RAPID COMMUNICATION

Telomerase activity in colorectal cancer, prognostic factor and implications in the microsatellite instability pathway

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Abstract

AIM: To determine whether the telomerase activity is related to the Microsatellite instability (MSI) genetic pathway and whether it means a difference in the survival.

METHODS: The population consisted of 97 colorectal cancer patients. MSI determination was performed in accordance with the NCI criteria using PCR and Genescan. Telomerase activity was determined by the TRAP-assay, an ELISA procedure based on the amplification of telomeric repeats sequences.

RESULTS: 6.2% showed high MSI (MSI-H), 10.3% showed low MSI (MSI-L) and 83.5% did not show this alteration (MSS). Positive telomerase activity was detected in 92.8% of the patients. 83.3% of MSI-H tumors showed positive telomerase against 93.8% of MSS tumors. In the overall survival analysis the absence of telomerase activity conferred a better prognosis.

CONCLUSION: Previous works have shown that tumors which develop via the MSI pathway present a better prognosis. No link between telomerase activity and MSI status is observed, although sample sizes are small. Patients with telomerase negative tumors had better overall survival than patients with telomerase positive tumors.

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INTRODUCTION

In colorectal tumorigenesis two different genetic pathways have been described: the pathway of chromosomic instability and the pathway of microsatellite instability (MSI). The MSI is involved in 10%-15% of sporadic colorectal cancers (CRC) and it is due to an alteration of the DNA repair genes (MMR)

These two genetic pathways give rise to two clinical colorectal tumor phenotypes, which differ in the characteristics of the tumor and, probably, in its clinical evolution. A high-frequency microsatellite instability (MSI-H) is associated with small insertions and deletions in repetitive sequences (microsatellites). MSI-H tumors are characterized by diploidy, right-sided location, mucinous histology and poor differentiation and this phenotype has been associated with a better prognosis.

Another characteristic of neoplastic transformation is uncontrolled cell proliferation. Telomeres are specialized segments of highly repetitive DNA placed at the end of chromosomes. They protect the end of the chromosome from degradation, aberrant recombination and terminal fusions. They also favour the binding of repair enzymes with DNA ends.

Immortalised cells with an indefinite replication potential show two mechanisms that maintain the length of the telomere. The activity of the telomerase enzyme is the most frequent mechanism of cell immortalisation in neoplastic transformation, as is proven by the fact that approximately 90% of tumor cells show telomerase activity. The telomerase enzyme is able to restore the sequence of the telomere, and therefore, to elongate the life of the cell, keeping its ability of multiplication and its immortality.

The second mechanism is the ALT pathway (Alternative Lengthening of Telomeres), based on homologous recombination. Owing to a telomere-specific flaw in the recombination, ALT cells critically recombine short telomeres to maintain cell proliferation.

In colorectal cancer (CRC), different authors...
have found high percentages of telomerase activity (80%-100%)\(^{[12-14]}\). At present, the clinical utility of drugs that inhibit the telomerase activity is being investigated\(^{[14]}\).

The objective of this study is to determine whether the telomerase activity is related to the MSI genetic pathway in colorectal tumorigenesis and whether it means a difference in the survival of patients with sporadic CRC.

**MATERIALS AND METHODS**

**Patients**

The population of our study consisted of a total of 97 patients undergoing surgery for colorectal cancer consecutively at the Hospital Clínico San Carlos in Madrid (Spain) between March 2001 and April 2003. It was a prospective cohort study. All the patients were operated on by the same surgeon who performed radical oncological surgery on the basis of the location of the tumor. The surgery was defined as curative when there was no evidence of macroscopic residual tumor after resection.

Following this criterion, a curative resection was performed on 76 patients (78.4%) and in 21 patients (21.6%) the primary tumor was resected as palliative treatment. Cases of metachronous carcinoma, familial polyposis, patients with a familial predisposition for hereditary nonpolyposis colon cancer (HNPCC) and those with inflammatory bowel disease were excluded from the study. None of the patients had received neoadjuvant treatment. Informed consent was obtained from each patient. The project was approved by the clinical research and ethics committee of this hospital.

Patients’ follow-up was performed according to the protocol designed by the present authors. The tumors were staged according to Dukes’ classification. Proximal tumors were defined as cecum through transverse colon; tumors in the splenic flexure, descending and sigmoid colon were defined as distal. 53.6% of the population were defined as distal.

**Sample processing**

Tumor and non-tumor tissue samples were obtained during the surgical procedure and immediately immersed in liquid nitrogen for storage in a freezer at -80°C with RNA later. The specimens were then independently examined especially for this study by two pathologists, who confirmed the samples had over 80% tumor cells.

