Prognostic and Diagnostic Significance of β-Catenin Nuclear Immunostaining in Colorectal Cancer

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ABSTRACT

In the present study, we investigated the prognostic and diagnostic significance of β-catenin nuclear immunostaining in 60 specimens of normal colorectal tissue; 180 specimens of colorectal polyps, adenomas, and carcinomas; and 40 specimens from patients with the simultaneous occurrence of polyps, adenomas, and carcinomas. Additional specimens from 59 patients with colorectal carcinoma and 14 patients with adenoma who subsequently developed carcinoma were examined for possible survival study. Immunohistochemical staining showed that the occurrence of nuclear β-catenin correlated with the sequential stages in colorectal carcinogenesis, in which positive staining was observed in 0% of normal tissues, 8% of polyps, 92% of adenomas, and 100% of carcinomas. High immunohistochemical scores in colorectal carcinoma were significantly associated with lymph node metastasis and poor survival. Adenomas associated with synchronous or metachronous carcinomas showed significantly higher levels of nuclear β-catenin compared with adenomas without associated carcinomas. Nuclear translocation of β-catenin was rare or absent in other types of cytokeratin 20 positive adenocarcinomas examined (99 cases). Thus, it was positive in only 7% of colonic mucinous adenocarcinomas, 3% of pancreatic adenocarcinomas, 8% of ovarian mucinous cystadenocarcinomas, and 0% of gastric adenocarcinomas. However, 100% of primary and metastatic colorectal adenocarcinomas were positive for nuclear staining for β-catenin. Thus, nuclear staining for β-catenin may serve as an additional parameter to help distinguish colorectal adenocarcinomas from adenocarcinomas of other tissue sites. Collectively, the present large-scale study has clearly addressed the clinical significance of β-catenin nuclear translocation with respect to tumor progression, survival, and differential diagnosis.

INTRODUCTION

The pathogenesis of colorectal cancer is a multistage process, starting as a benign polyp and subsequently progressing into adenoma and carcinoma. Throughout this process, several tumor suppressor genes and oncogenes are deleted or mutated sequentially (1). Among them, *APC*, the gene implicated in the genetic predisposition to familial adenomatous polyposis (FAP), is considered the most important "genome safeguard" for normal colonic tissues (2, 3). Intense screenings have revealed that the *APC* gene was mutated in ~80% of cases of both FAP and sporadic colon cancers (3, 4). Although the *APC* was identified in 1991 (4, 5), the precise functional significance of *APC* in tumorigenicity was not clear until the recent recognition of the β-catenin protein as the key mediator of Wnt signaling (6, 7). It is now well recognized that mutations in *APC* often lead to aberrant accumulation and deregulation of β-catenin signaling.

β-Catenin, a multifunctional protein, plays a dual role in the cell (8). It was first identified as a protein associated with E-cadherin in maintaining cell-to-cell interactions. In an apparently independent role in the Wnt signal transduction pathway, β-catenin acts as a transcription factor (7). Under normal conditions, β-catenin is under rigorous control of the upstream regulators of the Wnt-signaling cascade. At the cell surface, the interaction of Wnt and its frizzled receptor triggers activation of disheveled, which in turn inactivates glycogen synthase kinase-3β. When glycogen synthase kinase-3β is inactivated, it fails to phosphorylate the NH_2 terminus of β-catenin. The unphosphorylated β-catenin is not able to complex with *APC* to form an ubiquitin-mediated proteolytic complex. As a result, β-catenin accumulates in the cytoplasm, and presumably, this leads to translocation of this protein into the nucleus where it interacts with the DNA-binding T-cell factor complex and acts as a transcriptional activator, thus turning on target genes, including c-*myc* and cyclin D1 in the case of colorectal cancer (9, 10). Therefore, amplification of the *wnt* gene, mutations in *APC*, or mutations in β-catenin at sites of phosphorylation can activate β-catenin signaling, and thereby stimulate neoplastic growth. In addition to colorectal cancer, β-catenin deregulation is thought to also be a frequent event in a wide variety of tumors, including melanoma, liver cancer, prostate cancer, ovarian cancer, uterine endometrial cancer, medulloblastoma, pilamatricoma, and anaplastic thyroid cancers (7).

Although β-catenin signaling appears to play an important role in colorectal carcinogenesis, the contribution of β-catenin nuclear translocation to tumor progression is not known with certainty. In recent years, most of the studies have focused on the mutational events (11, 12), but a few have addressed the specific issue of nuclear translocation of β-catenin. Early studies suggested that nuclear translocation of β-catenin occurs early in

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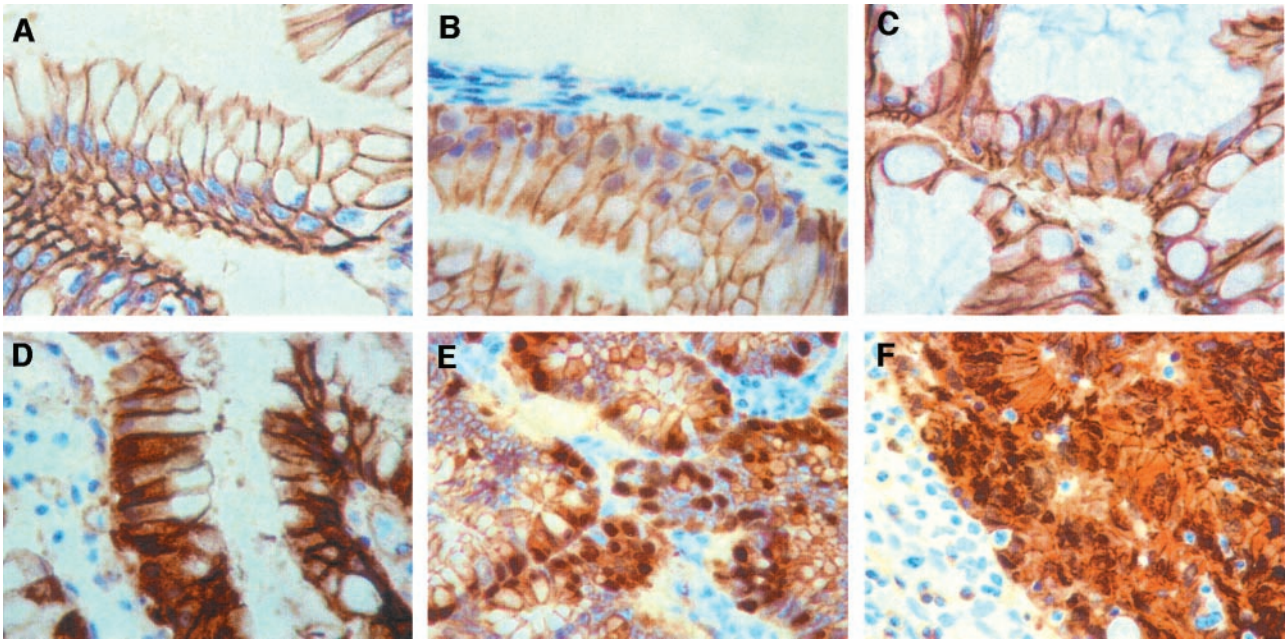

