Homozygous BUB1B Mutation and Susceptibility to Gastrointestinal Neoplasia

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A patient received a diagnosis of adenocarcinoma of the ampulla of Vater at 34 years of age. Two decades later, adenomatous polyps were found, followed by multiple primary invasive adenocarcinomas of both the colon and the stomach. Premature chromatid separation and mosaic variegated aneuploidy, combined with structural chromosomal abnormalities, were detected in his cells. We identified a germline homozygous intronic mutation, c.2386-11A→G, in the spindle-assembly checkpoint gene BUB1B, which creates a de novo splice site that is favored over the authentic (i.e., preferentially used) site. Our findings expand the phenotype associated with BUB1B mutations and the mosaic variegated aneuploidy syndrome to include common adult-onset cancers and provide evidence for the interdependency of the APC protein (encoded by the adenomatous polyposis coli gene) and the BUBR1 protein (encoded by BUB1B) in humans. (Funded by the Turner Family Cancer Research Fund and others.)

A ll cancers contain numerous alterations in DNA, some of which are heritable. Genomewide surveys of several tumor types have shown that most solid tumors accumulate a large number of mutations that affect several different pathways.¹ Tumors that have many mutations may have acquired them as a result of underlying genetic instability. It has been proposed that genomic instability can be divided into two types: microsatellite instability and chromosomal instability (see the Glossary in the Supplementary Appendix, available with the full text of this article at NEJM.org).² Microsatellite instability is a hallmark of colorectal cancers occurring in patients with the Lynch syndrome³; these cancers usually do not show substantial karyotypic abnormalities and are often diploid or nearly diploid.⁴ By contrast, tumors with chromosomal instability are aneuploid but do not often show instability at the nucleotide level.² Most colorectal cancers have a phenotype of chromosomal instability rather than microsatellite instability.

Chromosomal instability is defined as the accelerated mis-segregation of whole chromosomes in aneuploid cells. The idea that chromosomal instability might be an important, if not obligatory, step in carcinogenesis has been strongly supported by clinical observations of patients with a very rare childhood syndrome of autosomal recessive cancer susceptibility known as the mosaic variegated aneuploidy syndrome (Online Mendelian Inheritance in Man number, 257300).⁵,⁶ Although aneuploidy...
itself is not linked directly to chromosomal instability, in the mosaic variegated aneuploidy syndrome, chromosomal instability occurs in at least two cell types. In children with the syndrome, this leads to growth deficiency of prenatal onset (in 100% of patients), microcephaly (in 93%), mental retardation (in 72%), anomalies of the central nervous system (in 30%), mild physical dysmorphic features, and cancer.7

Worldwide, 37 cases of the mosaic variegated aneuploidy syndrome have been reported.7-11 Bi-allelic mutations in the spindle-assembly checkpoint gene BUB1B have been found in several cases of the syndrome, some of which involved premature chromatid separation as well.9,10 In cases involving premature chromatid separation, the mosaic variegated aneuploidy syndrome, and BUB1B mutations, cancer is usually diagnosed before 2 years of age. Wilms’ tumor, rhabdomyosarcoma, and leukemia are the only types of cancer that have been described in association with the mosaic variegated aneuploidy syndrome. By contrast, in children with the syndrome who have neither BUB1B mutations nor premature chromatid separation, cancer is rarely, if ever, present.7

CASE REPORT

The proband was healthy until 34 years of age, when an ulcerating, moderately differentiated carcinoma of the ampulla of Vater developed that was metastatic to 1 of 13 regional lymph nodes. He underwent a Whipple procedure and 14 months of chemotherapy (48 cycles of 500 mg of fluorouracil and 10 cycles of 120 mg of lomustine). At 56 years of age, a tubular adenoma of the transverse colon, with focal high-grade dysplasia, was diagnosed. Over the next 5 years, multiple colonic and gastric adenomas developed. At 61 years of age, a Dukes’ stage B invasive adenocarcinoma of the transverse colon developed, which was treated by a partial colectomy. This was followed by two separate invasive gastric adenocarcinomas, one at 64 years and one at 65 years of age, treated by partial gastric resections. More dysplastic lesions developed in the stomach, resulting in a total gastrectomy when the patient was 66 years of age. During that operation, additional gastric and colon adenocarcinomas were identified.

