American Journal of Pathology, Vol. 151, No. 2, August 1997

Short Communication

Increased β-Catenin Protein and Somatic *APC* Mutations in Sporadic Aggressive Fibromatoses (Desmoid Tumors)

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Sporadic aggressive fibromatosis (also called desmoid tumor) is a monoclonal proliferation of spindle (fibrocyte-like) cells that is locally invasive but does not metastasize. A similarity to abdominal fibromatoses (desmoids) in familial adenomatous polyposis and a cytogenetic study showing partial deletion of 5q in a subset of aggressive fibromatoses suggests that the adenomatous polyposis coli (APC) gene plays a role in its pathogenesis. APC helps regulate the cellular level of β-catenin, which is a downstream mediator in Wnt (Wingless) signaling. β-Catenin has a nuclear function (binds transcription factors) and a cell membrane function (is a component of epithelial cell adherens junctions). Six cases of aggressive fibromatosis of the extremities from patients without familial adenomatous polyposis, or a family history of colon cancer, were studied. Immunohistochemistry, using carboxy and amino terminus antibodies to APC, and DNA sequencing showed that three of the six contained an APC-truncating mutation, whereas normal tissues did not contain a mutation. Western blot and Northern dot blot showed that all six tumors had a higher level of β-catenin protein than surrounding normal tissues, despite containing similar levels of β-catenin mRNA. Immunohistochemistry localized β-catenin throughout the cell in tumor tissues, although it localized more to the periphery in cells from normal tissues. Reverse transcription polymerase chain reaction showed that the tumors expressed N-cadherin but not E-cadherin (a pattern of expression of proteins making up adherens junctions similar to fibrocytes), suggesting that the specific adherens junctions present in epithelial cells are not necessary for β-catenin function. Increased β-catenin may cause the growth advantage of cells in this tumor through a nuclear mechanism. The increased protein level, relative to the RNA level, suggests that β-catenin is degraded at a lower rate compared with normal tissues. In some cases, this is caused by a somatic mutation resulting in a truncated APC protein. ***(Am J Pathol 1997, 151:329–334)***

Aggressive fibromatosis occupies an unusual position in the progression of neoplasia, as its cells have lost local control of growth but do not have the capability of forming metastases. It is one of a group of clinically heterogeneous disorders composed of cytologically similar spindle-shaped (fibrocyte-like) cells termed fibromatoses.[1] The nomenclature of the fibromatoses is not uniform, and terms such as aggressive fibromatosis, deep fibromatosis, and desmoid tumors are all used to describe the same lesion. Aggressive fibromatosis is the most invasive of the fibromatoses; it infiltrates into surrounding structures, frequently recurs after surgical treatment, and often results in significant loss of function. The lesion causes loss of function due to impingement on surrounding structures and potentially causes mortality if it involves vital structures. Current treatments include chemotherapy, which is effective in only some cases, and radiation therapy, which is effective in many cases but associated with significant side effects. Operative resection with wide margins are needed to surgically eradicate the tumor, and occasionally it is necessary to perform an amputation for a tumor involving an extremity.[2–4] Study of clonality of aggressive fibromatosis shows that it is a

Supported in part by the Research Institute of the Hospital for Sick Children.

Accepted for publication May 20, 1997.

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monoclonal proliferation.[5,6] As it is due to a clone with a growth advantage, it is possible that a mutation increasing cell proliferation is responsible for its pathogenesis.

Ten to fifteen percent of patients with the familial cancer syndrome, familial adenomatous polyposis (FAP), develop abdominal fibromatoses (usually termed abdominal desmoids)[7] associated with a germ-line mutation in one allele and somatic mutation in the other allele of the adenomatous polyposis coli (APC) gene.[8,9] Somatic APC mutations in abdominal fibromatoses in patients with FAP tend to be located between codons 1309 and 1450, in an area termed the mutational cluster region.[10,11] They result in an early stop codon and a truncated protein product. Cytogenetic study of aggressive fibromatoses from patients without FAP show a number of anomalies, with trisomy 8,[12] trisomy 20,[13] and partial loss of the long arm of chromosome 5[14] occurring most frequently. The occurrence of aggressive fibromatoses in FAP, along with the cytogenetic demonstration of loss of a portion of 5q in some lesions in patients without FAP, suggests an APC mutation in some spontaneously occurring aggressive fibromatoses.

