Identification of a breast tumor with microsatellite instability in a potential carrier of the hereditary non-polyposis colon cancer trait

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Allelic expansion at microsatellite loci in colorectal tumor DNA indicates a genomic instability caused by defects in DNA mismatch repair. This is observed in a high proportion of tumors from individuals affected by hereditary non-polyposis colorectal carcinoma, but to a lesser extent in sporadic colorectal tumors. In this study we screened 46 colorectal tumors for replication errors (RER). Tumors from six patients were found to be RER positive, two of which had a marked family history of colon cancer. In both cases the RER+ phenotype was detected in colon tumors from other family members, suggesting a germline mutation in mismatch repair genes. Additionally, RER+ phenotype, distinct from that of the colon and sporadic breast tumors, was found in malignant breast tissue from the mother of one proband.

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Genes predisposing to colorectal cancer, have been mapped to chromosome regions 2p16 and 3p21, in families suffering from hereditary non-polyposis colorectal cancer (HNPCC) syndrome (Peltomaki et al. 1993a, Lindblom et al. 1993, Papadopoulos et al. 1994). Tumors derived from patients of families linked to 2p and 3p have also been found to possess significant shifts in the number of simple repeated DNA sequences. This phenomenon is thought to result from errors in the DNA-replication process (Parson et al. 1993), and the positive phenotype has consequently been termed RER+ (replication error). In addition, screening of sporadic colorectal tumors has revealed that 12–28% of cases are of the RER+ type (Thibodeau et al. 1993, Aaltonen et al. 1993, Ionov et al. 1993, Lohse et al. 1993). Both sporadic and familial RER+ tumors are preferentially located in the proximal colon. RER+ is also observed in other HNPCC-related tumor types, like gastric, pancreatic, and endometrial carcinoma (Han et al. 1993, Mironov

et al. 1994, Peltomaki et al. 1993b, Risinger et al. 1993).

Human homologues of the bacterial DNA mismatch repair genes *mutS* (*hMSH2*) and *mutL* (*hMLH1*) have been mapped to the smallest region of linkage on chromosomes 2p16 and 3p21 in HNPCC families. Moreover, mutations in conserved regions of these genes are detected in normal tissue of HNPCC patients and sporadic RER+ tumors as well (Papadopoulos et al. 1994, Leach et al. 1993, Bronner et al. 1994). Gene frequencies of 0.005 and 0.0025 have been suggested for the mutated form of *hMSH2* and *hMLH1*, respectively (Papadopoulos et al. 1994, Aaltonen et al. 1993).

The aim of this study was to estimate the prevalence of the RER+ phenotype in our collection of colorectal tumors. Secondly, we wanted to evaluate the role of hereditary factors in the onset of such tumors by exploring the family history of RER+ patients with regard to cancer,

and to see if the RER+ phenotype could be applied to tumors obtained from other family members as well.

Subjects and methods

Subjects

The initial screening for the RER phenotype included carcinomas from 46 patients with a mean age of 72.6 years at surgery (range: 44–94 years). The site of primary tumor was proximal (right site) in 22 cases, 22 were of distal (left site) origin and one was in the transverse colon. One case was a metastasis in the spleen.

Information regarding relatives of the probands was obtained from the Icelandic Genealogy Committee and checked with the Icelandic Cancer Registry, pathology records and death certificates obtained from the Statistical Bureau of Iceland.

Samples

Tumor and non-malignant tissue samples from colon were obtained on the day of surgery from the Department of Pathology and stored at -80°C . The same department provided paraffin-embedded tissue from which both tumor and normal samples were obtained for the family study. Samples were prepared for PCR analysis by the method described by Smith et al. (1992), or a standard DNA extraction method (Sambrook et al. 1989).