As of June 2010, the patient was alive and cancer-free at 68 years of age. His family history is shown in Figure 1A. He reported that his parents were distantly related, but the degree of relatedness was unknown. Clinical examination revealed age-appropriate nondysmorphic physical features, normal intellect, and no extracolonic signs consistent with a diagnosis of a known inherited gastrointestinal cancer syndrome. In particular, no café au lait spots, axillary freckling, or neurofibromas were noted. The occipito-frontal head circumference was 53.5 cm (21 in.), which is at the 3rd percentile after correction for the proband’s height (157 cm [62 in.]). The pattern of cancers seen in the proband does not completely fit with any known cancer-susceptibility syndrome.

METHODS AND RESULTS

INITIAL INVESTIGATIONS

Analysis of DNA, RNA, or protein (or a combination thereof) was performed in the proband for APC, the mutY orthologue gene MUTYH, and the DNA-mismatch-repair genes MLH1, MSH2, MSH6, and PMS2. No genetic lesions were found (data not shown). Cytogenetic analyses were performed with the use of peripheral-blood lymphocytes and skin fibroblasts. The mitotic index was low, but mosaic aneuploidies, predominantly trisomies, were noted. These involved multiple chromosomes. To a lesser extent, structural chromosomal abnormalities were also present. The resulting cytogenetic picture was similar (but not identical) to the mosaic variegated aneuploidy syndrome (Fig. 1B, and Fig. S1 and Table 1 in the Supplementary Appendix). In addition, premature chromatid separation12 (Fig. 1C) was present in 57% of the proband’s lymphocytes and 84% of the fibroblasts (Fig. 1A). Intermediate levels of premature chromatid separation, but not the mosaic variegated aneuploidy syndrome, were found to be present in all the proband’s siblings and children who were available for testing (Fig. 1A).

MICROARRAY ANALYSES

Using a microarray (the Human610-Quad BeadChip, Illumina) to study the DNA of blood cells obtained from the proband, we detected two regions of extended homozygosity (i.e., homozygosity across >1 Mb), one of which consisted of an uninterrupted stretch of 17.3 Mb on chromosome 15q, including BUB1B, previously found to be mutated in patients with the mosaic variegated aneuploidy syndrome.9-10 The proband’s lym-
Phocytes and fibroblasts showed decreased expression of BUB1B (data not shown but available at the National Council for Biotechnology Information’s Gene Expression Omnibus [www.ncbi.nlm.nih.gov/geo], accession number GSE22206).

**Mutation Identification**

Subsequent deep sequencing of 150 kb surrounding BUB1B, performed by amplifying lymphocyte DNA by means of a long-range polymerase-chain-reaction (PCR) assay and next-generation sequencing technology (Genome Analyzer II, Illumina), revealed a previously unreported homozygous intronic BUB1B mutation, c.2386-11A→G, which creates a de novo splice site that is favored over the authentic site (Fig. 2A). This mutation was present in the heterozygous state in the proband’s mother, two of his sisters (not all were available for testing), and his two children (Fig. 1A) but was absent in more distant relatives (data not shown).
was targeted by means of nonsense-mediated mRNA decay, such that no mutant protein was produced (Fig. S3 in the Supplementary Appendix). Levels of both BUB1B mRNA and derived BUBR1 protein in the proband were found to be slightly lower than those in persons who had BUB1B mutations as well as the mosaic variegated aneuploidy syndrome and were significantly lower than those in heterozygous relatives or in controls (Fig. 2B).

LOCALIZATION AND FUNCTION OF BUBR1
Immunofluorescence studies of fibroblasts obtained from the proband, involving antibodies against BUBR1 and CENP-A (a centromere-specific protein), indicated that the small amount of normal BUBR1 present was correctly localized to the kinetochores (Fig. 2C). Flow-cytometric analysis of the fibroblasts with and without the use of demecolcine, a microtubule-depolarization agent, showed that the small amount of normal BUBR1 present in the proband's fibroblasts was insufficient to maintain the spindle-assembly checkpoint activated at the metaphase–anaphase transition in the presence of demecolcine. The proband's cells completed mitosis without cytokinesis, and thus an excess of nuclei with 8C chromosomal content was seen (Fig. 2D).

