

Concurrent Overexpression of Cyclin D1 and Cyclin-dependent Kinase 4 (Cdk4) in Intestinal Adenomas from Multiple Intestinal Neoplasia (Min) Mice and Human Familial Adenomatous Polyposis Patients¹

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ABSTRACT

We postulated that increased expression of the cell cycle regulators cyclin D1 and cyclin-dependent kinase (Cdk) 4 may be involved in the development of intestinal adenomas associated with familial adenomatous polyposis (FAP). In the present study of multiple intestinal neoplasia (Min) mice and human FAP patients, the expression and distribution of cyclin D1, Cdk4, and cell proliferative activity (5-bromo-2'-deoxyuridine incorporation) in normal and adenomatous intestinal epithelium were investigated. Immunohistochemical analysis of Min mouse intestine revealed that cyclin D1 immunoreactivity in the intestinal epithelium was restricted to the adenomatous areas, with a significantly higher percentage of positively staining nuclei in high-grade dysplasia versus low-grade dysplasia ($54.8 \pm 18.4\%$ versus $34.6 \pm 16.9\%$, $P = 0.016$). Morphologically normal areas of intestinal epithelia were uniformly negative for cyclin D1 immunoreactivity. Cdk4 nuclear immunoreactivity was restricted to the crypt areas in morphologically normal small intestine and colon. Conversely, Cdk4 immunoreactivity was uniformly abundant in adenomatous areas regardless of the degree of dysplasia. Increased expression of cyclin D1 and Cdk4 in adenomas was accompanied by a significantly increased 5-bromo-2'-deoxyuridine incorporation rate in the same areas. Immunoblot analysis of lysates from surgical specimens revealed increased levels of cyclin D1 and Cdk4 in the majority of intestinal adenomas from human FAP patients in comparison to the adjacent grossly normal colonic mucosa. Our results indicate that overexpression of cyclin D1 and Cdk4 occurs in intestinal adenomas and is associated with increased cell proliferative activity in premalignant neoplastic cells. Increased cyclin D1 immunoreactivity is associated with more severe dysplasia. These data suggest that abnormal up-regulation of these important G₁ cell cycle proteins is a relatively early event in intestinal carcinogenesis and that these changes may contribute to malignant progression within those lesions.

INTRODUCTION

FAP³ is an autosomal dominant condition characterized by the development of numerous adenomatous polyps in the colon, and occasionally in the proximal gut. This genetic disease has attracted considerable interest because of the high rate of progression to colorectal carcinoma (1). Genetic linkage studies have shown that inactivation of the *Apc* gene located at chromosome 5q21 is responsible for

the abnormalities in FAP (2). Mutant *Apc* genes are also found in 60–80% of sporadic colorectal cancers and in a similar percentage of sporadic adenomas, including polyps smaller than 1 cm² in diameter (3–5). Therefore, FAP provides an excellent model for the study of multistep progression from adenoma to carcinoma, and studies of this model have led to significant advances in our understanding of colorectal carcinogenesis. The Min mouse serves as a murine model for FAP. These mice have a germline mutation in *Apc* alleles resulting in numerous intestinal adenomas throughout the intestine (6). Tumor formation in Min mice is similar to the human disease in that tumors form spontaneously in the absence of exogenous carcinogen.

Recent carcinogenesis studies have focused on cell cycle regulatory events, including Cdk4s, their positive regulators (cyclins), and their negative regulators (cyclin kinase inhibitors), and have revealed that derangements in the cell cycle machinery may contribute to inappropriate neoplastic cell growth (reviewed in Refs. 7–10). Growth factor stimulation of nonproliferating cells results in rapid induction of cyclin D and subsequent cell proliferation, whereas growth factor withdrawal results in the disappearance of cyclin D protein and G₁ growth arrest (7, 9, 10). Cumulative evidence indicates that, among G₁ cyclins, cyclin D1 is most strongly implicated in tumorigenesis, including colon cancer (11–15). It is important to know whether overexpression of cyclin D1 is correlated with increased proliferative activity in intestinal tumors and whether there is a concomitant overexpression of its Cdk partners Cdk4/Cdk6.

Cdk4 and Cdk6 are the major catalytic partners of D-type cyclins (10). The function of these Cdk4s is regulated through the following mechanisms: activation by binding of D cyclins and phosphorylation by cyclin-activated kinase, whereas inhibition results from binding of the Cdk inhibitors and from dephosphorylation. When activated by D cyclins and cyclin-activated kinase, Cdk4 (and Cdk6) phosphorylates its substrate, the product of the *rb* gene. Hyperphosphorylation of *rb* by Cdk4-cyclin D complex releases E2F, a necessary event for G₁-S transition (16, 17). Under- or unphosphorylated *rb* binds to the transcription factor E2F and prevents entry into S phase. Compared with cyclin D1, there are few studies that address the importance of *in vivo* expression of Cdk4 in tumorigenesis. The *Cdk4* gene maps to the long arm of chromosome 12, and amplification-associated overexpression of Cdk4 has been observed in small subsets of malignant human glial tumors, glial tumor cell lines (18), and human sarcoma cell lines (19). Increased expression of Cdk4 has also been reported in a study of carcinogen (*N*-methyl-*N*-nitrosourea)-induced primary rat mammary tumors (20). However, Cdk4 expression in the intestine and its clinicopathological significance in intestinal tumorigenesis have not been reported.

