Allele-Specific Expression of *APC* in Adenomatous Polyposis Families

ESTER CASTELLSAGUÉ,* SARA GONZÁLEZ,* ELISABET GUINÓ,‡ KRISTEN N. STEVENS,§ ESTER BORRÀS,* VICTORIA M. RAYMOND,‖ CONXI LÁZARO,* IGNACIO BLANCO,¶ STEPHEN B. GRUBER,# and GABRIEL CAPELLÁ*

*Translational Research Laboratory, ‡Bioinformatics and Biostatistics Unit, Department of Epidemiology, ¶Cancer Genetic Counseling Program, Institut d'Investigacions Biomédiques de Bellvitge–Institut Català d'Oncologia, Barcelona, Spain; §Department of Epidemiology, #Department of Internal Medicine, Epidemiology, and Human Genetics, Division of Molecular Medicine and Genetics, University of Michigan, Ann Arbor, Michigan; and ‖Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, Michigan

See related articles, Rozas DA et al, on page e85; and Ruys AT et al, on page 731 in *CGH*.

BACKGROUND & AIMS: Germline mutations in the *APC* gene cause of most cases of familial adenomatous polyposis (FAP) and a lesser proportion of attenuated FAP (AFAP). Systematic analysis of *APC* at the RNA level could provide insight into the pathogenicity of identified mutations and the molecular basis of FAP/AFAP in families without identifiable mutations. Here, we analyzed the prevalence of imbalances in the allelic expression of *APC* in polyposis families with germline mutations in the gene and without detectable mutations in *APC* and/or *MUTYH*. **METHODS:** Allele-specific expression (ASE) was determined by single nucleotide primer extension using an exon 11 polymorphism as an allele-specific marker. In total, 52 *APC*-mutation–positive (36 families) and 24 *APC/MUTYH*-mutation–negative (23 families) informative patients were analyzed. Seventy-six controls also were included. **RESULTS:** Of the *APC*-mutation–positive families, most of those in whom the mutation was located before the last exon of the gene (12 of 14) had ASE imbalance, which is consistent with a mechanism of nonsense-mediated decay. Of the *APC/MUTYH*-mutation–negative families, 2 (9%) had ASE imbalance, which might cause the disease. Normal allele expression was restored shortly after lymphocytes were cultured with puromycin, supporting a 'nonsense-mediated' hypothesis. **CONCLUSIONS: ASE analysis might be used to determine the pathogenesis of some cases of FAP and AFAP in which *APC* mutations are not found. ASE also might be used to prioritize the order in which different areas of *APC* are tested. RNA-level studies are important for the molecular diagnosis of FAP.**

Keywords: Allele-Specific Expression; Nonsense-Mediated Decay.

Familial adenomatous polyposis (FAP), an autosomal-dominant disease predisposing to colorectal cancer, is caused mainly by truncating germline mutations in the *APC* gene (5q21–22).[1] Widespread use of sequencing techniques has led to the identification of an increased number of missense mutations,[2] variants with a putative impact on messenger RNA (mRNA) splicing,[3] and cases of somatic and germline mosaicisms.[4] Exon dose analysis has detected gross rearrangements in a minority of cases.[5] However, in a lesser proportion of cases, attenuated FAP (AFAP) also is associated with *APC* mutations and with biallelic germline mutations in the *MUTYH* gene, showing an autosomal-recessive pattern.[6]

In other cancer predisposition genes such as *MSH2*, *MLH1*, *BRCA1*, *BRCA2*, and *NF1*, studies at the RNA level have shown that mutations causing a premature termination codon (PTC) usually trigger nonsense-mediated decay (NMD) of the mRNA.[7–11] This mRNA surveillance mechanism reduces the abundance of premature stop-codon–harboring mRNA and of the corresponding truncated proteins. NMD of the affected transcript occurs if the PTC is located approximately 55 base pairs upstream of the last intron-exon boundary.[12] Some RNA studies of the *APC* gene have been published, focused mainly on the presence of aberrant splicing caused by missense, silent, or unclassified variants.[3,13]

The proportion of FAP families with identifiable *APC* mutations has shown a slight increase with the incorporation of new techniques into diagnostic algorithms. However, a large subset has undetectable pathogenic changes (designated here as *APC*[−]/*MUTYH*[−]). Small decreases in *APC* mRNA have been detected in *APC*(−)[14,15] and *APC*(−)/*MUTYH*(−) families.[16–18] In contrast, high germline levels of an *APC* mRNA isoform resulting from an exon 10–15 connection have been observed in a case of *APC*(−)/*MUTYH*(−) AFAP.[19] Germline imbalances in allele-specific expression (ASE) of the *APC* gene have been detected in FAP and AFAP

Abbreviations used in this paper: AFAP, attenuated familial adenomatous polyposis; ASE, allele-specific expression; cDNA, complementary DNA; dNTP, deoxynucleoside triphosphate; F-UM, family code of the University of Michigan; FAP, familial adenomatous polyposis; ICO, Catalan Institute of Oncology; NMD, nonsense-mediated decay; PCR, polymerase chain reaction; PTC, premature termination codon; SD, standard deviation; SNP, single nucleotide polymorphism; UM, University of Michigan.

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families,[14,15,18,20] but their potential contribution to diagnostic yield is unknown.

