

Decreased Expression of DNA-dependent Protein Kinase, a DNA Repair Protein, during Human Colon Carcinogenesis

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Abstract

DNA-dependent protein kinase (DNA-PK), consisting of a catalytic subunit (DNA-PKcs) and the Ku70 and Ku86 proteins, participates in the repair of DNA double-strand breaks (DSBs). We assessed its expression immunohistochemically in normal human colon tissue, colon adenomas, colon carcinomas, and normal tissue distant from carcinomas. Normal colonocytes expressed all DNA-PK proteins. Compared with the expression in normal tissue [176.62 ± 18.56 (the intensity of expression \times the percentage of cells expressing this protein), mean \pm SE], the expression of Ku70 was significantly reduced in adenomas (36.62 ± 11.09 ; $P < 0.001$) and carcinomas (85.68 ± 15.76 ; $P < 0.01$), as was the expression of Ku86 [113.10 ± 10.22 versus 41.66 ± 14.71 in adenomas ($P < 0.01$) or versus 85.68 ± 15.76 in carcinomas ($P < 0.05$)]. The expression of DNA-PKcs was not significantly changed. The marked underexpression of Ku70 and Ku86 starting at the adenoma stage may be crucial to the development of colon cancer.

Introduction

DNA DSBs² are caused by endogenous free radicals such as the byproducts of oxidative metabolism and by exogenous agents such as ionizing radiation and chemotherapeutic agents (reviewed in Refs. 1; Refs. 2, 3). Unrepaired, they pose a major threat to the integrity of the genome and can cause cell death or can lead to cancer. Eukaryotic cells repair DSBs using a complex and sophisticated repair system, the nonhomologous (or illegitimate) end-joining apparatus (reviewed in Ref. 4). A hallmark of this system is that, in contrast to homologous recombination, it does not require sequence homology between the two recombining molecules. At least nine proteins are thought to participate in this pathway, critical components of which are conserved between yeast and mammalian cells. Although the details of this pathway have not been completely delineated to date, DNA-PK plays a crucial role. DNA-PK is a serine/threonine protein kinase consisting of a M_r 465,000 catalytic subunit, the DNA-PKcs, and a heterodimeric regulatory complex termed Ku, which is composed of a M_r 70,000 (Ku70) and a M_r 86,000 (Ku86) polypeptide. According to a model for mammalian nonhomologous end-joining, the Ku70 and Ku86 heterodimer binds to free DNA ends at the DSB and recruits DNA-PKcs, activating its kinase activity. A complex of proteins including DNA ligase IV is then recruited to the site of the DSB and interacts with DNA-PK to bring about the repair of the DNA DSB. Mutations in either DNA-PKcs or in Ku86 result in DSB repair defects manifesting as X-ray sensitivity and impaired V(D)J recombination, a process that requires DSBs to generate the vast range of

antigen-binding sites of antibody and T-cell receptor proteins. Moll *et al.* (5) recently surveyed the expression of DNA-PKcs and Ku86 in human tissues, observing that they are widely expressed in normal human tissues (exceptions were the liver and resting mammary tissue) and in most cancerous tissues. Not surprisingly, their greatest expression was in spermatogenic cells (5). DNA-PKcs-null mice showed severe immunodeficiency, radiation hypersensitivity, and, interestingly, hyperplasia, dysplasia, and aberrant crypt foci in the intestinal mucosa, which suggested a possible role in tumor suppression (6). A role of DNA-PK has been suspected in esophageal and head and neck tumors as well as in the response to chemotherapeutic agents causing DNA damage that may require the activity of DNA-PK for repair.

Given the potential role of DNA-PK in carcinogenesis, we undertook a systematic study of the expression of the three proteins comprising DNA-PK during human colon carcinogenesis. We assessed by immunohistochemistry the expression of Ku70, Ku86, and DNA-PKcs in normal human colon tissue, colon adenomas, colon carcinomas, and normal tissue distant from these carcinomas. We present here the results of our study, demonstrating dramatic changes in the expression of Ku70 and Ku86 during human colon carcinogenesis.

