Familial colorectal cancer in Ashkenazim due to a hypermutable tract in *APC*

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Approximately 130,000 cases of colorectal cancer (CRC) are diagnosed in the United States each year[1], and about 15% of these have a hereditary component[2,3]. Two well-defined syndromes, familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC), account for up to 5% of the total new cases of CRC[4]. Truncating *APC* mutations are responsible for FAP[5,6], and defective mismatch repair genes cause HNPCC[4,7,8]. However, the genes responsible for most of the familial cases are unknown. Here we report a mutation (T to A at *APC* nucleotide 3920) found in 6% of Ashkenazi Jews and about 28% of Ashkenazim with a family history of CRC. Rather than altering the function of the encoded protein, this mutation creates a small hypermutable region of the gene, indirectly causing cancer predisposition.

The current study was initiated during screening of a 39-year-old patient with eight colorectal adenomas and a family history of colorectal cancer. An analysis for microsatellite stability in his tumours excluded a diagnosis of HNPCC[4,7,9]. To test for germ-line *APC* mutations, the *in vitro* synthesized protein (IVSP) assay was employed[10,11]. A truncated APC polypeptide was identified with IVSP after *in vitro* transcription and translation of PCR products encompassing codons 1099–1693 (Fig. 1*a*). Surprisingly, sequencing of the relevant region of *APC* revealed no truncating mutations, as would be typically found in an FAP patient, but instead revealed a T-to-A transversion at nucleotide 3920 (Fig. 1*b*). This mutation resulted in a substitution of lysine for isoleucine at codon 1307 (I1307K). To confirm that the truncated polypeptide generated *in vitro* was due to this mutation, the relevant segment of *APC* was PCR amplified from the patient's DNA and cloned. IVSP assays performed on clones containing the I1307K mutation produced both full-length and truncated polypeptides in a ratio of 3:1, whereas clones containing the normal sequence produced only the full-length product (data not shown). No truncated proteins were observed on western blotting proteins from the patient's lymphocytes or from somatic cell hybrids in which the mutated allele had been isolated. Thus the truncation observed in the IVSP assay was apparently an *in vitro* phenomenon caused by the T-to-A transversion. Sequencing of the PCR products used for the IVSP assay showed that no amplification errors were introduced by *Taq* polymerase. The shorter polypeptides were therefore probably due to transcriptional or translational pausing or misincorporation during the IVSP reactions. Such failures of the transcriptional or translational machinery could have been the result of the extended mononucleotide tract created by the T to A transversion (AAATAAAA to $(A)_8$).

