Analysis of p53 Gene Polymorphisms and Protein Over-expression in Patients with Breast Cancer

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Clinical Significance of *p53*, K-*ras* and *DCC* Gene Alterations in the Stage I-II Colorectal Cancers

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Abstract

Background and aims. Genetic alterations of *p53*, K-*ras* and *DCC* genes have a pivotal role in the colorectal cancer progression. The aim of this study was to clarify the association between K-*ras* mutations, p53 aberrations and *DCC* loss of heterozygosity (LOH), with the patient outcome and tumor characteristics in 43 stage I-II colorectal cancer patients. **Methods.** Mutations in exons 5-8 of the *p53* gene and codon 12 and/or 13 of the K-*ras* gene were assayed by PCR-SSCP and then confirmed by DNA sequencing. *DCC* LOH was studied by PCR-RFLP, while p53 immunohistochemistry was also made. **Results.** Mutations of the *p53* gene were found in 14 (32.5%) tumors. Five (12%) cases showed mutation of the K-*ras* gene. Nuclear staining of p53 was found in 22 (51 %) cases. *DCC* LOH was found in 5 (12%) cases. Cases with guanine to thymine substitution that occurred in K-*ras* codon 12 and *DCC* LOH were found to be more aggressive than other cases with codon 12 mutations or *DCC* wild-type phenotype. Many tumors with p53 over-expression were localized on the left side of the colon (p=0.005). The stage of the tumor was higher in patients who died during the follow-up period, when compared to the ones who have survived. **Conclusions.** Although none of these genetic alterations showed a significant prognostic value, specific mutation of K-*ras* gene and *DCC* LOH phenotype might have a predictive prognostic implication in colorectal cancer. Furthermore, different etiopathogenetic mechanisms might be involved in the tumorigenesis of the left and right colon.

Key words

Colorectal cancer - p53 - K-*ras* - *DCC* LOH - prognosis

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Introduction

The development of colorectal cancer is a multi-step process, which can arise from accumulation of mutations in oncogenes, tumor suppressor genes or from epigenetic changes in DNA. Fearon and Vogelstein proposed a genetic model describing the transition from healthy mucosa to carcinoma and identified a number of key oncogenes and tumor suppressor genes (1). Recent progress in molecular biology has shown that there are different alternative pathways in colorectal carcinogenesis and also cross talk among these pathways (2,3). The heterogeneous nature of the genetic alterations determines the clinical feature of the patients and discloses new clinical implications.

The *p53* gene resides on chromosome 17p and encodes a protein, which maintains genomic integrity by inducing cell cycle arrest and apoptosis following DNA damage (4,5). Approximately half of all colorectal cancers show *p53* gene mutations that were proposed as a late event in the transition of an adenoma to carcinoma (6). Mutation of *p53* is thought to increase the protein half-life and is often associated with overexpression in the nucleus (7). Kato et al (8) have generated all possible mutations in p53 coding sequence by site-directed mutagenesis and interpreted their functions and structures in experimental studies. Functional definition of the *p53* mutations can provide a better understanding of its relationship with diseases. Some studies have reported that the presence of *p53* aberrations in colorectal cancer indicates a relatively poor prognosis (9), while others failed to show such a relationship (10).

The *ras* gene family consists of three homologous genes, *K*-, *H*-, and *N*-ras, which encode similar 21-kD protein (p21ras) involved in G protein-mediated signal transduction (11). *Ras* gene mutations have been found frequently in colorectal cancer especially in the *K-ras* gene (12). *K-ras* is activated most commonly by point mutations in codon 12, 13 or 61. These changes in *K-ras* lead to increased and uncontrolled cell proliferation and malignant transformation. Although different types of mutations in the gene were found to have a predictive value in the clinical outcome of colorectal cancer (13-17), some studies did not support this (18).

