[CANCER RESEARCH 53, 5849–5852, December 15, 1993]

Advances in Brief

Genomic Instability in Colorectal Cancer: Relationship to Clinicopathological Variables and Family History[1]

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Abstract

Recent reports have suggested that one or more genes may cause replication errors (RER) during colorectal tumorigenesis. Additional alleles are seen in the tumors when analyzing random microsatellite loci. We have studied seven dinucleotide repeat loci, located on seven different chromosomes, by use of polymerase chain reaction amplification and denaturing polyacrylamide gel electrophoresis. We found that 16.5% (40 of 243) colorectal cancers showed RER at one or several loci (RER+). This includes 31% (4 of 13) among cases with a strong positive family history according to previously published criteria and 17% (35 of 207) among cases with no history of familial cancer. Interestingly, no significant association was found between RER+ tumors and a general familial clustering of cancer. Microsatellite instability was significantly associated with DNA diploid status of the tumor ($P < 0.001$), with the location of the tumor in the proximal colon ($P < 0.001$), and with poorly differentiated tumor phenotype ($P < 0.001$). Patients with RER+ at ≥ 2 loci tumors had an increased survival ($P = 0.05$).

We further analyzed 84 breast cancers and 86 male germ cell cancers using the same seven markers. None of the tumors were RER+, indicating that this phenomenon may be specific to certain types of tumors.

Introduction

DNA alterations in colorectal tumors have previously been shown to occur at several chromosomal regions containing both oncogenes and tumor suppressor genes (1–4). Activation of oncogenes through point mutations or amplification and inactivation of suppressor genes by deletions and point mutations are the main mechanisms described. Recently, instability at repetitive loci scattered throughout the genome was reported in colorectal cancers, indicating a novel genetic mechanism in tumorigenesis (5–7).

Colorectal cancer occurs both as a hereditary disorder and as sporadic cases. Familial susceptibility to colorectal cancer includes familial adenomatous polyposis and HNPCC.[3] Germline mutations of the tumor suppressor gene *APC* (adenomatous polyposis coli) cause familial adenomatous polyposis. HNPCC includes several subgroups. Specific criteria for one of these subgroups have been described by Vasen *et al.* (8) and have been used to identify two large colorectal cancer families in which linkage to DNA markers on 2p was proved (9). In the majority of cancers from such families, instability at microsatellite loci was observed as additional new alleles in tumor DNA as compared to constitutional DNA (5). Therefore, it was suggested that this gene, now called *COCA1* (colon cancer 1), might maintain replication accuracy. This implies that a mutation in such a gene could cause RER throughout the genome.

In the current study, we have determined the frequency of RER+ colorectal cancers in a large series of tumors and investigated the relationship of this phenomenon to positive family history of cancer and specific clinicopathological variables. Finally, we have addressed the possibility that microsatellite instability is a specific or general phenomenon by studying breast and testicular cancers.

Materials and Methods

Tumor Samples. Tumor samples from 252 patients with colorectal carcinomas, 85 patients with breast carcinomas, and 96 patients with male germ cell tumors have been studied. One tumor from each patient is represented in this study. The colorectal tumors were mechanically minced in phosphate-buffered saline (pH 7.6) and then fixed and stored in 70% ethanol at 4°C prior to DNA extraction (10). All biopsies of the breast and testicular tumors were frozen and stored at −70°C prior to DNA extraction.

The colorectal cancers were collected from patients at 7 hospitals from the Oslo and Akershus regions during 1987–1989. Clinicopathological characteristics of the colorectal carcinomas are given in Table 1. The testicular tumors were classified as seminomas ($n = 44$) and various subtypes of nonseminomas ($n = 52$) according to the WHO recommendations (11). Tumor and node status on the breast carcinomas were determined based on the pathologists reports, according to the 1988 tumor-nodes-metastasis classification (12). Morphological diagnosis of the tumors were evaluated as ductal ($n = 62$), lobular ($n = 9$), intraductal ($n = 5$), and others ($n = 9$) according to WHO criteria.

Family History of Cancer. Written information on standardized questionnaires concerning cancer among the first and second degree relatives have been obtained from 250 of 252 colorectal cancer patients. Cases diagnosed after 1952 were checked through the Norwegian Cancer Registry.

