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Prognostic Significance of DNA Replication Errors in Young Patients With Colorectal Cancer

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Objective

To determine the DNA replication error (RER) status in young patients with colorectal cancer (CRC), and to compare the clinical and pathologic characteristics of RER-positive and RER-negative cases.

Summary Background Data

Recent studies suggest that patients with RER-positive CRC have an improved prognosis. Further data are required to confirm this observation in young CRC patients.

Methods

All patients 40 years of age and younger with CRC admitted to the National Naval Medical Center between 1970 and 1992 were considered for inclusion in the study. After review, 36 patients for whom the original archived pathology specimen could be retrieved served as the study population. The RER status was determined using a previously described polymerase chain reaction-based assay. The clinical and pathologic features and survival data were compared to RER status.

Results

RER-positive tumors were found in 17 cases (47%). There was no significant difference in Dukes' stage or histologic grade at the time of diagnosis between patients with RER-positive tumors compared to RER-negative tumors. Patients with RER-positive tumors were found to have an improved prognosis: the 5-year survival probability for patients with RER-positive tumors was 68%, as compared to 32% for patients with RER-negative tumors ($p < 0.05$).

Conclusions

RER-positive tumors are common in young patients with CRC, and patients with RER-positive tumors have a significantly improved prognosis. Because of their young age, survival data and prognosis play an important role in the overall treatment plan of young patients with CRC. Therefore, knowledge of RER status could affect initial therapy, postoperative chemotherapy, and follow-up.

More than 90,000 new cases of colorectal cancer (CRC) occur in the United States each year, and approximately 45,000 patients afflicted with CRC are expected to die of this malignancy.[1] The prognosis of CRC is largely determined by the extent of primary disease at the time of diagnosis.[2] Although some improvements in disease-free survival in node-positive CRC have been achieved with postoperative adjuvant therapy, the prognosis for patients with advanced disease is essentially unchanged with currently available treatments.[3] Earlier detection, improved therapy, and efforts to prevent the development of CRC are critical to improve overall survival.

Advances in the field of molecular genetics have improved our understanding of the pathogenesis of CRC. Recently, mutations in genes responsible for the repair of mismatched nucleotides in DNA have been found to be responsible for most cases of CRC in patients with hereditary nonpolyposis colorectal cancer syndrome (HNPCC), and a subset of sporadic CRC. The detection of alterations in these genes was facilitated by DNA microsatellite analysis. Microsatellites are short repeated sequences that are dispersed throughout the human genome. Typically, the sequences consist of DNA repeats of 6 base pairs or fewer, and the total length of the stretch is fewer than 100 base pairs. The important observation made in strains of yeast and bacteria and in the tumor cells of patients with HNPCC with mutated mismatch repair systems was that these repeated sequences often showed instability. This instability was manifested by marked variability in the number of repeats as ubiquitous somatic mutations, termed replication errors (RER), or microsatellite instability.[4]

RER-positive tumors are nearly a universal finding in CRC of patients with HNPCC and occur in approximately 15% of patients with the sporadic disease.[5] Recent studies suggest that patients with RER-positive tumors have an improved prognosis.[6,7] Further data are required to confirm this observation, especially in young CRC patients, who account for about 2% to 8% of CRC cases.[8] It remains unknown why patients with RER-positive CRC have improved survival, especially in light of experimental data showing that RER-positive tumor cell lines are resistant to certain chemotherapeutic agents.[9,10]

Little additional information exists about RER status in young CRC patients. One study of combined patient populations suggests that most young CRC patients harbor RER.[11] The clinical features, including family history and prognosis, of young patients with CRC relative to RER status have not been studied in detail. Therefore, the aim of this study was to determine the RER status of a well-defined group of young patients with CRC and to compare the clinical and pathologic characteristics of RER-positive and RER-negative cases.

MATERIALS AND METHODS

Study Subjects

All patients 40 years of age and younger with CRC admitted to the National Naval Medical Center between 1970 and 1992 were considered for inclusion in the study. These patients represented 5% of the CRC patients during the study period. Patients were excluded from the study if they had familial adenomatous polyposis syndrome. Information from the National Naval Medical Center Tumor Registry and chart review was used to determine clinical and pathologic features. An interview with the patient or family member was performed to obtain follow-up and family history data. After review, 36 patients for whom the original archived pathology specimen could be retrieved served as the study population.