Fig. 1 Immunostaining with β-catenin monoclonal antibody showing: *A,* normal colonic glandular epithelium with discrete membranous staining; *B,* glandular epithelium of hyperplastic polyp with weak nuclear staining; *C,* glandular epithelium of a nonadenomatous polyp with membranous and weak cytoplasmic stain; *D,* glandular epithelium of Peutz-Jeghers polyp with intense nuclear staining; *E,* dysplastic glandular epithelium of adenoma with nuclear staining; *F,* moderately differentiated adenocarcinoma with prominent nuclear stain; *G,* weak nuclear staining in colonic mucinous adenocarcinoma; *H–J,* negative nuclear staining in stomach adenocarcinoma, pancreas adenocarcinoma, and ovarian mucinous cystadenocarcinoma, respectively. Magnification: *A–D,* ×600; *E–J,* ×400.

colorectal adenomas in patients with colonic polyps and FAP (13, 14). However, in a later study, Samowitz *et al.* (15), on screening 202 sporadic colon tumors, found that β-catenin mutation is more common in small adenomas (12.5%) than in large adenomas (2.4%) and invasive tumors (1.4%). Therefore, based on their mutation analysis, they concluded that small adenomas with β-catenin do not appear to progress into larger adenomas and invasive carcinomas (15). On the other hand, a recent study suggests that nuclear translocation of β-catenin might be involved in the development of intramucosal and invasive colonic cancer but not adenomas (16).

To systematically address the involvement of β-catenin nuclear translocation in colorectal carcinogenesis, 60 specimens each of normal colorectal tissues, nonadenomatous polyps (referred to as polyps in the text), adenomas, and carcinomas were investigated. To additionally examine the correlation between β-catenin nuclear translocation and colorectal carcinogenesis, 40 additional specimens, obtained from patients who displayed the simultaneous occurrence of polyps, adenomas, and carcinomas, were also examined. Furthermore, the relationship among nuclear β-catenin, tumor metastasis, and survival rate was studied in 73 patients with colorectal carcinoma.

This study also investigated the potential value of nuclear β-catenin staining as a diagnostic marker in colorectal cancer. Currently, a cytokeratin 7 negative (CK7−)/cytokeratin 20 positive (CK20+) immunophenotype is often used to support the diagnosis of colorectal carcinoma for adenocarcinoma of uncertain origin (17, 18). CK7 labels a wide range of normal and cancerous epithelial cells, but not those of the gastrointestinal tract, whereas CK20 is selectively distributed in gastrointestinal epithelia, urothelia, Merkel cells, and tumors arising from these cells. Whereas a CK7−/CK20+ immunophenotype is characteristic of colorectal carcinomas, up to 25% of cases show a different immunophenotype, such as CK7+/CK20+, and other types of adenocarcinomas can show a similar immunophenotypic profile (17–22). To evaluate whether nuclear β-catenin can be used as an adjunct marker for distinguishing colorectal carcinomas from adenocarcinomas at other organ sites, CK+ tumors were selected for examination. These included 30 cases of colonic mucinous, 30 cases of gastric, 27 cases of pancreatic, and 12 cases of ovarian mucinous adenocarcinomas. The data obtained on nuclear staining for β-catenin were compared with those obtained with colorectal carcinoma used in the first part of the present study.

MATERIALS AND METHODS

Tissue Samples. In the first part of study, we examined two separate groups of specimens obtained from the Department of Pathology, Queen Elizabeth Hospital. Group 1 specimens consisted of 240 formalin-fixed, paraffin-embedded specimens, with 60 each of normal colorectal tissues, polyps (nonadenomatous polyps), adenomas, and carcinomas. The term "polyps" used in the present study is specifically defined as non-neoplastic or nonadenomatous polyps. Group 2 included specimens obtained from 40 patients with simultaneous occurrence of polyp, adenoma, and colorectal carcinoma. Both group 1 and 2 specimens were obtained during the period of 1998 to 2000.

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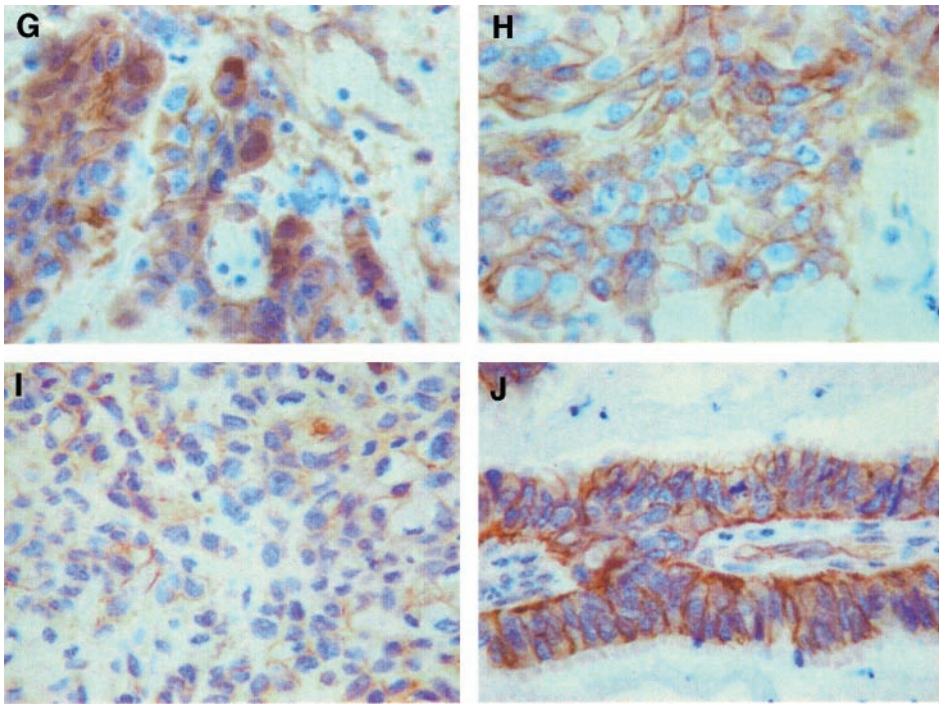

Fig. 1 Continued.