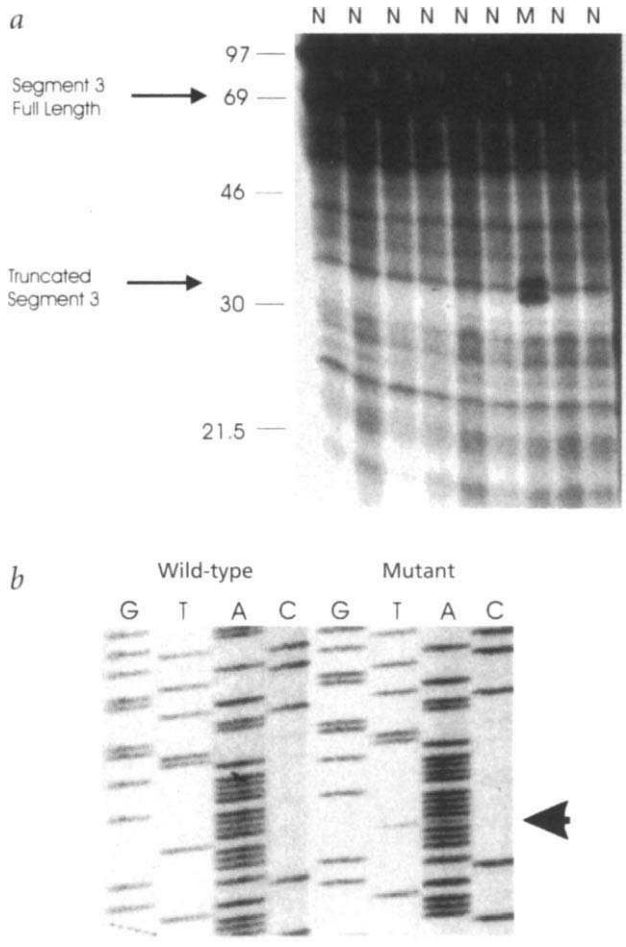

Fig. 1 IVSP assays of *APC* codons 1099–1693. ***a***, Lanes marked 'N' contain polypeptides from patients without a mutation, and lane 'M' contains polypeptides from the index patient who was found to be heterozygous for I1307K. The arrow points to the doublet of truncated polypeptides present in the patient with this mutation. The reason for the doublet at the indicated position is not clear. The numbers on the left represent molecular-weight standards in kilodaltons. ***b***, Sequence of PCR products from the index patient. The wild-type sequence is AGAAATAAAA, and the mutant sequence is AGAAAAAAAA, predicted to result in a substitution of lysine for isoleucine at codon 1307 (I1307K). The arrow points to the heterozygous mutation site.

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Table 1 • Incidence of the I1307K mutation

	I1307K +	Total	%
Non-CRC controls			
Non-Jewish	0	243	0[a]
Ashkenazi Jewish	47	766	6.1[b]
Ashkenazi-Jewish CRC patients			
All patients with CRC	22	211	10.4[c]
Patients < 66 years old	14	89	16[d]
Patients ≥ 66 years old	8	121	6.6[e]
Family History Positive	7	25	28[f]
Family History Negative	0	13	0[g]
Family History Unknown	15	173	8.7[h]

a versus b, $P<0.0001$ by χ^2; *b versus c*, $P<0.033$ by χ^2; *d versus e*, $P<0.033$ by χ^2 (the age of one patient with CRC was unknown); *b versus f*, $P<0.001$ by Fisher's exact test; *f versus h*, $P<0.02$ by Fisher's exact test; *b versus g* and *b versus h* are not significant by Fisher's exact test.

To evaluate epidemiological issues related to this presumptive mutation, an allele-specific oligonucleotide (ASO) hybridization assay was designed (see Methods). As the index patient was a member of a genetically homogeneous group (Ashkenazi Jewish), individuals of both Ashkenazi-Jewish and non-Jewish origins were examined. The I1307K mutation was not found in any of 243 non-Ashkenazim tested, but a remarkably high proportion of Ashkenazim (6.1%) were found to carry it (Table 1). The difference in mutation prevalence between the two populations was highly significant (Table 1; $P<0.0001$ by χ^2).

To determine whether the I1307K mutation was associated with colorectal cancer in Ashkenazi Jews, we examined 211 Ashkenazim with colorectal cancer. Using the ASO, it was found that 10.4% of such patients harboured I1307K. This elevated frequency was statistically significant when Ashkenazim with colorectal cancer and the general Ashkenazi-Jewish population were compared (Table 1; $P<0.033$ by χ^2). Upon stratifying the patients according to age, we found that the prevalence of the I1307K mutation was higher in younger patients, consistent with its causing cancer susceptibility (Table 1; 16% vs. 6.6%, $P<0.033$ by χ^2).

To further explore the cancer-susceptibility association, patients were stratified according to the presence of a family history of colorectal cancer (see Methods). Twenty-five probands were found to have a first- or second-degree relative with colorectal neoplasia (either cancer or adenomatous polyp). In the remaining 186 probands, either no first- or second-degree relative had colorectal cancer (n=13) or the family history was unknown (n=173). 28% of probands from the 25 familial cases carried I1307K—a frequency that was highly significant when compared either to the 6.1% found in the general Ashkenazi population (Table 1; $P<0.001$) or the 8.7% found in colorectal cancer patients with an unknown family history of CR neoplasia (Table 1; $P<0.02$ by χ^2).

