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Original Paper

Differential Prognosis of Replication Error Phenotype and Loss of Heterozygosity in Sporadic Colorectal Cancer

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Several distinct genetic alterations have been associated with colorectal tumorigenesis. This study investigated the frequency of microsatellite instability, also known as replication error (RER), and loss of heterozygosity (LOH) at six chromosome regions in sporadic colorectal cancer (CRC). Eighty-six tumour and paired normal mucosa samples were included in the study. A polymerase chain reaction (PCR)-based technique was performed to analyse six (CA)n dinucleotide repeats located near or within regions containing important genes implicated in the complex process of colorectal tumorigenesis (chromosomes 2p, 3p, 5q, 11p, 17p and 18q). Overall, LOH frequency was higher in RER^- tumours (25/46, 54.3%) compared with RER^+ tumours (9/40, 22.5) ($P = 0.04$). To investigate prognostic implications, survival analysis was performed for 66 patients. Compared with RER^- tumours, patients with RER^+ tumours at 2p, 3p, 5q, 11p or 18q were found to have an improved prognosis (overall survival, $P = 0.02$ and disease-free survival (DFS) $P = 0.005$) this variable being an independent prognostic factor by multivariate analysis ($P = 0.001$). Overall survival of patients whose tumours were LOH^+ was significantly shorter compared with those without LOH (overall survival, $P = 0.008$ and DFS, $P = 0.01$). Thus, tumours displaying RER^+ and LOH^+ phenotype, as established by microsatellite analysis, show a differential prognosis. These data indicate that this may be a useful tool for the identification of patients at different risks affected by CRC.

Key words: colorectal cancer, prognosis, replication error, loss of heterozygosity, microsatellite analysis
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INTRODUCTION

A SERIES of genetic alterations involving both proto-oncogenes and tumour suppressor genes occur in colorectal tumorigenesis [1]. Thus, it is widely accepted that multiple genetic alterations affecting proto-oncogenes, such as *ras*, and tumour suppressor genes, such as *APC* and *TP53*, are involved in the development and/or progression of colorectal cancer [1, 2].

Genome-wide searches for loss of heterozygosity (LOH) have been performed successfully to localise tumour suppressor gene loci [3, 4]. The observation that LOH may have prognostic implications has created a situation in which the study of this abnormality provides information significant enough to suggest that, for clinical purposes, tumours of certain types should be typed routinely for LOH at specific loci [5].

In the past few years, a new potential mechanism that could increase the usually rare rate of spontaneous mutations has been described. This potential 'mutator' defect has been referred to as either microsatellite instability (MSI) or the replication error (RER) phenotype [6]. This mechanism involves alterations on short tandem repeat DNA non-coding sequences (microsatellites). Although the function of these sequences is not yet clear, some researchers have speculated that such tandem repeats may be targets for certain proteins, which play a major role in the regulation of gene expression and DNA recombination [7, 8]. Moreover, four different mutant genes, all homologous to bacterial DNA repair genes, have been associated with the RER phenotype [9–11].

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RERs have been demonstrated to be a manifestation of a general underlying carcinogenic defect inherited in families with HNPCC (hereditary non-polyposis colorectal cancer) [12]. Simultaneous with the discovery of microsatellite instability in colon cancers of HNPCC families, it was shown that this defect also occurs in a number of sporadic colon cancers [6, 12–14]. The likely prognostic importance of recognising the RER phenotype in different tumour types was suggested in a few reports. This encouraged further studies to advance the application of this new knowledge to improve the identification of people at risk of cancer, and to open new strategies for treating the large subset of RER-type cancers [9].

In this study, we evaluated 86 colorectal cancer (CRC) patients, detecting RER and LOH by microsatellite analysis at (CA)n dinucleotide repeats on chromosomes 2p, 3p, 5q, 11p, 17p and 18q. Most of these microsatellite markers were chosen near or within regions containing mismatch repair genes, such as *MSH2* on chromosome 2p and *MLH1* on chromosome 3p, or thought to contain some tumour suppressor genes. The results of this study indicate that detection of RER and LOH tumour status by microsatellite analysis is a simple and reliable tool, prognostically useful in CRC patients.