To perform the DNA extraction, tumor and non-tumor samples were incubated overnight at 50°C in a lysis buffer (10 mmol/L tris HCl, 1 mmol/L EDTA, 100 mmol/L NaCl, 1% SDS and 500 µg/mL proteinase K). DNA was then isolated with Phenol: chloroform, and precipitated with ethanol.

**Telomerase activity determination**

Telomerase activity was determined in tumor specimens by the TRAP-assay, an ELISA procedure based on the amplification of telomeric repeat sequences\(^{[11]}\).

Frozen tissue specimens were homogenized in lysis buffer. Telomerase activity was determined in serial dilutions (1/10, 1/100, 1/1000) of a cell extract containing 3-6 µg total protein. Alkaline phosphatase activity was determined to check for possible protein degradation since this protein is the most labile\(^{[12]}\). All the protein extracts showed similar activity levels of this enzyme.

DNA integrity was evaluated by agarose gel (1%) electrophoresis in denaturing conditions.

Samples were amplified according to the procedure described by Kim et al\(^{[1]}\) in a final volume of 50 µL. Sample amplification was performed using specific primers labeled with a biotin molecule at the 3’ end and adjusting the PCR conditions.

Telomerase activity was determined through the amplification products by ELISA. The controls used for the telomerase assay were immortalized kidney cells (line 293) expressing telomerase (positive control) and a sample of these cells treated with 1.25 µg of RNase and incubated for 10 min at 65°C (negative control).

When analyzing the ELISA results, 0.25 OD was taken as the maximum absorbance (A\(_{540-650}\)) possible for the negative control, and an absorbance value above 1.5 OD was required for the positive control. Processed tissue specimens showing an absorbance above 0.2 OD were defined to be telomerase positive and those showing absorbances under this value were taken to be telomerase negative. Tumors showing a positive telomerase assay result were then classed according to absorbance values as: low (OD = 0.200-0.999), intermediate (OD = 1.000-1.999) or high (OD > 1.999) telomerase activity.

**MSI determination**

The DNA was amplified in a 25 µL final volume containing 100 ng of DNA, 200 µmol/L dNTP, 1-2 mmol/L MgCl\(_2\), 350 mmol/L of each primer and 2 U of Taq Polymerase. The PCR conditions were as follows: 25-35 cycles (95°C for 30 s, 50-58°C for 30 s depending on the marker and 72°C for 30 s). The primers used were BAT25, BAT26, D17S250, D5S346 and D2S123 in accordance with the NCI criteria\(^{[11]}\). Each pair of primers was tagged with a fluorochrome for later analysis (TIB MOLBIOL, Roche).

The size of the amplified products was determined using an ABI 310 PRISM sequencer and analyzed using Genescan and Genotyper software.

**Statistical analysis**

Quantitative variables were provided with their corresponding frequency distributions. Quantitative variables were expressed as their mean, standard deviation (SD) and range. Age was recoded into two groups according to median age (71 years). Associations between qualitative variables were evaluated using the χ² test or Fisher’s exact test when 25% of expected frequencies fell below 5. The overall survival (OS) was estimated by the Kaplan-Meier method and compared among groups using Breslow’s exact test. The event in OS was defined as deaths occurring as a consequence of the tumor, censuring live patients and those dying of another cause. OS was calculated as the time elapsed from the date of surgery until death or last follow-up. The data were fitted to Cox’s proportional risks regression model. The hazard ratio (HR) was given with a 95% (CI 95%) confidence interval. The variables included according to biological criteria
were age, sex, Dukes’ stage, tumor site, differentiation grade, histological type, adjuvant chemotherapy, MSI and telomerase. In each contrast, the null hypothesis was rejected when the type I error was less than 0.05. All statistical tests were performed using SPSS 11.5 software.

**RESULTS**

A total of 97 patients diagnosed with colon adenocarcinoma were analysed. 52.6% were male and 47.4% were female. The mean age was of 69.8 ± 11.3 (range 44-95) years, and median 71.0 years. The median age was established as the cutting point and 48.5% of the patients were below 71 years old. The clinicopathological variables of the study population are shown in Table 1. 72.2% of the tumors were located in the colon (37 in proximal colon and 33 in distal colon) and 27.8% (27 patients) in the rectum. 10.3% of the tumors were mucoid adenocarcinomas and the rest (89.7%) were adenocarcinomas. The cell differentiation grade could not be determined in 12 patients.

