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Molecular detection of TP53, Ki-Ras and p16^{INK4A} promoter methylation in plasma of patients with colorectal cancer and its association with prognosis. Results of a 3-year GOIM (Gruppo Oncologico dell'Italia Meridionale) prospective study

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Background: Despite the improvement in detection and surgical therapy in the last years, the outcome of patients affected by colorectal carcinoma (CRC) remains limited by metastatic relapse. The aim of this study was to investigate the presence of free tumor DNA in the plasma of CRC patients in order to understand its possible prognostic role.
Patients and methods: Ki-Ras, TP53 mutations and p16^{INK4A} methylation status were prospectively evaluated in tumor tissues and plasma of 66 CRC patients.
Results: In 50 of the 66 primitive tumor cases (76%) at least one significant alteration was identified in Ki-Ras and/or TP53 and/or p16^{INK4A} genes. Eighteen of the 50 patients presented the same alteration both in the plasma and in the tumor tissue. At univariate analysis, Ki-Ras mutations proved to be significantly related to quicker relapse (P <0.01), whereas only a trend towards statistical significance (P = 0.083) was observed for the TP53 mutations
Conclusions: Detection of Ki-Ras and TP53 mutation in plasma should be significantly related to disease recurrence. These data suggest that patients with a high risk of recurrence can be identified by means of the analysis of tumor-derived plasma DNA with the use of fairly non-invasive techniques.
Key words: colorectal carcinoma, free-cell DNA, Ki-Ras, TP53

introduction

Colorectal carcinoma (CRC) is one of the most common solid tumors in the Western world, with over 300 000 cases occurring every year in the USA and in Europe and a mean 5-year survival rate of 50% [1]. In spite of the progress made in the last few years with regard to early diagnosis and surgical treatment, 30%–50% of patients who undergo radical surgery develop a subsequent relapse leading to death [2].

The presence of fragments of circulating DNA has been described many times in different body fluids. A large number of studies conducted on the plasma and serum of patients with cancer of the pancreas, colon, breast, liver, head and neck, and esophagus, have shown that such subjects have a higher level of circulating DNA fragments than healthy subjects. No clear correlation has been established, however, either between circulating DNA levels and the size and site of the tumor, or, so far, between these levels and the clinical course of the disease [3, 4]. In addiction most of the circulating DNA would appear to originate from the primary tumor since the plasma shows the same genetic alterations [2].

Colorectal carcinogenesis is characterized by genetic alterations of the oncogenes and oncosuppressors, especially of the Ki-Ras and TP53 genes [5]. These alterations might become useful markers for the detection of tumor DNA in the plasma of patients with this disease.

It has recently been observed that the hypermethylation of the normally non-methylated CpG islands in the p16^{INK4A} gene promoter is correlated with its transcription level in various tumors. The inactivation of this gene subsequent to aberrant methylation has been reported in 20%–50% of CRCs [6]. The presence of epigenetic alterations of the oncosuppressor p16^{INK4A} might therefore be used as a tumoral marker in the plasma of patients with such tumors [7].

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The aim of this study was to support the diagnostic and prognostic value of circulating DNA detected in the plasma of patients affected by CRCs. The genetic alterations of the oncogene Ki-Ras, of the tumor suppressor gene TP53 and the methylation status of the $p16^{INK4A}$ gene promoter were analyzed in the primary tumors of 66 patients with CRC; in patients where such alterations were identified, analysis of their plasma was performed in order to detect the same genetic alterations. Finally, it has been assessed whether or not the presence of tumor-associated circulating DNA might be used as a marker for the identification of patients with a more unfavorable prognosis and/or disease relapse risk.

patients and methods

patient selection

A prospective study was performed on a consecutive series of 66 patients undergoing resective surgery for primary operable CRC at a single institution (Department of Oncology, University of Palermo).

