

Frequent Frameshift Mutations of the *TCF-4* Gene in Colorectal Cancers with Microsatellite Instability¹

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Abstract

About 15% of sporadic colorectal cancers show microsatellite instability (MSI) due to the inactivation of mismatch repair genes and are termed MSI-H tumors. In these tumors, frameshift mutations in coding repeats have been found within the *TGF β -RII*, *BAX*, and *IGFRII* genes that are probably involved in their progression. In the present work, we report frequent mutations in *TCF-4*, another target gene for instability. *TCF-4* codes for a transcription factor that is a crucial member of the adenomatous polyposis coli (APC)/ β -catenin/T-cell factor (TCF) pathway. Fifty percent (4 of 8) of human MSI-H colorectal cell lines and 39% (19 of 49) of MSI-H colorectal primary tumors were found to have a 1-bp deletion in an (A)₉ repeat within the coding region of this gene. In contrast, a frameshift mutation was found in only 1 of 56 non-MSI colorectal tumors and in none of 16 non-MSI colorectal cancer cell lines. These results suggest that *TCF-4* frameshift mutations are selected for and play a role in colorectal MSI-H tumorigenesis. Depending on different reading frames due to alternatively spliced *TCF-4* mRNA, the (A)₉ repeat normally codes for several isoforms that could serve as modulators of TCF-4 transcriptional activity. The deletion of one nucleotide in this repeat could change TCF-4 transactivating properties by modifying the respective proportions of the different isoforms.

Introduction

Microsatellite repeats are widely distributed throughout the genome. By failing to repair spontaneous errors that occur during replication, a subset of tumors accumulates frequent deletion and insertion mutations in these repetitive DNA sequences (1–3). Approximately 15% of sporadic colorectal carcinomas referred to as MSI-H³ exhibit this phenotype (4). Most MSI has thus far been described in noncoding DNA within introns or intergenic regions of the genome. Only a relatively small number of genes have been shown to contain alterations in mononucleotide repeat sequences within coding regions, and these mutations are probably involved in the progression of MSI-H tumors. The first target sequence identified within a coding region was a poly(A) nucleotide tract of the *TGF β -RII* gene (5). Other mutational targets of MSI-H tumors include repetitive sequences within genes involved in the regulation of cell growth, such as *IGFIR* (6), and *BAX*, which promotes apoptosis (7). In addition, MSI-H tumors contain frameshift mutations in repeat sequences located within the DNA mismatch repair genes *hMSH3* and *hMSH6* (8).

A systematic search of human sequence databases for mononucleotide repeats contained within coding regions identified an (A)₉

nucleotide tract in the *TCF-4* transcription factor. *TCF-4* is the most intensively expressed member of the TCF/lymphoid enhancer factor (LEF) family in colon tumor cell lines (9) and up-regulates the expression of *c-MYC* (10) as well as several other genes. Transcriptional activity of TCF-4 is enhanced by binding to β -catenin. This can occur either by inactivating mutations of *APC* that lead to the release of β -catenin or by activating mutations of β -catenin that make it insensitive to APC-mediated degradation (11). Because the APC/ β -catenin/TCF pathway is likely to play an important role in colorectal tumor progression, a search for mutations in the *TCF-4* repeat was initiated in a large series of cell lines and primary colorectal tumors with and without MSI-H.

Materials and Methods

Cell Lines and Tumor Samples. Twenty-four cell lines were obtained from Dr. B. Sordat (Institut Suisse de Recherches Expérimentales sur le Cancer, Epalinges, Switzerland) and Dr. A. Zweibaum (Villejuif, France) or purchased from the American Type Culture Collection. A total of 105 primary colorectal tumors from patients undergoing surgery for colorectal cancer were analyzed. These were selected to increase the number of MSI-H tumors and comprised 81 right-sided and 24 left-sided tumors. None of the patients included in this series had a known family history suggestive of hereditary nonpolyposis colorectal cancer. All cases were histopathologically confirmed as adenocarcinomas.