There was not enough data to do survival analysis. To study the association between survival and β-catenin expression, 59 colorectal carcinomas and 14 adenoma specimens from patients with subsequent development of carcinoma were retrieved from the archives of 1994 to 1997 (designated as group 3 specimens).

The polyps evaluated were juvenile (31%), hyperplastic (51%), Peutz-Jeghers (6%), and inflammatory (12%) polyps. Of the 100 cases with polyps, there was equal number of male and female patients, whereas the age distribution was 6, 19, 19, 25, and 31% for ages 21–30, 41–50, 51–60, 61–70, and 71–80, respectively. Of the 114 adenoma specimens examined, 63% was tubular, 34% was tubulovillous, and 3% was villous adenomas. The percentages of mild, moderate, and severe dysplasia in adenomas were 18, 62, and 20, respectively. The sex of the adenoma patients was at 1:1 distribution, and the age distribution was 3, 8, 21, 23, 37, 5, and 3% for ages 21–30, 41–50, 51–60, 61–70, 71–80, 81–90, and 91–100 respectively. Most cases of the adenocarcinomas were moderately differentiated (82%), whereas the rest were either well differentiated (3%) or poorly differentiated (15%). The sex distribution of adenocarcinoma patients was 61% male and 39% female. The age distribution was 5% for ages 41–50, 3% for ages 51–60, 29% for ages 61–70, 47% for ages 71–80, and 16% for ages 81–90.

In the second part of the study, adenocarcinomas of different organ origins, including 30 colonic mucinous adenocarcinomas, 30 gastric adenocarcinomas, 27 pancreatic adenocarcinomas, and 12 ovarian mucinous adenocarcinomas, were examined for β-catenin translocation.

Antibody. Monoclonal antibody to β-catenin (C19220) was purchased from Transduction Laboratories. The antibody, reactive to β-catenin of human, rat, and mouse species, was produced against the COOH terminus of a mouse β-catenin protein.

Immunohistochemical Staining and Evaluation. The staining procedures were carried out according Wong *et al.* (23). In brief, tissue sections of 4 μm thickness were placed on silane-coated (Sigma Chemicals, St. Louis, MO) glass slides, air dried overnight, and rehydrated with xylene and graded alcohol. Antigen retrieval was achieved by boiling the tissue sections in EDTA buffer (pH 8.0) in a pressure cooker for 2.5 min. After cooling in running water for 30 min, the immunochemical staining was performed in a Ventana-ES automated immunostainer at 37°C (Ventana, Tucson, AZ). Subsequent preparatory steps included inhibitor to quench endogenous peroxidase activity, β-catenin antibody at 1:200 dilution for 32 min, biotin-labeled secondary antibody, streptavidin-biotin peroxidase complex, diaminobenzidine tetrahydrochloride with hydrogen peroxide, and copper sulfate for enhancement of color. The sections were counterstained with Harris hematoxylin and mounted with Permount after dehydration in graded alcohol. Negative controls were done by replacing the primary antibodies with Tris-buffered saline for each tumor specimen. Positive signals were evaluated in four fields (~1000 cells) under a light microscope at 10 × 40 magnification, without knowledge of the clinical outcome by two independent teams of observers, including two pathologists (K. C. L. and J. K. C. C.). The scoring method was performed according to Mauri *et al.* (24) with slight modification. In brief, the staining intensity was scored as follows: 0 = no expression, 1 + = weak expression, 2 + = moderate expression, 3 + = strong expression, and 4 + = very strong expression. The final score was expressed as immunohistochemical staining score (IHC score) obtained by multiply-

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ing the percentage of positive cells (or nuclei) with the staining intensity according to Mauri *et al.* (24). This scoring method has been widely used to evaluate the results of immunohistochemistry staining (25–28).

Statistical Analysis. Statistical analysis was performed using the SPSS 10.0 software (SPSS Inc.). The correlation between β-catenin IHC scores and stages of progression of colorectal tumors was analyzed by Spearman's rank analysis, for which normal distribution is not a prerequisite. The χ^2 test was used to analyze the statistical significance of the relationship between β-catenin nuclear translocation and lymph node metastasis in colorectal carcinomas. The Mann-Whitney U test was used to analyze the correlation of the IHC score of β-catenin and the patient survival rate.

RESULTS

Level of Nuclear β-Catenin Expression Correlates with Stages of Progression of Colorectal Cancer. Immunostaining of group 1 specimens (Fig. 1, *A–F*) showed that 100% (60 of 60) of colorectal carcinomas and 92% (55 of 60) of colorectal adenomas were positive for nuclear staining of β-catenin (case was considered positive for β-catenin when the percentage of positive cells was ≥10% in our study). Only 8% (5 of 60) of the colorectal polyps was positive for nuclear β-catenin, and all of the positive cases were either Peutz-Jeghers (3 of 60) or hyperplastic polyps (2 of 60). Among cases with β-catenin nuclear translocation, the adenomas showed fewer positive cells and weaker staining compared with the carcinomas (Fig. 1, *E* and *F*). The normal colorectal tissues did not display nuclear or cytoplasmic, but rather membranous localization of β-catenin (Fig. 1*A*).