Hereditary non-polyposis colorectal cancer may be divided into two subcategories, Lynch syndromes I and II, based on the absence *versus* presence of extracolonic cancers (13). In an effort to define different subgroups within our material the following criteria have been used: group 1, at least three relatives should have colorectal cancer, one of them being a first degree relative of the other two. At least two successive generations should be affected, and one diseased should be diagnosed before age 50 [Amsterdam criteria (8)]; Group 2, four diseased who are first or second degree relatives, counting colorectal cancer, upper gastrointestinal tract cancer, and endometrial cancer as affected (14). Because of lack of definite diagnosis in old relatives, reported "abdominal cancer" and "female genital cancer" were accepted as meeting these criteria. In order to increase the sensitivity in our series two additional groups were constructed by reducing the number of affected with one in the first two groups: group 3, two colorectal cancer patients, first degree relatives, and one being diagnosed before age 50; group 4, three diseased who are first or second degree relatives, counting colorectal cancer, upper gastrointestinal tract cancer, and endometrial cancer as affected. Finally, we have searched for possible

Received 10/9/93; accepted 11/2/93.

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[1] Supported by grants from the Norwegian Research Council for Science and Humanities, the Norwegian Cancer Society, the Academy of Finland, the Finnish Cancer Society, and the Sigrid Juselius Foundation. Part of this study was done at the Folkhalsan Institute of Genetics.

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[3] The abbreviations used are: HNPCC, hereditary non-polyposis colorectal cancer syndrome; RER, replication errors; PCR, polymerase chain reaction.

representation of other inherited cancer syndromes like breast cancer, breast-ovarian cancer, or Li-Fraumeni syndrome.

Among the testicular cancer patients there were 19 with bilateral tumors and/or familial cases (at least one affected first or second degree relative).

The breast cancer patients were interviewed with respect to family history by standardized questionnaires, and all cancer diagnoses reported were confirmed by the Norwegian Cancer Registry. Familial clustering was considered if one or more first degree relatives had suffered from breast cancer. Eighteen of the breast cancer patients had either bilateral cancer or a positive family history for breast cancer. None of the breast cancer patients had any accumulation of colorectal cancer in the family.

DNA Analysis. DNA from blood leukocytes, representing constitutional DNA, and tumor DNA were extracted in an automated Nucleic Acid Extractor (Applied Biosystems) principally using the conventional method of phenol/chloroform extraction followed by ethanol precipitation (15).

Seven loci containing dinucleotide repeat sequences and representing different chromosomes (16) were studied in each case. The loci were (chromosomal localization): *D1S216* (1p), *D5S404* (5q), *D8S255* (8p), *D10S197* (10p), *D11S904* (11p), *D13S175* (13), and *D17S787* (17q). The markers were selected on the basis of two criteria: (*a*) the ability to combine primers so that all seven loci could be studied in only two PCR reactions, and (*b*) different chromosomal locations, representing regions that show a variable degree of alterations in the genesis of these cancers (17–19). The procedure for RER analysis have been described in detail previously (9). Briefly, primers specific for each locus were used to amplify the repeat and short flanking sequences in template DNA by PCR. The products were labeled by [^{32}P]dCTP during amplification reaction and separated by electrophoresis in 6% denaturing polyacrylamide gels and visualized through autoradiography. All the scorings were done independently by two of the authors (R. A. L. and P. P.). Information had to be obtained from at least three loci in order to score the case as RER–. All RER+ cases were analyzed twice by a new PCR and electrophoretic run.

Statistical Analyses. Associations between variables were tested by Pearson's χ^2 test or exact tests when appropriate. Cause-specific survival analysis (death by colorectal cancer) was performed by the Cox proportional hazard model, using the EGRET computer program. The effects were tested by a likelihood ratio test. $P < 0.05$ were considered statistically significant.

Results

After analysis of 252 matched pairs of constitutional DNA and tumor DNA at seven CA-repeat loci, 243 could be evaluated as RER– (information from at least three loci) or RER+. Among these, 16.5% (40 of 243) were RER+ for one or more loci. Examples of this microsatellite instability, observed as new fragments in the tumor, are illustrated in Fig. 1. These new fragments were of variable size, larger and/or smaller than the constitutional alleles. One or both constitutional alleles were always seen in addition to the new fragments, but the intensity ratio between the two normal alleles could be altered (allelic imbalance including loss of heterozygosity: data not shown). The average numbers of scorable loci among RER– tumors, RER+ at one locus, and RER+ at two or more loci were 6.2, 6.2, and 5.9, respectively.