PCR analysis

PCR was carried out in a volume of 30 μl containing 6–10 pmol of each primer, 0.2 mM of each dNTP, 0.5–1 μl (protease melted) or 50 ng (phenol extracted) template DNA, 0.3 units thermostable DNA polymerase (Finnzymes Oy) and 10 $\times$ buffer supplied with the enzyme. One primer in the reaction mixture was end-labelled with γATP^{32} using T_4 polynucleotide kinase (Amersham). Samples were processed through 35 cycles comprising 1 min at 94°C , 1 min at 55°C (except that 60°C was used for D3S1029) and 2 min at 72°C in a Techne PHC-3 thermocycler. Aliquots of the amplified DNA were electrophoresed on standard denaturing 6% polyacrylamide DNA sequencing gels, and subjected to autoradiography.

RER screening

All samples were analyzed initially by four different microsatellite primer pairs. RER+ phenotype was only assigned to those tumors with extra alleles detected at two different loci.

PCR primers

Microsatellite instability was examined using 18 separate (CA) $_n$ repeats localized at chromosomes 1q (D1S104), 2p16 (D2S123, D2S119, D2S136), 3p (THRB, D3S1210, D3S1029, D3S1217, D3S1293, D3S1298, D3S1283), 11 (D11S527, D11S907, D11S922) and 17 (THRA, TP53, D17S579, D17S588).

Results

Six out of 46 (13%) colorectal tumors showed microsatellite instability (Table 1 and 2). Five of these RER+ tumors were of proximal origin and one was from the transverse segment. Two of the six patients with the RER+ phenotype were shown subsequently to have a marked family history of cancer (Table 1; Fig. 1a and 1b).

In family A, two of the seven tumors in the main branch (Fig. 1a) were in the colon and three were breast carcinomas. The two other cancers were in the ovary and lung. Cancer in organs other than colon was, additionally, manifested in relatives of the father (II) (not shown in pedigree). His brother was diagnosed with leukemia at age 88 and various types of cancers were diagnosed in the children of three (out of seven) unaffected brothers. These included: cancer of the stomach, prostate, breast, lung, pancreas, and Hodgkins disease. The age at onset of cancer in these patients was, however, not particularly early compared to that typically observed in HNPCC.

RER+ phenotype was seen, at several microsatellite loci (Fig. 2 and Table 2), in tumors of pa-

Table 1. Patient information for individuals with RER+ colon tumors included in the original screening program (*); and affecteds in the two colon cancer families retrieved by reviewing family history of RER+ cases. Diagnosis was based on pathology records or death certificates (**)

Case no.	Site of primary tumor	Age at diagnosis	Family history
129*	colon/coecum	86	No
139*	colon/ascendes	83	No
220*	colon/coecum	81	No
234*	colon/coecum	78	No
121*/III 3	colon/coecum	50	Family A
A-II1	colon/transverse; lung	55, 64	Family A
A-II2	breast/bilateral 1)	33, 34	Family A
A-II3	breast; Ovary/bilateral	48, 61	Family A
219*/IV3	colon/transverse	44	Family B
B-II1	colon**	45	Family B
B-II2	colon**	67	Family B
B-II6	stomach**	87	Family B
B-II7	stomach**	55	Family B
B-III1	colon**	44	Family B
B-III2	colon**	37	Family B
B-IV2	colon/transverse	43	Family B

1) scirrhous type (sin) and adenocarcinoma (dx).

RER+ phenotype in hereditary colon and breast cancer

Table 2. RER+ tumors and familial tumors in this study assigned to type I or type II mutations according to Thibodeau et al. (1993). Type I is assigned to tumors with a change in allele size of more than one repeat unit. Type II indicates an allelic size reduction or expansion in tumor of one repeat unit. Zero indicates absence of extra alleles and a minus denotes that typing was not done. Samples are presented with sample number if sporadic or family number and site of tumor; *Proband

