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Specific TP53 and/or Ki-ras mutations as independent predictors of clinical outcome in sporadic colorectal adenocarcinomas: results of a 5-year Gruppo Oncologico dell'Italia Meridionale (GOIM) prospective study

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Background: Although Ki-ras and TP53 mutations have probably been the genetic abnormalities most exhaustively implicated and studied in colorectal cancer (CRC) progression, their significance in terms of disease relapse and overall survival has not yet clearly been established.

Patients and methods: A prospective study was carried out on paired tumor and normal colon tissue samples from a consecutive series of 160 previously-untreated patients, undergoing resective surgery for primary operable sporadic CRC. Mutations within the TP53 (exons 5–8) and Ki-ras (exon 2) genes were detected by PCR-SSCP analyses following sequencing.

Results: Mutation analyses of exons 5 to 8 of the TP53 gene showed mutations in 43% (68/160) of the cases, while mutation analyses of exon 2 of the Ki-ras gene showed mutations in 46% (74/160) of the cases. Multivariate analyses showed that clinical outcome were strongly associated with the presence of specific TP53 mutations in L3 domain alone (only in DFS) or in combination with specific Ki-ras mutations at codon 13.

Conclusion: Specific TP53 mutations in L3 domain alone (only in DFS) or in combination with specific Ki-ras mutations at codon 13 are associated with a worse prognosis in sporadic CRC.

Introduction

Colorectal Carcinoma (CRC) represents a major public health problem, especially in developed countries [1]. Numerous studies have already defined the genetic mechanisms of the adenoma-carcinoma sequence in CRC [2] and, although it has been suggested that in CRC progression the accumulation of multiple mutations is more relevant than the actual sequence of events, it has also been reported that Ki-ras mutations occur at the early stages of CRC development while TP53 mutations are usually predominant in advanced stages [3].

Ki-ras belongs to the ras gene family (H-, Ki- and N-ras) and encodes a protein with a molecular weight of 21 000 (p21) which is located within the inner plasma membrane and demonstrates GTPase activity and activation of gene products [4]. Oncogenic mutations of Ki-ras are involved in 40% (20–50%) of sporadic CRCs [5–7]. Most of the mutations (90%) are found in codons 12 and 13; less frequently they may also affect codon 61.

The TP53 tumor suppressor gene encodes a DNA sequence-specific transcription factor, a 53-kDa nuclear phospho-protein with multiple functions including gene transcription, DNA synthesis and repair, apoptosis, and angiogenesis [8]. Mutations in the TP53 gene are among the most frequently found genetic abnormalities in human cancers [9, 10]. In fact, mutations of the TP53 gene have been observed in more than 50% of sporadic CRCs [11]. The majority of these, occur in a highly conserved regions among species (area II, codons 112–141; area III, codons 171–181; area IV, 234–258; area V 271–286) that codify for the protein core domain. Several structural domains with distinct roles have been identified within these regions: the L2 loop (codons 163–195), required for the folding and stabilization of the central part of the protein; the L3 loop (codons 236–251) and the LSH (L1 loop-sheet-αhelix) motif (codons 273–286) within which at least two residues (241, 248 and 273, 280 respectively) contact the DNA directly [12].

Although a large number of primary sporadic CRC contain a mutated Ki-ras and/or TP53 genes, their relationship to

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the development of a metastatic phenotype and hence to patients' outcome is still not clear [13–17]. However, TP53 and Ki-ras mutations affecting regions which are important for particular functions of the proteins may have a stronger prognostic impact. In fact, it has recently been observed that the most frequent types of Ki-ras mutations in CRC are G → A transition [18] and G → T transversion [19], that the presence of a codon 12 glycine to valine mutation may predispose to more aggressive biological behaviour in patients with advanced CRC [20] and that specific codon 13 Ki-ras mutations are predictive of clinical outcome in CRC patients, whereas codon 12 K-ras mutations are associated with a mucinous histotype [21]. Moreover, it has been suggested that TP53 mutations in exon 7 [22], codon 245 [23], conserved areas [24] and the L3 structural domain [25–26] were associated with more CRC aggressive phenotype, presumably because these mutations have a particularly strong negative effect on the biological activity of the protein. Over the last few years, different studies have simultaneously analyzed Ki-ras and TP53 genes point mutations in CRC patients and their potential clinical usefulness [17, 27–28] but, to our knowledge, no other study has analyzed the prognostic role of specific Ki-ras and TP53 mutations.