Family history was obtained from 250 of 252 patients. Two families satisfied the Amsterdam criteria for HNPCC (group 1), and 11 fami-

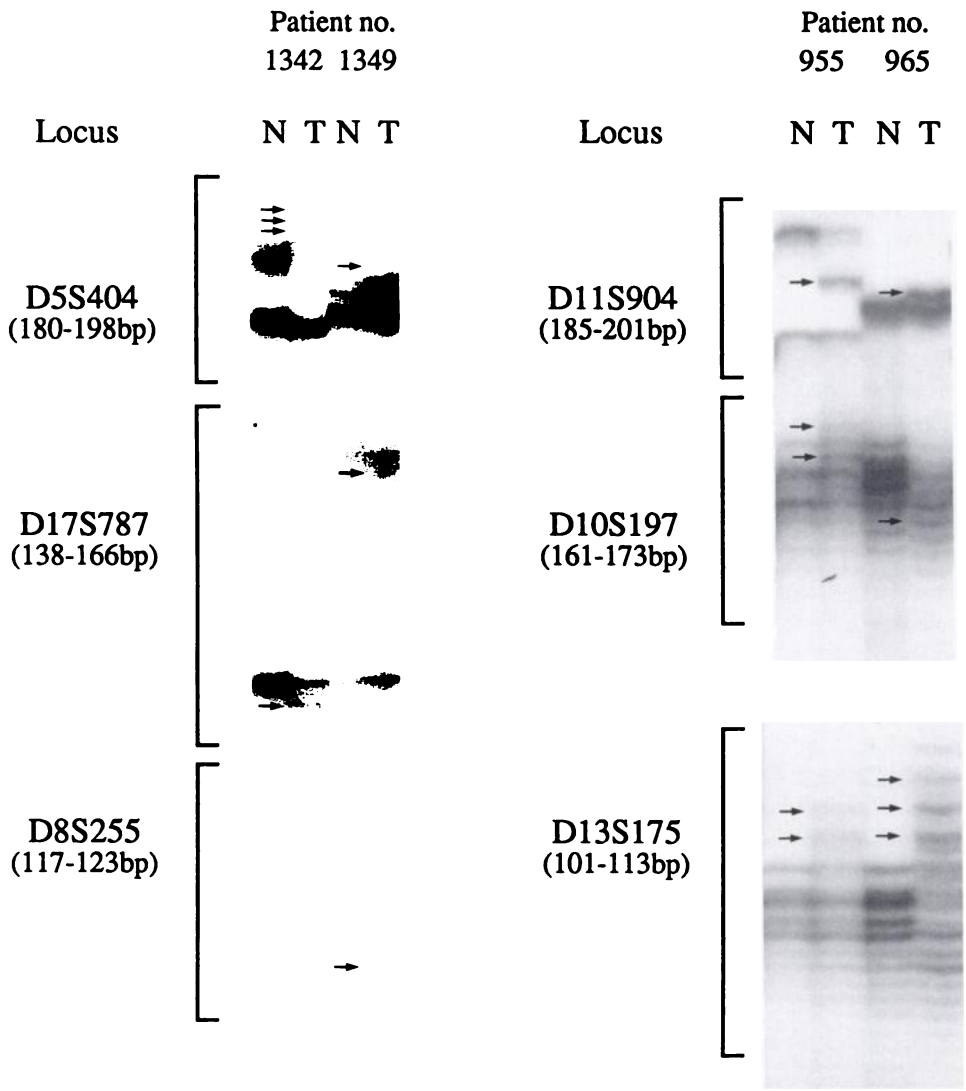

Fig. 1. Microsatellite instability in colorectal cancers. *N*, normal (blood) DNA; *T*, tumor (carcinomas) DNA. Microsatellite repeat patterns are shown for six loci (D-numbers). RER+ tumors show deviations from the normal pattern (*arrows*). The base pair range of each locus is shown and confirmed on every gel by use of *Hpa*II (*Msp*I) digested pBR322 as a size standard (not shown).

lies belonged to group 2. Several families (n = 28) were scored according to the less stringent criterias (group 3 and 4). Among the families negative for the group 2 criteria, three families with four affected members including ovarial, pancreas, and renal cancer were recorded. Two of these probands had RER- colorectal cancers, and one was not scorable for RER. Finally, in the total material one breast cancer family and one prostate cancer family were noted. These two probands had both RER- colorectal tumors.

