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Advances in Brief

β-Catenin Expression Is Altered in Human Colonic Aberrant Crypt Foci[1]

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Abstract

The aberrant expression of β-catenin in colon tumors and the discovery of β-catenin mutations in small adenomas suggest that alterations of β-catenin are early events in human colorectal carcinogenesis. Here, we describe the expression of β-catenin in human aberrant crypt foci (ACF), the earliest identified neoplastic lesions in the colon. Paraffin-embedded sections of 94 ACF, 12 adenomas, and 10 carcinomas were evaluated for β-catenin expression by immunohistochemistry. Normal colonic epithelial cells adjacent to these lesions showed strong membranous expression of β-catenin and lacked cytoplasmic and nuclear expression. Cytoplasmic expression of β-catenin was seen in 25 of 46 ACF with dysplasia and in 2 of 48 ACF with atypia. In ACF with dysplasia, reduced membranous expression of β-catenin was associated with increased nuclear ($P = 0.0013$) and cytoplasmic ($P = 0.0247$) expression. The membranous ($P = 0.0003$) expression of β-catenin was reduced, and the cytoplasmic ($P = 0.0016$) and nuclear ($P = 0.0266$) expressions increased in ACF according to their degree of dysplasia. Likewise, membranous ($P = 0.0007$) expression of β-catenin was reduced, and the cytoplasmic ($P = 0.0050$) and nuclear ($P = 0.0001$) expressions increased from ACF to adenoma to carcinoma. These data suggest that ACF and their aberrant expression of β-catenin play a role in colon tumorigenesis.

Introduction

β-Catenin is a M_r 92,000 protein that originally was found complexed with E-cadherin, α-catenin, and γ-catenin (1); its NH_2-terminal region appears to be necessary for cell-cell adhesion (2). β-Catenin also forms a complex with the protein product of *APC*, glycogen synthase kinase 3β, and conductin (3), which leads to the degradation of β-catenin by proteosomes. When the genes for β-catenin or *APC* are mutated or the *Wnt* signaling pathway is activated, β-catenin accumulates in the cytosol, binds proteins of the T-cell factor family of transcription factors, and moves to the nucleus (reviewed in Refs. 4 and 5). This results in the up-regulation of several genes such as c-*myc* (6), c-*jun*, *fra-1* (4), and cyclin D (7) that may be important in tumorigenesis. Activation of the *APC*/β-catenin pathway plays an important role in colon tumorigenesis (reviewed in Ref. 5), and mutations of *APC* (8) and β-catenin (9) occur early in this process, *i.e.*, by the adenoma stage. In the present study, we determined whether β-catenin expression is altered in human ACF,[3] putative premalignant lesions identified microscopically in unembedded pieces of colon (10). Cytoplasmic expression of β-catenin was the most frequently observed alteration of β-catenin; this was seen in 54% of ACF with dysplasia and in over 80% of tumors.

Materials and Methods

Specimens. Human ACF (78 ACF from 42 patients with sporadic colon cancer and 16 ACF from 6 patients with familial adenomatous polyposis) were identified microscopically in methylene blue-stained preparations of grossly normal colonic mucosa and marked with permanent ink as described previously (11). The ACF had 60 ± 58 (mean ± SD) crypts/focus (range, 7–255 crypts/focus) that had a mean size of 2.0 ± 1.7 mm^2 (range, 0.2–8.3 mm^2). In addition, 10 carcinomas and 12 adenomas were included for comparison. All human colonic tissues were provided by the Western Division of the Cooperative Human Tissue Network of the National Cancer Institute at Case Western Reserve University. The tissues were fixed in phosphate-buffered 10% formalin (Fisher Scientific, Pittsburgh, PA) and embedded in paraffin. Multiple 5-μm sections were cut, mounted on the Superfrost/Plus slides (Fisher Scientific), and stored at either 4°C or room temperature. H&E-stained sections of ACF near to those used for immunostaining were evaluated for atypia or dysplasia (mild, moderate, or severe) as described previously (11).