DNA Extraction

Formalin-fixed, paraffin-embedded sections of tissues prepared for routine histopathology were used for DNA extraction. Slides were stained with hematoxylin and eosin, dehydrated in ethanol, and dried without a coverslip. Regions containing neoplastic cells were inked with a black marker (Sharpie; Sanford Corp., Bellwood, IL) under a dissecting microscope. Tissue sections from at least 10 slides, each containing a region of tumor 0.2 to 1 cm^2 in area, were scraped from the slides with a razor blade and transferred to a microfuge tube. Nonneoplastic tissue from the same slide was then marked with ink and placed in a different tube. After deparaffinizing through xylene, DNA was extracted from these samples after SDS (sodium dodecyl sulfate)-proteinase K digestion and phenol chloroform (pH 8.0) extraction.

RER Assessment

Five different microsatellite markers for dinucleotide repeats on the long arm of chromosome 18 and a polyadenine tract in the transforming growth factor beta type II receptor gene were used to evaluate the tumors. Primers were chosen so as to amplify fragments of less than 180 base pairs in size because larger fragments did not amplify

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Table 1. MICROSATELLITE MARKERS USED IN THE STUDY

Marker	Type	Primer Sequence
D18S55	CA dinucleotide repeat	5′-GGGAAGTCAAATGCAAAATC-3′ 5′-AGCTTCTGAGT AATCTTATGCTGT-G-3′
D18S58	CA dinucleotide repeat	5′-GCTCCCGGCTGGTTTT-3′ 5′-GCAGGAAATCGCAGGAACTT-3′
D18S61	CA dinucleotide repeat	5′-ATTTCTAAGAGGACTCCCAAACT-3′ 5′-ATATTTTGAAACTCAGGAGCAT-3′
D18S64	CA dinucleotide repeat	5′-AACTAGAGACAGGCAGAA-3′ 5′-ATCAGGAAATCGGCACTG-3′
TGF-BETA R11	Polyadenosine tract	5′-CTTTATTCTGGAAGATGCTGC-3′ 5′-GAAGAAAGTCTCACCAGGC-3′

consistently using DNA templates prepared from paraffin-embedded tissues. The primers used are shown in Table 1. The conditions used for polymerase chain reaction (PCR) have been previously described.[12,13] Tumors were classified as RER-positive if at least two of the markers revealed PCR fragments in the neoplasm not found in the control colon tissue of the same patient.

Statistical Analysis

The clinical and pathologic features were compared to RER status, and the significance of the associations was determined using the chi square test. The survival data were used to generate Kaplan-Meier product limit survival curves, and the curves were compared using the log-rank test.

RESULTS

RER-positive tumors were found in 17 cases (47%). The clinical and pathologic characteristics are summarized in Table 2. There was no difference in mean age at presentation between RER-positive and RER-negative tumors. There was a predominance of males in the study population, but the prevalence of RER-positive tumors was not significantly different between males and females ($p = 0.18$). The majority of patients denied significant family history of colon cancer, with 26 patients (72%) developing apparently sporadic CRC (no family history for CRC), and only 4 patients (11%) meeting the criteria for HNPCC. Twelve (46%) of the 26 sporadic CRC cases proved to be RER-positive, and 3 of the 4 tumors in HNPCC patients were RER-positive difference (difference not significant). No synchronous tumors were found in the study population, and only one patient was found to develop a metachronous tumor (RER-positive, glioblastoma) during this study period.

Most patients were classified as Dukes' stage B or C at diagnosis (see Table 2). There was no statistically significant difference between RER-positive and RER-negative tumors with regard to stage at diagnosis. The tumors were predominantly right-sided neoplasms, with 20 patients (56%) having neoplasms proximal to the splenic flexure. RER-positive tumors were more frequently located in the right colon ($p = 0.023$).