Possible associations between the RER types and family history and the different clinicopathological characteristics were analyzed in 241 cases from whom we had information about all variables (Table 1). There was a trend towards more RER+ tumors among familial cases when only groups 1 and 2 were included, but the difference was not statistically significant: 31% (4 of 13) *versus* 17% (35 of 207) (P = 0.18). No significant association was found between RER+ and possible genetic predisposition including both stringent and less stringent criteria (groups 1–4).

Table 1 *Relation between RER+ colorectal cancers and family history and clinicopathological variables*

Family history and clinicopathological characteristica	Number of cases with (+) or without (-) RER				
	RER-	RER+ at 1 locus	RER+ at ≥ 2 loci	Total	P
Family history[a]					
Group 1	1	0	1	2	
Group 3	3	0	0	3	
Negative for group 1 and 3 criteria	197	17	22	236	NS[b]
				241	
Group 2	8	0	3	11	
Group 4	18	1	1	20	
Negative for group 2 and 4 criteria	175	16	19	210	NS
				241	
Group 1–4	29	1	4	34	
Negative for group 1–4 criteria	172	16	19	207	NS
				241	
Mean age SD	69.1 11.5	72.1 14.3	68.1 14.7		NS[c]
Sex					
Female	97	7	11	115	
Male	104	10	12	126	NS
				241	
Dukes' classification[d]					
A	33	3	3	39	
B	84	7	11	102	
C	58	2	7	67	
D	26	5	2	33	NS
				241	
Histological grade[e]					
Well differentiated	11	1	0	12	
Moderate differentiated	172	14	11	197	
Poorly differentiated	18	2	12	32	<0.001
				241	
Location[f]					
Right colon	55	3	19	77	
Left colon	50	6	3	59	
Rectum	96	8	1	105	<0.001
				241	
Ploidy[g]					
Diploid	69	6	21	96	
Aneuploid	132	11	2	145	<0.001
				241	

[a] Classification into different subgroups is defined in "Materials and Methods".
[b] NS, not significant.
[c] Determined by analysis of variance.
[d] According to the modified Dukes' classification (22, 23).
[e] According to the WHO criteria (24).
[f] Carcinomas in the coecum and ascending and transverse colon are classified as right sided, and carcinomas in splenic flexure, the descending and sigmoid part of the colon are left sided.
[g] Ploidy status was obtained by flow cytometry as described previously (25).

Significant associations were found between RER+ tumors and poor differentiation, diploidy, and right sided location (Table 1). These associations are mainly caused by the tumors with RER+ at two or more loci. From Table 1 it can be seen that the group with only one RER+ locus has a distribution similar to that of the RER- group.

Univariate cause specific (death by colorectal cancer) analysis of the 238 patients revealed a significant association between RER+ tumors at two or more loci and prolonged survival (P = 0.05, hazard ratio = 0.3) (Fig. 2). Patients dying within 30 days after surgery were excluded from the survival analysis. Bivariate analysis including Dukes' stages suggest the same trend but is not statistically significant (P = 0.21).

All scorable (information for at least three loci) cancers of the breast (84 of 85) and testis (86 of 96) revealed only RER- results.

Discussion

In an unselected material of primary colorectal cancers, 16.5% (40 of 243) showed microsatellite instability, detected as mobility shifts in the tumor DNA compared to constitutional DNA. Aaltonen *et al.* (5) reported alterations at ≥2 loci in 77% (10 of 13) of colorectal cancers from HNPCC patients (Amsterdam criteria), while only 13% (6 of 46) in sporadic cases. Among the patients reported here only two belonged to such families, and one of them had an RER+ tumor. However, we found no significant association between RER+ cases and general familial clustering (including all cases with positive family history: groups 1–4). RER is therefore not a common feature of tumors from genetically predisposed individuals but might be associated with a subgroup.