In the present study, we investigated cyclin D1 and Cdk4 expression and distribution and their relationship with cell proliferation (as determined by BrdUrd incorporation) in intestinal adenomas and histologically normal intestine in Min mice. We also compared the

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³ The abbreviations used are: FAP, familial adenomatous polyposis; *Apc*, adenomatous polyposis coli; Min, multiple intestinal neoplasia; Cdk, cyclin-dependent kinase; *rb*, retinoblastoma; IHC, immunohistochemistry; PCNA, proliferating cell nuclear antigen; BrdUrd, 5-bromo-2'-deoxyuridine.

levels of cyclin D1 and Cdk4 expression in colonic adenomas and in grossly normal colonic mucosa from human FAP patients.

MATERIALS AND METHODS

Min Mice. All of the mice used for this study were (AKR X B6)F1^{Min/+} mice produced by mating B6-Min *Apc*^{Min}/*Apc*⁺ males with AKR females. Individual Min mice developed adenomas in small and large intestine with a tumor size range of 1–4 mm². The mice were bred and maintained at the McArdle Laboratory for Cancer Research and fed with Purina Breeder Blox. The mice were euthanized with CO₂, and the large and small intestine were removed for tumor examination and collection.

Min Mouse Intestinal Tissue Collection. Twenty-nine intestinal adenomas and 15 adjacent normal intestinal samples were obtained from the small and large intestine of 15 Min mice. The entire small intestine was divided into three equal-length portions (proximal, middle, and distal). Among the 29 adenomatous tissues, 7 were from proximal, 9 from middle, 4 from distal small intestine, and 9 from the large intestine. The samples were immediately fixed in 4% paraformaldehyde at 4°C for 24 h and embedded in paraffin.

Human FAP Tissue Collection. Thirteen adenomas from large intestine and 10 paired adjacent normal large intestinal mucosal samples were collected from seven FAP patients undergoing total proctocolectomy. Precautions were taken to avoid tissue ischemia before processing. The mucosal samples and polyps from either the left colon or rectum were dissected from the muscularis propria within 5 min of removal from the patients. The samples were immediately frozen in liquid nitrogen, and within 24 h were homogenized and lysed in lysis buffer (150 mM NaCl, 1% NP40, 0.5% deoxycholic acid, and 0.1% SDS) on ice. After sedimentation, the cellular debris was removed, and the supernatants containing cellular soluble proteins (cellular lysates) were stored at –80°C until further analysis.

Intestinal Cell Proliferative Index. For studies of intestinal cell proliferation, 75 mg/kg BrdUrd (Sigma Chemical Co., St. Louis, MO) dissolved in PBS was injected i.p. into Min mice 2 h before sacrifice. The incorporation of BrdUrd into intestinal cell nuclei was determined by IHC as described in detail below. The BrdUrd incorporation was also compared with nuclear immunoreactivity for the PCNA, as determined by IHC.

IHC of Min Mouse Intestinal Tissues. Histological sections (5 μm) were affixed to glass slides, dewaxed, and rehydrated. The sections were then incubated in 1% hydrogen peroxide for 15 min at 25°C to quench endogenous peroxidase activity and digested in 0.1% trypsin for 5 min at 37°C. After being blocked in 1.5% normal horse/goat serum for 30 min at 25°C, the serial sections were incubated with one of the following primary antibodies, respectively: (a) mouse anti-cyclin D1 monoclonal antibody (Novocastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom); (b) rabbit anti-Cdk4 polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); (c) rat anti-BrdUrd monoclonal antibody (Sera Laboratory, Sussex, England); or (d) mouse anti-PCNA monoclonal antibody (Boehringer Mannheim, Indianapolis, IN) for 18 h at 4°C. After rinsing, the biotinylated secondary antibodies and horseradish peroxidase-conjugated anti-biotin antibody (Vectastain ABC-peroxidase kits, Vector Laboratories, Burlingame, CA) were applied to the sections according to the manufacturer's instruction. The peroxidase activity for cyclin D1, Cdk4, and PCNA was visualized by applying diaminobenzidine chromogen containing 0.05% hydrogen peroxide for 5–10 min at 25°C. The sections were then counterstained with hematoxylin, dehydrated, cleared, and mounted. The peroxidase activity for BrdUrd was visualized by applying 3-amino-9-ethylcarbazole chromogen (AEC kit, BioGenex, San Ramon, CA), counterstained with hematoxylin, and mounted with Immu-mount (Shandon Lipshaw, Pittsburgh, PA). The sections were examined and photographed on an Olympus AH Vanox light microscope. The visual images were captured by Nikon scanning system (Nikon Inc., Melville, NY) and reproduced by Adobe Photoshop software (Adobe Systems, Inc., Mountain View, CA).

Specificity of Immunostaining. The cyclin D1 primary antibody used for IHC was a mouse monoclonal antibody that has no cross-reactivity with cyclin D2 or D3 (12). The Cdk4 polyclonal antibody has no cross-reactivity with Cdk6 or any other CDKs. A human breast cancer section that was known to be positive for cyclin D1 and a rat normal intestinal epithelium cell line (IEC-6) that expresses abundant levels of Cdk4 were used as the positive controls for cyclin D1 and Cdk4, respectively, within each immunostaining run. For

negative controls, normal mouse/rabbit IgG (Pierce, Inc., Rockford, IL), normal horse/goat serum (Vectastain ABC-peroxidase kits), or PBS alone was applied to the sections to substitute for primary antibody. Additionally, PBS was used as a substitute for secondary antibodies. These rigorous negative controls were used to delineate the possibilities of false-positive responses from either endogenous peroxidase or antibodies.

Histopathological and IHC Assessment of Min Mouse Intestine. Serial sections from each block were stained with H&E for morphological examinations. Dysplasia was defined and further classified as low-grade dysplasia or high-grade dysplasia according to WHO criteria (21) by a pathologist (L.L.) who was unaware of the results of the IHC. The adenomas were also classified as tubular, villous, or tubulovillous.

