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Genetic Mapping of the Hereditary Mixed Polyposis Syndrome to Chromosome 6q

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Summary

Hereditary mixed polyposis syndrome (HMPS) is characterized by atypical juvenile polyps, colonic adenomas, and colorectal carcinomas. HMPS appears to be inherited in an autosomal dominant manner. Genetic linkage analysis has been performed on a large family with HMPS. Data did not support linkage to the APC locus or to any of the loci for hereditary nonpolyposis colorectal cancer. Evidence that the HMPS locus lies on chromosome 6q was, however, provided by significant two-point LOD scores for linkage between HMPS and the *D6S283* locus. Analysis of recombinants and multipoint linkage analysis suggested that the HMPS locus lies in a 4-cM interval containing the *D6S283* locus and flanked by markers *D6S468* and *D6S301*.

Introduction

Hereditary mixed polyposis syndrome (HMPS) is a rare condition in which individuals develop characteristic polyps of the large bowel. These polyps closely resemble juvenile polyps but show significant histological differences (S. C. Whitelaw, unpublished data). Affected individuals also develop colonic adenomas and colorectal carcinomas. The natural history of the disease is, however, incompletely characterized (Murday and Slack 1989): HMPS polyps may progress to adenomas and thence to carcinomas, or polyps and adenomas may arise and progress separately. HMPS patients may also have an increased propensity to develop inflammatory and metaplastic polyps.

The similarity of HMPS polyps to juvenile polyps and the possible progression of HMPS polyps to adenomas and carcinomas means that ascertainment of HMPS is unlikely to be accurate or complete. However, one large HMPS family (SM96) has been identified by St. Mark's Hospital, London (fig. 1). Accurate clinical details are available from most family members (S. C. Whitelaw, unpublished data). HMPS in this pedigree appears to be inherited as an autosomal dominant syndrome. Most older individuals presented with colorectal carcinoma; younger individuals, most of whom have undergone screening by colonoscopy, tend to present with benign colonic lesions, often the characteristic HMPS polyp (S. C. Whitelaw, unpublished data).

By analogy to familial adenomatous polyposis (FAP) (Miyaki et al. 1994), loci at which mutations predispose to rare, inherited bowel tumors may also be important to sporadic colorectal tumors (Solomon et al. 1987). We have performed genetic linkage analysis on 46 members of family SM96 (fig. 1), in order to determine the possible location of the HMPS gene. Allele loss (loss of heterozygosity [LOH]) at the putative HMPS locus has also been studied in the colorectal carcinomas of three members of family SM96 and in 100 sporadic colorectal cancers.

Methods

Collection and Preparation of Samples

Peripheral blood was collected from 43 members of the family and was Epstein-Barr virus–transformed to form permanent lymphoblastoid cell lines. DNA was extracted by use of standard techniques. Paraffin-embedded archival material was obtained from the normal tissues of two members of the family and from the colorectal carcinomas of three patients. After microdissection of the appropriate tissue and digestion in 400 μg proteinase K/ml, DNA was extracted from the par-