The prevalence of imbalances in *APC* allelic expression was analyzed in 2 sets of polyposis families: *APC* mutation-positive, designated here as *APC*(+), and *APC*(−)/*MUTYH*(−). Here we show that mutations outside exon 15 are associated with a clear allelic imbalance at the RNA level that is secondary to NMD, and that these imbalances also are detected in a low proportion of *APC*(−)/*MUTYH*(−) families.

Patients and Methods

Patients

A total of 127 FAP and AFAP families from the Catalan Institute of Oncology (ICO) and the University of Michigan (UM) initially were tested for germline *APC* and *MUTYH* mutations by comprehensive diagnostic methods (sequencing or single-strand conformation polymorphism-analysis of all exons and exon-intron boundaries, gross deletion analysis by quantitative multiplex polymerase chain reaction [PCR] of short fluorescent fragments, and/or multiplex ligation-dependent probe amplification, and/or Southern blot for the *APC* gene, presence of germline epimutations in *APC* promoter in conjunction with sequencing of the 2 most prevalent variants of the *MUTYH* gene—Y165C and G382D). Mutations were found in 76 families, although the molecular pathogenic cause of the disease remained unknown in 51 families. The mutation detection rates were 83% for FAP and 25% for AFAP.

Individuals from 59 of the families tested heterozygous for the rs2229992 *APC* coding single nucleotide polymorphism (SNP) in exon 11 of the *APC* gene and were the informative population of the present study. These families were divided according to mutational status as follows: (1) 36 *APC*(+) families (24 classic FAP, 34 carrier individuals; 12 AFAP, 18 carrier individuals); and (2) 23 *APC*(−)/*MUTYH*(−) families (5 FAP, 6 individuals; 18 AFAP, 18 individuals).

A total of 76 heterozygous controls were included: 29 noncarriers from *APC* mutation families, 6 carriers and 24 noncarriers from *MSH2* and *MLH1* mutation families, and 17 Ashkenazi Jewish individuals diagnosed with sporadic microsatellite-unstable colorectal cancer that formed part of the Molecular Epidemiology of Colorectal Cancer study. This was a population-based, case-control study of incident colorectal cancer, including histopathologically confirmed cases from all incident colorectal cancer cases diagnosed in northern Israel from March 31, 1998, onward. Informed consent was obtained from all of the subjects who participated in the study.

Genotyping: Analysis of Genomic DNA

Peripheral blood lymphocyte DNA was obtained using the FlexiGene DNA kit (QIAGEN, Hilden, Germany). A total of 25 ng of genomic DNA was amplified in a final volume of 25 μL containing 0.13 μmol/L primers, 0.2 mmol/L deoxynucleoside triphosphate (dNTP), 2.5 mmol/L $MgCl_2$, and 1.25 U *Taq* polymerase (Thermoprime Plus DNA Polymerase; ABgene, Rochester, NY). After an initial denaturing step, samples underwent 5 initial cycles (1 min at 94°C, 30 s at 65°C, and 30 s at 72°C), 20 cycles (1 min at 94°C, 30 s at 65°C decreasing 0.5°C/cycle, and 30 s at 72°C), and 10 final cycles (1 min at 94°C, 30 s at 55°C, and 30 s at 72°C). Primers in exon 11 (forward: 5′-GGGACTACAGGCCATTGCA-3′, and reverse: 5′-CAAGTTTGTCAAAGCCATTCCAGC-3′) were used to amplify the rs2229992 SNP. To remove unincorporated primers and dNTPs, PCR fragments were purified using illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Boston, MA). For the single nucleotide primer extension reaction, primer extension was performed with the SNaPshot Multiplex Kit (Applied Biosystems, Carlsbad, CA) with 5′-ATTGCAAGTGGACTGTGAAATGTA-3′ according to the manufacturer's instructions. Briefly, reactions were performed in a total volume of 10 μL containing 1.5 μL treated PCR product, 4.5 μL SNaPshot Ready Reaction Mix, and 0.2 μmol/L extension primer. Primer extension thermocycling conditions consisted of 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 30 seconds. SNaPshot reaction products were treated with 1 U shrimp alkaline phosphatase (USB Corporation, Cleveland, OH) for 60 minutes at 37°C and then 15 minutes at 75°C. Products were run in an ABI Prism 3130 DNA sequencer and analyzed by GeneMapper v4.0 (Applied Biosystems). Heterozygous samples showed a profile with 2 peaks (black and red peaks represent C and T alleles, respectively), whereas only one peak was observed for homozygous samples.

Measuring ASE: Analysis of Complementary DNA

Total RNA was isolated from frozen lymphocytes using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. A total of 250 ng of RNA were reverse-transcribed into complementary DNA (cDNA) using random hexa-nucleotide mixture primers and MMLV reverse transcriptase (Invitrogen). To specifically amplify rs2229992 SNP in cDNA, we used the same exon 11 forward primer as for DNA amplification and a reverse primer targeting the exon 11–12 junction (5′-ATAGAGCATAGCGTAGCCTTGTTG -3′). PCR reactions were performed in a final volume of 25 μL containing 2 μL of cDNA, 0.2 μmol/L primers, 0.2 mmol/L dNTPs, 2.5 mmol/L $MgCl_2$, and 1.25 U *Taq* polymerase (Thermoprime Plus DNA Polymerase; ABgene). After a denaturing step, 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 30 seconds at 72°C were performed. The remaining steps were the same as described for genotyping, including purification, SNaP-