The majority of the tumors were classified histopathologically as moderately differentiated at the time of diagnosis, and nearly half of these tumors were RER-positive. Two thirds of the well-differentiated tumors were RER-positive; only 25% of poorly differentiated tumors were RER-positive. A mucinous component was seen in 5 tumors (14%); only 1 was RER-positive. There were no statistically significant differences in differentiation between RER-positive and RER-negative tumors.

The overall cumulative 5-year survival probability for the study population was 50%. Patients with RER-positive tumors were found to have an improved prognosis: their 5-year survival probability was 68%, compared to 32% for patients with RER-negative tumors (Fig. 1, $p < 0.05$).

Nineteen patients (53%) were treated postoperatively with chemotherapy based on 5-fluorouracil (5-FU). There was no significant difference in survival with 5-FU therapy. Of the patients treated with 5-FU, 8 had RER-positive tumors and 11 had RER-negative tumors. There was a suggestion that patients with RER-positive tumors who were treated with 5-FU had excellent survival: their 5-year survival probability was 85% compared to 73%, 55%, and 30%, respectively, for patients with RER-negative tumors and 5-FU therapy; RER-positive tumors and no 5-FU therapy; and RER-negative tumors and no 5-FU therapy (Fig. 2, $p = 0.19$).

DISCUSSION

Previous studies reported that 10% to 15% of sporadic CRC cases showed RER but that as much as 60% of young CRC patients had the RER phenotype.[11] Our study,

Table 2. CLINICAL AND PATHOLOGIC CHARACTERISTICS OF PATIENT POPULATION: PERCENT OF PATIENTS (NUMBER OF PATIENTS)

	All Patients (n = 36)	RER + CRC (n = 17)	RER − CRC (n = 19)	p Value (RER+ *vs.* RER−)
Clinical characteristics				
Mean age (±SD) (yr)	32 ± 6	32 ± 6	32 ± 7	NS
Age range	20–40	22–40	20–40	
Male gender	72 (26)	82 (14)	63 (12)	NS
Family history of CRC	28 (10)	29 (5)	26 (5)	NS
HNPCC (ICG criteria)	11 (4)	20 (3)	5 (1)	NS
Synchronous/metachronous	3 (1)	6 (1)	0	NS
Stage				
Dukes' B	33 (12)	41 (7)	24 (5)	NS
Dukes' C	36 (13)	35 (6)	37 (7)	NS
Disseminated	30 (11)	24 (4)	37 (7)	NS
Site				
Right sided	56 (20)	76 (13)	37 (7)	0.023
Grade of differentiation				
Well	25 (9)	35 (6)	16 (3)	NS
Moderate	53 (19)	53 (9)	53 (10)	NS
Poor	22 (8)	12 (2)	32 (6)	NS
Mucinous component	14 (5)	6 (1)	21 (5)	NS

NS = not significant; RER = DNA replication error status; CRC = colorectal cancer; HNPCC = hereditary nonpolyposis colorectal cancer; ICG = International collaborative group criteria.

specifically directed at a young patient population, also showed that RER-positive tumors are common among young patients with CRC (47%). Young patients with CRC often share several features previously documented in the familial tumors, such as proximal location, poor differentiation, and improved prognosis. These similarities have led to speculation that young CRC patients with RER-positive tumors represent a less expressive form of HNPCC or an as-yet-unidentified familial syndrome. RER-positive tumors have a defective DNA mismatch repair system.[5] Although a majority of RER-positive HNPCC kindreds had been shown to harbor germline mutations in one or more of the known mismatch repair genes,[14,15] this has not been found in RER-positive patients with sporadic disease. In fact, only about 10% to 15% of RER-positive patients with sporadic RER and CRC have RER positive CRC proved to have germline mutations in known mismatch repair genes.[6,14,15] In a previous study of patients under 35 years old with RER-positive CRC, only 5 of 12 (42%) were found to have a germline mutation in the mismatch repair genes hMSH2 or hMLH2.[11] It is possible patients have a mutation in a yet-to-be-identified mismatch repair gene. Excluding the four patients who met International Collaborative Group criteria for HNPCC, only two of our patients with RER-positive tumors had a family history of CRC. Our findings support a previous report that the RER phenotype was not a useful clinical indicator of familial CRC.[16]

