

# Pak-1 Expression Increases with Progression of Colorectal Carcinomas to Metastasis

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## ABSTRACT

**Purpose:** The p21-activated kinase-1 (Pak-1) promotes cell motility and invasiveness. Pak-1 is activated by the Rac, Rho, and Cdc42 small GTPases in response to a variety of stimuli including ras and phosphatidylinositol 3'-kinase/AKT pathway activation. Because Pak-1 plays a central role in regulating cell motility and invasiveness, we sought to determine whether Pak-1 may be involved in the malignant progression of colorectal carcinoma.

**Experimental Design:** Pak-1 expression was examined by immunohistochemistry in archived tissues from normal human colons, tubular and tubulovillous adenomas, invasive adenocarcinomas (stages I-III/IV), and lymph node metastases (184 total specimens from 38 patients). Specific cytoplasmic immunostaining was evaluated for overall intensity and uniformity to derive a combined histoscore (stain intensity × percentage of epithelium stained).

**Results:** Pak-1 expression was increased significantly with colorectal cancer progression from normal tissue to lymph node metastases ( $P < 0.0001$ ). Furthermore, Pak-1 expression was increased significantly in adenomas, invasive carcinomas, and lymph node metastases compared with normal colon ( $P < 0.0001$ ). Strikingly, Pak-1 expression was significantly higher in lymph node metastases than in invasive cancers, adenomas, or normal colon ( $P < 0.0001$ ). Moreover, in patients with multiple lesions representing different stages of disease, Pak-1 expression was increased specifically in the most advanced lesions.

**Conclusions:** This study demonstrates that Pak-1 expression is increased significantly with malignant progression of human colorectal carcinoma. These data, along with

numerous functional studies demonstrating a central role for Pak-1 activity in tumor invasiveness and motility, implicate Pak-1 as an exciting target for therapy of colorectal carcinoma.

## INTRODUCTION

Colorectal carcinoma (CRC) is the second leading cause of death from cancer in the United States population with 56,730 deaths and 146,940 new cases of invasive CRC anticipated for 2004 (1). Most invasive CRCs develop from intraepithelial neoplasms (adenomas), although the adenoma to carcinoma sequence may take 10 years (2). Neoplastic progression to the invasive phenotype is associated with mutational activation of oncogenes (most notably *ras* and  $\beta$ -*catenin*), loss of function of tumor suppressor genes, and increased genetic instability (3–8). CRC is frequently a systemic disease with 50% of CRC patients requiring treatment of macro- or micrometastases for survival. Thus, elucidation of both the process of development for the metastatic phenotype and the biology of CRC metastases is required for the ultimate control of CRC (9).

The molecular events that give rise to the metastatic phenotype are complex (10), clearly requiring enhanced cell motility and invasiveness (11). Cell motility and invasiveness require cytoskeletal reorganization, including formation of filopodia/lamellipodia and changes in focal adhesion complexes (12). These processes are regulated by the Cdc42, Rac1, and RhoA small GTPases (13–15). Indeed, cell motility and invasiveness are enhanced in epithelial cells that have activated forms of Rac1 and Cdc42 (16). The mechanism by which these GTPases regulate cytoskeletal changes is not fully understood but involves activation of the p21-activated kinase (Pak)-1 (17–21).

Pak-1 was the first Pak to be cloned and is homologous to other group I Paks, including Pak-2 and Pak-3. Group II Paks (Pak-4, Pak-5, and Pak-6) share only limited similarity to the group I Paks (21). Among normal tissues, Pak-1 is expressed largely in the brain, muscle, and spleen (21). Recent reports have now implicated increased Pak-1 expression in breast cancer. Pak-1 is overexpressed in 55% of human breast cancer tissues in concordance with increased cyclin D1 expression. Moreover, transgenic mice engineered to overexpress Pak-1 in mammary tissue developed mammary hyperplasia, again in accord with enhanced cyclin D1 expression (22). In addition, *Pak-1* gene amplification has been demonstrated recently in both breast and ovarian cancers (23, 24).