DETERMINATION OF CONSTITUTIVE ANEUPLOIDY
The spindle-assembly checkpoint dysfunction is mirrored by the presence of aneuploidy (Fig. 1B, and Fig. S1 and Table 1 in the Supplementary Appendix) and cellular abnormalities (centrosome amplification and extra micronuclei) (Fig. 3A). On chromogenic in situ hybridization involving probes mapping to chromosomes 7, 8, 17, and X and three-dimensional fluorescence in situ hybridization involving a chromosome 8 probe, we did not find any evidence of increased levels of aneuploidy in the normal colon of the proband (data not shown). In addition, neither multiplex ligation-dependent probe amplification nor array comparative genomic hybridization of DNA from the proband's peripheral-blood lymphocytes, performed with the use of a high-resolution platform, revealed consistent copy-number variations or a clonal population of aneuploid cells (Fig. S4 in the Supplementary Appendix).

RNA AND PROTEIN STUDIES OF BUB1B
We confirmed that the mutant messenger RNA (mRNA) generated in persons with the mutation was targeted by means of nonsense-mediated mRNA decay, such that no mutant protein was produced (Fig. S3 in the Supplementary Appendix). Levels of both BUB1B mRNA and derived BUBR1 protein in the proband were found to be slightly lower than those in persons who had BUB1B mutations as well as the mosaic variegated aneuploidy syndrome and were significantly lower than those in heterozygous relatives or in controls (Fig. 2B).

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IMMUNOHISTOCHEMICAL ANALYSIS
Immunohistochemical analysis showed that, as compared with controls, BUBR1 was found at very
A

Genomic DNA

Wild-Type mRNA

Mutant mRNA

Stop at codon 1050

Premature stop at codon 820

Lymphoblastoid Cell Lines

Fibroblasts

Mutant mRNA

Wild-type mRNA

Mutant mRNA (%)

Control

Proband

Heterozygote

B

Relative Expression Normalized to Control Level

Lymphoblastoid Cell Lines

Fibroblasts

C

BUBR1 CENP-A DAPI Merged

Control

Proband

D

No. of Cells

2C 4C

2C 4C

2C 4C

2C 4C

Control

Control, with Demecolcine

Proband

Proband, with Demecolcine
Figure 2 (facing page). Analyses of the c.2386-11A→G Mutation in BUB1B and Its Effects on the Spindle-Assembly Checkpoint.

