Abstract

Some colorectal tumors with wild-type adenomatous polyposis coli gene have activating mutations in β-catenin (encoded by CTNNB1) that result in decreased phosphorylation by GSK-3β and increased signaling through the Tcf/Lef transcription factors. To investigate the relationship between CTNNB1 mutations and underlying pathways of genomic instability, we examined 80 colorectal cancers stratified by the presence or absence of microsatellite instability (MSI). CTNNB1 mutations were identified in 13 (25%) of 53 cancers with high frequency MSI (MSI-H), including 12 point mutations at exon 3 phosphorylation sites (codons 41 and 45) and one deletion of the entire exon 3 degradation box. No CTNNB1 mutations were identified in 27 microsatellite stable or low frequency MSI (MSI-L) colorectal cancers (P ≤ 0.01). In contrast, CTNNB1 mutations were identified in 3 of 9 (33%) MSI-H and 10 of 20 (50%) MSS/MSI-L endometrial carcinomas, suggesting a more generalized involvement in these tumors. Only six (46%) of the endometrial carcinoma CTNNB1 mutations occurred at residues directly phosphorylated by GSK-3β, and only one of these was at either codon 41 or 45. All point mutations in MSI-H cancers were transitions, whereas 64% of those in MSS/MSI-L cancers were transversions (P < 0.01). The differences in the mutation profiles suggest that there may be molecular fingerprints of CTNNB1 mutations, determined by biological factors related to both tumor type and underlying pathways of genomic instability.

Introduction

The Wnt signal transduction pathway was first implicated in mammalian tumorigenesis when the Drosophila gene, wingless, was shown to be the homologue of the mouse mammary oncogene, int-1 (later renamed Wnt-1; Refs. 1 and 2). The generalized importance of this pathway was realized when APC3 was found to regulate Wnt signaling by acting in a complex with GSK-3β to control phosphorylation and degradation of β-catenin (3–5). Inactivating APC mutations occur in about 85% of colorectal carcinomas, resulting in β-catenin stabilization and increased signaling through the Tcf/Lef transcription factors (6–8). Recently, up to 50% of colorectal carcinomas without APC mutations were found to contain CTNNB1 mutations (9–12). These mutations alter APC-dependent exon 3 serine/threonine phosphorylation residues, leading to β-catenin stabilization and activation of signaling (10, 13). Interestingly, about 50% of primary colorectal carcinomas with CTNNB1 mutations are MSI-H, suggesting that CTNNB1 mutations may be more common in the DNA MMR-deficient pathway of tumorigenesis (10, 12, 14, 15). CTNNB1 mutations are found in a number of other cancers, including about 13% of endometrial carcinomas; however, there has been no investigation of associations with underlying pathways of genomic instability (16–19). In the present study, we examined the relationship between CTNNB1 mutations and DNA MMR deficiency and found that CTNNB1 mutations were significantly more common in MSI-H colorectal carcinoma as compared to MSS/MSI-L colorectal carcinoma. Surprisingly, we found a high frequency of CTNNB1 mutations in endometrial carcinoma, regardless of the presence or absence of microsatellite instability. Comparison of these cancers suggests that CTNNB1 mutation profiles may reflect different underlying carcinogenic pathways.

Materials and Methods

Tissue Samples. Tumor samples were obtained from an ongoing, unlinked, population-based study of the frequency of MSI in human cancer. Ethics approval was obtained from The University of Toronto Human Ethics Committee prior to commencement of the study. Resected colorectal and endometrial carcinomas in patients <50 years of age were identified by The Ontario Cancer Registry, and paraffin blocks were retrieved from the treating hospitals. A blinded histopathological review was performed on all cases, and the results were entered into a coded database. For each case, normal and invasive carcinoma samples of at least 50% neoplastic cellularity were microdissected from two to three 10-μm unstained slides. Adjacent adenomas (colorectal carcinoma cases) or hyperplasias (endometrial carcinoma cases) were dissected separately when available. If cancers had more than one discrete morphological pattern, multiple tissue samples were also dissected separately. Genomic DNA was extracted as follows. Samples were incubated in 50–100 μl of lysis buffer [10 mM Tris-Cl (pH 8), 100 mM KCl, 2.5 mM MgCl2, and 0.45% Tween 20] for 10 min at 95°C. Proteinase K (15–35 μl, 20 mg/ml) was added, and samples were incubated overnight at 65°C.

