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Reduced C-Terminal Src Kinase Activity Is Correlated Inversely with pp60$^{c\text{-}src}$ Activity in Colorectal Carcinoma

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BACKGROUND. Specific kinase activity of the proto-oncogene product pp60$^{c\text{-}src}$ is reported to be elevated in patients with carcinoma of the colon, and a novel cytoplasmic protein-tyrosine kinase, C-terminal Src kinase (Csk), has been found to inactivate the members of the Src family protein-tyrosine kinase. In this study, Csk activity and pp60$^{c\text{-}src}$ activity were examined in colorectal tumors as well as in colon carcinoma cell lines.

METHODS. Colorectal carcinoma tissue and adjacent nonneoplastic tissue from 24 patients, from 8 colon carcinoma cell lines, and from 1 normal colon cell line were used. The levels of pp60$^{c\text{-}src}$ and Csk in colorectal tissue and cell lines were analyzed by Western and/or Northern blot analysis, and their kinase activity levels were measured by in-gel kinase assay.

RESULTS. In the samples from 24 patients with colorectal carcinoma, pp60$^{c\text{-}src}$ kinase activity and protein levels were increased by 7.8 $\pm$ 0.55 and 2.6 $\pm$ 0.13 times the control levels, respectively. Conversely, the Csk protein level and its kinase activity were reduced by 0.53 $\pm$ 0.08 and 0.53 $\pm$ 0.09 times the control levels, respectively. pp60$^{c\text{-}src}$ kinase activity was correlated inversely with Csk activity (correlation coefficient = -0.71; $P < 0.0001$). Of the cell lines, pp60$^{c\text{-}src}$ kinase activity and protein levels, respectively, were 7.4 $\pm$ 1.22 and 1.86 $\pm$ 0.28 times greater than normal control levels. Csk protein level and kinase activity, respectively, were 0.54 $\pm$ 0.13 and 0.52 $\pm$ 0.11 times less normal control levels and were correlated with mRNA amount.

CONCLUSIONS. Csk mRNA, protein, and its kinase activity were reduced in colorectal carcinoma and were correlated with pp60$^{c\text{-}src}$ kinase activity level. The reduced activity of Csk may be involved in the transformation of a subset of colorectal carcinoma. ***Cancer* 2001;92:61–70.** *© 2001 American Cancer Society.*

KEYWORDS: pp60$^{c\text{-}src}$, C-terminal Src kinase, colon carcinoma.

Protein phosphorylation in signal transduction is related to proliferation, differentiation, and carcinogenesis of cells.[1–6] Src family proteins have tyrosine kinase activities, and some of their members, like pp60$^{c\text{-}src}$, have viral homologues that cause malignant transformation of cells.[7,8] The kinase activity of Src family members is regulated by phosphorylation and dephosphorylation of a tyrosine kinase residue located close to the C-terminus, corresponding to tyrosine 527 (Tyr-530) in chicken c-src.[7–12] The pp60$^{v\text{-}src}$, or other mutationally activated variants of pp60$^{c\text{-}src}$ that lack Tyr-530, are capable of transforming fibroblast cell lines in vitro,[13,14] and the constitutive reduction of phosphate on Tyr-530 of pp60$^{c\text{-}src}$ by overexpression of protein tyrosine phosphatase causes activation of pp60$^{c\text{-}src}$ as well as transformation of rat embryonic fibroblasts.[15]

Recently, it has been found that C-terminal Src kinase (Csk) is a

TABLE 1
pp60$^{c\text{-}src}$ and C-Terminal Src Kinase in Patients with Sporadic Colorectal Carcinoma

Patient	Age (yrs)	Gender	Location	Degree of differentiation	Lymph meta	Duke classification	T/N ratio				
							pp60$^{c\text{-}src}$ protein	pp60$^{c\text{-}src}$ activity	pp60$^{c\text{-}src}$ [pY^{530}]	Csk activity	Csk protein
1	62	M	A	Mod	−	A	1.8	4.2	0.94	0.72	0.88
2	66	M	C	Well	−	A	2.1	4.4	0.92	0.87	0.95
3	52	M	A	Mod	−	A	2.3	7.9	0.01	0.15	0.01
4	76	F	S	Mod	−	A	1.7	3.8	0.96	1.04	1.28
5	74	F	A	Mod	−	B	3.1	6.9	0.79	0.59	0.51
6	75	M	S	Well-Mod	−	B	1.9	10.1	0.01	0.01	0.02
7	58	M	S	Mod	−	B	2.9	6.2	0.84	0.61	0.65
8	72	M	A-T	Mod	−	B	2.1	9.8	0.01	0.01	0.02
9	55	F	C	Mod	−	B	3.2	6.2	0.64	0.51	0.55
10	74	F	C	Mod	−	B	2.7	7.9	0.35	0.46	0.22
11	47	F	A	Mod	−	B	2.0	6.8	0.01	0.15	0.04
12	70	M	R	Mod	−	B	2.1	5.9	0.01	0.11	0.01
13	72	F	A	Mod-Poor	+	C	2.4	13.4	0.01	0.03	0.01
14	73	F	C	Mod-Poor	+	C	4.2	7.8	0.92	0.80	0.86
15	76	F	R	Mod	+	C	3.5	11.2	0.43	0.48	0.21
16	68	M	C	Mod	+	C	2.3	6.4	0.94	0.88	1.15
17	79	F	C	Mod	+	C	2.8	6.2	0.96	1.00	1.00
18	72	F	R	Mod-Poor	+	C	3.1	12.3	0.01	0.19	0.18
19	64	M	R	Well	+	C	2.8	9.8	0.46	0.32	0.39
20	68	M	R	Mod	+	C	2.7	5.8	0.89	1.07	1.24
21	62	F	A	Well	+	C	1.8	4.7	0.92	1.18	0.95
22	62	F	S	Well	+	C	3.4	8.2	0.61	0.51	0.57
Average (mean ± SE)	—	—	—	—	—	—	2.58 ± 0.13	7.84 ± 0.55	0.5 ± 0.08	0.51 ± 0.08	0.50 ± 0.09

Csk: C-terminal Src kinase; pp60$^{c\text{-}src}$ [pY^{530}]: phosphorylated pp60$^{c\text{-}src}$; T/N: tumors tissue/nontumours tissue; M: male; F: female; A: ascendent; C: cecum; T: transverse; R: rectal; S: sigmoid; Mod: moderately differentiated; meta: metastasis; +: positive; −: negative; SE: standard error.