Functional studies have implicated Pak-1 in cell motility and tumor cell invasiveness. Kinase-dead mutants of Pak-1 suppress invasiveness of human breast cancer cell lines (25), suppress breast epithelial cell migration in response to heregulin (26), and inhibit ras-mediated transformation of rat Schwann cells (27). In rat fibroblasts, Pak-1 activation is necessary for cooperative transformation by Ras, Rac, and Rho (28). In PC12 cells, Pak-1 acts as a downstream effector of Rho family GT-

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Pases driving the polarized outgrowth of the actin cytoskeleton in the developing neurite (29). Transfection of constitutively active Pak-1 results in the dissolution of stress fibers and reorganization of focal complexes in HeLa cells and fibroblasts (30). NIH 3T3 cells that inducibly express either wild-type Pak-1, a kinase-dead, or constitutively active form of this enzyme, show dramatic changes in actin organization. Fibroblasts expressing constitutively active Pak-1 form large polarized lamellipodia at the leading edge, have increased motility, and display enhanced directional movement (31).

These studies have clearly implicated Pak-1 in the regulation of cell motility and tumor cell invasiveness. The relationship of Pak-1 to malignant progression of CRC has not been examined. Therefore, we sought to determine whether Pak-1 expression might be related to human CRC development, progression, and increased metastatic potential. We have now evaluated Pak-1 expression in 184 archived specimens from 38 patients by immunohistochemistry. Our results show that Pak-1 expression is increased with progression through the adenoma to carcinoma sequence, with the most dramatic increases in invasive and metastatic CRCs. Furthermore, in patients with multiple lesions, the most advanced lesions showed the highest Pak-1 expression further underscoring the link between Pak-1 expression and CRC progression. These data implicate increased Pak-1 expression in CRC progression.

## MATERIALS AND METHODS

**Tissues.** Archival, formalin-fixed, paraffin-embedded surgical specimens of non-neoplastic and neoplastic colorectal tissues were obtained from St. Elizabeth Medical Center (Covington/Edgewood, KY). The study was approved by the Institutional Review Board of St. Elizabeth Medical Center. The specimens examined in this study had been fixed in 10% neutral-buffered formalin overnight and routinely processed into low melting point paraffin. Serial 5  $\mu$ m tissue sections were cut and fixed onto ProbeOn Plus microscope slides (Fisher Scientific, Pittsburgh, PA).