Sample chromatograms from a control, the proband (a homozygote for the mutation), and a heterozygous relative of the proband confirm the results of deep sequencing (Panel A, top). The location of the c.2386-11A→G mutation is indicated by the vertical arrows. Below the chromatograms, the effects of the mutation at the level of DNA and messenger RNA (mRNA) are shown. The numbered rectangles indicate exons, and the black lines introns. The italicized g indicates the mutation. On the wild-type allele, the introns are spliced following the patterns indicated by pairs of lines (dashed and solid) connecting the exons (top corners of the rectangles). For example, after splicing, exons 19 and 20 are consecutive at the mRNA level. On the mutant allele, the presence of the c.2386-11A→G mutation (in the intron, 11 base pairs upstream of the exon 19 splice site) creates a stronger acceptor splice site than the authentic site (with the bold pair of lines depicting the mutant splicing pattern, and the dotted pair of lines the wild-type splicing pattern), resulting in the production of a greater amount of mutant mRNA than wild-type mRNA from that allele. The resultant mutant mRNA retains the 10 intronic bases upstream of exon 19, causing a frame shift (the extent of which is shown by gray shading) and a premature stop codon in exon 19, making the transcript susceptible to nonsense-mediated mRNA decay (shown in Fig. S2 in the Supplementary Appendix). The results of analysis of BUB1B mRNA in the proband (P), who is homozygous for the mutation, relatives of the proband who are heterozygous carriers (HC), and controls (unrelated noncarriers [NC]) are also shown (Panel A, bottom). The region from exon 18 to exon 20 of the complementary DNA (cDNA), synthesized from RNA, isolated from lymphoblastoid cell lines obtained from each person and fibroblasts obtained from the proband were amplified by means of a polymerase-chain-reaction (PCR) assay (with 30 cycles) and visualized on a 5% agarose gel. The top and bottom bands represent cDNA corresponding to mutant and wild-type mRNA, respectively. The last lane shows results for a sample of water, as a negative control. The amount of BUB1B mutant mRNA present, relative to the total BUB1B mRNA (mutant + wild type), was quantified with the use of Image J software; the resulting mean (±SD) percentages from three independent replicates are shown below the gel. Levels of BUB1B mRNA and its protein product, BUBR1, were measured in lymphoblastoid cell lines (Panel B, top) for the proband, four heterozygotes related to the proband, and four controls and in fibroblasts (Panel B, bottom) for the proband, three patients with the mosaic variegated aneuploidy syndrome (MVA), and six controls. Measurements were obtained in triplicate on the basis of a real-time PCR assay and Western blotting and are reported as means of three replicates. P values were calculated with the use of a t-test, with P<0.001 for all comparisons except for protein levels in lymphoblastoid cell lines between heterozygotes and controls, for which P=0.001, and mRNA and protein levels in fibroblasts between patients with MVA and the proband, for which P=0.08 and P=0.16, respectively. I bars indicate standard deviations. The very low levels of mRNA and protein in the proband as compared with controls, despite equivalent amounts of mutant and wild-type BUB1B mRNA in the proband’s cells (Panel A), indicate that only about half the total BUB1B mRNA measured in the proband is wild type, corresponding to approximately 10 to 15% of the levels in controls. This confirms that the mutation creates a de novo acceptor splice site that plays a much stronger role than the authentic site. Heterozygotes have an intermediate level of BUB1B mRNA, but their BUBR1 level is only slightly lower than the control level, which may explain why the level of premature chromatid separation in their blood lymphocytes is approximately 5%, although it would have been predicted to be much higher, as reported previously. Localization of functional BUBR1 in fibroblasts from a control and the proband was assessed by means of immunofluorescence involving anti-BUBR1 antibodies (green; 612502, BD Biosciences) and an antibody against CENP-A, a centromere-specific protein (red; 2186, Cell Signaling) (Panel C). Also shown are fibroblasts in which DNA is stained with 4’,6-diamidine-2-phenylidole dihydrochloride (DAPI), which forms a fluorescent complex (blue) with double-stranded DNA. The “Merged” image corresponds to the overlay of the previous three images in each row. BUBR1 and CENP-A proteins are perfectly colocalized during metaphase. Flow-cytometric analysis was performed on three independent, short-term cell cultures of fibroblasts from a control and the proband, with and without treatment with demecolcine (1 μg per milliliter), a microtubule-depolarization agent, for 72 hours (Panel D). The chromosomal content during the cell cycle is indicated on the x axis as multiples of the amount of DNA in a haploid genome (represented by the constant C), with 2C indicating a diploid genome (i.e., 46 total chromosomes) and 4C and 8C indicating polyploid genomes. Control fibroblasts treated with demecolcine show an arrest in mitosis (4C), whereas treated fibroblasts from the proband appear to escape the mitotic arrest and commence a new cell cycle without cytokinesis, as indicated by a relative accumulation of 8C cells. The proportion of fibroblasts with 2C detected after demecolcine treatment, measured in an independent experiment, indicates substantially reduced mitotic activity in the proband (0.5%) as compared with the control (20%).

low levels in the normal colon (Fig. 3B) and stomach (Fig. S5 in the Supplementary Appendix) of the proband. In tumor cells from the proband and from controls, BUBR1 was up-regulated, suggesting that the elevated levels of BUBR1 observed is a secondary phenomenon, most likely attributable to the higher fraction of tumor cells that are proliferating, as compared with normal cells.
**APC–BUBR1 INTERACTION STUDIES**

The relation between BUBR1 and APC was studied by immunoprecipitating proteins from the proband's fibroblasts with the use of the anti-APC antibody Ab-5 (Calbiochem), followed by Western blotting with an antibody targeting BUBR1 (612502, BD Biosciences), before and after infecting the fibroblasts with a retrovirus containing wild-type BUB1B complementary DNA (cDNA) to make them stably express this cDNA. Modified fibroblasts and control fibroblasts were found to have similar BUBR1 levels (Fig. 3C). Before infection, the interaction between APC and BUBR1 in the proband's cells was minimal, despite normal levels of APC (data not shown). After infection, the normal interaction was restored.
Figure 3 (facing page). Cellular Abnormalities Associated with the c.2386-11A→G Mutation in BUB1B.