Assessment of MSI. The MSI status of the 24 colorectal cancer cell lines has been described previously (12). Microsatellite alterations of the 105 primary colorectal tumors were analyzed by PCR. MSI-H tumors were defined as those with somatic deletion mutations in the mononucleotide repeats

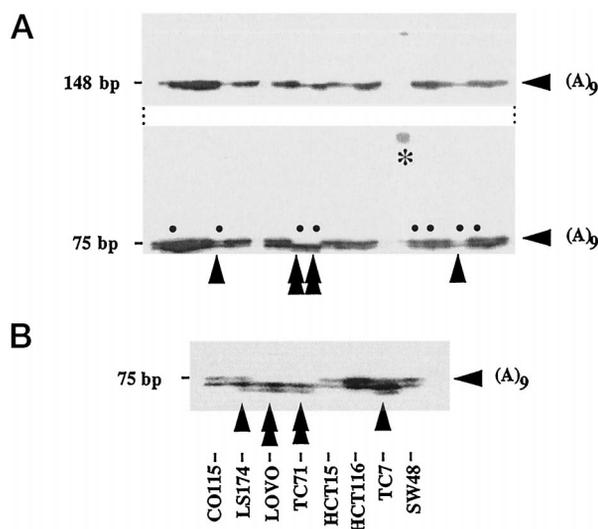


Fig. 1. *A*, mutations of *TCF-4* in 24 colorectal tumor cell lines. Two RT-PCR products (75 and 148 bp in size) of *TCF-4* containing the (A)₉ tract are shown. The wild-type fragment is indicated by arrowheads at the right. Arrowheads pointing up indicate deletions of one nucleotide. ●, MSI-H cell lines. *, the only non-MSI cell line with a *TCF-4* mutated allele (Colo320). *B*, details of the mutations in the 75-bp *TCF-4* RT-PCR product detected in four of eight MSI-H colorectal tumor cell lines.

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³ The abbreviations used are: MSI-H, high frequency MSI; MSI, microsatellite instability; RT-PCR, reverse transcription-PCR; BAC, bacterial artificial chromosome; CEPH, Centre d'Etude du Polymorphisme Humane; TCF, T-cell factor.

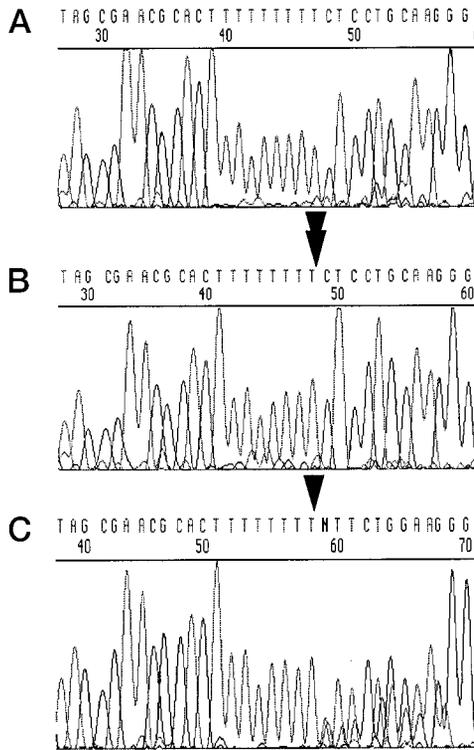


Fig. 2. Nucleotide sequence analysis of *TCF-4*. Reverse sequences from three tumor cell lines are shown. A, Co115 with two normal alleles containing a poly(T)9. B, Lovo with a homozygous T deletion. C, TC7 heterozygous for the same deletion.

BAT-26 in intron 5 of *hMSH2* and BAT-25 in intron 16 of *c-kit* that have been shown to establish MSI status with an accuracy of greater than 99.5% (13, 14).

RT-PCR Amplifications and Mutation Analysis of the *TCF-4* Coding (A)9 Repeat. Total RNA from colorectal tumor cell lines was reverse-transcribed using murine leukemia virus reverse transcriptase. PCR was performed with primers P13S (5'-AACTGGTGCGGCCCTTGAG-3') and P13AS (5'-TGGGCTGAG-GCAGCTGCCTT-3') at an annealing temperature of 66°C. The RT-PCR products were separated on a 7 M urea/32% formamide/7% polyacrylamide gel, transferred overnight on a Hybond N+ nylon membrane, and hybridized with an internal ³²P-labeled probe, P22AS (5'-GTGCGTTTCGCTACATACAAGGT-3').

