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Close linkage to chromosome 3p and conservation of ancestral founding haplotype in hereditary nonpolyposis colorectal cancer families

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ABSTRACT **A susceptibility to hereditary nonpolyposis colorectal cancer (HNPCC) was recently shown to be due to mutations in the *MSH2* gene on chromosome 2p. A second susceptibility locus has been mapped to chromosome 3p in two families. The present report describes the results of a genetic study of Finnish HNPCC kindreds. Of 18 apparently unrelated families living in different parts of the country, 11 could be genealogically traced to a common ancestry dating at least 13 generations back in a small geographic area. Linkage studies were possible in 9 families, revealing conclusive or probable linkage to markers on 3p in 8. Five of these were among those having shared ancestry. The location of the gene was refined by a linkage study comprising 12 marker loci. By analysis of recombinations in such families, the HNPCC locus could be assigned to the 1-centimorgan interval between marker loci *D3S1561* and *D3S1298*. A haplotype encompassing 10 centimorgans around the HNPCC locus was conserved in five of the pedigrees with shared ancestry and present in 2 further families in which linkage analysis was not possible. Our results suggest the presence of a widespread single ancestral founding mutation. Moreover, the map position of the 3p gene for HNPCC susceptibility was greatly refined.**

Hereditary nonpolyposis colorectal cancer (HNPCC) is a dominantly inherited trait that accounts for up to 13% of all colorectal cancers in industrial nations (1, 2). In predisposed individuals, adenocarcinomas of the colorectum and other organs, notably the endometrium, typically develop at <50 years of age (2, 3). To date, two susceptibility loci for HNPCC have been mapped, one on 2p (4) and another on 3p (5). The 2p gene was recently isolated based on homology to bacterial and yeast mismatch repair genes (6, 7) and shown to cause HNPCC by virtue of heritable germ-line mutations that segregate with the disease phenotype (7). The 3p locus has so far been defined only by linkage to *D3S1029* in two Swedish HNPCC families (5).

Over the past 10 years, extensive clinical and genealogical studies have led to the identification in Finland of about 40 HNPCC families (3) that meet the internationally accepted criteria for the disorder (8). In the present investigation, we focused on 18 of these families. Since convincing evidence of 2p linkage had not been found in Finnish families (9), this study was undertaken to determine the role of the proposed locus in 3p. By linkage analysis in 8 families, the position of the HNPCC susceptibility gene could be narrowed to a region of 1 centimorgan (cM). Genealogical tracing and the conservation of a disease-associated haplotype suggested that a single founding mutation is widespread and accounts for the great majority of, but probably not all, HNPCC mutations in Finland.

MATERIALS AND METHODS

Kindreds. This investigation was performed on 18 Finnish HNPCC families meeting the international criteria for HNPCC (8). All families were genealogically studied. Kindreds 2, 3, 6, 8, 10, 11, 39, 56, and 59 were included in the linkage study as well as haplotype and allele association analyses. More detailed clinical data on the patients from these families are available in two previous publications (10, 11). Haplotype analyses were carried out on an additional 4 kindreds, nos. 1, 9, 26, and 51 (one to four individuals studied per kindred).

Genealogic Study. The ancestries of 18 families were traced using data from the church parish registers. These data include dates and places of birth and death and the identification of parents and children. Church parish registers have the status of official population registers in Finland; therefore, their coverage and accuracy is high (12).

Genotyping. We studied 12 microsatellite markers from 3p specified in Fig. 2. Marker *D3S1029* was developed by Jones *et al.* (13) and all others were developed by Weissenbach *et al.* (ref. 14 and unpublished). Alleles were numbered consecutively according to decreasing size. DNA samples were amplified by the polymerase chain reaction using primers specific for the above-mentioned markers, and the amplification products were separated by electrophoresis through polyacrylamide gels. A detailed description of the method is available in ref. 4.