cytoplasmic protein tyrosine kinase that inactivates members of the Src family tyrosine kinases in vitro.[16–19] Csk contains SH3 and SH2 regions at the upstream of the kinase domain, and the primary structure has a high similarity to Src family kinases. However, Csk has several unique characteristics; although it does not have a myristoylation site and lacks an autophosphorylation site (Tyr-416 in chicken c-src) as well as a negative regulatory tyrosine residue equivalent to Tyr-530 in pp60$^{c\text{-}src}$, it can phosphorylate pp60$^{c\text{-}src}$ specifically at Tyr-530, thereby inhibiting its kinase activity.[16–19]

Altered gene products are observed in patients with colon carcinoma with respect to their disease stage.[20] Protooncogene product pp60$^{c\text{-}src}$ is one such gene product, and its tyrosine kinase activity is elevated in patients with colon carcinoma and in patients with some types of adenoma and ulcerative colitis who present with a high risk of developing malignant disease.[21–25] However, the mechanisms responsible for the increased tyrosine kinase activity have not been clarified, except for our recent report on hepatocellular carcinoma.[26] Thus, we analyzed the amount of mRNA, protein, and/or its kinase activity of both pp60$^{c\text{-}src}$ and Csk using specimens form patients with colorectal carcinoma as well as colorectal carcinoma cell lines.

MATERIALS AND METHODS

Patients

Samples containing tumor tissue from the colon (T) and adjacent nontumorous colorectal tissue (N) were obtained during surgery from 24 patients (11 men and 13 women; mean age, 66 ± 9 years; range, 47–79 years) (Table 1). None of the patients had received any chemotherapy or radiotherapy before surgery. The stage of each colorectal tumor was determined according to the classification of Dukes[27]: Four patients had Stage A tumors, 8 patients had Stage B tumors, and 12 patients had Stage C tumors. Informed consent was obtained from each patient according to the Helsinki declaration.

Isolation of Tumor Cells

Resected tissues were frozen immediately at −80 °C. At first, we distinguished the N (normal) and T (tumor) tissues in colorectal specimens by the naked eye. Portions of N and T tissues were fixed with formalin and embedded in paraffin. Sections of colorectal speci-

mens were stained by hematoxylin and eosin and were reviewed by a pathologist (T.S.). We confirmed that > 80% of cells in T tissues obtained in this way consisted of tumor cells, whereas > 80% of cells in N tissues consisted of normal epithelial cells. The remaining 20% of cells consisted of mesenchymal cells.

Cell Lines

Colon carcinoma cell lines LoVo, LS-174-T, HCT 116, HT 29, HCT 15, SW 48, SW 837, and WiDr were obtained from the American Type Culture Collection (Baltimore, MD). The epithelial-like colon cell line, CCD 841 CoN, was a gift from Dr. Yuasa Y (Tokyo Medical and Dental University, Tokyo, Japan). Cells were cultured in Dulbecco modified Eagle medium for the LS 174-T and WiDr cell lines, in RPMI 1640 medium for the HCT 15 and CCD 841 CoN cells lines, in Mc Coy 5A medium for the HCT 116 and HT 29 lines, in Hamm F-12 medium for the LoVo cell line, and in Leibovitz 15 medium for the SW 48 and SW 837 cell lines, which were supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L glutamine, 1% nonessential amino acids, 50 mg/mL penicillin, and 50 mg/mL streptomycin. Cultured cells were processed for 2 days or for 10 days after reaching confluence, as specified. Cultures were tested routinely for mycoplasma contamination using a DNA hybridization method (Dai Nippon Seiyaku, Tokyo, Japan), and contaminated cultures were discarded.

Tissue Lysate

The tissue and cell line samples were frozen on dry ice within 20 minutes of collection. The samples were homogenized in a buffer containing 10 mM Tris HCl, pH 7.5; 1.0 mM ethylenediamine tetraacetic acid; 150 mM NaCl; 1.0 mM Na3VO4; 50 mM Na2MoO4; 1% nonidet P-40; and 100 U/mL aprotinin (TNE buffer); and lysates were centrifuged at ×29,000 g for 30 minutes at 4 °C. Protein concentration was measured by using the bicin choninic acid protein assay.

Chemicals

Chemicals were obtained from Sigma Chemical Company (St. Louis, MO) and from Wako Pure Chemical Company (Osaka, Japan). The $pp60^{c\text{-}src}$ antibody was purchased from Oncogene Laboratory (Cambridge, MA; mouse monoclonal antibody [mAb]; clone 327). The polyclonal anti-Src[pY^{530}] phosphospecific antibody was purchased from Biosource International. The polyclonal and monoclonal Csk antibodies were purchased from Transduction Laboratories Co. Ltd. and from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. The Csk polyclonal antibody (C-20) is an affinity purified rabbit polyclonal antibody raised against a peptide corresponding to amino acids 431–450, which map at the C-terminus of Csk p50 of human origin and differs from the corresponding mouse sequence by two amino acids.[16,17] The Csk mAb is an affinity purified mouse mAb raised against a peptide corresponding to amino acids 1–156, which map at the N-terminus of Csk p50 of rat origin.[18,19] These antibodies react with Csk of mouse, rat, and human origin in Western blot analysis and immunoprecipitation, and they are noncross reactive with other tyrosine kinases. For controls, we used actin (C-11), a goat polyclonal antibody purchased from Santa Cruz Biotechnology, Inc. The β-actin cDNA control probe was obtained from Clontech Laboratories Inc. (Palo Alto, CA).