Immunohistochemical Analysis. Sections were heated at 60°C for 75 min, deparaffinized in xylene twice for 7 min, and rehydrated through graded alcohols. Antigen retrieval was carried out by heating sections in 0.01 M citrate buffer (pH 6.6) in a pressure cooker, as we have done previously (12). Slides stored at 4°C were held at full pressure in a pressure cooker for 3 min; slides kept at room temperature were held at full pressure for 10–15 min to obtain optimal results (membranous expression of β-catenin in normal adjacent colonic mucosa). To prevent nonspecific staining, the sections were incubated in a blocking solution of 10% normal horse serum in PBS [0.01 M phosphate (pH 7.4) and 0.137 M NaCl] for 15 min. Sections were incubated for 1 h at 37°C in a humidified chamber with mouse monoclonal anti-β-catenin antibody (IgG1; Transduction Laboratories, Lexington, KY) diluted 1:2000 in blocking solution. Control sections were incubated with mouse monoclonal anti-bromodeoxyuridine (IgG1; Chemicon, Temecula, CA) or normal horse serum at the same concentration as the primary antibody for a negative control in every set of slides stained. The remaining procedures took place at room temperature. Sections were washed in PBS, incubated for 30 min with biotinylated horse antimouse IgG (Vector Laboratories, Burlingame, CA) diluted 1:200 in blocking solution, and treated with 3% hydrogen peroxide in 30% methanol for 10 min to stop endogenous peroxidase activity. After washing in distilled water, the sections were incubated for 30 min in streptavidin-biotinylated horseradish peroxidase complex (Amersham Corp., Arlington Heights, IL) diluted 1:100 in blocking solution, washed in PBS, and incubated with the substrate, 3′-diaminobenzidine (Sigma Chemical Co.). The slides were counterstained with 0.1% methyl green for 3 min, dried, and mounted with 50% Clearium/50% xylene (Surgipath Medical Industries, Inc., Richmond IL).

Evaluation of the Staining. The membranous expression of β-catenin in the colonic epithelial cells was evaluated as described previously (12, 13). Membranous expression for β-catenin was scored as follows: (*a*) 0, <5% of the epithelial cells in the respective lesions; (*b*) 1, 5–25% of the epithelial cells in the respective lesions; (*c*) 2, 26–50% of the epithelial cells in the respective lesions; (*d*) 3, 51–75% of the epithelial cells in the respective lesions; and (*e*) 4, >75% of the epithelial cells in the respective lesions. The intensity was graded as follows: (*a*) 0, negative; (*b*) 1+, weak; (*c*) 2+, moderate; and (*d*) 3+, strong (as intense as normal mucosa). A final score between 0 and 12 was achieved by multiplication of the extent of positivity and intensity. Scores of 9–12 were defined as "strong expression," scores of 5–8 were defined as "reduced expression," and scores of 0–4 were defined as "markedly reduced expression." Nuclear and cytoplasmic staining for β-catenin was scored as follows: (*a*) 0, negative (no cytoplasmic or nuclear staining, *i.e.*, normal); (*b*)

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[3] The abbreviation used is: ACF, aberrant crypt foci.

