[CANCER RESEARCH 60, 4049–4052, August 1, 2000]

Advances in Brief

Novel Transcripts of Fibroblast Growth Factor Receptor 3 Reveal Aberrant Splicing and Activation of Cryptic Splice Sequences in Colorectal Cancer[1]

Jun-Hyeog Jang, Ki-Hyuk Shin, Young-Jin Park, Richard J. Lee, Wallace L. McKeehan, and Jae-Gahb Park[2]

Laboratory of Cell Biology, Cancer Research Institute, Seoul National University College of Medicine, Seoul 110-744, Korea [J-H. J., K-H. S., Y-J. P., J-G. P.]; National Cancer Center, Koyang, Kyunggi 411-351, Korea [J-G. P.]; Department of Developmental and Molecular Biology, Albert Einstein Cancer Center, Albert Einstein College of Medicine, Bronx, New York 10461 [R. J. L.]; and Center for Cancer Biology and Nutrition, Institute of Biosciences and Technology, Texas A&M University System Health Science Center and Department of Biochemistry and Biophysics, Texas A&M University, Houston, Texas 77030-3303 [W. L. M.]

Abstract

A nested reverse transcription-PCR analysis of *FGFR3* from human colorectal carcinomas revealed novel mutant transcripts caused by aberrant splicing and activation of cryptic splice sequences. Two aberrantly spliced transcripts were detected with high frequency in 50% of 36 primary tumors and in 60% of 10 human colorectal cancer cell lines. Most transcripts used normal splice sites but skipped or included exons 8 and 9. Two mutant transcripts arose from cryptic splice donor sites in exon 7 that spliced to exon 10. The predicted translation products would exhibit frameshifts and a premature termination codon in exon 10. We propose that dysregulation of mRNA splicing frequently generates an aberrant *FGFR3* transcript that may confer a selectable advantage on clones of cells in colorectal tumorigenesis.

Introduction

Fibroblast growth factor signaling plays an important role in a variety of processes, including cellular proliferation, cellular differentiation, wound repair, angiogenesis, and carcinogenesis (1–3). The FGFR[3] family of membrane-spanning tyrosine kinase receptors consists of four members (FGFR1–4) that differ in their tissue expression, specificity for ligand, signal pathways, and biological effects (1, 4, 5). FGFR3 has been demonstrated to either stimulate or prohibit cell proliferation, depending on the tissue type (6, 7). Missense mutations in the transmembrane domain of FGFR3 that are found in dominant heritable skeletal dysplasias result in constitutive activation of FGFR3 (8). Transgenic and knockout mouse studies have confirmed an inhibitory role for FGFR3 in bone growth (9). Several recent reports have demonstrated that activated FGFR3 can inhibit chondrocyte proliferation via STAT signaling pathways, with up-regulation of the $p21^{WAF1/CIP1}$ or Ink4 cell cycle inhibitors (10, 11). FGFR3 may exert a negative effect on cellular proliferation through the activation of growth-inhibitory pathways. Indeed, FGFR3 expression has been associated with differentiation in Caco-2 intestinal epithelial cells (12), suggesting that FGFR3 may maintain the differentiated state in colorectal tissue. Thus, FGFR3 may play a different role than the other FGFR family members, such as FGFR1, which is overexpressed in many tumor types (2, 13). In this report, we have analyzed FGFR3 expression in 36 primary colorectal tumors and 10 cancer-derived cell lines. Here we show that FGFR3 is frequently inactivated by aberrant splicing and activation of cryptic splice donor sites within exon 7.

Materials and Methods

Patients and Cell Lines. Human CRC cell lines were grown in RPMI 1640 supplemented with 10% bovine serum. The origin of the cell lines used in this study has been described previously (14). Surgically resected tumors and corresponding normal tissue were obtained from patients with primary CRC at the Seoul National University Hospital (Seoul, Korea).