Markers	129 Colon	139 Colon	220 Colon	234 Colon	FamA III3* Colon	Fam A II1 Colon	Fam A II2 Br.sin	Fam A II2 Br.dxt	Fam A II3 Breast	Fam A II3 Ovary	Fam B IV3* Colon	Fam B IV2 Colon
D1S104	-	-	-	-	I	I	0	0	II	-	-	-
D2S136	-	-	-	-	I	I	-	-	II	-	-	-
D2S123	I	-	-	-	I	I	-	-	II	0	I	I
D2S119	-	-	-	-	II	I	-	-	II	-	-	-
THRB	-	-	0	-	0	-	-	-	-	-	I	-
D3S1029	I	-	I	-	I	I	-	-	II	0	I	I
D3S1217	-	I	-	0	I	-	-	-	-	-	II	-
D3S1293	-	I	-	I	I	-	0	0	-	-	-	-
D3S1298	-	I	I	I	I	-	-	-	-	-	I	-
D3S1283	-	-	I	I	I	-	-	-	-	-	II	-
D3S1210	-	-	-	-	I	I	0	0	0	-	0	I
D11S527	-	-	-	-	I	I	-	-	II	0	I	I
D11S907	-	-	-	-	I	I	-	-	0	0	I	II
D11S922	-	-	-	-	I	I	-	-	II	0	I	I
TP53	-	I	-	II	I	-	0	0	-	-	I	-
THRA	I	-	I	-	I	I	0	0	II	0	I	I
D17S579	I	II	-	II	I	I	0	0	I	-	II	-
D17S855	-	-	-	-	-	-	0	0	II	-	-	-
Total	4/4	5/5	4/5	5/6	16/17	11/11	0/7	0/7	10/12	0/6	12/13	7/7

tients III1 (in colon; lung tumor was not obtainable), II3 (RER+ in breast but not ovary tumor) and III3. Moreover, two tumors (breast cancer, age 55; pancreatic cancer, age 62) from the more distant relatives of the proband were found to be RER negative. We cannot exclude that the absence of RER in breast tumors from individual II2 is due to the high fraction of normal cells in the tissue examined, as PCR products from reactions with these samples were low in quantity and faint extra bands in gels could have been missed. The RER- ovary tumor from individual II3 showed a strong allelic imbalance with a marker located on chromosome 17q (data not shown), which indicates a high tumor cell fraction; this diminishes the probability of a false negative result.

In family B (Fig. 1b) six colon and two stomach cancers were detected in three generations. A strong RER+ phenotype was observed in colon tumors of individuals IV2 and IV3. Tissue samples were not available from tumors in other family members.

The RER+ phenotype seen in the four familial colon tumors corresponded, most often, to the type I mutation described by Thibodeau et al. (1993), and was defined by an increase or decrease in normal allele size of more than one repeat unit. The number of extra alleles in the colon tumors was usually large and frequently a ladder of alleles was produced. The largest number of extra alleles was found with the marker TP53, which detected

12 extra alleles in sample III3 (family A). The phenotype in the breast tumor was much weaker and typically only one extra allele was detected. Usually this extra band was in one repeat unit distance from a normal allele which corresponded to type II changes (Table 2 and Fig. 2).

Discussion

The observed frequency of RER+ (13%) in our collection of colorectal tumors and the bias towards RER+ tumor location in the proximal colon is in line with the results of others (Thibodeau et al. 1993, Aaltonen et al. 1993, Ionov et al. 1993, Lothe et al. 1993). Two individuals were identified as potential HNPCC gene carriers, which implies that the RER criteria can be used effectively to find potential HNPCC kindreds. Early age at onset of colon cancer in these two patients is also consistent with HNPCC.

The possibility of a hereditary susceptibility in family A is supported by the finding of double primaries and a relatively early age at onset in three siblings, II1-3, and by the appearance of extra alleles in three tumors of the colon and breast. This is unlikely to be caused by chance, since RER is infrequently seen in sporadic breast tumors (Eiriksdottir et al. 1995, Han et al. 1993, Wooster et al. 1994) or colon tumors. Breast cancer cases have been reported in some Lynch II syndrome kindreds (Lynch et al. 1988), although stat-

Fig. 1a and b. Pedigrees of families A and B. Squares denote males and circles females. A circle inside a square denotes male and female siblings, and the number within this symbol denotes the number of siblings. A diagonal slash through a symbol denotes a deceased individual. Arrows point to probands. An asterisk is used to mark individuals with RER+ phenotype in tumor. □ healthy individual; ■ colon cancer; ○ malignancies at extra-colonic site; ● two primary cancers at extra-colonic sites; site of primary tumor and age at diagnosis is given below symbols for affected individuals, and age at death (d) or last observation (age) for unaffected individuals.

