TP53 mutations and loss of heterozygosity of chromosome 17 in colorectal tumors

Lidia Yamamoto[1], Ademar A. Lopes[2], Angelita Harb-Gama[3] and Maria A. Nagai[1]

ABSTRACT

The incidence of TP53 point mutations and loss of heterozygosity (LOH) of chromosome 17 in colorectal tumors was determined in a group of Brazilian patients. We screened DNA samples from tumors and distal normal mucosa of 39 patients with colorectal cancer, for TP53 mutations by PCR-SSCP (single-strand conformation polymorphism) analysis. Chromosome 17 LOH was investigated using six PCR-based polymorphic markers and one VNTR probe. TP53 mutations were demonstrated in 15/39 of the cases. Mutations were distributed among all exons examined (five to eight), the majority of them being G/C to A/T transitions. LOH of chromosome 17p and 17q was detected in 70 and 46% of the tumors, respectively. There was a significant association between TP53 mutations and LOH in chromosome 17p (P = 0.0035) and 17q (P = 0.03). Although no correlation was observed between TP53 genetic alterations and clinical/pathological characteristics, the association of TP53 mutations with loss of both chromosome 17 arms may indicate that TP53 inactivation provokes an unstable phenotype in tumor cells in colorectal tumors.

INTRODUCTION

Genetic alterations leading to loss of normal function of the TP53 tumor suppressor gene are the most common genetic event observed in human cancers (Soussi et al., 1994). The TP53 gene is located on chromosome 17p13.1 and consists of 11 exons, coding for a nuclear phosphoprotein which can bind to specific DNA sequences, and acts as a transcription factor (Levine et al., 1994). Most TP53 gene mutations are missense mutations that tend to cluster within exons five to eight, spanning the evolutionary conserved region of the protein (Hollstein et al., 1991). This highly conserved region encompasses aminoacids 102 to 292,

and contains the DNA binding domain of the protein (Cho et al., 1994). Many of these point mutations can alter the TP53 wild-type conformation, resulting in protein stabilization and accumulation in cells (Finlay et al., 1988; Gannon et al., 1990; Bartek et al., 1991). TP53 biological activities are complex and have not been completely elucidated. However, evidence indicates that induction of DNA damage by physical or chemical agents results in wild-type TP53 protein accumulation, which transiently arrests the cell cycle in G1, preventing entry into S-phase to allow repair of the damage, or inducing apoptosis if DNA repair fails (Levine et al., 1994; Lane, 1994).

A molecular model for tumor progression has been delineated in colon cancer. The progression from adenoma to carcinoma is correlated with an accumulation of specific genetic alterations (Fearon and Vogelstein, 1990). TP53 mutations are among the most frequent of these genetic changes (Scott et al., 1991; Cunningham et al., 1992; Soussi et al., 1994). In colon

[1] Disciplina de Oncologia, Departamento de Radiologia, Faculdade de Medicina, Universidade de São Paulo, Av. Dr. Arnaldo 455, 4° andar, 01246-093 São Paulo, SP, Brasil. Fax: (55) (11) 282-6580. Send correspondence to M.A.N.
[2] Fundação Antônio Prudente, São Paulo, SP, Brasil.
[3] Departamento de Gastroenterologia, Faculdade de Medicina, Universidade de São Paulo, São Paulo, SP, Brasil.

cancer TP53 inactivation is predominantly a result of a point mutation in one allele, followed by deletion of the remaining wild-type allele (Baker *et al.*, 1990), and has been associated with the transition from adenoma to carcinoma (Fearon and Vogelstein, 1990; Kikuchi-Yanoshita *et al.*, 1992; Hasegawa *et al.*, 1995).

MATERIAL AND METHODS

Tissue samples

Samples of colorectal tumors and adjacent normal colonic mucosa were obtained from 39 patients at Osvaldo Cruz Hospital (31 cases) and A.C. Camargo Hospital (eight cases), São Paulo, Brazil. The tumors consisted of carcinomas localized on the splenic flexure (one case), sigmoid colon (11 cases), right colon (three cases), left colon (one case) and rectum (23 cases). Nineteen patients were males. The clinical stage of the patients was determined using Dukes' classification.

DNA extraction

Tissue was ground to a powder using a Frozen Tissue Pulverizer (Termovac). The powder was resuspended in 1 ml of lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA and 0.6% SDS) and 100 µg/ml proteinase K, and incubated at 37°C overnight. High molecular weight DNA was extracted with phenol-chloroform and precipitated with ethanol.

