ABSTRACT

Hereditary non-polyposis colorectal carcinoma (HNPPC) syndrome is characterized by early onset and multiple cancers of predominantly the proximal colon and occasionally other organs. The mode of transmission is compatible with autosomal dominant inheritance, but the location and characteristics of the putative susceptibility gene are unknown. We performed linkage analyses with the aim of proving or excluding the existence of a susceptibility locus on 18q. This hypothesis was based on the frequent involvement of the DCC gene in colorectal carcinoma and on the previously reported linkage between HNPPC and the Kidd blood group locus (JK) also on 18q. Seven HNPPC families were tested with eight polymorphisms, including three from within DCC. The DCC locus could be excluded as the HNPPC susceptibility locus in five families in which the two point logarithm-of-odds scores were −3.66, −3.63, −4.12, −7.90, and −3.74 at the recombination fraction of 0.00. In the remaining two families linkage could be neither excluded nor confirmed. The added pairwise logarithm-of-odds score for all seven families was −22.65 at the recombination fraction of 0.00. Multipoint analyses of linkage in the seven families suggested exclusion of some 60 cM in the region D18S18-D18S22-D18S7 as the site for HNPPC susceptibility locus. In addition to DCC, the excluded portion comprises JK.

INTRODUCTION

The HNPPC syndrome constitutes the most common form of hereditary colorectal cancer. It comprises at least 5% of all cases of colorectal cancer (1, 2). Diagnostic criteria include: (a) familiality: a minimum of three affected first-degree relatives in at least two generations; (b) early onset: mean age about 45 years as opposed to over 60 years in cases with sporadic colon cancer; (c) multifocality and multiplicity of primary cancers, particularly in the right-sided colon but also in the endometrium and other intraabdominal organs (3, 4). Vertical transmission of the susceptibility is compatible with autosomal dominant inheritance. Colon cancer associated with extracolonic cancers [Lynch syndrome II = cancer family syndrome; McKusick No. 114400 (5)] is sometimes distinguished from site-specific nonpolyposis colon cancer [Lynch syndrome I; McKusick No. 114500 (5)] even though the clinical basis for this distinction is not unequivocal (6). Thus far, there is no genetic evidence for such a distinction. In the present context we made no effort to distinguish between the two proposed entities based on clinical findings.

The molecular pathogenesis of HNPPC is unknown. There is a report suggesting linkage (lod score 3.19 at θ = 0.00) of the Lynch syndrome II subgroup of HNPPC to the Kidd blood group (7, 8). This result was based on nine families. The locus for the Kidd blood group (JK) has been mapped to the long arm of chromosome 18 (9).

Allelic deletions involving chromosome 18 occur in 70–80% of colorectal cancers (10, 11). According to Knudson’s two-hit hypothesis (12) both familial and nonfamilial forms of a given neoplasm result from mutations of the same critical (tumor suppressor) gene locus. A gene with tumor suppressor properties termed DCC was recently identified on the long arm of chromosome 18 (13). The DCC gene showed allelic deletions in 71%, reduced expression in 88%, and somatic mutations in at least 13% of colorectal carcinoma biopsies or cell lines. Further evidence of an important role of chromosome 18 in colon carcinogenesis was recently provided by Tanaka et al. (14) who showed that the introduction of a normal chromosome 18 into a human colon carcinoma cell line suppressed its tumorigenicity.

The present investigation was undertaken to determine if the putative gene predisposing to HNPPC cosegregates with DCC or is genetically linked to 18q specific markers close to this gene. Here we report linkage studies in seven families. Evidence supporting exclusion of DCC as the locus for HNPPC was obtained in five families while two families were uninformative. By multilocus analysis exclusion of a portion of 18q was also suggested.