PATIENTS AND METHODS

Patients and tumour samples

Eighty-six freshly resected colorectal adenocarcinomas were obtained from 86 patients (38 females and 48 males, with an average age of 66.2 ± 12.3 years) undergoing radical surgery between 1990 and 1995 at San Carlos Hospital in Madrid. Genomic alterations were analysed in samples containing more than 80% of tumour cells. In all cases non-tumour tissues selected from macroscopically normal areas of surgical specimens were used as control.

Tumours were pathologically staged as Dukes A–D according to Turnbull's modification of Dukes original staging [15] and consisted of 8 Dukes A, 35 Dukes B, 27 Dukes C and 16 Dukes D. Twenty-seven tumours were located in the left colon, 20 in the right colon and 39 in the rectum. The histological classification of tumours was established according to previous criteria [16].

DNA extraction

In order to confirm the presence of more than 80% of tumour cells, cryostat sections stained with haematoxylin and eosin from each tumour block were microscopically examined by two independent pathologists. Representative sections from the same blocks previously examined were submitted to DNA extraction as described by Blin and Stafford [17]. By the same method, corresponding normal mucosa from all 86 patients were also included.

Polymerase chain reaction (PCR) analysis

DNA samples were analysed by PCR for RER and LOH at six loci (Table 1). Primer pairs were synthesised in an Oligonucleotide synthesiser (Gene Assembler Plus, Pharmacia LKB, Uppsala, Sweden). The microsatellites studied consisted of CA dinucleotide repeats and their heterozygosity indices were D2S119 (73.6%), G219511 (91.9%), D5S299 (73.6%), D11S904 (83.9%), G29672 (95.4%) and G18S58 (89.6%).

PCRs were performed in a Thermocycler Perkin Elmer (Gene Amp PCR System 2400) and were carried out in a 20 µl volume containing: 50 ng of genomic DNA, 1 µM of each primer, 0.2 mM of each deoxynucleoside triphosphate, 10 mM Tris HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.5 µl (α^{32}P)dCTP (3,000 Ci/mmol) (Amersham, Buckinghamshire, U.K.) and 1 U of Dynazyme Thermostable DNA Polymerase (Finnzymes OY, Helsinki, Finland). PCR conditions were as follow: initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 0.5 min, 55°C for 0.5 min and 72°C for 1 min. Final extension was 72°C for 7 min. PCR products were denatured by 95% formamide and electrophoresed on 7 M urea polyacrylamide gels for 3 h at 43 W followed by autoradiography. RER appeared as a change in the length of microsatellite sequences (expansions or contractions in tumour DNA compared with constitutional DNA), and LOH was considered when the complete loss of one or both alleles of the repeated locus appeared.

Statistical analysis

RER and LOH at each locus were assessed for potential associations with a number of clinicopathologic parameters, including gender and sex of the patients, and Dukes stage and site of the primary tumour. Associations of categorical variables were assessed using the chi-square test. A P value < 0.05 was judged to be significant.

Distributions of overall survival (defined as the time from initial diagnosis to death) and disease-free survival (DFS) (defined from the date of surgery until the time of tumour recurrence) were estimated with the Kaplan–Meier method, and comparisons were made with log-rank statistics. Results

Table 1. *Microsatellite markers used in the study*

Marker	Chromosome location	Primer pairs	Product length (bp)	Reference
D2S119	2p16	s 5′CTTGGGGAACAGAGGTCATT3′ a 5′GAGAATCCCTCAATTTCTTTGGA3′	221	[18]
G219511	3p23	s 5′TGTGAATTATATGAAGAGAT3′ a 5′TATGTAATATGTCTGTGGTG3′	197	[19]
D5S299	5q15-21	s5′GTAAGCAGGACAAGATG3′ a 5′GCTATTCTCTCAGGATCTTG3′	182	[20]
D11S904	11p14-11p13	s 5′ATGACAAGCAATCCTTGAGC3′ a 5′CGTTCTTATATCCCTAAAGTGGTGA3′	198	[21]
G29672	17p13.1	s 5′ACTGCCACTCCTTGCCCCATTC3′ a 5′AGGGATACTATTCAGCCCGAGGTG3′	118	[22]
D18S58	18q22.3-18q23	s 5′GCTCCCGGCTGGTTTT3′ a 5′GCAGGAAATCGCAGGAACTT3′	160	[23]

s, sense; a, antisense.