The aim of our prospective study was to evaluate, in 160 patients who had undergone curative surgical resection for primary sporadic CRC, whether specific TP53 and/or Ki-ras genes mutations might have a particular prognostic significance in CRC progression. For this reason, we performed a multivariate analyses considering two different models in which specific TP53 and /or specific Ki-ras are evaluated for both distinct and combined variables.

Materials and methods

Patient features

A prospective study was performed on paired tumor and normal colon tissue samples from a consecutive series of 160 previously untreated patients, undergoing resective surgery for primary operable sporadic CRC at a single institution (Department of Oncology, University of Palermo) from January 1988 to December 1992.

Briefly, none of the patients had any history of previous neoplasias, synchronous or metachronous CRC. This series of patients comprised 84 females and 76 males with a median age of 66 years (range 31–88). A total of 137 patients were potentially cured by means of radical surgical tumor resection with regional *en bloc* lymphadenectomy proximally up to the origin of the vascular trunks. Twenty-three patients had either non-radical surgery or distant metastasis.

All tumors were histologically confirmed to be colorectal adenocarcinomas. Patients with Dukes' stage A and B sporadic CRC were treated with surgery alone, whereas only 10 patients with Dukes' stage C received adjuvant chemotherapy with 5-FU, leucovorin and levamisole since in the period previous to 1991 hardly any of the patients had received adjuvant treatment. Patients with non-radical surgery and/or distant metastasis were treated with 5-FU and leucovorin. Postoperatively, all patients were checked at 3-monthly intervals for the first 2 years, at 6-monthly intervals for the next 2 years, and annually thereafter. Written informed consent was obtained from all patients included in this study.

Tissue handling

Multiple samples of the primary tumor tissue were taken from different tumor areas (including the core and the invasive edge of the tumor). Furthermore, multiple samples of normal mucosa (as confirmed by histology) were taken from macroscopically uninvolved areas 20–40 cm away from the tumor site, to be used as a control for biomolecular and flow-cytometric analyses. The tissues were bisected, one half of each sample was processed for pathological examination, and the remaining half of the sample pool was immediately frozen and stored at −80°C until analyzed. The adequacy of the material was checked on frozen tissue sections and only tissue samples with >80% tumor content were utilized in subsequent biomolecular analyses. Evaluation of each biomolecular sample was performed independently by researchers who had no knowledge of the clinical data regarding the samples.

Detection of TP53 and Ki-ras gene mutations

High molecular weight genomic DNA was extracted as previously described [29] from primary sporadic CRC and normal colon specimens. Mutations within the TP53 (exons 5–8) and Ki-ras (exon 2) genes were detected by PCR-SSCP (Single Strand Conformation Polymorphism) analyses following sequencing, performed as described previously [30]. The quality and the concentration of the amplification products were verified by 1.5% agarose gel electrophoresis and ethidium bromide staining. 100 mg aliquots of the amplified DNA fragments, purified and concentrated by filtration through Microcon 50 columns (Amicon, Beverly, MA) were denatured and analyzed by SSCP analyses. DNA of normal colon tissue from each patient was also amplified and run in parallel with matched tumoral DNA samples on SSCP gels, to evaluate the occurrence of germline mutations or polymorphisms. Individual ssDNA fragments with shifted mobilities, compared to normal controls, were electroeluted from polyacrylamide gel, reamplified and subjected to automated sequencing by 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) as described previously [30].

Statistical analyses

Fisher's exact test (StatXact Turbo, Cytel Software Corporation, Cambridge, MA, USA) was used to evaluate the associations between biological variables. The relationship of different prognostic variables to disease-free survival (DFS) and overall survival (OS) was assessed univariately by means of the Kaplan and Meier method. Survival time was calculated from the date of surgery to the date of death (cancer-related causes) or last follow-up, with times censored for patients dying of causes unrelated to CRC and those surviving. DFS was measured from the day of primary surgery to the date of the first relapse (locoregional or metastatic). Significant differences among survival curves were checked by the log-rank test and Wilcoxon test, or a test for trend when appropriate. Multivariate analyses was carried out by means of Cox proportional hazards model, using a backward procedure [24]. P values <0.05 were considered significant.

