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Evidence that the *MCC-APC* Gene Region in 5q21 Is Not the Site for Susceptibility to Hereditary Nonpolyposis Colorectal Carcinoma[1]

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ABSTRACT

Hereditary nonpolyposis colorectal carcinoma (HNPCC) is the most common form of hereditary colon cancer. Autosomal dominant inheritance is evident from pedigrees but the genetic basis of the disorder is otherwise unknown. Recently, two genes in 5q21 involved in colon carcinogenesis, *APC* and *MCC*, were identified, and *APC* was shown to be the gene predisposing to familial adenomatous polyposis. To determine if these genes also confer susceptibility to HNPCC we performed linkage analyses in nine affected families. The *MCC-APC* region could be formally excluded as the locus for HNPCC in seven families. In one family the results were suggestive of exclusion, although they were not conclusive. The remaining family was uninformative. We used two alternative definitions of affected status. Based on haplotypes for *MCC* and *APC* the added pairwise logarithm-of-odds score for all nine families was −22.57 at the recombination fraction of 0.00 using more stringent criteria for the HNPCC phenotype and −22.67 for less stringent criteria. In addition to blood DNA samples from living family members, DNA from formaldehyde-fixed archival pathology specimens from deceased individuals contributed to these linkage results.

INTRODUCTION

The HNPCC[3] syndrome comprises about 5% of all colorectal carcinomas (1, 2). The minimum criterion of HNPCC is that colorectal carcinoma is diagnosed and histologically verified in at least three relatives (one of whom is a first-degree relative to the other two) belonging to two or more successive generations. Moreover, the age of onset should be less than 50 years in at least one patient (3). In addition to colon (most often right-sided), organs commonly affected with cancer include the endometrium, stomach, biliopancreatic system, and urinary tract (4).

The molecular basis of HNPCC is unknown. Recently, two genes termed *MCC* (5) and *APC* (6, 7) were identified in 5q21, a region considered critical for colorectal tumor development (8, 9). *MCC* and *APC* are located some 150 kilobases apart (6) and both show somatic mutations in colorectal cancer (10). Furthermore, germ-line mutations and deletions of the *APC* gene occur in patients with familial adenomatous polyposis (and Gardner syndrome), and therefore, *APC* is regarded as the gene predisposing to this hereditary colon cancer syndrome (10, 11).

This study was carried out to determine if the putative gene predisposing to HNPCC cosegregates with *MCC* or *APC* in any of the nine families subject to our investigation. We also tested the applicability of fixed tissue stored in paraffin blocks in our experiments and found that DNA extracted from paraffin sections from deceased family members can be of great potential use in linkage studies of HNPCC and other similar disorders.

MATERIALS AND METHODS

Subjects

Of 40 families with HNPCC identified in Finland (4) nine were selected on the basis that they had the greatest possibilities of being informative in linkage analysis. Clinical features of seven of the families were described in detail previously (12). The two additional families (nos. 11 and 56; Fig. 1) had essentially similar main characteristics. The number of family members studied was 117, including 36 patients with carcinoma considered typical of HNPCC (groups I and II in Table 2) and 7 patients with adenoma. In addition, fixed tissue in paraffin blocks was available from 20 deceased cancer patients.

Methods

Established procedures were used to isolate DNA from blood leukocytes. DNA from paraffin sections was extracted by the method described by Wright and Manos (13). Following amplification by PCR (14), polymorphisms were revealed by size fractionation on agarose gels or hybridization of slot-blotted DNA with allele-specific oligonucleotides which were end-labeled with [γ-^{32}P]ATP.

DNA Polymorphisms

We studied polymorphisms from the *MCC* gene and the *APC* gene (Table 1). All PCR amplifications were performed in a buffer containing fixed concentrations of KCl (50 mM), Tris-Cl (10 mM, pH 8.9), and bovine serum albumin (0.01%), while the concentration of $MgCl_2$ varied in different reactions. Reaction conditions for individual polymorphisms were as follows.

MCC **Deletion Polymorphism.** The procedure comprised 30 cycles consisting of 1.5 min at 95°C, 1.5 min at 55°C, and 2 min at 72°C, followed by 10 min at 72°C. The optimal concentration of Mg^{2+} in the amplification buffer was 1.5 mM. The alleles were detected by agarose gel electrophoresis followed by ethidium bromide staining of gels.

