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AKT proto-oncogene overexpression is an early event during sporadic colon carcinogenesis

Hemant K.Roy[1,4], Bola F.Olusola[1], Dahn L.Clemens[1], William J.Karolski[1], Anne Ratashak[1], Henry T.Lynch[2] and Thomas C.Smyrk[3]

[1]Department of Internal Medicine, University of Nebraska Medical Center, Omaha, NE, [2]Department Creighton University, Department of Hereditary Cancer, Omaha, NE, [3]Department of Pathology, Mayo Clinic, Rochester, MN, USA

[4]To whom correspondence should be addressed at: University of Nebraska Medical Center/Eppley Cancer Institute, 982000 Nebraska Medical Center, Omaha, NE 68198-2000, USA
Email hroy@unmc.edu

The inhibition of apoptosis is a critical event in the development of colorectal malignancies, although the mechanism(s) remain incompletely understood. The anti-apoptotic proto-oncogene, AKT, has been implicated in the molecular pathogenesis of a variety of human malignancies; however, no data exist on the role of AKT in colon carcinogenesis. We therefore evaluated the presence of *AKT* in human and experimental colon neoplasms by immunohistochemistry. Normal colonic mucosa and hyperplastic polyps exhibited no significant AKT expression, in marked contrast to the dramatic AKT immunoreactivity seen in colorectal cancers (57% positive) and in both human colorectal cancer cell lines examined. Importantly, AKT was also detected in 57% of the adenomas examined, implicating overexpression of this proto-oncogene as an early event during colon carcinogenesis. Moreover, in the rodent-carcinogen model, azoxymethane (AOM)-treatment induced AKT expression in premalignant rat colonocytes. Tumors that evolve via different genetic pathways displayed a lower incidence of AKT overexpression. Indeed, only 22% of mismatch repair defective tumors and 42% of AOM-induced rodent tumors upregulated AKT. Staining with an antibody specific for AKT 2 duplicated findings with the AKT 1&2 antibody, suggesting that AKT 2 was the predominant isoform involved in colon carcinogenesis. Furthermore, utilizing an antibody that specifically recognizes the serine-473 phosphorylated form of AKT, we observed that activated AKT was detectable in the neoplastic but not normal epithelium.

In summary, our immunohistochemical analysis indicates AKT overexpression occurs frequently during human colon carcinogenesis, but is less common in colon cancers with microsatellite instability. The early inhibition of apoptosis during sporadic colon carcinogenesis may be related, at least partly, to the overexpression of AKT.

Abbreviations: AOM, azoxymethane; APC, adenomatous polyposis coli; GSK, glycogen synthase kinase; HNPCC, hereditary non-polyposis colorectal cancer; MSI, microsatellite instability; PI, phosphoinositol; PTEN, phosphatase and tensin.

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Colon carcinogenesis is characterized by distinct morphological, genetic and cellular events. The transition from normal colon mucosa to an invasive colorectal carcinoma is mirrored by an orderly accumulation of mutations in critical genes (1). In order for the typically short-lived colonocytes to acquire these mutations, apoptosis needs to be inhibited early during neoplastic transformation (2). The mechanism(s) through which apoptosis is dysregulated is incompletely understood, but generally involves either overexpression of anti-apoptotic proteins or inactivation of pro-apoptotic proteins. The specific molecular alterations are largely dependent on the genetic background of the individual tumor. For instance, most sporadic colon cancers inhibit apoptosis through truncation of the adenomatous polyposis coli (APC) protein, along with overexpression of a variety of anti-apoptotic proteins including cyclooxygenase 2 and BCL-2 (2). However, in colorectal cancers that evolve through DNA mismatch repair deficiency, the pro-apoptotic BAX protein is frequently inactivated (2). DNA mismatch repair inactivation, with resultant microsatellite instability (MSI), occurs in 6–15% of sporadic colon cancers and in the vast majority of tumors from patients with hereditary non-polyposis colorectal cancer (HNPCC) (3). MSI-stable and MSI-high colon cancers also employ some similar strategies to inhibit apoptosis such as dysregulation of WNT signaling with overexpression of the potentially anti-apoptotic β-catenin protein and c-MYC (4).