Cellular abnormalities in a fibroblast obtained from the proband (Panel A, top) are shown by means of immunofluorescence involving anti-γ-tubulin antibody (GTU-88, Sigma Aldrich) and antibody against CENP-A, a centromere-specific protein, and 4',6-diamidine-2-phenylindole dihydrochloride (DAPI). Centrosome amplification can be seen (green dots), which is similar to that detected in patients with classic mosaic variegated aneuploidy syndrome (MVA) (not shown). A total of 17.5% of the proband's fibroblasts had more than two centrosomes, as compared with 3% of control fibroblasts (not shown). Micronuclei containing centromeres (red dots) are also visible. This cellular abnormality, a consequence of defects at the spindle-assembly checkpoint, was present in 14% of fibroblasts from the proband but in only a small proportion of fibroblasts from the control and a patient with classic MVA (Panel A, bottom). Micronuclei were increased in patients with classic MVA only after treatment with demecolcine. Immunohistochemical analysis of BUBR1, the protein encoded by BUB1B, was performed in colon-tissue specimens obtained from the proband and an age-matched control who had a diagnosis of colorectal cancer (but not familial adenomatous polyposis or the Lynch syndrome) (Panel B). The sections were stained with the anti-BUBR1 antibody 612502 (BD Biosciences), and the chromogen used was 3,3′-diaminobenzidine. (Additional images of colon and stomach tissues are available in Fig. S5 in the Supplementary Appendix.) The amount of BUBR1 is reduced in normal tissue in the proband as compared with the control; in the control, but not the proband, the amount of protein is substantially increased in the tumor tissue, as compared with the normal tissue. BUBR1 expression was found to be restored in fibroblasts from the proband after they were made to have stable expression of BUB1B complementary DNA (cDNA), by means of infection with retroviral vectors (prepared with the use of the Phoenix and Invitrogen Gateway systems) (Panel C). The BUBR1 levels were similar in the modified fibroblasts from the proband and in the control fibroblasts. Immunoprecipitation with the use of anti-APC antibody (Ab-5, Calbiochem) shows that the interaction between APC and BUBR1 is compromised in the proband's fibroblasts as compared with control fibroblasts, but is restored after retroviral infection (bottom). The experiment was repeated with the use of a different BUBR1 antibody (3F2, Novus Biological), and the results were similar (data not shown). The effect of the restoration of BUBR1 expression on the spindle-assembly checkpoint (by means of infection with the retrovirus) was measured in the proband's fibroblasts (Panel D). Restoration of the expression of BUBR1 with wild-type BUB1B cDNA resulted in a decrease in the percentage of metaphases showing premature chromatid separation (PCS). P values in Panels A and D were calculated with the use of the t-test, with P<0.001 for all comparisons in both panels except for the comparison, in Panel D, between the proband's fibroblasts modified by the wild-type BUB1B cDNA and the control, for which P=0.005. The I bars in Panels A and D indicate standard deviations. Panels C and D depict results from experiments performed on three independent cell cultures.

(Fig. 3C). Fibroblasts with a restored BUBR1 level, as compared with those with unmodified levels, had a significantly reduced level of premature chromatid separation (Fig. 3D).

**DISCUSSION**

The proband was referred to the genetics service because of his history of multiple primary gastrointestinal cancers. Analysis of APC, MLH1, MSH2, MSH6, and PMS2, as well as the genes encoding them, and analysis of MUTYH, failed to show any abnormalities. Karyotypes of both lymphocytes and fibroblasts from the patient revealed a cytogenetic picture closely resembling that seen in cases of the mosaic variegated aneuploidy syndrome caused by biallelic BUB1B mutations. This is surprising, since in many other ways, the findings in the proband differ from those in patients described in previous reports — in particular, by the presence of centrosome abnormalities (Fig. 3A) and structural chromosomal abnormalities seen in nontransformed lymphocytes (Fig. S1 and Table 1 in the Supplementary Appendix). Moreover, the proband's clinical presentation was completely different, with an absence of all features previously reported in association with the mosaic variegated aneuploidy syndrome. In fact, the occurrence in the proband of adult-onset, multisite gastrointestinal neoplasia more closely resembles the pattern of tumors seen in patients with germline APC mutations.