Loss of heterozygosity (LOH) analyses

Chromosome 17 analysis was performed using six PCR-based polymorphic markers and one VNTR probe. LOH for TP53 was analyzed using two polymorphic markers (Futreal *et al.*, 1991; Jones and Nakamura, 1992) to ensure the detection of a maximum number of informative patients for this locus. The markers used for LOH analysis on the long arm of chromosome 17 were: mfd15/D17S250 (Weber *et al.*, 1990), mfd191/D17S776 (Genomic Data Bank), 248yg9/D17S855 (Weissenbach *et al.*, 1992), and nm23/NME1 (Hall *et al.*, 1992). PCR reactions were performed in 25-µl volumes using 50-100 ng of genomic DNA template, 1 µM of each primer, 1.5 mM MgCl$_2$, 200 µM of each deoxynucleotide triphosphate, 0.5 pmol of one end labelled primer [γ-^{32}P-dATP] (Amersham, specific activity, 5000 Ci/mmol), 50 mM KCl, 10 mM Tris-HCl, pH 8.0, and 0.5 unit of Taq DNA polymerase (Pharmacia, NJ, USA). Samples were overlaid with mineral oil and amplified for 35 cycles of denaturation,

annealing and extension optimized for each primer set. The reactions were performed with an automated Perkin Elmer 580 Thermal Cycler. The PCR products were diluted 1:2 in 90% formamide, 10 mM EDTA, 0.3% bromophenol blue, 0.3% xylene cyanol, boiled for 5 min and resolved by electrophoresis through 6% denaturing polyacrylamide gels. Alleles were detected by autoradiography of dried gels using Kodak X-Omat XAR film for 12-48 h at -70°C, with intensifying screens.

LOH for D17S5 was analyzed by Southern blot using the probe YNZ22.1 (Nakamura *et al.*, 1988), as described previously (Nagai *et al.*, 1994). Allelic losses were determined as complete or significantly decreased by the intensity of one allele relative to the other in constitutively heterozygous patients. Most of the cases with LOH showed partial allelic loss.

PCR-SSCP analysis

PCR reactions were carried out as described for LOH analysis, but using primers for the most highly conserved regions of the TP53 gene and 0.5 µCi of [α^{32}P-dCTP] (Amersham, specific activity, 3000 Ci/mmol). Oligonucleotide primers used to amplify exons 5, 6, 7 and 8 were the same as described by Murakami *et al.* (1991). Amplification products (1 µl) were diluted 10-fold in a buffer containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol, heated at 83°C for 5 min and applied (3 µl/lane) on two 6% polyacrylamide nondenaturing gels, one containing 5% and the other 10% glycerol. Electrophoresis was performed at 6W for 14-16 h at room temperature, with two cooling fans. Band shift mobility was detected by autoradiography of dried gels using Kodak X-Omat XAR film with an intensifying screen for 12 to 48 h at -70°C.

Direct DNA sequencing

DNA samples with TP53 mutations detected by single-strand conformation polymorphism (SSCP) gels were amplified for those exons which showed a mobility shift. The PCR products obtained were purified on MicroSpin^{TM} columns (Pharmacia, Biotech, USA) according to the manufacturer's procedure. Three-5 µl of the purified DNA was subjected to a dideoxy chain termination reaction using a double stranded DNA cycle sequencing kit (Pharmacia, USA) for both sense and antisense primers. Sequencing reaction products were denatured and resolved on 6% denaturing urea/polyacrylamide gels. Gels were fixed for 15 min in a 10% methanol/10% acetic acid solution, dried and an X-ray film exposed overnight.

Table I - Loss of heterozygosity of chromosome 17 in colorectal tumors.