RNA Extraction and RT-PCR. All tissue samples were frozen in liquid nitrogen after surgery, and RNA was isolated. Total RNA was extracted from the cell lines and tissues using Tri-Reagent according to the manufacturer's protocol (Molecular Research Center, Inc., Cincinnati, OH). The extracted RNAs (2 μg of each) from tissues were reverse transcribed to synthesize cDNA using random hexamers in a final volume of 20 μl using the Superscript kit (Life Technologies, Inc., Grand Island, NY). The reaction was incubated at 42°C for 90 min and boiled for 5 min.

The first PCR was carried out in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates, and 0.5 unit of Taq polymerase in a 12.5 μl final volume with 1 μl of cDNA and 0.5 μM of each primer. The amplifications were performed in a GeneAmp PCR system 9600 (Perkin-Elmer, Norwalk, CT). The reaction profile consisted of one cycle at 94°C for 1 min, followed by 25 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min with external primers 5′-GGCAGCATCCGGCAGACG-3′ (sense primer) and 5′-GCGTCACAGCCGCCACCACC-3′ (antisense primer). The second PCR was carried out in the same way using 1 μl of the first PCR-amplified reaction product but 30 cycles with internal primers 5′-CGCACCGGCCCATCCTG-3′ (sense primer) and 5′-GCGTCACAGCCGCCACCACC-3′ (antisense primer). The PCR products were then resolved on 2% ethidium bromide-stained agarose gel. For direct sequencing, RT-PCR fragments were purified from agarose gel using a gel extraction kit (Qiagen, Hilden, Germany).

Mutation Analysis of the *FGFR3* Gene. Genomic DNA samples from tumors were extracted using standard procedures with proteinase K and chloroform-phenol. DNA samples were amplified for SSCP analysis of the *FGFR3* gene using PCR under the same conditions as reported previously (15).

DNA Sequencing. Automated sequencing was performed using dideoxy terminator cycle sequencing (Applied Biosystems) and an Applied Biosystems model 377 DNA sequencer (Perkin-Elmer, Foster City, CA).

Western Blot Analysis. Cells ($5–10 \times 10^6$) were lysed in electrophoresis sample buffer [80 mmol/liter Tris-HCl (pH 6.8), 2% SDS, 2% 2-mercaptoethanol, and 10% glycerol] and boiled for 5 min. Fifty μg of total protein samples were subjected to electrophoresis on 8% SDS-polyacrylamide gels and transferred to nitrocellulose filters (Novex, San Diego, CA). The membranes were blocked with 2.5% dried milk in TBST [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20] for 1 h, washed three times with TBST, and incubated overnight with the FGFR3 antibody (1:2000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). After three final washes, the FGFR3 protein was detected by incubation of blot with a 1:2000 dilution of peroxidase-labeled antirabbit antibody using the enhanced chemiluminescence system (Amersham, Zürich, Switzerland).

Received 12/28/99; accepted 6/16/00.

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[1] Supported by a postdoctoral fellowship from the Korea Science and Engineering Foundation (KOSEF; Post Doc. 98–99) through the Cancer Research Institute of Seoul National University.

[2] To whom requests for reprints should be addressed, at Cancer Research Institute, Seoul National University College of Medicine, Seoul 110-744, Korea. Phone: 82-2-760-3380; Fax: 82-2-742-4727; E-mail: jgpark@plaza.snu.ac.kr.

[3] The abbreviations used are: FGFR, fibroblast growth factor receptor; RT-PCR, reverse transcription-PCR; SSCP, single-strand conformation polymorphism; CRC, colorectal cancer; ORF, open reading frame; AT, aberrant transcript; MT, mutant transcript; NMD, nonsense-mediated decay.