The *deleted in colorectal cancer* (*DCC*) gene is located on chromosome 18q21.2 and encodes a neural cell adhesion-like transmembrane protein that has roles in regulating cell survival. The loss/reduction of DCC expression represents a selective advantage for tumor development. Although some studies have demonstrated that the loss of heterozygosity (LOH) and lack of mRNA or protein expression of the gene are related to poor differentiation, metastases and poor prognosis in colorectal cancer (19-21), others did not find any relation of DCC protein expression with colonic tumor progression (22).

The purpose of this study was to evaluate the associations between *K-ras* mutations, p53 aberrations and *DCC* LOH status and the patient outcome and tumor characteristics in early stage colorectal cancer patients. In addition, we investigated the relationship between observed mutations in the *p53* gene and the overexpression of the p53 protein, detected by immunohistochemical (IHC) analysis.

Material and methods

Patients

Tissue specimens from 43 sporadic colorectal cancers were obtained with consent from patients who underwent curative surgical resection from 1990 to 2002 at the Marmara University Hospital Department of General Surgery. The median follow-up time was 76 months (ranges 6-168). No patient received postoperative adjuvant therapy. The patient group included 24 women and 19 men with ages ranging from 42 to 86 years (median 64 years). The anatomical distribution of the tumor was as follows: right bowel (cecum, appendix, ascending colon, hepatic flexure, transverse colon and splenic flexure) and left bowel (descending colon, sigmoid colon and rectosigmoid junction and rectum) defined by ICD-C-2 codes. Overall, 33 tumors were localized in the colon and 10 in the rectum. The study protocol was approved by the Marmara University Faculty of Medicine Research Ethics Committee.

Specimen handling and DNA extraction

Archival materials were reviewed by an experienced pathologist, to select a representative tumor block and a surrounding normal mucosal tissue block for each case. All the tumors were adenocarcinomas staged as stage I-II according to the TNM system of UICC. The distribution of cases using WHO grading system was as follows: 8 low, 27 moderate, and 8 high-grade adenocarcinomas.

DNA analysis and IHC were performed on the same selected samples, 10% formalin-fixed-paraffin-embedded tissue blocks. Genomic DNA was extracted from stored tissues using a method described previously (23).

Screening for K-ras and p53 mutations

The region containing codons 12 and 13 of *K-ras* gene and exons (5,6,7, and 8) of the *p53* gene were amplified using specific oligonucleotide primers. After 35 cycles of PCR, the products analyzed by 1.2% agarose gel electrophoresis.

Single-stranded DNA for SSCP analysis was produced by combining 3 μL PCR product and 18 μL formamide loading buffer (95% formamide, 10 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and heating at 95şC for 10 minutes. Silver staining was conducted by use of a modification of the method described previously (24).

Samples identified as mutated by screening for *p53* and *K-ras* mutations were subject to automated sequencing by ABI PRISM 310 (Applied Biosystem) and analyzed with Sequencing Analysis software programs.

Immunohistochemistry

Immunohistochemical stain was performed by the avidin-biotin-peroxidase technique using DO-7 monoclonal antibody (Lab Vision) as primary and diaminobenzidine (DAB) as the chromogen, according to manufacturers' instructions, running in parallel with the known positive and negative controls. p53 protein immunoexpression was evaluated by counting the number of stained nuclei in at least 500 tumor cells in five different tumor fields. If the percentage of positive tumor nuclei to the total number of counted tumor nuclei was 10% or more, the slide was scored as positive. If 10% or less of the nuclei were stained, the slide was scored as negative. The staining was evaluated by two observers simultaneously and a consensus was reached for each sample.

DCC LOH analysis

Two different (M2 and M3) *Msp*I polymorphic sites in the intron 5 of the gene were used for the analysis of *DCC* LOH. After these two regions were amplified by PCR, *DCC* LOH was evaluated using RFLP. For RFLP analysis 10 μL PCR product was digested overnight with 10 U of MspI enzyme in a volume of 20 μL. Then the samples were run on 2% agarose gel electrophoresis. Normal DNA from heterozygous patients showed three bands at M2 polymorphic: a 367 bp "uncut" band and two "cut" bands (227 and 140 bp); at the M3 site: a 240 bp "uncut" band and two "cut" bands (137 and 103 bp). DCC M2 or M3 LOH was demonstrated when the tumor DNA showed loss of either the single "uncut" band or of the "cut" bands. A positive LOH of *DCC* was judged by LOH at one or any combination of these two sites. When the normal DNA showed homozygote, it was described non-informative on this locus.