Patients and Methods

Patients and Tissues

Colon biopsies were obtained from 23 unselected subjects (12 men, 11 women; average age, 64 years; range, 45–74 years) who had a normal colonoscopy on a screening examination; none had a personal or family history of colonic neoplasia. Tissue was properly oriented under a dissecting microscope. Portions of colonic adenomas removed endoscopically were obtained from eight patients (four men, four women; average age, 69 years; range, 42–89 years). In four patients, the adenoma was located in the sigmoid, in three in the transverse, and in one in the descending colon. No patient had a synchronous colon cancer and apparently normal colonic mucosa at least 10 cm away from the tumor were obtained during surgery from 11 patients (5 men, 6 women; average age, 64 years; range 57–78 years). In four patients, the cancer was located in the sigmoid, in one in the transverse, in three in the cecum, in two in the rectum, and in one in the descending colon. Nine carcinomas were moderately differentiated, one poorly differentiated, and one well differentiated. Six were classified as stage B and five as stage C (Dukes/Astler-Coller). All of the tissues were fixed in 10% buffered formalin phosphate and embedded in paraffin wax. The study was approved by the appropriate Committees for Human Rights in Research.

Immunohistochemistry for DNA-PK Proteins

Four- μ m tissue sections mounted on pretreated microscope slides were stained by indirect immunoperoxidase. Briefly, tissue was deparaffinized and rehydrated in decreasing alcohols, incubated for 15 min in 3% H₂O₂ (Fisher Scientific) to quench endogenous peroxidase activity, microwaved in the presence of Antigen Retrieval Solution (Biogenex, San Ramon CA), cooled down at room temperature, then washed with PBS, and incubated for 15 min with 10% complement-inactivated nonimmune serum-containing aprotinin 7.1

Received 8/17/01; accepted 10/17/01.

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² The abbreviations used are: DSB, double-strand break; DNA-PK, DNA-dependent protein kinase; DNA-PKcs, DNA-PK catalytic subunit.

TIU/ml (Sigma Chemical Co.). After draining the immune serum, and incubating in PBS for 2 min, sections were incubated overnight with the primary antibody diluted in 1% complement-inactivated non-immune serum containing aprotinin at 2°C-8°C in a humidified chamber. After equilibration to room temperature for 30 min, the primary antibody was rinsed with PBS and incubated with the appropriate secondary biotinylated antibody for 30 min. The avidin-peroxidase complex (Vector Laboratories, Burlingame, CA) was added for 30 min, followed by the addition of DAB (3,3'-diaminobenzidine tetra-chloride, Grade II; Sigma Chemical Co.), the final coloring agent. Tissue was counterstained with hematoxylin and examined under the microscope.

Antibodies. The following polyclonal antibodies were used (all from Santa Cruz Biotechnology, Inc.): anti-Ku-70 (C-19) goat polyclonal IgG, epitope mapping at the carboxyl terminus of the M_r 70,000 subunit of human Ku protein; anti-Ku-86 (C-20) goat polyclonal IgG, epitope mapping at the carboxyl terminus of the M_r 86,000 subunit of human Ku protein; anti-DNA-PKcs goat polyclonal IgG, epitope mapping at the NH_2 terminus of human DNA-PKcs.

Controls. Negative control: nonspecific goat IgG (Santa Cruz Biotechnology, Inc.), the same class as the primary antibodies. To minimize intra-assay variability, sections of tumor and histologically normal mucosa from the same patient were processed on the same microscope slide. Also the slides were processed in sequential groups of four, consisting of normal, adenoma, the slide with tumor and histologically normal mucosa from the same patient, and the negative control.

Evaluation. The intensity of staining was rated according to the following scale: 3 = most intense brown staining; 2 = staining intensity between 1 and 3; 1 = light brown staining; 0 = no staining. In each sample the percentage of colonocytes expressing each protein was determined. To obtain a numerical assessment of the expression of each protein, we calculated for each sample the multiple of the intensity of expression by the percentage of cells expressing a protein. This was termed the *expression score*. Thus the expression score provided a semi-quantitative assessment of the expression of each protein in a given sample. Two observers rated the samples blindly; differences in their scoring did not exceed 10%; we used the average of their score as the final score.

Statistical Methods

Group means of expression scores of DNA-PK antigens were compared using ANOVA (ANOVA) followed by Fisher's protected *t* test; $P < 0.05$ was statistically significant.