Results

Premature Termination of *FGFR3* Transcripts by Aberrant Splicing in CRC Tissues. FGFR3 normally exists in two forms, IIIb and IIIc, which arise from alternative splicing in which either exon 8 (*FGFR3IIIc*) or exon 9 (*FGFR3IIIb*) is skipped. *FGFR3IIIb* was first cloned from a colon cancer cell line and is the predominant form expressed in epithelial cells (16). We examined 36 primary human colorectal carcinomas and 10 cell lines to elucidate the role of FGFR3IIIb in CRC. In 10 cases, matched normal colorectal tissues were also analyzed. We used a nested RT-PCR assay to screen for the expression of FGFR3 as shown in Fig. 1*A*. The amplified products were separated by electrophoresis, and the cDNA fragments were purified and sequenced. Of the 36 primary tumors, 18 cases (50%) revealed the presence of two distinct products in tumor samples, whereas 10 normal colorectal tissues showed a normal-sized transcript (Figs. 1*A* and 2*A*). As expected, sequence analyses of normal-sized transcript revealed the FGFR3IIIb isoform (Fig. 1*B*). However, sequence analyses of the aberrant cDNAs revealed abnormally spliced transcripts. The lower FGFR3 ATs (Fig. 1*B*, FGFR3 *AT-I*) encode a form of FGFR3 missing the second half of the immunoglobulin-like-III domain, whereas the upper FGFR3 ATs (Fig. 1*B*, FGFR3 *AT-II*) encode both exons 8 and 9 in tandem array. FGFR3 AT-I was observed in 5 of 36 primary carcinomas, whereas FGFR3 AT-II was observed in 15 of 36 cases. Wild-type *FGFR3IIIb* that encodes an intact ORF was expressed in 15 of 36 cases (42%). In 5 of 15 cases, the ATs coexisted with expression of wild-type *FGFR3IIIb* transcripts. Of the 36 cases, 9 exhibited poor amplification of FGFR3 transcripts.

The predicted translation products of both FGFR3 AT-I and AT-II would end prematurely due to a nonsense mutation introduced by a frameshift (Fig. 3*A*). Wild-type splicing of FGFR3IIIb creates a codon from the final base of exon 8 and the first 2 bases of exon 10 (Fig. 1*C*). Similarly, FGFR3 IIIc uses a codon created by the final base of exon 9 and the first 2 bases of exon 10. In FGFR3 AT-I, exon 7 is spliced to exon 10, introducing a 1-base (−1) frameshift and resulting in a premature termination sequence at base 1217. In FGFR3 AT-II, exons 7–10 are included in the transcript, introducing a 1-base (+1) frameshift and a premature termination codon at base 981 (Fig. 3*A*).