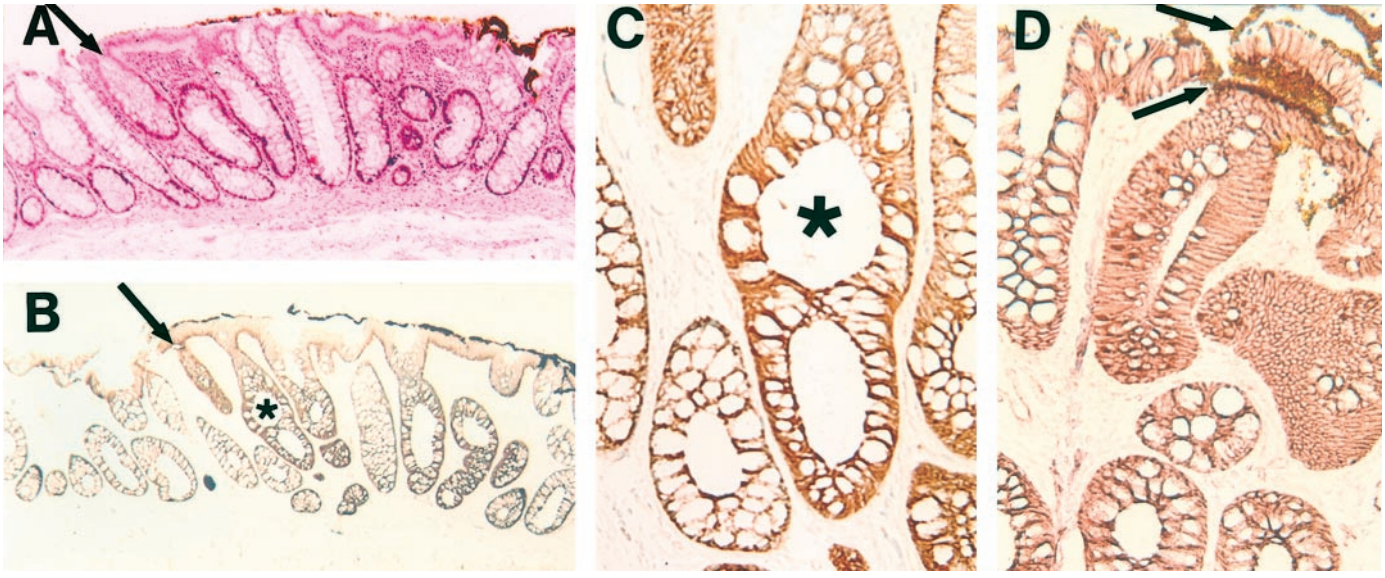

Fig. 1. Expression of β-catenin in human colonic mucosa embedded in paraffin by immunohistochemical staining with a methyl green counterstain. *A*, H&E-stained section of mucosa with an ACF with mild dysplasia marked with *yellow ink* at the *top* and to the *right* of the *arrow* (×50). *B*, a nearby section demonstrating strong cytoplasmic expression of β-catenin in the same ACF marked with *yellow ink* at the *top* and to the *right* of the *arrow* compared with strong membranous expression in the adjacent normal colonic epithelium; an *asterisk* marks the same gland here and in *C* (×50). *C*, a higher magnification of the same ACF shown in *B* (×120). *D*, *yellow ink* at the *top* (*arrows*) marks an ACF with dysplasia that shows nuclear and cytoplasmic expression of β-catenin (×120).

1+, <5% of the epithelial cells in the respective lesions; (*c*) 2+, 5–25% of the epithelial cells in the respective lesions; and (*d*) 3+, >26% of the epithelial cells in the respective lesions.

Statistical Analyses. Poisson log-linear model (SAS Version 6; SAS, Cary, NC) was used to assess trends between increasing dysplasia or histopathology and the expression of β-catenin. Fisher's exact test was used to access the associations between β-catenin expression in different cellular locations. A $P < 0.05$ was considered significant.

Results

***β*-Catenin Expression in Normal Mucosa, Adenomas, and Carcinomas.** Colonic epithelial cells in all histologically normal mucosa adjacent to ACF, adenomas, and carcinomas clearly showed membranous expression of β-catenin from the bottoms to the tops of the crypts; this served as an internal positive control for every sample (Fig. 1). No cytoplasmic or nuclear expression of β-catenin was observed in normal colonic epithelial cells. In adenomas and carcinomas, three phenomena were observed: (*a*) reduced membranous expression of β-catenin; (*b*) the appearance of cytoplasmic expression of β-catenin; and (*c*) the appearance of nuclear expression of β-catenin (Table 1). Fifty-eight percent of adenomas and 60% of carcinomas showed reduced membranous expression of β-catenin; 83% of adenomas and 80% of carcinomas displayed cytoplasmic expression of β-catenin; and 58% of adenomas and 50% of carcinomas showed nuclear expression of β-catenin as observed previously (13).

***β*-Catenin Expression in ACF.** As in tumors, cytoplasmic expression of β-catenin was the most frequent abnormality observed in ACF (Fig. 1; Table 1). Twenty-five of 46 (54.3%) ACF with dysplasia and 2 of 48 (4.2%) ACF with atypia exhibited cytoplasmic expression of β-catenin (Table 2). None of the 48 ACF with atypia displayed alterations in membranous or nuclear expression of β-catenin (Table 2). Of the dysplastic ACF with cytoplasmic expression of β-catenin, six displayed nuclear expression of β-catenin (Fig. 1), and six showed reduced membranous expression. Reduced membranous expression ($P = 0.0003$) and increased cytoplasmic ($P = 0.0016$) and nuclear ($P = 0.0266$) expressions of β-catenin in ACF were associated with advanced degree of dysplasia from mild to moderate to severe dysplasia (Table 2; Poisson log-linear model). Reduced membranous expression of β-catenin was associated with increased nuclear ($P = 0.0013$) and cytoplasmic ($P = 0.0247$) expression of β-catenin in ACF, but it was associated only with nuclear expression of β-catenin ($P < 0.0001$) in adenomas and carcinomas (Table 3; Fisher's exact tests). In addition, reduced membranous expression ($P = 0.0007$) and increased cytoplasmic ($P = 0.0050$) and nuclear ($P = 0.0001$) expressions of β-catenin were associated with progres-