Therefore, the mechanisms involved in the early inhibition of apoptosis during colon carcinogenesis are multifactorial and incompletely elucidated. Recently, attention has focused on the novel proto-oncogene, *AKT* (protein kinase B), which has profound anti-apoptotic activities. This 58 kDa serine-threonine kinase is overexpressed in numerous human malignancies (5). At present, three distinct AKT isoforms have been identified which share homology in ~80% of their amino acids (5). Several lines of evidence indicate that AKT signaling is important in neoplastic transformation. AKT controls a variety of critical cellular pathways including those leading to both apoptosis inhibition and increased cell proliferation (6). Since no data exist for the role of AKT in colorectal carcinogenesis, we investigated AKT protein expression in both human (sporadic and MSI-high) and experimental colon cancers.

Archived blocks were utilized for this study. Human MSI-high tumors were represented by a series of HNPCC tumors obtained through the Creighton University Hereditary Cancer Center Registry. Premalignant rat colonocytes and tumors were obtained 10 and 35 weeks, respectively, after azoxymethane (AOM) treatment of Fisher 344 rats. Deparaffinized tissue sections (3–5 μM) first underwent antigen retrieval with target retrieval solution at pH 6.1 (Dako, Carpinteria, CA), then incubated with AKT antibody for 1 h at 37°C and developed with the Vectastain A, B, C kit (Vector Laboratories, Burlingame, CA). We utilized a polyclonal antibody raised against the complete (480 AA) human AKT 1 molecule, but that also cross-reacts with AKT 2 (SC-1619 Santa Cruz