Fig. 2. Dinucleotide repeat polymorphism of paired normal and tumor tissue from breast and colon cancer patients of family A (a) and B (b). The markers used in the analysis were THRA (a) and D11S527 (b). Additional alleles in tumors are indicated by arrows.

istical evidence linking breast cancer to HNPCC was not obtained in an independent study (Watson et al. 1993).

The RER+ breast tumor in family A was altered in most of the repeats examined. However, of the RER+ sporadic breast tumors studied by Wooster et al. (1994) none had instability at more than one locus (of 12). Glebov et al. (1994) have reported a high frequency of RER+ in breast cancers from individuals with a positive family history of the disease. In their series RER+ was seen in 5 of 14 (35%) cases of sporadic breast tumors and 15 of 18 (83%) in the familial tumors. However, they found no tumor altered at more than four of the nine loci tested. These results are inconsistent with the findings of Peltomaki et al. (1993b) who found no RER+ phenotype in 86 breast tumors examined. Further, we have analyzed 35 familial breast tumors with microsatellite markers (Bergthorsson et al. 1995) and found none with a RER+ phenotype.

One possible explanation for the appearance of extra alleles in sporadic breast cancer is that the instability observed is not caused by mismatch repair gene defects, but rather reflects the ability of this repair system to work in the environment provided by the tumor cell. Secondly, sporadic RER+ tumors could be caused by milder mutations, and perhaps in different genes. Finally, sporadic tumors may have a defective mismatch repair system but need an additional environmental or genetic cofactor to result in a strong RER+ phenotype.

Defective mismatch repair is perhaps not the main source of genome instability in RER+ breast tumor cells, as has been suggested for colon tumors in HNPCC. The absence of mismatch repair in the minority of breast tumors could be a secondary event and the result of unrelated chromosomal changes. The localization of the *hMLH1* gene on chromosome 3p21, in a region frequently altered in breast tumors (Sato et al. 1991), may be important in this respect.

The mode of inheritance of cancer susceptibility in family A is complicated by the fact that both grandparents (I1 and I2) were free of cancer and they had no further family history of colon cancer. The discovery of RER+ in both breast and colon tumors from the family probably reflects the transmission of a defective mismatch repair gene. The alternative, that an unidentified environmental factor may specifically promote RER+ tumor formation, is unlikely. Haplotypes from 2p16 but not 3p21 are shared in individuals II1 and III3 in family A, and a germ line defect in the *hMSH2* gene is therefore possible.

Family B is characterized by early age at onset of colon cancer (onset before age 50) in five indi-

viduals of three consecutive generations, and is compatible with the autosomal dominant model of inheritance proposed for HNPCC. An interesting feature of the two cases from this family for which the histology was known (cases IV3 and IV2) is that both are from the transverse colon. Altogether, three out of four informative familial tumors originate at this site, including the tumor in case II1 in family A. The tumor incidence in the transverse segment is generally low compared to other regions in the colon: hence, the possibility of a connection to family history should be considered in future studies.

The two families reported here are the first Icelandic HNPCC families that have been described. The small population size, successful cancer registration, and the availability of genealogical and pathological information make it possible to identify the majority of HNPCC families in the whole population. A screening program based on identification of early-onset colon cancer patients, presence of the RER phenotype, and proximal location of the tumor would also permit an estimation of the fraction of HNPCC caused by mismatch repair gene defects.

With this report we contribute to the growing body of evidence suggesting a hereditary factor in the etiology of colon tumors. To our knowledge, family A is the first reported family where both colon and breast tumors demonstrate an RER+ phenotype.

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