APC binds β-catenin, a protein involved in Wnt signal transduction.[15–19] Wnt signaling in humans is homologous to the Wingless signaling pathway in *Drosophila* and functions in normal development.[20] Wnt signaling is also activated in some human malignancies, likely resulting in cell proliferation.[21,22] Wild-type APC binds β-catenin and, when β-catenin is present at high concentrations, binds to the serine-threonine kinase, GSK3β. GSK3β phosphorylates APC, activating a second binding site for β-catenin, resulting in β-catenin degradation.[19] APC truncations due to early stop codons in the mutational cluster region may lack the GSK3β binding site, resulting in a higher β-catenin level.[23] Wnt expression results in increased β-catenin, potentially due to decreased GSK3β activity.[18] β-Catenin is a key mediator in Wnt signalling, and, in Drosophila, beta-catenin mimics Wnt activity.[24] The β-catenin protein level can also be increased by mutations in β-catenin itself that interfere with normal protein degradation. Thus, there are a number of mechanisms resulting in an increased β-catenin level, including Wnt activation and APC truncation.

β-Catenin binds nuclear transcription factors in the lymphoid enhancer-binding factor-1 (LEF-1)[25–28] family. LEF-1 is an architectural binding factor, causing a bend in DNA, allowing other nuclear proteins to bind. β-Catenin bound to LEF-1 causes a bend in DNA that may be different than the bend formed by LEF-1 alone.[27] Although LEF-1 is expressed only during development and by adult lymphoid tissues, other members of this family, Tcf-1,-2, and -4, are expressed by colonocytes.[15,28] β-Catenin is also a member of the cell membrane adherens junctions. These junctions play a role in cell-cell communication and cell adhesion.[16,29] In epithelial cells, during wounding, or when tumors metastasize, the junctions disassociate. The disassociation and association of adherens junctions is hypothesized to mediate the contact inhibition signal. Proteins making up adherens junctions, such as E-cadherin, act as tumor invasion suppressors, with more invasive tumors lacking the protein.[30] APC competes with E-cadherin for binding to β-catenin.[31] β-Catenin, therefore, has potential functions in cell adhesion and in nuclear transcription.

There is a higher frequency of colonic neoplasms than tumors elsewhere in FAP, suggesting that APC truncation plays a role in only select cell types. This could be due to differences in expression of APC or differences in how APC functions in different cell types. Differences in expression could be related to alternative splicing of the 5′ noncoding portion, although all alternative sequences, except for one found in the brain, are expressed by a wide variety of tissues.[32] Differences in cell type may be related to differences in adherens junctions. The quantity and protein content of adherens junctions differs between cell types, with fibrocytes having a smaller number compared with colonocytes, containing N-cadherin rather than E-cadherin.[33–35]

We aimed to determine whether β-catenin protein is elevated in sporadic aggressive fibromatoses compared with surrounding normal tissues and whether somatic mutations causing APC truncations are present in a subset of the tumors. In addition, we will determine the expression of E-cadherin and N-cadherin (to determine whether this tumor contains epithelial or fibroblast-like adherens junctions) and localize the site of β-catenin in the cell to determine whether β-catenin more likely acts through a cell membrane or nuclear function.

Materials and Methods

Six cases of aggressive fibromatoses of the extremities were studied. All were deep tumors of the extremities (two of the upper and four of the lower extremity). The patients had isolated tumors without evidence of colonic polyps and without a family history of colonic neoplasia or FAP. Samples were obtained from the initial operative procedure before undergoing chemotherapy or radiation therapy. Surrounding normal tissue was also obtained for study and processed in an identical manner. Tissue was snap-frozen as soon as possible after surgical excision and stored in liquid nitrogen. In four cases, tissue was also cryopreserved in OCT before storage in liquid nitrogen. Medical records, radiographs, and formalin-fixed, paraffin-embedded materials were available to confirm the diagnosis. Colon cancers containing an APC mutation and the SW480 colon cancer cell line (American Type Culture Collection, Rockville, MD), which contains an APC-truncating mutation, were used as positive controls.