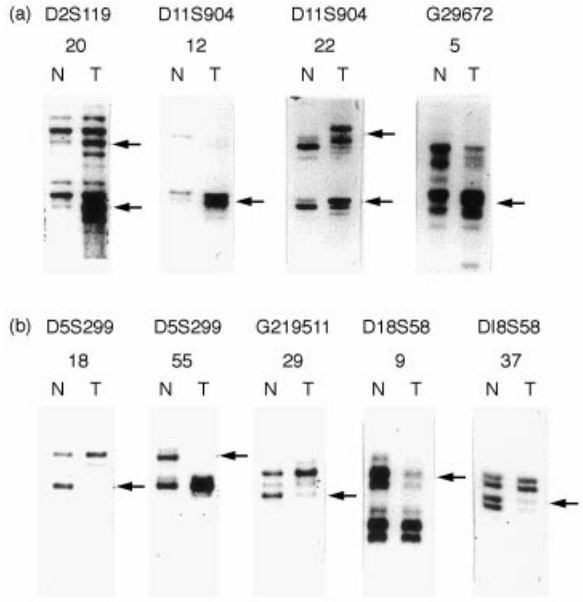

Figure 1. (a) Examples of microsatellite instability. Arrows refer to alterations in electrophoretic mobility of PCR products from tumour compared with normal tissue DNA. (b) Examples of loss of heterozygosity in colorectal cancer. Arrows refer to absent polymorphic repeat indicating LOH in the tumours. Chromosome locus and patient number are indicated in each case. N, normal tissues; T, tumour tissues.

were considered significant for P values <0.05. For survival analysis, only patients who had had potentially curative surgery (patients who had Duke's stages A, B and C tumours) were considered. Also excluded were 4 patients who died in the postoperative period. Thus, the number of patients included in the survival study was 66. The median follow-up period was 63.6 months. The Cox proportional hazards model was used to identify which independent factors jointly had a significant influence on survival.

RESULTS

We examined 86 primary sporadic colorectal tumours obtained from 86 patients, for RER and LOH, by microsatellite analysis at six loci. DNA of normal tissue from each patient was simultaneously analysed. Representative results are shown in Figures 1 and 2.

Table 2. Frequencies of microsatellite instability for each one of the markers considered

Marker	RER$^+$ tumours No. (%)
D2S119 ($n=64$)	4 (6.2)
G219511 ($n=80$)	10 (12.5)
D5S299 ($n=64$)	9 (14.1)
D11S904 ($n=73$)	9 (12.3)
G29672 ($n=83$)	16 (19.3)
D18S58 ($n=78$)	13 (16.7)

RER, replication error.

To detect RER phenotype, a total of 516 analyses were performed. Overall, 40 (46.5%) tumours showed RER at least in one of the locus investigated; 10 (11.6%) in two or more loci, and 5 (5.8%) in three or more of the markers considered. Tumours showing instability at two or more loci were considered as the group of high instability. The frequency of RER$^+$ was random among various loci without clear evidence of clustering (Table 2). In Table 3, the frequencies of LOH for each of the loci analysed for informative tumours only are shown. In comparison with normal tissue, LOH was detected for one or more loci in 34 cases (39.5%). Eight tumours were LOH$^+$ for two or more loci, whilst the remaining 26 tumours were LOH$^+$ for only one locus. Overall, the LOH frequency was higher in RER$^-$ tumours (25 of 46, 54.3%) than in RER$^+$ tumours (9 of 40, 22.5%) ($P=0.04$). Within the group of high instability, we only found one tumour with allelic imbalance.

Regarding associations between RER phenotype and other abnormalities that had been previously detected in our tumour population, such as *K-ras* and *TP53* mutations, no significant correlations were found. Of 40 RER$^+$ tumours, 11 (27.5%) harboured *K-ras* mutations, and of 46 RER$^-$ tumours, 14 (30.4%) showed activating mutations ($P=0.824$). Moreover, 15 (37.5%) of 40 RER$^+$ tumours and 8 (17.4%) of 46 RER$^-$ tumours had mutations affecting the *TP53* gene ($P=0.111$).