APC **Exon 11, T/C Polymorphism.** The procedure comprised 35 cycles consisting of 1.5 min at 94°C, 1.5 min at 53°C, and 2 min at 72°C, followed by 8 min at 72°C. The Mg^{2+} concentration in the amplification buffer was 1.5 mM. The polymorphism was detected by two different methods: *Rsa*I digestion and agarose gel electrophoresis or, alternatively, hybridization of slot-blotted amplification products with allele-specific oligonucleotides. In the latter case prehybridization, hybridization, and filter washes were performed at 50°C for ASO1 and 52°C for ASO2. The prehybridization and hybridization solution contained 1 mM EDTA, 0.5 M phosphate buffer (pH 6.8), and 7% sodium

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[3] The abbreviations used are: HNPCC, hereditary nonpolyposis colorectal carcinoma; MCC, mutated in colorectal carcinoma; APC, adenomatous polyposis coli; PCR, polymerase chain reaction; ASO, allele-specific oligonucleotide; lod, logarithm of odds.

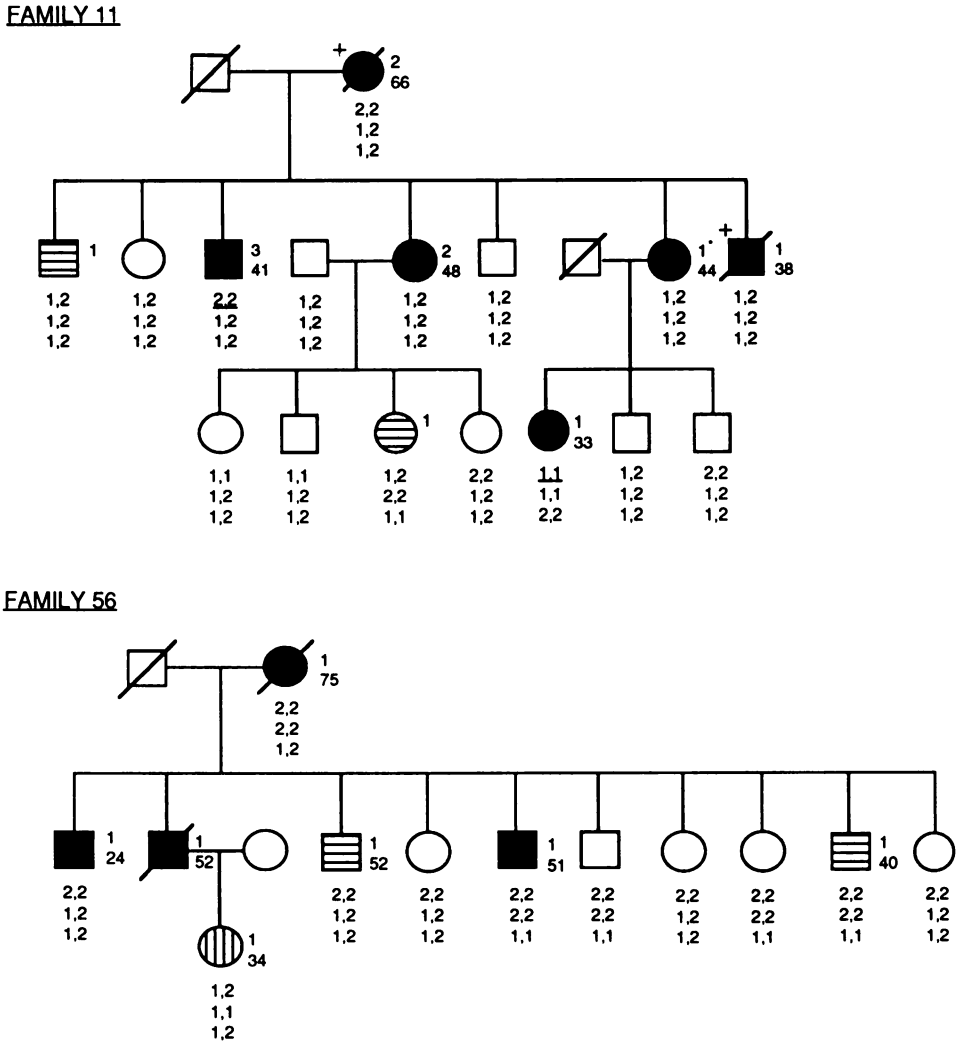

Fig. 1. Pedigrees of families 11 and 56. ○, ●, female; □, ■, male; ⊘ (and all symbols with diagonal), deceased. *Open symbol*, no neoplasm detected; *filled symbol*, affected with neoplasm. ■, HNPCC carcinoma (group I in Table 2); ⊜, ⊟, adenoma; ◍, any malignancy not typical of HNPCC (group III in Table 2); $^{+}$●, $^{+}$■, DNA derived from paraffin blocks; ■^{2}, number of primary neoplasms; ■$_{48}$, age at diagnosis of first cancer. □, alleles. The polymorphisms are (top to bottom): *MCC* deletion (*1*, deletion present; *2*, deletion absent); *APC* exon 11, thymine/cytosine polymorphism (*1*, thymine; *2*, cytosine); *APC* exon 13, adenine/guanine polymorphism (*1*, adenine; *2*, guanine). Allelic patterns indicating recombination between *HNPCC* and *MCC* in family 11 are *underlined*. This conclusion is based on the observation that two affected family members do not have any allele in common.

dodecyl sulfate. Filter washing was done starting with 3× standard saline citrate + 0.1% sodium dodecyl sulfate, and final washes were in 0.1× standard saline citrate + 0.1% sodium dodecyl sulfate.