As an additional test of the association between I1307K and colorectal-cancer predisposition, the segregation of this mutation was

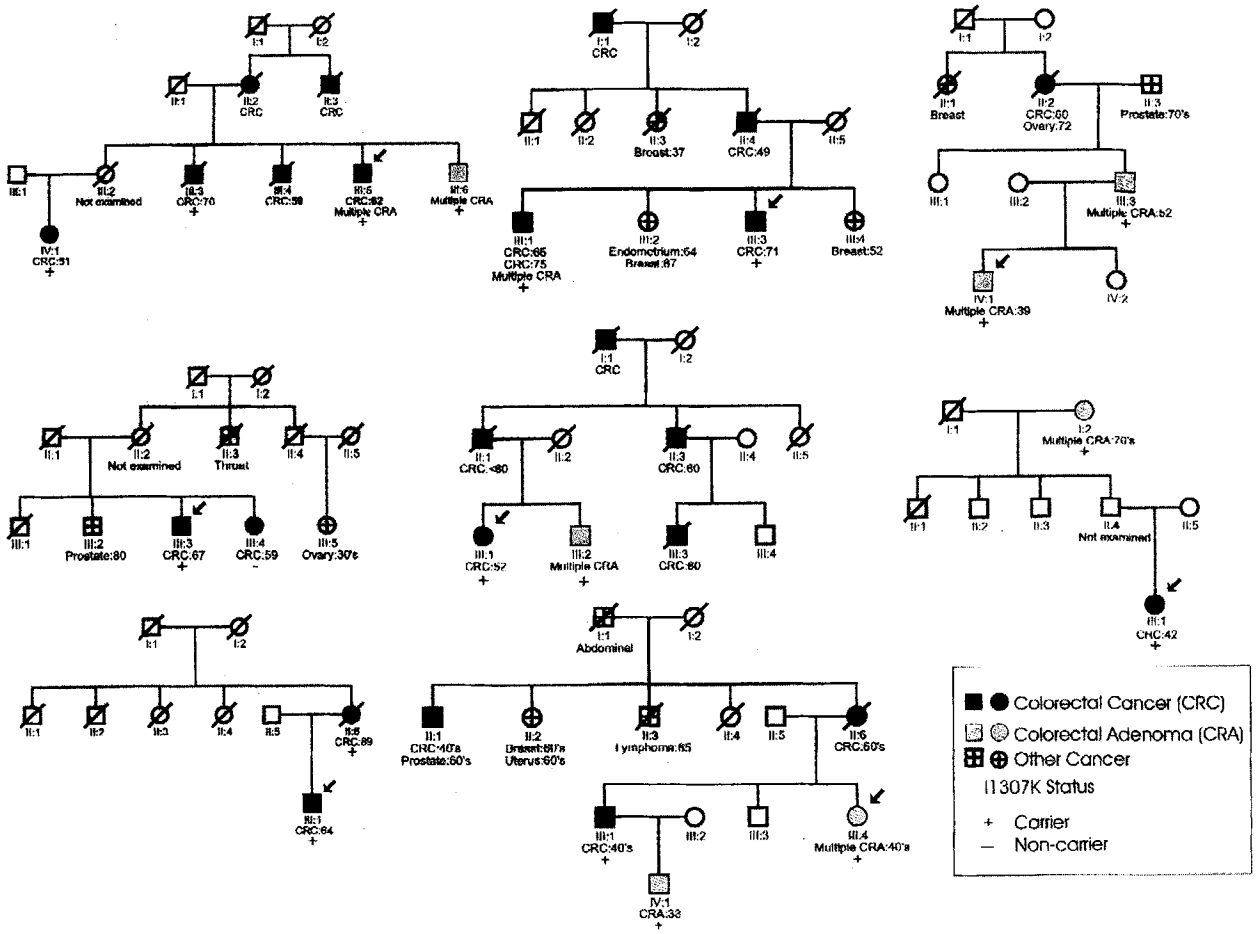

Fig. 2 Pedigrees of probands with a family history of CRC and I1307K. Patients affected with CRC are denoted by filled symbols, patients with adenomatous polyps are denoted by shaded symbols, patients with other cancers are denoted by crosses and probands are indicated with arrows. Cancer types and age at diagnosis are indicated when known. Individuals with the I1307K mutation are denoted as '+', and the affected individual who was found not to carry the I1307K mutation is indicated as '–'. None of the patients studied met the clinical criteria for FAP. In patients with 'multiple colorectal adenomas', on whom detailed pathologic records were available, the number of adenomatous polyps varied between two and twenty.