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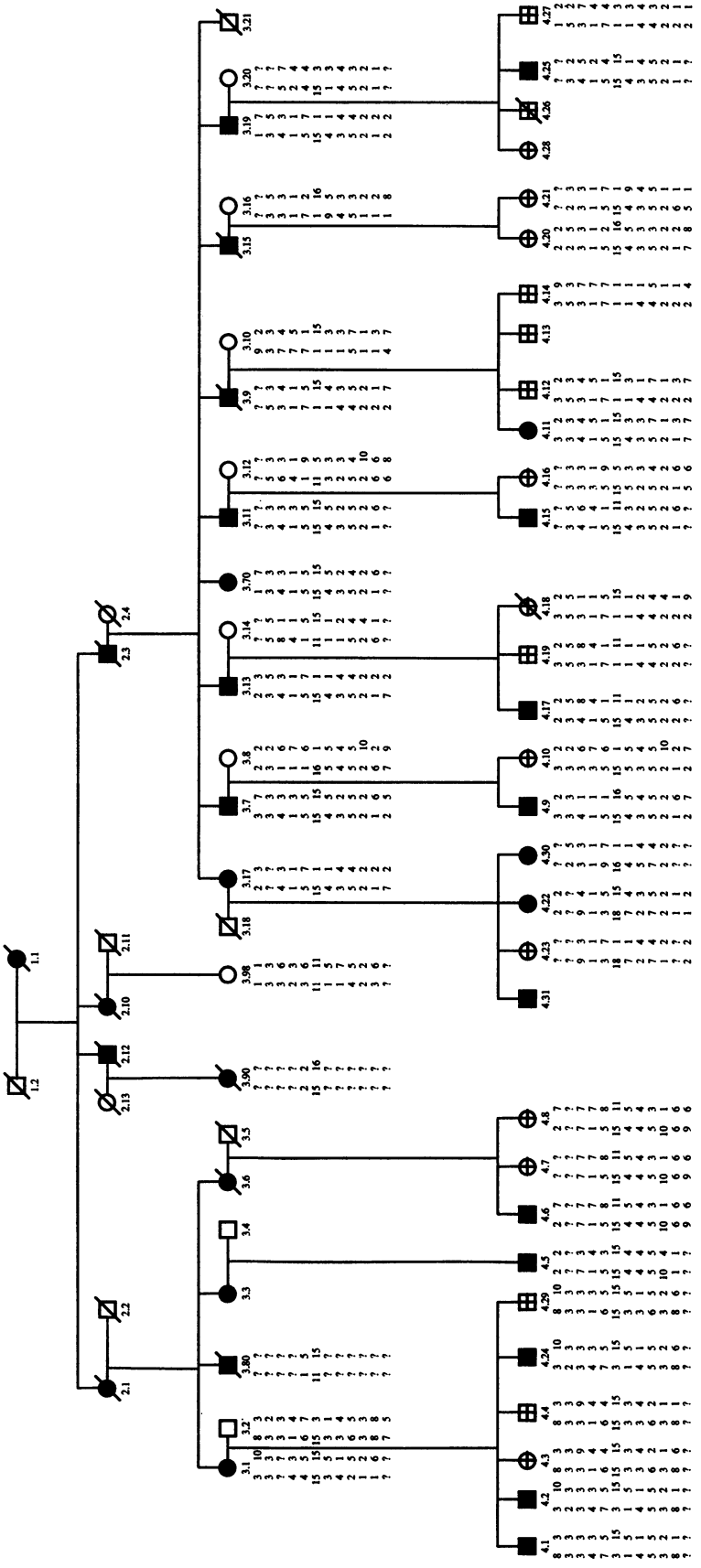

Figure 1 Pedigree of family SM96, showing haplotypes at loci near the putative HMPS locus. The full pedigree is much larger than that shown, but all genotyped individuals are illustrated. Loci are shown in the map order given in fig. 2. Affected individuals are denoted by blackened symbols, unaffected individuals are denoted by unblackened symbols, and individuals of unknown affection status are denoted by symbols that have a cross. The disease-associated haplotype 5-15-4 (or 5-15-5) at D6S283-D6S434-D6S301 can be followed throughout the family, with the exception of the postulated phenocopy 4.30. We assume that the allele "5" at D6S301 arose either by a recombination (which would place the putative HMPS locus proximal to D6S301; see multipoint LOD score analysis) or by mutation of an allele "4."

affin-embedded tissue by use of a Nucleon kit (Scotlab). Fresh samples of peripheral blood were collected from one affected family member, and cells were grown for karyotyping and FISH analysis. DNA had previously been extracted from 100 paired, frozen samples of colorectal carcinoma and blood/normal tissue, by standard methods.

Determination of Genotypes

Genotyping was performed by two methods, depending on the type of marker involved. For RFLP markers, 5–10 μg genomic DNA was digested with an appropriate restriction endonuclease and buffer. Restricted DNAs were electrophoresed for 8–24 h on 0.8%–1% agarose gels, followed by Southern transfer overnight onto Hybond N+ membranes (Amersham). α^{32}P-dATP–labeled (Amersham) DNA probes were hybridized overnight to the membranes, which were washed according to standard protocols and exposed to film (Hyperfilm) for 1–3 d.