Western Blot Analysis of $pp60^{c\text{-}src}$, $pp60^{c\text{-}src}[pY^{530}]$, and Csk

Fifty micrograms of total cell tissue lysate prepared as described above were separated by 8.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8.5% SDS-PAGE) according to the method of Laemmli.[28] After blotting onto PVDF filters, Western blot was analysis performed using either a mouse mAb to $pp60^{c\text{-}src}$ (Oncogene Laboratory) in a 1:400 dilution, a rabbit polyclonal antibody to $pp60^{c\text{-}src}[pY^{530}]$ (Biosource International) in a 1:1000 dilution, a rabbit polyclonal antibody to Csk (Transduction Laboratories Co. Ltd.) in a 1:500 dilution, or a mouse mAb to Csk (Santa Cruz Laboratories) in a 1:100 dilution following the method described by Towbin et al.[29]

Immunoreactive proteins were detected using a goat antirabbit secondary antibody (for $pp60^{c\text{-}src}[pY^{530}]$ and Csk polyclonal antibody) or a goat antimouse secondary antibody (for $pp60^{c\text{-}src}$ and Csk mAb) conjugated to horseradish peroxidase (Pierce, Rockford, IL) in a 1:1000 dilution and were visualized with an enhance chemiluminescence (ECL) detection system (Amersham, Buckinghamshire, England) on X-ray film. The exposure time with the ECL method lasted for 30 seconds at room temperature in all samples. All membranes were stripped with 2 M glycine, pH 2.8, for 30 minutes and were incubated with actin goat polyclonal antibody in a 1:100 dilution followed with incubation using an antigoat secondary antibody.

Measurement of Kinase Activity

$pp60^{c\text{-}src}$ activity

We used an assay kit from Upstate Biotechnology (Lake Placid, NY) that is designed to measure the phosphotransferase activity of Src kinase in immunoprecipitates and column fractions. The assay kit is based on phosphorylation of a specific substrate peptide (KVEKIGEGTYGVVYK)[30] using transfer of the γ phosphate of adenosine-5′-radioactive phosphorous ([^{32}P]) triphosphate ([γ-^{32}P] ATP) by $pp60^{c\text{-}src}$ kinase.

In brief, 200 μg of protein were immunoprecipitated with Src specific mAb and mixed in a microcentrifuge tube with 10 μL of pp60$^{c\text{-}src}$ kinase reaction buffer. Then, 10 μL of the substrate peptide were added at a concentration of 150 μM and incubated for 10 minutes at 30 °C with 10 μL of [γ-^{32}P] ATP. Twenty microliters of 40% TCA were added to precipitate peptide and incubated for 5 minutes at room temperature.

Then, 25 μL were spotted onto the center of a P81 phosphocellulose paper square and washed three times for 5 each minutes with 0.75% phosphoric acid and then once for 3 minutes with acetone. The squares were transferred to a scintillation vial with 5 mL of scintillation cocktail and read in a scintillation counter, and the activity of enzyme samples was compared with the activity in control samples that contained no enzyme (background control). To certify the quality of the Src kinase kit, we used purified Src kinase expressed in Sf9 insect cells by recombinant baculovirus containing the human pp60$^{c\text{-}src}$ gene (Upstate Biotechnology).

Csk activity

Aliquots of lysate containing 500 μg of cellular proteins in colorectal carcinoma tissues, normal adjacent colorectal mucosa, and cell lines were incubated with 2 μL of rabbit polyclonal antibody (C-20) for 4 hours on ice. After absorption to pansorbin, immunoprecipitates were washed five times with TNE buffer and twice with kinase assay buffer (50 mM Tris-HCl, pH 7.4; 3 mM $MnCl_2$; and 0.1 mM Na_3VO_4). Immunoprecipitated proteins on beads were suspended in 25 μL of kinase buffer (50 mM HEPES, pH 8.0; 10 mM $MgCl_2$; 1 mM dithioreitol; 2 mM glutathione [reduced form]; 10 mM b-glycerophosphate; 1 mM NaF; 0.1 mM sodium orthovanadate; and 20 mM ATP) and then incubated at 30 °C for 30 minutes with 2.0 μg of tyrosine-containing polyamino acids, poly(Glu,Tyr)[31] and 4 nmol/0.74 MBq of [γ -^{32}P] ATP (Daiichikagaku, Co. Ltd., Tokyo, Japan). After incubation, phosphoproteins were resolved by 12.5% SDS-PAGE followed by autoradiography or image analysis with the BAS 2000 system (Fuji Film, Tokyo, Japan). Under these assay conditions, phosphorylation against poly(Glu,Tyr) was linear for up to 30 minutes (data not shown).

Extraction of RNA and Northern Blot Analysis

Total RNA fractions were extracted from colorectal carcinoma cell lines using acid-guanidinium thiocyanate-phenol-chloroform.[32] The probe used for Northern blot analysis was the one for Csk from the complementary DNA (cDNA) clone pME18S, which has an open reading frame of 1350 nucleotides and encodes a protein of 450 amino acids. This cDNA was provided by Dr. M. Okada (Division of Protein Metabolism, Institute for Protein Research, Osaka University, Osaka, Japan). Total RNA isolated from colorectal carcinoma cell lines was denatured and separated by electrophoresis in formaldehyde agarose gels. RNA was transferred to Hybond N membranes (Amersham). After prehybridization at 42 °C for 4 hours, the membranes were incubated at 42 °C for 15 hours in the hybridization mixture containing 50% formamide; 5 times standard saline citrate; 10 mmol/L sodium phosphate, pH 6.8; 0.5% SDS; 5 times Denhart medium; 20 μg/mL salmon sperm DNA; and the ^{32}P-labeled cDNA probe. The membranes were washed three times with 0.2 $\times$ standard saline citrate containing 0.1% SDS for 30 minutes at 42 °C, air dried, and analyzed with the BAS 2000 system. The membrane was stripped with 0.5% SDS at 90 °C for 10 minutes and incubated with β-actin cDNA as a control.

Densitometric Analysis

The density of the Western blot protein bands and the phosphorylated smear of poly(Glu,Tyr) obtained on autoradiography were analyzed by means of an image analyzer (Intelligent Quantifier I-D; Bio Image, Tokyo, Japan). Data were expressed as relative ratios compared with the ratio in adjacent normal mucosa.

Statistical Analysis

Data were expressed as the mean $\pm$ standard error of the mean. A Pearson rank-order correlation coefficient was calculated for correlation analysis. A Fisher coefficient value < 0.05 denoted the presence of statistical significance.