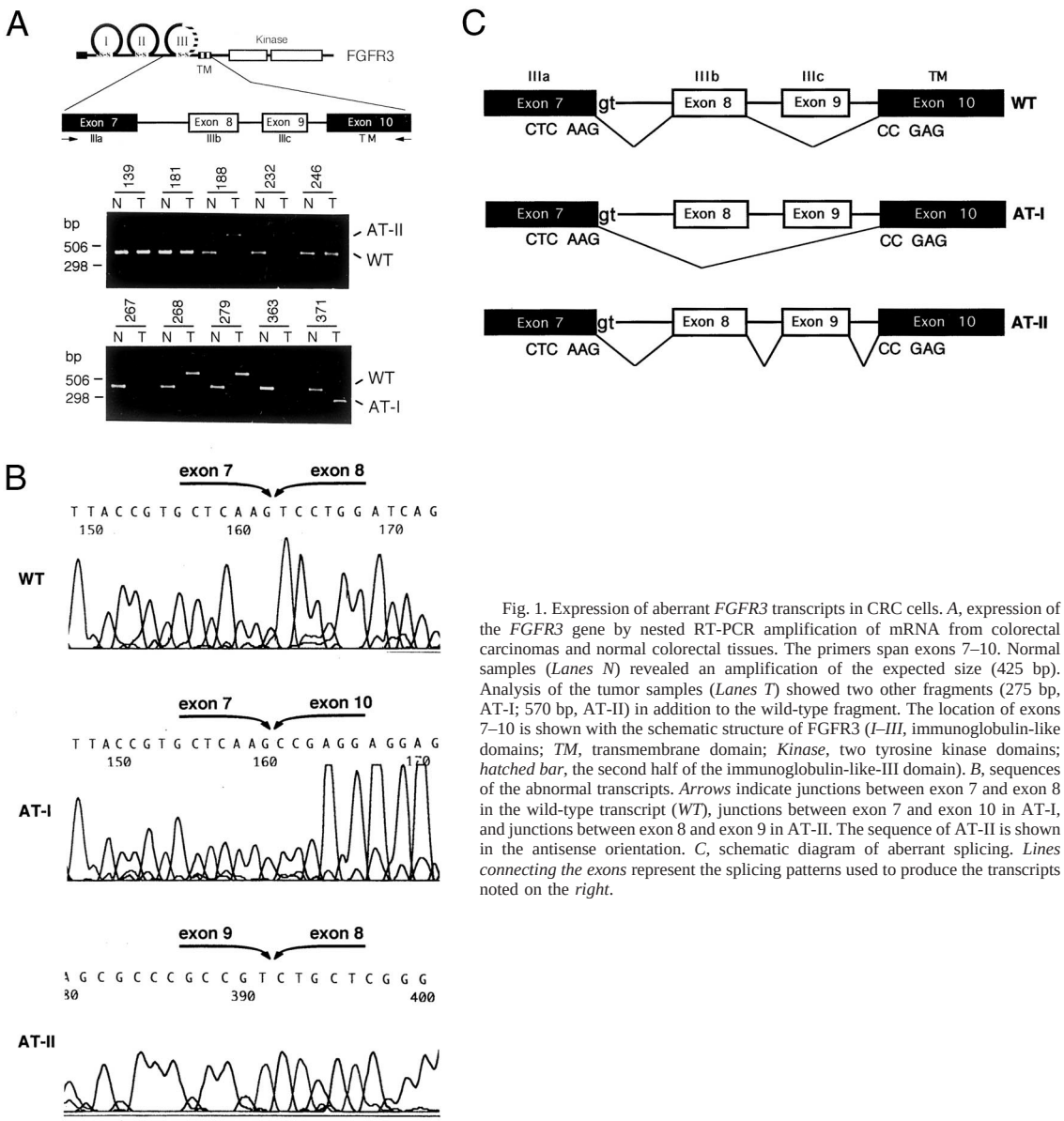

Fig. 1. Expression of aberrant *FGFR3* transcripts in CRC cells. *A*, expression of the *FGFR3* gene by nested RT-PCR amplification of mRNA from colorectal carcinomas and normal colorectal tissues. The primers span exons 7–10. Normal samples (*Lanes N*) revealed an amplification of the expected size (425 bp). Analysis of the tumor samples (*Lanes T*) showed two other fragments (275 bp, AT-I; 570 bp, AT-II) in addition to the wild-type fragment. The location of exons 7–10 is shown with the schematic structure of FGFR3 (*I–III*, immunoglobulin-like domains; *TM*, transmembrane domain; *Kinase*, two tyrosine kinase domains; *hatched bar*, the second half of the immunoglobulin-like-III domain). *B*, sequences of the abnormal transcripts. *Arrows* indicate junctions between exon 7 and exon 8 in the wild-type transcript (*WT*), junctions between exon 7 and exon 10 in AT-I, and junctions between exon 8 and exon 9 in AT-II. The sequence of AT-II is shown in the antisense orientation. *C*, schematic diagram of aberrant splicing. *Lines connecting the exons* represent the splicing patterns used to produce the transcripts noted on the *right*.