Outcomes

The primary outcome measures were the incidence of each gene alteration and the relation between presence of each gene alteration and the rate of disease relapse and death rate in the studied patients. Secondary outcome measure was the relationship between demographic features, as well as conventional histologic parameters, including p53 over-expression and disease outcomes (relapse and death rate).

Statistical analysis

Patients were assessed according to their disease relapse and overall survival. Initial analysis was made between the groups of patients who had disease relapse (locoregional or distant) and those without any relapse. Secondly, assessment was done between patients who survived and the ones who died before or during the study time.

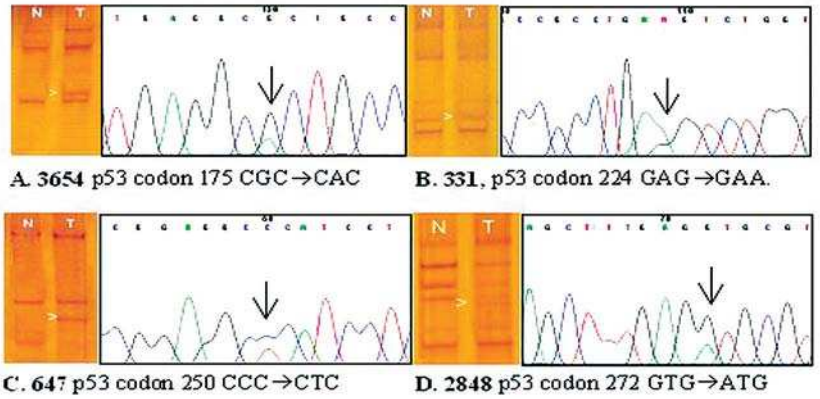

Fig.1 Analysis of p53 mutations by SSCP and sequencing. **A.** Exon 5; **B.** Exon 6; **C.** Exon 7; **D.** Exon 8 samples. Arrows show mutant bands (N, normal; T, tumor tissue.

Sample size calculation for the study was assessed for relapse rates, as it is the major outcome of the study. Briefly, a 25% of relapse rate in patients without any gene mutation was assumed to occur in 40% in patients with mutation. Therefore, a 15% of difference between two study groups with an alpha error level of 5% and a beta error level of 50% required 53 patients/samples in each study group.

In univariate analysis, all categorical variables such as gender and age of the patients (>70 vs <70), location of the tumor (left or right sided), histological grade (I, II or III), p53 protein overexpression (negative or positive), *p53* and *K-ras* mutation (absent or present) and *DCC* LOH (negative or positive) were compared using Pearson, chi-squared or Fisher's exact tests whenever appropriate. Results were considered statistically significant when two-sided *p* value was less than 0.05. Odds ratio calculation with 95% confidence intervals (CI) was done only for 2x2 tables. Independent multivariate analysis based on logistic regression was performed for multiple comparisons. Cancer-specific survival curves were constructed using the Kaplan-Meier method and differences between curves were evaluated using the log-rank test.

Results

p53 analysis

The *p53* mutational status for 43 colorectal cancers was determined by SSCP analysis, followed by sequencing of aberrant bands (Fig.1). Mutations were detected in 14 (32.5 %) samples; 3 silent mutations in 3 cases, and 17 missense mutations in 12 cases have been found. Twenty genetic changes were found and the distribution of these mutations is summarized in Table I. Among all mutations, the most common types were transition (85%) and missense (85%) mutations. We checked our mutations by using MUT-TP53 program (25). The rate of disease relapse and death was not different between patients with wild or mutant *p53* gene (0.92 95% 0.60-1.40; *p*=1.0 or 1.39 95% 0.97-2.00; *p*=0.24 respectively) (Table II, and III). Our results also demonstrated a codon 213 CGA → CGG polymorphysm in 3 cases with a frequency of 7%.