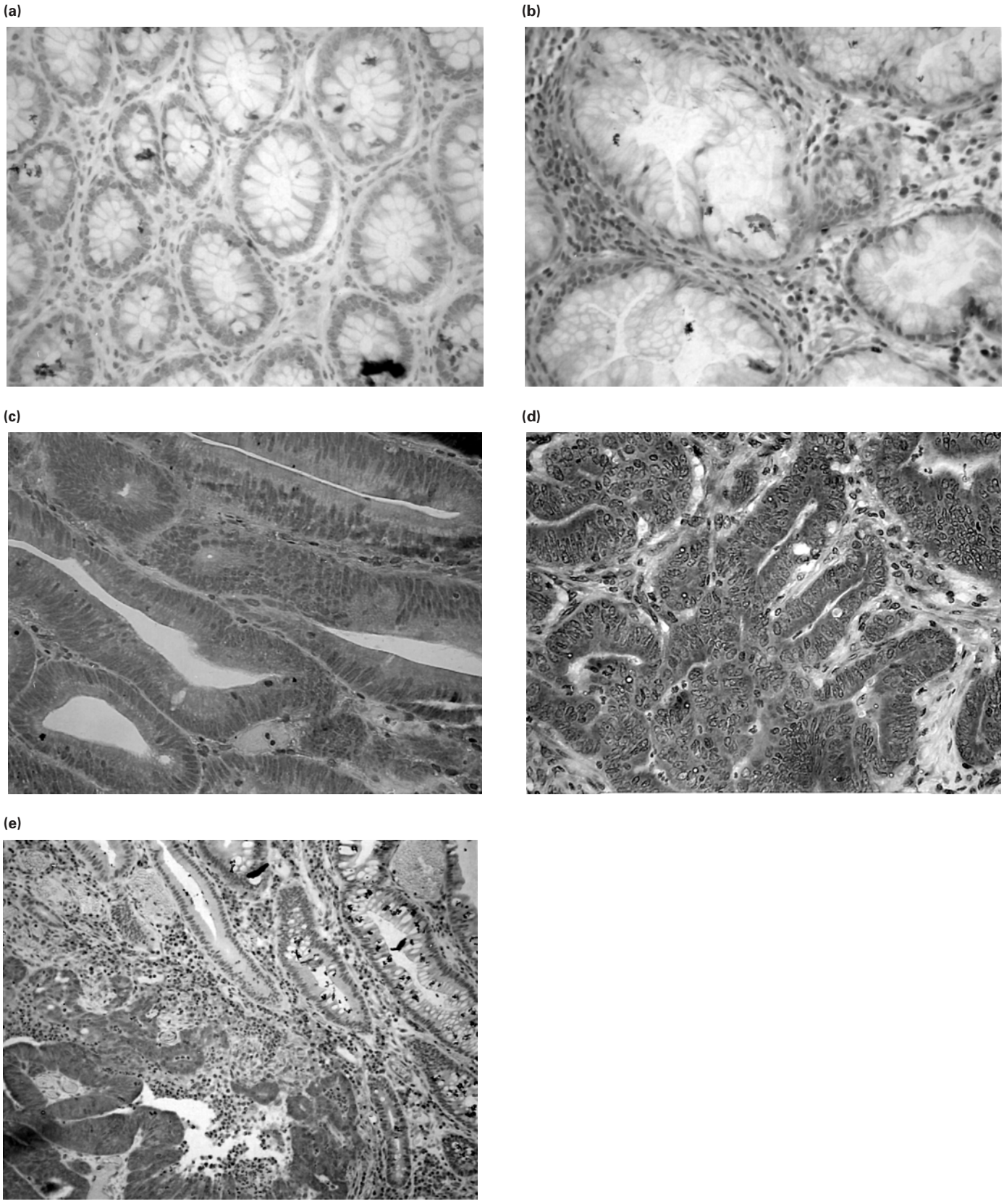

Fig. 1. Representative sections of AKT immunohistochemistry utilizing a polyclonal antibody that recognizes both AKT 1&2. (**a**) Negative control: sections incubated with secondary alone without primary antibody. No significant staining is noted. (**b**) Hyperplastic human colon polyp. (**c**) Sporadic colon adenomatous polyp. (**d**) Sporadic colon carcinoma. (**e**) HNPCC-related colon carcinoma.

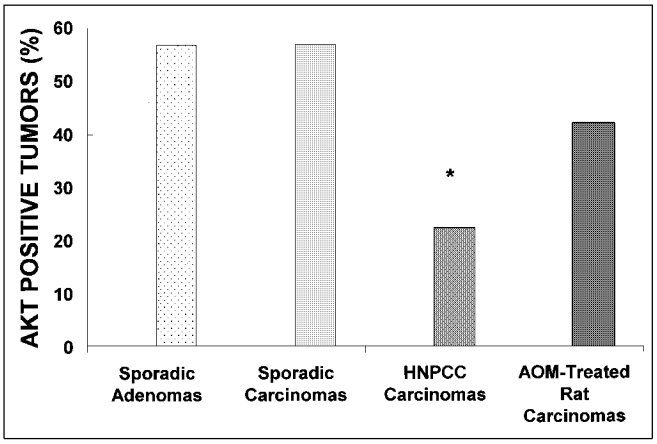

Fig. 2. Prevalence of AKT overexpression (utilizing polyclonal antibody to AKT 1&2) Number of samples analyzed for sporadic adenomas, sporadic carcinomas, HNPCC-related carcinomas, AOM-treated rat tumors were 30, 37, 36 and 12 respectively. *$P = 0.0036$ by 2-tailed Fisher's exact test of HNPCC vs. sporadic tumors.

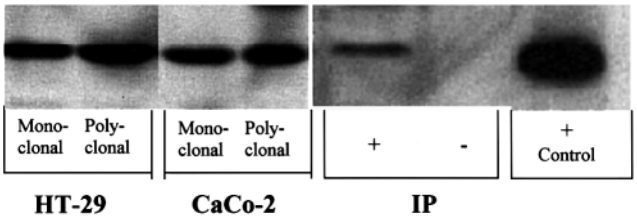

Fig. 3. Representative western blot analysis on two human colon adenocarcinoma cell lines, HT-29 and CaCo-2. The IP represents immunoprecipitation studies on HT-29 cells. HT-29 cell lysates (500 μg) of HT-29 were incubated with a goat polyclonal AKT 1&2 (plus lane) (SC 7127 from Santa Cruz Biotechnology, Santa Cruz, CA) or with an equal amount of protein A-agarose without primary antibody (minus lane). The resulting precipitant was probed with a rabbit polyclonal AKT 1&2 antibody (SC 8312, Santa Cruz Biotechnology, Santa Cruz, CA). Positive control was HCT 8 cell lysate recommended/obtained from Transduction Laboratory (Lexington, KY).