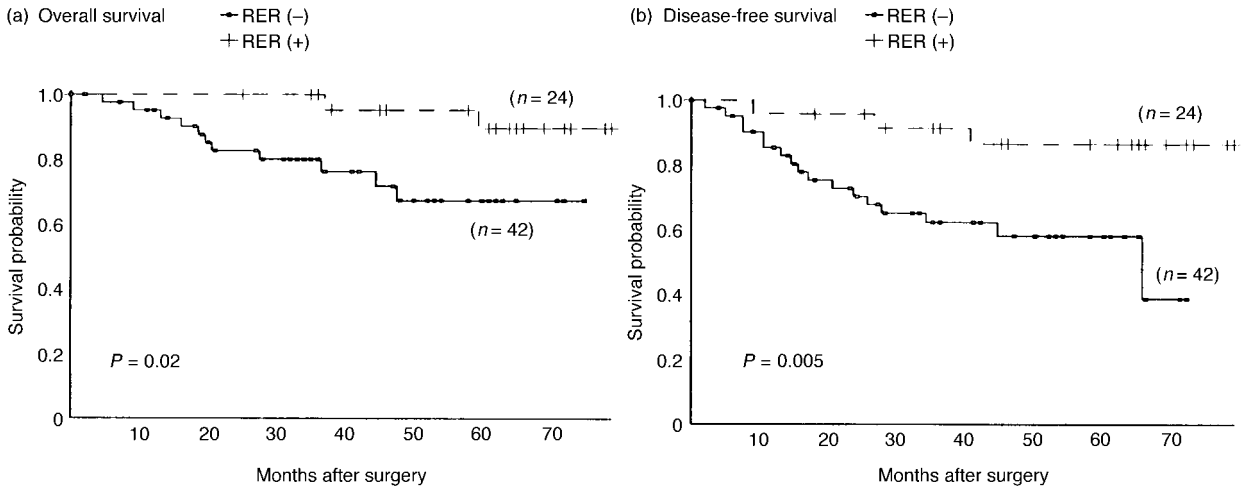

Figure 2. Kaplan–Meier survival curves showing the association of replication error (RER) in 2p, 3p, 5q, 11p or 18q with overall survival (a) and disease-free survival (DFS) (b).

Table 3. Loss of heterozygosity at various loci

Marker	No. informative tumours	LOH$^+$ tumours No. (%)
D2S119	64	2 (3.1)
G219511	80	8 (10.0)
D5S299	64	12 (18.7)
D11S904	73	3 (4.1)
G29672	83	14 (16.9)
D18S58	78	6 (7.7)

The potential impact of RER and LOH on tumour progression was determined by examining the association with a variety of clinical and pathological characteristics of the primary colorectal cancers. No significant correlations were found between the molecular alterations examined and age or gender of patients. Considering other clinicopathological variables, such as Duke's stage or location, the group of tumours with high instability (that is instability at two or more loci) was significantly correlated with proximal colon locations ($P = 0.02$). When we established these relationships individually for each of the markers evaluated, we found borderline or significant associations between RER at D2S119, D5S299 or D11S904 and tumours located on the right side of the colon ($P = 0.05$, $P = 0.01$ and $P = 0.02$, respectively). With regard to LOH phenotype, no associations with the clinicopathological features evaluated in this work were found.

In addition, RER and LOH at each locus were assessed for their association with overall survival and DFS. Patients with RER$^+$ tumours on 2p, 3p, 5q, 11p or 18q were found to have an improved prognosis: their median survival was 76 months compared with 59.6 for patients with RER$^-$ tumours at that loci (overall survival, $P = 0.02$, and DFS, $P = 0.005$, Figure 2). DFS for the group of patients with high instability tumours was 100%. Moreover, when we focused the survival analysis on the two chromosome regions containing mismatch repair genes (RER at 2p or 3p), a trend toward a better prognosis for patients showing RER$^+$ tumours was found (overall survival, $P = 0.07$, and DFS, $P = 0.02$, Figure 3).

Survival of patients whose tumours were LOH$^+$ was significantly shorter than that of patients whose tumours did not show LOH (median survival of 73.7 months for LOH$^-$, and 57.4 for LOH$^+$ patients), (overall survival, $P = 0.008$, and DFS, $P = 0.01$, Figure 4). The worst prognosis was found for