MSI Testing. For the colorectal carcinomas, MSI was tested using the Bethesda Consensus Conference reference panel of 5 markers (BAT-25, BAT-26, D2S123, D5S346, and D17S250; Ref. 20), with conditions as described previously (21). Colorectal carcinomas were classified as MSI-H if two or more loci displayed MSI, or as MSS, if no loci displayed MSI. If one locus had MSI, up to five additional loci were tested (BAT-40, BAT-RH, D18S58, D18S59, and D17S787); these reevaluated tumors were then classified as MSI-H if ≥40% of loci displayed MSI, or as MSI-L if ≥30% of loci displayed MSI (20). For the endometrial carcinomas, MSI was tested using at least five markers from the Bethesda reference panel and the Bethesda alternative marker list (BAT-26, BAT-40, BAT-RH, D2S123, D5S1611, D5S346, D17S787, D18S59, and D18S69). Definitions for MSS, MSI-L, and MSI-H were the same as for the colorectal carcinomas.
**CTNNB1** Mutation Detection. For the **CTNNB1** mutation analysis, we selected cases from the ongoing population-based study, including all available MSI-H cancers, all MSI-L cancers, and a sampling of MSS cancers. In total, we studied 80 colorectal carcinomas, including 53 MSI-H cancers, 19 MSI-L cancers, and 8 MSS cancers, and 29 endometrial carcinomas, including 9 MSI-H cancers, 2 MSI-L cancers, and 18 MSS cancers. Exon 3 of **CTNNB1** was amplified by PCR in five separate reactions using six primers as shown in Fig. 1A. The primer sequences were as follows: P1, 5'-AGTCACTGGCACTTGGGAC-3'; P2, 5'-TCTTCTCTAGGAGCCTT-3'; P3, 5'-GATTTCGATGGAGTTGGACATGG-3'; P4, 5'-TGGTCTTGAGTGAAGGACT-3'; P5, 5'-TACAACTGTTTTGAATGCGCGTGGAC-3'; and P6, 5'-TCGAGTATTGCATACTGTCC-3'. Using this approach, a small product including codons 30–48 (product P1-2) was amplified from all tumors for direct sequence analysis. Remaining PCR products were designed to screen for intragenic deletions beginning outside of the P1-2 primer, and including the β-catenin regulatory region of exon 3 (codons 32–45). Fragments >250 bp are not reliably amplified from paraffin tissue; therefore, overlapping fragments were chosen to increase the likelihood of detecting deletions. Because the P5-4 and P5-6 PCR products are too large for successful amplification in the absence of an intragenic deletion, DNA extracted from peripheral blood lymphocytes was included as a separate control for PCR set-up. Although this approach would be expected to identify most of the deletions reported previously in **CTNNB1** (11, 12, 19), it is not possible to rule out deletions in the absence of a full-length PCR product spanning P5-6. Therefore, the results may be an underestimate of the actual **CTNNB1** intragenic deletion frequency. For each PCR reaction, 2 μl of DNA was combined with 1 unit of Perkin-Elmer AmpliTaq DNA polymerase in a 15-μl PCR mixture, with PCR buffer, 1.5 mM MgCl₂, 0.13 mM deoxynucleotide triphosphates, and 0.4 μM of each primer. Samples were heat denatured at 94°C for 2 min, followed by 35 PCR cycles as follows: 94°C for 15 s, annealing temperature for 15 s, and 72°C for 20 s (in a DNA Engine, model PTC-200; MJ Research, Watertown, MA). The annealing temperatures were as follows: P1-2, 52°C; P3-4, 62°C; P5-4, 52°C; P3-6, 56°C; and P5-6, 46°C. To screen for intragenic deletions, PCR products (P3-4, P3-6, and P5-6) were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining. To detect nucleotide sequence alterations, the 98-bp P1-2 PCR product was electrophoresed on a 2% agarose gel, visualized by ethidium bromide staining, and excised from the gel, and DNA was purified using QIAquick Gel extraction kit (Qiagen, Mississauga, Ontario, Canada) as per the manufacturer’s instructions. Purified PCR products were sequenced using ThermoSequenase Radiolabeled Terminator Cycle Sequencing kit (Amersham, Cleveland, OH) as per the manufacturer’s instructions. Reactions were run on a 6% sequencing gel, dried onto filter paper, and exposed to Kodak Biomax film.