RESULTS

Colorectal Carcinoma Specimens

pp60$^{c\text{-}src}$ protein

Protein levels of pp60$^{c\text{-}src}$ were determined by immunoblot analysis (Fig. 1A). Three bands were detected in each line: The upper band represented pp60$^{c\text{-}src}$ (60 kilodaltons [kD]), the middle band was mAb 327 heavy-chain immunoglobulin (50 kD), and the lower band was mAb 327 light-chain immunoglobulin (25 kD).[23–25,33] The average level of pp60$^{c\text{-}src}$ protein in 24 patients was increased by 2.6 $\pm$ 0.13 times the level in adjacent normal counterpart samples (Table 1).

pp60$^{c\text{-}src}$ activity

The activity of pp60$^{c\text{-}src}$ is shown in Table 1. The mean value of pp60$^{c\text{-}src}$ activity in colorectal neoplasms was 7.8 $\pm$ 0.55 times greater than the activity level in the adjacent normal tissue. For a positive control, we used a purified Src kinase expressed in Sf9 insect cells by recombinant baculovirus containing the human *c-src* gene: Twenty units of this lot of Src kinase were trans-

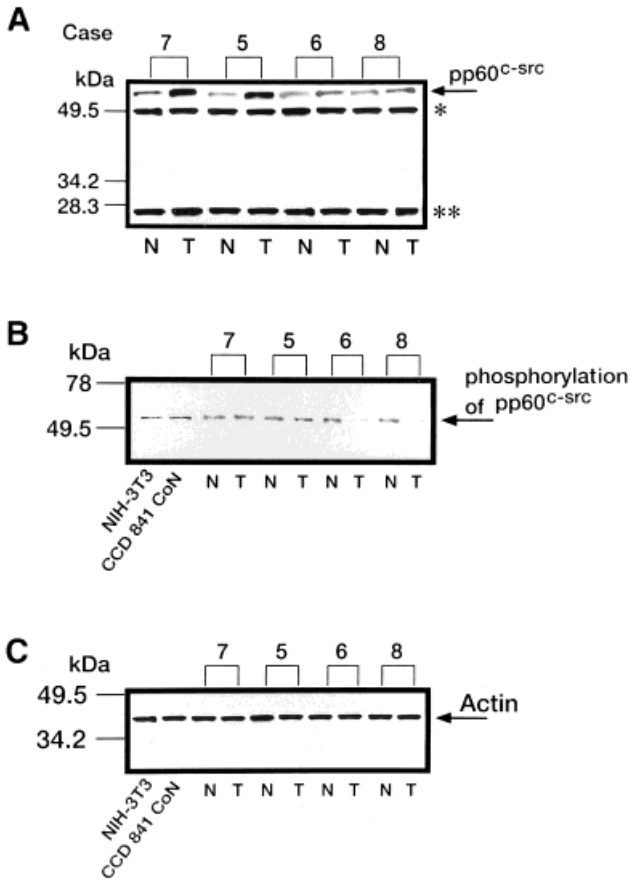


FIGURE 1. pp60$^{c\text{-}src}$ analysis in colorectal carcinoma specimens. (A) Representative Western blot of pp60$^{c\text{-}src}$ immunoprecipitated in nontumorous (N) and tumorous (T) tissues from a patient with carcinoma of the colon. Two hundred micrograms of protein from total tissue lysate were immunoprecipitated with pp60$^{c\text{-}src}$ specific monoclonal antibody, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and analyzed as described in the text (see Materials and Methods). Arrows indicate bands corresponding to pp60$^{c\text{-}src}$, a single asterisk indicates a band representing heavy-chain immunoglobulin; and double asterisks indicate a band representing light-chain immunoglobulin. (B) Representative Western blot of pp60$^{c\text{-}src}$[pY^{530}] in nontumorous (N) and tumorous (T) tissues from a patients with carcinoma of the colon. Fifty micrograms of tissue lysate of N and T regions of colon carcinoma were subjected to SDS-PAGE and analyzed as described in the text (see Materials and Methods). The NIH-3T3 and CCD 841 CoN cell lines were used as positive controls. Note the decrease rate of pp60$^{c\text{-}src}$ phosphorylated in Patients 6 and 8. (C) Actin Western blot analysis after stripping the membrane. Note that the same amount of protein was applied in all lines.

ferred at 0.85 pmoles of phosphate per minute per unit to Src kinase substrate peptide, as established previously.[30] Fifteen units of this purified Src kinase produced a major band at approximately 60 kD on a silver-stained SDS-PAGE gel (data not shown). Colon tumors from patients who were classified with Dukes Stage C disease showed greater pp60$^{c\text{-}src}$ activity compared with tumors from patients who were classified with Dukes Stage A and B disease (relative pp60$^{c\text{-}src}$ ratio, 9.0 $\pm$ 0.83 vs. 6.7 $\pm$ 0.59, respectively; $P < 0.05$). The specific activity of pp60$^{c\text{-}src}$ in colon carcinoma (estimated by dividing the total protein kinase activity by the amount of pp60$^{c\text{-}src}$ protein) was 3.04 times greater than the activity found in normal colonic mucosal cells.

pp60$^{c\text{-}src}$[pY^{530}]

The amount of phosphorylated pp60$^{c\text{-}src}$[pY^{530}] was assessed by immunoblot analysis using a phosphospecific antibody (Fig. 1B). All tissue samples exhibited a pp60$^{c\text{-}src}$[pY^{530}] band with a molecular weight of approximately 60 kD. An average amount of pp60$^{c\text{-}src}$[pY^{530}] in neoplasm tissue was reduced compared with normal tissue (relative ratio, 0.50 $\pm$ 0.08) (Table 1). We confirmed that the same amounts of protein were applied in each case by stripping the membrane and incubating with actin antibody (Fig. 1C).

Csk protein

The expression of Csk protein was measured by Western blot analysis using two Csk antibodies (Fig. 2). All nontumorous tissue samples exhibited a Csk band with a molecular weight of 50 kD using an mAb (Fig. 2A, arrow) described by Nada et al.;[18] however, the band of Csk in a subset of tumorous tissue samples was not detected (Fig. 2A, Patients 6 and 8). We conducted an additional Western blot analysis using a Csk polyclonal antibody, which recognizes an epitope different from that of the mAb (Fig. 2B). The results shown in both Figure 2A and Figure 2B are similar. Table 1 shows that the average Csk protein level in tumors tissue was reduced by 0.53 $\pm$ 0.08 times of the protein level in adjacent normal tissue. The same amounts of protein were found after stripping the membrane and incubating with actin antibody (Fig. 2C).