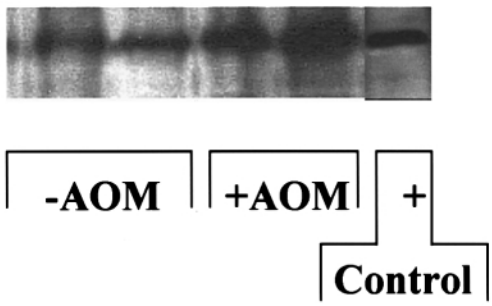

Fig. 4. Western blot analysis of uninvolved rat colonocytes. Fisher 344 rats were injected with either saline or AOM (15 mg/kg s.c.) twice separated by one week. Animals were killed 15 weeks after the second injection and 50 μg of isolate colonocyte lysate was subjected to Western blot analysis utilizing a polyclonal antibody that recognized Akt 1&2. Equal loading of gel was confirmed by both India ink staining membranes and by probing for β-tubulin. The positive control is HCT 8 cells lysate (recommended/ supplied by Transduction Laboratories, Lexington, KY). The immunoblot of individual animals are representative of at least eight animals per group and demonstrates that AOM treatment resulted in a marked induction in AKT expression in the uninvolved colonocytes.

Biotechnology, Santa Cruz, CA). We also used a monoclonal antibody that recognized all AKT isoforms (Transduction Laboratory, Lexington, KY) to duplicate part of the data. To ascertain the specific AKT isoform involved, a polyclonal antibody was employed that targeted the carboxy terminus of human AKT 2, but did not cross-react with AKT 1 (SC-7127 Santa Cruz Biotechnology, Santa Cruz, CA). Specificity was confirmed by a peptide competition assay, pre-incubating the AKT 2 antibody with a 5-fold excess (by weight) of its immunogenic peptide. To assess AKT activity, an antibody specific for serine-473 phosphorylation (#9277S, Cell Signaling Technology, Beverly, MA) was utilized. All slides were scored in a blinded fashion by a gastrointestinal pathologist (TCS) utilizing a four-point scale of intensity (0: no staining; 1: faint; 2: moderate; and 3: strong). Grades 0 and 1 were considered negative whereas grades 2 and 3 were scored as positive. AKT expression was assessed by western blot in two human adenocarcinoma cell lines and premalignant rat colon lysates isolated via modified Weiser technique (7). Fifty μg of protein from cellular lysates were separated by polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% nonfat milk, probed with either a polyclonal or monoclonal antibody to AKT 1&2 and developed with enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). Statistical analysis was performed with a two-sided Fischer's exact test.

Marked AKT immunoreactivity was detected in both human and experimental colon carcinomas (Figure 1). The staining pattern was predominantly cytoplasmic, consistent with reports in pancreatic cancer (8). In contrast, no significant AKT staining was observed in non-neoplastic mucosa or hyperplastic polyps. AKT upregulation was detected in 57% of sporadic carcinomas (21 of 37 tumors) but only 22% of MSI-high tumors (8 of 36 HNPCC samples) ($P < 0.004$) (Figure 2). Forty-two percent of AOM-tumors were positive for AKT and this protein was also detected in both of the human colon cancer cell lines examined (Figure 3).

The observation that a similar proportion of adenomas (17 out of 30) and sporadic carcinomas overexpressed AKT (both 57%) leads us to suggest that this molecular event occurs during the initial phases of colorectal carcinogenesis. Furthermore, AOM-induced AKT expression in premalignant colonic mucosa indicates the potential role of this proto-oncogene in the initiation of neoplastic transformation (Figure 4). Thus, both the human and experimental data suggest that AKT induction is one of the early events during colon carcinogenesis.

Several lines of evidence support the specificity of the immunostaining in this study. The panel of antibodies to AKT 1&2 (both monoclonal and polyclonal) yielded similar results in western blot analysis (Figure 3). The frequency of AKT detection was also similar between these two antibodies, although the staining pattern was less marked with the monoclonal antibody (data not shown). Moreover, we were able to abrogate AKT 2 reactivity by peptide competition, demonstrating the specificity of the immunostaining (Figure 5). We clearly demonstrate that AKT 2 is upregulated; however our data does not completely exclude the possibility that AKT 1 is also overexpressed (Figure 5). Analysis of a subgroup of the sporadic adenomas and carcinomas did not demonstrate any significant correlation between AKT protein expression and the clinical characteristics. To assess AKT activity, we utilized the phosphorylation status of the proto-oncogene as a surrogate marker. Phosphorylation of serine-473 is important for AKT kinase function. Furthermore, because serine-473 is autophosphorylated by AKT, it is a valuable marker of AKT activity (9). Using antibodies specific for serine-473 phosphorylated