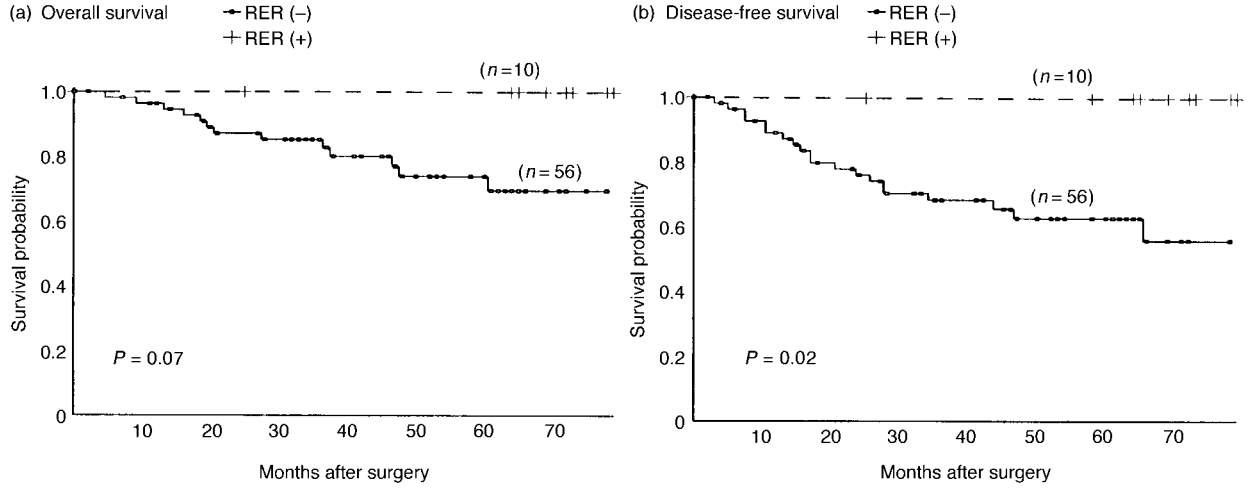

Figure 3. Kaplan–Meier survival curves showing the association of replication error (RER) in D2S119 (chromosome 2p) or G219511 (chromosome 3p) with overall survival (a) and disease-free survival (DFS) (b).

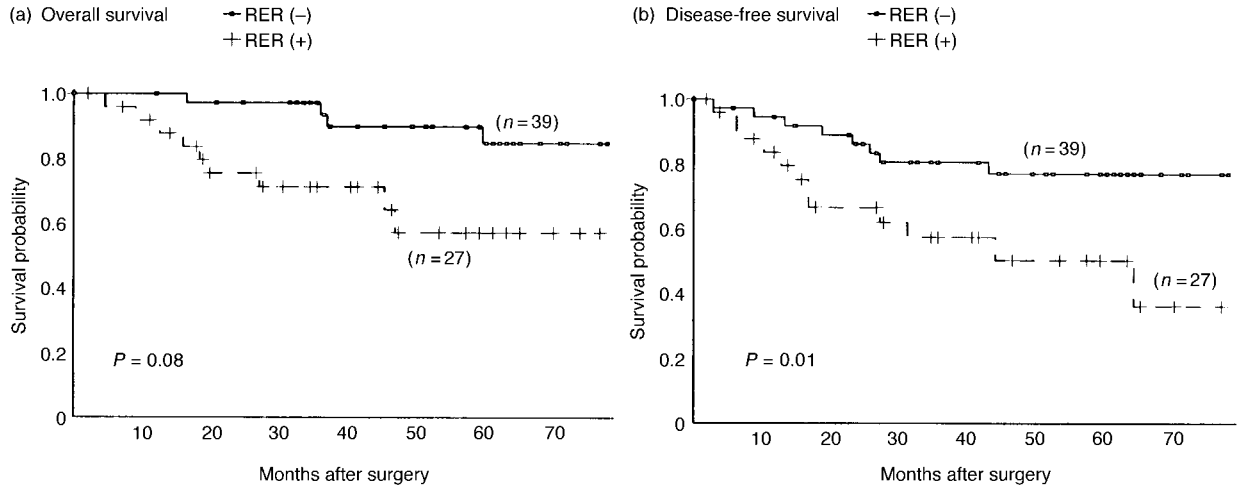

Figure 4. Relationship between loss of heterozygosity (LOH) status and overall survival (a) or disease-free survival (DFS) (b).