Overexpression of p53 protein was found in 22 (51%) colorectal cancer patients. There was a concordance in the results of the DNA sequencing and the IHC analysis in 30 (70%) patients. Three patients with one silent and two

Table I *p53* aberrations among 43 patients with stage I-II colorectal cancer

Patient No	Exon/ Codon	Mutation	Aminacid changes	Activity	IHC (DO7)
566	5/154	GGC → GGT	-	unknown	+
3007	5/163	TAC → AAC	Tyr → Asn	11,38	+
2079	5/175	CGC → CAC	Arg → His	12,41	+
	5/181	CGC → TGC	Arg → Cys	26,10	
	8/273	CGT → TGT	Arg → Cys	0,91	
	8/283	CGC → CAC	Arg → His	0,46	
3654	5/175	CGC → CAC	Arg → His	12,41	+
5367	5/175	CGC → CAC	Arg → His	12,41	+
2729	5/177	CCC → CTC	Pro → Leu	15,13	-
5195	6/205	TAT → CAT	Tyr → His	6,44	+
	6/224	GAG → GAA	-	unknown	
2848	8/261	AGT→ AGG	Ser → Arg	59,13	+
	8/272	GTG → ATG	Val → Met	8,79	
331	6/224	GAG → GAA	-	unknown	+
752	7/245	GGC → AGC	Gly → Ser	0	-
2640	7/245	GGC → AGC	Gly → Ser	0	+
647	7/250	CCC → CTC	Pro → Leu	0	+
2416	7/254	ATC → ACC	Ile → Thr	0,85	+
1619	8/261	AGT→ AGG	Ser → Arg	59,13	+
	8/282	CGG → TGG	Arg → Trp	0,55	

Table II The variables in patients with and without relapse

Variables	Patients with relapse n=14	Patients without relapse n=29	OR (95% CI)*	p
Gender; n (%)			1.09 (0.64-1.86)	1.0
Female	8 (57)	18 (62)		
Male	6 (43)	11 (38)		
Age n (%)			1.01 (0.68-1.51)	1.0
<70 years	10 (71)	21 (72)		
≥70 years	4 (29)	8 (28)		
Stage n (%)			0.87 (0.36-2.11)	1.0
I	5 (36)	9 (31)		
II	9 (64)	20 (69)		
Differentiation n (%)				0.49
Grade I	4 (29)	4 (14)		
Grade II	8 (57)	19 (66)		
Grade III	2 (14)	6 (20)		
Localization n (%)			2.41 (0.61-9.58)	0.28
Right	2 (14)	10 (34)		
Left	12 (86)	19 (66)		
p53 IHC** n (%)			0.97 (0.51-1.84)	1.0
negative	7 (50)	14 (48)		
positive	7 (50)	15 (52)		
p53 mut*** n (%)				
absent	10 (71)	19 (66)		
present	4 (29)	10 (34)		
k-ras mut*** n (%)				
absent	12 (86)	26 (90)		
present	2 (14)	3 (10)		
DCC LOH****n (%)			1.17 (0.7-2.78)	0.60
negative	5 (71)	15 (83)		
positive	2 (29)	3 (17)		

* Odds Ratio (95% Confidence Interval); **Immunehistochemistry; ***Mutation;
****Data from 25 available patients