Linkage and Allele Association Analyses. We used programs of the LINKAGE program package (15). Marker *D3S1029* was tested in eight Centre d'Etude du Polymorphisme Humain (CEPH) families (nos. 1331, 1332, 1347, 1362, 1413, 1416, 884, and 102). The location of this marker between *D3S1260* and *D3S1289* represents the best placement (odds > 10^7) on a fixed map of 10 markers (ref. 14 and unpublished), computed using subprogram CILINK. In linkage analyses of the HNPCC families, individuals with colorectal or endometrial carcinoma or other cancer typical of HNPCC (2, 3) were considered as affected. Patients with colorectal adenoma or cancers not considered to belong to the HNPCC tumor spectrum were treated as having an unknown status. We used age-

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Abbreviations: HNPCC, hereditary nonpolyposis colorectal cancer; cM, centimorgan; CEPH, Centre d'Etude du Polymorphisme Humain.

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dependent penetrance values with four liability classes (4). The frequency of the HNPCC gene was set as 0.001.

Pairwise linkage analyses were carried out as described (16) with minor modifications. Briefly, allelic association was tested using the ILINK subprogram to determine haplotype frequencies for the disease and marker locus combination and maximizing over θ (recombination fraction), p (disease allele frequency), q (marker allele frequency), and δ (deviation from linkage equilibrium). The maximum likelihood estimate was then compared by the $-2 \ln$ likelihood ratio test (17) with an estimate obtained assuming $\delta = 0$ (equilibrium). For markers showing significant departure from equilibrium the haplotype frequencies were adjusted according to the population frequency of 0.001 of the disease allele and incorporated in linkage analyses by program ILINK (16). For markers not showing allele association the pairwise haplotype frequencies were determined by ILINK at $\theta = 0.5$ and the allele frequencies were calculated only from haplotypes not segregating with HNPCC. The obtained frequencies were adjusted to unity and used in conventional pairwise analyses by the MLINK program. This method of counting alleles was expected to minimize the bias from the relatedness of the families.

RESULTS

Genealogic Study. Eighteen families were chosen for genealogic study. They comprised all 9 families in which linkage studies were possible. The remaining 9 families were chosen randomly among those in which DNA samples were available from at least one affected individual. Ancestries were traced back from the proband or the oldest generation having affected individuals. The Finnish population has been genetically isolated for the past 2000 years (18, 19). People lived in rural isolates from which they started to move into cities, particularly to the extended Helsinki region, only at the time of the Second World War.

Most probands or other patients described in this study were treated close to where they live, at the Helsinki University Hospital or the Jyväskylä Central Hospital in south-central Finland. The genealogical study revealed that among the 18 families, 11 descended from ancestors who had lived in a small rural region located in south-central Finland some 400 km from Helsinki and 100 km from Jyväskylä. The nucleus of this region comprises three parishes (shaded on the map in Fig. 1) that today have a total population of ≈24,000. In 7 families (nos. 1, 2, 9, 10, 19, 59, and 61) the ancestors from this region were <5 generations removed from the present generation; in 3 kindreds (nos. 6, 12, and 30), up to 13. Family 11 apparently came from a region 150 km to the north but may have originated in the nuclear region based on the family name of the ancestors. At least 5 kindreds (nos. 1, 6, 9, 10, and 30) shared a single ancestor born in 1505. One family (no. 51) originated <100 km east of the small region. All in all, the ancestries of 11 families are compatible with their sharing one ancestral mutation expanding in that region for at least 13 generations, and perhaps much longer. Interestingly, 3 families (nos. 3, 8, and 65) originated from another limited region some 200 km to the southeast. As shown below, the conserved haplotype did not occur in these.

Of the 18 families whose genealogy was studied in detail, only 3 clearly originated from areas different from the two main regions described above. Two families (nos. 26 and 56) had their roots in the densely populated region around Helsinki while the third (no. 39) lived in Helsinki but came from a foreign country.

Constructing a Linkage Map of the 3p Region. The position of *D3S1029*, the closest marker showing linkage to HNPCC in the previous study (5), was determined using the data available in the CEPH data base (version 6.0) as described in *Materials and Methods*. *D3S1029* mapped between *D3S1260* and *D3S1289*, 5.7 cM proximal of *D3S1260* (Fig. 2).