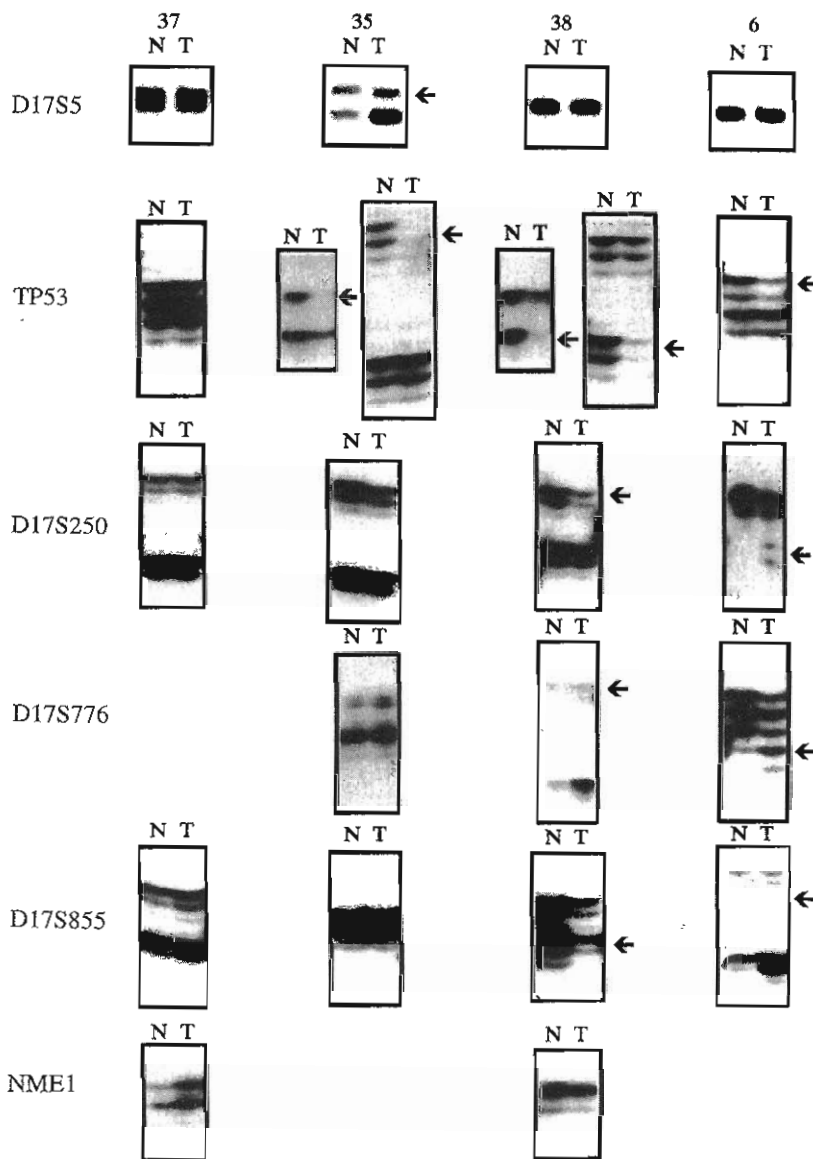| Marker	Locus	Number of cases analyzed	Allele loss/ informative cases
YNZ22.1	D17S5	39	11/15
TP53	TP53	37	22/31
mfd15	D17S250	39	13/31
mfd191	D17S776	39	11/27
248yg9	D17S855	33	10/27
nm23	NME1	38	12/26

Figure 1 - Loss of heterozygosity at loci on chromosome 17 in representative colorectal tumors. Patient identification numbers are shown at the top and markers are indicated on the left. Arrows indicate the reduced or deleted alleles at D17S5 (case 35), TP53 (cases 35, 38 and 6), at D17S250, D17S776 and D17S855 (case 38) and microsatellite expansion at D17S250, D17S776 and D17S855 (case 6). N, Normal tissue DNA; T, tumor tissue DNA.

Statistical methods

Analyses of statistical significance between the TP53 genetic alterations and the clinicopathological characteristics of the patients were performed by the chi-square test and Fisher exact test for frequency data in contingency tables.

RESULTS

Table I summarizes the LOH data. The highest frequencies of LOH were found for loci at the short arm of chromosome 17 (17p), D17S5 and TP53. Representative results of our LOH analysis are shown in Figure 1 and a schematic representation of the allele loss pattern observed is shown in Figure 2. Among 33 tumors constitutively heterozygous for at least one marker for each chromosome 17 arm analyzed, nine showed allelic losses on 17p alone and 13 showed allelic losses on both 17p and 17q. None of the tumors analyzed showed LOH at 17q alone. Replication error was found in just one tumor (tumor 6) (Figure 1).

The incidence of mutations in exons 5, 6, 7 and 8 of the TP53 gene was examined using PCR-SSCP analysis. Mutations were detected in 15/39 of the tumors. Twelve out of 15 tumors with mutations were analyzed by direct DNA sequencing. Table II shows the TP53 mutations and LOH results in those tumors. Mutations were observed in all exons examined, with no predominance of any specific codon. Eleven mutations were missense and one was nonsense. Nine of the mutations were transitions (8, $G \rightarrow A$; 1, $A \rightarrow G$) and three were transversions (1, $C \rightarrow G$; 1, $T \rightarrow A$; 1, $A \rightarrow C$). Representative results of SSCP and DNA sequence analyses are shown in Figure 3.