Csk activity

Enzymatic activity of Csk was measured using an in-gel kinase assay (Fig. 2D). Black grains representing ^{32}P incorporation into poly(Glu,Tyr) ranged between 20 kD and 50 kD. In the control, representative Csk activity in immunoprecipitate product using nonimmune rabbit serum was very low (Patient 7).

The relative ratio of ^{32}P incorporation into the poly-(Glu,Tyr) in tumors to that incorporated in normal adjacent mucosa was reduced by 0.53 $\pm$ 0.09 times (Table 1). Markedly decreased Csk activity ($<$ 20% of that in adjacent normal mucosa) was seen in eight specimens (33%), moderately decreased CSK activity (20–60% of control) was seen in seven specimens (29%), and there was no significant change in nine specimens (38%) compared with control (Table 1).

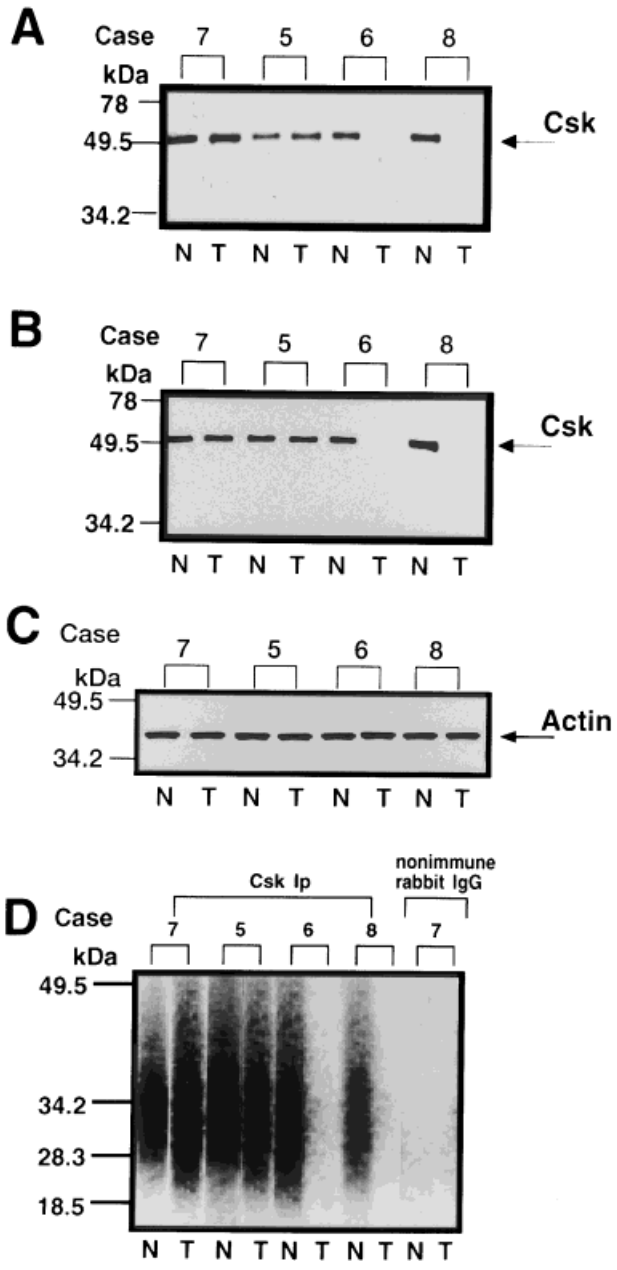
Correlation of Csk and pp60$^{c\text{-}src}$

Csk activity was correlated directly with Csk protein amounts (r = 0.96; $P < 0.0001$) (Fig. 3A) and with the level of phosphorylated pp60$^{c\text{-}src}$ (r = 0.96; $P < 0.0001$) (Fig. 3B), but Csk activity was correlated inversely with pp60$^{c\text{-}src}$ kinase activity levels in patients with colorectal carcinoma (r = −0.71; $P < 0.0001$) (Fig. 3C).

Colorectal Cell Lines

pp60$^{c\text{-}src}$ protein

The protein level of pp60$^{c\text{-}src}$ also was determined in cell lines by Western blot analysis (Fig. 4A). The average pp60$^{c\text{-}src}$ protein level was increased by 1.86 ± 0.28 times compared with the level in the CCD 841 CoN cell line (Table 2).



pp60$^{c\text{-}src}$ activity

Src activity was increased in all carcinoma cell lines compared with the activity in the epithelial-like colon cell line CCD 841 CoN. The average relative ratio of ^{32}P incorporated into the Src kinase substrate peptide (KVEKIGEGTYGVVYK) in colon carcinoma cell lines was increased by 7.4 ± 1.22 times (Table 2).

pp60$^{c\text{-}src}$[pY^{530}]

Phosphorylated pp60$^{c\text{-}src}$[pY^{530}] in colorectal carcinoma cell lines was reduced by 0.41 ± 0.15 times compared with the levels in the epithelial-like colon cell line CCD 841 CoN (Fig. 4B, Table 2). The same amounts of protein were found in each case after stripping the membrane and incubating with the actin antibody (Fig 4C).

Csk protein

The average value of Csk protein level was reduced by 0.54 ± 0.13 times compared with the value in the epithelial-like colon cell line CCD 841 CoN (Fig. 5A, Table 2). The same amounts of protein were found after stripping the membrane and incubating with the actin antibody (Fig. 5B).

FIGURE 2. C-terminal Src kinase (Csk) analysis in colorectal cancer specimens. Representative Western blot of Csk in nontumorous (N) and tumorous (T) tissues using two kinds of antibodies—rabbit polyclonal antibody (A) and mouse monoclonal antibody (B)—by means of an enhanced chemiluminescence detection system. Fifty micrograms of tissue lysate fraction of tumorous (T) and nontumorous (N) regions of samples from patients with colorectal carcinoma were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed as described in the text (see Materials and Methods). Note the 50-kD Csk band in N and T tissues (Patients 7 and 5). A Csk band was not seen in Patients 6 and 8 (note that the case numbers shown here correspond to the patient numbers listed in Table 1). (C) Western blot analysis of actin after stripping the membrane. Note that the same amount of protein was applied in all lines. (D) Representative activity of Csk in nontumorous (N) and tumor (T) portions of colorectal carcinoma samples. Lysates containing 500 μg of total cellular protein were prepared as described in the text (see Materials and Methods). Proteins were immunoprecipitated with excess Csk polyclonal antibody; then incubated for 30 minutes with 2.0 μg of tyrosine-containing polyamino acids, poly(Glu,Tyr), and 4 nmol/0.74 MBq of γ-phosphate of adenosine-5′-radioactive phosphorus ([^{32}P]) triphosphate ([γ-^{32}P] ATP) in 25 μL of kinase assay buffer; then resolved on 12.5% SDS-PAGE gels. Note the low ^{32}P incorporation into poly(Glu,Tyr) in the N portions of a subset of colorectal carcinomas. Note also that the activity of Csk was very low in Patients 6 and 8. IgG: immunoglobulin G.