Linkage Analyses with 3p Markers. The pedigrees of the HNPCC families studied for linkage are shown in Fig. 3. Haplotype analysis with 12 markers from 3p was compatible with linkage in 8 of 9 families. Two marker loci, *D3S1277* and *D3S1611*, showed no recombination in any of the 8 families, whereas recombination was observed with the other markers tested. Table 1 shows results from pairwise linkage analysis of the three most informative marker loci. The highest two-point lod scores were obtained for family 2 (4.95, 4.67,

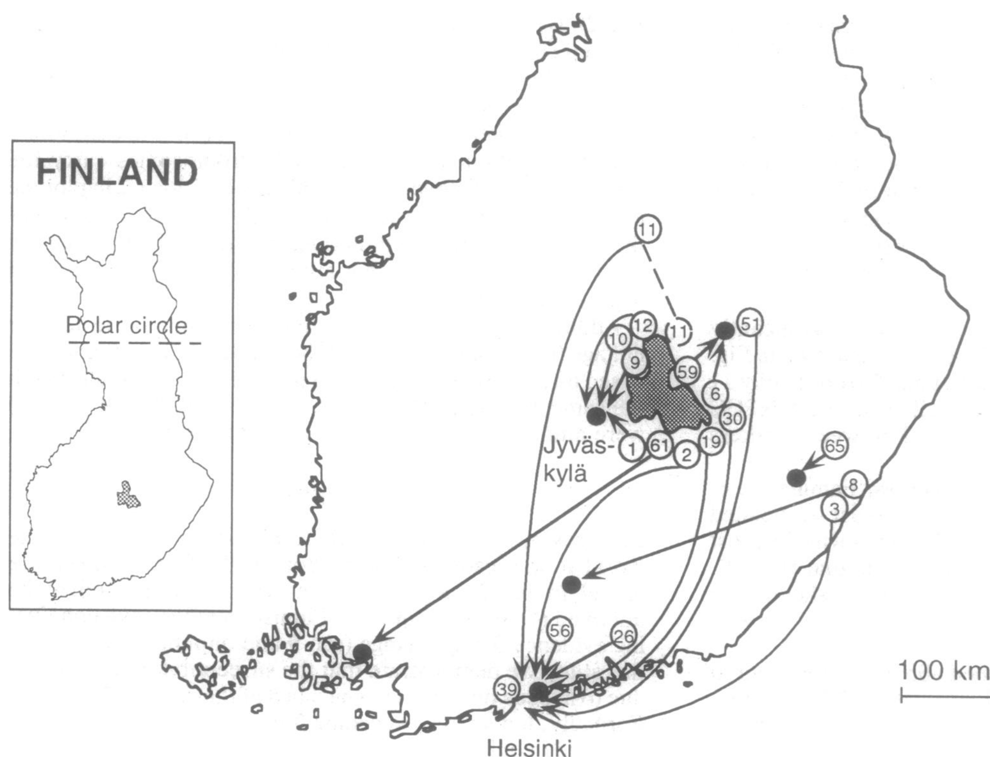

FIG. 1. Left, map of Finland showing the region where the ancestry of the HNPCC families originated. Right, enlarged map of southern Finland showing the region to which the ancestors of 11 families could be traced (shaded) and the geographical origin of the remaining 7 families. Numbers in circles refer to HNPCC family numbers in the text and Table 1. Circles surrounding the shaded area refer to families originating inside the area. Arrows show where present-day affected members of the families live. One partly dashed arrow line indicates probable but unconfirmed connection. Filled circles denote the location of hospitals in which the probands and many of their relatives have been diagnosed and treated.