In these patients it was possible to take a pre-operative blood sample, the primary tumor tissue and normal mucosa. Inclusion criteria were: histologically-confirmed CRC diagnosis; and patients undergoing radical surgery with resection margins histologically negative for neoplastic infiltration. Clinicopathological variables of the patients were age, sex, tumor stage, histological grading and tumor size. The mean age of the patients at diagnosis was 66 ± 11 years and the group was made up of 34 men and 32 women. Up to the present time, all the patients are undergoing regular follow-up. Staging was performed according to the TNM classification. The tumors were divided into three groups according to the WHO criteria: well-differentiated (grade 1), moderately differentiated (grade 2) and poorly differentiated (grade 3). Twenty blood samples of healthy subjects and seven non-neoplastic gastrointestinal tissue were analyzed for the same alterations as controls.

sampling, DNA extraction and analysis of the genic status of Ki-Ras and of TP53 in the primary tumor tissue

The samples of primary tumor and normal tissue were stored at –80°C until required. Genomic DNA was extracted by means of the QIAamp kit (Qiagen, Hilden, Germany), following the standard protocol. The DNA samples were used to screen for mutations within the TP53 and Ki-Ras genes using Single Strand Conformational Polymorphism (SSCP) analysis. PCR amplification of the exons 5–8 for TP53 and exon 1 for Ki-Ras (Table 1) was performed as previously described [9]. The abundance and integrity of the amplification products were verified by 1.5% agarose gel electrophoresis and ethidium bromide staining. One hundred nanogram aliquots of the PCR products, purified and concentrated by filtration through Microcon 50 columns (Amicon, Beverly, MA), were denatured and used for SSCP analysis. Individual ssDNA fragments exhibiting shifted mobilities relative to normal controls, were electroeluted from polyacrylamide gel, as described previously [10], reamplified and sequenced. The characterization of single mutations was performed by automated sequencing using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and the model 3100 GeneticAnalyzer sequencer (Perkin-Elmer, Foster City, CA).

sampling, DNA extraction and mutational analysis of the plasma

The plasma sample was obtained by centrifuging 3 ml of peripheral blood at 1550 *g* for 30 min. An aliquot was prepared from the resulting supernatant and was stored at –20°C until required. Genomic DNA was extracted by means of an Ultrasense Virus Kit (Qiagen, Hilden Germany), according to the standard protocol. Direct automatic sequencing was used to detect the same genetic alterations in the plasma.

Table 1. Oligonucleotide primers for p53, H-ras, K-ras and p16 genotyping

Exon	Primer sequences	Anneal (°C)
ex5 p53	5′-caa cca gcc ctg tcg tct ctc-3′	58
	5′-ctg ttc act tgt gcc ctg ac-3′	
ex6 p53	5′-cct cac tga ttg ctc tta gg-3′	50
	5′-agt tgc aaa cca gac ctc a-3′	
ex7 p53	5′-caa gtg gct cct gac ctg ga-3′	58
	5′-tcc tag gtt ggc tct gac-3′	
ex8 p53	5′-tcc tgc ttg ctt acc tcg-3′	54
	5′-tcc tat cct gag tag tgg t-3′	
h-ras	5′-ctg agg agc gat gac gga ata taa gc-3′	68
	5′-ctc tat agt ggg gtc gta ttc gtc ca- 3′	
k-ras	5′-gtg tga cat gtt cta ata tag tca ca-3′	58
	5′-gaa tgg tcc tgc acc agt aa -3′	
p16M	5′-tta tta gag ggt ggg gcg gat cgc-3′	61
	5′-gac ccc gaa ccg cga ccg taa-3′	
p16U	5′-tta tta gag ggt ggg gtg gat tgt-3′	66
	5′-cca ccc caa acc aca acc ata a-3′	

methylation status of $p16^{INK4A}$ locus

Genomic DNA obtained from plasma, normal and tumor tissue samples was modified with the CpGenome DNA Modification kit (Intergene Company) following the manufacturer's instructions [10]. Peripheral blood leukocytes (L) were used as negative controls and universal methylated DNA, UMD (ONCOR, Geithersburg, MD) was used as a positive control. The modified DNA was amplified by PCR using specific primers (Table 1) to distinguish between methylated and unmethylated regions. The size and integrity of the amplification products were verified by 2% agarose gel electrophoresis and ethidium bromide staining.