Results

Mutation analysis of TP53 gene

Mutation analysis of exons 5 to 8 of the TP53 gene showed mutations in 43% (68/160) of the cases (Figure 1). In particular, 84 TP53 mutations were identified in 68 of the 160 screened CRCs, and sequence data were obtained for 81. Of the 84 mutations, 19% (16 of 84) were in exon 5, 32% (27 of 84) in exon 6, 36% (30 of 84) in exon 7 and 13% (11 of 84)

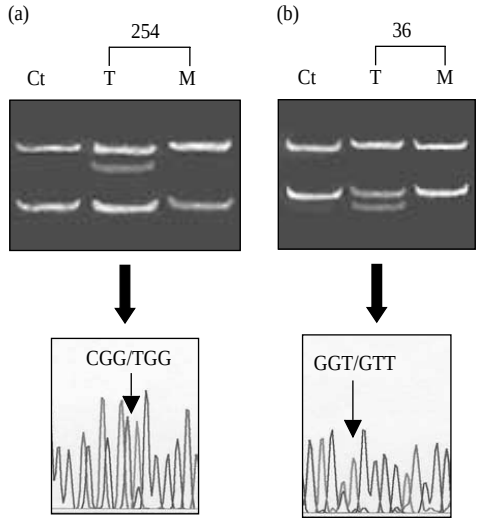

Figure 1. (a) SSCP analyses of exon 7 of the TP53 gene, amplified from colorectal carcinoma and mucosa genomic DNA of 1 patients (254). The extra bands visualized in lanes 2 correspond to ssDNA molecules harbouring mutations in codon 248 (CGG → TGG); (b) SSCP analyses of exon 1 of the k-ras gene, amplified from colorectal carcinoma and mucosa genomic DNA of 1 patients (36). The extra bands visualized in lanes 2 correspond to ssDNA molecules harbouring mutations in codon 12 (GGT → GTT).

in exon 8. Fourteen tumor samples presented two (9 in the same exon and 3 in two different exons) or three (2 in two different exons) different TP53 mutations. Nineteen of the 81 sequenced mutations (23%) were found to be frameshifts (15 microdeletions and 4 microinsertions) whereas 62 (77%) were single-nucleotide substitutions. 82% (51 of 62) of the latter were mis-sense (45 of 62) or nonsense (6 of 62) mutations, while silent mutations were found in 11 cases (9 of them being in codon 213, a previously identified site of polymorphism). Moreover, transitions (81%, 50 of 62) were far more frequent than transversions (19%). No germ-line mutations were found indicating that in every case the change was somatic. Tumors with TP53 mutations were classified into two groups: 18/68 cases (26%) with mutations of the L3, 11/68 cases (16%) with mutations of the LSH motif and 39/68 cases (58%) with mutations outside L3 and LSH. Since silent mutations do not determine any amino acid change in the protein, they have been included in the wild type group for statistical analyses.

Mutation analyses of the Ki-ras gene

Mutation analyses of exon 2 of the Ki-ras gene showed mutations in 46% (74/160) of the cases (Figure 1). Eighty Ki-ras mutations were identified in 74 of the 160 screened CRCs. Of the 80 mutations, 57% (46 of 80) were found in codon 12, and 43% (34 of 80) in codon 13, (68/74 tumors presented single mutations, 3/80 a double mutation in codon 12, and 3/80 a mutation in codon 12 and one in codon 13). The most frequent mutation in codon 12 was GGT to GAT (Gly→Asp), which was observed in 17/46 cases (37%).

Table 1. Patient characteristics ($n = 160$)

Characteristic	No. Pts
Sex:	
Male	76
Female	84
Age:	
<55	29
55–75	96
>75	35
Site:	
Proximal tumor	31
Distal tumor	129
Tumor size (cm):	
≤5	60
>5	100
Dukes' Stage:	
A	40
B	51
C	41
D	28
Node status:	
Negative	101
Positive	59
Tumor growth:	
Expansive	20
Infiltrative	140
Tumor grade:	
Well differentiated (G1)	23
Mod differentiated (G2)	104
Poorly differentiated (G3)	33
Tumor type:	
Adenocarcinoma NOS	137
Mucinous	23
Lymphoematives invasion:	
None	45
Present	115
Lymphocytic infiltrate:	
Prominent	48
Non-prominent	112
Surgery:	
Curative resection	137
Non curative resection	23

The most frequent mutation in codon 13 was GGC to GAC (Gly → Asp) which was detected in 32/34 cases (94%).