Point mutations and allelic losses at TP53 were significantly associated (Table III). However, in nine of the 22 informative tumors with TP53 LOH we were not able to detect any mutation for the exons analyzed. TP53 mutations were also significantly associated with 17q allelic losses (Table III).

To assess the significance of genetic alterations on chromosome 17 in the development of the colorectal tumors

analyzed we compared clinicopathological characteristics (age, Dukes' stage, differentiation grade, tumor size and lymph node involvement) of the patients with tumors showing TP53 mutations (Table III), TP53 LOH, 17p LOH and 17q LOH alone or together (combined) with those patients with tumors without any of these genetic alterations. No significant associations were found (data not shown).

DISCUSSION

This study demonstrated that TP53 mutations and chromosome 17 allelic losses are common events in colorectal tumors of Brazilians. The frequency of TP53 mutations detected by PCR-SSCP analysis in our panel of tumors was slighter lower than that reported in the literature (Soussi *et al.*, 1994). The mutations were distributed throughout all exons examined and the majority of these mutations were G → A transitions within CpG dinucleotides. This is consistent with the literature data demonstrating that in colon cancer most of the TP53 mutations are G/C to A/T transitions in CpG sites, which are hot-spots for spontaneous mutations (Coulondre *et al.*, 1978; Harris and Hollstein, 1993; Tornaletti and Pfeifer, 1995). The frequency of loss of heterozygosity on chromosome 17p is also in accordance with previous reports (Khine *et al.*, 1993; Goh *et al.*, 1994). As postulated for the classical model of tumor suppressor gene inactivation (Knudson, 1971), in colorectal tumors TP53 gene point mutations are followed by a loss of the remaining wild-type allele of the gene (Baker *et al.*, 1990; Rodrigues *et al.*, 1990). In our study, all colorectal tumors with TP53 mutations showed LOH while nine tumors with TP53 LOH showed no detectable TP53 gene mutations. As we did not investigate the entire coding region (exons 2 to 11) or intronic sequences of the gene, those tumors may have mutations outside the highly conserved region examined (exons 5 to 8), which could have been missed in this study.
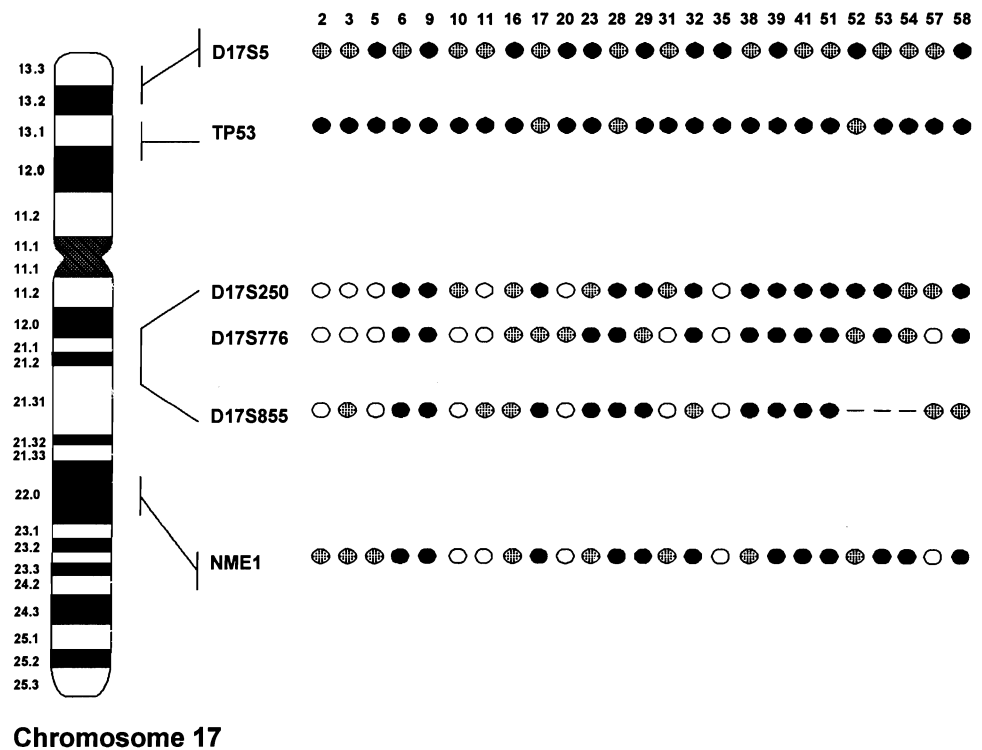

Chromosome 17

Figure 2 - Allelic deletion map of 25 colorectal tumors with loss of heterozygosity (LOH) for loci at chromosome 17. Patient identification numbers are shown at the top. Open circles - informative markers with retention of both alleles; closed circles - informative markers with loss of heterozygosity; shaded circles - markers not informative; bars - not determined.