Although previous studies found that many RER tumors are poorly differentiated or mucinous,[12] we did not find this association in our young patient population. The majority of RER-positive tumors were either well or moderately differentiated. The explanation of this difference is not evident. There was no evidence in our study that young patients with RER-positive CRC were different with regard to age or Dukes' stage. Additionally, synchronous CRC and metachronous CRC, common in HNPCC patients, were not found during the study period. Only one patient with RER-positive CRC developed an extracolonic tumor (a glioblastoma), suggesting Turcot's syndrome due to germline mutation of a mismatch repair gene.[17]

Several previous studies indicated that RER is predominantly a characteristic of right-sided colonic tumors,[6,7,12,18] and we found a proximal distribution of RER-positive tumors in young patients as well: 76% of RER-positive tumors were right-sided. Thus, the mechanism responsible for mutations of mismatch repair genes during tumorigenesis in the right colon is active in young as well as older patients.

It appears that there is a distinct survival advantage in patients whose tumors have the RER phenotype.[6,19,20] However, before this study there were few data to support this statement with regard to young CRC patients. Our study shows a clear survival advantage in young patients with RER-positive tumors. It remains unknown why pa-

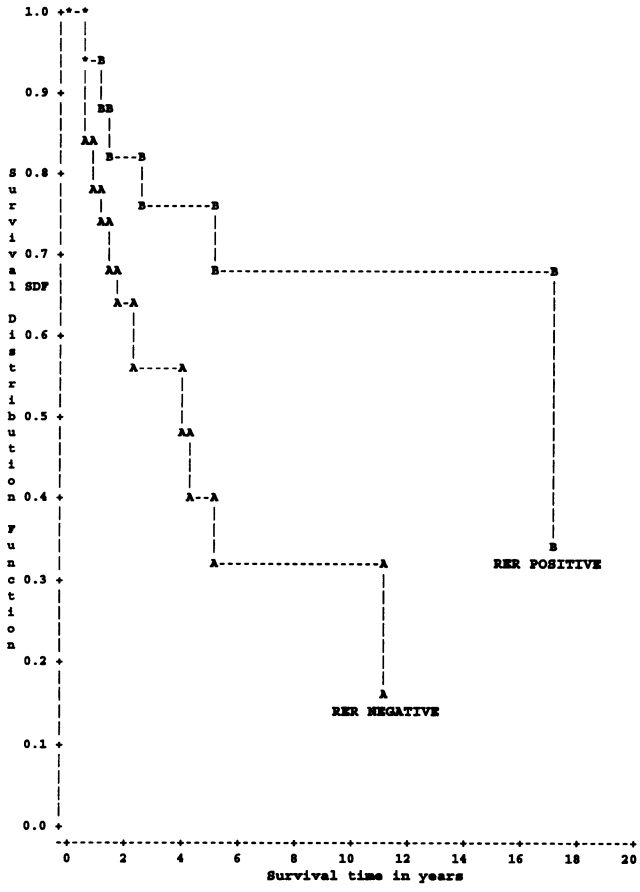

Figure 1. Kaplan-Meier survival curves for the study population based on replication error (RER) status. Curve A represents all patients with RER-negative tumors, curve B all patients with RER-positive tumors. The 5-year survival probability for patients with RER-negative tumors was 32% *versus* 68% for patients with RER-positive tumors (log-rank test, $p < 0.05$).

tients with RER-positive tumors have an improved prognosis. There is compelling evidence that the RER phenotype is associated with abnormal DNA repair function. This repair function is critical for normal cellular function, and its dysfunction is associated with the neoplastic state. Tumor cells incapable of repairing DNA replication errors (RER-positive tumor cells) accumulate an escalating number of mutations throughout their genome, including mutations in known oncogenes and tumor suppressor genes that can lead to tumorigenesis.[4] Theoretically, the immortalized tumor cell continues to develop mutations in its DNA, eventually accumulating mutations in genes important for cell function or growth. This could lead to an apoptotic response and programmed cell death. This hypothesis has experimental support; several investigators have shown that RER-positive human cell lines develop mutations in important genes in addition to simple microsatellite noncoding sequences.[21–24] Therefore, it is plausible that tumor cells with the RER phenotype sometimes contribute to their own demise, which may lead to improved patient survival.