Immunohistochemistry

OCT-embedded cryopreserved tissues were cut into 12-μm sections and fixed in 0.3% hydrogen peroxide in absolute methanol at room temperature for 30 minutes. Frozen sections from the additional cases were embedded in OCT and then sectioned. A sporadic colonic carcinoma with an APC mutation and sections of normal colonic mucosa from the same patient were used as positive and negative controls and were processed along

with the fibromatosis tissue. The sections were washed in PBS, blocked with 1% horse serum for 30 minutes at room temperature, and washed again in PBS. The sections were incubated with monoclonal antibody to the amino terminus of APC (APC-3, Oncogene Science, Cambridge, MA) or the carboxy terminus of APC (APC-4, Oncogene Science) overnight at 4°C. Additional sections were incubated in control mouse immunoglobulins. Protein digestion was performed using Pronase, 50 μg/ml, for 5 minutes at room temperature for the APC-4 antibody before incubation to enhance antigen detection, after initial experience with the colonic carcinoma and normal mucosa. Antibodies were used at 1.0 μg/ml, 1.5 μg/ml, and 2.0 μg/ml. The tissues were washed with PBS and incubated with a secondary anti-mouse immunoglobulin and detected using immunoperoxidase staining (Vector Laboratories, Burlingame, CA). Hematoxylin was used as a counterstain.

Immunohistochemistry for β-catenin was performed on paraffin-embedded, formalin-fixed materials. A previously published microwave retrieval technique was used.[36] Briefly, tissues were dewaxed and quenched in a hydrogen peroxide/methanol solution, immersed in 10 mmol/L citric acid, and heated 15 minutes in a 750-W microwave oven. After washing and blockade using 1% bovine serum albumin, slides were incubated with a monoclonal anti-β-catenin antibody (Transduction Laboratories, Lexington, KY) at 10 μg/ml overnight at 4°C and detected using the same technique as for APC. Hematoxylin was used as a counterstain.

DNA Analysis

DNA was extracted from cryopreserved tissues using proteinase K. The mutational cluster region of APC occurs within exon 15, and this exon was chosen for sequencing. Exon 15 was amplified using polymerase chain reaction (PCR) and a series of 20 overlapping primer pairs.[37] An aliquot of each PCR product was electrophoresed, stained with ethidium bromide, and observed under ultraviolet light to ensure that a single product of appropriate size was amplified. Additional aliquots were purified using the QIAquick PCR purification column (Quiagen, Chatsworth, CA) and sequenced using an automated sequencer, based on the PCR. The PCR primers were used as the primers for sequencing.

RT-PCR

RNA was extracted using guanidinium isothiocyanate homogenization followed by cesium trifluoride ultracentrifugation. mRNA was converted to cDNA using reverse transcriptase with a poly(T) primer. PCR was performed using specific oligonucleotide primers for GAPDH, E-cadherin, N-cadherin, and select primer pairs for exon 15 of APC. RNAse-treated controls were also used. Primers and conditions for GAPDH and E-cadherin were previously published.[33,38] The upstream primer for N-cadherin was selected from base pairs 80 to 100 (ATAGAGATAAAAACCTTCA) and downstream primer from base pairs 479 to 498 (ATTGTTGATTGTAAACATGT). An annealing temperature of 50°C was used. The resultant products were electrophoresed, stained with ethidium bromide, and photographed under ultraviolet light.

Northern Dot Blot

Equal amounts of extracted total RNA (4 μg) from each sample, and from control cell lines, were prepared on a nylon membrane for dot blot. Probes for β-catenin and GAPDH were digoxigenin labeled from specific cDNA (available from American Type Culture Collection) using the Dig Easy Hyb kit according to the manufacturer's specifications (Boehringer Mannheim, Laval Quebec, Canada). Hybridization was carried out overnight at 50°C and detected using an anti-digoxigenin antibody and chemiluminescence (Boehringer Mannheim). Northern blot using a larger quantity of RNA (15 μg) was also performed on one sample to verify the dot blot results.

Western Blot

Protein was extracted from tissue samples by homogenization in lysis buffer (1% SDS, 10 mmol/L Tris/HCl, pH 7.4), followed by 10 seconds of microwaving and 5 minutes of centrifugation (12,000 × *g*). Equal amounts of total protein were electrophoresed on an SDS-polyacrylamide gel, transferred to a polyvinylidene diflouride membrane, and stained to verify an equal amount of transferred protein from each sample. Western blot was performed using a monoclonal antibody to amino acid residues 571 to 781 of β-catenin (Transduction Laboratories). Hybridization was carried out overnight at 4°C and detected using an anti-mouse IgG-horseradish peroxidase secondary antibody and chemiluminescence.

Results

Immunohistochemistry showed the amino-terminal portion of APC present in tumor tissue, adjacent vascular tissue from the same slides, and normal tissues. The antibody to the carboxyl-terminal portion of the protein stained the cytoplasm of the vascular and normal tissue cells but was absent in the aggressive fibromatosis cytoplasm (Figure 1) in two cases. In two additional cases, there was staining using both the carboxyl-terminal and amino-terminal antibodies. The remaining two cases did not have tissues cryopreserved in OCT available, and the poor preservation of cellular morphology made the results impossible to interpret. Two of the four cases with reliable data contained a truncated protein.