MATERIALS AND METHODS

Subjects. Of 40 families with HNPPC identified in Finland (6), 7 well verified families (families 2, 3, 6, 8, 10, 39, and 59) were selected on the basis that they had the greatest possibilities of being informative by linkage analysis. The total number of family members studied was 94 including 29 patients with carcinoma and 3 patients with colonic adenoma. Most of the patients diagnosed during the last 10 years were seen by us. In affected individuals not seen by us the origin and histology of the cancer were ascertained through the medical records and pathology reports.

The mean age at onset of colorectal cancer was 42.4 ± 2.7 (SD) years in six of the families while in family 59 it was 53 years. In three-fourths of all colon carcinoma patients belonging to these families the proximal (i.e. ascending plus transverse) colon was affected whereas in one-fourth carcinoma was located only in the distal part of the colon. Multiple primary cancers were common (with a maximum of eight primary neoplasms observed in one person). Extracolonic cancers occurred in all families and with the exception of families 3 and 39 these cancers were found in several members per family. The most common
EXCLUSION OF DCC AND PART OF 18q IN HNPCC

extracolonic malignancies were endometrial, gastric, and biliopancreatic carcinoma.

Samples. Venous blood (20–40 ml) was obtained from each individual after informed consent. Part of the sample was used to establish a lymphoblastoid cell line as a permanent source of material. Another part of the sample was used for DNA extraction.

Southern Blot Hybridization. High-molecular-weight DNA was isolated from blood leukocytes according to standard procedures (16). DNA samples were digested with restriction endonucleases (from Promega, Madison, WI, or Gibco-BRL, Gaithersburg, MD) under conditions recommended by the manufacturer. DNA fragments were separated by electrophoresis on 0.7% agarose gels and transferred by the method of Southern (17) to nylon membranes (Zeta-Probe; Bio-Rad, Richmond, CA). DNA probes were labeled with [α-32P]dCTP to a high specific activity by nick translation (18) or oligolabeling (19). The membranes were prehybridized and hybridized in solutions recommended by the supplier. After hybridization the membranes were washed at appropriate stringencies and autoradiographed at -70°C for 1–10 days using intensifying screens.

DNA Probes. Eight probes from the long arm of chromosome 18 were used in the linkage analyses (Table 1). The most likely order of the respective loci (from proximal to distal) is D18S7-D18S22-D18S18-DCC-BCL2-D18S14 (data not shown).

Linkage Analysis. We used programs of the LINKAGE program package (25). Pairwise linkage analyses were carried out by programs ILINK and MLINK and four-point analyses by LINKMAP assuming no sex difference. Marker distances and order were based on published data (26) or multipoint linkage calculations (program CILINK) using information from the Centre d’Etude du Polymorphisme Humain public database (version 3.1, 1990) and DCC marker p15-65 and pSAM1.1 hybridization results produced by our laboratories on DNA samples from a panel of 40 Centre d’Etude du Polymorphisme Humain families.

RESULTS

There was no evidence for linkage of HNPCC to the markers specific for 18q in any of the seven families. Summation of the pairwise lod scores obtained for individual families using different probes gave maximum lod scores <0.5 (at varying values of θmax). The maximum total pairwise lod score for DCC was 0.00 at θ = 0.50.

Evidence supporting exclusion of DCC as the locus for susceptibility to HNPCC was obtained in five of the seven families. The pairwise lod score values for these families (families 2, 3, 8, 10, and 59) were -3.66, -3.63, -4.12, -7.90, and -3.74, respectively, at θ = 0.00. (Table 2). Families 6 and 39 were uninformative and showed evidence neither for nor against linkage. Added lod scores were below -2, the conventional threshold for rejection of linkage, for all studied markers except