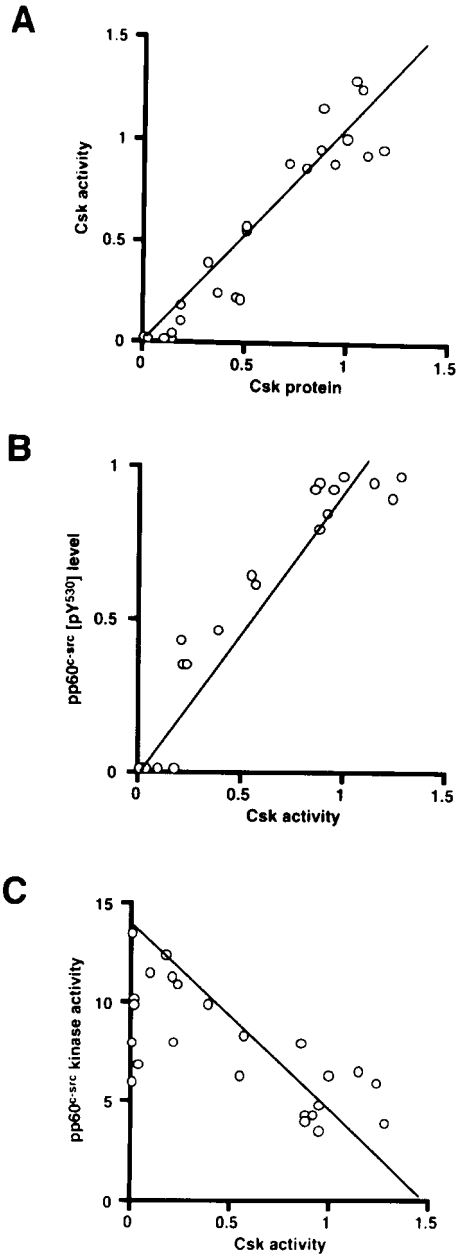


FIGURE 3. Correlation analysis of $pp60^{c\text{-}src}$ and C-terminal Src kinase (Csk). (A) Csk activity was correlated with Csk protein amount (correlation coefficient [r] = 0.96; $P = 0.0001$). (B) Phosphorylation of $pp60^{c\text{-}src}$ in tyrosine 530 (pY^{530}) was dependent on the Csk activity (r = 0.96; $P = 0.0001$). (C) $pp60^{c\text{-}src}$ kinase activity is correlated inversely with Csk activity (r = −0.71; $P = 0.0001$).

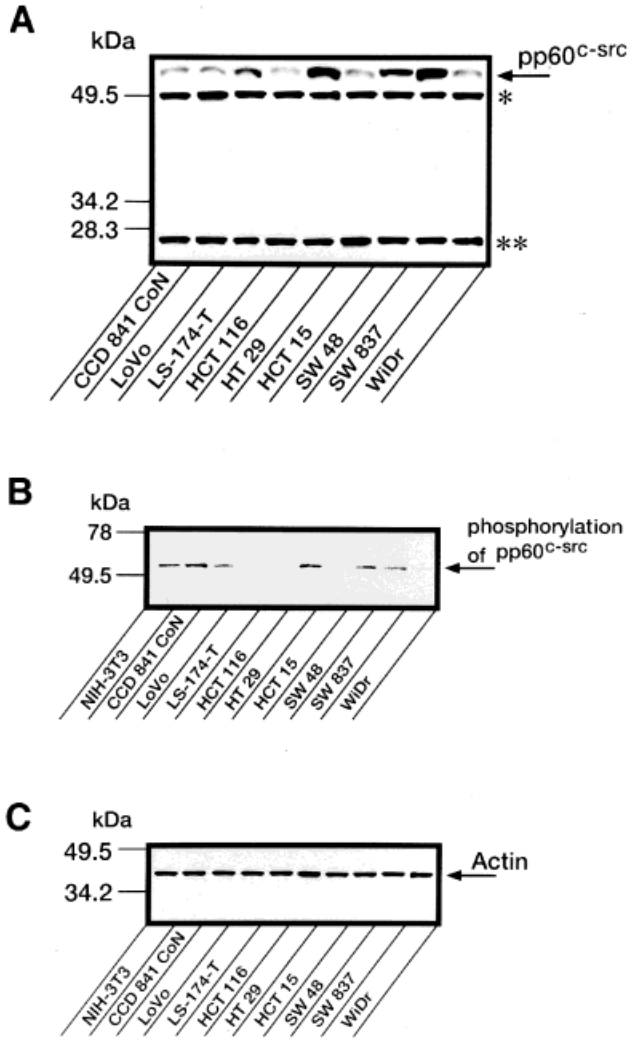


FIGURE 4. $pp60^{c\text{-}src}$ analysis in colorectal carcinoma cell lines. (A) Western blot analysis of $pp60^{c\text{-}src}$ immunoprecipitated in colon carcinoma cell lines and in a colon epithelial cell line. Arrowheads indicate bands corresponding to $pp60^{c\text{-}src}$, a single asterisk indicates a band representing heavy-chain immunoglobulin; and double asterisks indicate a band representing light-chain immunoglobulin. (B) Western blot analysis of $pp60^{c\text{-}src}[pY^{530}]$ in colon carcinoma cell lines and in a colon epithelial cell line. Fifty micrograms of tissue lysate of colon carcinoma cell lines were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed as described in the text (see Materials and Methods). NIH-3T3 and CCD 841 CoN cell lines were used as positive controls. (C) Western blot analysis of actin after stripping the membrane. Note that the same amount of protein was applied in all lines.

Csk activity

The average Csk activity level was reduced by 0.52 ± 0.11 times compared with the activity level in the epithelial-like colon cell line CCD 841 CoN. Reduced Csk activity was observed in all carcinoma cell lines except for HT 29, SW 48, and SW 837 (Fig. 5C, Table 2).