The number of cyclin D1, Cdk4, or BrdUrd positive and negative epithelial cells in a minimal total of 600 cells in (a) areas with adenomatous changes (within unequivocal areas of high- or low-grade dysplasia), (b) the crypts of small intestine, and (c) the lower two-thirds of the crypts of colon was determined by an independent histologist using a BIOQUANG OS/2 computerized video image analysis system. The quantity of the IHC was expressed as positive percentage (mean ± SD)/total cell number. Student's unpaired *t* test was applied to the comparison between groups, and *P* < 0.05 was defined as a statistically significant difference.

Immunoblot Analysis of Human FAP Tissues. The protein concentration of the cellular lysates was determined by the Bio-Rad method (Bio-Rad Laboratories, Hercules, CA). Equal amounts (50 μg/lane) of protein were resolved by electrophoresis on 10% polyacrylamide gels containing 1% SDS and transferred to the nitrocellular membrane (Millipore Corp., Bedford, MA) using a semidry transfer cell (Bio-Rad). The membrane was probed with rabbit anti-cyclin D1 or Cdk4 polyclonal antibody (Santa Cruz) and then with horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz). The positive bands were detected by the enhanced chemiluminescence system (Amersham Life Science Inc., Arlington Heights, IL) and quantified by PhosphorImager computerized digital image analysis system.

RESULTS

Histopathological Assessment of Min Mouse Intestine. Twenty-nine intestinal adenomas and 15 adjacent normal intestinal samples from the small and large intestine of 15 Min mice were included in this study. Among the 29 adenoma samples from the Min mice, dysplasia was defined and further classified as low-grade dysplasia (*n* = 16) or high-grade dysplasia (*n* = 13). The adenomas were also classified as tubular (*n* = 9), villous (*n* = 6), or tubulovillous (*n* = 14).

Cyclin D1 IHC. Cyclin D1 immunoreactivity was restricted to the nuclei of abnormal epithelial cells within the adenomatous areas from all of the mouse adenomatous tissues. The percentage of cyclin D1-positive cells ranged from 2.9 to 76% with an average of 41.9 ± 20.9% (Table I). Cells with cyclin D1 immunoreactivity were predominantly confined to the area with high-grade architectural and cytological alterations (Fig. 1A–C, *large arrow*). Although the immunostaining intensity varied within an individual adenomatous lesion (Fig. 1A), the areas of the “normal” intestinal epithelium (whether adjacent to or remote from dysplastic areas) were consistently negative with regard to cyclin D1 immunoreactivity (Fig. 1, *D* and *E*, *small*

Table I Immunoreactivities of cyclin D1, Cdk4, and BrdUrd in Min mouse intestine

IHC	Index of positive IHC		
	Normal intestine		
	Crypts	Noncrypt	Adenomas
Cyclin D1	0	0	41.9 ± 20.9 ^a
Cdk4	63.0 ± 8.2 (small) 65.8 ± 8.2 (colon)	0	73.7 ± 17.9
BrdUrd	32.5 ± 2.5 (small) 19.3 ± 2.4 (colon)	0	44.3 ± 1.1 ^a

^a Significant difference compared with the normal intestine (*P* < 0.05).