**Statistical Methods.** Mutation frequencies, mutation profiles, and pathological parameters were compared by χ² or Fisher exact test.

**Results**

Mutations in the **CTNNB1** gene were identified in 13 (25%; 95% CI, 14–39%) of the MSI-H colorectal carcinomas and none (95% CI, 0–10%) of the MSS/MSI-L colorectal carcinomas (**P** 0.01; Figs. 1B and 2; Table 1). All point mutations were amino acid substitutions at

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Fig. 1. Identification of **CTNNB1** mutations. A, PCR strategy for **CTNNB1** mutation detection. Primer pairs used to generate each of the five PCR products are as indicated. B, representative sequencing gel revealing point mutations in **CTNNB1** exon 3. Colorectal carcinoma sample 54 (Lane 5) has an A to G substitution (S45P), and colorectal carcinoma sample 56 (Lane 6) has a G to A substitution (S45F). Sequencing reactions were run using the reverse primer P2. C, representative 2% agarose gel revealing intragenic deletion of **CTNNB1** in PCR fragment P5-6. Carcinoma sample 128 (Lane 4, Del) has a 653-bp deletion. The deletion is not present in the adjacent adenoma sample (Lane 3). The full-length normal P5-6 PCR product is not successfully amplified from either of the paraffin DNA samples (Lanes 3 and 4). Lanes 1 and 6, 123-bp ladder; Lane 2, negative control; Lane 5 (Norm), full-length P5-6 PCR fragment from DNA extracted from peripheral blood lymphocytes.
the threonine and serine residues at codons 41 and 45 (Fig. 2). A single 653-bp intragenic deletion was identified (Fig. 1C) that joined nucleotide 27 of intron 2 to nucleotide 5 of intron 3, resulting in complete deletion of exon 3. **CTNNB1** mutations predicting amino acid substitutions were identified in 3 (33%; 95% CI, 8–53%) of the MSI-H endometrial carcinomas and 10 (50%; 95% CI, 27–73%) of the MSS/MSI-L endometrial carcinomas (Fig. 2; Table 1). Only six of the endometrial carcinoma amino acid substitutions involved the threonine and serine residues at codons 33, 37, and 41 (Table 1). The remaining seven substitutions were at codons 32 and 34, flanking the serine at codon 33 (Table 1). No intragenic deletions were identified in the endometrial carcinomas. Silent nucleotide substitutions were also identified in two endometrial carcinomas (one MSI-H and one MSS; Table 1). These changes were not present in the normal tissues from these cases or in a separately dissected neoplastic sample, indicating that they were focal somatic alterations. All 16 of the nucleotide substitutions present in MSI-H cancers were transitions, whereas only 4 (36%) of the substitutions in MSS/MSI-L cancers were transitions (P < 0.01).

Adjacent adenoma samples were available from five MSI-H colorectal carcinomas with **CTNNB1** mutations, and three of these contained the identical mutation as was present in the invasive component (Table 1). A second distinct histopathological subtype of invasive carcinoma was available in three MSI-H colorectal carcinomas with **CTNNB1** mutations, and all three contained the same mutation in both components. Four endometrial carcinomas with **CTNNB1** mutations predicting amino acid substitutions had an adjacent complex hyperplasia sample available, and two of these had the same mutation as was present in the invasive component. Both cases with mutation-positive complex hyperplasia were MSS, whereas the two cases with mutation-negative complex hyperplasia were MSI-H. Adjacent simple hyperplasia was also present in one of the cases with mutation-positive complex hyperplasia; however, no mutation was identified in this case.

