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Advances in Brief

Microsatellite Instability and Mutations of the Transforming Growth Factor β Type II Receptor Gene in Colorectal Cancer[1]

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Abstract

The TGFβ type II receptor (*RII*) was found to be mutated within a polyadenine tract in 100 of 111 (90%) colorectal cancers with microsatellite instability. Other polyadenine tracts of similar length were mutated in these samples but not as frequently as *RII*. In most cases, the polyadenine tract mutations affected both alleles of *RII*, and in four tumors heterozygous for the polyadenine mutations, three 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 CR[3] 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, HNPCC, accounts for approximately one-fourth of these 21,000 cases, and is due principally to germline mutations of the MMR genes, *hMSH2* and *hMLH1*, *hPMS1*, or *hPMS2*(5–8). Somatic mutations of MMR genes also account for a significant fraction of 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 ^{32}P-labeled primer and one unlabeled primer. Reactions were resolved on urea-formamide polyacrylamide gels and exposed to film. The primers that were used for the amplification of BAT-RII were 5′-CTT TAT TCT GGA AGA TGC TGC-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′-ATC AGC AGC ATG AAG CCA G-3′ and 5′-TCT TTT ACA AAA GAT TCA AAA TTC-3′; for BAT-26, 5′-TGA CTA CTT TTG ACT TCA GCC-3′ and 5′-AAC CAT TCA ACA TTT 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 3-β-hydroxysteroid dehydrogenase gene, respectively, and were identified by searching the Genbank human data base with polyadenine tracts. The primers for BAT-10A were 5′-GA TAA TAT AGC ATT ATA ACA CTG-3′ and 5′-GAA CAC AAA GGA AGT GTC TG-3′; for BAT-10B, 5′-ATG TCG TAT TAT GAC CAT CAC-3′ and 5′-GGT CAA GGC TAC AGT AAG C-3′; for BAT 25, 5′-TCG CCT CCA AGA ATG TAA GT-3′ and 5′-TCT GCA TTT TAA CTA TGG CTC-3′; and for BAT-40, 5′-ACA ACC CTG CTT TTG TTC CT-3′ and 5′-GTA GAG CAA GAC CAC CTT G-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 mutation within RII, the entire coding region was amplified using RT-PCR in two overlapping segments. Products were directly sequenced with ^{32}P-labeled primers using SequiTherm polymerase. The primers used for amplification and sequencing of RII are available from the authors upon request.

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. 1*A*). 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. 1*B*). 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

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[3] The abbreviations used are: CR, colorectal; HNPCC, hereditary nonpolyposis colorectal cancer; RER, replication error; MMR, mismatch repair; TGF-β, transforming growth factor β; RII, TGFβ type II receptor; pA, polyadenine; RT-PCR, reverse transcription-PCR.

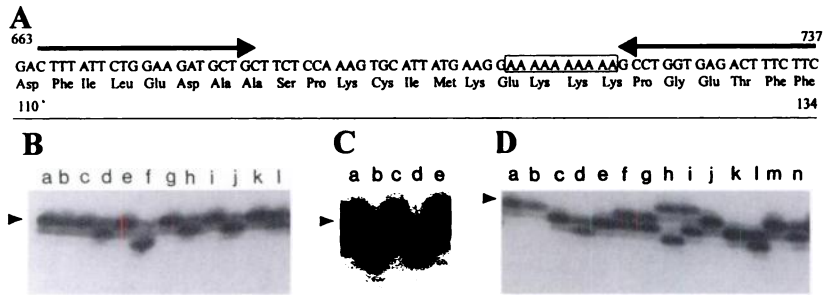

Fig. 1. pA tract of RII. *A*, sequence of RII cDNA encompassing BAT-RII and including amino acids 110–134. *Arrows*, primers used for genomic amplification. The stretch of 10 A residues is boxed. *B*, analysis of primary tumors. Genomic DNA from sporadic primary RER CR cancers and paired normal colon samples. *Lanes a, c, e, g, i,* and *k*, 73-bp PCR products from normal DNA resolved on a sequencing gel. *Lanes b, d, f, h, j,* and *l*, products from tumor samples. Deletions of pA tracts are present in all tumor samples except that in *Lane b*. *C*, analysis of xenografts. Genomic amplification of 5 xenografted CRCs. *Lanes a, c,* and *e*, amplified products of three non-RER xenografts; *Lanes b* and *d*, products for two RER samples. Deletions can be seen in *Lanes b* and *d*. *D*, analysis of cell lines. *Lanes a* and *b*, products from normal DNA controls. The products of RER cell lines are shown in *Lanes c* (SW48), *d* (LoVo), *e* (LS-180), *f* (DLD-1), *g* (HCT15), *h* (VACO5), *i* (VACO6), *k* (HCT116), *l* (C), and *n* (KM12). Products of non-RER cell lines are shown in *Lanes j* (VACO8) and *m* (VACO410). Deletions can be seen in cell lines except *Lanes a, b, j,* and *m*. *Arrowheads*, position of the wild-type 73-bp PCR product.

primary tumors described above, the band representing the wild-type allele was significantly decreased in intensity compared to the mutant BAT-RII alleles (Fig. 1*B*). 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 frameshifts 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 RII, 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 χ^2). Additionally, we found that pA tracts rarely became smaller than 10 nucleotides in RER tumor cells,