Table 1 *β-Catenin expression in dysplastic ACF, adenomas, and carcinomas*

Membranous expression of β-catenin is shown as the product of the intensity and percentage of colonic epithelial cells with immunohistochemically demonstrable β-catenin in membranes; cytoplasmic and nuclear expressions are scored on the basis of the percentage of colonic epithelial cells with those phenotypes (see "Materials and Methods").

		Membranous[a]			Cytoplasmic[b]				Nuclear[c]		
	Total *N* (%)	9–12[d] (%)	5–8 (%)	0–4 (%)	−[d] (%)	1+ (%)	2+ (%)	3+ (%)	−[d] (%)	1+ (%)	2+ (%)
Dysplastic ACF	46 (100)	87	11	2	46	26	22	6	87	13	0
Adenoma	12 (100)	42	50	8	17	25	33	25	42	25	33
Carcinoma	10 (100)	40	50	10	20	30	10	40	50	30	20

[a] Membranous expression of β-catenin decreases as lesions proceed from dysplastic ACF to adenomas to carcinomas ($P = 0.0007$, Poisson log-linear model).
[b] Cytoplasmic expression of β-catenin increases as lesions proceed from dysplastic ACF to adenomas to carcinomas ($P = 0.0050$, Poisson log-linear model).
[c] Nuclear expression of β-catenin increases as lesions proceed from dysplastic ACF to adenomas to carcinomas ($P = 0.0001$, Poisson log-linear model).
[d] β-Catenin shows strong membranous expression and lacks cytoplasmic and nuclear expression in normal colonic epithelial cells.

Table 2 *β-Catenin expression in colorectal ACF*

Membranous expression of β-catenin is shown as the product of the intensity and percentage of colonic epithelial cells with immunohistochemically demonstrable β-catenin in membranes; cytoplasmic and nuclear expressions are scored on the basis of the percentage of colonic epithelial cells with those phenotypes (see "Materials and Methods").

		Membranous[a]			Cytoplasmic[b]				Nuclear[c]		
	Total ACF *N* (%)	9–12[d] (%)	5–8 (%)	0–4 (%)	−[d] (%)	1+ (%)	2+ (%)	3+ (%)	−[d] (%)	1+ (%)	2+ (%)
Atypia	48 (100)	100	0	0	96	4	0	0	100	0	0
Dysplasia	46 (100)	87	11	2	46	26	22	6	87	13	0
Mild	34 (100)	97	3	0	59	26	9	6	94	6	0
Moderate	7 (100)	86	14	0	14	29	43	14	71	29	0
Severe	5 (100)	20	60	20	0	20	80	0	60	40	0

[a] Membranous expression of β-catenin in ACF decreases as lesions proceed from mild to moderate to severe dysplasia ($P = 0.0003$, Poisson log-linear model).
[b] Cytoplasmic expression of β-catenin in ACF increases as lesions proceed from mild to moderate to severe dysplasia ($P = 0.0016$, Poisson log-linear model).
[c] Nuclear expression of β-catenin in ACF increases as lesions proceed from mild to moderate to severe dysplasia ($P = 0.0266$, Poisson log-linear model).
[d] β-Catenin shows strong membranous expression and lacks cytoplasmic and nuclear expression in normal colonic epithelial cells.

sive deviation from normal, *i.e.*, from ACF to adenoma to carcinoma (Table 1; Poisson log-linear model).