The study of the MSI and the telomerase activity was carried out in 97 patients. 6.2% (6 patients) showed high MSI (MSI-H), 10.3% (10 patients) showed low MSI (MSI-L) and 83.5% (81 patients) did not show this alteration (MSS). Positive telomerase activity was detected in 92.8% of the patients (90 patients).

The relationship between the telomerase activity and the MSI was related to age and tumor location; MSI-H was more frequent in patients older than 71 and in patients with proximal colon tumors than in those with distal colon or rectal tumors. The telomerase activity was related to the Dukes’ stage. Patients at Stage A showed a lower percentage of positivity than the ones at stages B, C or D (P = 0.001). Tumors localized in colon showed a higher percentage of positivity than the tumors localized in the rectum (P = 0.09).

The relationship between the telomerase activity and the MSI is shown in Table 2. 83.3% of MSI-H tumors showed positive telomerase against 93.8% of MSS tumors (P = 0.89).

**Overall survival**

The median follow-up period time in this study was 40 mo (3 years), with an interquartile range between 32 and 48 mo. In our population of patients the OS at 40 mo was of 69.8%. All the survival analyses correspond with our follow-up median. During the follow-up period 36 patients died; 25 resulting from neoplasia.

The univariate analysis of the OS is shown in Table 3. None of the patients with negative telomerase died during the follow-up period (P = 0.04). In the stratified analysis of the OS of the clinicopathological variables with respect to the two studied genetic alterations, no significant differences were found in the different groups.

The stratified analysis of OS of genetic determinations is described in Table 4. In patients with positive telomerase activity, the MSI does not establish significant differences in OS (P = 0.99). With respect to the adjuvant chemotherapy treatment, when it was stratified by stages, no significant differences were found between the group of treated patients and the group of non-treated patients (data not included).

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Table 1  Relationship of telomerase activity and MSI with clinicopathological variables of the 97 patients with colorectal carcinoma a (%) 

| Variable          | MSS (76.3) | MSI-L (46.4) | MSI-H (24.7) | P   | Telomerase positive | Telomerase negative |
|-------------------|------------|--------------|--------------|-----|---------------------|---------------------|
| Sex               | Men: 51 (52.6) | 40 (78.4)   | 7 (13.7)     | 4 (7.8) | 0.20 | 46 (90.2) | 5 (9.8) | 0.44 |
|                  | Female: 46 (47.4) | 41 (89.1)   | 3 (6.5)     | 2 (4.3) | 44 (95.7) | 5 (9.8) | 0.44 |
| Age               | ≥ 71 yr: 50 (51.5) | 39 (78.0) | 5 (10.0) | 6 (12.6) | 0.03 | 47 (94.0) | 3 (6.0) | 0.70 |
|                  | < 71 yr: 47 (48.5) | 42 (89.4) | 5 (10.0) | 0 (0) | 43 (91.5) | 5 (8.5) | 0.05 |
| Dukes stage       | A+B: 47 (48.5) | 40 (85.1) | 4 (8.5) | 3 (6.4) | 0.90 | 42 (89.4) | 5 (10.6) | 0.13 |
| Site              | Colo: 70 (72.2) | 56 (80.0) | 8 (11.4) | 6 (8.6) | 0.08 | 67 (95.7) | 3 (4.3) | 0.09 |
|                   | Rectum: 27 (27.8) | 25 (92.6) | 2 (7.4) | 0 (0) | 23 (85.2) | 4 (14.8) | 0.001 |
| Colon site        | Proximal: 37 (52.8) | 26 (70.3) | 5 (13.5) | 6 (16.2) | 0.01 | 34 (91.9) | 3 (8.1) | 0.04 |
|                  | Distal: 33 (47.2) | 30 (92.6) | 3 (9.1) | 0 (0) | 33 (100) | 0 (0) | 0.05 |
| Grade             | I: 56 (65.9) | 47 (83.9) | 6 (10.7) | 3 (5.4) | 0.32 | 27 (93.1) | 2 (6.9) | 1.00 |
|                   | II + III: 29 (34.1) | 22 (75.9) | 4 (13.8) | 3 (10.3) | 0.52 | 52 (92.9) | 4 (7.1) | 0.001 |
| Histological type | Adenocarcinoma: 87 (89.7) | 72 (82.8) | 9 (10.3) | 6 (6.9) | 0.44 | 80 (92.0) | 7 (8.0) | 1.00 |
|                  | Mucoid: 10 (10.3) | 9 (90.0) | 1 (10.0) | 0 (0) | 10 (100) | 0 (0) | 0.05 |
| Chemo             | Yes: 52 (53.6) | 44 (84.6) | 6 (11.5) | 2 (3.8) | 0.50 | 49 (94.2) | 3 (5.8) | 0.70 |
| therapy           | No: 45 (46.4) | 37 (82.2) | 4 (8.9) | 4 (8.9) | 41 (91.1) | 9 (8.9) | 0.05 |

MSS: absence of microsatellite instability; MSI-L: low instability; MSI-H: high instability. Statistically significant: P < 0.05.