Comparison of pathological features (Table 2) revealed that MSI-H colorectal carcinomas with **CTNNB1** mutations were more likely to be T4 tumors, right-sided, and of unusual histological subtypes. None of these trends were statistically significant. Irrespective of **CTNNB1** mutation status, MSI-H colorectal carcinomas were more likely to be associated with right-sided location, marked tumor-infiltrating lymphocytes, marked Crohn’s-like lymphoid reactions, and nonmetastatic disease (Table 2). Although mutations had no apparent associations with pathological features in the endometrial carcinomas, the number of cases was too small for a comprehensive analysis (data not shown).
The CTNNB1 mutation frequency in MSI-H colorectal cancers (25%; 95% CI, 14–39%) compared with MSS/MSI-L colorectal cancers (0%; 95% CI, 0–10%) suggests that CTNNB1 mutations are relatively specific to the MMR-deficient genomic instability pathway. Although this difference is quite striking, it is perhaps not entirely unexpected. Activating β-catenin mutations are present in up to 50% of colorectal carcinomas with wild-type APC (9, 11, 12), and several studies have suggested that MSI-H colorectal cancers are less likely to have APC mutations (6, 22, 23). Furthermore, the frequency identified in our study is similar to previous observations of CTNNB1 mutations in selected MSI-H colorectal carcinoma cell lines (12). This contrasts the findings in primary melanomas, where CTNNB1 mutation frequencies are much lower than predicted from mutation screens of melanoma cell lines and suggests that this latter discrepancy could reflect differences in underlying genomic instability pathways (13, 24). The existence of MSI-L colorectal carcinomas as a distinct pathogenetic entity is controversial (20), and the absence of activating β-catenin mutations suggests that the pathogenesis of this group of tumors is quite unlike MSI-H cancers.

Endometrial carcinoma is the second most common cancer in individuals with germ-line MMR gene mutations, suggesting that the molecular pathogenesis has similarities with colorectal carcinoma. In endometrial cancers, however, we found that CTNNB1 mutations were not specific to the MMR-deficient pathway. Furthermore, the high mutation frequency raises the possibility that activation of Wnt signaling may be universally important in a subset of these neoplasms. Although previous studies have found a low frequency of loss of heterozygosity at 5q21 in endometrial carcinomas, we are not aware of any rigorous searches to identify APC mutations (25, 26).

CTNNB1 mutations were reported previously in only 13% of endometrial carcinomas (16). Most of our cancers were well to moderately differentiated endometrioid carcinomas, a subtype that had a higher CTNNB1 mutation frequency (18%) and a higher frequency of β-catenin stabilization (46%) in the previous study (16). Most CTNNB1 mutations reported in ovarian carcinomas are also found in endometroid carcinomas, supporting a specific morphogenetic association (17). Our cases were also selected for early age of onset, which could be associated with differences in tumor genetic profiles. Finally, the mutation frequencies could reflect differences in carcinogenic influences between Japan and North America. For instance, intragenic deletions of CTNNB1 were the predominant mutations reported in one series of Japanese colorectal cancers (11).

The difference in the spectrum of missense mutations between colorectal and endometrial carcinomas was unexpected. All of the colorectal carcinoma amino acid substitutions were at known phosphorylation sites (codons 41 and 45), and three of the four mutations have been reported previously (10, 12, 13). Although the final substitution, T41I, has not been described in colorectal carcinoma, it was present in one endometrial cancer (16). Most CTNNB1 mutations in colorectal and endometrial carcinomas was unexpected. All of the colorectal carcinoma amino acid substitutions were at known phosphorylation sites (codons 41 and 45), and three of the four mutations have been reported previously (10, 12, 13). Although the final substitution, T41I, has not been described in colorectal carcinoma, it was present in one endometrial cancer (16). Most CTNNB1 mutations in colorectal carcinomas, however, we found that CTNNB1 mutations reported in ovarian carcinomas are also found in endometrial carcinomas, supporting a specific morphogenetic association (17). Our cases were also selected for early age of onset, which could be associated with differences in tumor genetic profiles. Finally, the mutation frequencies could reflect differences in carcinogenic influences between Japan and North America. For instance, intragenic deletions of CTNNB1 were the predominant mutations reported in one series of Japanese colorectal cancers (11).

