Analysis of masked mutations in familial adenomatous polyposis

STEVEN J. LAKEN*, NICKOLAS PAPADOPOULOS†, GLORIA M. PETERSEN‡, STEPHEN B. GRUBER§, STANLEY R. HAMILTON§, FRANCIS M. GIARDIELLO∥, JILL D. BRENSINGER∥, BERT VOGELSTEIN**††, AND KENNETH W. KINZLER*

*The Johns Hopkins Oncology Center and *Howard Hughes Medical Institute, 424 North Bond Street, Baltimore, MD 21231; †Department of Pathology, Columbia University College of Physicians and Surgeons, 630 West 168th Street, New York, NY 10032; ‡Department of Epidemiology, Johns Hopkins School of Public Health, 615 North Wolfe Street, Baltimore, MD 21205; §Division of Molecular Medicine and Genetics, University of Michigan, 4301 MSRB3, Ann Arbor, MI 48109; ∥Division of Pathology and Laboratory Medicine, M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030; and †Department of Medicine, Johns Hopkins Hospital, 600 North Wolfe Street, Baltimore, MD 21287

ABSTRACT Familial adenomatous polyposis (FAP) is an autosomal-dominant disease characterized by the development of hundreds of adenomatous polyps of the colorectum. Approximately 80% of FAP patients can be shown to have truncating mutations of the APC gene. To determine the cause of FAP in the other 20% of patients, MAMA (monoallelic mutation analysis) was used to independently examine the status of each of the two APC alleles. Seven of nine patients analyzed were found to have significantly reduced expression from one of their two alleles whereas two patients were found to have full-length expression from both alleles. We conclude that more than 95% of patients with FAP have inactivating mutations in APC and that a combination of MAMA and standard genetic tests will identify APC abnormalities in the vast majority of such patients. That no APC expression from the mutant allele is found in some FAP patients argues strongly against the requirement for dominant negative effects of APC mutations. The results also suggest that there may be at least one additional gene, besides APC, that can give rise to FAP.

Experience with FAP also illustrates the technical problems associated with genetic testing. APC is a large gene, encoding a protein of 2,843 aa contained within 15 exons (8, 9). Sequencing the entire gene, including introns, untranslated, and promoter regions, is impractical. Fortunately, all confirmed FAP-causing mutations detected to date result in truncations of the protein (4, 5). One major consequence of these truncating mutations is the disruption of APC’s ability to inhibit the function of β-catenin (10, 11). It is believed that APC normally binds to β-catenin (12, 13) and promotes its degradation (14), thereby preventing activation of growth-promoting genes, such as c-myc (15), by a β-catenin/Tcf-4 transcription complex (16, 17). This mutation spectrum has stimulated the development of testing approaches that can reveal truncated APC proteins. In particular, the most commonly used test [called in vitro synthesized protein (IVSP) or protein truncation test (PTT)] involves in vitro transcription and translation of APC PCR products. Gel electrophoretic analysis of the translated polypeptides reveals truncated proteins indicative of mutations (3, 18).

Extended analyses of FAP kindreds with IVSP and direct or indirect DNA sequencing methods have been used to identify more than 200 different mutations (4, 5). The frequency of APC mutations detected among FAP kindreds varies with the technique used, but in no case has it been more than 80%. The basis for the inability to identify mutations in a substantial proportion of such kindreds is unclear. One possibility involves the existence of APC mutations that are difficult to detect by standard mutational analyses. Indeed, the patient whose analysis originally led to the chromosome 5 localization of APC had a large deletion that would have been impossible to detect with any standard sequencing or IVSP assay (19). Furthermore, some FAP patients without truncating APC mutations appeared to express significantly reduced levels of APC transcript from one allele (3). A second possibility is that some cases of FAP are caused by mutations in genes other than APC.

We have described previously a mutation detection approach called monoallelic mutation analysis (MAMA). Chromosomes from an affected individual are isolated in hybrid cells formed from fusion of the patient’s cells with a suitable rodent recipient (20). Because each allele can be examined independently, mutations are not obscured by the wild-type product from the normal allele, as can occur with standard analyses of patients’ cells. For example, mutations in promoter regions, which decrease expression from the corresponding allele in cis fashion, are very difficult to detect with routine assays. However, because patterns of expression are faithfully preserved in such hybrids (20, 21), mutations that affect

Abbreviations: MAMA, monoallelic mutation analysis; FAP, familial adenomatous polyposis; PTT, protein truncation test; IVSP, in vitro synthesized protein.
††To whom reprint requests should be addressed. e-mail: vogelbe@welchlink.welch.jhu.edu.