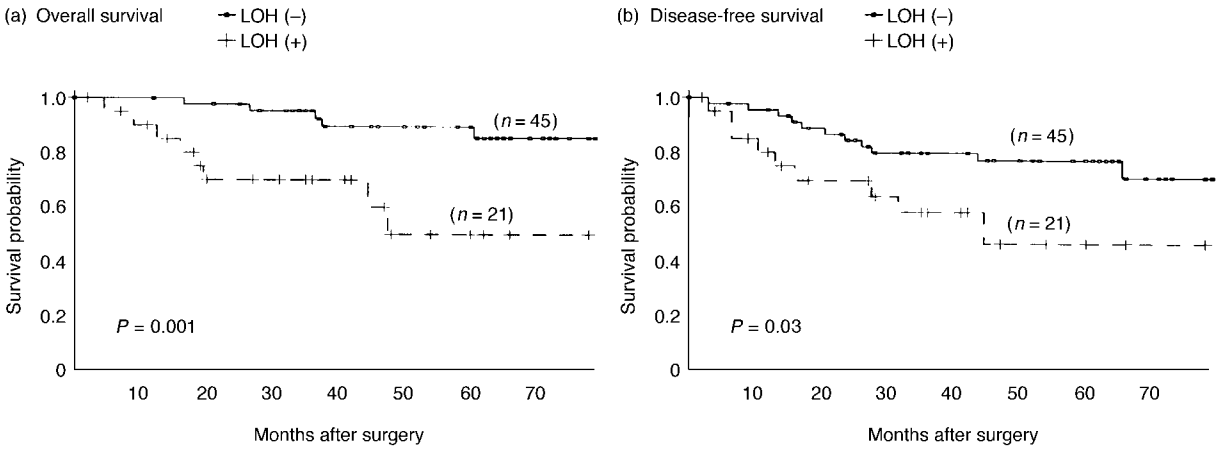

Figure 5. Relationship between loss of heterozygosity (LOH) status at D5S299 (chromosome 5q), G29672 (chromosome 17p) or D18S58 (chromosome 18q) and overall survival (a) or disease-free survival (DFS) (b).

patients whose tumours had LOH on markers located at chromosomes 5q, 17p or 18q (overall survival, $P = 0.001$ and DFS, $P = 0.03$, Figure 5). The median survival was 73.4 months for patients with LOH^- tumours at D5S299, G29672 or D18S58, and 48 months for patients LOH^+ at those loci.

By multivariate analysis, the only independent predictors of survival found were the presence of RER at 2p, 3p, 5q, 11p or 18q ($P = 0.001$) and Duke's stage (C versus A/B) ($P = 0.007$). The decreased and increased hazards were 6.22 (95% confidence interval (CI), 1.7–22.7) and 5.32 (95% CI, 1.1–24.9), respectively.

DISCUSSION

Colorectal cancer was the first type of human malignancy in which RER was described [6, 12–14]. Genomic instabilities reported by these authors occurred in hereditary non-polyposis as well as sporadic CRC. Subsequently, RER was described in sporadic cancers of different origins [24–28].

Our approach consisted of examining six microsatellite markers located near or within regions containing important genes implicated in the complex process of colorectal tumorigenesis. Two of these markers were selected in relation to mismatch repair genes: *MSH2* (chromosome 2p) and *MLH1* (chromosome 3p). Four more markers, located in the regions of *APC* (chromosome 5q), *H-ras* (chromosome 11p), *TP53* (chromosome 17p) and *DCC* (chromosome 18q) were also analysed.

In the present series of patients with resected primary sporadic CRCs, the frequency of MSI at multiple loci was similar to data reported previously [6, 13]. In relation to LOH, the number of tumours assessable for LOH for each chromosome arm varied, since the number of informative tumours for each marker were different. Although the frequency of LOH in this study was similar to those reported previously [29], they are lower compared with what has been published in other series of CRCs [30, 31]. A variety of reasons could explain these discrepancies. Firstly, allelic losses in general have been associated with advanced colorectal tumours, the highest incidences being described in resected Duke's stage D tumours [31]. In our tumour population only 18.6% of cases were classified as Duke's stage D. Secondly, we considered LOH^+ status when the complete loss of one or both alleles of the repeated locus appeared. By contrast, other authors assign LOH^+ status when the relative intensity of the two alleles in the tumour DNA differs from the relative intensity in the normal DNA by a factor, considering that small fractions of normal cells may appear as contaminants in tumour tissues.