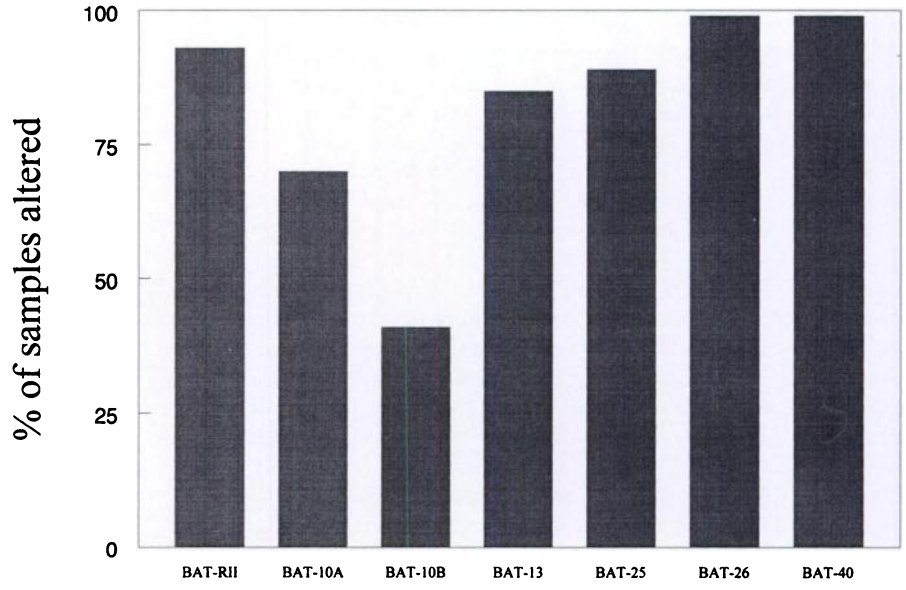

Fig. 2. Frequency of alterations in pA tracts of different lengths. RER CR cancers were tested for alterations in BAT-RII, BAT-10A, BAT-10B, BAT-13, BAT-25, BAT-26, and BAT-40. BAT-RII, BAT-10A, and BAT-10B have 10-bp pA tracts; BAT-13 has a 13-bp PA tract; and BAT-25, BAT-26, and BAT-40 have pA tracts of approximately 25, 26, and 40 bp, respectively. Each marker was tested with the same panel of 27 RER CR lines (see text).

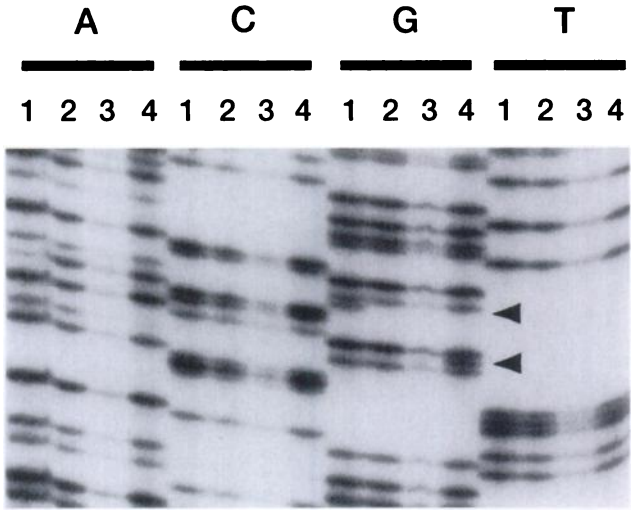

Fig. 3. Point mutations of RII. RII cDNA was amplified by RT-PCR and subjected to sequence analysis using an antisense primer. *Lane 1*, DLD-1, *Lane 2*, RKO; *Lane 3*, control xenograft; and *Lane 4*, KM12. The ddA mixes from each sequencing reaction were loaded in adjacent lanes to facilitate comparison, as were those for C, G, and T. Different A to G mutations are present in *Lanes 1* and *4 (arrowheads)*.

even with continued passage *in vitro* for several years, suggesting a minimum threshold for instability.[4]

The fact that the BAT-RII pA tract was altered at elevated frequency, compared to BAT-10A and BAT-10B, supported the idea that mutations in BAT-RII provide a selective growth advantage rather than simply reflecting nonspecific microsatellite instability. To independently confirm that RII mutations are selected for, we searched for RII mutations outside of the BAT-RII tract in RER tumors having one wild-type and one mutant BAT-RII 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 RII-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 RII (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 RII was determined in three RER lines with no wild-type BAT-RII sequences and in three non-RER lines; no mutations were identified in any of these samples.

In summary, the BAT-RII pA tract is mutated in the great majority of RER CR cancers, whether from HNPCC or sporadic cases. Although all pA tracts are unstable in these tumors, the BAT-RII tract is more unstable when compared to pA tracts of identical size in the same tumors. Moreover, both alleles of RII are mutated in most RER cases, either through alterations of both BAT-RII alleles or by mutation of BAT-RII 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 RII mutations are critical for RER-associated CR tumorigenesis. Like RER tumors, many non-RER CR cancers are resistant to the growth-inhibitory affects of TGF-β (24). Because such tumors do not have BAT-RII mutations, it will be of interest to determine whether other components of the TGF-β pathway are responsible for this insensitivity.

[4] R. Parsons *et al.*, unpublished results.

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