APC and BUBR1 are both implicated in regulation of mitosis. BUB1B is encoded by BUB1B and has a critical role in regulating the spindle-assembly checkpoint, by means of three apparently independent mechanisms: it acts as a diffusible inhibitor, it facilitates catalysis at the kinetochore, and it is a protein required for chromosomal alignment during metaphase. BUBR1 is a component of the mitotic checkpoint complex, which contains the spindle-assembly proteins MAD2L1 and BUB3, as well as CDC20. Other spindle proteins are required for amplification of the signal and control of the rate of formation of the mitotic checkpoint complex. BUB proteins can exist in a complex containing APC, the product of the gene responsible for familial adenomatous polyposis, APC forms a protein partnership with MAPRE1 (previously known as EB 1...
[end-binding protein 1]), and it is thought that the combined proteins attach to the “plus” ends of the microtubules (which extend toward the cell periphery and are preferred for assembly over the “minus” ends, near the centrosome), whereby they interact directly with kinetochore-bound BUBR1.21 In vitro, BUBR1 binds to APC and can directly phosphorylate the protein.16,21

Our results show that the expected interaction between BUBR1 and APC occurred in the proband’s fibroblasts (Fig. 3C). The amount of BUBR1 present, however, may have been insufficient to stabilize the association of the microtubules with the kinetochore.23 Human cells with artificially reduced levels of APC have been reported to have a reduced accumulation of BUBR1 at the kinetochores, an observation that is consistent with our findings.18 This limited interaction between APC and BUBR1 could underlie the observed susceptibility to gastrointestinal neoplasia. Modifying the proband’s fibroblasts to stably express wild-type BUB1B cDNA restored the interaction between APC and BUBR1 and reduced the level of premature chromatid separation (Fig. 3C and 3D). The data from the proband and his relatives are consistent with data from experiments wherein reduction in the levels of full-length BUBR1 protein first resulted in premature chromatid separation and then, when reduced to less than 50% of the normal level, resulted in aneuploidy.13

Somatic mutations have been reported in colorectal cancer in several groups of genes implicated in chromosome segregation in mitosis,23 including the spindle-assembly checkpoint genes BUB1,20 ZW10, and KNTC1.24 Possibly deleterious BUB1B mutations were also previously noted.20 The finding of widespread gastrointestinal neoplasia in a patient with a nonclassic form of the mosaic variegated aneuploidy syndrome is therefore particularly intriguing. When the Min mouse strain, which is heterozygous for a truncating Apc mutation, is crossed with mice that are heterozygous for a Bub1b mutation, the offspring with both mutations have 10 times as many colorectal polyps as do their littermates, and in these double heterozygotes, colonic tumorigenesis appears to be linked to an increased frequency of premature chromatid separation. However, the double heterozygotes have fewer small intestinal polyps,25 illustrating that the effect of BUBR1 deficiency may depend on the physical and genetic context. Experimental models have suggested that low Bub1b levels can be lethal to colon-cancer cells.26

To our knowledge, no cases of the mosaic variegated aneuploidy syndrome have been reported to be caused by a homozygous mutation in BUB1B that results in complete BUBR1 deficiency; our patient had only partial deficiency. Since mice in which Bub1b is knocked out do not survive embryogenesis,27 homozygosity for complete BUB1B deficiency has been postulated to be lethal in humans as well.9 However, we show here that homozygosity for a splice-site mutation permitting some expression of full-length BUBR1 protein is compatible with a normal age span.

In conclusion, at least one BUB1B mutation can result in autosomal recessively inherited susceptibility to gastrointestinal cancer, as do mutations in MUTYH and the mismatch-repair genes. Whether other rare germline BUB1B mutations will be found to predispose persons to the common gastrointestinal cancers of adulthood will be of considerable interest.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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