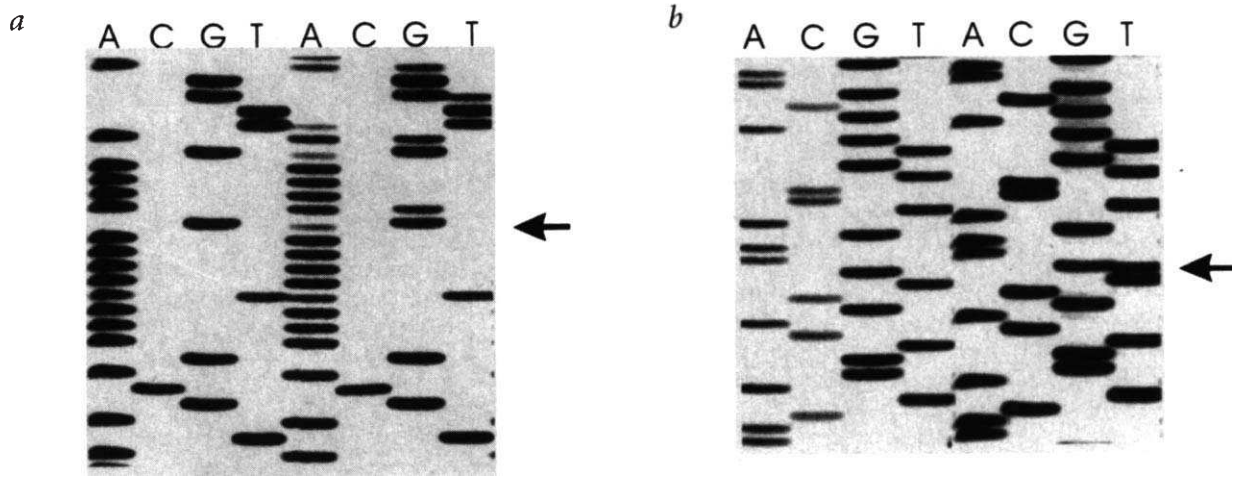

Fig. 3 *Somatic mutations observed in tumours from patients who were heterozygous for the I1307K mutation.* ***a***, *The four left lanes illustrate sequencing results from a tumour that did not exhibit a somatic alteration between codons 1296 and 1322. The arrow next to the right lanes denotes a somatic 1-bp insertion within the $(A)_8$ tract from a different tumour.* ***b***, *The four left lanes show a result from a tumour without a somatic mutation between codons 1296 and 1322. The arrow on the right indicates a transition of G to T, which creates a stop at codon 1317, in a different colorectal tumour.* ***c***, *Coding strand of the wild-type and I1307K alleles of APC. The bold 'A' indicates the position of the I1307K mutation. Arrows below the I1307K allele indicate somatic mutations in the I1307K allele, and the numbers in parentheses indicate the number of times each mutation was observed.*

examined in eight Ashkenazi-Jewish colorectal cancer families in which the proband carried I1307K. Eleven relatives affected with colorectal cancer or adenomas were examined with the ASO assay, and ten were found to carry the I1307K mutation; each was confirmed by sequencing (Fig. 2). Bayesian statistical analysis indicated that this result was very unlikely to be due to chance ($P<0.005$; see Methods).

Although these data strongly suggested that I1307K was intrinsically related to colorectal-cancer predisposition, it remained possible that this mutation was in linkage disequilibrium with another mutation in *APC*. To rule this out, sequencing of the entire coding region of *APC* was performed on two unrelated probands with familial colorectal neoplasia who carried the I1307K mutation. Only two previously identified silent polymorphisms, at codons 486 and 1678, were found in both individuals in addition to the I1307K mutation.