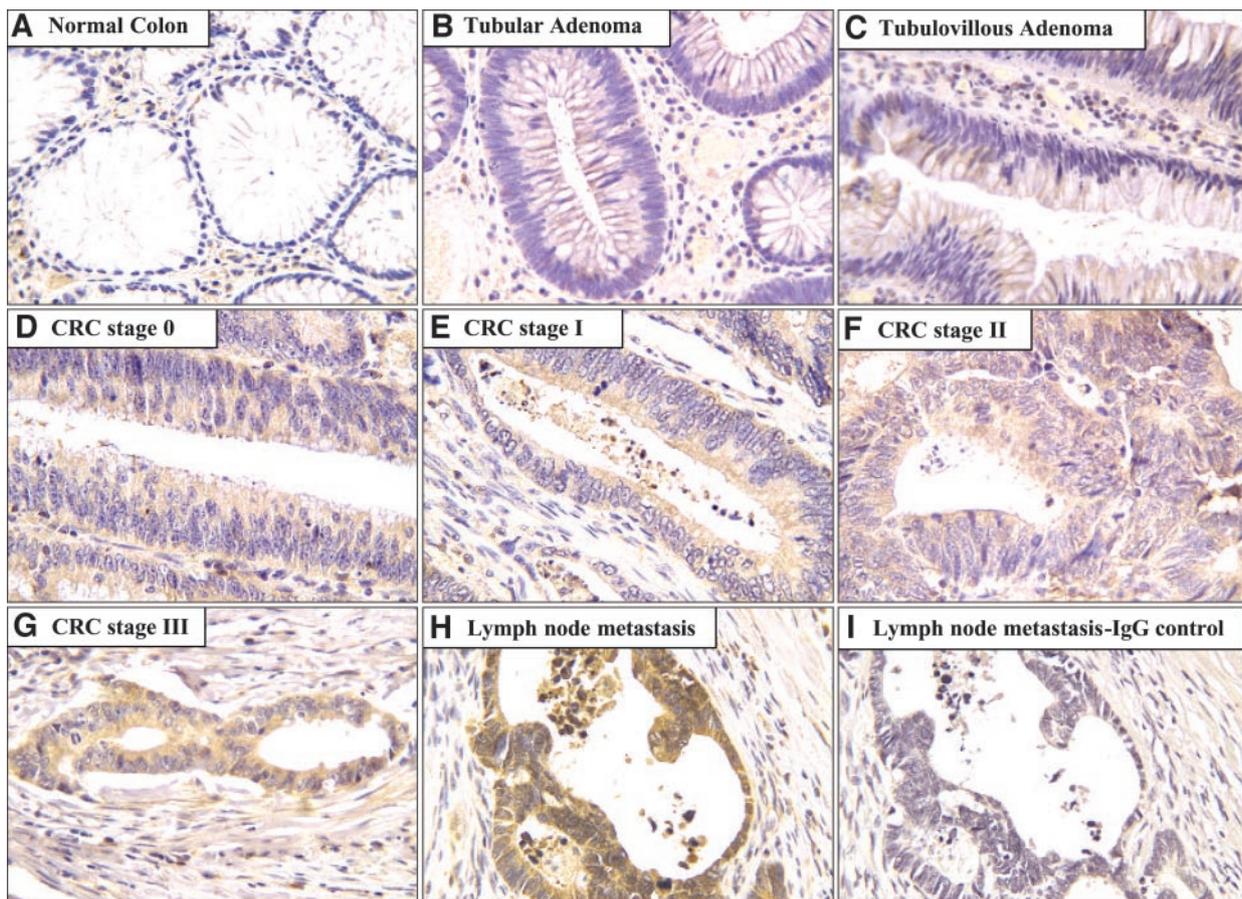
**Pathological Staging.** The histopathology of each specimen was studied on an H&E-stained tissue section by a board-certified pathologist (L. E. D.). Tissue Consultation Reports from the Department of Laboratory Medicine, St. Elizabeth Medical Center, were used to confirm patient Tumor-Node-Metastasis status (32). According to this classification, stage 0 represents carcinoma *in situ*; stage I represents tumors that invade the submucosa or the muscularis propria (and corresponds to Dukes' stage A); stage II represents tumors that invade through the muscularis propria into the subserosa or nonperitonealized pericolic or perirectal tissues, or tumors that perforate the visceral peritoneum or directly invade other organs or structures (and corresponds to Dukes' stage B); stage III represents any tumor with regional lymph node metastases (and corresponds to Dukes' stage C); stage IV represents tumors with confirmed distant metastases. All specimens of normal colon were from patients with hyperplastic polyps, adenomas, or carcinomas. Clinical follow-up was obtained from the Tri-State Tumor Registry database, which was given to Wood Hudson Cancer Research Laboratory by St. Elizabeth Medical Center.

**Pak-1 Immunohistochemistry.** Antibody staining was performed on 5- $\mu$ m histological sections of formalin-fixed, paraffin-embedded surgical specimens baked for 1.5 h at 60°C onto Probe-On Plus slides (Fisher Scientific). Sections were deparaffinized in Clear-Rite 3 (Richard-Allan Scientific, Detroit, MI) and hydrated through a graded series of alcohol to distilled water before antigen retrieval. Slides were briefly rinsed in PBS (pH 7.5) and immersed in preheated Dako Target antigen retrieval solution (DakoCytomation, Carpinteria, CA) at 90–95°C in a steamer for 20 min then cooled to room temperature. Endogenous peroxidase was blocked by incubation in 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for 30 min. Nonspecific binding was blocked by incubating slides with Dako Serum-free Protein Block (X0909) for 45 min at room temperature. Slides were stained using the capillary gap method.

Sections were incubated overnight at 4°C with a 1:250 dilution of rabbit anti-Pak-1 antibody (Zymed Laboratories, Inc., South San Francisco, CA) diluted in Dako Antibody Diluent (S0809). This antibody is specific for the 68 kDa Pak-1 protein and does not react with Pak-2 or Pak-3 proteins according to the manufacturer (Zymed). We confirmed this specificity. Immunoreactivity of this Pak-1 antibody on tissue sections was blocked by incubation with the immunizing peptide. Similarly, Western blotting with this Pak-1 antibody was also blocked by incubation with the immunizing peptide (data not shown).