Simple-sequence-length polymorphic (SSLP) markers (microsatellite markers, CA repeats) were derived from the Généthon set (Gyapay et al. 1994). PCR reactions were performed with 50–200 ng genomic DNA in 1 × PCR buffer (with 1.5 mM $MgCl_2$) (Promega), 0.4 mM dNTPs, and 0.4 μM of each specific oligonucleotide, according to the standard Généthon protocol: once at 94°C for 4, 35 cycles at 94°C for 1 min and at 55°C for 1 min, and once at 72°C for 5 min. Products were electrophoresed for 3–5 h on 6%–8% denaturing polyacrylamide gels. PCR products were transferred by blotting onto Hybond N+ membranes for 4–24 h. Membranes were hybridized either to a CA-repeat oligonucleotide probe or to a specific PCR-primer oligonucleotide extended with random nucleotides and labeled by use of the glutaraldehyde/horseradish peroxidase system (Amersham). Hybridization and detection of bound probes was performed by use of the enhanced chemiluminescence (ECL) method (Amersham). All autoradiographs/ECL films were scored by inspection using DNAs of known genotype as standards.

Allele loss at SSLP loci in sporadic colorectal carcinomas was scored by eye, in order only to count unambiguous cases as having lost an allele. Scoring by eye also avoided spurious cases of allele loss produced by "stutter" or conformational bands that were different, by chance, between tumor and normal DNAs. Microsatellite instability and allele loss in HMPS carcinomas were also scored by eye.

Karyotyping and FISH

Metaphase spreads were prepared from phytohemagglutinin-stimulated normal human lymphocytes by use of standard cytogenetic techniques. After 72 h of incubation, cells were exposed to 200 μg bromodeoxyuridine/ml for 17 h. The DNA synthesis block was released by the addition of 2.5 μg thymidine/ml, and the cells were harvested after 5 h. The slides were baked at 65°C for 2–3 h, then denatured in 70% formamide and 2 × SSC pH 7.0 at 75°C for 2 min and dehydrated through an ethanol series of cold 70%, 95%, and absolute ethanol.

Probe DNA was biotinylated by use of BRL's Bionick kit, then purified through a Sephadex G50 column and precipitated with 50 μg salmon sperm DNA and 50 μg *Escherichia coli* tRNA. Hybridization and detection used a modification of the technique described by Fidlerova et al. (1993). Two hundred nanograms labeled probe was mixed with 2 μg human cot-1 DNA, in order to compete out repetitive elements in the probe DNA. This mixture was dried in a vacuum centrifuge and was resuspended in 11 μl hybridization mix. After heat denaturation and quenching on ice, the mixture was preincubated at 37°C for 30 min. The preincubated probe was applied to the denatured slide under a 22 × 22-mm coverslip, which was then sealed and placed in a moist chamber for 24 h.

Posthybridization washes were performed essentially as described by Fidlerova et al. (1993). For probe detection, all antibodies were diluted in 4 × SSC, 0.05% Tween 20, 5% low-fat dried milk (Marvel), pH 7.0, and incubated for 30 min at 37°C in the following order: (1) avidin-Texas red (Vector Laboratories) (2 μg/ml), (2) biotinylated anti-avidin (Vector Laboratories) (5 μg/ml), (3) avidin-Texas red (2 μg/ml), and finally (4) with anti-BrdU-fluorescein (Boehringer) (10 μg/ml), to obtain fluorescein isothiocyanate (FITC)–stained R-bands. Slides were dehydrated and mounted in Citifluor containing 4,6-diamidino-2-phenylindole (DAPI) (0.06 μg/ml).

Slides were viewed by use of a Zeiss Axiophot fluorescence microscope. FITC and Texas red were visualized simultaneously by use of a dual pass filter set (Omega), and DAPI counterstain was visualized with filter block set 02 (Zeiss G365, FT395, and LP420). Metaphase spreads were processed by use of a Zeiss Axioscop microscope equipped with a CCD camera (Photometrics). Separate images of probe signal, banding pattern, and counterstain were captured and colored. These images were then merged by use of an Apple Macintosh IIci computer with software developed by T. Rand and D. C. Ward (Yale University, New Haven).

Linkage Analysis

Individuals were classified as "affected" if they had developed one or more of the following during their lifetime: colorectal carcinoma, colorectal adenoma, or HMPS polyp. Since the oldest affected patient presented (with colorectal cancer) at the age of 63 years, a conser-

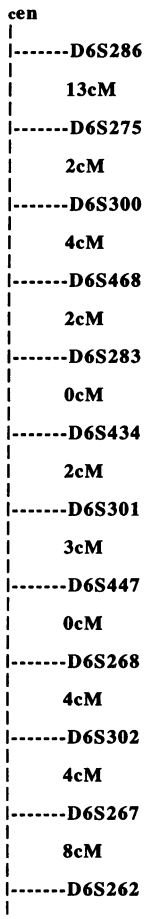

Figure 2 Genetic map of proximal part of chromosome 6q, showing markers studied.