Table 1 Probes from chromosome 18 used to study linkage

<table>
<thead>
<tr>
<th>Probe (locus)</th>
<th>Restriction enzyme</th>
<th>Allele size (kilobases)</th>
<th>Allele frequency</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>OLVIA8 (D18S7)</td>
<td>MspI</td>
<td>3.8</td>
<td>0.56</td>
<td>20</td>
</tr>
<tr>
<td>pEFZ10 (D18S22)</td>
<td>PvuII</td>
<td>5 alleles between 2.5 and 5.0</td>
<td>Heterozygosity 0.70</td>
<td></td>
</tr>
<tr>
<td>CRI-L84 (D18S18)</td>
<td>MspI</td>
<td>6.4</td>
<td>0.72</td>
<td>22</td>
</tr>
<tr>
<td>pSAM1.1 (DCC)</td>
<td>EcoRI</td>
<td>9.0</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>p15-65 (DCC)</td>
<td>MspI</td>
<td>10.5</td>
<td>0.17</td>
<td>13</td>
</tr>
<tr>
<td>pJOSH4.4 (DCC)</td>
<td>PstI</td>
<td>15</td>
<td>0.50</td>
<td>B. Vogelstein et al., unpublished</td>
</tr>
<tr>
<td>pB6 (BCL2)</td>
<td>EcoRI</td>
<td>10</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>CRI-L1156 (D18S14)</td>
<td>MspI</td>
<td>12.5</td>
<td>0.50</td>
<td>22</td>
</tr>
</tbody>
</table>

Table 2 Two-point analysis of linkage between HNPCC and different loci from 18q

Lod score values are shown separately for each family.

<table>
<thead>
<tr>
<th>Locus/recombination fraction</th>
<th>D18S7</th>
<th>D18S22</th>
<th>D18S18</th>
<th>DCC*</th>
<th>BCL2</th>
<th>D18S14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family</td>
<td>0.00</td>
<td>0.01</td>
<td>0.05</td>
<td>0.00</td>
<td>0.01</td>
<td>0.05</td>
</tr>
<tr>
<td>2</td>
<td>-0.11</td>
<td>-0.11</td>
<td>-0.09</td>
<td>-3.02</td>
<td>-1.14</td>
<td>-0.50</td>
</tr>
<tr>
<td>3</td>
<td>-3.63</td>
<td>-1.50</td>
<td>-0.78</td>
<td>-4.08</td>
<td>-1.36</td>
<td>-0.69</td>
</tr>
<tr>
<td>6</td>
<td>-0.03</td>
<td>-0.02</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>8</td>
<td>-4.31</td>
<td>-1.40</td>
<td>-0.72</td>
<td>0.42</td>
<td>0.41</td>
<td>0.36</td>
</tr>
<tr>
<td>10</td>
<td>-3.50</td>
<td>-1.46</td>
<td>-0.76</td>
<td>-4.58</td>
<td>-1.55</td>
<td>-0.84</td>
</tr>
<tr>
<td>39</td>
<td>0.10</td>
<td>0.09</td>
<td>0.07</td>
<td>0.06</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>59</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Haplotype based on pSAM1.1, p15-65, and pJOSH4.4 polymorphisms. ND, not determined.

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D18S14 at $\theta = 0.00$ suggesting exclusion of these regions as the site of the putative gene for HNPCC.

Fig. 1 shows pedigrees of the families and the alleles representing D18S22, D18S18, and DCC (polymorphic sites for EcoRI, MspI, and PstI). At least one obligate recombination between HNPCC (status affected) and DCC was observed in each of families 2, 3, 8, and 59 while family 10 had at least two obligate recombinations. The fact that the lod scores (Table 2) do not unequivocally indicate recombination (i.e., lod score $\sim 0$) is due to reduced penetrance.

Multipoint analysis of linkage versus HNPCC was performed on the seven families. We studied a region of about 60 cM which included the following markers (from distal to proximal): DCC, D18S18, D18S22, and D18S7. The analysis was carried out in two steps. We first performed four-point linkage analysis using DCC and the two markers closest to it, D18S18 and D18S22. To extend our studies to encompass JK another four-point analysis was then performed using D18S18, D18S22, and D18S7; JK maps between the last two markers (27). The former analysis (Fig. 2a) suggested exclusion of DCC and a region spanning about 35 cM around the marker loci as the site for the HNPCC susceptibility locus. The latter analysis (Fig. 2b) suggested extension of the excluded portion about 10 cM proximal to D18S7, i.e., to a total genetic length of some 60 cM. Consequently, exclusion of JK residing in the excluded interval was also suggested.