The mean nuclear β-catenin IHC scores were highly correlated with stages of progression, from normal epithelia, to polyp, adenoma, and carcinoma (Fig. 2*A*). Similar results were obtained for group 2 specimens with simultaneous occurrence of polyp, adenoma, and carcinoma. Nuclear staining for β-catenin was observed in 100% (40 of 40) of the carcinomas, 90% (36 of 40) of the adenomas, and 8% (3 of 40) of polyps that showed weak to moderate staining. (Fig. 1, *B* and *C*). The IHC scores are shown in Fig. 2*B*. Of interest, the IHC scores of adenomas with synchronous carcinomas were significantly higher than those of pure adenomas (Fig. 2, *A* and *B*). Statistical analysis showed that nuclear translocation signals were highly correlated with the stages of progression of colorectal cancer ($r = 0.875$, $P < 0.0001$ for group 1 specimens; and $r = 0.723$, $P < 0.0001$ for group 2 specimens). The nuclear β-catenin IHC scores did not correlate with age or histology or the degree of dysplasia of adenomas evaluated in the present study

Relationship between Membrane, Cytoplasmic, and Nuclear β-Catenin, and Stages of Progression of Colorectal Cancer. To additionally examine the significance of subcellular distribution of β-catenin in colorectal cancer development, we compared group 1 and group 3 specimens (retrieved from the archives of 1994 to 1997) for membranous, cytoplasmic and nuclear staining. In general, the levels of β-catenin in all three of the subcellular localizations were elevated from normal to polyp, adenoma, and carcinoma (Table 1 and Fig. 3). Statistically, all of the subcellular expressions of β-catenin were significantly associated with stages of progression of colorectal cancer (Table 1); however, the most striking difference was observed with nuclear β-catenin staining in carcinomas *versus* adenomas and polyps (Fig. 3).

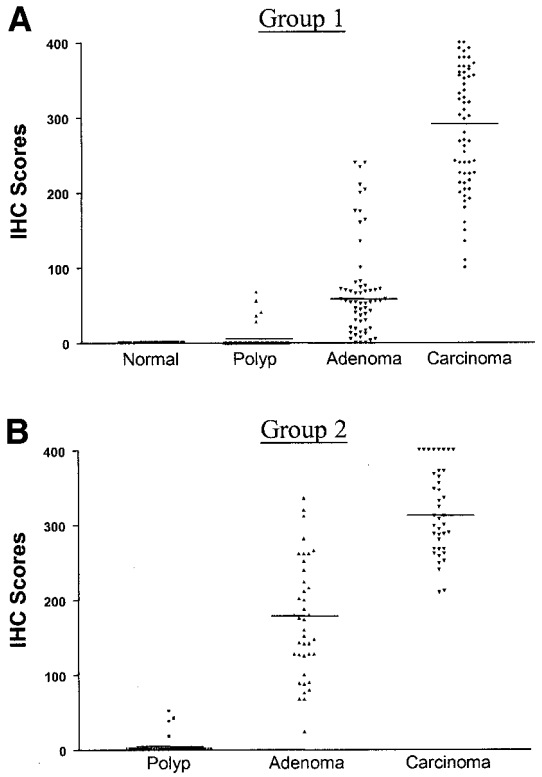

Fig. 2 The mean immunohistochemical staining scores (*IHC scores*) of nuclear β-catenin staining in (*A*) 60 paraffin-embedded group 1 specimens each of normal colorectal tissues, polyps, adenomas, and colorectal carcinomas; *B*, 40 paraffin-embedded group 2 specimens with simultaneous occurring polyp, adenoma and colorectal carcinoma. IHC scores = percentage of positive cells × staining intensity (for details, see "Materials and Methods").

Correlation between Nuclear β-Catenin and Lymph Node Metastasis. To explore the prognostic significance of β-catenin translocation in colorectal carcinomas, the lymph node involvement, one of the most important prognostic markers for colon cancer, was analyzed among group 1 carcinoma patients who had IHC scores >300 and those who had IHC scores <200 for nuclear β-catenin. Results showed that 85% (23 in 27 cases) of the high IHC group but only 17% (2 in 12 cases) of the low IHC group showed lymph node involvement. The association between β-catenin nuclear signal and lymph node metastasis was highly significant by χ^2 test ($\chi^2 = 16.99$; $P = 0.003$).

To verify the correlation between lymph node involvement and the level of nuclear β-catenin expression observed with group 1 specimens, analysis was also performed on group 3 samples. Among the 59 patients, 46 showed metastasis to lymph nodes, whereas 13 did not. The mean nuclear β-catenin IHC scores were 310 and 194, respectively ($P < 0.001$), for those with and without metastasis (Fig. 4*A*).

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Table 1 Relationships between subcellular β-catenin expression and the stages of progression of colorectal cancer[a]
The correlation coefficiency (r) and *P* value were calculated by Spearman's rank analysis.

	Membranous β-catenin	Cytoplasmic β-catenin	Nuclear β-catenin
	Mean IHC ± SEM	Mean IHC ± SEM	Mean IHC ± SEM
Normal[b] n = 60	102 ± 3	57 ± 3	0 ± 0
Polyp[b] n = 60	211 ± 15	108 ± 13	0 ± 0
Adenoma[b] n = 60	220 ± 8	128 ± 9	57 ± 15
Carcinoma I[b] n = 60	282 ± 13	226 ± 15	293 ± 14
Carcinoma II[c] n = 59	324 ± 12	243 ± 13	327 ± 19
Spearman's rank correlation	r = 0.7779 $P < 0.0001$	r = 0.7598 $P < 0.0001$	r = 0.8753 $P < 0.0001$

[a] Stages of progression refers to normal-polyp-adenoma-carcinoma sequence of colorectal carcinogenesis.
[b] Obtained from Group 1 specimens.
[c] Obtained from Group 3 specimens.

Correlation between Nuclear β-Catenin and Survival. Because follow-up duration was short for both group 1 and group 2 patients (diagnosed between 1998 and 2000), we retrieved specimens of 59 colorectal cancer patients diagnosed from 1994 to 1997 for survival analysis. Thirty one of these patients have died, and 28 are still alive; of the survivors, 79% (22 of 28) are relapse-free, and 21% (6 of 28) have relapsed. The mean nuclear β-catenin IHC score was substantially lower among the survivors (IHC was 125 for the entire group and 121 for the relapse-free group) than those who died of disease (IHC = 293; Fig. 4*B*). Thus, the correlation between nuclear β-catenin staining and survival rate is highly significant when analyzed by the nonparametric Mann Whitney *U* test ($P < 0.0001$). The nuclear β-catenin IHC score did not correlate with Dukes' stage ($r = 0.04924$; $P = 0.7369$) or age ($r = 0.1379$; $P = 0.3448$) using multivariate Spearman's rank analysis. The lack of correlation between the nuclear β-catenin IHC score and Dukes' stage is due to fact that most of the carcinoma specimens were strongly positive for nuclear translocation.