vative approach was used to define affection status: individuals were only classified as "unaffected" if they were >65 years of age and disease free. All individuals <65 years of age were classified either as "affected" (according to the above criteria) or "unknown." Two-point LOD scores between the hypothetical disease locus and each test locus were computed by the MLINK program in the LINKAGE package, by use of published allele frequencies and standard techniques (Terwilliger and Ott 1994). Complete dominance of the disease allele (A) was assumed. LOD scores were computed at the following penetrances for each genotype at the disease locus: AA = .95; Aa = .95; and aa = .075. These values recognized the possibility of incomplete penetrance of HMPS (there being very little evidence for or against this). They also took into account the possible existence of phenocopies, at least of adenomas and carcinomas (although phenocopies of the HMPS polyp would be most unlikely). A LOD score >3.0 (corresponding to a level of significance of 5%) was considered as significant evidence of genetic linkage between disease and test loci. A LOD score <−2.0 was considered as significantly excluding linkage at that recombination fraction. Multipoint LOD scores were calculated by use of the LINKMAP program at and around loci showing significant two-point LOD scores (Terwilliger and Ott 1994).

Results

RFLP and SSLP analysis excluded close linkage of HMPS to the *APC, hMSH2, TP53,* and *DCC* loci (data not shown). No good evidence was found in favor of linkage to the *hMLH1* locus or 50 other loci associated with allele loss in sporadic colorectal carcinomas (data not shown). A genomewide search with the Généthon 1992 set of microsatellite markers (Weissenbach et al. 1992) was then performed. The only significant positive LOD score obtained was at the *D6S283* locus on the proximal part of chromosome 6q (details are given in fig. 2 and table 1). Genotyping was then performed by use of microsatellite markers closely linked to *D6S283,* in an attempt to refine the location of the putative HMPS locus. A further near-significant LOD score was found at the *D6S301* locus, 2 cM distal to *D6S283* (table 1). Analysis of haplotypes and recombinants showed the probable location of the HMPS locus as being between *D6S468* and *D6S268/D6S447* (fig. 1). Multipoint linkage analysis between and around markers *D6S468, D6S283, D6S301,* and *D6S447* gave a maximum LOD score of 3.93, between *D6S283* and *D6S301* (fig. 3), suggesting this interval as the most likely location of the putative HMPS locus. The maximum LOD score of 3.29, between *D6S468* and *D6S283* (fig. 3), also provided support for this region as a possible location for the HMPS gene.

One individual (4.30; fig. 1) was classified as "af-

Table 1

Maximum Two-Point LOD Scores at Loci on Chromosome 6q

Locus	Maximum LOD Score	Recombination Fraction
D6S468	.18	.28
D6S283	3.32	.06
D6S434	2.60	.03
D6S301	2.97	.05
D6S447	1.06	.08

NOTE.—HMPS locus penetrances are as shown in the text, and genetic distances between the marker loci are as given in fig. 2. Within realistic limits, changes in the penetrances used in the two-point LOD-score analysis (or the incorporation of age-dependence penetrances) have little effect on the values of maximum LOD score or recombination fraction obtained (details not shown).