Northern blot analysis of Csk

Using equal amounts of total RNA (Fig. 6A), analysis with the BAS 2000 system revealed a single band of 1.35-kb mRNA of Csk in all colorectal carcinoma cell lines examined. The cell lines with low protein expression showed decreased levels of mRNA compared with the levels in the epithelial-like colon cell line CCD 841 CoN (Fig. 6B). After stripping the membrane and using

TABLE 2
$pp60^{c\text{-}src}$ and C-Terminal Src Kinase in Colorectal Carcinoma Cell Lines Compared with Cell Line CCD 841 CoN[a]

Cell line	Ratio of $pp60^{c\text{-}src}$ protein	Ratio of $pp60^{c\text{-}src}$ activity	Ratio of phosphorylated $pp60^{c\text{-}src}$	Ratio of Csk protein	Ratio of Csk activity
CCD 841 CoN	1	1	1	1	1
LoVo	1.30 ± 0.8	4.8 ± 1.1	0.72 ± 0.05	0.60 ± 0.09	0.42 ± 0.06
LS 174T	1.84 ± 0.9	10.5 ± 2.1	0.01 ± 0.01	0.14 ± 0.04	0.27 ± 0.03
HCT 116	1.10 ± 0.6	13.5 ± 0.9	0.01 ± 0.01	0.47 ± 0.04	0.32 ± 0.04
HT 29	2.90 ± 2.4	4.7 ± 1.4	0.94 ± 0.30	0.97 ± 0.01	1.00 ± 0.01
HCT 15	1.16 ± 0.8	9.4 ± 1.9	0.01 ± 0.01	0.15 ± 0.05	0.28 ± 0.05
SW 48	2.14 ± 1.9	3.8 ± 0.8	0.78 ± 0.06	0.88 ± 0.02	0.63 ± 0.10
SW 837	3.10 ± 1.5	5.2 ± 1.2	0.69 ± 0.10	0.95 ± 0.02	0.94 ± 0.05
WiDr	1.33 ± 0.8	7.2 ± 1.9	0.08 ± 0.02	0.15 ± 0.04	0.33 ± 0.01
Mean ± SE	1.86 ± 0.28	7.4 ± 1.22	0.41 ± 0.15	0.54 ± 0.13	0.52 ± 0.11

Csk: C-terminal Src kinase; SE: standard error.
[a] The data represent the mean ± SE of three independent experiments.

β-actin cDNA as a control probe, a single band of the same intensity was detected in all cell lines (Fig. 6C). Similar results were obtained when RNA from colorectal carcinoma specimens was examined (data not shown).

DISCUSSION

Elevated levels of $pp60^{c\text{-}src}$ kinase activity have been reported to be implicated in colon carcinoma[21–23] as well as in adenoma[24] and ulcerative colitis,[25] both of which carry a high risk for developing malignant disease. However, the mechanism responsible for an increased $pp60^{c\text{-}src}$ protein kinase activity in these tissues remains unclarified. To address this question, the mutation of $pp60^{c\text{-}src}$ in colorectal carcinoma has been studied on a large scale; however, no mutations of $pp60^{c\text{-}src}$ at codons 98, 381, 444, and 530 were detected by means of RNAase protection and restriction fragment length polymorphism assays.[34] Recently, a truncating mutation in $pp60^{c\text{-}src}$ at codon 530 was observed in 12% of patients with advanced colon carcinoma,[35] although this does not explain the high frequency of up-regulated kinase activity of $pp60^{c\text{-}src}$ in patients with colorectal malignancies.[22–23]

Previous reports showed that the $pp60^{c\text{-}src}$ specific kinase activity (estimated by dividing the total protein kinase activity by the amount of $pp60^{c\text{-}src}$ protein) was increased in colorectal tumors.[22–24] In fact, we found that the average specific activity of $pp60^{c\text{-}src}$ in colon carcinoma is three times greater than that of $pp60^{c\text{-}src}$ from normal colonic mucosal cells. Thus, in concordance with the previous report,[23] an increase in $pp60^{c\text{-}src}$ kinase activity in tumor tissues and in cultured colon tumor cells cannot be explained only by an increase in the expression of $pp60^{c\text{-}src}$ protein but also must be explained by an increase in its intrinsic activity.

Csk is a novel cytoplasmic protein tyrosine kinase that inactivates members of the Src family of protein-tyrosine kinases in vitro and in vivo.[15–19] It negatively regulates $pp60^{c\text{-}src}$ activity by specifically phosphorylating the tyrosine residue (Tyr-530) of $pp60^{c\text{-}src}$.[15,16] There has been no information on the role of Csk in human carcinogenesis except for our previous report on patients with hepatocellular carcinoma.[26]

The major finding in the current study is a reduced activity of the tyrosine kinase of Csk in 60% of colorectal malignancies. Furthermore, the expression levels of Csk mRNA and protein also were reduced in most of the colon carcinoma cell lines studied, (LoVo, LS174-T, HCT116, HCT 15, and WiDr). Because we detected the Csk gene in these cell lines using Southern blot analysis (data not shown), further studies are necessary to clarify the regulation of the Csk gene at the transcriptional level. In addition, it is not clear how Csk activity is regulated. In this study, we found that Csk activity was correlated directly with Csk protein levels. Conversely, the Csk activity level was directly proportional to the level of phosphorylated $pp60^{c\text{-}src}$ but was correlated inversely with the $pp60^{c\text{-}src}$ kinase activity level in patients with colorectal carcinoma, reflecting the important role of Csk in regulating the activity of $pp60^{c\text{-}src}$. In addition, like what was seen in the cell lines HT 29 and SW 837, we found some sporadic colorectal tumors that presented normal Csk activity with high $pp60^{c\text{-}src}$ activity. It is possible that either a mutation in the regulatory tail of $pp60^{c\text{-}src}$ or other mechanisms may be responsible for the increased activity in these tumors.[35] One of the mechanisms may involve the Csk binding protein Cbp, a recently discovered transport protein that is involved in the membrane localization of Csk and in the inhibition of $pp60^{c\text{-}src}$.[36]