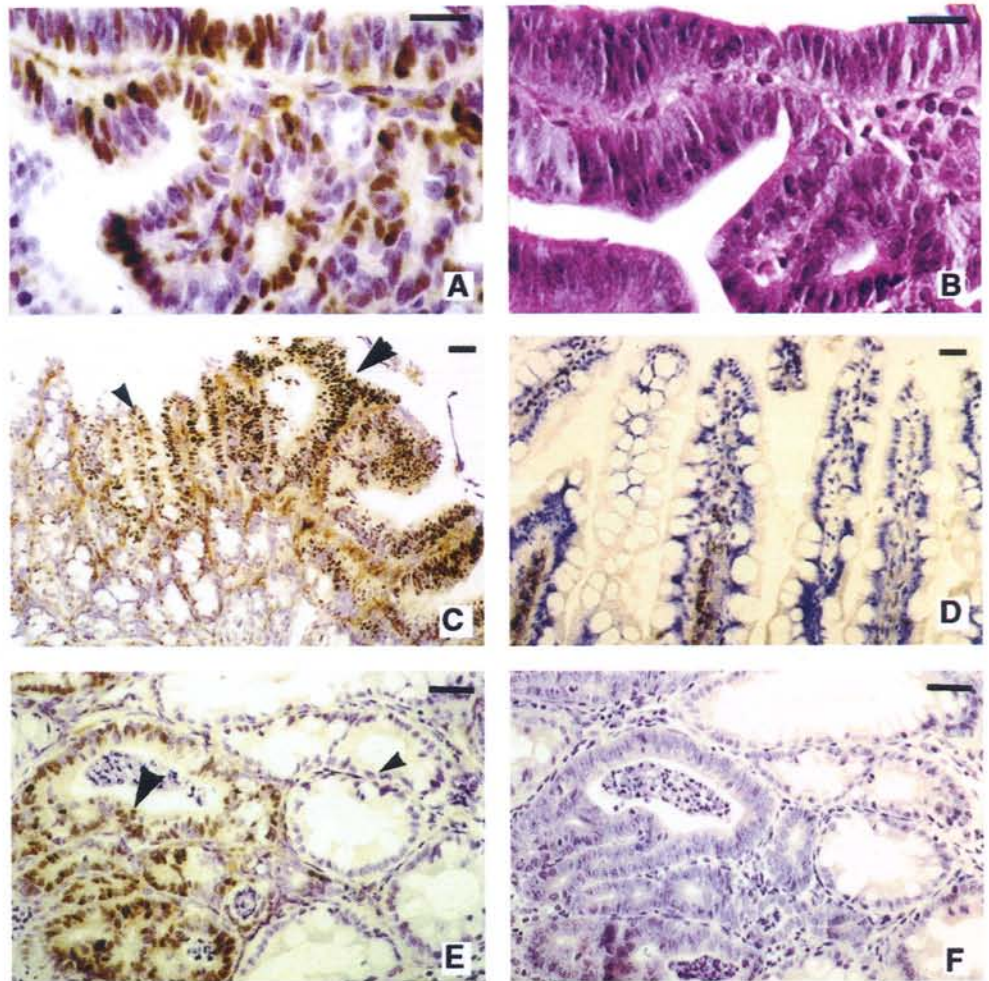


Fig. 1. Cyclin D1 IHC in Min mouse intestine. **A**, Cyclin D1 immunoreactivity in a highly dysplastic area. Cyclin D1 immunoreactivity is located in the nuclei (*brown nuclei*), and staining intensity varies from moderate to very strong. **B**, H&E section corresponding to **A**. **C**, Cyclin D1 immunostaining in a dysplastic area with both severe (*large arrow*) and mild (*small arrow*) architectural and cytological alterations. The positive nuclei are predominantly confined to the severe dysplastic compartment (*large arrow*) compared with the mild dysplastic compartment (*small arrow*). **D**, Cyclin D1 immunostaining in normal intestinal mucosa. Intestinal epithelial cells are negative for cyclin D1. A few positive mesenchymal cells (possibly lymphocytes or fibrocytes) are present in the lamina propria. **E**, Cyclin D1-positive nuclei are located in the dysplastic (*large arrow*) area, but the cells in the adjacent normal glands (*small arrow*) are negative for cyclin D1. **F**, Control immunostaining with normal mouse IgG on the serial section to **E**. Bars, 25 μ m.

arrow). An occasional mesenchymal cell nucleus in both dysplastic and normal areas stained positive for cyclin D1, and examination of serial H&E sections revealed that those cells appeared to be lymphocytes or fibroblasts. The sections incubated with normal mouse IgG (Fig. 1F), PBS, or normal horse serum as substitutes for antibodies were all negative for cyclin D1 immunostaining. As an additional control, the cyclin D1 nuclear immunostaining was consistently present in breast cancer cells, and the normal cells in a benign breast cyst on the same sections were negative (data not shown).

A direct correlation was noted between the number of cyclin D1-positive cells and the grade of dysplasia (Fig. 2). Cyclin D1 nuclear immunoreactivity was observed with greater frequency in the adenomas with high-grade dysplasia ($54.8 \pm 18.4\%$, $P = 0.016$) compared with adenomas with low-grade dysplasia ($34.6 \pm 16.9\%$). The proportion of cells that are cyclin D1-positive in villous adenomas ($51.5 \pm 10.4\%$) was marginally higher than tubular adenomas ($27.5 \pm 6.4\%$, $P = 0.078$). No differences were found either between tubulovillous ($44.5 \pm 5.4\%$) and villous or between tubulovillous and tubular adenomas. Cyclin D1 immunoreactivity in the adenomas did not appear to vary with adenoma location: proximal ($47.1 \pm 4.6\%$), middle ($41.3 \pm 18.6\%$), or distal ($40.4 \pm 23.9\%$) small intestine, or large intestine ($43.5 \pm 27.5\%$).

Cdk4 IHC. In normal small intestinal mucosa, Cdk4-positive cells were restricted to the nucleus of the cells in the crypt region, where the proliferating cells are located (Fig. 3A, *arrows*). In normal colonic mucosa, Cdk4-positive cells were observed predominantly in the bottom one-third of the crypt (Fig. 3B, *arrows*), which is the proliferative zone. Scattered Cdk4-positive cells were also found in the

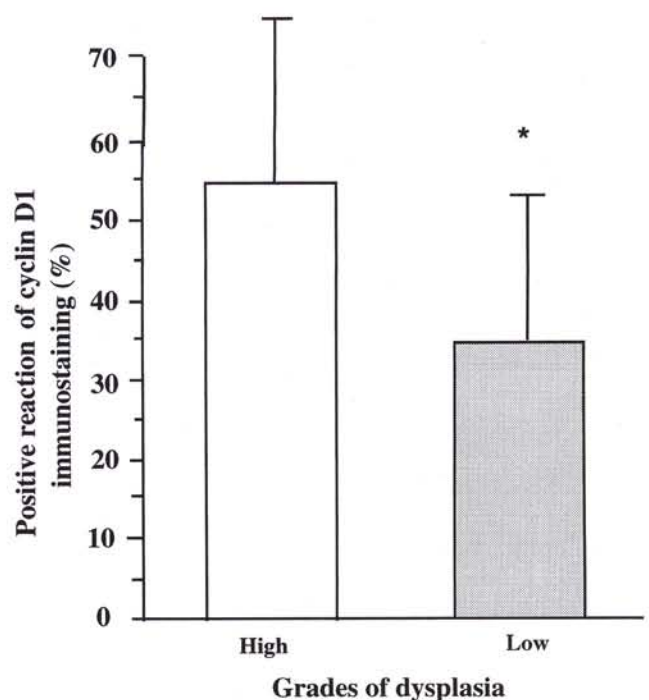
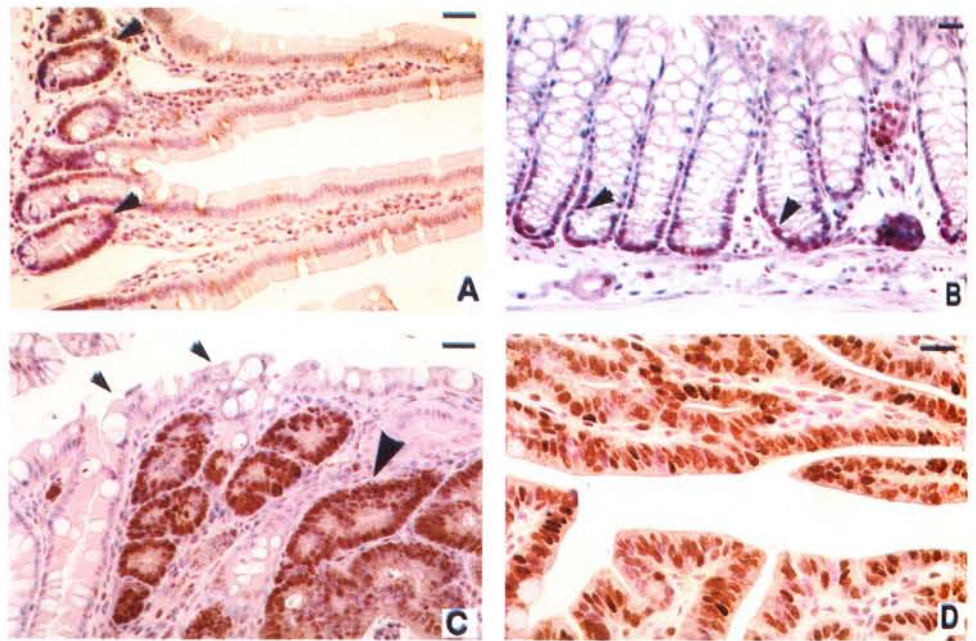