Our results have indicated a significant negative association between the two molecular events investigated. This negative correlation may be due to two different groups of tumours, promoted through two distinct pathways, occuring in our tumour population. In fact, mutations (insertions and deletions of one or a few nucleotides) in microsatellites are the distinctive feature that characterises the 'microsatellite mutator phenotype' (MMP) pathway for cancer [32]. According to this and other authors, cancer genes in tumours of the MMP are generally different from those found in tumours of the classical suppressor pathway [6]. Therefore, strictly speaking, cancer genes are directly involved in cell proliferation, having a positive or a negative role in cell growth, differentiation, senescence or survival. However, genes related to the MMP pathway—mutator genes and cancer susceptibility genes—are involved in the repair of DNA mutations or in any of the multiple types of DNA transactions, mutations in which influence the probability of occurrence of mutations in cancer genes [32].

Moreover, our results show a lack of correlation between the presence of ubiquitous microsatellite mutations and mutations in *K-ras* and *TP53* genes. These and similar data previously published [10, 11] indicate that two apparently mutually exclusive pathways have to be considered in colorectal tumorigenesis.

With regard to biological features of tumours, none of the tested associations was found to be statistically significant, except for the association of high instability (RER^+ at two or more loci) and right-colon location. When analysed separately, considering abnormalities affecting each one of the six markers, our data showed a borderline or significant association between RER at D2S119, D5S299 or D11S904 and tumours located in the right colon. These results agree with previous studies that indicated an association of RER+ phenotype with tumours of the right colon [6]. Considering that mutations affecting classical cancer genes have been considered to be prevalent in left-sided colorectal tumours,

our study supports the theory that carcinogenesis of left and right-sided colorectal tumours may involve different mechanisms [33]. Nevertheless, since our results indicate different sensitivities according to the marker considered, it seems interesting to investigate in further studies the importance of the locus analysed, in order to clarify whether RER phenotype might be associated with a specific group of colorectal tumours, according to their biological features. Moreover, tumours of the right colon have been correlated with less aggressiveness [24] and our data predict a better prognosis for patients affected by RER^+ tumours.

Overall, we observed an improved prognosis in patients showing RER^+ tumours, this alteration being an independent factor by multivariate analysis when we considered RER at 2p, 3p, 5q, 11p or 18q. In addition, the usefulness of RER phenotype in predicting the likelihood of developing recurrent disease after surgical resection was confirmed by analysing DFS for patients with tumours displaying high instability, since none of them showed tumour recurrence during the follow-up period. Similar observations have been made previously by other authors [6, 13, 34], pointing out that the presence of RERs could be the result of defective DNA repair genes located on chromosomes 2p and 3p (*MSH2* and *MLH1*). In the model of the MMP for cancer, *MSH2* and *MLH1* are assumed to be primary mutator mutations. *MSH3* and *MSH6*, two other members of the *Mut S* subfamily of DNA mismatch repair genes, are considered secondary mutator mutations, according to the absence or presence of slippage targets in their coding regions [32]. Recent results indicate that somatic mutational inactivation of known mismatch repair genes does not account for the great majority of sporadic CRC with microsatellite instability. A significant fraction of these cases may instead be causally associated with hypermethylation of the MLH1 promoter [35]. Other causes, apart from mismatch repair gene defects, may be considered in order to explain the presence of the RER^+ phenotype. Mechanisms involving cell oxidative stress have also been suggested [36], indicating deficient pathways other than mismatch repair genes defects. In addition, Liu and associates [37] observed that only three of seven sporadic colorectal tumour cell lines with RER^+ phenotype had mutations in known mismatch repair genes, suggesting that sporadic tumours with RER may have alterations in genes other than those already known to participate in mismatch repair. This multiplicity of repair genes could reflect differentiation with respect to function or that these genes function in a tissue-dependent or developmentally controlled manner in different systems [38]. Therefore, mutations associated with RER^+ phenotype in the tumours in our study could involve any of the numerous loci responsible for DNA fidelity.

We also found a significant unfavourable outcome in patients whose tumours showed LOH. The worst prognosis was found in patients with LOH in 5q, 17p or 18q. Several reports have shown that large chromosomal regions are involved in many of the allelic deletions observed in CRC, particularly those affecting chromosomes 5, 17 and 18 [3, 39]. In spite of some discrepancies in the literature, most studies indicate poor outcome in the presence of these alterations in CRC. These are unsurprising results considering the presence, in those regions, of tumour suppressor genes (*APC*, *TP53* and *DCC*), with a crucial role in the colorectal tumorigenesis process.

In conclusion, the results confirm that sporadic colorectal tumours have RER^+ and LOH^+ phenotypes, as established by microsatellite analysis, and show a differential prognosis, which may be useful in identifying groups of patients with different risks.

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