***APC* Exon 13, A/G Polymorphism.** The procedure comprised 30 cycles consisting of 1.5 min at 94°C, 1.5 min at 50°C, and 2 min at 72°C, followed by 8 min at 72°C. The Mg^{2+} concentration was 1.0 mM in the amplification buffer. Prehybridization, hybridization, and filter washes of slot blots were performed in solutions described above at 48°C for ASO1 and 50°C for ASO2.

Analysis of DNA from Fixed Paraffin Block-derived Tissue

DNA extracted from paraffin sections of fixed tissue was subjected to two successive amplification reactions consisting of 35 cycles each. Amplification was carried out in a volume of 50 or 100 μl, and 2 μl from the first reaction were used for reamplification. Conditions in each of the reactions were the same as for blood DNA except that annealing temperatures were decreased by 1 or 2°C. We used either the same primers in both reactions (*MCC* deletion and *APC* exon 11 polymorphisms) or two nested sets of primers (*APC* exon 13). In the nested primer system the reaction conditions were similar for inner and outer primers, and the basic reaction was as described above.

Linkage Analysis

Pairwise linkage analyses were carried out by the MLINK program from the LINKAGE program package (15). In linkage calculations, individuals with different neoplasms were treated as detailed in Table 2. Colorectal and endometrial carcinoma (the two clearly most common cancers in HNPCC family members; Refs. 4 and 16) were considered as definitive manifestations of the disorder if diagnosed at 60 years of age or below. Individuals with (a single) carcinoma of the ovary, stomach, hepatobiliary system, small intestine, kidney, or ureter were treated in one of two alternative ways, either as having an unknown status or being affected. Similarly, patients with colorectal adenoma were treated in one of these two alternative ways.

Phenotypes for HNPCC were coded as affected with an autosomal dominant mode of inheritance and four liability classes (penetrances) according to the age at the time of observation (healthy) or at the time of diagnosis. Penetrances for heterozygotes were set as 0.15 at age 30 years or below (liability class 1), 0.40 at age 31–45 years (liability class 2), 0.70 at age 46–60 years (liability class 3), and 0.90 at age 61 or over (liability class 4). The frequency of the *HNPCC* gene was estimated to be 0.001. For efficient linkage calculations the *MCC+APC* markers were haplotyped familywise.

