Homozygous BUB1B Mutation and Susceptibility to Gastrointestinal Neoplasia

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SUMMARY

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.)
itsel it 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-assemble 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).
The allelic status correlates with the percentage of premature chromatid separation, which was higher in heterozygotes than in persons who did not have the mutation and was highest in the homozygote (Fig. 1A). In addition to the proband and family members, we tested DNA samples obtained from 590 unaffected, anonymous persons of the same reported population group as the proband. One of these persons was found to be heterozygous for the mutation. This person had the same haplotype as the proband (Fig. S2 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\textsuperscript{10} 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).

IMMUNOHISTOCHEMICAL ANALYSIS
Immunohistochemical analysis\textsuperscript{14} showed that, as compared with controls, BUBR1 was found at very
Relative Expression Normalized to Control Level

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0

Proband
Heterozygotes
Controls

Lymphoblastoid Cell Lines

Fibroblasts

BUB1 mRNA
BUBR1

HC
HC
P
HC
HC
NC
P
NC

Control
Proband
Patients with MVA
Controls

C

BUBR1
CENP-A
DAPI
Merged

Control
Proband

D

No. of Cells

2C
4C
8C

Control
Control, with Demecolcine
Proband
Proband, with Demecolcine
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.

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.16 Immunohistochemical analysis of BUB1, 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-BUB1 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 BUB1 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. BUB1 expression was found to be restored in fibroblasts from the proband after they were made to have stable expression of BUB1 complementary DNA (cDNA), by means of infection with retroviral vectors (prepared with the use of the Phoenix and Invitrogen Gateway systems) (Panel C). The BUB1 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 BUB1 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 BUB1 antibody (3F2, Novus Biological), and the results were similar (data not shown). The effect of the restoration of BUB1 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 BUB1 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 BUB1 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.9 This is surprising, since in many other ways, the findings in the proband differ from those in patients described in previous reports7-11 — 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 germ-line APC mutations.

APC and BUB1 are both implicated in regulation of mitosis.16-19 BUB1 is encoded by BUB1B20 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.21 BUB1 is a component of the mitotic checkpoint complex, which contains the spindle-assembly proteins MAD2L1 and BUB3, as well as CDC20.22 Other spindle proteins are required for amplification of the signal and control of the rate of formation of the mitotic checkpoint complex.19 BUB proteins can exist in a complex containing APC, the product of the gene responsible for familial adenomatous polyposis.16 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 BUB1 deficiency may depend on the physical and genetic context. Experimental models have suggested that low Bub1 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 BUB1 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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