**DISCUSSION**

Since Lynch et al. (28, 29) described two large families with hereditary non-polyposis colorectal carcinoma the existence of a gene conferring susceptibility to this condition has been proposed. However, as long as no reliable biochemical or other marker exists the diagnosis is based on the family history and phenotype of cancer alone. The situation is different from APC in which linkage to a locus in 5q21-22 has firmly established the existence of a susceptibility gene with very high penetrance; moreover, no heterogeneity appears to occur (30, 31).

The present investigation was carried out to determine whether the DCC gene or another gene in its vicinity acts as a gene which, when mutated and inherited, confers susceptibility to HNPCC. DCC is a good candidate gene for two reasons: (a) it is altered in unselected colorectal carcinoma tumors; and (b) it is located on 18q not far from the gene for the Kidd blood group to which Lynch et al. (7) reported linkage in their HNPCC families. In spite of its inherent problems we chose the linkage analysis approach mainly because it is practically the only method that is presently feasible (see below).

Our study suggested exclusion of DCC as the gene predisposing to HNPCC in five families while two families were uninformative. Moreover, multilocus analysis suggested exclusion of JK. Our results apparently contrast with those of Lynch et al. (7). This could be due to several factors which are discussed below.

Recent findings in a few common dominantly inherited disorders indicate that genetic heterogeneity should not be overlooked. Presenile and senile onset forms of familial Alzheimer's disease seem to be genetically separate entities since the former has shown linkage to chromosome 21 while the latter has not (32). Similarly, some families with early onset breast cancer have shown linkage to 17q21 (33) while lack of linkage to markers from the same region has characterized other families with breast cancer of early onset (34) as well as those with late-onset breast cancer (33).

In HNPCC the absence or presence of extracolonic cancer might define distinct subgroups [Lynch syndromes I and II (3)] but we are not aware of any unequivocal criteria for such a separation. Interestingly, phenotypically intermediate forms between HNPCC and APC were recently reported (35, 36). These variant families showed evidence for genetic linkage to the APC locus on chromosome 5; therefore the conditions may well be caused by different mutations of the APC gene.

The seven HNPCC families studied by us were clinically quite typical and uniform. The organ distribution of the cancers in this study was highly typical of that commonly reported in HNPCC (3, 6, 37). In particular, the cancers of those patients who were obligate recombinants between HNPCC and DCC (see above) were typical. A possibility remained that the weakly positive (although nonsignificant) lod scores for linkage between HNPCC and DCC obtained in families 6 and 39 might reflect genetic heterogeneity. In the absence of firm positive linkage in any of the families the question of homogeneity versus heterogeneity could not be statistically examined. The positive lod scores in these two families could obviously turn negative as well once more information (more informative meioses and/or markers) becomes available.

Parameters used in statistical calculations can cause variation in linkage results. An important parameter is the age-dependent penetrance. On the basis of studies of 14 Finnish families Mecklin et al. (4) observed a very high (89%) penetrance when, apart from colorectal carcinoma, carcinoma of the uterus and undefined intraabdominal carcinoma were regarded as manifestations of the disorder. The mean age of onset is approximately 45 years but the range is from below 20 years to over 70 years (37-39). In the seven families we studied the range was from 19 to 83 years. This necessitates the use of several liability classes. The age-dependent penetrance values used in the present study can be considered conservative. Furthermore, moderate changes in the parameters (including the penetrance) did not considerably alter the final conclusions. Modification of parameters could, however, have more important consequences if there were positive evidence for linkage as illustrated by linkage studies in manic depressive illness (40). This led Robertson (41) to suggest that lod score values higher than 3 should be required as proof of linkage in diseases with complex inheritance patterns.