Results

The Expression of DNA-PK in Normal Human Colon. As shown in Fig. 1 and 2, and Table 1, normal human colonocytes expressed all three of the proteins comprising DNA-PK. Ku70 was expressed almost exclusively in the nuclei and usually throughout the colonic crypt or predominantly in its luminal one-third. In 74% of the subjects, the expression of Ku70 was uniform. In almost all of the remainder subjects (22%), its expression was more prominent in the luminal (upper) one-third of the crypt, and in only one subject (4%), was it more prominent in the basal (lower) two-thirds of the crypt. In nearly one-half (48%) of the subjects, all of the cells expressed Ku70, in 35% of these, 60–90% of the cells were positive, and in 17% of them, less than 60% of the cells were positive. The intensity of expression varied among individual subjects, being maximal or near maximal in 43% and low in 35% (data not shown).

Ku86 was expressed in the cytoplasm following morphologically either a homogeneous or a speckled pattern. Eight subjects (35%) had concomitant nuclear and cytoplasmic expression of Ku86. In 52% of the subjects, Ku86 was expressed uniformly throughout the crypt, whereas in the remaining 48%, its expression was predominant in the luminal (upper) one-third of the crypt. Although the expression of Ku86 was significant, it was never apparent in the entire population of colonocytes. In 30% of the subjects, this antigen was detected in nearly 90% of the cells. In the remaining 70% of the subjects, <60%

Table 1 *The expression of the components of DNA-PK in normal human colon*

	Ku70	Ku86	PKcs
Colonocytes	Nuclear	Cytoplasmic ^b	Cytoplasmic
Crypt			
Entire crypt	74%	52%	43%
Luminal one-third ^a	22%	48%	57%
Basal two-thirds ^a	4%	0%	0%
Positive cells			
All	48%	0	58%
60–90%	35%	30%	30%
<60%	17%	70%	13%

^a Only or predominantly in this segment.

^b 1/3 staining was both nuclear and cytoplasmic.

of the colonic epithelial cells were positive for Ku86. As was the case for Ku70, the intensity of expression varied among individual subjects. Although it was never maximal, it was intermediate in 78% of the cases and low in 22% (data not shown).

DNA-PKcs was expressed exclusively in the cytoplasm of the colonic epithelial cells. In 9 (43%) of 23 subjects, this protein was expressed throughout the crypt, whereas in the remaining 57%, its expression was more pronounced in the luminal (upper) one-third of the crypt. In over one-half of the subjects (58%), all of the cells expressed DNA-PKcs and with the same intensity. In 30% of the subjects, 60–90% of the cells expressed DNA-PKcs, whereas in the remaining 13% of the subjects, the percentage of positive cells was <60%. Among individuals, the intensity of expression of this protein varied, with almost two-thirds showing an intermediate intensity of expression (data not shown).

The expression of these antigens did not change appreciably in normal colonic mucosa distal from colon cancer (Table 2, Fig. 1). In the few instances in which there was normal tissue contiguous to cancer, there was a tendency for reduced expression of these antigens, in particular, Ku70 and DNA-PKcs, compared with normal tissue from normal controls. Proper assessment of this question was, however, not possible, given the small number of tumors with contiguous normal mucosa and the small size of such normal mucosa.

The Expression of DNA-PK Is Markedly Reduced in Human Adenomas. In adenomas, the expression of Ku70 was markedly reduced compared with normal. The expression score for Ku70 was 36.62 ± 11.09 (mean \pm SE for this and all subsequent values), and this was significantly reduced compared with that of normal mucosa from normal controls (176.62 ± 18.56 ; $P < 0.001$), and of normal mucosa distant from colon cancer (189.06 ± 27.31 ; $P < 0.01$). In addition, the expression of Ku70 in adenomas was also significantly lower than in cancers (85.68 ± 15.76 ; $P < 0.01$), in which it was lower than normal. In the majority of adenomas, there was no expression of Ku70, except for a rim of cells in the surface, as demonstrated in Fig. 1. The staining pattern was only nuclear in 50% of the cases, only cytoplasmic in 25%, and both nuclear and cytoplasmic in 25%. In two-thirds of the cases, the adenomatous tubules (distorted crypts) stained only in their luminal (upper) one-third, whereas in the remaining one-third, the staining extended to the basal (lower) part of the tubules as well.

The expression of Ku86 in adenomas was also reduced compared with normal. The expression score for adenomas was 41.66 ± 14.71 , and this was significantly reduced compared with that of normal mucosa from normal controls (113.10 ± 10.22 ; $P < 0.01$). In contrast, the expression of Ku86 in adenomas did not differ significantly from that in cancer (63.57 ± 15.97) or in normal mucosa distant from colon cancer (96.55 ± 25.64). All of the other differences in the expression of this protein were also statistically not significant. The staining was often limited to the top one-third of the (distorted) crypts or to the brush border.