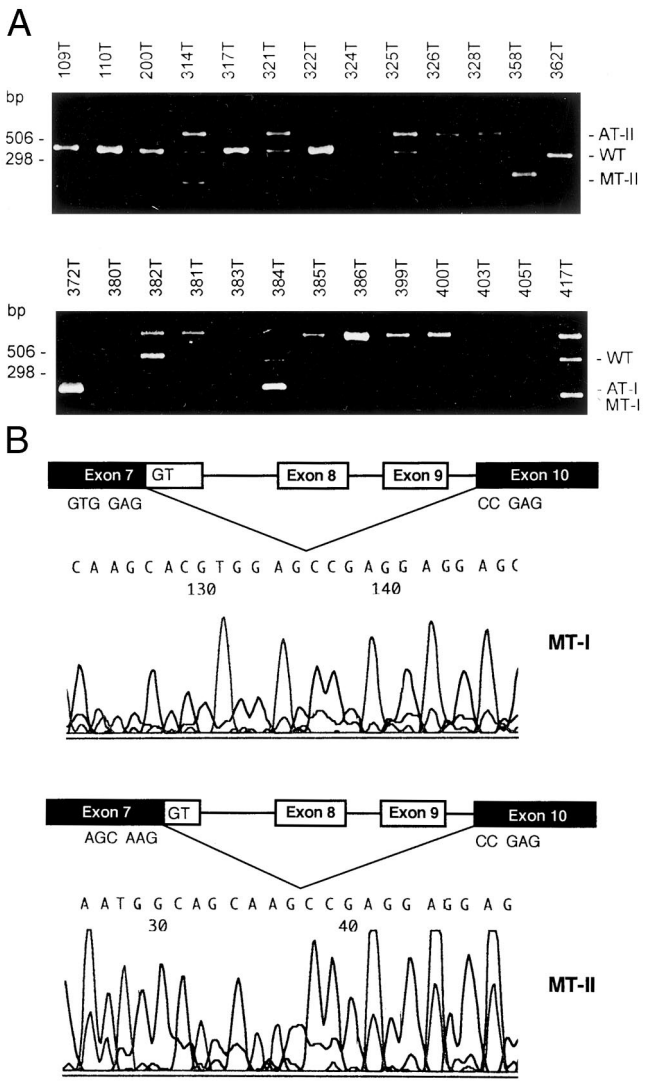

Fig. 2. Activation of cryptic splice sites within exon 7. *A*, expression of the MTs of the *FGFR3* gene by nested RT-PCR amplification of mRNA from colorectal carcinomas. Analysis of the tumor samples showed, in addition to the wild-type fragment, AT-I and AT-II and other fragments (236 bp, MT-II; 221 bp, MT-I). *B*, sequences of the MTs with the schematic diagram of aberrant splicing by the activation of cryptic splice sequences. The sequences of junctions between the internal site (54 bp upstream) within exon 7 and exon 10 in MT-I and between the internal site (39 bp upstream) within exon 7 and exon 10 in MT-II are shown.

Novel MTs of FGFR3 by Activation of Cryptic Splicing Sequences within Exon 7. Of the 36 primary tumors, 4 showed additional truncated transcripts (Fig. 2*A*). Sequencing analysis of the faster-migrating products revealed novel MTs spliced from internal sites within exon 7 to exon 10 (Fig. 2*B*). The MTs revealed deletions of 204 bases and 189 bases, respectively (designated MT-I and MT-II). In FGFR3 MT-I (Thr^{372} and Thr^{417}), we found that the use of a cryptic splice donor sequence at position 877 resulted in the deletion of 54 bases of exon 7 and skipping of exon 8, deleting the COOH-terminal portion of the immunoglobulin-like-III domain at the cDNA level. In FGFR3 MT-II (Thr^{314}), another internal site 15 bases downstream of the cryptic site from MT-I within exon 7 was used, resulting in a deletion of 39 bases of exon 7 and skipping of exon 8 (Fig. 3*B*). Translation of both FGFR3 MT-I and MT-II ends prematurely due to a nonsense mutation introduced by a frameshift (Fig. 3*A*). Overall, 19 of 36 primary tumors (53%) showed the presence of abnormal *FGFR3* transcripts, demonstrating a significantly high frequency in CRC.

Lack of Correlation between the Expression of Various Nonsense FGFR3 Transcripts and FGFR3 Mutations. Recent studies have suggested that inappropriate nonsense mutations inserted within the coding region of various genes may result in altered splice site selection (17). To investigate whether mutation is responsible for the various nonsense *FGFR3* transcripts, we examined the corresponding genomic DNA from tissues and CRC cell lines that displayed MTs. PCR-SSCP analysis of all coding region (exon 2–18) of *FGFR3* including adjacent splicing sites on genomic DNA was performed. There was no apparent abnormal band by PCR-SSCP analysis. An additional examination of the genomic DNA sequences of the surrounding the cryptic splice sites confirmed no mutations or deletions in the *FGFR3* sequence by sequencing analysis. However, the sequences surrounding the cryptic splice donor sites within exon 7 are identical by 5 bases (AG*GT*G, MT-I) and 11 bases (CAAG*GT*-GGGCC, MT-II) to the normal boundary (CAAG*gt*gggcc) of exon 7 and intron 8, respectively (Fig. 3*B*). This suggested that the MTs arose due to the activation of cryptic splicing sites and that such errors of exon definition occurred mechanistically in *trans* (opposite of *cis*).