A potentially important source of confusion in linkage studies is that sporadic forms of the cancers that occur in HNPCC are common in the general population. Thus, it cannot be ruled out that some cancers in members of the HNPCC families could be “sporadic” and thus independent of the putative HNPCC susceptibility gene. An illustrative example was provided by Srivastava et al. (42) in a family with Li-Fraumeni syndrome where bilateral breast cancer in a woman belonging to a cancer-prone lineage was apparently independent of the heritable defect occurring in her affected brother and his affected children. False assignment of a single individual as affected could lead to erroneous linkage results in some cases. For example, in our family 59 a male in the second generation was diagnosed with colon cancer at age 63. The linkage results presently suggest at least one recombination between DCC and the disease phenotype. However, if the cancer in the male in question was sporadic instead of being due to the putative susceptibility gene occurring in his sister and apparently passed on to three of her seven children, then there is no evidence of recombination in the pedigree. In family 6 there was a married couple with colorectal cancer in both spouses. In the absence
Fig. 1. Pedigrees of the seven HNPCC families. ●, O, female; □, male; □ (and all symbols with diagonal), deceased; □, number of siblings (within ○) without HNPCC. Open symbols, no neoplasm detected; filled symbol, affected with neoplasm. □, carcinoma considered typical of HNPCC (i.e., carcinoma of colon, endometrium, or other intraabdominal organs); ✗, other cancer; □, adenoma; □, diagnosis uncertain; □, number of primary neoplasms; □, age at diagnosis of first cancer. □, alleles. The probes (polymorphisms) are (top to bottom): pEFZ10 (PvuII), CRI-L84 (MspI), pSAM1.1 (EcoRI), p15-65 (MspI), and pJOSH4.4 (PciI). Alleles are numbered consecutively according to decreasing fragment size. ND, not determined. Allelic patterns of DCC from which it is concluded that a recombination between HNPCC and DCC must have occurred are underlined. This conclusion is based on the observation that two affected family members do not have any allele in common within an allele system or is inferred from haplotype analysis (families 10 and 59).
of positive family history we regarded the cancer of the wife as sporadic but the possibility that she, too, had a susceptibility gene (e.g., through consanguinity) could not be totally excluded. However, in this case erroneous assignment would result in neither confirmation nor rejection of linkage. These conditions indicate that interpretations should be regarded with some caution. On the other hand, as long as defined mutations of a candidate gene cannot be tested for cosegregation with the disease phenotype it will not be possible to use approaches other than linkage analysis to prove or disprove a candidate gene.

A major problem in attempts to demonstrate statistically significant linkage in HNPCC is the malignant nature of the disease and the ensuing loss of linkage information through the death of several key family members. This leads to scarcity of informative pedigrees comprising two generations and the virtual absence of informative three-generation families. This drawback may at least partially be overcome by using paraffin blocks of tissue from dead individuals for DNA extraction. Another approach is to search for a heritable mutation in a suitable candidate gene. This strategy was successful in Li-Fraumeni syndrome, a rare cancer syndrome characterized by multiple neoplasms of mesenchymal and epithelial origin. In these families germ-line transmission of a mutated TP53 gene was shown (42, 43). The DCC gene is too large to allow a similar direct search for mutations at present. Once a consistent genetic linkage or a heritable mutation is found in the hereditary non-polyposis colorectal carcinoma syndrome it will provide a means of identifying family members who have inherited the susceptibility. This, in turn, will enable more effective surveillance programs for early detection and treatment of cancer in members of these families.

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Evidence Supporting Exclusion of the DCC Gene and a Portion of Chromosome 18q as the Locus for Susceptibility to Hereditary Nonpolyposis Colorectal Carcinoma in Five Kindreds

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