Fig. 2. Comparison of positive cyclin D1 immunostaining index from Min mouse intestines between high ($n = 13$) and low ($n = 16$) grades of dysplasia (mean \pm SD). *, significant difference compared with high-grade dysplasia ($P < 0.05$). Bars, SD.

Fig. 3. Min mouse intestine immunostained with Cdk4 rabbit polyclonal antibody. *A* and *B*, Cdk4 expression in the normal small intestine (*A*) and colon (*B*). In the epithelial cells, Cdk4 immunoreactivity (dark nuclei) was located in the nuclei and was restricted to the crypt region in small intestine (*A*, arrows). In normal colonic mucosa, Cdk4-positive cells were located predominantly in the bottom one-third of the crypt (*B*, arrows). Scattered Cdk4-positive cells also were found in the middle one-third of the crypt. No Cdk4-positive cells were identified in the villus epithelium outside the crypt area in the small intestine or in the upper one-third of the colonic crypt and surface epithelium in the colon. *C* and *D*, Cdk4 expression in the adenomatous area and adjacent normal epithelium. Cdk4 immunoreactivity was dramatically increased and uniformly distributed in the adenomatous area (*C*, large arrow, and *D*) compared with the adjacent normal epithelium in which Cdk4-positive cells were rarely found (*C*, small arrows). Bars, 25 μ m.



middle one-third of the crypt. No Cdk4-positive cells were identified in the villus epithelium outside the crypt area in the small intestine or in the upper one-third of the colonic crypt and surface epithelium in the colon (Fig. 3, *A* and *B*). The sections incubated with PBS, normal rabbit IgG, or normal goat serum as substitutes for antibodies were all negative for Cdk4 immunostaining, and cultured rapidly proliferating IEC-6 cells were consistently positive for Cdk4 nuclear immunoreactivity (data not shown).

Abundant Cdk4-expressing cells were found in the adenomatous areas from all the Min mouse adenoma tissues compared with the adjacent normal epithelium, in which Cdk4-positive cells were rarely identified (Fig. 3, *C* and *D*). The Cdk4 immunoreactivity in adenomatous areas was uniformly present whether the cells were near the crypt or near the luminal surface. The index of Cdk4 nuclear immunoreactivity in adenomatous area was similar to that in the proliferative crypt regions of the normal intestine ($73.7 \pm 17.9\%$ in adenoma versus $63.0 \pm 8.2\%$ in crypts of normal small intestine and $65.8 \pm 8.2\%$ in crypts of normal colon; Table 1). Distinct from the pattern of cyclin D1 expression, Cdk4 expression was more uniformly distributed in the adenomas, and there was no significant correlation between the levels of Cdk4 expression and the degree of dysplasia ($75.9 \pm 16.2\%$ in high-grade dysplasia and $68.7 \pm 24.6\%$ in low-grade dysplasia). No differences were found either between different pathological types of adenomas or between different adenoma locations (data not shown). The serial sections immunostained for cyclin D1 and Cdk4 indicated that there was a high frequency of the cells that coexpressed cyclin D1 and Cdk4 in adenomatous areas. This pattern was not observed in the normal intestine. Fewer cells exhibited cyclin D1 immunoreactivity compared with Cdk4 immunostaining. Cdk6 is another catalytic partner for cyclin D1, but we did not detect Cdk6 immunoreactivity in the either normal or adenomatous mouse intestinal epithelium (data not shown).