Table 2 The expression^a of the DNA-PK proteins in normal human colon, adenoma, carcinoma, and normal tissue distant from cancer^a

	Ku70	Ku86	DNA-PKcs
A) Normal (n = 23)	176.62 ± 18.56	113.10 ± 10.22	129.90 ± 17.99 ^b
B) Adenoma (n = 7)	36.62 ± 11.09	41.66 ± 14.71	79.36 ± 20.47
C) Cancer (n = 11)	85.68 ± 15.76	63.57 ± 15.97	78.91 ± 13.79
D) Normal from cancer (n = 11)	189.06 ± 27.31	96.55 ± 25.64	133.79 ± 21.94

^a Expression score (mean ± SE). The expression score, described in "Patients and Methods," is the product of the intensity of expression multiplied by the percentage of cells expressing a given protein, and assesses numerically the expression of each protein. Statistically significant differences: Ku70: A versus B, $P < 0.001$; A versus C, $P < 0.01$; B versus D, $P < 0.001$; C versus D, $P < 0.01$. Ku86: A versus B, $P < 0.01$; A versus C, $P < 0.05$.

^b n = 22.

An interesting feature of the expression of Ku86 was that in four of seven adenomas, only goblet cells stained for Ku86 (Fig. 2). The possibility of nonspecific binding of the antibody to the mucous content of the goblet cells has been ruled out by performing careful control studies on adjacent tissue sections. Even when we used a 5-fold excess of nonspecific antibody of the same immunoglobulin class, there was no staining in these cells. Such exclusive staining of goblet cells was not observed for either of the other two antigens.

The expression of DNA-PKcs was reduced in adenomas (79.36 ± 20.47) compared with normal controls (129.90 ± 17.99) or normal tissue from cancer patients (133.79 ± 21.94), but these differences did not reach statistical significance. The expression of DNA-PKcs was virtually identical to that of cancers (78.91 ± 13.79).

The Expression of Ku70 and Ku86 Is Reduced in Human Colon Cancers. In cancer tissue, the expression of Ku70 was variable. There were areas totally devoid of Ku70 expression, whereas in

others, Ku70 was adequately expressed (Fig. 1). The area of tumor in each sample that stained positive for Ku70 varied between 25 and 90% (average 51%). All of the patients had nuclear staining; three (27%) of them showed, in addition, cytoplasmic staining. The expression score was also decreased to about one-half compared with controls (85.68 ± 15.76 versus 176.62 ± 18.56 ; $P < 0.01$) or with normal mucosa away from cancer (85.68 ± 15.76 versus 189.06 ± 27.31 ; $P < 0.01$). This expression score, however, was higher compared with that of adenomas (85.68 ± 15.76 versus 32.62 ± 11.09), but this difference was not statistically significant.

The expression of Ku86 was also significantly reduced in colon cancer, being about two-thirds of that of normal tissue (113.10 ± 10.22 versus 63.57 ± 15.97 ; $P < 0.05$). There was no clear association between the degree of tumor differentiation and the expression of this antigen (or either of the other two antigens).

The expression of DNA-PKcs was reduced in colon cancer compared with that in normal controls (78.91 ± 13.79 versus 129.90 ± 17.99) or with that in normal tissue from cancer patients (78.91 ± 13.79 versus 133.79 ± 21.94), but neither difference was statistically significant. Goblet cells were seen in a few samples, but only in two of them, did they stain positive for this antigen.

Of 11 cancers, one showed staining of goblet cells for Ku70 but nongoblet cells stained as well; two demonstrated exclusive staining of goblet cells for Ku86; and for DNA-PKcs, one showed exclusive staining of goblet cells, whereas one showed staining of both goblet and nongoblet cells (Fig. 2).