Table III The variables in patients who survived and who did not survive

Variables	Patients survived n=35	Patients not survived n=8	OR (95% CI)*	p
Gender; n (%)			0.80 (0.38-1.67)	0.69
Female	22 (63)	4 (50)		
Male	13 (37)	4 (50)		
Age n (%)			0.84 (0.48-1.49)	0.67
<70 years	26 (74)	5 (63)		
≥70 years	9 (26)	3 (37)		
Stage n (%)			1.67 (1.27-2.18)	0.04
I	14 (40)	0		
II	21 (60)	8 (100)		
Differentiation n (%)				0.81
Grade I	7 (20)	1 (12)		
Grade II	22 (63)	5 (63)		
Grade III	6 (17)	2 (25)		
Localization n (%)			0.40 (0.06-2.65)	0.41
Right	11 (31)	1 (13)		
Left	24 (69)	7 (87)		
p53 IHC** n (%)			1.03 (0.48-2.23)	1.0
negative	17 (49)	4 (50)		
positive	18 (51)	4 (50)		
p53 mut*** n (%)			1.39 (0.97-2.00)	0.24
absent	22 (63)	7 (88)		
present	13 (37)	1 (12)		
k-ras mut*** n (%)			0.99 (0.74-1.32)	1.0
absent	31 (89)	7 (88)		
present	4 (11)	1 (12)		
DCC LOH****n (%)			0.58 (0.22-1.58)	0.17
negative	18 (86)	2 (50)		
positive	3 (14)	2 (50)		

* Odds Ratio (95% Confidence Interval); **Immunehistochemistry; ***Mutation;
****Data from 25 available patients

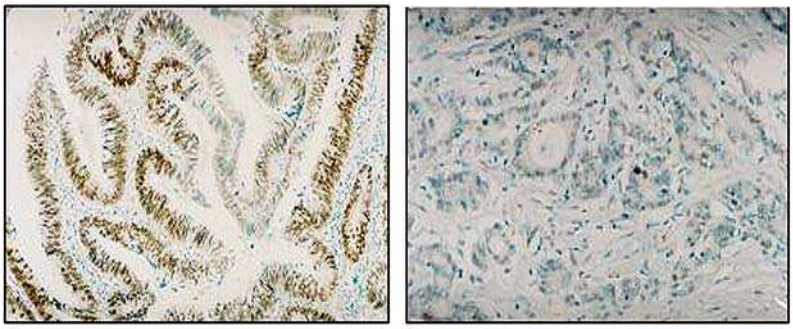

Fig.2 Immunohistochemical staining for p53 protein in colon tissues from patients with stage I-II colorectal cancer. **A**. Strong staining for p53 protein in a well differentiated adenocarcinoma, **B.** Weak p53 immunostaining in a moderately differentiated adenocarcinoma (original magnification X20).

missense mutations were positive by DNA sequencing but negative by IHC analysis. Twelve patients were positive by both DNA sequencing and IHC analysis. Fig.2 shows an example of immunohistochemical staining for p53 protein. The rates of disease relapse and death were not different between patients in whom a *p53* overexpression was or was not detected (0.97 95% 0.51-1.84; p=1.0 or 1.03 95% 0.48-2.23; p=1.0, respectively) (Table II and III).

K-ras analysis

K-ras mutations at codons 12 and 13 were observed in 5 (12%) colorectal cancer patients. Four mutations occurred at codon 12; GGT to GAT transition was identified in two cases, GGT to GTT transition was identified in two cases. The only one with GGC to GAC change was found at codon 13. All the mutations were missense. Fig.3 shows an example of K-*ras* mutations. One patient with *K-ras* codon 12 mutation showed both p53 overexpression and *p53* mutation at codon 254. The rate of disease relapse and death was not different between patients with wild or mutant *K-ras* gene (1.05 95% 0.82-1.34; p=1.0 or 0.99 95% 0.74-1.32; p=1.0, respectively) (Table II and III).

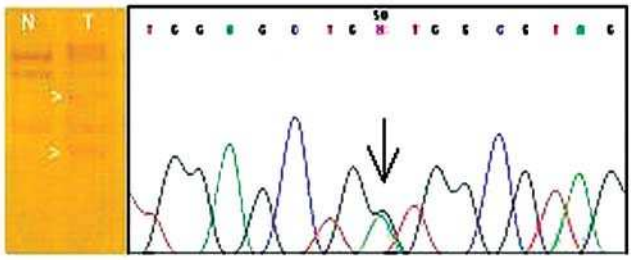

Fig.3 Example of K-ras mutations identified by SSCP and sequencing (**1168,** codon 12, GGTŕGAT) (N, normal; T, tumor tissue).