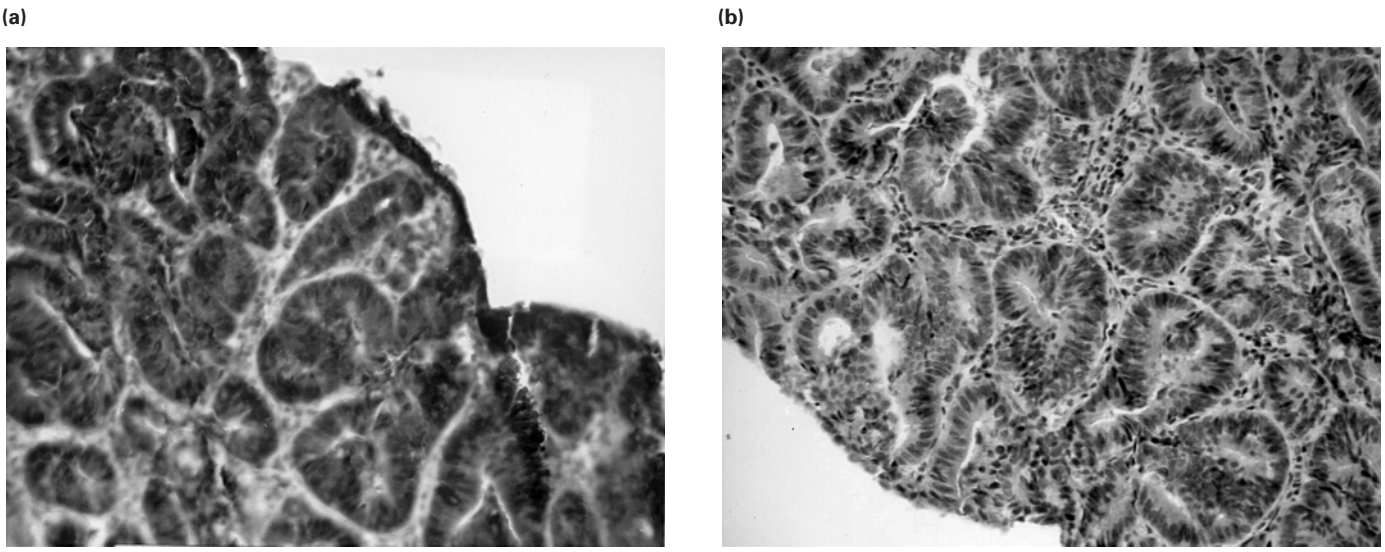

Fig. 5. Representative immunohistochemistry with an antibody specific to AKT 2, without cross-reactivity to AKT 1. (**a**) AKT 2 staining of a sporadic human colon cancer which demonstrates cytoplasmic staining comparable to results obtained with the AKT 1&2 antibody. (**b**) AKT 2 staining is abolished by pre-incubating AKT 2 antibody with a five-fold excess of its immunogenic peptide.

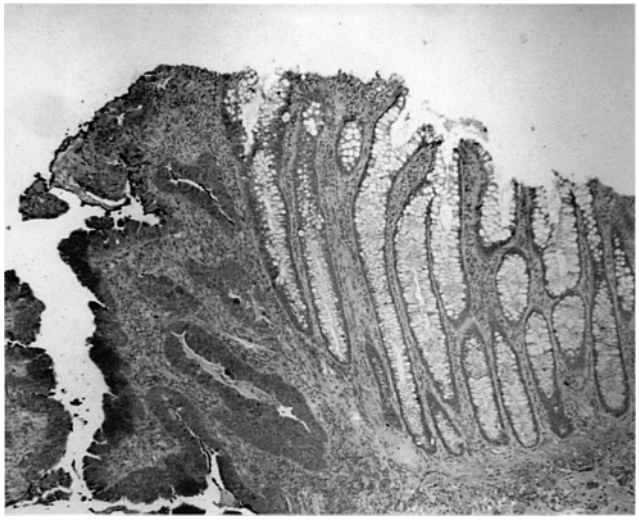

Fig. 6. Representative immunohistochemistry of a sporadic colon cancer probed with an antibody specific for serine-473 phosphorylated AKT ($n = 10$). The section demonstrates no significant staining in the uninvolved colonic mucosa whereas the colon cancer demonstrated marked cytoplasmic immunoreactivity.