Discussion

Our results describe the pattern of expression in the normal human colon of the three proteins comprising the DNA repair protein

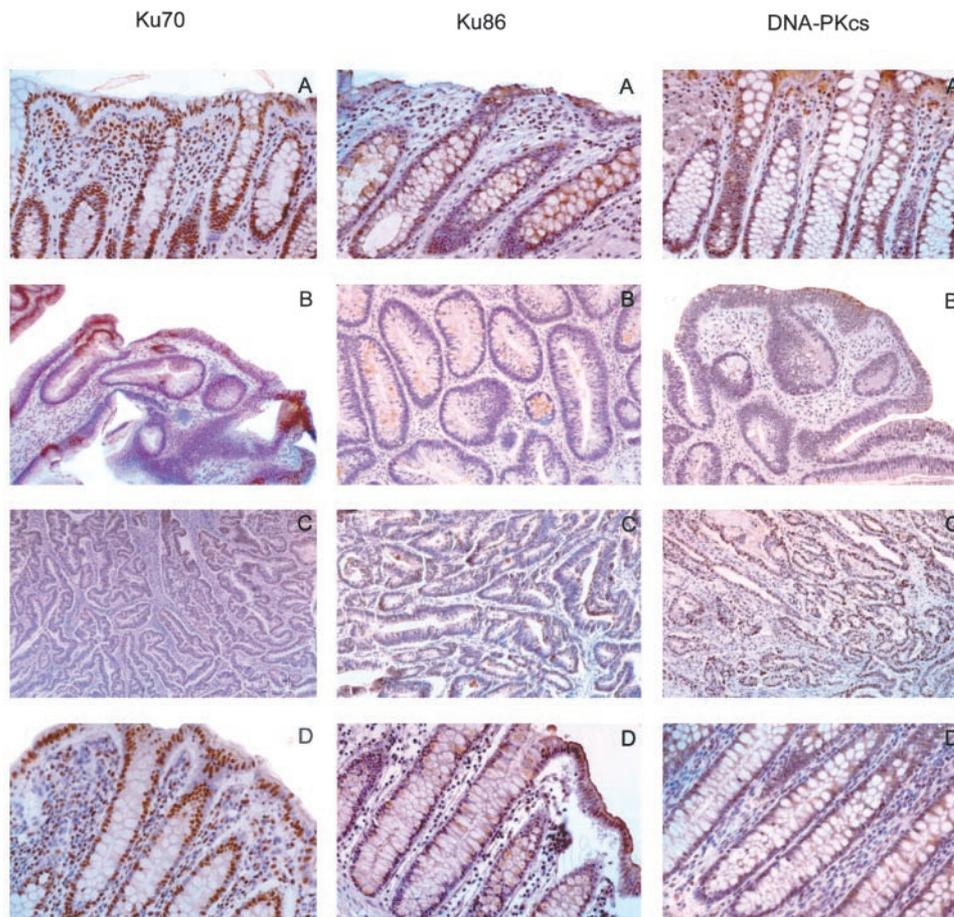


Fig. 1. The expression of DNA-PK proteins in human colon tissues. Tissue sections have been stained by immunohistochemistry for each of the three proteins comprising DNA-PK, as described in "Patients and Methods." A, normal control tissue; B, adenoma; C, carcinoma; D, normal colon tissue distant from the adenoma. The expression of Ku70 in the adenoma (B) is restricted to cells in the outer perimeter of the tissue (the "rim-effect" described in the text). The carcinoma (C) displays an area expressing Ku70, whereas the remaining area does not express any detectable Ku70; this is typical of the cancer samples displaying a striking heterogeneity of antigen expression, as described in the text.