If the I1307K mutation were sufficient to cause disease, this allele would be predicted to be altered in tumours, whereas the wild-type allele should be inactivated as a result of somatic mutation. To determine whether this was the case, microdissection was performed on neoplastic tissues from 23 paraffin-embedded tumours of patients with the I1307K mutation (see Methods). DNA was purified from the tissues and PCR used to amplify the region between *APC* codons 1296 and 1322. Unexpectedly, sequencing of the PCR products revealed sequence changes within this small region of *APC* in nearly half of the tumours analysed (11 of 23, 48%). These changes were all somatic, as none was found in DNA extracted from normal peripheral blood or non-neoplastic tissues scraped from the same slides used for microdissection of tumour tissue. All sequence changes (one in each of eleven different tumours) produced a stop codon, either as a result of a 1-bp insertion, 1-bp deletion or a nonsense mutation (Fig. 3 and Table 2). The

Table 2 • Somatic mutations in tumours from patients with an I1307K allele

Tumour	Grade	Codon	Mutation
1	adenocarcinoma	1314[a]	AGG→AG (and loss of wt allele)
2	tubulovillous adenoma	1306–1308 [a]	AAAAAAAA→AAAAAAAAA
3	adenocarcinoma	1309[a]	GAA→TAA
4	tubulovillous adenoma	1309[a]	GAA→TAA
5	adenocarcinoma	no mutation	(LOH of 1307K allele)
6	adenocarcinoma	no mutation	(LOH of 1307K allele)
7	tubular adenoma	no mutation	
8	adenocarcinoma	no mutation	
9	tubular adenoma	no mutation	
10	tubulovillous adenoma	no mutation	
11	tubular adenoma	no mutation	
12	adenocarcinoma	1306–1308[a]	AAAAAAAA→AAAAAAAAA
13	adenocarcinoma	1317[a]	GAA→TAA
14	villous adenoma	1309[a]	GAA→GGAA
15	tubular adenoma	1306 -1308[a]	AAAAAAAA→AAAAAAAAA
16	tubular adenoma	no mutation	
17	tubular adenoma	no mutation	
18	villous adenoma	1306–1308[a]	AAAAAAAA→AAAAAAAAA
19	tubulovillous adenoma	1306–1308[a]	AAAAAAAA→AAAAAAAAA
20	tubular adenoma	no mutation	
21	tubular adenoma	1306–1308[a]	AAAAAAAA→AAAAAAAAA
22	adenocarcinoma	no mutation	
23	tubular adenoma	no mutation	

[a]Somatic mutation occurred in the 1307K allele.

PCR products were cloned and at least ten clones were sequenced from each patient to determine which allele contained the truncating mutation. It was found that each of the somatic mutations occurred in the I1307K allele rather than in the wild-type allele, a result very unlikely to be due to chance ($P<0.001$ by χ^2).

The data described above document a common *APC* alteration in Ashkenazi Jews. To our knowledge, this mutation is the most common cancer-associated mutation known in a specific population. Several lines of evidence suggest that this alteration is pathogenic. First, alterations of *APC* are thought to initiate colorectal neoplasia in both familial and sporadic settings[4]. Second, it is found at a higher frequency in CRC patients than in the general Ashkenazi population, and at particularly high frequency in younger Ashkenazi CRC patients (Table 1). Third, its frequency is markedly higher in familial colorectal-cancer patients than in the general Ashkenazi-Jewish population (Table 1). Fourth, within such families, the mutation was found to segregate with disease (Fig. 2). Fifth, analysis of the tumours occurring in patients with I1307K revealed somatic truncating mutations closely surrounding the germ-line mutation (Table 2). It is unlikely that such a small region of *APC* harbours so many mutations by chance; previous studies of *APC* have identified somatic mutations within this region in less than 5% of alleles[12,13], whereas the patients we studied acquired these mutations in nearly 50% of their I1307K alleles. Finally, one of the best tests of the association between the I1307K mutation and truncating mutations in the vicinity was provided by the allelic analysis of the mutations. Somatic mutations in the region occurred exclusively in the I1307K allele.