***DCC* LOH analysis**

Tumor DNA was informative at one or both *DCC* loci in 25 of the 43 patients (58%); five cases (20%) showed LOH at *DCC* locus. Four out of five cases, who were *DCC* LOH positive, coexisted with at least one extra genetic alteration: p53 overexpression, a *K-ras* mutation, a *p53* mutation or a p53 overexpression with a *p53* mutation. Only one case that was *DCC* LOH positive had no other genetic changes. The rates of disease relapse and death were not different between patients with or without LOH at DCC loci (1.17 95% 0.70-2.78; p=0.60 or 0.58 95% 0.22-1.58; p=0.17, respectively) (Table II and III).

Demographics and conventional histologic variables

No correlation was found between other demographic as well as conventional histologic parameters, including p53 overexpression and disease outcomes (relapse and death rate), except the stage II patients who were found to survive significantly less when compared to stage I patients (1.67 95% 1.27-2.18; p=0.04) (Table II and III). However, multivariate logistic regression analysis identified no single independent risk factor for disease relapse and death.

Survival analysis

The 5-year overall (OS) and disease free (DFS) survival rates with genetic alterations are shown in Table II and III. Patients with *DCC* LOH negative tumor phenotype showed a better overall survival than those harboring a *DCC* LOH positive phenotype (log-rank test; p=0.11). Neither the *K-ras* mutations nor the p53 aberrations correlated with prognosis.

Discussion

Colorectal cancers are characterized by multiple chromosomal abnormalities. Recent studies addressing the characterization and identification of distinct pathways of tumor progression suggest that there are several important correlations between selection of any specific type of genetic pathway and variations of clinical outcome in the stage I-II colorectal cancer patients.

Besides molecular genetic analysis, p53 abnormalities can also be detected by IHC analysis. Mutations are mostly missense, leading to a protein with a prolonged half-life, which makes it immunohistochemically detectable. One of the objectives of our study was to search for a correlation between p53 overexpression and *p53* gene mutation. p53 overexpression was observed in 22 (51%) patients. Among our cases, concordance between the IHC and sequencing techniques were found to be statistically significant ($p<0.05$). Interestingly, p53 overexpression was more frequent in tumors located in the left bowel compared to the right (p=0.005). This has been suggested as a reflection of different etiological factors involved in the pathogenesis of right and left sided colorectal cancers (26). Besides, type of the genetic instability plays an important role both in the tumor location and prognosis (27).

Information on *p53* database has indicated that 80% are GC to AT transitions occurring predominantly at CpG islands.

Mutations in five hotspots codons (175, 245, 248, 273 and 282) accounted for approximately 43% of all p53 mutations in colorectal cancer (26). In this study, transition mutations were more common than transversion (85% vs. 15%), and 60% of the mutations occurred at CpG islands. Hotspots mutations were found to be 45%. We also detected two mutations (codon 154 and 224) which do not change the amino acid residue, but which can change splicing, translation or RNA stability. These mutations have never been described so far. We know that silent mutations of p53 especially G:C A:T transitions can modify its methylation capacity and determine genetic instability (28). Codon 254, 261, 205 mutations which were detected in 4 cases, are infrequent. Our results show that mutations occurring in the conserved regions of p53 were more frequent in proximal (60%) than distal (40%) colon cancers. In the prognostic view, neither the *p53* gene mutations nor the p53 overexpression had any impact on the survival rates. The same conclusion was drawn by Dix et al (10), but one large population-based study has indicated that specific classes of mutations, namely, the G245 hotspot mutation and mutations in proximal tumors were related to poor survival (9). Iatopotta et al (29) showed in an international collaborative study that inactive *p53* mutations which block its transactivational ability, had only prognostic significance in Dukes' stage D tumors. Our data have unique *p53* mutations profile, with multiple missense mutations in the same patient and also show infrequent p53 mutations. It could be possibly due to different environmental effects and genetic background of Turkish people.