Slides were washed twice with PBS-BRIJ and incubated with Dako LSAB2 System Peroxidase Goat Antirabbit Link Antibody for 10 min. Slides were washed in PBS-BRIJ, then incubated with Dako LSAB2 System Peroxidase Streptavidin-horseradish peroxidase reagent for 10 min. Immunoreactivity was detected using Vector Laboratories 3,3'-diaminobenzidine substrate kit (Vector Laboratories, Burlingame, CA). Slides were counterstained with hematoxylin (Sigma-Aldrich, St. Louis, MO). Negative controls included serial sections incubated without antibody and sections incubated with nonspecific rabbit IgG (DakoCytomation, Carpinteria, CA) at an equivalent protein concentration as the primary anti-Pak-1 antibody (1  $\mu$ g/ml).

**Semiquantitative Evaluation of Stain.** The vast majority of stain was cytoplasmic, although there was evidence for limited nuclear staining. For this study, only the cytoplasmic staining was scored. The intensity of stain and the percentage of epithelial cells with stain were estimated by two investigators (L. E. D. and J. H. C.) at a two-headed microscope. Stain intensity was given a numerical score on a scale of 0–3, with 0 = negative, 0.5 = trace, 1 = light, 2 = moderate, and 3 = intense. The percentage of the epithelium stained was also assigned a numerical value. Areas that were negative were given a value of 0, areas with <25% stain were given a value of 0.1, areas with 26–50% stain were given a value of 0.4, areas with 51–75% stain were given a value of 0.6, and areas of near homogeneous staining (76–100%) were given a value of 0.9. The average stain intensity and the average percentage area stained are represented individually. To gauge both stain intensity and uniformity simultaneously, the average values for intensity for each tissue were multiplied by the average values for percentage area stained in each tissue to derive a composite histoscore (*i.e.*, histoscore = area  $\times$  intensity). A tissue with intense, uniform staining would be assigned the maximum histoscore of 2.7,



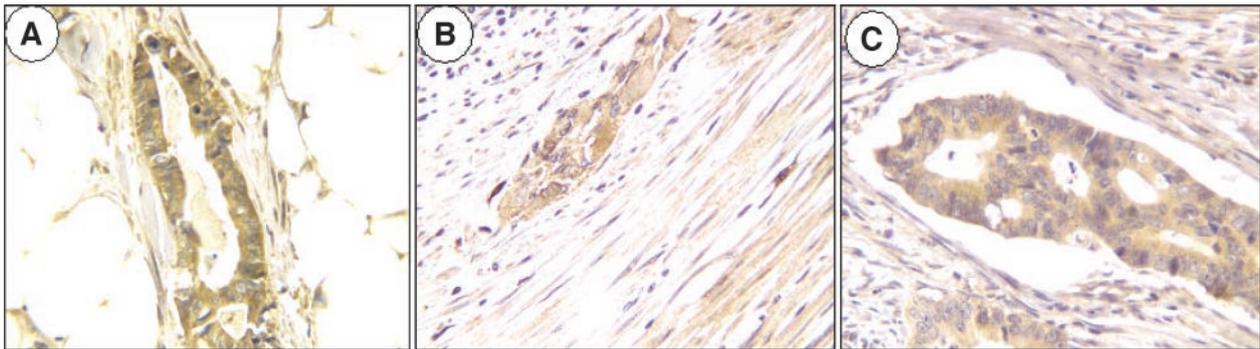
**Fig. 1** Immunohistochemical detection of p21-activated kinase-1 in normal, neoplastic, invasive, and metastatic colon tissues. *A*, normal-appearing colonic epithelium near the tubulovillous adenoma seen in *C* is lightly stained; *B*, tubular adenoma with focal, light-to-moderate staining; *C*, tubulovillous adenoma with focal, light-to-moderate staining; *D*, *in situ* carcinoma with piling-up of pleomorphic, atypical nuclei and diffuse, moderate staining; *E*, diffuse, moderate staining in a carcinoma that has invaded, but not yet penetrated the smooth muscle of the bowel wall (stage I carcinoma); *F*, diffuse, moderate staining in a stage II carcinoma that has completely penetrated the smooth muscle of the bowel wall; lymph nodes were negative for metastasis; *G*, stage III carcinoma penetrating muscle wall of colon is strongly stained; metastatic carcinoma was present in regional lymph nodes (see *H*); *H*, lymph node metastasis is intensely stained throughout; same patient as *G*; *I*, same lymph node metastasis as in *H* but incubated with nonspecific IgG (Control). Original magnifications,  $\times 400$ .

whereas a tissue with light staining intensity (a value of 1) in only 26–50% of the tissue (a value of 0.4) would get a histoscore of 0.4. Assigning a histoscore is now a commonly used method for evaluating both stain uniformity and intensity in tissues to better relate results between multiple samples from immunohistochemical studies (33).