statistical analysis

Association between biological and clinicopathological variables was evaluated by means of the chi-square test and, where appropriate, Yates' correction. Disease-free survival (DFS) was measured from the day of primary surgery to the date of first relapse (locoregional or metastatic) and overall survival (OS) from the day of surgery to the day of death. If patients did not relapse or die, they were censored at the time of their last follow-up. Clinical and morphobiological variables were examined by means of the Kaplan–Meier method; significance of differences for each prognostic factor was assessed by the log rank and Wilcoxon tests or trend tests where appropriate.

results

detection of genetic alterations in colorectal cancer tissue

Genetic alterations in Ki-Ras and TP53 were performed with the PCR-SSCP techniques. The sequence analysis of the DNA fragments with altered electrophoretic mobility helped to establish the exact site and nature of the genetic alteration. Hypermethylation of the $p16^{INK4A}$ gene promoter were performed with MSP of bisulfite modified DNA.

In 50 of the 66 cases (76%) at least one significant alteration was identified. Twenty-nine of the 66 cases (44%) showed

hot-spot mutations of Ki-Ras gene after sequencing: 21 in codon 12 and eight in codon 13.

Aberrantly migrating bands of exons 5–8 of the TP53 gene were found in 41% (27/66) of the cases; sequence analysis was performed in only 20 tumor samples (Figure 1). The 27 nucleotidic alterations were made up of five in exon 5 (19%), six in exon 6 (22%), nine in exon 7 (33%) and seven in exon 8 (26%). Two mutation were frameshifts while the majority of the mutations were single nucleotide substitutions. Sixty-seven per cent of the latter were missense (12/18), 28% were silent mutations or polymorphisms (5/18) and 5% were nonsense mutations (1/18). Fifty per cent of the mutations (10/20) occurred in conserved domains (areas II–V). In addition, by taking into account the specific functional and structural domains of TP53 affected by the mutations, the latter were classified as follows: two of 20 cases (10%) with mutations of the L2, three of 20 cases (15%) with mutations of the LSH motif and five of 20 cases (25%) with mutations of the L3 (Figure 2). Since the polymorphism do not determine any amino acid change in the protein, for statistical analysis they have been included in the wild-type group.

The analysis of the $p16^{INK4A}$ gene promoter showed that 14/66 cases (21%) presented hypermethylation; the analysis of the normal tissue of the same patients did not reveal hypermethylation (Figure 3).

Multiple alterations were found in 20% (13/66) of the analyzed CRC cases. Eleven cases presented double mutations; four cases (200/110, 200/18, 200/187 and 200/1817) presented the hypermethylation of the $p16^{INK4A}$ promoter and Ki-Ras mutation, two cases (200/48, 200/71) presented the hypermethylation of the $p16^{INK4A}$ promoter and TP53 mutation, and four cases (200/70, 200/267, 200/275 and 200/288) presented both TP53 and Ki-Ras mutations. One case (200/65) was found to harbor two mutations in two different exons (7–8) of TP53. Two cases (200/01 and 200/142) presented a triple mutation: the hypermethylation of the $p16^{INK4A}$ promoter and mutations of Ki-Ras and TP53. Table 2 shows the results of the genetic alterations analyzed in all the patients.

Exactly the same analyses were performed on the control group; no genetic or epigenetic alterations were detected.

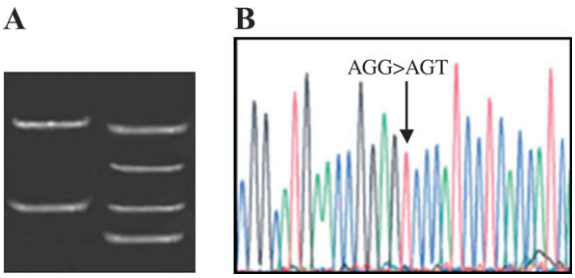

Figure 1. SSCP analysis of exon 7 of TP53 gene amplified from DNA of primitive tumor (case 200/65) and normal tissue of the same patient: the normal tissue DNA is on the left; tumor DNA is on the right (**A**). The extra bands correspond to ssDNA molecules harboring mutations in exon7 R249S AGG>AGT as confirmed by sequencing (**B**).

detection of tumor DNA in the plasma

The second step of this study was to detect in plasma samples the same alteration found in the corresponding tumor tissues; only 76% of patients (50/66) were therefore selected.