The frequency of RER+ tumors in this material 16.5% (40 of 243) is within the range of previously published figures. Ionov *et al.* (7) found 12% (16 of 137), Aaltonen *et al.* (5) found 13% (6 of 46 sporadic cases), and Thibodeau *et al.* (6) reported 28% (25 of 90). In the paper by Thibodeau *et al.* the observations were divided into alterations at one locus and two or more loci, and an even distribution between these two was found. This is also observed by us. However, the percentage of alterations is lower in this report than reported by Thibodeau *et al.* (6): 7% (17 of 243) and 10% (23 of 243) *versus* 13% (12 of 90) and 14% (13 of 90). It is noteworthy that the tumors with RER+ at only one locus have a distribution similar to that of the RER- group with regard to other variables (see Table 1) and that they are quite distinctly different from the RER+ at ≥2 loci group. This phenomenon of a single locus change seems to be characteristic of colorectal cancer. Among the tumor types defined as negative regarding RER, we observed this only twice among 85 lung cancers (see accompanying paper), none among 84 breast cancers and 86 testicular cancers. Among the other types of RER+ cancers, gastric cancer and endometrial cancer, alterations were always observed at more than one locus (20). However, here the number of tumors studied were quite small.

RER+ colorectal cancers were clearly associated with certain clinicopathological variables (Table 1). RER+ is much more frequent in the tumors from the proximal part of the colon than the distal part, consistent with other reports (5–7). Right sided location of a cancer is considered typical of hereditary cases and is inversely associated with a general loss of heterozygosity. This suggests that the inherited cases are predisposed to acquire replication errors and accumulate other genetic changes later in tumor progression. However, this is not com-

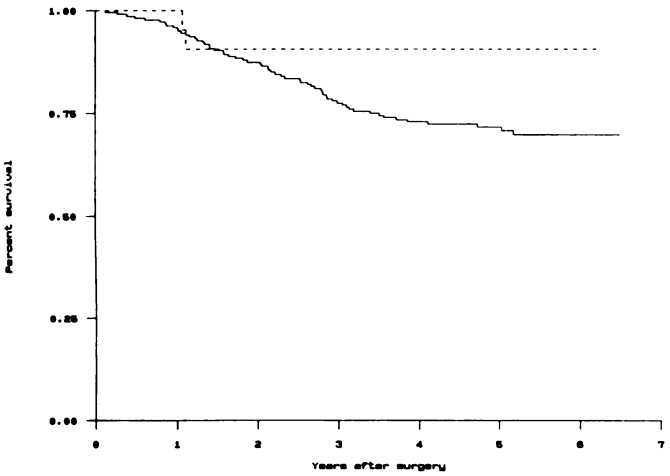

Fig. 2. Cause specific survival (death by colorectal cancer; Kaplan-Meier plot) for 238 patients with relation to microsatellite instability. —, RER– and RER+ at one locus (n = 217); - - -, RER at ≥ 2 loci (n = 21).

patible with our observation that RER+ tumors are not significantly associated with familial cases. If we look at the RER+ cases from whom we have family information, 2 of 19 (10%) of the patients with proximal tumors have a positive family history, while this is seen in 2 of 4 (50%) among patients with distal tumors. Possibly due to the small numbers this difference is not statistically significant, but the suggestion that one-half of the RER+ distal tumors seem to be genetically predisposed is intriguing (compatible with unpublished data from Aaltonen *et al.*). It should also be noted that among the 16 RER+ cases reported by Ionov *et al.* (7), 2 were distally located and resected from the 2 of the 3 youngest patients, suggesting genetic factor(s).

We found a striking association between RER+ tumors and DNA diploid status. This suggest that replication errors occur earlier in the tumor development than other genetic changes like amplification and loss of heterozygosity, which were mostly found in the aneuploid tumors (10, 21). Therefore information about the RER status in benign tumors from the colon would be of great interest.

Patients with RER+ (≥2 loci) cancers seem to have a longer survival, but microsatellite instability does not seem to be an independent prognostic indicator. Correction for staging reduces the P value to a nonsignificant level, although a tendency is still seen. A similar observation was made by Thibodeau *et al.* (6).

Among the families of the colorectal cancer patients there was only one family with clustering of breast cancer. The breast cancer patient series reveal none with clustering of colorectal cancer. Therefore, the breast-colon cancer families cannot be very frequent, although the breast cancer series is biased to some extent, since the tumors were partly selectively collected from patients with positive family history of breast cancer.

Our results provide further evidence for the hypothesis that inaccurate replication is an important contributor to the genesis of colorectal cancer. This phenomenon might be highly frequent in a subgroup (8) among the familial cases, but as shown here it is not associated with familial clustering in general. From a substantial number of testicular and breast cancers, that all were negative with regard to the described microsatellite instability we conclude that this phenomenon is not a general mechanism in carcinogenesis.

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