A final area with important potential clinical applications is that of cancer chemotherapy. Recently, investigators have begun to evaluate tumor cells with the RER phenotype and their responsiveness to certain chemotherapeutic agents.[9,10,25] In our study, it appears that patients with RER-positive CRC who are treated with 5-FU therapy have improved survival, although the differences were not statistically significant because of the number of patients studied. Interestingly, Kat et al.[9] and Branch et al.[25] have shown that RER-positive cell lines are not more sensitive to alkylating chemotherapeutic agents but are, in fact, more resistant to these type of agents. It has also been shown that RER-positive cell lines are more resistant to cisplatin and doxorubicin.[25] It has been postulated that the process of repairing DNA damaged by these agents is crucial for the drug's cytotoxic effect.[5] RER-positive cells may not recognize the level of DNA damage

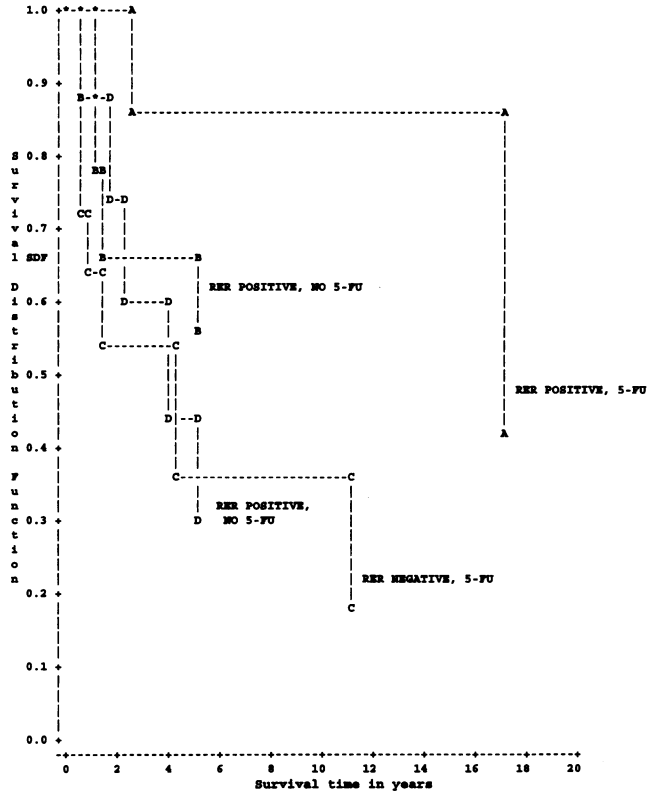

Figure 2. Kaplan-Meier survival curves for the study population based on replication error (RER) status and treatment with or without 5-fluorouracil (5-FU). Curve A represents patients with RER-positive tumors treated with 5-FU; curve B represents patients with RER-positive tumors not treated with 5-FU; curve C represents patients with RER-negative tumors treated with 5-FU; and curve D represents patients with RER-negative tumors not treated with 5-FU. The 5-year survival probability for curves A, B, C, and D was 85%, 55%, 73%, and 30% respectively (log rank test, $p = 0.19$).

that would induce apoptosis. In contrast, antimetabolite chemotherapy (*e.g.*, 5-FU), by acting as competitive inhibitors for substrates critical for DNA synthesis, may work in concert with RER-positive cells lacking normal DNA repair mechanisms, using a "two-hit" mechanism to illicit a rapid apoptotic response.

Although patients under 40 years of age make up a small percentage of the total number of patients with CRC, these patients are a particularly important subgroup with regard to molecular genetic prognostic markers. Because of their young age, survival data and prognosis play an important role in their overall treatment plan. If a molecular genetic aspect of these tumors, such as the RER phenotype, becomes recognized as associated with increased survival and improved response to chemotherapy, then young patients with the molecular genetic marker could receive more aggressive initial therapy, postoperative chemotherapy, and closer follow-up care.

Finally, if 5-FU or other chemotherapeutic agents are more effective against RER-positive tumors, this could have significant clinical implications in the treatment of all patients with tumors harboring the RER phenotype.

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