**Statistical Analysis.** Tissues were grouped according to histopathology and Tumor-Node-Metastasis pathological stage, colonic region (proximal colon: cecum and ascending and transverse segments; or distal colon: splenic flexure, descending, and sigmoid and rectum segments), sex, and if the patient died or had a recurrence within 5 years or survived without a recurrence. For statistical analyses, tissues from patients with and without CRC were assigned tissue codes according to the current understanding of neoplastic progression in the colon (32). In total, 184 specimens were stained from 38 patients (18 female and 20 male). In some cases, we stained and evaluated numerous specimens of the same lesion (for instance, numerous areas of a large tumor may have been stained). In these cases, the

mean histoscore from these lesions was used for statistical analyses to ensure that only 1 value was included per lesion for statistical evaluation. Therefore, although 184 total specimens were stained and evaluated, 125 values were included in our statistical evaluation for Pak-1 staining in the adenoma to carcinoma sequence (38 normal colon tissues, 27 adenomas, 35 primary CRCs, and 25 CRC lymph node metastases).

Repeated measures ANOVA was used to determine differences among tissue type (normal colons, adenomas, invasive CRCs, and lymph node metastases), tumor progression (expressed as tissue codes 2–12), region, and sex for Pak-1 composite histoscores (which were the continuous outcome variables). Patient was considered a random effect. *Ps* were determined using the Tukey multiple comparison method. *Ps* < 0.05 were considered significant. For each individual tissue type, the usual *t* test was used to determine differences in region and sex. The usual *t* test was used to assess differences between patients who survived 5 years with those who died using either the primary or metastatic tumor with the highest histoscore from



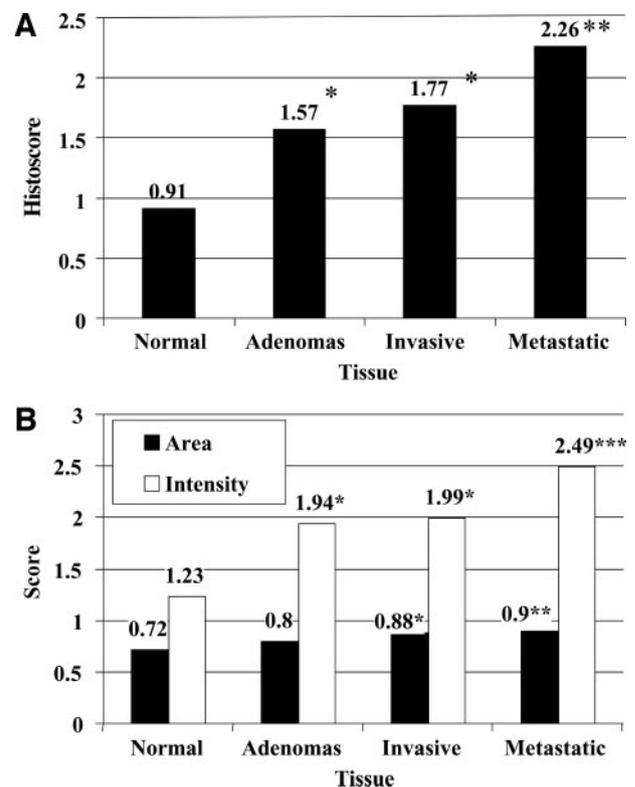
**Fig. 2** Intense p21-activated kinase-1 immunostaining in colorectal carcinoma (CRC) cells spreading, surviving, and growing outside the colon. **A**, CRC cells invading and growing in fat; **B**, CRC cells invading and growing in smooth muscle; **C**, CRC cells in a tissue space resembling a lymphatic. Original magnifications (A–C),  $\times 400$ .

each patient or the average histoscore of all stage II and III/IV primary tumors from each patient. The coefficient of variation (CV) between samples was calculated from:  $CV = (SD/\text{mean}) \times 100$ .