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these sites are observed frequently in human and carcinogen-induced rat tumors (16–19, 28, 29) and may affect phosphorylation by altering recognition sequences or tertiary protein structure. Furthermore, these two residues are important for β-catenin ubiquitination and proteasome–dependent degradation (30).

It is possible that the DNA sequence surrounding codons 41 to 45 of CTNNB1 is specifically hypermutable in the setting of MMR deficiency. The predominance of transition mutations supports this possibility and parallels the mutation profile of some MMR-deficient cell lines (31). Although codon 45 lies within a homopolymer tract, which is known to be hypermutable (32), the specific sequences that predispose to transitions in the setting of MMR are not known (33). The presence of CTNNB1 mutations at codons 41 and 45 in small MSS colorectal adenomas (34) and the differences in the mutation profile of MSI-H endometrial cancers, however, suggest that sequence susceptibility is not the only factor. The presence of two silent CTNNB1 exon 3 alterations in the endometrial carcinomas also raises the possibility of a tissue-specific hypermutability or carcinogenic influence. An alternative explanation for the MSI pathway specificity of CTNNB1 mutations in colorectal cancer is that APC mutations and CTNNB1 mutations may not be biologically equivalent in tumorigenesis. Both genes may have functions outside of Wnt signaling that are important for clonal selection, and differences in the genetic targets of neoplastic progression between MSI-H and MSS colorectal cancers and between MSI-H colorectal and endometrial cancers are well established (6, 23, 35, 36). The recent finding that CTNNB1 mutations are frequent in small MSS adenomas suggests these neoplasms are less likely to progress and supports tumorigenic differences compared with APC inactivation (34).

Adjacent adenomas contained the same CTNNB1 mutation as the invasive carcinomas in three of five cases. Although this is generally consistent with the hypothesized role of the APC pathway as a neoplasia gatekeeper (6), at least two of the CTNNB1 mutations occurred during neoplastic progression from adenoma to carcinoma. In the endometrial carcinomas, we also found that two of the adjacent complex hyperplasias did not contain CTNNB1 mutations, suggesting that these alterations are not necessarily gatekeeper-type events. When multiple paired tumor samples were examined, however, the uniform presence of CTNNB1 mutations in both colorectal and endometrial cancers suggests that all have occurred during the early stages of malignant transformation. This contrasts findings in prostate cancer, where focal mutations suggest a late event during the advanced progression of subclones (18).

If activating β-catenin mutations are associated with specific biological attributes not present in tumors with APC inactivation, differences in the pathological features of these tumors might be expected. Although there was a tendency for MSI-H colorectal carcinomas with CTNNB1 mutations to be right-sided, higher stage, and of unusual histological subtypes, the number of cancers analyzed does not allow for definitive conclusions to be made. In comparison, many of the features known to be associated with MSI-H colorectal carcinomas (37) were uniformly present in tumors with and without CTNNB1 mutations. Although no associations between CTNNB1 mutations and pathological features of endometrial carcinomas were apparent, our series was very homogeneous in terms of the spectrum of grade, stage, and histological subtypes.

In summary, our findings confirm that CTNNB1 mutations are particularly common in MSI-H colorectal carcinomas. These mutations consist almost entirely of transitions at codons 41 and 45, revealing a relatively specific molecular fingerprint compared with CTNNB1 mutation profiles in other cancers. We also found that CTNNB1 mutations are very common in endometrial carcinomas; however, there is no association with the presence or absence of underlying microsatellite instability. Additional studies will be required to determine whether functional differences between β-catenin activation and APC inactivation may explain some of these findings.

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References


β-Catenin Mutations Are Specific for Colorectal Carcinomas with Microsatellite Instability but Occur in Endometrial Carcinomas Irrespective of Mutator Pathway

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