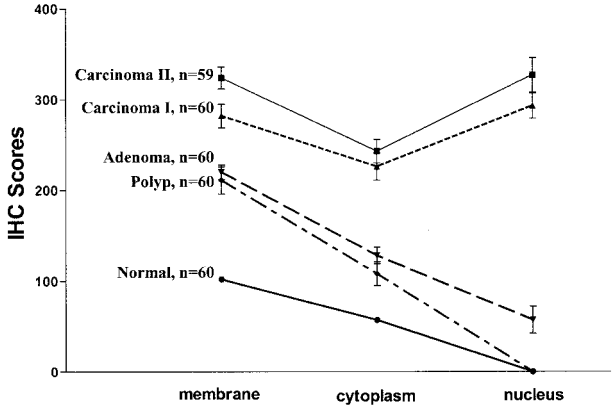

Fig. 3 Relationships between subcellular distribution of β-catenin and stages of progression of colorectal cancer. The graphs were drawn based on the mean immunohistochemical staining (*IHC scores*) presented in Table 1. *n*, number of cases in each group of specimens. IHC scores = percentage of positive cells × staining intensity; *bars*, ±SD.

High Level of Nuclear β-Catenin in Adenomas May Be Associated with Disease Progression. To additionally explore the significance of nuclear translocation of β-catenin as a prognostic marker in adenomas, we examined 14 adenoma specimens from patients who later developed carcinomas. All but one of the adenomas showed a high level of nuclear β-catenin with IHC scores ranging from 50 to 255 (Fig. 4*C*). The mean IHC score of all 14 of the specimens was 132, which is high when compared with a mean score of 57 for adenomas from group 1, but is comparable with that for group 2 patients (mean IHC score = 180; Fig. 2). It is worth noting that the case with the highest IHC score (= 255) was derived from a 32-year-old patient with FAP.

β-Catenin Nuclear Translocation in Other Types of Adenocarcinoma. To investigate β-catenin translocation in other types of adenocarcinoma and to compare it with that in colorectal tumors, we selected a total 99 CK20+ tumor specimens and subjected them to β-catenin staining. Results showed that 2 in 30 (7%) colonic mucinous adenocarcinomas, 1 in 27 (4%) pancreatic adenocarcinomas, 1 in 12 (8%) ovarian mucinous cystadenocarcinomas, and none of the gastric adenocarcinoma showed positive β-catenin signals in the nucleus (Fig. 1, *G–J;* Fig. 5). In the sole positive case of pancreatic and ovarian mucinous tumors, β-catenin was focally present in only a small percentage (~10%) of the cells. The results contrasted with positive nuclear staining for β-catenin in all cases of conventional colorectal adenocarcinomas (Fig. 1*F*), often with high intensity of staining.

In view of the above results, we additionally analyzed β-catenin expression in 18 cases of metastatic colorectal carcinoma, including 3 in lymph nodes, 2 in bladders, 3 in vaginas, 2 in pericardium, 3 in uterus, 2 in peritoneum, and 3 in liver. All of the cases were strongly positive for β-catenin, indicating persistent nuclear labeling for β-catenin in metastatic deposits.

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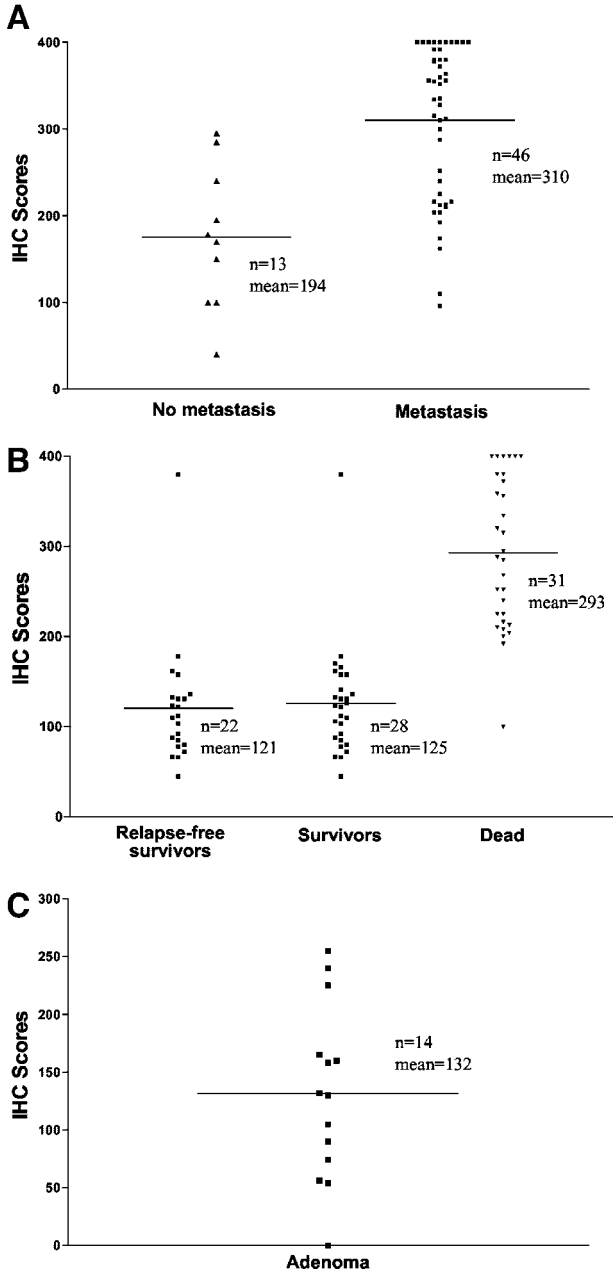

Fig. 4 Correlations between nuclear expression of β-catenin with (*A*) metastasis and (*B*) survival in colorectal carcinomas. A total of 59 specimens from patients diagnosed from 1994 to 1997 were analyzed. *C*, levels of nuclear β-catenin in adenomas from patients diagnosed with carcinomas in subsequent years. *n*, number of cases in each group of specimens. IHC scores = percentage of positive cells × staining intensity.