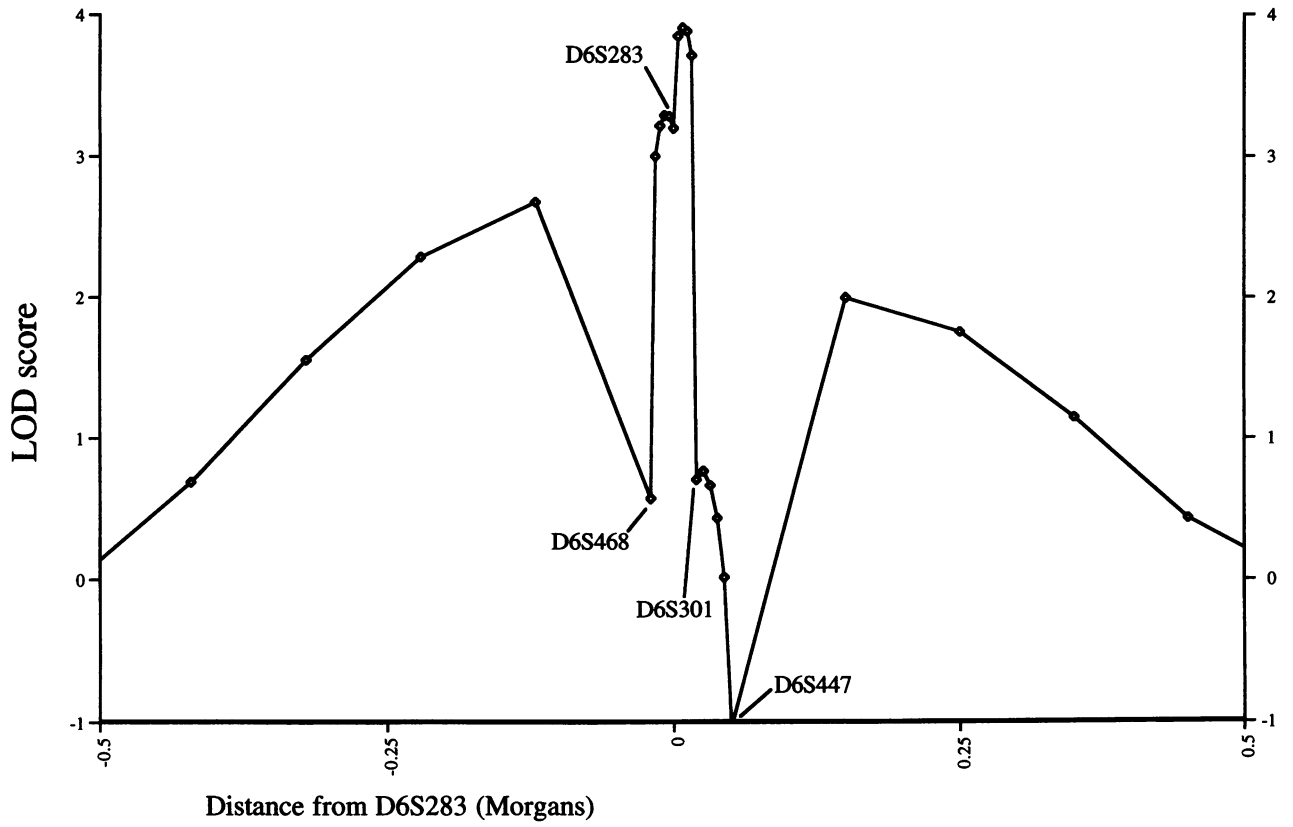

Figure 3 Multipoint linkage analysis between and outside markers D6S468 and D6S447. HMPS-locus penetrances are as shown in the text. Genetic distances between the marker loci (x-axis) are as given in fig. 2. LOD scores for the location of the HMPS locus are shown on the y-axis.

fected" for the purposes of linkage analysis but had probably inherited an "unaffected" haplotype. This individual was homozygous at the *D6S301* locus and might therefore represent a double recombination either side of *D6S301*. Given the low probability of two recombinations occurring in this interval of just 5 cM, it is possible that individual 4.30 is a phenocopy. This person was screened regularly, from age 20 years, by colonoscopy, and she developed a colonic serrated adenoma at what is probably an early age (29 years) relative to that of the general population. Most important, however, no characteristic HMPS polyp has been found in this individual, and she may therefore be a phenocopy.

By means of comparison with the *APC* locus (Solomon et al. 1987), we reasoned that significant allele loss might be expected both in HMPS tumors and in sporadic colorectal cancers near the HMPS locus. LOH was therefore searched for use of the highly polymorphic *D6S434* marker. Of three colorectal carcinomas from members of SM96, none showed LOH at *D6S434;* further analysis also showed no allele loss at *D6S301* or *D6S268* in these tumors. Of 94 sporadic carcinomas studied, 77 were informative, and only 10 (13%) had lost a *D6S434* allele. These samples had previously shown allele loss, at frequencies $\geq$40%, at other loci (I. P. M. Tomlinson, unpublished data). The three HMPS carcinomas were also analyzed for microsatellite instability, by use of the *D6S434, D6S301, D6S268, D11S29, DRD2,* and *D3S1611* loci. No allelic bands characteristic of microsatellite instability were seen.