Down-Regulation of FGFR3 Protein Expression in CRC Cell Lines. To look for abnormalities in *FGFR3* transcripts from cancer-derived cell lines, we further tested 10 cancer-derived cell lines and amplified *FGFR3*, exon 7–10. Of the 10 human CRC cell lines, 9 cell lines exhibited a normal-sized *FGFR3* transcript, whereas in 5 cell lines, abnormal transcript AT-I was detected as well. In addition, the SNU-503, SNU-769A, SNU-769B, and SNU-1033 cell lines showed expression of MT-I. HCT-116 cell lines exhibited a lack of wild-type *FGFR3* transcripts but expressed AT-I and MT-I (Fig. 4*A*). These observations showing that abnormal transcripts are frequently ex-

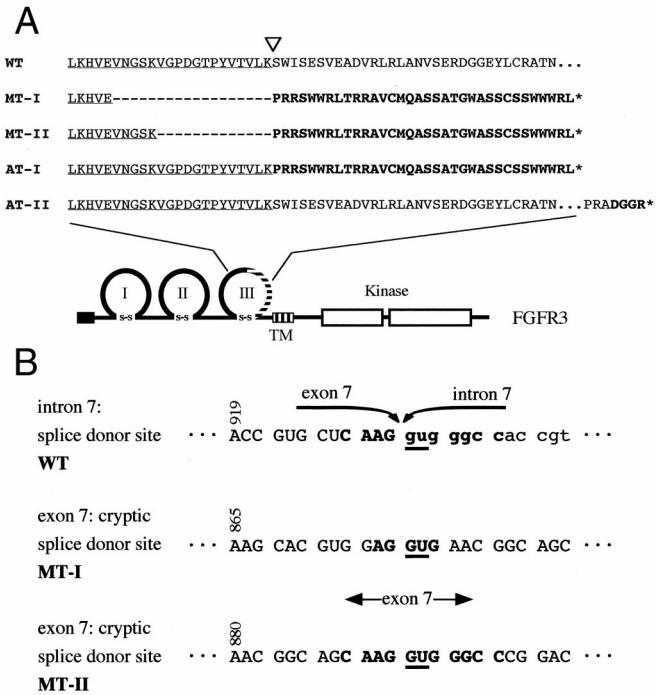

Fig. 3. Frameshift introduction and premature termination of translation due to aberrant splicing in the nonsense *FGFR3* transcripts. *A*, amino acid sequence of processed AT-I, AT-II, MT-I, and MT-II. The use of cryptic splice sites introduces frameshift of the ORF and a downstream stop codon, resulting in the encoding of a novel 34-amino acid peptide sequence (indicated in *bold*) that shares no sequence homology to any other gene in the database. The boundary of exon 7/8 is indicated by an *arrowhead*. The COOH-terminal amino acid sequence of exon 7 is *underlined*. ∗, stop codon. *B*, the sequences surrounding the cryptic splice donor sites. The identical sequences at the wild-type exon 7/intron 8 boundary (*WT*) and the surrounding cryptic sites within exon 7 are indicated in *bold*. The intron sequence is given in *lowercase letters*. The 5′ splice donor sites are *underlined*.