DISCUSSION

β-Catenin is currently believed to be involved in the development of colorectal cancer. Aberrant expression of the *APC* gene and mutations at the site of phosphorylation of β-catenin are the two major factors that account for the overexpression and subsequent cytoplasmic/nuclear translocation of β-catenin found in colorectal carcinoma cells. According to *in vivo* and *in vitro* data collected by others and us (23, 26), the accumulation of cytoplasmic β-catenin does not always correlate with nuclear expression of the protein. Because the nuclear entry of β-catenin is an obvious step required for gene activation induced by the β-catenin/T-cell factor/Lef-1 transcriptional complex, we used nuclear staining as primary evidence for β-catenin overexpression. Our data revealed a remarkable correlation between β-catenin nuclear translocation and the purported sequential stages of colorectal cancer development in both Group 1 (240 cases) and group 2 (40 cases) specimens ($P < 0.0001$ for both groups; Fig. 2, *A* and *B*). In that, 8% (8 of 100) of nonadenomatous polyps, 91% (91 of 100) of adenomas, and 100% (100 of 100) of adenocarcinomas showed β-catenin nuclear translocation. Our results are in agreement with those reported by Valizadeh *et al.* (14), who confined his study to colorectal polyps and found that 5% (2 of 40) of nonadenomatous polyps and 65% (13 of 20) of adenomas cases with intense nuclear stain; and by Hao *et al.* (29), who found that among the sporadic colorectal cancer patients, 45.9% (34 of 74) of adenomas and 84.1% (44 of 52) of adenocarcinomas displayed nuclear staining. The discrepancy in percentage of positive nuclear signals may be related to the differences in antigen retrieval and staining procedures used by each laboratory. In our study, there is no significant correlation among nuclear expression of β-catenin, degree of dysplasia, histology, or age of patients. Similar findings were observed by Valizadeh *et al.* (14). The failure of Kobayashi *et al.* (16) to

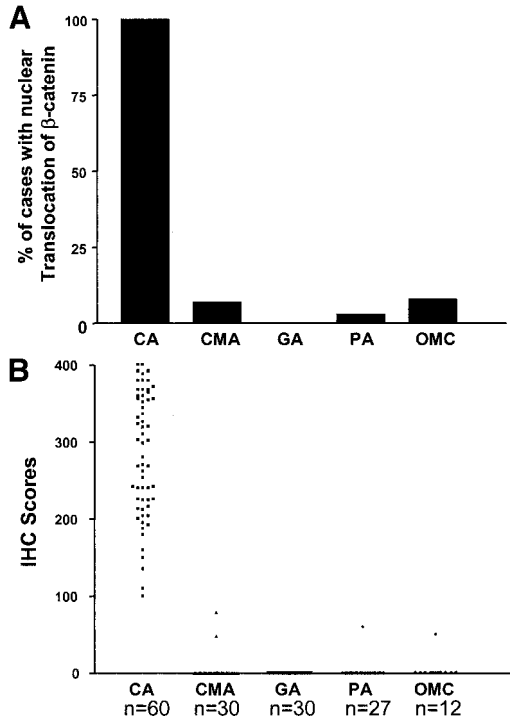

Fig. 5 Comparisons of the (*A*) percentage and (*B*) immunohistochemical staining (*IHC scores*) of β-catenin nuclear signals among cytokeratin 20 positive adenocarcinomas, including colorectal adenocarcinomas (*CA*), colonic mucinous adenocarcinoma (*CMA*), gastric adenocarcinoma (*GA*), pancreas adenocarcinoma (*PA*), and ovarian mucinous cystadenocarcinoma (*OMC*). *n*, number of cases in each group of specimens.

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find β-catenin nuclear translocation in colorectal tubular adenomas from either sporadic or FAP cases remains unclear. However, when we tested our positive adenoma specimens using the antigen retrieval procedures according to Kobayashi *et al.* (16), an extremely weak nuclear signal was obtained. This result may explain, at least in part, the lower nuclear β-catenin signal observed in their studies. The other possible explanation for the disparity may be because of the difference in histological interpretations of clinical specimens by pathologists of different regions (30). The specimens used in the study by Kobayashi *et al.* (16) might belong to the early stage of adenomas. In our study, the majority of the nonadenomatous polyps were negative for nuclear staining.

The other interesting finding is that the mean IHC score (=180) of the adenomas associated with synchronous or metachronous carcinomas (Fig. 2*B*) is much higher than that of the group consisting of adenomas without synchronous carcinomas (mean IHC score = 55; Fig. 2*A*), suggesting that intense β-catenin expression in the nucleus of adenomas may signify a higher malignancy potential. This hypothesis is substantiated by the 14 additional adenoma specimens, each of which came from an individual patient who subsequently developed colorectal carcinomas; 13 of 14 show high levels of nuclear signal for β-catenin with scores ranging from 50 to 255, with a mean of 132 (Fig. 4*C*).

In addition to the nuclear signal, we also demonstrated that both membranous and cytoplasmic β-catenin signals steadily increased through the purported stages of colorectal carcinogenesis. High levels of membranous and cytoplasmic β-catenin were detected as early as in the nonadenomatous polyp stage. The nuclear translocation, on the other hand, began at a later stage, when adenoma supersedes and is additionally escalated into carcinoma. This view is shared by others (16, 31). These step-wise alterations in level and subcellular localization of β-catenin may reflect a series of genetic "hits" along the Wnt-signaling or cell-cell adhesion pathways (7, 32, 33) as the normal epithelium progresses into colorectal carcinoma.

The prognostic significance of nuclear β-catenin in colorectal tumor is additionally supported by three lines of evidence from this study. First, among group 1 patients with carcinoma, the subset with high β-catenin nuclear signaling (IHC $>$ 300) had higher incidence of lymph node metastasis than the subset with lower nuclear signaling (IHC $<$ 200; Fig. 2*A*). Second, among group 3 patients, the correlation between nuclear β-catenin IHC score and survival rate was highly significant (Fig. 4*B*). 3). Finally, among group 3 patients, the high level of nuclear β-catenin and the lymph node metastasis in colorectal cancer was closely associated (Fig. 4*A*). Thus, β-catenin nuclear translocation can potentially be a valuable tool for the prognosis of colorectal cancer. Our data were supported by a recent study of 111 colorectal carcinomas and showed that nuclear β-catenin was significantly related to higher mortality rates in colorectal cancer patients (34).