Eighteen of the 50 patients (36%) presented the same alteration both in the plasma and in the tumor tissue, eight of which mutated in Ki-Ras and eight in TP53. In four cases the amplifications with the primers for Ki-Ras and/or TP53 failed and the alterations were thus considered unreliable.

Hypermethylation of $p16^{INK4A}$ gene promoter was detected in the plasma of three of 18 patients (17%) (Table 2). Multiple alterations were found in plasma of three cases.

relationship between plasma biomolecular indicators and clinical data

No significant correlations were observed between gender, age, tumor site and tumor stage and the detection of free-circulating tumor-associated DNA (data not shown).

relationship between detection of tumor DNA in plasma and prognosis

At the time of this report, 21/50 patients have relapsed, 18 with distant metastases and three with locoregional recurrence, while 10/50 have died from tumor-related causes. The median follow-up time in our study group was 26 months (range 2–48 months). At univariate analysis, Ki-Ras mutations proved to be significantly related to quicker relapse ($P < 0.01$), whereas only a trend towards statistical significance ($P = 0.083$) was observed for the TP53 mutations (Figure 4). No significant association has been identified between the same genetic alteration and the

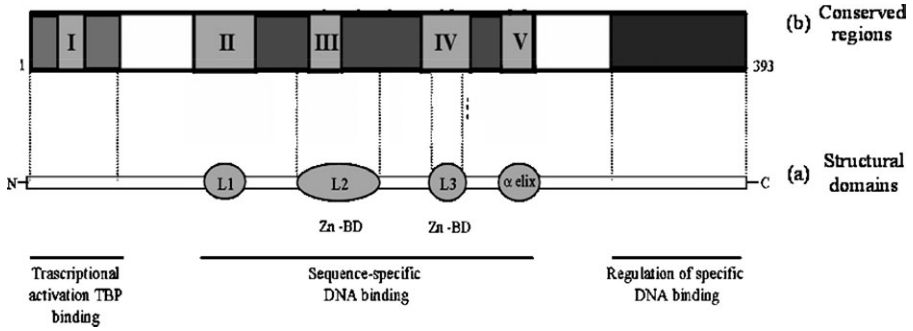

Figure 2. TP53 structure showing the five areas highly conserved during evolution and the most important functional domains of the protein [5].

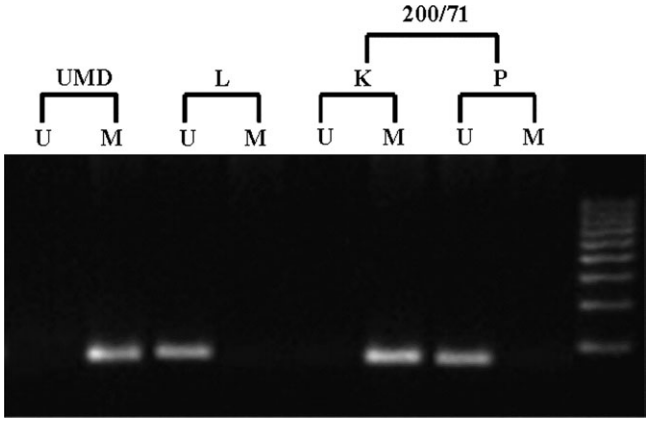

Figure 3. $p16^{INK4a}$ analysis using MSP in case 200/71. MSP results are expressed as unmethylated (U) or methylated (M) $p16^{INK4A}$ specific bands. Normal lymphocytes (L) and *in vitro* universal methylated DNA (UMD) are used as negative and positive controls, respectively. Case 200/71 shows hypermethylated of $p16^{INK4A}$ promoter within the primitive tumor (K), but not in plasma (P). The last lane contains 100 bp DNA ladder.