BrdUrd Incorporation and the Relationships between Cyclin D1, Cdk4, and Proliferation. To determine whether there is an increased proliferative rate in intestinal adenomas and whether cyclin D1 and/or Cdk4 expression is correlated with the increased proliferation, we examined BrdUrd incorporation in the Min mouse intestine. BrdUrd is a halogenated 5-substituted derivative of deoxyuridine, a thymidine analogue, that is specifically incorporated in the DNA of

proliferative cells during DNA synthesis. Consistent with the published data from normal intestine (22), BrdUrd-labeled cells were located predominantly in the crypt region of normal small intestine and the bottom one-third of the colonic crypt (Fig. 4, *A* and *B*, arrows). This distribution was similar to that for Cdk4 (Fig. 3, *A* and *B*, arrows). The frequency of BrdUrd-labeled cells in adenomatous areas ($44.3 \pm 1.1\%$) was significantly higher than that of adjacent normal epithelium, in which there was no BrdUrd labeling [Fig. 4, *C* (small arrow) and *D*]. In addition, BrdUrd nuclear labeling was significantly higher in adenoma than in the crypt areas of either small intestine or colon (32.5 ± 2.5 and $19.3 \pm 2.4\%$ in crypts of small intestine and colon, respectively; $P < 0.001$; Table 1). There was good agreement between Cdk4 immunoreactivity and BrdUrd nuclear labeling when serial sections were compared. Most cells that were positive for cyclin D1 or BrdUrd labeling were also positive for Cdk4.

Nuclear PCNA Immunoreactivity. To further confirm that there was an increased proliferative activity in the adenomas and to determine the relationship with cyclin D1 and Cdk4 expression, we also immunostained Min mouse serial sections with another proliferative marker, PCNA. Similar to Cdk4 and BrdUrd immunostaining, cells with PCNA nuclear immunoreactivity were located predominantly in the crypt regions of normal small and large intestine. The index of PCNA-positive cells was increased dramatically in adenomatous areas (Fig. 4, *E* and *F*, large arrows) compared with the adjacent normal epithelium (Fig. 4, *E* and *F*, small arrows). Areas with increased PCNA expression coincided with increased immunoreactivity for cyclin D1, Cdk4, and BrdUrd.

Immunoblot Analysis of Human FAP Tissues. To determine the clinical relevance of the findings in Min mice, the protein lysates from adenomas (0.5–1.5 cm in diameter) and the grossly normal adjacent tissue from freshly obtained proctocolectomy specimens from seven FAP patients were examined for cyclin D1 and Cdk4 levels by immunoblotting. The adenomas were obtained from the left colon and the proximal rectum from the first patient, and cyclin D1 levels were increased in all four adenoma tissues. Cdk4 levels also were increased in two of four adenomas compared with adjacent grossly normal mucosa (Fig. 5). Additional protein lysates were examined from nine adenomas and from paired grossly normal colonic mucosal samples from six additional FAP patients. The cyclin D1 signals were readily

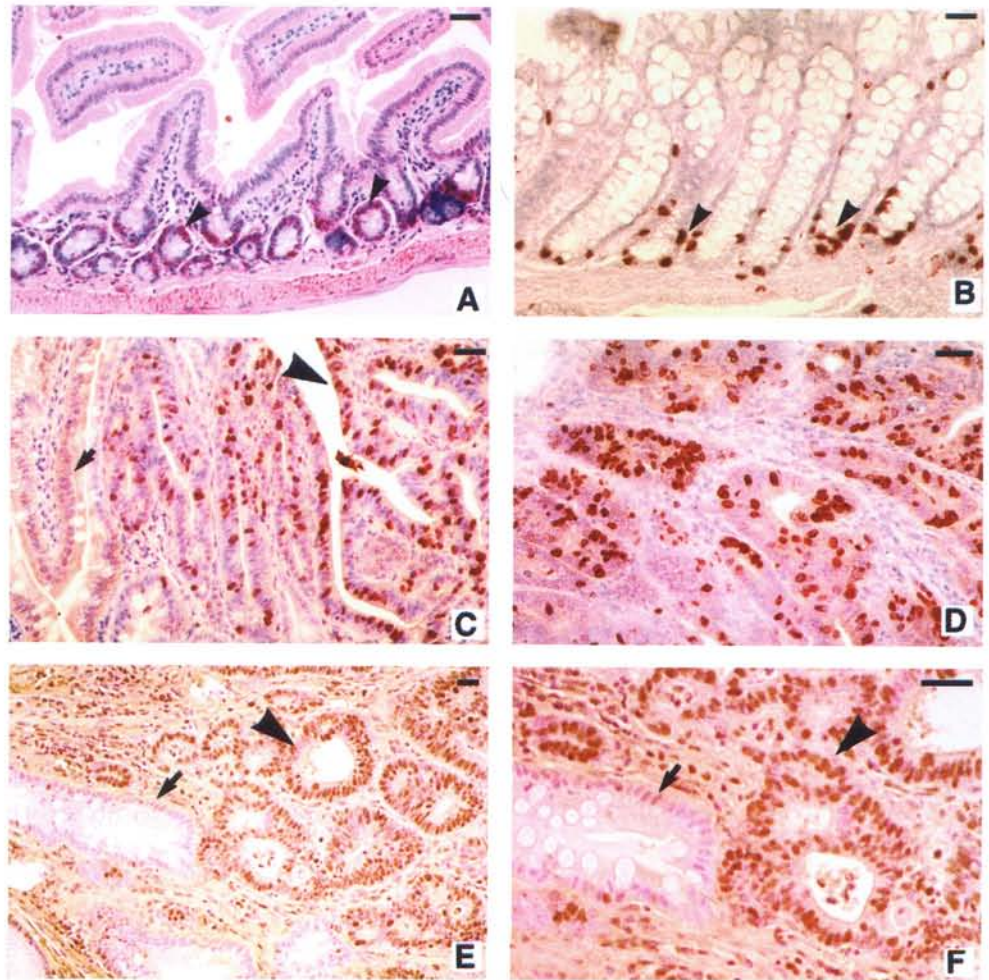


Fig. 4. Min mouse intestine immunostained with BrdUrd rat monoclonal antibody. *A* and *B*, BrdUrd labeling in the normal small intestine and colon. BrdUrd-labeled cells (dark nuclei) were located predominantly in the crypt region of normal small intestine and the bottom one-third of the colonic crypt (arrows). *C* and *D*, BrdUrd labeling in adenomatous area. The frequency of BrdUrd labeling cells in adenomatous areas (*C*, large arrow, and *D*) was dramatically increased compared with the adjacent normal epithelium, in which there was no BrdUrd labeling (*C*, small arrow). *E* and *F*, A Min mouse adenoma immunostained with PCNA mouse monoclonal antibody. PCNA nuclear immunoreactivity (dark nuclei) was dramatically increased in the adenomatous area compared with the adjacent normal epithelium. Bars, 25 μ m.