Mutation Analysis of Genomic *TCF-4*. The CEPH human BAC library was screened for *TCF-4* sequences with primer sets specific for exons 5 and 10 (sequences available on request). Subclones in pUC9 of a positive BAC were screened for exon 10 sequences containing the (A)9 repeat by hybridization with ³²P-labeled P22AS primer and sequenced with primer 13AS, allowing a partial intron 9 determination. Genomic DNA was amplified with primers P40S (5'-GCCTCTATTACAGATAACTC-3') in intron 9 and P40AS (5'-GTTACCT-

TGTATGTAGCGAA-3') in exon 10 with an annealing temperature of 56°C. PCR products were then analyzed for mutations as described for RT-PCR products.

Sequencing Analysis of *TCF-4* Transcripts. The RT-PCR products were purified and sequenced with primers P13S and P13AS using a terminator cycle sequencing kit (Perkin Elmer).

Results

RT-PCR and Mutational Analysis of *TCF-4* in Colorectal Cancer Cell Lines. Twenty-four colorectal cancer cell lines (8 of which were MSI-H) showed two DNA fragments upon RT-PCR of *TCF-4*. These fragments were excised from agarose gel, reamplified with the same primer set, and sequenced. The smaller product was 75-bp long and contained the 5' primer in a putative exon 9, as deduced from comparison with the closely related *TCF-1* gene (15), followed by a putative exon 10 containing the (A)9 repeat. The larger, 148-bp product contained an alternative, putative exon 9' that will not be discussed in further detail in this report. Four of the eight MSI-H cell lines showed size alterations in both the 75- and 148-bp cDNA products (Fig. 1, A and B). Sequencing of these altered PCR products showed a deletion of one A of the (A)9 repeat. A normal *TCF-4* allele was absent in the Lovo and TC71 cell lines but present in the TC7 and LS174T cell lines (Fig. 2, A–C). With the exception of the Colo320 cell line, all MSI-negative cell lines had only normal 75- and 148-bp DNA fragments without any size variation (Fig. 1A). The Colo320 cell line showed two additional RT-PCR products, which, when sequenced, revealed a new 25-bp sequence (5'-TCTTT-GAATTTGGAATATTACAATG-3') adjacent to the 3' ends of exon 9 and exon 9', respectively.

Mutational Analysis of Genomic *TCF-4*. Using the above-mentioned primer set flanking the (A)9 repeat, we were unable to amplify genomic DNA, suggestive of the presence of an intronic sequence close to the repeat. To analyze genomic DNA, the CEPH human BAC library was screened for *TCF-4* sequences. One positive BAC was subcloned and partially sequenced to obtain information on intron 9 of the *TCF-4* gene. This allowed us to choose a sense primer which, together with an antisense primer in exon 10, was able to amplify the (A)9 repeat from genomic DNA. To avoid unsystematic, nontemplated nucleotide addition leading to problems in allele calling, we added a G to the 5' ends of each primer (16). Identical (A)9 size variation observed in the RT-PCR experiments was found in the cell lines with improved accuracy, confirming the specificity of the primers (Fig. 3A). DNA from 105 primary colorectal tumors (49 of which were MSI-H) was then analyzed. One of the 56 non-MSI tumors showed a 1-bp insertion, whereas a 1-bp deletion was observed in 19 of the 49 (39%) MSI-H tumors (Fig. 3B). Due to the presence of normal cells, it was not possible to determine for the majority of tumors whether these mutations were homozygous or heterozygous, although in two cases, normal (A)9 allele appeared to be totally absent.

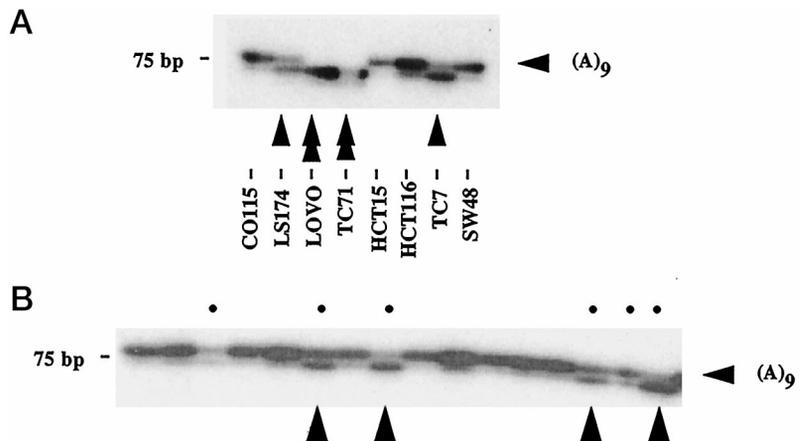
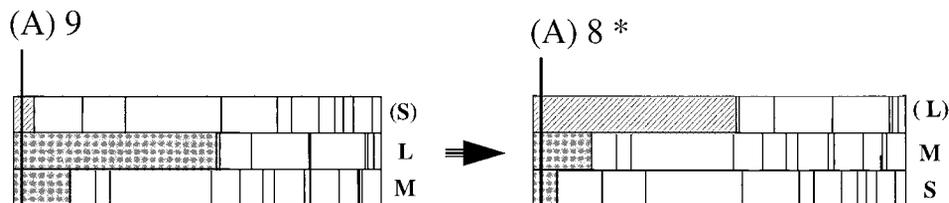