OS. $p16^{INK4a}$ promoter hypermethylation and the clinicopathological parameters analyzed were not significantly associated.

discussion

The mechanism leading to the presence of free tumor DNA in the plasma of cancer patients is still not fully understood. It might be caused by the lysis of circulating cancer cells or by DNA leakage resulting from tumor necrosis or apoptosis [11]. Recent studies have confirmed that somatic genetic or epigenetic alterations in tumors are potential targets for molecular investigation in plasma [12]. The aim of this study was to confirm the hypothesis that molecular markers may be used today in the early diagnosis and prognosis of patients affected by CRC. Therefore, the genetic and epigenetic alterations characterized in the CRC were analyzed in plasma in order to identify a possible association.

The most commonly used target was Ki-Ras, which is mutated in a large number of gastrointestinal tumors.

Table 2 Aberrant p16 methylathion and TP53 and K-Ras mutation in the tumors and plasma DNA of colorectal cancer patients

Patient no.	Stage (Dukes)	TP53		Ki-Ras		p16	
		DNA Tumor	DNA Plasma	DNA Tumor	DNA Plasma	DNA Tumor	DNA Plasma
200/01	1	Mut ex5	Mut ex5	Mut ex1 G12N	Mut ex1 G12N	M	U
200/13	1	WT		WT		M	U
200/141	1	WT		WT		M	U
200/145	1	WT		WT		U	
200/146	1	WT		WT		U	
200/16	1	Pol ex6 R213R		Mut ex1 G12V	WT	U	
200/28	1	Pol ex6 R213R		Mut ex1 G12V	Mut ex1 G12V	U	
200/196	1	Mut ex5	WT	WT		U	
200/202	1	WT		WT		U	
200/77	1	WT		WT		M	U
200/87	1	WT		WT		U	
200/91	1	Pol ex7 D228D		WT		U	
200/240	1	WT		WT		U	
200/245	1	WT		Mut ex1 G12s	WT	U	
200/257	1	WT		Mut ex1 G12V	WT	U	
200/267	1	Mut ex7 R249S	WT	Mut ex1 G12N	WT	U	
200/275	1	Mut ex7 A244D	Mut ex7 A244D	Mut ex1 G12V	np	U	
200/293	1	WT		Mut ex1 A13N	np	U	
200/66	2	Mut ex8 R282P	Mut ex8 R282P	WT		U	
200/69	2	WT		Mut ex1 G13C		U	
200/70	2	Mut ex7	Mut ex7	Mut ex1 G12V	Mut ex1 G12V	U	
200/71	2	Mut ex7	Mut ex7	WT		M	U
200/80	2	WT		WT		U	
200/105	2	Pol ex6 R213R		Mut ex1 G12V	WT	U	
200/123	2	WT		WT		U	
200/26	2	WT		Mut ex1 G12V	WT	U	
200/1817	2	WT		Mut ex1 G13C	WT	M	M
200/182	2	WT		Mut ex1 G13C	WT	U	
200/1822	2	WT		WT		M	M
200/58	2	WT		Mut ex1 G12S	WT	U	
200/265	2	Mut ex8 792delCTA	WT	WT		U	
200/276	2	Mut ex8 R306X	WT	WT		U	
200/288	2	Mut ex8 R282W	WT	Mut ex1 G12N	np	U	

Table 2. (*Continued*)