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expression can be detected as easily as those that affect the structure of the gene. We now have used MAMA to analyze nine FAP patients in whom no APC mutations could be detected with standard methods. The results show that more than 95% of FAP patients have inactivating mutations in APC and that a combination of MAMA and standard genetic testing can identify APC abnormalities in the vast majority of FAP patients. Additionally, the results suggest that there may be at least one other gene besides APC that can give rise to FAP.

**MATERIALS AND METHODS**

**Cell Culture.** Lymphoblastoid lines were established by Epstein–Barr virus infection of peripheral blood leukocytes from patients diagnosed with FAP who had no evidence of APC mutation upon IVSP analysis. These lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and grown at 37°C and 5% CO2. The UCW-56 hamster cell line (22) was grown in DMEM supplemented with 10% fetal bovine serum and 60 µg/ml L-proline and grown at 32°C and 5% CO2.

**Cell Fusions.** Fusions initially were performed with polyethylene glycol as described (20). In later experiments, electrofusion was employed because a greater yield of viable fusion clones could be obtained. UCW-56 cells were combined with lymphoblastoid cells in FS (0.3 M mannitol/0.1 mM MgCl2/0.1 mM CaCl2) at a ratio of 3:1. The cells were washed and centrifuged three times in FS before resuspending in FS at a final concentration of 5 × 10^7 cells/ml. Thirty microliters of this solution then was mixed and pipetted into a 0.5-mm gap microfusion plate (BTX Microslide 450; BTX, San Diego). Fusions were performed by using a BTX Electrocell Mani- pulator, ECM 200. The settings that yielded the greatest number of fusion clones were 15 V (AC) for 10 sec followed by two 100-V (DC) pulses of 30 µsec each.

The cells from one 30-µl fusion were plated into four wells of a 48-well plate (Costar) in DMEM supplemented with 10% fetal bovine serum and 60 µg/ml L-proline, and grown at 32°C. To obtain a sufficient number of clones, we generally performed three fusions from each line, employing a total of 1.8 × 10^7 UCW-56 cells and 6 × 10^6 lymphoblastoid cells. After 24 hr, the medium was changed and the 48-well plates were transferred to 39°C. Medium was changed every 4 days for approximately 2 weeks, at which time individual clones were genotyped and expanded for immunoblotting (20). Immunoblotting was performed as described (23) except that cells were lysed in 8 M urea/2 M thiourea/0.05 M Tris base/0.075 M DTT/3% SDS/0.004% bromophenol blue and adjusted to a final pH of 6.8 (24), and APC was detected with an antibody (catalog no. OP47L; Oncogene Science) reactive with the C terminus of APC (23).

**RESULTS**

**Generation of Monoallelic Clones.** Isolation of human chromosome 5 alleles was accomplished by fusing lymphoblastoid cells from FAP patient lines with the UCW-56 hamster cell line (Fig. 1). UCW-56 has a temperature-sensitive tRNA-leucyl synthetase mutation, which prevents growth at 39°C unless complemented by the human tRNA-leucyl synthetase gene on chromosome 5. Because lymphoblastoid cells are nonadherent, clones derived by fusion are identified easily after growth at 39°C. In the original MAMA technique, fusions were performed with polyethylene glycol. We subsequently found that electrofusion was considerably more efficient at generating fusion clones. After electrofusion with a total of 1.8 × 10^7 UCW-56 cells and 6 × 10^6 lymphoblastoid cells, an average of 3% SDS

**FIG. 1.** Schematic of MAMA. The hamster cell line, UCW-56, is fused to an FAP lymphoblastoid line, and clones are subsequently selected at 39°C. Only clones that retain human chromosome 5 can grow at this temperature. During the expansion process, hybrids retain at least one human chromosome 5 and usually lose the other copy. Clones then are genotyped to determine which chromosome 5 they contain. Proteins from the fusion clones are used for Western blotting with antibodies reactive with the amino or carboxyl ends of the APC protein. The N-terminal antibody (Human/Rodent APC) reacts with human and rodent APC protein and serves as a loading control. The C-terminal antibody (Human APC) reacts against human but not hamster APC and is used to determine whether full-length APC expression occurs.