The percentage of *K-ras* gene mutation in our sample was 12%, which was lower than the expected level (25-40%). There are some possible explanations for this difference. First of all, we analyzed only mutations in codon 12 and 13 of *K-ras* gene; therefore, mutations at the other sites, such as those rarely observed in codon 61, might have also been affected. Secondly, mutation detection rate of SSCP ranges between 50-90% (30), so it is possible that we have missed some mutant samples. But most importantly, alternative *K-ras* independent genetic pathways, as indicated by Frattini et al (31) might have been involved in our cases with colorectal cancer.

In our study, presence of *K-ras* gene mutations was not statistically related to the poor survival. Previous studies evaluating the relation between *K-ras* mutations and prognosis showed no significant relationship with prognosis (18) or an association with poor prognosis (13,14). Collaborative RASCAL studies reported a correlation between *K-ras* mutation and poor prognosis (15,16). This project also showed that one mutation on codon 12, glycine to valine, found in 8.6% of all the patients with colorectal cancer, increases the risk of recurrence or death by 30% (16). In our study, we determined codon 12, glycine to valine, mutations only in two cases and these cases also showed recurrence. On the other hand, recurrence was not observed in other *K-ras* mutations. These results, which are in concordance with RASCAL studies, show that *K-ras* specific mutations in the patients with colorectal cancer may predispose to more aggressive biological behavior.

Smith et al (32) analyzed the coexistence of the mutations of the three key genes, namely, *APC*, *K-ras* and *p53* in a large cohort of colorectal cancer patients. Only 6.6% of the tumors were found to contain combinations of mutations in these genes. The most common combination was *p53* with *APC* (27.1%), whereas mutations in both *p53* and *K-ras* were extremely rare. Our findings revealed that the most common combination of alterations was *DCC* and p53 (7%), whereas a combination of *p53* and *K-ras* mutations was evidenced only in one case (2.3%). This is possibly due to the fact that there are many different alternative pathways in colorectal cancer progression.

Losses of specific chromosomal regions show high prevalence in epithelial cancers, including colorectal cancer. At the molecular level, the allelic imbalance is often associated with losses of heterozygosity (LOH) (33). Zhou et al (34) indicated that imbalances of chromosomes 8p and 18q were a better predictor of prognosis than histopathological stage in the early stage colorectal cancer patients. We studied *DCC* gene LOH on the chromosome 18q by PCR-RFLP. LOH at the *DCC* locus was observed in 5 (20%) of 25 (58%) informative cases. These findings were similar with the results of another study in which the ratio of informative cases and *DCC* LOH were reported as 75% and 26%, respectively (35). The 5-year survival rate was 60% in the group with the *DCC* LOH compared with 90% in the group without the *DCC* LOH (p=0.11). Previous studies have demonstrated that losses of *DCC* gene are related with poor prognosis and the presence of metastases (19-21). The most possible reason for not finding significance in our group might be the small number of informative patients.

We also found that *DCC* LOH tumors frequently coexisted with one extra abnormality. Four of five patients with *DCC* LOH phenotype had one more genetic alteration. This suggests that *DCC* LOH may be encountered with multiple genetic abnormalities and may also be associated with poor prognosis.

In summary, different genetic pathways, which could play important roles in the colorectal tumorigenesis, may lead to varying clinical outcomes in colorectal cancer patients. Specific mutations in the *K-ras* gene and *DCC* LOH status might have a predictive value in the outcomes of colorectal cancer patients. Although no role of *p53* mutations and p53 immunoexpression could be determined with respect to survival rate, these were related with the colorectal tumor localization.

Conflicts of interests

None to declare.

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