Patient no.	Stage (Dukes)	TP53		Ki-Ras		p16	
		DNA Tumor	DNA Plasma	DNA Tumor	DNA Plasma	DNA Tumor	DNA Plasma
200/294	2	Mut ex5 R175P	WT	WT		U	
200/304	2	Mut ex6 R213L	np	WT		U	
200/327	2	Mut ex5 R175C	WT	WT		U	
200/03	3	WT		Mut ex1 G13A	Mut ex1 G13A	U	
200/06	3	WT		WT		M	U
200/09	3	WT		WT		U	
200/65	3	Mut ex8 R283P	Mut ex8 R283P	WT		U	
		Mut ex7 R249S	Mut ex7 R249S				
200/101	3	Mut ex8	WT	WT		U	
200/110	3	WT		Mut ex1 G12N	WT	M	U
200/142	3	Mut ex7 R248W	WT	Mut ex1 G12N	WT	M	U
200/143	3	Mut ex8	WT	WT		U	
200/18	3	WT		Mut ex1 G12N	Mut ex1 G12N	M	U
200/1816	3	WT		WT		U	
200/1821	3	WT		WT		M	M
200/1845	3	WT		WT		U	
200/187	3	WT		Mut ex1 G13A	Mut ex1 G13A	M	U
200/48	3	Mut ex8 S576H	WT	WT		M	U
200/52	3	Mut ex7	Mut ex7	WT		U	
200/53	3	WT		Mut ex1 G12N	Mut ex1 G12N	U	
200/56	3	WT		Mut ex1 G13C	Mut ex1 G13C	U	
200/209	3	WT		WT		U	
200/218	3	WT		WT		U	
200/219	3	WT		Mut ex1 G12A	WT	U	
200/228	3	WT		WT		U	
200/229	3	WT		Mut ex1 G13A	WT	U	
200/260	3	WT		Mut ex1 G12N	WT	U	
200/262	3	WT		Mut ex1 G12V	WT	U	
200/290	3	Pol ex6 R213R		Mut ex1 G12S		U	
200/305	3	Mut ex6 634delTT	WT	WT		U	
200/83	4	WT		Mut ex1 G12V	WT	U	
200/211	4	WT		WT		U	
200/227	4	Mut ex7 R248W	WT	WT		U	
200/270	4	WT		WT		U	

WT, wild type; Mut, mutation; Pol, polymorphism; U, unmethylated; M, methylated; np, no product; ex, exon.

In particular, the CRC showed Ki-Ras mutations in 20%–50% of the cases [13]. In agreement with reported data, the results of our study show that 44% of Ki-Ras mutations in primary tumors are detected in the hot spots.

Point mutations in the TP53 tumor suppressor gene are the most common cancer-related genetic abnormality in human malignancy. Previous analyses of different types of tumors have shown that most of TP53 mutations affect exons 5–8, especially residues 130–286 encoding TP53 DNA-binding domain. Only rarely have mutations outside exons 5–8 been identified, including those in the introns of the splicing sites, which probably give rise to an aberrant RNA splicing [14]. In agreement with reported data, our study shows 41% of TP53 mutations in primitive tumors.

Methylation changes are sometimes associated with the ageing of normal epithelium but a new generation of studies is beginning to investigate the prognostic significance of the methylation of multiple genes in cancer. Only those methylation markers, which are always unmethylated in normal cells such as the oncosuppressor genes, might, therefore, be potentially useful for cancer detection [15]. Hypermethylation of the $p16^{INK4A}$ promoter has been reported in many primary tumors such as those of the esophagus, the lung, the cervix, and the colon, where it was found in 20%–40% [16]. In our study, 21% of the patients show p16 ^{INK4A} hypermethylation in tumor DNA. Furthermore, the hypermethylation observed by us did not appear to be age-related, since the normal tissue of the same patients showed no alteration. All the results of the analyses performed were in agreement with reported data.

The use of the three molecular markers seems to be extremely useful since this made it possible to detect free-circulating tumor-associated DNA in 76% of the patients. The results of our study confirm that it is possible to identify Ki-Ras, TP53 and the hypermethylation of the $p16^{INK4A}$ promoter alterations in DNA extracted from the pre-operative plasma of patients with colorectal cancer, and that PCR-SSCP and MSP are extremely sensitive methods for this [17, 18]. In fact, 38% of the patients with mutated