## RESULTS

**Immunohistochemical Detection of Pak-1 Expression in Normal and Neoplastic Colon.** Using immunohistochemistry, Pak-1 was found to be specifically expressed in the cytoplasm of colonic epithelial cells. Specimens evaluated for Pak-1 staining included normal colon near the neoplasms and at the surgical margins, as well as hyperplastic polyps, intraepithelial neoplasms (adenomas) without dysplasia, intraepithelial neoplasms with carcinoma *in situ*, and invasive neoplasms with increasing penetration of the bowel wall, as well as lymph node metastases (stages I - III/IV). Distant metastases were not available for study in this tissue repository, although review of surgical reports and the tumor registry indicated that 4 patients included in this study had stage IV disease (*i.e.*, disease with distant metastases).

Normal-appearing epithelial cells adjacent to colonic neoplasms stained less intensely with anti-Pak-1 than cells within neoplasms (Fig. 1). As shown in Fig. 1, *B–H*, stain was increased in the epithelial cells of colonic neoplasms, including adenomas, invasive carcinomas, and lymph node metastases relative to the normal epithelia. The most intense stain was found in lymph node metastases (Fig. 1*H*). Intense stain was also found in some CRC cells spreading locally by extension into the fat or the smooth muscle, or into tissue spaces resembling lymphatics (Fig. 2). Thus immunohistochemically detected Pak-1 expression was increased in both intraepithelial and invasive neoplasms relative to normal colon and was additionally increased in colon cancer cells capable of surviving in tissues outside the colon, in pericolonc tissues, and in regional lymph nodes. Serial histological sections treated identically but incubated with nonspecific rabbit IgG at the comparable protein concentration (1  $\mu\text{g}/\text{ml}$ ) were negative for all of the samples (for example, Fig. 1*I*), indicating that 3,3'-diaminobenzidine staining was specific to the Pak-1 antibody.



**Fig. 3** p21-activated kinase-1 staining in normal, neoplastic, invasive, and metastatic human colon tissues. **A**, average cytoplasmic composite histoscore (area stained multiplied by intensity). \* denotes that staining was significantly increased relative to that in normal colon,  $P < 0.0001$ ; \*\* denotes that staining was significantly increased relative to normal colon, adenomas, and invasive carcinomas ( $P < 0.0001$ ,  $0.0001$ , and  $0.0005$ , respectively). **B**, average cytoplasmic area and intensity scores. \* indicates significantly increased *versus* normal colon ( $P < 0.0001$ ); \*\* indicates significantly increased *versus* normal and adenoma ( $P < 0.0001$  and  $P = 0.0497$ , respectively); \*\*\* indicates significantly increased relative to normal colon, adenomas, and invasive carcinomas ( $P = 0.0001$ ,  $0.0001$ , and  $0.0007$ , respectively).

**Table 1** Pak-1<sup>a</sup> expression during the adenoma to carcinoma sequence in the human colon: cytoplasmic histoscores (ScC)

Tissue codes represent the adenoma to carcinoma sequence.<sup>b</sup> Pak-1 antibody staining was performed on histologic sections of formalin-fixed, paraffin-embedded surgical specimens. For each tissue, the most and least intensely stained areas were evaluated for reactivity. The overall area and intensity was calculated for each tissue and multiplied to give a composite histoscore (i.e. histoscore = area × intensity) for cytoplasmic stain.

Tissue codes	Tissue (n)	Pak-1 ScC <sup>c</sup> mean ± SD
2	Normal, adenoma pt. (8)	0.91 ± 0.38
3	Normal, ca pt. (30)	0.91 ± 0.42
5	Tubular adenomas (12)	1.44 ± 0.50
6	Tubulovillous adenomas (8)	1.69 ± 0.47
7	Villous adenomas (1)	1.64
8	Adenomas w/ca <i>in situ</i> (6)	1.65 ± 0.35
9	CRC stage I (10)	1.59 ± 0.61
10	CRC stage II (9)	1.85 ± 0.40
11	CRC stage III/IV (16)	1.84 ± 0.33
12	Lymph node metastases (25)	2.26 ± 0.48

<sup>a</sup> Pak-1, p21-activated kinase-1; ScC, composite histoscore; CRC, colorectal cancer.

<sup>b</sup> Normal tissue from patients without inflammatory bowel disease, adenomas, or carcinomas (tissue code 1) was not available.