Sequencing data (Figure 2) for exon 15, which contains the mutational cluster region, showed mutations resulting in an early stop codon in three of the six cases. Two cases showed loss of heterozygosity, with the mutation present in both alleles. The third case demonstrated two base substitutions at codons 1492 and 1493, with a mixture of wild-type and mutant sequences observed. Normal tissue DNA from all three

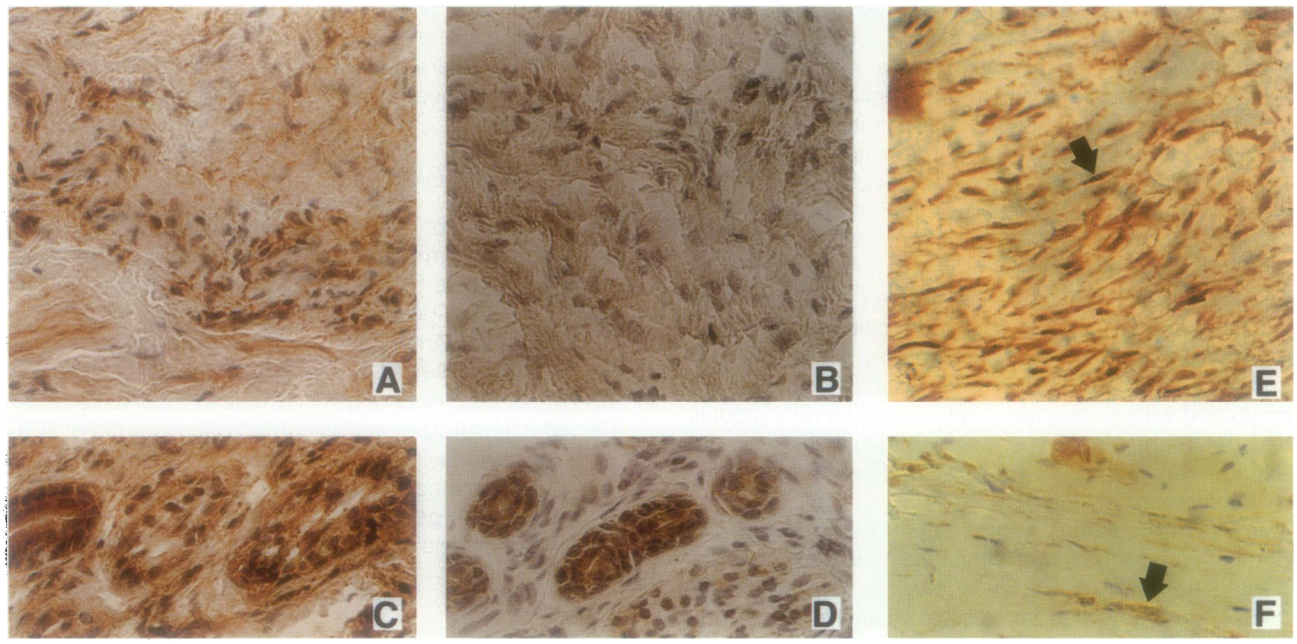

Figure 1. Immunohistochemistry for APC and β-catenin. APC protein was detected using antibodies specific to the amino terminus (**A** and **C**) and carboxyl terminus (**B** and **D**). **C** and **D** are from aggressive fibromatosis tumor tissue. **A** and **B** are from blood vessels, infiltrated by the fibroproliferative process, located on the same slide. Immunostaining (brown color) of the cytoplasm is demonstrated by both the aggressive fibromatosis tissue and vascular tissue for the amino-terminus-specific antibody. Immunostaining for the carboxyl-terminal portion of the protein demonstrates cytoplasmic staining of the vascular tissue but absent staining in the aggressive fibromatosis cytoplasm. This suggests that a truncated APC protein is produced by cells from aggressive fibromatosis. β-Catenin (**E** and **F**) immunohistochemistry showed staining through the cell in tumor tissues (**E**) and staining more in the cell periphery in surrounding normal fascial tissues (**F**). Representative cells are indicated by **arrows**. This same pattern was observed in tumors containing a truncated APC and tumors containing a wild-type sequence.