Table 2  Relationship between telomerase activity and MSI n (%) 

| Telomerase positive | Telomerase negative |
|---------------------|---------------------|
| MSS                 | 76 (93.8) | 3 (6.2) | 0.89 |
| MSI-L               | 9 (90.0)  | 1 (10.0) | 0.05 |
| MSI-H               | 5 (83.3)  | 1 (16.7) | 0.05 |
DISCUSSION

Two genetic pathways have been described for the neoplastic transformation in sporadic colorectal cancer. In our study population, 6.2% develops through MSI. We have already explained the involvement of repairing genes alteration in this pathway and mainly the hMLH1 promoter methylation[14]. Independently of the genetic pathway involved in the tumorigenesis, the tumor cell has to restore its proliferative ability and avoid senescence. Activation of telomerase enzyme to maintain the telomeres length has been frequently found in tumor cells.

Our study showed positive telomerase activity in 92.8% of tumors. This activity is more frequent in the chromosome instability pathway than in the MSI pathway. Telomerase positive percentage in MSI-H group (83.3%) is lower than in MSS group (93.8%), although this relationship is not statistically significant (P = 0.89), probably due to the reduced population size. These results could suggest the non dependence of the MSI tumor of telomerase activity to maintain their proliferative ability and are coincident with the results obtained by Unate et al[17].

Although the telomerase activity is the most frequent mechanism for the telomeres lengthening in the neoplastic transformation, a second mechanism has been described, it is the ALT pathway. In cells that have the ALT mechanism activated, recombination can occur in telomeres and this way the cell proliferation is maintained[14]. The machinery involved in this pathway is not known in detail but it is thought that recombination of telomere repeated sequences can be the mechanism underlying telomeres stabilization in absence of telomerase activity[17].

MMR proteins inhibit recombination between non-matching sequences. This is the reason why, in case of MMR system failure, recombination between diverged sequences is increased[18]. Rizki et al[19] demonstrated that the loss of mismatch-repair function promotes cellular proliferation in the absence of telomerase. This would corroborate the relationship between MSI and MMR defects and the no dependence of the telomerase activity.

Lanend et al[20] observed a lower frequency of telomerase activity as a mechanism to avoid senescence in MSI-H ovarian tumors and they hypothesized the presence, in this kind of tumors, of an alternative mechanism for the maintenance of the telomeres’ length. Bechter et al[21] demonstrated telomerase activity inhibition and ALT pathway activation in colon cancer cell lines when there were mutations in the MSI system.

This association between the non-telomerase-dependent cell proliferation and the presence of MSI-H can be related to the alteration of MMR genes. It has been demonstrated that MSH2 and MSH3 act together with the Rad1-Rad10 endonucleases complex avoiding non-homologous recombination[22]. In the ALT pathway, recombination seems to be increased and when the MMR system is undamaged it can prevent this mechanism. This is the reason why the loss of MMR system function could allow telomeres lengthening, even when there is no telomerase activity[20].

However, Cerone et al[23] showed some evidences which suggest that some tumors can have the ALT pathway activated although they show telomerase activity. This would mean that these mechanisms are not mutually exclusive, and they can coexist in the same cell.

It has been established that ALT cells show lower tumorigenic potential than cells with telomerase activity[24]. Bechter et al[25] observed that, at least in cell lines, the ALT pathway is not able to replace telomerase function in neoplastic proliferation and its metastatic potential.

It has been demonstrated that MSI tumors show a better prognosis in sporadic CRC[14]. Previous studies showed that OS of those patients with no telomerase activity reaches the level of 100%[26]. These results are coincident with the ones published by Tsutsumoto et al[27] who observed a lower OS when the activity of this enzyme was high.

Previous works have shown that tumors which develop via the MSI pathway present a better prognosis. Furthermore, if instead of using telomerase activity to
maintain cell proliferation in these tumors, they use the alternative ALT mechanism, which shows less tumorigenic potential, this could improve significantly the patients’ prognosis. These genetic events, MSI and ALT, show low frequency in sporadic CRC, for this reason larger population size studies are needed to demonstrate its association and its influence in the outcome.

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