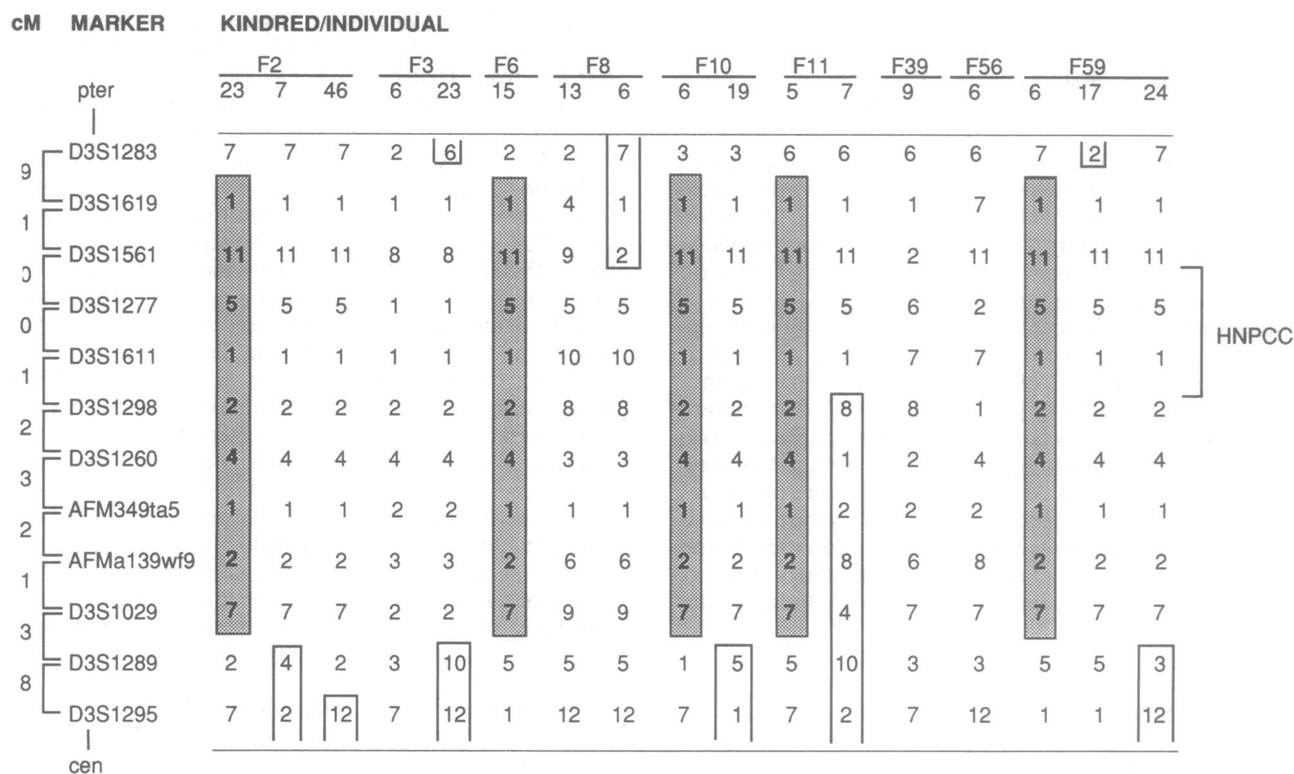

FIG. 2. Results of haplotype analysis in families 2, 3, 6, 8, 10, 11, 39, 56, and 59. Left, the order of markers studied and their relative distances in cM. The orientation is from telomere (pter) to centromere (cen). Right, the alleles for the markers in a panel of affected representatives of each family. The first individual to the left in each kindred shows the disease haplotype segregating in the family. Other individuals illustrate critical recombination events that have occurred between the HNPCC locus and particular markers (open boxes). Shaded boxes indicate the haplotype shared by families 2, 6, 10, 11, and 59. In family 56 one affected member had inherited from the affected parent a haplotype that was totally different from that shown here (see text). The frequencies of the disease-associated alleles in haplotypes not associated with HNPCC, as determined from ≈50 chromosomes, were as follows: 0.27 (allele 1, *D3S1619*), 0.43 (allele 11, *D3S1561*), 0.27 (allele 5, *D3S1277*), 0.00 (allele 1, *D3S1611*), 0.00 (allele 2, *D3S1298*), 0.15 (allele 4, *D3S1260*), 0.30 (allele 1, *AFM349ta5*), 0.05 (allele 2, *AFMa139wf9*), and 0.23 (allele 7, *D3S1029*).