RESULTS

We found no evidence for linkage of HNPCC to the markers from *MCC* or *APC* in any of the nine families. A formal exclusion of the *MCC-APC* region as the site of the putative gene for HNPCC was obtained in seven families. In each of these families the pairwise lod score values for *MCC+APC* haplotypes were below −2, the conventional threshold for rejection of linkage, at a recombination fraction = 0.00 (Table 3). In one family not providing formal exclusion, family 2, a lod score of −1.10 at a recombination fraction = 0.00 also suggested exclusion. Family 39 was uninformative. Different ways of status assignment for group II individuals (Table 2) did not have any major influence on linkage results. The added pairwise lod score for

Table 1 *DNA polymorphisms studied from MCC and APC*

Locus	Polymorphism/primers and allele-specific oligonucleotides	Alleles (in base pairs)	Reference
MCC	14-base pair deletion polymorphism/ 5′-TAC GAA TCC AAT GCC ACA GC-3′ 5′-CAC TTC TAC CCT GAA GTA GC-3′	89, 75	Unpublished data
APC	Exon 11, T/C polymorphism/ 5′-ATT AGG GGG ACT ACA GGC CA-3′ 5′-AGC CAT TCC AGC ATA TCG TC-3′	After *RsaI* digestion: 108, 46 + 62	7
	ASO1: 5′-TGA AAT GTA TGG GCT TAC-3′ ASO2: 5′-TGA AAT GTA C̲GG GCT TAC-3′	108 (T), 108 (C)	
	Exon 13, A/G polymorphism/ 5′-GCA ACT AGT ATG ATT TTA TGT A-3′ 5′-TCT TTT TAC TAT TTA CAT CTG C-3′		Unpublished data
	Nested primers Outer primers 5′-GCA TTA AAA ACA AAA AAG CA-3′ 5′-CAC ACT TCC AAC TTC TCG AC-3′ Inner primers 5′-GCA ACT AGT ATG ATT TTA TGT A-3′ 5′-TCT TTT TAC TAT TTA CAT CTG C-3′		
	ASO1: 5′-GTT ATT GCA AGT GTT TTG-3′ ASO2: 5′-GTT ATT GC̲G AGT GTT TTG-3′	110 (A), 110 (G)	

Table 2 *Categories applied to HNPCC family members with different neoplasms in regard to affection status*

Group I Affected
- a. Colorectal or endometrial carcinoma diagnosed at ≤60 years
- b. Two or more primary carcinomas from groups I or II diagnosed at any age
- c. Anyone from group II with offspring in group I

Group II. Treated in one of two alternative ways, either as having an unknown status or as being affected
- a. Colorectal or endometrial carcinoma diagnosed at >60 years
- b. Carcinoma of the ovary, stomach, hepatobiliary system, small intestine, kidney, or ureter
- c. Colorectal adenoma

Group III. Unknown
Other malignancies or uncertain diagnosis

MCC+APC in all nine families was −22.57 *versus* −22.67 at a recombination fraction = 0.00 when individuals from group II were considered as having an unknown status *versus* being affected. Information derived from paraffin blocks was included in the above-mentioned linkage results.

Fig. 1 shows pedigrees of families 11 and 56 that were not included in our previous study in which the pedigrees of the other seven families were depicted (12). Alleles representing the *MCC* deletion polymorphism and the single nucleotide polymorphisms of exons 11 and 13 of *APC* are shown. At least one obligate recombination between *HNPCC* (status affected) and *MCC* was observed in family 11.

DISCUSSION

The present study was undertaken with three principal aims. First, we wanted to find out if susceptibility to HNPCC was linked to the putative candidate genes in 5q21. Second, we considered it important to determine how the stringency of definitions for HNPCC would affect the linkage results. Third, we wanted to test whether DNA samples originating from fixed tissue stored in paraffin blocks could be successfully used in linkage studies.