Table II - TP53 gene mutations and loss of heterozygosity (LOH) in colorectal tumors.

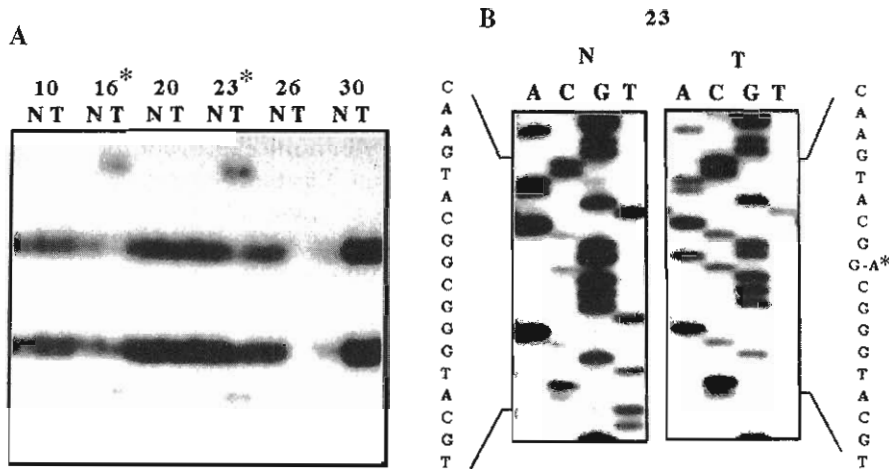| Tumor	Exon	Codon	Base change	Amino acid change	LOH
2-1	8	279	GGG → GAG	Gly → Glu	+
3-1	5	175	CGC → CAC	Arg → His	+
9-1	8	282	CGG → CAG	Arg → Gln	+
11-1	6	194	CTT → CAT	Leu → His	+
16-1	7	245	GGC → AGC	Gly → Ser	+
23-1	7	245	GGC → AGC	Gly → Ser	+
28-1	5	154	GGC → AGC	Gly → Ser	Not informative
29-1	5	175	CGC → CAC	Arg → His	+
35-1	6	-	-	-	+
38-1	5	163	TAC → TAG	Tyr → Stop	+
51-1	6	190	CCT → CTT	Pro → Ala	+
52-1	6	204	GAG → GCG	Glu → Ala	Not informative
53-1	7	248	CGG → CAG	Arg → Gln	+
57-1	6	-	-	-	+
58-1	6	-	-	-	+

Figure 3 - Examples of TP53 mutations. In A, PCR-SSCP analysis of exon 7 of the TP53 gene. *Tumors that showed mobility shift. In B, DNA sequence autoradiograph of exon 7 of the TP53 gene showing point mutation at codon 245 in tumor 23. N, Normal tissue DNA; T, tumor tissue DNA.

Table III - Relationship between p53 gene mutations and clinico-pathological characteristics of the patients with colorectal tumors.

Characteristics	Category	p53		
		mut-	mut+	P value
Age	≤ 50 years	4	5	0.43[a]
	> 50 years	19	10	
Duke's stage	A	1	4	0.11[b]
	B	7	5	
	C	9	3	
Grade of differentiation	Poorly	2	0	0.36[b]
	Moderately	9	7	
	Well	6	7	
Tumor size	≤ 4 cm	11	3	0.12[a]
	> 4 cm	6	8	
Lymph-node involvement	Negative	9	10	0.35[a]
	Positive	11	5	
p53	LOH-	9	0	0.0035[a]
	LOH+	9	13	
17q	LOH-	16	3	0.03[a]
	LOH+	7	8	

mut-, Tumors without p53 gene mutations; mut+, tumors with p53 gene mutations. LOH-, Informative tumors with heterozygosity retained; LOH+, informative tumors with loss of heterozygosity.
[a]Fisher exact test.
[b]Chi-square test.