Discussion

ACF in humans, identified microscopically in grossly normal colonic mucosa, are monoclonal proliferations (10) that exhibit varying degrees of pathology from minor atypia to severe dysplasia (11). The discovery of aberrant expression of β-catenin in a significant proportion (54%) of dysplastic human ACF suggests that dysfunction of β-catenin is a very early event in the development of human colonic tumors. The fact that all three phenotypic alterations (*i.e.*, decreased expression of membranous β-catenin, increased cytoplasmic β-catenin, and increased nuclear β-catenin) increased significantly as dysplasia increased suggests that each of these phenotypic alterations reflects the role of β-catenin in the progression of ACF. In addition, these phenotypic alterations were further increased with progression from dysplastic ACF to adenoma to carcinoma (Table 1). These data extend the observations of Hao *et al.* (13), who reported a significant increase in nuclear expression and a decrease in membranous expression of β-catenin in carcinomas compared with their adjacent adenomas.

In addition to nuclear expression of β-catenin in colorectal carcinomas (13–15) and adenomas (13, 15, 16), some studies have previously reported reduced membranous expression of β-catenin (13, 16) and cytoplasmic expression of β-catenin (15, 16) in tumors. In a recent study of abnormal expression of P-cadherin in 23 human ACF, 5 ACF were found with cytoplasmic expression of β-catenin (17). Cytoplasmic β-catenin expression has also been reported in dysplastic lesions in azoxymethane-treated rats (18) that appear to us to be dysplastic ACF (19).

The mechanisms leading to the cytoplasmic accumulation and nuclear expression of β-catenin are unclear. Mutations of *APC* or the gene for β-catenin occur in approximately 80% of human colon cancers and are known to result in cytoplasmic accumulation of β-catenin (reviewed in Ref. 5). The current data available suggest that these mutations are unlikely to account for the β-catenin accumulation that we noted in ACF because mutated *APC* has been found only rarely in human ACF (20, 21), and only a small percentage of human colonic tumors harbor β-catenin mutations (9). More recently, Iwamoto *et al.* (15) reported immunohistochemically demonstrable β-catenin expression in both the cytoplasm and nucleus of "100% of the cells in all [58] of the [human] adenomatous polyps" examined. Immunohistochemically demonstrable APC protein was seen in 62% of these same polyps, and only 29% of the polyps showed a complete absence of APC protein (15). In both those polyps (15) and ACF (20, 21), a loss of normal APC protein does not appear to account for most of the dysregulation of β-catenin observed. However, until mutational and immunohistochemical assays with similar sensitivities are performed on the same ACF for *APC* and β-catenin, this question cannot be resolved fully.

Table 3 *Associations of membranous expression of β-catenin with cytoplasmic and nuclear expression of β-catenin*

	Dysplastic ACF[a]			Adenomas and carcinomas[b]		
		Membranous expression			Membranous expression	
	Total	Normal	Reduced	Total	Normal	Reduced
Total	46	40	6	22	9	13
Cytoplasmic normal	21	21	0	4	3	1
Cytoplasmic increased	25	19	6	18	6	12
Nuclear normal	40	38	2	10	9	1
Nuclear increased	6	2	4	12	0	12

[a] Membranous expression of β-catenin is associated with cytoplasmic ($P = 0.0247$) and nuclear ($P = 0.0013$) expression of β-catenin in dysplastic ACF.
[b] Membranous expression of β-catenin is associated only with nuclear ($P < 0.0001$) and not with cytoplasmic ($P = 0.2643$) expression of β-catenin in adenomas and carcinomas.

As suggested by Uthoff *et al.* (22), *Wnt* proteins and earlier members of the *Wnt* signaling pathway could control β-catenin expression and may be involved in human colon tumorigenesis. Also, nitric oxide has been demonstrated to enhance the level of cytoplasmic and nuclear β-catenin in mouse colonic epithelial cells in culture (23) and may account for some of the modulation of β-catenin that we observed. Inducible nitric oxide synthase, the enzyme that is mainly responsible for the production of nitric oxide, was reduced in the epithelial cells of >50% of human colon tumors and ACF studied (12); however, an additional source of the nitric oxide could be the stromal cells that are frequently present in high numbers in colonic mucosa and cancers.

The demonstration of cytoplasmic expression of β-catenin in 54% of dysplastic human ACF and its association with increasing dysplasia suggest that this is one of the earliest alterations in human colon tumorigenesis. Whereas the current data suggest that mutations in the *APC* or β-catenin genes are unlikely to be mechanisms that control this aberration in ACF, the role of these mutations cannot be ruled out until mutational and immunohistochemical studies with similar sensitivities are carried out on the same ACF.

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