Overall, transitions (70% 56 of 80) were the predominant alteration. No germ-line mutations were found indicating that in every case the change was somatic.

Uni- and multivariate analyses of prognostic factors

The median follow-up time in our study group was 71 months (range 34–115 months). The median survival of the whole group was 43 months. At univariate analyses, distal cancers, infiltrative tumor growth, advanced Dukes' stage, node status positive, lymphohematic invasion, DNA-aneuploidy, high SPF, the presence of TP53 mutations in L3, Ki-ras mutations in codon 13 and TP53 and/or Ki-ras in L3 and/or in codon 13, proved to be significantly related to quicker relapse whereas these same factors, and in addition non-curative resection were significantly related to shorter overall survival (Table 2).

At multivariate analyses we considered a model with TP53 and/or Ki-ras as a distinct variable (Model DV) and a model with TP53 and/or Ki-ras as a combined variable (Model CV) (Table 3). Multivariate analyses in both models was adjusted for DFS according to Dukes' stage, lymphohematic invasion, DNA-aneuploidy and high SPF; while for OS according to Dukes' stage, surgical resection, DNA-aneuploidy and high SPF.

The major significant predictors for both disease relapse and death were TP53 mutations affecting the L3 loop, while Ki-ras mutations affecting codon 13 were the only independent factors for death (Table 3). Moreover, when the TP53 and Ki-ras mutations were considered together, mutations affecting p53 in the L3 loop and/or Ki-ras in codon 13 were independent factors for both disease relapse and death.

Table 2. Univariate (distribution of pts only for the significant TP53 and/or Ki-ras variables) disease-free and overall survival analyses

	No. Pts	DFS (%) 5 yrs	*P*	No. pts	OS (%) 5 yrs	*P*
[a]TP53						
No. mutations	87	56		100	49	
Mutations outside LSH/L3	26	27		29	34	
Mutations in LSH	9	33		11	27	
Mutations in L3	13	15		18	11	
[b]Ki-ras						
No mutations	77	53		86	47	
Mutations in cod.12	34	56		40	47	
Mutations in cod. 13	27	22		34	18	
TP53 and/or Ki-ras						
No mutations	54	60		60	54	
Mut. Outside L3 and/or cod.13	46	52		53	45	
Mutations in L3 and/or cod.13	36	22		46	17	

[a] All mutation subgroups are compared with patients with no mutations (wild type TP53).

[b] All mutation subgroups are compared with patients with no mutations (wild type Ki-ras).

Pts: Patients; DFS: Disease-Free Survival; OS: Overall Survival.

Table 3. Multivariate disease-free and overall survival analyses

	DFS ($n = 138$)		OS ($n = 160$)	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
1. Model DV[a]				
P53:				
L3 vs no mutation	2.14 (1.06–4.32)	<0.05	2.26 (1.21–4.21)	<0.05
Ki-ras:				
Mut. in codon 13 vs no mutation			1.86 (1.10–3.17)	<0.05
2. Model CV[b]				
p53 and/or Ki-ras:				
No mutations	1		1	
Mutations in L3 and/or codon13 vs no mutations	2.42 (1.32–4.43)	<0.01	2.24 (1.30–3.84)	<0.01

[a]model with TP53 and/or Ki-ras as Distinct Variable;

[b]model with TP53 and/or Ki-ras as Combined Variable; Pts: Patients; DFS: Disease Free Survival; OS: Overall Survival; HR: Hazard Ratio; CI: Confidence Interval.

Multivariate analyses was adjusted for DFS according to Dukes' stage, lymphatic invasion, DNA-ploidy and SPF; while for OS according to Dukes' stage, surgical resection, DNA-ploidy and SPF. Mutations in codon 12, outside L3 and mutations outside L3 and/or codon13 are not independent variables for DFS and OS both in DV and CV models.