The results of the current study suggest that de-

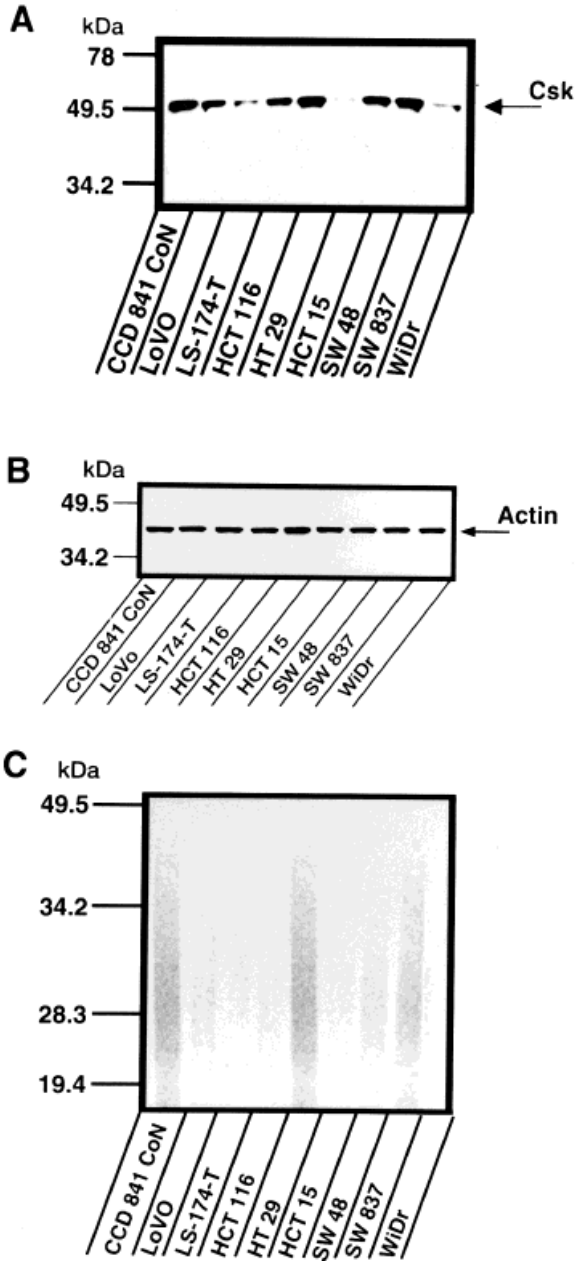


FIGURE 5. C-terminal Src kinase (Csk) analysis in colorectal carcinoma cell lines. (A) Western blot analysis of Csk in the various colon carcinoma cell lines using the monoclonal antibody by means of an enhanced chemiluminescence detection system. Fifty micrograms of tissue lysate fraction from carcinoma cell lines were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and analyzed as described in the text (see Materials and Methods). Note that the expression of Csk protein in a subset of carcinoma cell lines (LS174-T, HCT15, and WiDr) was very low. (B) Western blot analysis of actin after stripping the membrane. Note that the same amount of protein was applied in all lines. (C) Csk activity in the various carcinoma cell lines. Proteins were immunoprecipitated with excess Csk polyclonal antibody; then incubated for 30 minutes with 2.0 μg of tyrosine-containing polyamino acids, poly(Glu,-Tyr), and 4 nmol/0.74 MBq of [γ-^{32}P] ATP in 25 μL of kinase assay buffer; and then resolved on 12.5% SDS-polyacrylamide gels. Note that Csk activity was down-regulated in most carcinoma cell lines except for HT29 and SW 837.

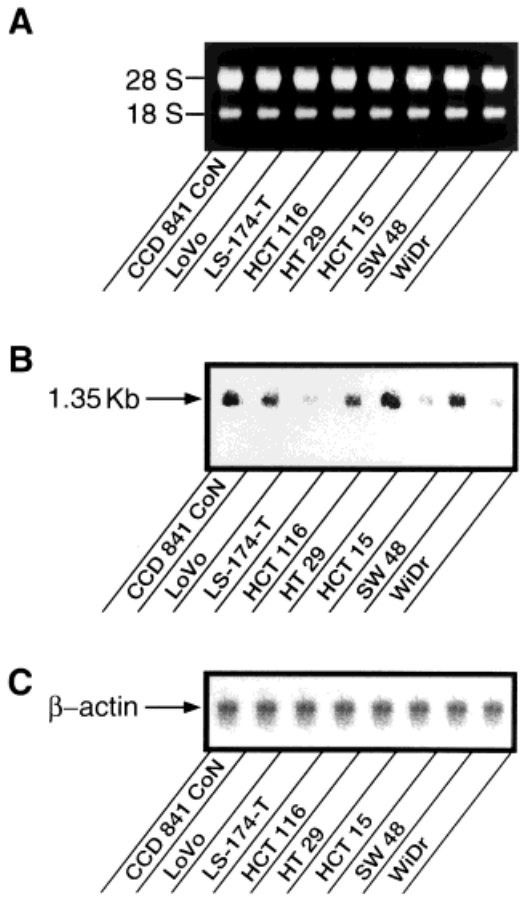


FIGURE 6. Northern blot analysis of C-terminal Src kinase (Csk) in colorectal carcinoma cell lines. (A) Twenty micrograms of RNA were loaded in each lane. Agarose gel stained with ethidium bromide and visualized under ultraviolet illumination shows bands of 28S and 18S ribosomal RNA. (B) Northern blot analysis of Csk mRNA using Csk cDNA as a probe. A single band of Csk mRNA was detected in all colorectal carcinoma cell lines examined. Note the low expression levels of Csk mRNA in some colorectal carcinoma cell lines compared with the level in the epithelial-like colon cell line, CCD 841 CoN. (C) Northern blot analysis using β-actin cDNA as a control probe. A single band of the same intensity was detected in all lines.

creased Csk activity may exert its function in the process of carcinogenesis of colorectal tissues by up-regulating pp60$^{c\text{-}src}$ activity. These data are consistent with a previous report indicating the possibility that an increased Csk activity in normal tissue may be related to the inhibition of disease progression in patients with colorectal carcinoma, because Sobe et al.[37] reported the inhibition of viral-induced transformation by Csk overexpression in fibroblasts.

In conclusion, a decrease in Csk activity may be one of the important factors involved in the malignant transformation and progression in a subset of colorectal tumors. Further studies are necessary to clarify the exact role of Csk in malignancy.

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