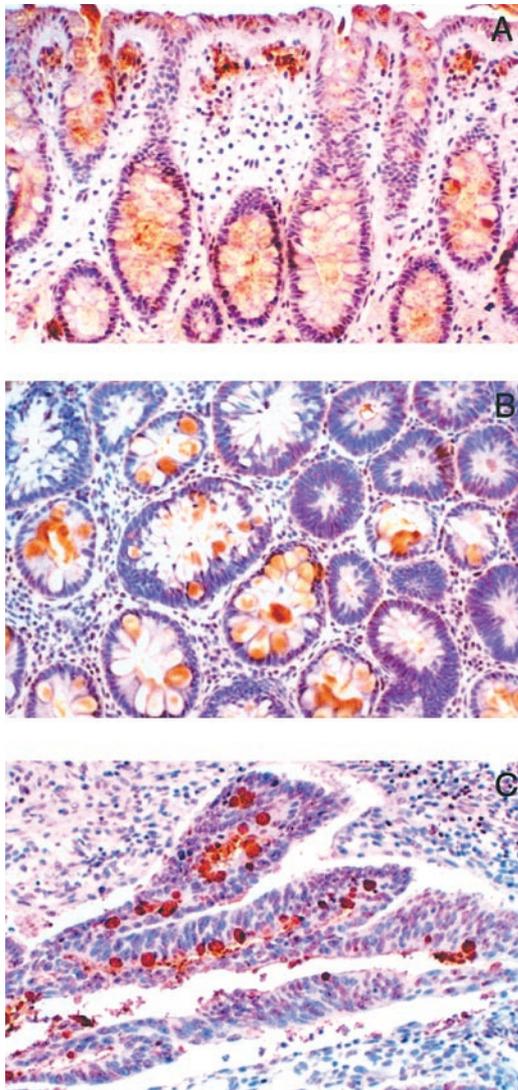


Fig. 2. The expression of DNA-PK proteins in goblet cells. Human colon tissue sections have been stained by immunohistochemistry for Ku86, as described in "Patients and Methods." In these examples, protein expression is either predominant in (A) normal control tissue or limited to (B) adenoma or (C) carcinoma goblet cells.

DNA-PK and demonstrate extensive down-regulation during human colon carcinogenesis.

DNA-PK is expressed amply in normal human colonocytes, perhaps indicating its biological significance. Of the three components of DNA-PK, Ku70 is the one expressed most prominently and is frequently evident throughout the colonic crypt. This pattern of expression is consistent with the current model for mammalian nonhomologous end-joining (4). Ku70 is the first to respond to DSB, forming a heterodimer with Ku86, which binds to free DNA ends at the DSBs and then recruits DNA-PKcs. Thus, this pattern of expression may suggest that, for this function, the cell produces second-line proteins on demand.

Ku70 is expressed in the nuclei, whereas Ku86 and the catalytic subunit are expressed either exclusively or predominantly in the cytoplasm. Such distribution is not inconsistent with their role in repairing damaged DNA in the nucleus. NF- κ B is but one example of many proteins residing in the cytoplasm and transported into the nucleus for their action (7).

An interesting aspect of the distribution of these proteins is that

whenever their expression in the crypt was asymmetric, they tended to be expressed in the luminal part of the crypt. This finding suggests, however tenuously, that repair of DSB catalyzed by DNA-PK takes place at a later stage in the life of the colonocyte (when DNA damage requiring repair will have occurred) and prior to the stage when damaged or senescent cells are extruded into the lumen of the colon by apoptosis.

The second interesting finding of our study is that the expression of Ku70 and Ku86 is severely diminished during colonic carcinogenesis. Quantitatively, the greatest reduction in the expression of these proteins occurs at the adenoma stage. For each of these two subunits, the amount of protein is about one-half to two-thirds of that in cancer, which in turn is lower than in normal tissue. There is suppressed expression of the catalytic subunit of DNA-PK, to the same extent in both adenomas and carcinomas, but these changes do not reach statistical significance. The absence of these two antigens, especially Ku70, is often dramatic, being present only in a rim of cells at the luminal side of the adenoma. The apparent "partial escape" that occurs at the carcinoma stage is of unknown etiology or significance.

The known function of DNA-PK in the repair of DSBs in nonmammalian and in some mammalian systems provides for a central biological role of DNA-PK in carcinogenesis. DSBs, a dramatic damage in a chromosome, when left unrepaired can have major biological consequences (1). Indeed, DSBs occur during colon carcinogenesis, some of them appearing at an early stage (8). Lack of an adequate repair system, as exemplified by our observation concerning components of DNA-PK, may be an important contributor to the propagation of the carcinogenic cascade in the colon. That the reduction in Ku70 and Ku86 is an early event in colon carcinogenesis argues for a pathogenetic role in cancer. In fact, the observation that these changes are maximal at the adenoma stage makes it less likely that they are simply an epiphenomenon of an already altered genotype. This notion is supported by evidence that changes in DNA-PK can play a role in carcinogenesis (6).

In conclusion, our findings demonstrate that normal colonocytes express amply DNA-PK, a protein complex involved in the repair of DSBs. Human colon carcinogenesis is associated from its early stages with severely reduced expression of two of the three proteins that constitute DNA-PK. The biological role of DNA-PK in maintaining DNA integrity and the features of these changes suggest that their underexpression can be crucial to the development of colon cancer.

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Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

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Cancer Res 2001;61:8381-8384.

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