cases demonstrated only the wild-type sequence. One case showed a frameshift mutation in both alleles at codon 1324 (tumors demonstrate CCCA; wild-type sequence is CCA), resulting in a downstream stop codon (TAG) at codon 1331. The second case demonstrated a frameshift mutation in both alleles at codon 1371 (AT for AGT) with a downstream stop codon (TAA) at 1414. The third demonstrated a substitution of T for G in one allele causing a stop codon (TAA) at 1493. The remaining three cases demonstrated the wild-type sequence. The cases demonstrating a frameshift mutation with loss of heterozygosity showed immunohistochemical staining to only the amino-terminal antibody, whereas two of the three cases with a wild-type sequence showed staining with antibodies

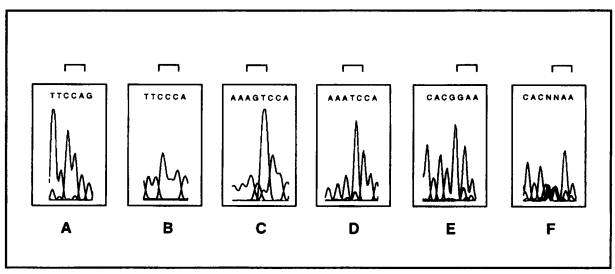

Figure 2. Sequencing data for DNA from aggressive fibromatosis and from normal tissues from the same patients. **A** and **B:** One case showed a frameshift mutation in both alleles at codon 1324 (wild-type sequence is CCA; tumors demonstrate CCC) resulting in a downstream stop codon (TAG) at codon 1331. **C** and **D:** A second case demonstrated a frameshift mutation in both alleles at codon 1371 (AT for AGT) with a downstream stop codon (TAA) at 1414. **E** and **F:** The third case demonstrated a substitution of a T for G in one allele causing a stop codon (TAA) at 1493.

to both the carboxy and amino termini. The remaining two cases did not have adequately preserved tissues available for immunohistochemistry.

Western blot showed that all six cases exhibited increased β-catenin protein relative to adjacent, normal, fibrous tissues (Figure 3). Northern dot blot showed a similar level of β-catenin RNA. The increased protein level, without increased transcription, suggests decreased protein or mRNA degradation. Immunohistochemistry for β-catenin (Figure 1) localized β-catenin in the tumor tissue throughout the cell, whereas in adjacent normal tissues, β-catenin was localized principally in the cell periphery. This localization pattern was the same for tumors containing an APC truncation and for tumors with a wild-type APC sequence.

Reverse transcription PCR demonstrated that the fibromatoses expressed N-cadherin but not E-cadherin, whereas the SW480 colon cancer cell line expressed E-cadherin but not N-cadherin (data not shown). Thus, aggressive fibromatosis expresses the same proteins making up adherens junctions as fibrocytes. All of the cases expressed RNA for exon 15 of APC. The case with both a mutant and wild-type sequence identified also expressed both the mutant and wild-type sequences.

Discussion

All of the tumors exhibited increased β-catenin protein compared with normal tissues, despite containing similar levels of mRNA, suggesting that tumors exhibit de-

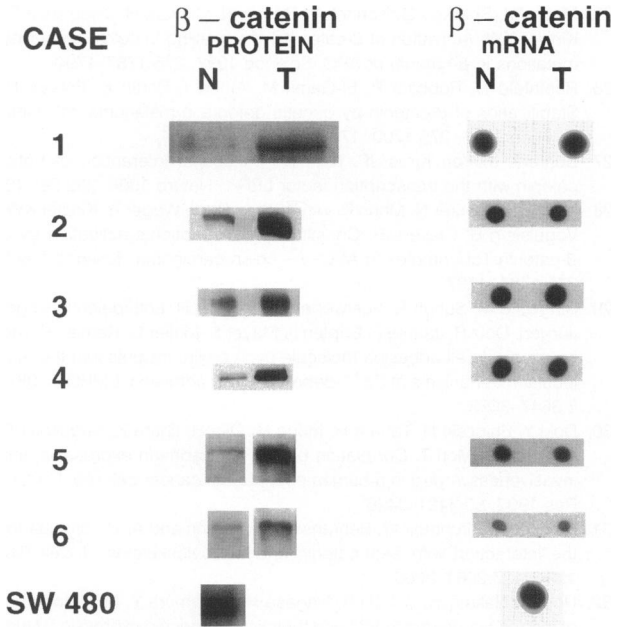

Figure 3. Western blot and Northern dot blots for β-catenin for all six tumors. **Lane N**, loaded with normal tissue; **Lane T**, tumor tissue. Increased β-catenin protein in the tumors compared with normal tissue despite similar mRNA levels is illustrated in all cases. SW 480 is loaded with protein and mRNA from the SW480 colon cancer cell line. The increased protein level in tumors is likely due to decreased degradation.

