Microsatellite Instability and Mutations of the Transforming Growth Factor β Type II Receptor Gene in Colorectal Cancer

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Abstract

The TGFβ type II receptor (RII) was found to be mutated within a polyadenylate tract in 100 of 111 (90%) colorectal cancers with microsatellite instability. Other polyadenylate tracts of similar length were mutated in these samples but not as frequently as RII. In most cases, the polyadenylate tract mutations affected both alleles of RII, and in four tumors heterozygous for the polyadenylate mutations, there had additional mutations that were expected to inactivate the other RII allele. These genetic data support the idea that RII behaves like a tumor suppressor during CR cancer development and is a critical target of inactivation in mismatch repair-deficient tumors.

Introduction

Of the 160,000 new CR3 cancers diagnosed this year, approximately 21,000 will have widespread mutations in microsatellite sequences characteristic of the RER phenotype (1–4). An inherited form of CR cancer, HNPPCC, accounts for approximately one-fourth of these 21,000 cases, and is due principally to germline mutations of the MMR genes, hMSH2 and hMLH1. Other polyadenylate tract mutations affected both alleles of RII, and in four tumors heterozygous for the sporadic CR cancers exhibiting the RER phenotype (9).

We have recently shown that RII was mutated in 8 of 11 RER tumors (10). TGF-β inhibits the growth of colon epithelial cells and is the primary ligand for RII (11). The TGF-β-RII complex interacts with the TGF-β type I receptor, which in turn activates parallel pathways that inhibit cellular proliferation (12). Genetic analysis of these pathways has shown that both type I and type II receptors are absolutely required for growth inhibition (13).

Materials and Methods

Primary Tumors. Serial 6-μm paraffin sections of HNPCC tumors were microscopically dissected to separate neoplastic from nonneoplastic elements. Whenever possible, normal and tumor components were prepared from the same paraffin block (14). DNA was prepared from cryostat sections of other CR tumors as described (15). In these cases, DNA was also obtained from separate pieces of uninvolved colonic mucosa. Of the primary tumors in this study, DNA was prepared from paraffin sections in 69 cases and from frozen sections in 15 cases.

Cell Lines and Xenografts. CR cancer cell lines (n = 94) were obtained from ATCC or established as described (16). Xenografts (n = 55) were generated from primary CR cancers and either blood or colonic mucosa samples were used to purify corresponding normal DNA (10). RER status was assessed as described previously using the microsatellite markers BAT-26, BAT-40, D2S123, and D18S58 (17). In some cell lines, RER status could not be assessed directly because of the absence of corresponding normal DNA, and the presence of MMR gene mutations or microsatellite instability was used as a surrogate to indicate RER status.

PCR and Sequencing Analysis. Marker loci were PCR amplified for 30 cycles using one 32P-labeled primer and one unlabeled primer. Reactions were resolved on 2% agarose gels and exposed to film. The primers that were used for the amplification of BAT-RII were five 3′-CTT TAT TCT GGA AGA TGC CTC-3′ and 5′-GAA GAA AGT CTC ACC AGG C-3′. BAT-13 and BAT-26 were identified serendipitously while sequencing introns 1 and 5 of hMSH2, respectively. The primers for BAT-13 were 5′-ATG AGC AGC ATG AAC AGA TCA AAA GAT GTA ACA TTC-3′; for BAT-26, 5′-TGA CTA GCT TTG ACT TCA GAC-3′-3′ and 5′-ACC CAT GCA TTA TTA TTA ACC C-3′. BAT-10A, BAT-10B, BAT-25, and BAT-40 tracts were derived from introns of the dystrophin gene, APP gene, c-kit oncogene, and 3β-hydroxysteroid dehydrogenase gene, respectively, and were identified by searching the Genbank human data base with polyadenylate tracts. The primers for BAT-10A were 5′-GAA TAA TAT TAC AGT AAG CAC AAA GAC GTG TTC-3′ and 5′-GAA GAA AGT CTC ACC AGG C-3′; for BAT-10B, 5′-ATG TCG TAT TAT GAC CAT CAC C-3′ and 5′-GAA TAA TAT TAC AGT AAG CAC AAA GAC GTG TTC-3′; for BAT-25, 5′-TGG CCT CCA AGA ATG TAA GT-3′ and 5′-TCG CTT GCA TTA TTA GTA TGG TTC-3′; and for BAT-40, 5′-ACC CTG CCT TTG TTC CT-3′ and 5′-GTA GAG CAA GAC CAC GTT C-3′.

The search for 10-bp pA tracts in the human genome led to the identification of six cDNA clones (RMSA-1, human glucose transporter 2, interleukin 5, human mitochondrial transcription factor 1, DNA polymerase accessory protein A1, and ISL-1). The search also identified the cDNA for RII. To screen for mutational status, the entire coding region was sequenced on the ABI prism 373 automated DNA sequencer.

Results and Discussion

RII has a 10-bp pA tract at codons 125–128 of its 565-codon open reading frame. Of the 8 CR cancers with RII mutations demonstrated previously, 7 were caused by small insertions or deletions within this pA tract (BAT-RII). To examine the generality of these results, we designed a PCR-based assay for analyzing BAT-RII at the genomic level. A 73-bp genomic DNA fragment encompassing BAT-RII was amplified from 84 primary tumors known to exhibit the RER phenotype (Fig. 1A). Of these, 78 were from HNPCC patients and 6 were sporadic tumors. Seventy-five of these tumors (89%) had at least one altered BAT-RII allele (Fig. 1B). All of the alterations were due to 1–2-bp insertions or deletions that would result in a frameshift in the encoded protein. These results are in contrast to the results found in non-RER tumors, in which 0 of 40 were mutated.