**MAMA in a Patient with a Known APC Mutation.** FAP patient C1 was tested by IVSP and found to have a truncating mutation of APC resulting in the removal of ~2,000 aa from the C terminus. DNA genotyping was performed on clones derived from the fusion of lymphoblasts of patient C1 with UCW-56. Using a polymorphism at the LNS-CA locus, just telomeric of APC, it was found that fusion clones contained either one of two individual chromosomal 5 alleles arbitrarily designated “A” and “B” (examples in Fig. 2). These clones were expanded further and protein was isolated for immunoblotting with a human specific antibody reactive against the C terminus of APC. All clones containing chromosome “B” failed to produce full-length APC protein, whereas those with chromosome “A” produced a protein of the expected wild-type size (Fig. 3A). Thus, the germ-line mutation in this patient resided on the “B” chromosome. Using an N-terminal antibody, the cells with chromosome “B” revealed no truncated protein of the expected size. This is not unusual, because the mRNA or protein from APC genes with truncating mutations often is unstable and thereby undetectable by Western blot assays (23).
MAMA in Patients Without APC Mutations Identifiable by IVSP. Nine unrelated FAP patients in whom no APC mutations could be identified by IVSP were analyzed by MAMA. Each patient had at least 100 adenomas (verified by pathologic records), and each patient had a family history of FAP, ruling out germ-line mosaicism as a cause of the negative IVSP results (Table 1). Upon fusion to UCW-56, hybrids were expanded and genotyped with LNS-CA and other chromosome 5 polymorphic markers to identify monoallelic clones. Of 165 clones analyzed, 101 were found to contain one allele, whereas 64 were found to contain both maternal and paternal alleles. Monoallelic clones then were expanded and their proteins were used for immunoblotting. A minimum of two clones representing each allele from each patient was used to exclude clonal variability as an explanation for failure to detect full-length protein. Analysis of the nine patients revealed three classes of expression; no detectable APC expression from one allele (six patients; Fig. 3B), reduced APC expression from one allele (one patient, Fig. 3C), or equal APC expression from both alleles (two patients, Fig. 3D).

Confirmatory Studies. There was little doubt about the mutational basis of FAP in patients with no detectable expression of APC. However, we were less confident about the patient with detectable but reduced expression. This patient was from a reasonably large kindred, and linkage analysis was carried out with markers closely surrounding APC. Two significant results were evident from this linkage analysis. First, a multipoint logarithm of odds (lod) score of 2.06 was obtained at a $\theta$ of 0.1 by using markers D5S82, LNS-CA, and MCC (25), strongly suggesting that the disease was caused by an APC mutation in this family. Second, the haplotype that was associated with disease in the linkage analysis was also the haplotype of the chromosome that showed reduced expression. Haplotypes are extremely easy to define with MAMA and can be used as diagnostic tools for genetic testing of other members of the family; only the proband needs to be tested by MAMA.

In the two cases in which full-length APC protein was expressed by both alleles, it was possible that missense mutations or small in-frame insertions or deletions of APC were responsible for the disease. To evaluate this possibility, both APC alleles were normal in these two patients. No other members of these two families were available to compare haplotypes or to exclude linkage to APC.

DISCUSSION

Several important conclusions can be made on the basis of this study. First, it is now clear that at least 95% of FAP patients have mutations in APC. In other studies (ref. 3 and unpublished data) we have observed that $\approx 80\%$ of FAP patients have
APC mutations detectable with IVSP. In the other 20% of patients, our new results show that seven of nine (78%) contain mutations detectable with MAMA. In total, it can be estimated that 95% of FAP patients harbor APC mutations. These figures are consistent with linkage analyses showing that disease in FAP kindreds generally is linked to chromosome 5q markers (26). Most previous failures to detect APC mutations in FAP kindreds therefore are likely to have been a result of the insensitivity of the assays used rather than the absence of APC mutations.

Second, these results have significant implications concerning the relationship between specific APC mutations and phenotype. In particular, it has been shown that the precise number of FAP probands do not inherit a mutant APC allele, yet many must be screened with colonoscopy and some will suffer the emotional consequences of uncertainty. The cost of alternative techniques such as MAMA must be judged against the medical and human costs of having no definitive diagnoses in such cases.

Finally, two of nine FAP patients analyzed harbored alleles from which full-length, completely wild-type sequence was expressed at normal levels. These patients may have contained mutations in noncoding regions of the gene that specifically diminished their expression in colonic stem cells but that did not lead to decreased expression in the hybrids generated upon fusion to UCW-56 cells. Alternatively, the FAP in these kindreds may be a result of a gene different from APC. Unfortunately, neither of these two patients came from a kindred large enough for linkage analysis, so these possibilities cannot be distinguished at present. It is interesting that a homolog of APC, located on chromosome 19p13.3, recently has been described (33, 34). This homologue as well as the functionally related family of axin genes (35–40) are good candidates for FAP causation in these kindreds. Elucidation of other genes responsible for FAP might shed considerable additional light on the mechanisms through which APC causes polyposis, just as mutations in β-catenin in sporadic cancers have illuminated the pathway (10, 41).

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<th>IVSP result</th>
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<th>No. of polyps</th>
<th>No. of affected family members</th>
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NA, not available.