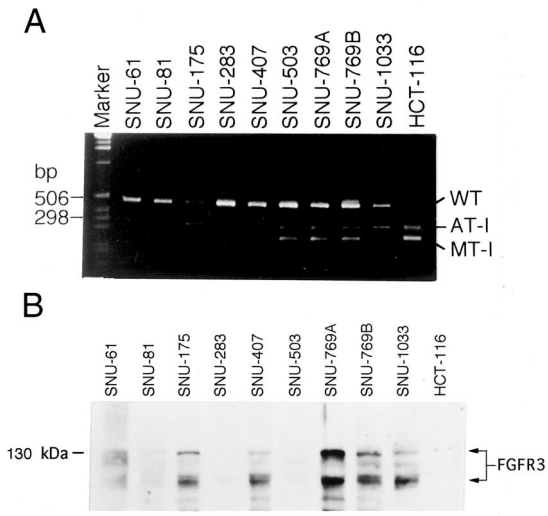

Fig. 4. FGFR3 expression by CRC cell lines. *A*, a nested RT-PCR analysis of the FGFR3. *B*, Western analysis of FGFR3 protein expression in CRC cell lines using a polyclonal rabbit anti-FGFR3 IgG antibody.

pressed in cancer-derived cell lines (6 of 10 cell lines) support the results from primary tumors discussed earlier that show that FGFR3 is frequently inactivated in CRC cells.

Finally, we examined expression of the FGFR3 protein in 10 CRC cell lines. FGFR3 protein of the expected size was present in six cell lines expressing the wild-type *FGFR3IIIb* transcript. Four cell lines (SNU-81, SNU-283, SNU-503, and HCT-116) expressed barely detectable levels of FGFR3 protein (Fig. 4*B*). With the exception of SNU-503, four cell lines in which wild-type *FGFR3* transcript was detected together with abnormal transcripts showed full-length FGFR3 protein expression. Because the FGFR3 antibody recognized the COOH-terminal portion of the FGFR3 tyrosine kinase, the presence of the truncated translation products predicted by the ATs could not be evaluated. The results confirm that wild-type FGFR3 is deficient in CRC frequently, regardless of whether or not aberrantly spliced transcripts are expressed.

Discussion

The disruption of regulated mRNA processing has emerged as a cellular function that is disturbed during neoplastic transformation of cells (2, 18, 19). Here we show that four novel aberrantly spliced transcripts of *FGFR3* containing nonsense codons with respect to translation occur in both primary CRC tissues and cell lines. The abnormal nonsense transcripts can be classified into two groups (class I and class II). In class I transcripts, the beginning and the end of the transcripts coincided with splice junctions where the abnormal splicing occurred exon to exon. Class II transcripts arose from internal sites within exon 7 that were used for splice donor sites. Both class I and class II transcripts would produce a prematurely terminated translation product due to a frameshift. Only the wild-type transcript has an intact ORF and can encode fully functional protein.

Recent studies have demonstrated that cells have evolved elaborate mechanisms to rid themselves of ATs by a pathway referred to as NMD. The NMD pathway has been suggested as a surveillance mechanism to ensure that transcripts containing premature nonsense codons are degraded rapidly, thus preventing synthesis of incomplete and potentially deleterious proteins. However, it has been observed that several human genetic diseases are a consequence of stabilizing the nonsense-containing transcript by inactivating the NMD pathway (20). Currently, it is unclear whether the apparent stability of the nonsense FGFR3 transcripts results from escape of the NMD pathway due to its alteration in the carcinomas or because they are remnants of major transcription products that have been largely degraded by NMD detected by our sensitive PCR methods. In any event, our results suggest that fidelity of alternate splicing has been perturbed in the carcinomas.

In 13 of the 36 primary tumor samples, we have observed FGFR3 AT-I or AT-II alone without any detectable wild-type *FGFR3* transcript, suggesting that different types of ATs resulted during the multistep RNA processing mechanism. Regardless of whether or not the ATs are significantly expressed as products, the dominant expression of the ATs at the expense of wild-type FGFR3 at transcription would have the same end result of dampening or reducing an unfavorable negative regulation of progression of the carcinomas to malignancy.

FGFR3 has been shown to transduce a different signal that either inhibits or stimulates cell proliferation, depending on the cell type. The potential of FGFR3 to negatively regulate colorectal carcinoma cell proliferation and bypass that regulation during progression to malignancy is an area that requires further investigation. Our results strongly support the idea that the dysregulation of *FGFR3* expression by aberrant splicing of mRNA in a significant subset of CRC is an alternative pathway to neoplastic transformation.

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Cancer Res 2000;60:4049-4052.