Inactivation of a single RII allele by a BAT-RII frameshift mutation would not be predicted to abrogate RII function in the presence of an unaltered, second RII allele. It was, therefore, of interest to determine how often both RII alleles were mutated in RER tumors. In most of the
primary tumors described above, the band representing the wild-type allele was significantly decreased in intensity compared to the mutant BAT-RII alleles (Fig. 1B). This suggested that both alleles were mutant, and that residual wild-type signal arose from contaminating nonneoplastic cells within the tumor specimens. However, the presence of nonneoplastic cells made it difficult to unambiguously interpret these data. To answer the question more definitively, we analyzed 27 RER colorectal cancer cell lines, passaged in vitro or as xenografts in nude mice, for mutations in BAT-RII sequences. At least 1 BAT-RII alteration was found in 25 of these samples. In 19 of the 25, both alleles were altered, whereas only 1 allele was altered in 6. As in the primary tumors, 1–2-bp insertions or deletions causing frame shifts were always observed. The combined frequency of BAT-RII mutations in the primary and cultured CR cancer was 90% (100 of 111). In contrast, BAT-RII was not altered in any of 122 samples from non-RER CR cancer lines (75 cell lines and 47 xenografts).

Alterations of numerous pA sequences, distributed widely and randomly throughout the genome, was the characteristic first used to define tumors with microsatellite instability (1, 18). In the context of such widespread changes, it is difficult to ascribe significance to alterations of any single pA tract, such as those in BAT-RII. To further address this issue, we studied several pA tracts in the tumors described above. There was a gradual decrease in the fraction of tumors exhibiting pA tract length alterations as the length of the tract decreased from 40 to 13 bp (Fig. 2). This is consistent with other studies showing that the probability of maintaining a microsatellite in MMR-deficient cells is inversely proportional to the length of the microsatellite (4). To determine whether smaller pA tracts, like those in RI!, were often mutated in RER cancers, we searched Genbank (Version R86.0; December, 1994) for 10-bp pA tracts within genes. No 10-bp pA tracts were identified within coding exons of genomic DNA entries, but six were identified within coding regions of cDNA entries (See “Materials and Methods”). Of these, four were examined, using multiple primer combinations by PCR, and each failed to yield the expected amplification product. Presumably, this was because introns were present within or closely adjacent to the pA tracts. This suggested that long pA tracts are rare in coding sequences. A search for 10-bp pA tracts within noncoding DNA revealed numerous tracts in intronic and intergenic genomic DNA, as well as in untranslated portions of cDNAs. PCR-based amplification was then performed on two randomly chosen intronic 10-bp pA tracts (BAT-10A and BAT-10B) using genomic DNA as template (see “Materials and Methods”). BAT-10A and BAT-10B were altered less frequently (70 and 41%, respectively) than were longer pA tracts. Moreover, BAT-10A and BAT-10B were altered less frequently than BAT-RII in the same tumors (P < 0.03 for each by χ²). Additionally, we found that pA tracts rarely became smaller than 10 nucleotides in RER tumor cells.
even with continued passage in vitro for several years, suggesting a minimum threshold for instability.4

The fact that the BAT-R1I pA tract was altered at elevated frequency, compared to BAT-10A and BAT-10B, supported the idea that mutations in BAT-R1I provide a selective growth advantage rather than simply reflecting nonspecific microsatellite instability. To independently confirm that BAT-R1I mutations are selected for, we searched for R1I mutations outside of the BAT-R1I tract in RER tumors having one wild-type and one mutant BAT-R1I allele. As noted above, six of the RER cell lines exhibited this heterozygosity, of which we were able to obtain cDNA from four (VACO481, DLD1, KM12, and RKO). The complete sequence of the R1I-coding region was determined in each line from RT-PCR products (Fig. 3). The VACO481 line had a 2-bp insertion at codon 533, as reported previously, resulting in a frameshift in the highly conserved serine/threonine protein kinase catalytic domain XI (10, 19). In DLD1, a T to C transition at codon 452 resulted in a substitution of proline for leucine. In KM12, a T to C transition at codon 454 also resulted in a substitution of proline for leucine. Both of these mutations fell within the highly conserved protein kinase domain IX of R1I (19). Crystallography of serine/threonine kinases has suggested that this domain is organized as an α-helix, and proline substitutions would be expected to strongly disrupt this structural feature (20–22). As controls, the sequence of domain IX of R1I was determined in three RER lines with no wild-type BAT-R1I sequences and in three non-RER lines; no mutations were identified in any of these samples.

In summary, the BAT-R1I pA tract is mutated in the great majority of RER CR cancers, whether from HNPPC or sporadic cases. Although all pA tracts are unstable in these tumors, the BAT-R1I tract is more unstable when compared to pA tracts of identical size in the same tumors. Moreover, both alleles of R1I are mutated in most RER cases, either through alterations of both BAT-R1I alleles or by mutation of BAT-R1I in conjunction with a separate mutation elsewhere in the gene. These data, in combination with studies of endometrial cancer provided in the accompanying paper (23), lend strong support to the argument that R1I mutations are critical for RER-associated CR tumorigenesis. Like RER tumors, many non-RER CR cancers are resistant to the growth-inhibitory effects of TGF-β (24). Because such tumors do not have BAT-R1I

4 R. Parsons et al., unpublished results.

References


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