AKT we demonstrated that the activated AKT was located in the neoplastic but not uninvolved mucosa (Figure 6). The present study is limited in that only AKT protein expression was evaluated; however, reports in other malignancies suggest that *AKT* DNA amplification and immunohistochemistry are closely correlated (8).

Our observation that AKT immunostaining was more common in sporadic than MSI-high tumors indicates that the genetic mechanism of tumor initiations may determine AKT upregulation. The 57% AKT overexpression rate in sporadic colon cancer is markedly higher than reports in other malignancies. In a report on human and experimental pancreatic cancer, AKT 2 was elevated in only 20% of cases (8). Additionally, AKT 2 DNA was amplified in 2.8% of breast and 12.1% of ovarian cancers (10) and was correlated with lack of differentiation in ovarian cancers.

While AKT overexpression may occur infrequently in many other tumor types, AKT signaling can still occur through alterations in different facets of its cascade. For instance, AKT activation was detected in 16 out of 17 non-small cell lung cancers (11). Moreover, mutations in the upstream regulator of AKT, phosphatase and tensin homologue (*PTEN*) (6), is one of the earliest genetic events in endometrial cancer (12). PTEN loss activates phosphoinositol (PI)-3 kinase, which generates phosphoinositol 3,4,5-trisphosphate, thereby allowing AKT, through its pleckstrin homology domains, to be recruited to the plasma membrane (6). PI-3 kinase has been implicated in colon carcinogenesis by the observation that mice lacking the catalytic subunit of PI (3) kinase γ develop spontaneous intestinal adenocarcinomas (13).

Several lines of evidence underscore the critical nature of AKT in carcinogenesis. For example, AKT expression correlated with disease progression in an experimental model of prostate cancer (14). Moreover, inhibition of this serine-threonine kinase with antisense oligonucleotides dramatically improved the neoplastic phenotype in pancreatic cancer cells (15). The mechanism by which AKT signaling promotes tumorigenesis is multifactorial, however inhibition of apoptosis appears to be of paramount importance. Transfection studies confirm the profound anti-apoptotic effect of AKT and suggest a myriad of potential molecular mechanisms (16). AKT can phosphorylate and inactivate pro-apoptotic members of the Bcl-2 family, such as Bad (17), and also induce expression of the anti-apoptotic Bcl-2 protein (18). In some systems, the anti-apoptotic effect of AKT appears to be mediated through nuclear factor-κB (19). AKT has also been shown to block several crucial steps in apoptosis including mitochondrial cytochrome *c* release (20) and activation of caspases 9 and 13 (21). AKT has been shown to impact on WNT signaling, a pathway central in the initiation of colorectal carcinogenesis. AKT activation leads to inhibition of the pro-apoptotic glycogen synthase kinase (GSK) 3β, with resultant increase in the levels of the anti-apoptotic β-catenin protein (22). Moreover, AKT can act synergistically with the Ras (23) and Raf (24) cascades, both critical in colorectal carcinogenesis (2). In colon

cancer cell lines, AKT has been demonstrated to regulate expression of the anti-apoptotic COX 2 protein (25) and modulates prostaglandin E2-induced cellular motility (26). AKT has recently been demonstrated to regulate cell cycle progression (27) and angiogenesis (28), providing other potential pro-neoplastic effects in colon cancer. Finally, AKT may represent an important therapeutic target in that its inhibition has been shown to be important in both NSAID (29) and chemotherapy (30) induced apoptosis.

In summary, we demonstrate, for the first time, that the AKT proto-oncogene is overexpressed in colorectal cancer. The upregulation of this proto-oncogene occurred at the premalignant stage, leading us to suggest that AKT overexpression may be important in the early inhibition of apoptosis during colon carcinogenesis. The genetic pathway leading to tumorigenesis seems to be of considerable importance in the induction of AKT expression. Future studies need to address the diagnostic, prognostic and potential therapeutic implications of AKT upregulation in colon cancer.

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