These studies reveal an intriguing mechanism through which a germ-line mutation can predispose to neoplasia. The I1307K mutation is unlikely to cause such predisposition by directly altering the function of the encoded protein for two reasons. First, missense mutations in *APC* do not appear to cause a phenotypic effect, perhaps because of the internal repetition of motifs that mediate its function[4]. Second, the I1307K allele is frequently mutated or lost in the tumours, strongly suggesting that the unmutated I1307K allele does not directly participate in the tumorigenic process. Instead, the I1307K allele appears to create an unstable sequence that is hypermutable, leading to somatic, truncating mutations occurring at adjacent sequences. Although the mechanism underlying this hypermutability is unclear, several observations are relevant. The mutation substitutes an $(A)_8$ tract for the $(A)_3T(A)_4$ tract normally present at this position. Mononucleotide tracts, like other microsatellite sequences, are known to be highly mutable[14,15]. The basis for this instability appears to be 'slippage' during the replication process, resulting in gains or losses of repeat units[16]. Interestingly, the types of somatic mutations found in the I1307K allele were not those most commonly associated with microsatellites[15]. For example, mutations within poly(A) tracts are almost always deletions rather than insertions[15], whereas the mutations we observed within the $(A)_8$ tract were all insertions. Moreover, several of the somatic mutations in our patients were substitutions closely surrounding, rather than within, the $(A)_8$ tract. The $(A)_8$ tract not only was unstable *in vivo*, leading to somatic mutation, but also appeared to be unstable *in vitro* during the enzymatic manipulations employed in the IVSP assay (Fig. 1*a*). Together, these results suggest that the $(A)_8$ tract creates an unusual secondary structure that is processed incorrectly both by the polymerases present in human cells and by those used in the IVSP assay. Further studies will be required for an understanding of how this mutation results in hypermutability, and in particular whether the $(A)_8$ tract alone or (more likely) the $(A)_8$ in conjunction with surrounding sequences is responsible.

One of the most important implications of these results concerns screening for colorectal cancer predisposition in Ashkenazi Jews. Other disease-associated mutations have been observed in this population, including mutations in *BRCA1* and *BRCA2* genes predisposing to breast cancer[17]. The I1307K mutation is the most common mutation found so far in such individuals, occurring in about 1/16 Ashkenazim. The cancer risk for carriers can be approximated from two independent sources of data. First, the mutation was identified in 10.4% of Ashkenazim with colorectal cancer, as against 6% of unselected Ashkenazim. Second, a somatic mutation surrounding the I1307K allele was found in 48% of the tumours arising in these patients; we conservatively assume that in the other 52% of tumours, the I1307K allele did not contribute to the neoplasms. Both results suggest that the I1307K causes about a twofold increased risk for CRC. As the lifetime incidence of CRC in the general Ashkenazi population has been estimated to be 9–15% (refs 18–20), the lifetime risk for CRC in patients with I1307K is likely to be in the range of 18–30%. Moreover, our data suggest that 28% of familial CRC in Ashkenazim is associated with the I1307K mutation. In patients with a family history of CRC and the I1307K mutation, this risk may be higher, owing to the influence of other genetic and environmental factors. Future studies with larger numbers of patients can address the influence of such factors and arrive at more precise figures for age-adjusted risks. As effective clinical measures are available for limiting CRC morbidity in individuals at high risk through inherited mutations[21–24], further evaluation of this issue seems warranted.

The results also have potential implications for human cancer biology. Numerous studies have indicated that relatives of patients with cancer have a twofold increase in cancer risk[22,24]. Our data indicate that subtle or unconventional mutations in cancer-predisposition genes may be responsible for part of this increased risk. The I1307K results provide an example of the way in which somatic mutations could cause such risks, namely by creating mutation 'hotspots'. Many common polymorphisms in cancer-predisposition genes have been noted. However, because they are common or because they are not expected to functionally alter the predicted proteins, they have not been thought to be related to disease. The new data raise the possibility that some of these mutations, even silent or non-coding ones, may not be innocuous.

Methods

IVSP. IVSP was performed essentially as has been described[10]. Genomic DNA was amplified with primers 5′–GGAGGATCCTGTAGGAATGGTATCTCG–3′ and 5′–GGATCCTAATACGACTCACTATAGGGAGACCACCATGGTTTCTCCATACAGGTCACGG-3′ (DNAgency). PCR products were then transcribed and translated *in vitro* with the use of reagents available from Promega. For cloning the I1307K region into *E. coli*, the PCR products were purified from agarose gels and cloned into the pZero vector (Invitrogen). Clones were sequenced with ThermoSequenase (Amersham). PCR products from the clones were then used as templates for IVSP reactions.