Discussion

Although Ki-ras and TP53 mutations have probably been the genetic abnormalities most exhaustively implicated and studied in sporadic CRC progression [3], their significance in terms of disease relapse and overall survival has not yet been clearly established. This variability can be explained by several factors, such as the different methods used to assess TP53 or Ki-ras mutations (SSCP; DGGE, Denaturing Gradient Gel Electrophoresis; TGGE, Temperature Gradient Gel Electrophoresis; direct sequencing), the type of tumor storage (fresh/frozen tissue and paraffin embedded blocks) and an intrinsic tumoral heterogeneity that might mask, mainly in the cases with Dukes' stages A-B CRC, the lower percent of single cells with these genetic alterations. Furthermore, the specific features of the patient cohorts enrolled in the study may give rise to artifacts in mutational and, thus, in statistical analyses. For example, it has been reported that specific Ki-ras mutations in codon 12 were associated with mucin histotype [21], while it is well known that patients at an advanced Dukes' stage (C and D) generally present a higher rate of TP53 mutations [31, 32]. Another source of discrepancy concerning TP53 mutations and prognosis of CRC may be related to differences between immunohistochemical and molecular genetic analysis of TP53. Several factors have been taken into consideration as an explanation for the fact that TP53 mutations are mostly mis-sense leading to a protein with a prolonged half life which makes it immunohistochemically detectable only in 50–80% of cases [33]. Finally, it should be

considered that the majority of the studies have used a retrospective design, so it has not been possible to draw final recommendations about the application of such aberrations as prognostic factors.

In the present study, we have prospectively evaluated the prognostic value of Ki-ras and TP53 gene mutations detection in a series of 160 patients with sporadic CRC, taking special care in using a methodologic approach that is suitable for reproduction in other laboratories. In accordance with several other reports [11, 26, 32], 43% of all the mutations observed in our series (68/160) were found in a region of the gene which includes two important regions for TP53 binding to DNA. One of these contains the amino acids needed for DNA interaction, in particular those which are part of the L3 Zinc-binding domain and of the LSH motif. In our own series, 26% (18/68) of the mutations occurred in L3 and 16% (11/68) in LSH, in accordance with the results reported by Borresen-Dale [25].

For Ki-ras gene, we found a mutation rate in exon 2 of 46% (74/160) for a total of 80 mutations: 57% (46/80) in codons 12 and 43% (34/80) in codon 13; this is hardly surprising since most of the mutations found in human tumors involve these two codons, coding for two adjacent glycines located in proximity of the catalytic site of RAS. This means that different Ki-ras mutations resulting in the incorporation of different amino acids at these positions leads, albeit to a different degree, to a reduction of the intrinsic GTPase activity of RAS. In accordance with several other studies [13–21] these results show that not all mutations are equal and that different mutational types may can confer different biological properties.

Our report showed that specific TP53 in the L3 domain and/or specific Ki-ras mutations at codon 13 are strong prognostic indicators of worse survival, while only specific TP53 in L3 domain, alone or in combination with Ki-ras mutations at codon 13, were an independent prognostic variable of DFS in pts with primary sporadic CRC. These results suggest that specific Ki-ras mutations in codon 13 have an effect on DFS only when the mutation in the L3 domain of TP53 gene is present. These findings also show that specific TP53 in L3 domain and Ki-ras mutations at codon 13 maintain their predictive role even the presence of established traditional prognostic variables such as Dukes' stage, lymphatic invasion or surgical resection.

In conclusion, we have provided evidence that specific TP53 and Ki-ras mutations have a divergent effect on the structure, stability and function of the protein. In the analyses of the prognostic value of Ki-ras and TP53 on sporadic CRC, the type of mutations and its effect on biological function should be considered. Multivariate analyses showed that clinical outcome were strongly associated with the presence of specific TP53 mutations in L3 domain alone (only in DFS) or in combination with specific Ki-ras mutations at codon 13. Unfortunately, to our knowledge, no other study has analyzed the prognostic role of specific Ki-ras and TP53 mutations in CRC progression. If our results on these biological indicators (specific TP53 mutations in L3 domain and/or Ki-ras mutations at codon 13) are confirmed by large prospective studies, it will be possible to provide additional and important useful information for prognosis and a rational basis for the development of more specific clinical treatments (more radical surgery, and/or adjuvant chemotherapy or radiotherapy). In particular, efforts should therefore be directed towards subsets of sporadic CRC patients with a high risk of relapse or death, especially those with Dukes' stages B (Astler-Coller B2) and C.

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