35 to 45% in late-staged tumors with poor differentiation. Messinetti (18) reported expression of nm23-H1 protein as 73% in 41 enrolled patients in the study, but no significant relation could be determined between nm23-H1 protein expression and stage, size differentiation, vascular and perineural invasion, localization and aggressiveness of the tumor. Dusonchet et al. (19) reported that no association was observed between

Table 3. Expression of protein markers in relation with PO morbidity and disease-free and overall survival

		C-erb B-2 N=55	Nm23 N=51	Bcl-2 N=25	P53 N=73	Overall N=124
	Pulmonary Complication	1	3	2	4	8.1%
Early PO morbidity	Leakage	1		3	2	4.8%
	Mechanical Bowel Obstruction				1	0.8%
Involved surgical margin		2	5	5	14	26
		(3.6%)	(9%)	(20%)	(19.2%)	(20%)
Recurrence in 2 years		7 3	3 2	1 4	9 4	26.6%
Disease free survival in 2 years		88.7%	94.4%	99.2%	87%	67.4%
Overall survival (about 27 months) (N=exitus in months)		N=9 92.7%	N=2 98.4%	N=3 97.5%	N=12 90%	N=2679%

PO: Postoperative

nm23-H1 protein expression and clinicopathological features and survival of patients with either moderate or strong nm23-H1 expression. They also stated that the results indicate that nm23-H1 activity is tissue-specific, and that in CRCs the expression of the protein is not associated with tumor progression or patient prognosis. Dursun et al. (20) stated that the well-differentiated adeno-CRC showed strong expression of nm23-H1, yet advanced tumor stages were associated with reduced nm23-H1 expression. They also noticed an inverse correlation with angio-lymphatic invasion, nodal metastasis and liver metastasis. In this study, tumors expressed nm23 in 41.1% of patients with CRC mostly located in the rectum. The tumors more often had well and moderate differentiation and were classified in stage B CRC; less vascular and lymphatic invasion was observed than in patients with tumors expressing c-erb B-2 and p53 protein markers.

Some authors, like Lee, Osako, Nakae (21-23), have reported a relationship between c-erb B-2 protein marker expression and histopathologic differentiation and staging. Kay et al. (24) also noticed a correlation between c-erb B-2 gene and poor prognosis of CRC like in breast, ovarian and stomach cancers. Yang (25) in his study determined that metastasizing CRC and liver metastases expressed the c-erb B-2 protein marker. All authors have reported expression of c-erb B-2 as varying from 11 to 95%, and associated with the advanced stage of cancer and the prediction of metastases. We determined the expression of the c-erb B-2 protein marker as 44.3% for the tumors mostly located in the rectum and more than 5 cm in size. The significant overexpression was consistent with histological differentiation and vascular invasion. The tumors expressing c-erb B-2 demonstrated a higher percentage of N1 and N2 lymphatic invasion, though the variables were not statistically significant (Table 2). Considering the relationship between behavior of the tumor expressing c-erb B-2 and recurrences, DFS and OS, recurrences were seen in nine patients (27.3%) of 33; 88.7% of the patients had DFS in two years; and about 92% of patients with CRC expressing c-erb B-2 were alive during the follow-up, which covered a reasonable (27 months) time period.

Mutations in p53, a tumor suppressor gene located on chromosome 17p, are the most frequent genetic alterations found in human cancers. Increased intracellular concentration of p53 has been projected to be associated with poor prognosis in some tumor types. In CRC, this significance is still a matter of debate (26). Diez (27) reported that p53 expression was more common in distal than in proximal tumors. p53 positivity and distal localization are significantly correlated with high recurrence. p53 exhibited different prognostic values in the distal versus proximal colon. Tumors located in the rectum have loss of heterozygosity (LOH)+ phenotype, but the replication error (RER) + phenotype is rare (28, 29). Although Sauter (30) highlighted the suggestion that p53 expression may serve as a marker of improved survival in patients with upper gastrointestinal tract tumors, in the study performed by Kinki Cooperative Study Group of Chemotherapy for Colorectal Carcinoma (KCSGCCC), Tomita et al. (31) emphasized that p53 LOH was positive in 40% of cases and they exposed it as having a significant association with left-sided localization and histology (well to moderate differentiation). When we assessed p53 protein marker expression, the majority of tumors were located in the rectum (64.4%), more than 5 cm in size, had dreadful histology with moderate to poor differentiation and were Astler-Coller C2 stage. Although the relationship between staging and p53 expression was not significant, a statistical difference between tumor size and p53 expression

was determined. The tumor expressing p53 demonstrated significant vascular invasion. Although 74.2% of cases with p53 expression had invasion to LNs, the relation was not statistically significant in that series (p=0.301). In this series, we found 11 patients (33%) of 33 had recurrences in the first two years after the surgical procedure. In the same time period, 87% of patients with CRC were disease-free. During 27 months when OS was evaluated, 90% of the patients were living.

bcl-2 protein marker expression was determined in 20.2% of cases. In the literature, bcl-2 has been frequently investigated in adenomas and was expressed at rates from 5 to 55% of tumors, with poor prognosis, which was not related to advanced-staged tumors (32). Pereira (33) stated that there was no correlation between bcl-2 expression and staging, grading and vascular invasion. Bossari (5) in his study stressed that bcl-2 protein marker expression played a role in the early colorectal carcinogenesis but had no prognostic value. In our study, we did not find any significant characteristics regarding expression of the bcl-2 protein marker predicting prognosis.

Regarding the two different carcinogenetic pathways for the rectum and colon, expression of some proteins may indicate characteristics of the tumors like stage, aggressiveness and invasion to adjacent tissue and lymphatic basin. Some authors have worked on several protein markers to define the tumor progression and the patient's survival. We found that c-erb B-2 and p53 were frequently expressed in the Astler-Coller stage C tumors, which were usually more than 5 cm in size, located in the rectum, and had a high degree of vascular and lymphatic invasion. Moreover, when recurrence was evaluated, it was observed more frequently in the patients with CRC expressing c-erb B-2 and p53 (21/26 patients, 81%). DFS and OS of these patients were shorter than in the patients with tumors expressing nm23 and bcl-2. Thus, two different protein markers should be taken into consideration when evaluating the clinical outcome of patients with CRC.

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