creased β-catenin protein degradation. Somatic mutations resulting in a truncated APC that are present in some of the tumors likely cause the elevated β-catenin level, as the premature stop codon is located in the mutational cluster region and probably interferes with GSK3β function. In the other cases, an alternative mechanism increasing β-catenin protein, such as a mutation stabilizing β-catenin or activation of Wnt signaling, is likely present.

There are a number of potential roles for β-catenin in neoplasia. β-Catenin is present in normal cells, and in some epithelial cell tumors, it is present at decreased levels compared with normal cells.[39] This decrease is thought to play a role in increased cell mobility by disrupting adherens junctions. In contrast, β-catenin may be elevated in other tumors, such as by APC truncation in colonic neoplasia, by mutations in β-catenin itself, or by Wnt activation. Expression of at least one Wnt is demonstrated in some breast, lung, and prostate carcinomas and some melanoma.[22] Wnt is also expressed at high levels in the benign breast lesion fibroadenoma.[21] Although Wnt activation is not demonstrated by our data, the elevated β-catenin levels and Wnt expression in fibroadenoma suggest a common role for Wnt signaling in fibroproliferative lesions.

Somatic mutations causing APC truncations are also present in a subset of another locally invasive sporadic lesion, colon polyps. In both aggressive fibromatosis and colonic polyps, the APC truncation is likely responsible for the local growth of the tumor cells, as transfection of APC into cells decreases proliferation.[40] There are clinical differences between these two lesions. For instance, unlike aggressive fibromatosis, colonic polyps containing APC truncations progress to malignancy. Another difference between the two lesions is the type of adherens junction present, as aggressive fibromatosis expresses N-cadherin rather than E-cadherin. Thus, the specific epithelial cell adherens junctions are not necessary for APC truncation to allow cell proliferation. The localization of β-catenin throughout the cell in tumor tissues suggests a nuclear role as opposed to a cell membrane role. Thus, APC truncation and elevated β-catenin likely function to alter proliferation through a nuclear mechanism.

Demonstration of somatic APC mutations in one allele with germline mutations in the other allele in abdominal fibromatoses in individuals with FAP gave evidence that the Knudson hypothesis holds true for APC truncation to cause cell proliferation in fibroblasts.[8,9] Despite this, the majority of evidence from colonic neoplasia supports a dominant negative role for APC truncation. Evidence for this is the correlation of site of mutation with disease severity in FAP,[41] the occurrence of mutations in a single allele in sporadic colonic neoplasm (tumors exhibit either a loss of heterozygosity or a mutation in only one allele),[10] and the demonstration that APC forms a dimer of wild-type and mutated protein.[42,43] Unlike the abdominal fibromatoses in FAP, one of the sporadic tumors exhibited both a mutant and wild-type sequence, presumably resulting in both a truncated protein and a full-length product. The two sequences are most likely due to the mutation occurring in only one allele, with a wild-type sequence to the other allele. An alternative explanation for this sequencing result is that there are normal cells within the sample studied. As aggressive fibromatosis is a monoclonal disorder, the tissue studied should be homogeneous and, thus, less likely to contain normal cells. APC has the potential to act in a dominant negative fashion in aggressive fibromatosis. A mutation in a single allele alone, however, is not sufficient to cause aggressive fibromatosis. If this were true, then all individuals with FAP would have the tumor, whereas approximately 10% exhibit the lesion.

Elevated β-catenin protein, caused by a somatic APC mutation resulting in a truncated protein in some cases, is present in sporadic aggressive fibromatosis. β-Catenin protein functions through an epithelial cell adherens-junction-independent mechanism. The localization of β-catenin protein throughout the cell (including the nucleus) further suggests a nuclear role for the elevated β-catenin protein. Somatic mutations resulting in a truncated APC increase cell proliferation and potentially act in a dominant negative manner in fibrocytes as well as epithelial cells. Current treatments for aggressive fibromatosis are less than satisfactory. Adjuvant treatments based on manipulation of cellular growth control may improve the outcome for individuals with this disorder. Perhaps a treatment based on modulation of the function of a truncated APC, or elevated β-catenin, by manipulating a downstream mediator could be devised.

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