Fig. 3. Mutations of *TCF-4* in colorectal cell lines and primary tumors. PCR of genomic DNA was analyzed (A) in MSI-H cell lines and (B) in primary tumors. ●, MSI-H tumors. Arrowheads pointing up indicate deletions of one nucleotide.

Fig. 4. Open reading frames within the (A)9-containing exon of the *TCF-4* gene. The three reading frames of the *TCF-4* exon containing the (A)9 repeat encode a short (*S* = 11 amino acids), a long (*L* = 138 amino acids), and a medium (*M* = 25 amino acids) COOH terminus, respectively. The unused (▨) and used (▣) reading frames in a normal [(A)9] and mutated [(A)8] *TCF-4* allele are shown.



Discussion

Taken together, alterations of the (A)9 repeat within the *TCF-4* coding region were observed in 4 of 8 (50%) and 19 of 49 (39%) MSI-H cell lines and primary tumors, respectively, compared to 0 of 16 and 1 of 56 non-MSI cell lines and tumors, respectively ($P = 0.0001$, Fisher's exact test). In contrast, an (A)8 repeat and an (A)9 repeat in the coding region of the *BRCA2* gene and epidermal growth factor-stimulated protein kinase *p56* gene, respectively, remained intact in all eight MSI-H colorectal cell lines and in nine of the MSI-H tumors analyzed (data not shown). MSI-H cell lines and tumors were also analyzed for size alterations in coding mononucleotide repeats of the *TGF- β RII* (46 of 49 mutations, 94%), *IGF1R* (8 of 30 mutations, 27%), and *BAX* (20 of 55 mutations, 36%) genes (Refs. 17 and 18).⁴ Frameshift mutation of *TCF-4* and mutations in these latter genes may be of comparable functional significance in that frameshift mutation is selected for during tumorigenesis of MSI-H colorectal tumors.

In the APC/ β -catenin/TCF pathway, TCF-4 transcriptional activity is increased after alterations to the APC or β -catenin proteins (9, 11) and results in enhanced expression of *c-MYC* (10) and other genes. In a previous study, Sparks *et al.* (19) did not find *TCF-4* mutations in three MSI-H cell lines known to have intact APC and β -catenin. Of the five cell lines found to be mutated for *TCF-4* in the present study, four (Lovo, TC71, TC7, and Colo320) are mutant for APC, and one (LS174T) is mutant for β -catenin.⁵ Our results suggest that mutations of *TCF-4* stimulate its transcriptional activity synergistically with APC or β -catenin gene alterations. This hypothesis awaits confirmation by an exhaustive screening for *TCF-4*, APC, and β -catenin alterations in primary colorectal MSI-H tumors.

The (A)9 repeat is localized downstream of both the β -catenin binding domain and the HMG box DNA-binding region of the TCF-4 protein (9); therefore, mutations are unlikely to interfere with these activities. A number of alternatively spliced mRNA coexist for the *TCF-4* gene (9, 19);⁶ consequently, the (A)9 repeat can normally code for two different COOH-terminal ends (the *L* and *M* isoforms shown in Fig. 4). These could serve as modulators of transcription and have either agonist or antagonist activities, as proposed for the closely related *TCF-1* gene (20).

In MSI-H tumors, the frequently observed deletion of one nucleotide in the (A)9 repeat alters the reading frames such that the short and medium (*S* and *M*) isoforms are encoded (Fig. 4). Whether this mutational event exacerbates TCF-4 transactivating properties in colon tumors remains to be determined.

Acknowledgments

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