We then investigated whether a mutation at the chromosomal level might be responsible for HMPS in family SM96. The karyotypes of two affected individuals were normal. The fact that two alleles were observed at 6q loci in all affected members of SM96 showed that a deletion of 6q (other than a very small one) was not present. FISH was then performed by use of YACs 931A10 and 961G3, which map close to the centromere on the short and long arms, respectively, of chromosome 6 (Généthon data; D. Markie, unpublished data). These YACs hybridized to their expected positions in an affected family member (3.13), thereby excluding a pericentric inversion or other gross rearrangement of chromosome 6 that was likely to involve the HMPS locus. YAC 963D6 (containing the *D6S283* locus) also

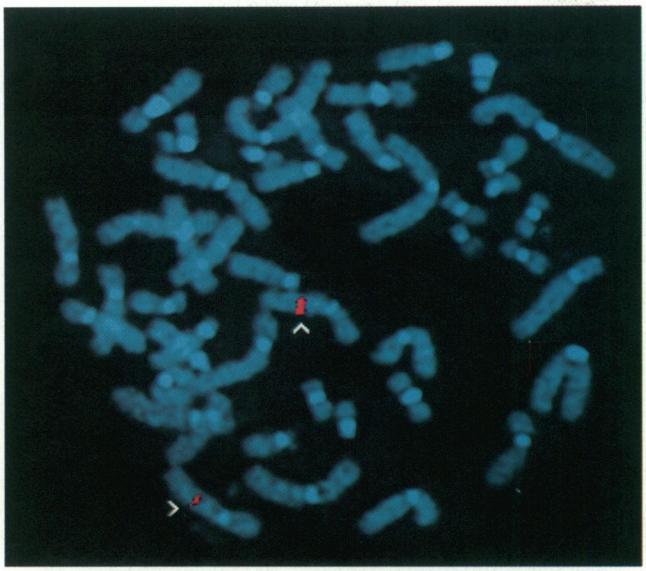

Figure 4 FISH analysis of affected member of SM96 (3.13), with use of YAC 963D6. The YAC maps to band 6q21 on both copies of this individual's chromosome 6. This position is the same as that in a normal individual (not shown).

mapped to its expected position (fig. 4) on both copies of chromosome 6 in individual 3.13.

Discussion

The results presented suggest that a gene for HMPS exists on the proximal part of the long arm of chromosome 6. Linkage analysis and study of recombinants suggest that the HMPS locus lies within a 7-cM interval flanked by the markers *D6S468* and *D6S447*, with the most probable location somewhere in the 4-cM interval between *D6S468* and *D6S301*. HMPS is not caused by mutations at either the loci responsible for FAP or the loci known to cause hereditary nonpolyposis colorectal cancer.

It is unclear whether HMPS is a variant of juvenile polyposis or a distinct disease (Murday and Slack 1989; S. C. Whitelaw, unpublished data). Certainly, it will be interesting to look for linkage between the disease and the putative HMPS locus in juvenile polyposis families. If HMPS is indeed a variant of juvenile polyposis, then the distinctive phenotype of family SM96 may result from a specific mutation in a juvenile polyposis gene, from environmental effects, or from the effects of modifier loci. Clearly, we cannot exclude the first of these possibilities. The second possibility also remains plausible. However, the family is distributed across the world and subject to many different environments, yet distant relatives have produced the characteristic HMPS polyp. The third possibility is interesting: an FAP modifier locus, *mom-1*, has been identified in the mouse (Dietrich et al. 1993; Macphee et al. 1995), showing that polyposis syndromes are affected by genetic influences additional to the locus primarily responsible. Certainly, the phenotype of juvenile polyposis is highly variable (Desai et al. 1995) and may in theory be influenced by modifying loci. Family SM96 is too small to allow us to detect the influence of modifying loci, and phenotypes are confounded by factors such as the availability of colonoscopic screening in recent years. Nevertheless, the high penetrance of HMPS in this family (in the early generations at least) and the possible phenocopy 4.30 with a colonic adenoma at a relatively early age are both factors consistent with the existence of modifying loci.

Although HMPS predisposes strongly to colorectal cancer—as do all known gastrointestinal polyposis syndromes—it does not follow that the HMPS locus is important in the development of sporadic colorectal tumors. A low proportion of sporadic colorectal carcinomas in this study showed LOH at *D6S434*. However, none of three HMPS carcinomas showed LOH at this locus. Therefore, the HMPS gene presumably acts in some way that is not analogous to that of *APC* or other colorectal tumor-suppressor genes. Its importance for colorectal tumors in general remains unknown.

Acknowledgments

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