In this study, all of the nonadenomatous polyps did not show nuclear expression of β-catenin, except occasional Peutz-Jeghers or hyperplastic polyps. This observation is in line with the study by Back *et al.* (35), who found the intestinal polyps from Peutz-Jeghers syndrome and juvenile polyposis were positive for β-catenin nuclear translocation. Both syndromes are associated with an increased risk for colorectal cancer (36, 37). Hyperplastic polyps have also been associated with adenomas and carcinomas, with frequent k-*ras* and p53 mutations (38). Whether hyperplastic polyps with positive nuclear staining for β-catenin have a propensity to develop into higher grades of neoplastic lesions remains to be tested.

In the second part of this study, the low frequency of nuclear translocation of β-catenin in gastric adenocarcinomas, pancreatic ductal adenocarcinomas, ovarian mucinous carcinomas, and colonic mucinous adenocarcinomas, and the consistent nuclear translocation in colorectal carcinoma suggests that this immunostaining can be used in the diagnosis of colorectal carcinoma. Even in the small number of the above tumors (excluding colorectal carcinoma) that are positive, the staining is focal and weak (Fig. 1, *G–J*). These findings also suggest that β-catenin signaling pathway does not play an important role in the development of the above tumors. Use of CK7 and CK20 in conjunction with β-catenin as markers may provide a new tool to confirm the colorectal origin of adenocarcinoma when there are uncertainties about the tissue origin. It is worthy to note that, as described by several recent studies, colonic mucinous adenocarcinoma is associated with favorable prognostic features, including microsatelle instability and negativity in β-catenin nuclear stain (38–42). These findings support the prognostic value of β-catenin nuclear stain in colorectal cancer.

In conclusion, β-catenin nuclear translocation is closely correlated with colorectal carcinogenesis, and can potentially serve as a new prognostic indicator. In combination with CK20 marker, it can aid in the diagnosis of colorectal cancer of primary and metastatic sites. These data collectively present clear evidence for the involvement of β-catenin in colorectal cancer development and progression.

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REFERENCES

1. Kinzler, K. W., and Vogelstein, B. Lessons from hereditary colorectal cancer. Cell, *87:* 159–170, 1996.

2. Polakis, P. The adenomatous polyposis coli (*APC*) tumor suppressor. Biochim. Biophys. Acta, *1332:* 127–147, 1997.

3. Goss, K. H., and Groden, J. Biology of the adenomatous polyposis coli tumor suppressor. J. Clin. Oncol., *18:* 1967–1979, 2000.

4. Groden, J., Thliveris, A., Samowitz, W., Carlson, M., Gelbert, L., Albertsen, H. Joslyn. G., Stevens, J., Spirio, L., and Robertson, M. Identification and characterization of the familial adenomatous polyposis coli gene. Cell, *66:* 589–600, 1991.

5. Nishisho, I., Nakamura, Y., Miyoshi, Y., Miki, Y., Ando, H., Horii, A., Koyama, K., Utsunomiya, J., Baba, S., and Hedge, P. Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients. Science (Wash. DC), *253:* 665–669, 1991.

6. Willert, K., and Nusse, R. β-catenin: a key mediator of Wnt signaling. Curr. Opin. Genet. Dev., *8:* 95–102, 1998.

7. Morin, P. J. β-catenin signaling and cancer. Bioessays, *21:* 1021–1030, 1999.

8. Ben-Ze'ev, A. The dual role of cytoskeletal anchor proteins in cell adhesion and signal transduction. Ann. N. Y. Acad. Sci., *886:* 37–47, 1999.

9. He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawal, L., da Costa, L. T., Morin, P. J., Vogelstein, B., and Kinzler, K. W. Identifi-

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cation of c-MYC as a target of the *APC* pathway. Science (Wash. DC), *281:* 1509–1512, 1998.

10. Chen, R. H., Ding, W. V., and McCormick, F. Wnt signaling to β-catenin involves two interactive components. Glycogen synthase kinase-3β inhibition and activation of protein kinase C. J. Biol. Chem., *275:* 17894–17899, 2000.

11. Kitaeva, M. N., Grogan, L., Williams, J. P., Dimond, E., Nakahara, K., Hausner P., DeNobile, J. W., Soballe, P. W., and Kirsch, I. R. Mutations in β-catenin are uncommon in colorectal cancer occurring in occasional replication error-positive tumors. Cancer Res., *57:* 4478–4481,1997.

12. Iwao, K., Nakamori, S., Kameyama, M., Imaoka, S., Kinoshita, M., Fukui, T., Ishiguro, S., Nakamura, Y., and Miyoshi, Y. Activation of the β-catenin gene by interstitial deletions involving exon 3 in primary colorectal carcinomas without adenomatous polyposis coli mutations. Cancer Res., *58:* 1021–1026, 1998.

13. Inomata, M., Ochiai, A., Akimoto, S., Kitano, S., and Hirohashi, S. Alteration of β-catenin expression in colonic epithelial cells of familial adenomatous polyposis patients. Cancer Res., *56:* 2213–2217, 1996.

14. Valizadeh, A., Karayiannakis, A. J., El-Hariry, I., Kmiot, W., and Pignatelli, M. Expression of E-cadherin-associated molecules (α-, β- and γ-catenins and p120) in colorectal polyps. Am. J. Pathol., *150:* 1977–1984, 1997.

15. Samowitz, W. S., Slattery, M. L., Potter, J. D., and Leppert, M. F. BAT-26 and BAT-40 instability in colorectal adenomas and carcinomas and germline polymorphisms. Am. J. Pathol., *154:* 1637–1641, 1999.

16. Kobayashi, M., Honma, T., Matsuda, Y., Suzuki, Y., Narisawa, R., Ajioka, Y., and Asakura, H. Nuclear translocation of β-catenin in colorectal cancer. Br. J. Cancer, *82:* 1689–1693, 2000.

17. Miettinen, M. Keratin 20: immunohistochemical marker for gastrointestinal, urothelial and Merkel cell carcinomas. Mod. Pathol., *8:* 384–388, 1995.

18. Tot, T. Adenocarcinomas metastatic to the liver: The value of cytokeratins 20 and 7 in the search for unknown primary tumors. Cancer (Phila.), *85:* 171–177, 1999.

19. Wang, N. P., Zee, S., Zarbo, R. J., Bacchi, C. E., and Gown, A. M. Coordinate expression of cytokeratins 7 and 20 defined unique subsets of carcinomas. Appl. Immunohistochem., *3:* 99–107, 1995.