Subject selection. Blood samples without personal identifiers from a group of 766 Ashkenazim undergoing genetic testing for Tay-Sachs disease were used as the non-CRC control group. Age data were available on 237 people from this group, and the mean age was 34.2. Family history of CRC was unavailable for this group. Another set of 243 blood samples, also without identifiers, was obtained from non-Ashkenazi Jews who had contributed samples for diverse reasons unrelated to colorectal cancer (Tay-Sachs, lung cancer cases and a variety of non-cancer controls); age data were available on 77 people from this group, and the mean age was 60.7. Two groups of Ashkenazi-Jewish colorectal-tumour patients were evaluated. One group represented a consecutive series of 172 Ashkenazim who had been treated for colorectal cancer at Memorial Sloan-Kettering Cancer Center between 1986 and 1996 and from whom paraffin-embedded tissue blocks were available (mean age, 68.1). Patient identifiers and family-history information were not available for these samples. The second group consisted of 39 colorectal-tumour patients who had participated in IRB approved family studies at the Johns Hopkins Hospital, Memorial Sloan-

Kettering Cancer Center or Cedars-Sinai Medical Center (mean age, 59 y). Of these, 25 patients had a positive family history of CRC. Eligibility criteria for these studies included Ashkenazi-Jewish status (by self-identification) and documented personal history of colorectal neoplasia. Informed consent was obtained from all studied subjects, and information about personal and family history of cancer was obtained from tested family members. A positive family history was defined as one in which at least two first- or second-degree relatives had colorectal neoplasia (polyps or cancer), with at least one of these relatives having had colorectal cancer.

Statistical methods. Comparison of carrier frequencies in the sampled groups was performed with the χ^2 test or Fisher's exact test. Bayesian methods were used to estimate the probability of observing the I1307K mutation in eight affected first degree relatives (7/8 I1307K+) and three affected second-degree relatives (3/3 I1307K+) of probands from the eight families in Fig. 2. Two alternative hypotheses were compared: i) I1307K mutation status is causally related to colorectal neoplasia; ii) I1307K mutation status and colorectal neoplasia are unrelated. The probabilities of a positive and negative I1307K mutation result were first calculated for each relative, conditioned on degree of relationship to the proband, a phenocopy rate of colorectal neoplasia of 0.2 and 0.06 frequency of the I1307K mutation. The products of the eleven outcomes under each hypothesis were then compared, resulting in a posterior probability of 0.996 that I1307K is causally related to colorectal neoplasia in these families.

Mutation analyses. Genomic DNA was used as template for PCR with the following primers: 5′–GATGAAATAGGATGTAATCAGACG–3′ and 5′–CTTCGCTCACAGGATCTTCAGC–3′. The PCR product was slot-blotted onto nylon filters and hybridized with oligonucleotides corresponding to the wild-type or mutant sequence at codon 1307, 5′–AATAGCAGAAATAAAAGAAAAGAT–3′ or 5′–AAATAGCAGAAAAAAAAGAAAAGAT–3′, respectively. Hybridizations were performed at room temperature for 1 h, then washed for 15 min in 2× SSC, 0.1% SDS at room temperature, followed by a 2.5-min wash at 56 °C in 2× SSC, 0.1% SDS. To confirm the hybridization results, all PCR products that were positive in the ASO assay were sequenced using the primer 5′–GATGAAATAGGATGTAATCAGACG–3′. Sequencing of the entire coding region of *APC* was performed on two probands who had colorectal cancer and were from two I1307K-positive familial colorectal-cancer families. Sequence of the primers used for PCR amplification of *APC* sequences from cDNA and genomic DNA as well as the primers used for sequencing are available from the authors upon request.

Isolation of DNA from paraffin-embedded tumours. DNA was isolated from paraffin-embedded colorectal tumours as described previously[25]. To examine the sequence of the region surrounding the I1307K mutation from this DNA, one of the following two primer sets was used for PCR-based amplification: 5′–GCAGATTCTGCTAATACCCTGC–3′ and 5′–CTTCGCTCACAGGATCTTCAGC–3′ (82-bp PCR product) or 5′–CCCTGCAGTCTGCTGGATTTGG–3′ and 5′–TGTGTAGAAGATACTCCAATATG–3′ (230-bp PCR product). Sequencing was performed with either 5′–CTTCGCTCACAGGATCTTCAGC–3′ or 5′–TGTGTAGAAGATACTCCAATATG–3′. An evaluation for microsatellite instability at other loci was performed with the primers and conditions described elsewhere[9].

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