and 3.20 at $\theta = 0.0$ for markers *D3S1611*, *D3S1298*, and *AFMa139wf9*, respectively). A highly significant ($P < 0.001$) allelic association was detected with markers *D3S1611* and *D3S1298* and this was incorporated in the linkage analysis. Combining the lod scores for the 8 linked families resulted in the maximum lod scores of 14.67 and 12.11 at $\theta = 0.0$ and $\theta = 0.03$ for markers *D3S1611* and *D3S1298*, respectively, and 6.52 at $\theta = 0.03$ for *AFMa139wf9*. In family 56 one affected individual did not share the disease haplotype occurring in other affected members and this resulted in mainly negative lod scores for this family. Of the 8 families showing proven or probable linkage to the locus on 3p, five (nos. 2, 6, 10, 11, and 59) were among those 11 who had shared ancestry, while three were not.

Critical recombination events that led to a significantly refined assignment of the HNPCC locus are illustrated in Fig. 2. Individual 6 from family 8 and individual 7 from family 11 showed recombination patterns that allowed the HNPCC locus to be assigned to the 1-cM interval between markers *D3S1561* and *D3S1298*.

Haplotype and Allele Association Analyses. Families 2, 6, 10, 11, and 59 exhibited an identical disease-associated haplotype with nine markers of which *D3S1619* was the most telomeric and *D3S1029* the most centromeric one (Fig. 2). The haplotypes of the four remaining families showed no significant similarity with the conserved haplotype or with the haplotypes of each other.

The conserved haplotype described above did not occur in any chromosome not expected to carry the HNPCC mutation. Conservation of a haplotype for markers spanning as much as 10 cM might not be expected *a priori* over 13 or more generations. However, this may in part be due to chance, as several of the outlying markers of this haplotype had relatively high population frequencies of these alleles (legend to Fig. 2). By contrast, the disease-associated alleles of either of the two central markers closely linked to HNPCC (*D3S1611* and *D3S1298*) did not occur at all in the sample of 50 normal chromosomes studied. The conservation of the 1-2 haplotype for these markers in association with HNPCC is therefore a strong indication that the affected chromosomes carry the same mutation. Conversely, the absence of this 1-2 haplotype in 3p-linked families such as nos. 8, 39 could indicate either that they carry different mutations or, alternatively, that they carry the same mutation but are genealogically far removed from each other.

Additionally, two kindreds that could not be tested for linkage (nos. 1 and 19) showed the conserved large haplotype based on a study of 2–4 affected members. All 7 families with the same disease haplotype (nos. 1, 2, 6, 10, 11, 19, and 59) were among those 11 who had shared ancestry.

DISCUSSION

In a previous study of 16 HNPCC kindreds from all over the world it was estimated that about half of the families are linked to the 2p locus, whereas the other half might be unlinked (4, 9). None of the 9 Finnish families gave convincing evidence for 2p linkage (ref. 9 and unpublished data). In this study we demonstrate that the susceptibility to cancer is due to a locus on chromosome 3 in 8 of these same 9 families. Formally, lod scores exceeding 3 in support of linkage were obtained in only one family (no. 2). However, on the basis of