DNA aneuploidy, which is considered a prognostic indicator, has been associated with TP53 genetic alterations in colon cancer (Remvikos et al., 1992; Meling et al., 1993; Goh et al., 1994). Meling et al. (1993)

demonstrated a strong association between TP53 allelic loss and DNA aneuploidy but only when both chromosome arms 17p and 17q were lost, suggesting that genetic alterations in the TP53 gene are the "target" for the loss of chromosome 17 in colorectal tumors. In agreement with this, in our series of tumors there was a significant association between TP53 mutations and chromosome 17q allelic losses. This may indicate that, in a subgroup of colorectal tumors, point mutations at the TP53 locus are followed by the complete loss of chromosome 17, possibly leading to a more aggressive phenotype. Genetic alterations in the nm23 metastatic tumor suppressor gene located at 17q22 have been reported in 50% of the colorectal tumors examined, being significantly associated with distant metastasis (Cohn et al., 1991; Wang et al., 1993). Based on that, we cannot exclude the possibility that other genes, such as nm23, or another candidate tumor suppressor gene located near it, could be the "target" of the chromosome 17 deletions observed.

Evidence provided by immunohistochemical and molecular studies indicates that TP53 mutations could play an important biological role in the tumorigenic process of colon cancer, however the relationship among TP53 genetic alterations and clinicopathological characteristics or disease outcome is still not well characterized (Scott et al., 1991; Cunningham et al., 1992; Remvikos et al., 1992; Bosari et al., 1994; Hasegawa et al., 1995). In this study, no correlation was observed between TP53 genetic alterations and clinicopathological characteristics, such as age, Dukes' stage, grade of differentiation, tumor size or lymph node involvement. However, the limited number of tumors examined did not permit us to discuss these findings properly.

The TP53 tumor suppressor gene has been recently considered a "guardian of the genome" because induction of DNA damage leads to an accumulation of TP53 protein, which transiently arrests the cell cycle at G1 allowing repair of the damage or inducing apoptosis (Lane, 1994). Evidence indicates that the TP53 gene is a positive modulator of apoptosis (Hoffman and Liebermann, 1994). In this context, TP53 inactivation may promote inhibition of apoptosis, leading to growth advantages for tumor cells, including colorectal tumors (Hoffman and Liebermann, 1994;

Bedi *et al.*, 1995). Cells with TP53 mutations have been postulated to be genetically unstable, facilitating the occurrence of new genetic alterations, as found recently in cultured tumor cells and primary breast tumors (Yin *et al.*, 1992; Livingstone *et al.*, 1992; Eyfjord *et al.*, 1995). In colon cancer the association of TP53 genetic changes in the conversion of adenoma to carcinoma indicates that TP53 inactivation is an initial event in the malignant transformation, leading to genetic instability, and that progression to a more aggressive phenotype by different tumors is promoted by the acquisition and accumulation of further genetic alterations.

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RESUMO

Este trabalho teve como objetivo determinar a incidência de mutações no gene TP53 e de perdas de heterozigose (LOH) no cromossomo 17 em carcinomas coloretais de pacientes brasileiros. Analisamos amostras de DNA do tumor e da mucosa normal de 39 pacientes com câncer coloretal. As mutações no gene TP53 foram analisadas pela técnica de PCR-SSCP (polimorfismo decorrente da conformação da fita simples de DNA) e a análise de perda de heterozigose para o cromossomo 17 foi feita com a utilização de seis marcadores de DNA polimórfico do tipo microsatélite e uma sonda para minisatélite. Mutações no gene TP53 foram observadas em 15/39 dos casos analisados. As mutações observadas estão distribuídas por todos os exons examinados (exons 5 a 8), sendo a maioria das mutações transições $G/C \rightarrow A/T$. Perdas de heterozigose nos segmentos cromossômicos 17p e 17q foram observadas em 70 e 46% dos tumores, respectivamente. Observamos uma associação significativa entre a ocorrência de mutações para o gene TP53 e as perdas de heterozigose nos segmentos cromossômicos 17p ($P = 0,0035$) e 17q ($P = 0,03$). Embora nenhuma correlação estatisticamente significativa tenha sido observada entre a ocorrência de alterações genéticas no gene TP53 e as características clinicopatológicas dos pacientes, a associação entre a ocorrência de mutação no gene TP53 e a perda de heterozigose em ambos os braços do cromossomo 17 pode indicar que em um subgrupo de tumores coloretais a inativação do gene TP53 resulte em células com maior instabilidade genética.

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