detectable in five adenoma samples from four different FAP patients with a 6–12-fold increase of cyclin D1 expression compared with their paired normal intestinal tissues (Fig. 5). Increased Cdk4 levels (2–8-fold) also were detected in seven adenoma samples from the same four patients who had concurrent overexpression of cyclin D1 (Fig. 5). Overall concurrent overexpression of cyclin D1 and Cdk4 was detected in 9 (69%) of 13 human adenomas compared with normal colonic mucosa in these seven FAP patients.

DISCUSSION

Tumor growth can occur as a result of uncontrolled cell proliferation and/or a decreased capacity to undergo apoptosis, and these characteristics may be associated with aberrant cell cycle regulation. Consistent with this hypothesis, our present studies have demonstrated that cyclin D1 was dramatically increased in Min mouse adenomas compared with the nondetectable levels in adjacent normal tissues. Cdk4, the major catalytic partner for cyclin D1, was found to be expressed in the normal intestine, but normal expression was restricted to the proliferative (crypt) zones and was not observed in the differentiated villus cells of the small intestine or in the upper one-third of the colonic mucosa. In addition, Cdk4 expression was observed in all areas of adenomatous change regardless of whether these cells extended to the luminal surface of the Min mouse gut. Furthermore, the coexpression of cyclin D1 and Cdk4 was accompanied by a significant increase in the number of proliferating cells within the adenomas, as indicated by BrdUrd incorporation and PCNA immunostaining. The proportion of cyclin D1-positive cells was signifi-

cantly increased in adenomas with high-grade dysplasia compared with low-grade dysplasia. Furthermore, within an individual adenoma, more abundant cyclin D1 immunoreactivity was observed in areas with severe architectural and cytological alterations. Along with the absence of cyclin D1 in any area of the normal mouse intestinal epithelium, these findings indicate that cyclin D1 is not a marker of proliferation in the normal intestine, but that expression is associated with neoplastic cell proliferation. The immunoblot analysis of the specimens from FAP patients demonstrated increased levels of cyclin D1 and Cdk4 in 69% of adenomas compared with adjacent grossly normal colonic mucosa, thus supporting the clinical relevance of our findings in the Min mouse. Taken together, our results strongly suggest a relationship between overexpression of cyclin D1, inappropriate expression of Cdk4, and the increased cell proliferation that was observed in intestinal adenomas. These associated abnormalities in cell growth regulation are likely to contribute to cell transformation and tumor progression.

Cyclin D1 is overexpressed in some parathyroid adenomas and in centrocytic B-cell lymphomas because of chromosomal rearrangement (9, 11), and it is frequently overexpressed in a subset of breast, esophageal, and head and neck squamous carcinomas as a result of amplification of the 11q13 region containing the *CCND1* gene (11, 13–15). Overexpression of cyclin D1 protein has also been observed in other tumors in the absence of any measurable increase in gene copy number (13, 23), indicating that multiple mechanisms may be involved in the expression of this oncogene in tumors. Although amplification of the cyclin D1 gene has not been observed in colo-

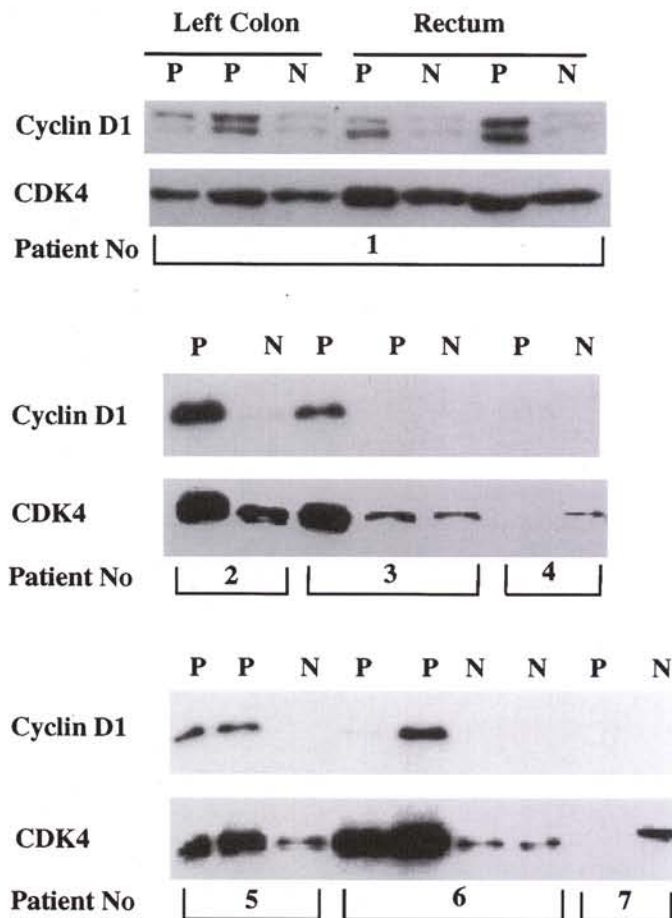


Fig. 5. Cyclin D1 and Cdk4 immunoblot analysis of colonic polyp (P) and paired normal colonic mucosa (N) from seven FAP patients. The polyps from patients 1, 2, 3, 5, and 6 have a 6–12-fold and a 2–8-fold increase of cyclin D1 and Cdk4 expression, respectively, compared with the adjacent normal colonic mucosa.