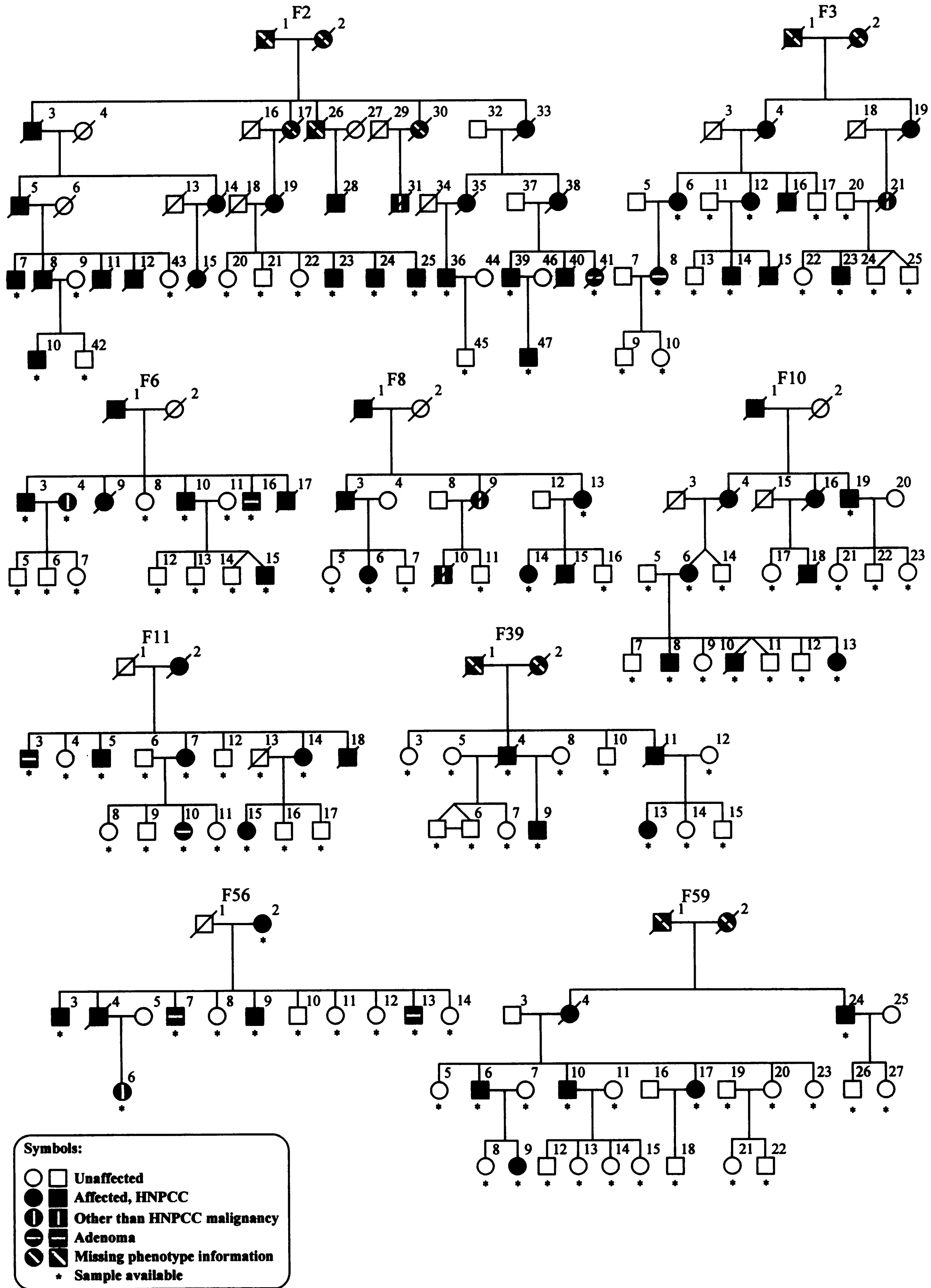

FIG. 3. Pedigrees of the HNPCC families studied for linkage.

both linkage and haplotype analyses it is likely that cancer susceptibility in 8 of the families is in fact due to mutations at the 3p locus.

The results of the present study suggest that a single ancestral mutation is widespread in the Finnish population. Some 40 HNPCC families have so far been diagnosed in this

Table 1. Pairwise lod scores for markers *D3S1611*, *D3S1298*, and *AFMa139wf9* in nine HNPCC families