Region 5q21 is a plausible candidate area for the putative *HNPCC* gene for several reasons. There is evidence suggesting that chromosome 5 contains genes which normally suppress colon carcinogenesis (see "Introduction" and Ref. 17). The properties of the *APC* and *MCC* genes are compatible with a role in colon tumor suppression. Furthermore, there are phenotypically intermediate forms between hereditary nonpolyposis colorectal carcinoma and familial adenomatous polyposis. Lynch *et al.* (18) described a hereditary right-sided colon carcinoma syndrome with flat adenomas. These are more numerous than in HNPCC but less so than in familial adenomatous polyposis. Unlike both HNPCC and familial adenomatous polyposis, this syndrome is characterized by late onset (mean, 62 years). A family with clinical features similar to this syndrome was studied by Leppert *et al.* (19). These variant families showed evidence for genetic linkage to *APC*. Although the nine

Table 3 *Two-point analysis of linkage versus HNPCC using haplotypes for MCC* and *APC*

The data are based on the deletion polymorphism of *MCC* and the single nucleotide polymorphisms of exons 11 and 13 of *APC*. lod scores are shown separately for each family. Individuals belonging to group II (Table 2) were scored in two alternative ways: either as having an unknown status (left) or as being affected (right).

	Recombination fraction									
Family	0.00		0.01		0.05		0.10		0.20	
2	−1.10	−0.78	−0.30	0.00	0.24	0.52	0.40	0.65	0.39	0.59
3	−1.69	−2.32	−0.75	−1.55	−0.18	−0.81	0.02	−0.46	0.10	−0.15
6	−2.72	−2.42	−2.42	−2.14	−1.35	−1.12	−0.82	−0.63	−0.35	−0.23
8	−2.71	−2.71	−1.34	−1.34	−0.67	−0.67	−0.40	−0.40	−0.16	−0.16
10	−2.22	−2.22	−1.26	−1.26	−0.63	−0.63	−0.37	−0.37	−0.15	−0.15
11	−3.89	−4.42	−2.49	−1.92	−1.61	−1.05	−1.12	−0.62	−0.59	−0.22
39	0.83	0.83	0.81	0.81	0.73	0.73	0.63	0.63	0.41	0.41
56	−5.49	−4.89	−2.30	−3.67	−1.40	−2.12	−0.93	−1.37	−0.43	−0.63
59	−3.57	−3.74	−1.54	−1.74	−0.85	−1.01	−0.55	−0.67	−0.26	−0.32
Total	−22.57	−22.67	−11.59	−12.81	−5.70	−6.16	−3.15	−3.24	−1.03	−0.85

families studied by us were clinically quite typical and uniform, the diversity of clinical manifestations in different families could reflect genetic heterogeneity. Therefore, it would not be surprising if some HNPCC families turned out to be genetically linked to *APC*.

None of the families included in the present investigation showed evidence of close linkage of the disease phenotype to *MCC* or *APC*, and the *MCC-APC* region could be excluded as the site of the putative gene for HNPCC in at least seven families. We previously provided data supporting the exclusion of DCC and part of chromosome 18q, another candidate region, in five of these families (12). Our results indicate that the hereditary susceptibility to HNPCC is independent of these genes in the families we have studied and that the putative gene predisposing to HNPCC is different from that causing familial adenomatous polyposis.

As discussed previously several factors may cause variation in linkage results in HNPCC (12). Our present results were not significantly influenced by the different definitions of HNPCC. As pointed out by others, changes in the affection status may, however, have important consequences if there is positive evidence for linkage (20, 21). In the present study we used a value of 0.001 for the frequency of the putative *HNPCC* gene in the population. This is likely to be a safe overestimate. However, since sporadic colorectal cancer is a common disorder, one might argue for using an even higher value for the frequency of the putative *HNPCC* gene in linkage calculations. Using a frequency of 0.01 instead, only three of the nine families showed lod score values below −2 for the *MCC-APC* region. These examples demonstrate that linkage calculations in HNPCC should regularly be carried out in several ways to test the effect of changes in the appropriate variables.

A major drawback in studies of HNPCC is the malignant nature of the disorder and the ensuing loss of linkage information through the death of several key family members. This problem can be partially overcome by using archival specimens as the DNA source. The DNA recovered from formaldehyde-fixed, paraffin-embedded pathology specimens is of lower molecular weight than DNA extracted from fresh or frozen blood or tissues (22, 23). However, it is usually sufficiently well preserved to allow amplification of short segments by PCR. In the present study we were able to successfully use 1- to 20-year-old paraffin blocks in PCR reactions which produced amplified fragments between 100 and 200 base pairs in size. The role of fixed tissue becomes even more important in future studies when statistically significant linkage rather than exclusion is to be demonstrated.

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