rectal cancer (24), cyclin D1 protein overexpression has been reported in colon carcinoma cell lines, in 30–46% of primary colorectal cancers (12, 25, 26), and in at least 33% of sporadic colorectal adenomatous polyps in humans (27). These studies, along with our present findings in Min mice and human FAP patients, indicate that cyclin D1 overexpression may be important, not only in growth of cancer cells, but also in the progression of premalignant intestinal lesions. The mechanism of cyclin D1 overexpression in intestinal adenomas is the subject of ongoing investigation.

The findings of cyclin D1 overexpression in human tumors, as described above, strongly suggest an oncogenic role for this protein. More direct evidence in support of the oncogenic role of cyclin D1 has been provided by the following findings. (a) Rat embryo fibroblastic cell lines overexpressing cyclin D1 induce fibrosarcomas when injected into nude mice (28). (b) Transgenic mice that overexpress cyclin D1 in the breast, under control of the mouse mammary tumor virus promoter, develop breast hyperplasia and carcinomas (29). Despite clear evidence that cyclin D1 can function as an oncogene, the precise role of cyclin D1 in tumorigenesis is still under investigation. The promoting role of cyclin D1 in G_1 progression is suggested from the observations that exogenous growth factors induce cyclin D1 expression, and cyclin D1 expression has been found to be essential for G_1 cell cycle progression in both nontransformed and transformed cells in which it is expressed, with inhibition of expression resulting in G_1 growth arrest (12, 15, 30–33). The regulation of the expression and function of the D-type cyclins provides the molecular basis

through which cells respond to extrinsic mitogenic signals (9, 10). Increased expression of cyclin D1 may reflect increased growth factor stimulation, or, if autonomous, may indicate that cells have lost the requirement for extrinsic growth factors. Increased expression of cyclin D1 also has been found to increase the likelihood of gene amplification and thereby may contribute to genetic instability in neoplastic cells (34).

In concert with cyclin D1, Cdk4 appears to have an important role in tumorigenesis. Cdk4 is one of the key regulators of G_1 progression in mammalian cells. We have found previously that expression of Cdk4 is greatly reduced in contact-inhibited, cultured intestinal epithelial cells; however, Cdk4, along with cyclins D1 and D3, is strongly induced within 6 h after release from contact inhibition in the presence of serum (35).⁴ The cyclin D/Cdk4 (or Cdk6) kinase complex is selectively inhibited by a family of cyclin kinase inhibitors that includes p16^{INK4a} and p15^{INK4b}, and failure to appropriately inhibit Cdk4 activity likely contributes to tumor cell proliferative capacity and to cell transformation (9, 10). Expression of p16^{INK4a} can inhibit proliferation and oncogenic transformation of cultured cells (36–38), and *Ink4a* gene inactivation has been observed with high frequency in numerous tumor cell lines and selected primary tumors (39–42). *Cdk4* gene amplification and overexpression also have been reported in small subsets of human glial tumors, glial tumor cell lines (18), and human sarcoma cell lines (19). Thus, abnormal increases in the level and/or activity of Cdk4 appear to be common events in tumorigenesis.

Our present findings of Cdk4 expression in the normal mouse intestinal crypts suggest that it may have an important role in normal intestinal crypt cell proliferation. We did not detect Cdk4 immunoreactivity outside of the expected normal proliferative (crypt) zone in the histologically normal intestinal epithelium, but expression was abundant in adenomatous epithelium, even at the luminal surface. Thus, Cdk4 expression in this ectopic location is inappropriately increased. Expression of Cdk4 throughout the adenomas may reflect expansion of the crypt proliferative zone. This would be consistent with recently reported findings that early adenomas appear to arise in the crypt regions of the intestine in transgenic mice bearing a truncated *Apc* gene (43). The absence of detectable cyclin D1 expression in the normal mouse intestinal crypt, which has a relatively high proliferative index, was surprising to us and argues against its role in cell cycle progression in the normal adult intestine. Our observation that cyclin D1 levels are very low or undetectable in the grossly normal human colon mucosal samples supports this postulate, and is consistent with the recently reported immunohistochemical findings of Arber *et al.* (27) in non-FAP patients. These findings suggest that either cyclin D2 or cyclin D3 functions to activate Cdk4, and thereby regulates G_1 progression in the intestinal crypt. Alternatively, it is possible that expression of cyclin D1 may occur transiently and for only a brief period, making detection difficult. Coexpression of Cdk4 and cyclin D1 in the intestinal adenomas may enable the cells to retain the capacity to proliferate beyond the intestinal crypt, and this contention is supported by our BrdUrd incorporation data.

In conclusion, our results suggest that inappropriate coexpression of cyclin D1 and Cdk4 occur at the relatively early premalignant stage of tumor development in the intestine. The expression of these G_1 regulatory proteins may account for the increased cell proliferation and the autonomous growth of intestinal adenomas. It will be of interest to determine the mechanisms involved in cyclin D1 and Cdk4 coexpression in intestinal adenomas, because such studies could lead to novel therapeutic strategies to prevent adenoma formation or progression.

⁴ R. D. Beauchamp, H. Sheng, and J. Shao, unpublished observations.

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