Family	Locus/recombination fraction							
	0.00	0.001	0.01	0.05	0.1	0.2	0.3	0.4
				D3S1611				
F2	4.95	4.94	4.88	4.59	4.20	3.35	2.38	1.27
F3	2.61	2.60	2.57	2.42	2.22	1.77	1.27	0.68
F6	1.34	1.34	1.32	1.24	1.13	0.90	0.64	0.34
F8	0.44	0.43	0.42	0.36	0.28	0.14	0.04	0.00
F10	2.81	2.81	2.77	2.59	2.35	1.85	1.30	0.69
F11	−0.02	−0.01	0.04	0.21	0.32	0.39	0.35	0.22
F39	1.03	1.03	1.01	0.92	0.80	0.57	0.33	0.13
F56	−1.57	−1.56	−1.47	−1.10	−0.73	−0.28	−0.05	0.03
F59	1.51	1.51	1.50	1.45	1.37	1.15	0.86	0.48
Total	13.10	13.09	13.04	12.67	11.94	9.85	7.12	3.85
				D3S1298				
F2	4.67	4.66	4.60	4.33	3.96	3.16	2.24	1.20
F3	2.61	2.60	2.57	2.42	2.22	1.77	1.27	0.68
F6	0.42	0.43	0.46	0.56	0.60	0.57	0.45	0.26
F8	0.87	0.87	0.85	0.79	0.70	0.52	0.34	0.17
F10	2.67	2.67	2.63	2.44	2.21	1.73	1.21	0.64
F11	−2.04	−1.99	−1.64	−0.97	−0.57	−0.17	0.00	0.05
F39	0.82	0.82	0.84	0.87	0.86	0.76	0.58	0.33
F56	−0.32	−3.17	−2.93	−2.15	−1.53	−0.79	−0.37	−0.12
F59	1.69	1.69	1.68	1.62	1.52	1.27	0.93	0.52
Total	8.51	8.58	9.05	9.90	9.98	8.82	6.67	3.72
				AFMa139wf9				
F2	3.20	3.19	3.14	2.90	2.58	1.89	1.17	0.50
F3	0.97	0.97	0.94	0.83	0.69	0.43	0.21	0.06
F6	−0.23	−0.23	−0.18	−0.05	0.02	0.05	0.04	0.01
F8	0.87	0.87	0.85	0.76	0.64	0.41	0.21	0.05
F10	1.95	1.95	1.91	1.76	1.56	1.15	0.74	0.34
F11	−2.21	−2.17	−1.94	−1.31	−0.88	−0.42	−0.17	−0.04
F39	0.99	0.98	0.96	0.86	0.74	0.48	0.23	0.06
F56	1.11	1.11	1.09	1.02	0.92	0.69	0.42	0.15
F59	0.75	0.75	0.74	0.72	0.67	0.52	0.33	0.14
Total	7.40	7.41	7.52	7.48	6.93	5.21	3.18	1.28

The observed allelic association was taken into account in linkage analyses using the first two markers. lod score, Logarithm of odds score.

population of 5 million, where the degree of ascertainment is high (20). Eighteen families were included in this study. Is the high proportion of shared ancestry and/or shared haplotypes (11/18) representative of all HNPCC families in Finland or was it due to a bias in the selection of the 18 families that were studied? The only basis for selection was large family size and availability of samples for molecular studies. We favor the hypothesis that this did not seriously bias the sampling in favor of linkage to 3p or shared ancestry. If this is correct, then the majority of Finland's HNPCC cases may be due to a single ancestral mutation.

We show here that the mutation may be old, as common ancestry was traced as far back as 1505. Then the apparent lack of selection against a cancer mutation needs to be considered. Two alternative explanations may account for this situation. First, the deleterious clinical effects of the mutation (cancer at young age) may not significantly affect reproduction as the mean age of first cancer is 42 years (21). Second, one might hypothesize that the heterozygous state of this mutation confers a selective advantage of unknown basis. Evidence regarding mutations in *MSH2* suggests that different germ-line mutations in different parts of the gene account for cancer susceptibility in different families (7). Thus, it is clearly possible that the high enrichment of one putative mutation in the Finnish population is a unique event due to chance rather than the effect of any selective advantage, in analogy with the enrichment of many other disease genes in the Finnish gene pool (19, 22).

Our linkage map of the region harboring the HNPCC gene extends over ≈30 cM. Physically the region can be tentatively anchored to band 3p21 through marker *D3S1298*, which has been mapped to that band by both genetic and physical methods (23). The location of the HNPCC gene could be narrowed to a 1-cM interval by linkage (two markers showing no recombinations) and by the analysis of rare recombinant chromosomes in affected individuals (Fig. 2). Efforts to physically map and clone the HNPCC gene can now be focused on this interval.

Note Added in Proof. A DNA mismatch repair gene in 3p21, *MLH1*, was recently cloned, and families 2, 3, 6, 8, 10, 11, and 59 showed an identical germ-line mutation amply confirming the conclusions suggested here (24).

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