20. Moll, R. Cytokeratins in the histological diagnosis of malignant tumors. Int. J. Biol. Markers, *9:* 63–69, 1994.

21. Sack, M. J., and Roberts, S. A. Cytokeratins 20 and 7 in the differential diagnosis of metastatic carcinoma in cytologic specimens. Diagn. Cytopathol., *16:* 132–136, 1997.

22. Chan, J. K., Suster, S., Wenig, B. M., Tsang, Y. W., Chan, J. B., and Lau, A. L. Cytokeratin 20 immunoreactivity distinguishes Merkel cell (primary cutaneous neuroendocrine) carcinomas and salivary gland small cell carcinomas from small cell carcinomas of various sites. Am. J. Surg. Pathol., *21:* 226–234, 1997.

23. Wong, S. C., Chan, J. K., Lee, K. C., and Hsiao, W. L. Differential expression of p16/p21/p27 and cyclin D1/D3, and their relationships to cell proliferation, apoptosis, and tumour progression in invasive ductal carcinoma of the breast. J. of Pathol., *194:* 35–42, 2001.

24. Mauri, F. A., Caffo, O., Veronese, S., Verderio, P., Boracchi, P., Bonzanini, M., Rossi, N., Perrone, G., Dalla Palma, P., and Barbareschi, M. Tissue carcinoembryonic antigen and oestrogen receptor status in breast carcinoma: an immunohistochemical study of clinical outcome in a series of 252 patients with long-term follow-up. Br. J. Cancer, *77:* 1661–1668, 1998.

25. Remmele, W., and Schicketanz, K. H. Immunohistochemical determination of estrogen and progesterone receptor content in human breast cancer. Computer-assisted image analysis (QIC score) vs subjective grading IRS. Pathol. Res. Pract., *189:* 862–866, 1993.

26. Saegusa, M., Hashimura, M., Hara, A., and Okayasu I. Up-regulation of pS2 expression during the development of adenocarcinomas but not squamous cell carcinomas of the uterine cervix, independently of expression of *c-jun* or oestrogen and progesterone receptors. J. Pathol., *190:* 554–563, 2000.

27. Baillie, R., Carlile, J., Pendleton, N., and Schor, A. M. Prognostic value of vascularity and vascular endothelial growth factor expression in non-small cell lung cancer. J. Clin. Pathol., *54:* 116–120, 2001.

28. Heideman, D. A., Snijders, P. J., Bloemena, E., Meijer, C. J., Offerhaus, G. J., Meuwissen, S. G., Gerritsen, W. R., and Craanen, M. E. Absence of *tpr-met* and expression of *c-met* in human gastric mucosa and carcinoma. J. Pathol., *194:* 428–435, 2001.

29. Hao, X., Tomlinson, I., Ilyas, M., Palazzo, J. P., and Talbot, I. C. Reciprocity between membranous and nuclear expression of β-catenin in colorectal tumours. Virchows Arch., *431:* 167–172, 1997.

30. Schlemper, R. J., Itabashi. M., Kato, Y., Lewin, K. J., Riddell, R. H., Shimoda, T., Sipponen, P., Stolte, M., and Watanabe, H. Differences in the diagnostic criteria used by Japanese and Western pathologists to diagnose colorectal carcinoma. Cancer (Phila.), *82:* 60–69, 1998.

31. Hao, X. P., Pretlow, T. G., Rao, J. S., and Pretlow, T. P. β-Catenin expression is altered in human colonic aberrant crypt foci. Cancer Res., *61:* 8085–8088, 2001.

32. Novak, A., Hsu, SC., Leung-Hagesteijn, C., Radeva, G., Papkoff, J., Montesano, R., Roskelley, C., Grosschedl, R., and Dedhar, S. Cell adhesion and the integrin-linked kinase regulate the LEF-1 and β-catenin signaling pathways. Proc. Natl. Acad. Sci. USA, *95:* 4374–4379, 1998.

33. Bienz, M., and Clevers, H. Linking colorectal cancer to Wnt signaling. Cell, *103:* 311–320, 2000.

34. Cheah, P. Y., Choo, P. H., Yao, J., Eu, K. W., and Seow-Choen, F. A. survival-stratification model of human colorectal carcinomas with β-catenin and p27kip1. Cancer (Phila.), *95:* 2479–86, 2002.

35. Back, W., Loff, S., Jenne, D., and Bleyl, U. Immunolocalization of β-catenin in intestinal polyps of Peutz-Jeghers and juvenile polyposis syndromes. J. Clin. Pathol., *52:* 345–349, 1999.

36. Spigelman, A. D., Murday, V., and Phillips, R. K. Cancer and the Peutz Jeghers syndrome. Gut, *30:* 1588–1590, 1989.

37. Wu, T. T., Rezai, B., Rashid, A., Luce, M. C., Cayouette, M. C., Kim, C., Sani, N., Mishra, L., Moskaluk, C. A., Yardley, J. H., and Hamilton, S. R. Genetic alterations and epithelial dysplasia in juvenile polyposis syndrome and sporadic juvenile polyps. Am. J. Pathol., *150:* 939–947, 1997.

38. Fenoglio-Preiser, C. M. When is a hyperplastic polyp not a hyperplastic polyp? Am. J. Surg. Pathol., *23:* 1001, 1999.

39. Furlan, D., Tibiletti, M. G., Taborelli, M., Albarello, L., Cornaggia, M., and Capella, C. The value of microsatellite instability in the detection of HNPCC families and of sporadic colorectal cancers with special biological features: an investigation on a series of 100 consecutive cases. Ann. Oncol., *9:* 901–906, 1998.

40. Salahshor, S., Kressner, U., Pahlman, L., Glimelius, B., Lindmark, G., and Lindblom, A. Colorectal cancer with and without microsatellite instability involves different genes. Genes Chromosomes Cancer, *26:* 247–252, 1999.

41. Wong, N. A., Morris, R. G., McCondochie, A., Bader, S., Jodrell, D. I., and Harrison, D. J. Cyclin D1 overexpression in colorectal carcinoma *in vivo* is dependent on β-catenin protein dysregulation, but not k-ras mutation. J. Pathol., *197:* 128–135, 2002.

42. Jass, J. R., Barker, M., Fraser, L., Walsh, M. D., Whitehall, V. L., Gabrielli, B., Young, J., and Leggett, B. A. APC mutation and tumour budding in colorectal cancer. J. Clin. Pathol., *56:* 69–73, 2003.

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