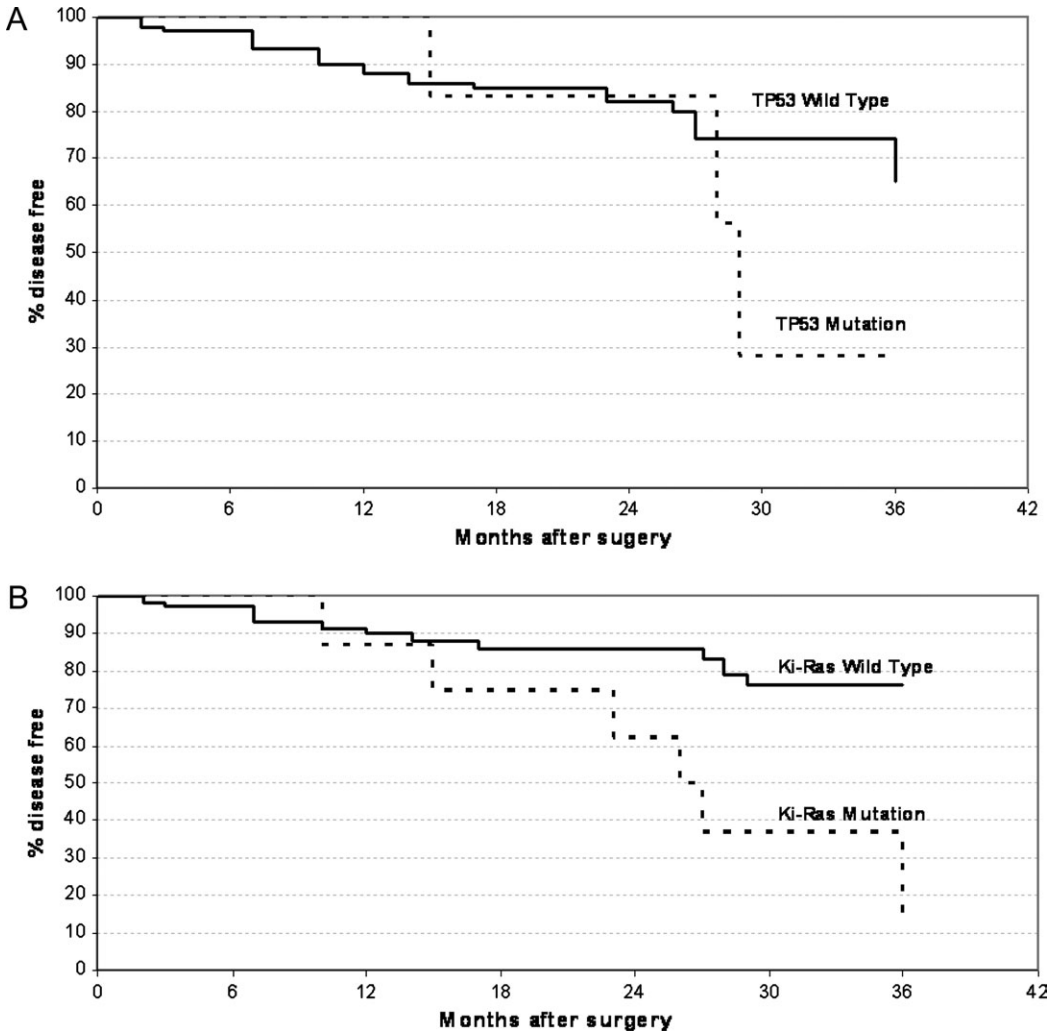

Figure 4. Disease-free survival (DFS) according to TP53 mutations (**A**) and Ki-Ras mutations (**B**).

Ki-Ras in the tumor tissue showed the same alteration in the plasma, 30% showed an alteration in TP53 and 17% presented hypermethylation of the $p16^{INK4A}$ gene promoter.

Very few data are available concerning the prognostic impact of plasma tumor DNA in cancer patients. Several studies, in fact, have demonstrated that plasma tumor-associated alterations in the Ki-Ras gene could predict surgical resectability and prognosis in pancreatic cancer patients [19]. A preliminary study conducted in CRC patients showed who were positive for Ki-Ras mutations in plasma DNA, were more likely to have large tumors and were less likely to have curative resection than those who were negative for plasma DNA mutations [12]. In our study the statistical analysis performed on the three variables considered independent has shown that Ki-Ras and TP53 mutations are significantly related to disease recurrence.

In conclusion, our data suggest that patients with high risk of recurrence can be identified by means of the analysis of tumor-derived plasma DNA with the use of fairly non-invasive techniques. Nevertheless, further studies are needed to reach a clearer understanding of the mechanisms leading to the presence of free-circulating tumor-associated DNA in the plasma. Genetic alterations in cancer DNA can be used to identify early cancers and it would be interesting to study the variation in circulating tumor DNA levels in relation to cancer treatment and patient prognosis [20].

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