<sup>c</sup> The relationship between Pak-1 ScC and the adenoma to carcinoma sequence (tissue codes 2–12) is positive and the *P* as determined by repeated measures ANOVA is <0.0001.

### Semiquantitative Evaluation of Pak-1 Expression during the Adenoma to Carcinoma Sequence in Human Colon.

Staining was heterogeneous, particularly in normal epithelia. In normal tissue, only the tips of the crypts had moderate stain, with light or no stain further down in the crypts. In adenomas, stain intensity decreased deep in the crypts. Clones of cells within invasive tumors had variable stain intensity. Lymph node metastases showed nearly uniform staining. To account for both stain intensity and the uniformity of stain, a composite histoscore (percentage epithelium stained × stain intensity) was calculated (Fig. 3A). The individual evaluations for stain intensity and percentage area stained are also graphically represented (Fig. 3B). Results from multiple samples of normal colon from the same patient or of large tumors were averaged, as were

multiple evaluations of the same specimen that had been used for quality control between staining runs. Colon tumor development and progression in the colon were associated with increased cytoplasmic Pak-1 expression (see Table 1). Using repeated measures ANOVA, increasing cytoplasmic Pak-1 immunoreactivity (Pak-1 composite histoscore) was highly positively correlated with progression from normal epithelium to intraepithelial neoplasms and to invasive and metastatic cancers (*P* < 0.0001; Table 1). Both percentage of area and intensity of stain increased with disease progression (Fig. 3).

Both intraepithelial and invasive neoplasms showed increased cytoplasmic expression of Pak-1 relative to normal colonic epithelium. Pak-1 expression was additionally increased in stage II tumors, corresponding to Dukes' stage B, that showed invasion through the muscularis propria into the subserosa or nonperitonealized pericolic or perirectal tissues, or that perforated the visceral peritoneum or directly invaded other organs or structures. Pak-1 expression was also increased in stage III tumors (tumors with regional lymph node metastases) relative to intraepithelial neoplasms (tissue codes 5–8, Table 1; *P* = 0.0180). Colon cancer cells metastatic to regional lymph nodes had the highest Pak-1 expression (Table 1; Fig. 1H).

### Correlation between Pak-1 Expression and CRC Progression.

The average composite histoscores from tissue codes 2–3 (normal colonic epithelium from patients with adenomas or with cancer), 5–8 (adenomatous polyps including tubular adenomas, tubulovillous adenomas, villous adenomas, and large tubulovillous/villous adenomas with carcinoma *in situ*), 9–11 (stages I–III invasive neoplasms), and 12 (regional lymph node metastases) were analyzed by ANOVA (Table 2; Fig. 3A). Cytoplasmic expression of Pak-1 was significantly higher in adenomas and invasive neoplasms compared with normal colon (*P* < 0.0001). Pak-1 expression was similar in adenomas and invasive neoplasms and was significantly lower in these lesions than in lymph node metastases (*P* < 0.0001 and *P* = 0.0005, respectively). Lymph node metastases had the highest Pak-1 expression of all of the tissues examined (*P* < 0.0001 *versus* normal colon and *versus* other colonic neoplasms).

Increases in the average composite histoscores during the adenoma to carcinoma sequence and in lymph node metastases

**Table 2** Cytoplasmic Pak-1<sup>a</sup> expression in human colon tissue types (averaged tissue codes) representing neoplastic progression [cytoplasmic histoscores (ScC)]

Pak-1 antibody staining was performed on histologic sections of formalin-fixed, paraffin-embedded surgical specimens. The overall area and intensity of stain was calculated for each tissue and multiplied to give a composite histoscore (i.e. histoscore = area × intensity) for the cytoplasmic stain. Tissues were grouped according to stages of neoplastic progression: normal tissue, intraepithelial neoplasms, invasive neoplasms, and metastases. Repeated measures ANOVA was used to determine differences among tissue type (normal colons, adenomas, invasive CRCs, and lymph node metastases).

Tissue codes averaged <sup>b</sup>	Tissue type (n)	Pak-1 ScC mean ± SD	Adjusted <i>P versus</i> normal <sup>c</sup>	Adjusted <i>P versus</i> adenomas <sup>c</sup>	Adjusted <i>P versus</i> invasive <sup>c</sup>
2, 3	Normal (38)	0.91 ± 0.41	–	<0.0001	<0.0001
5, 6, 7, 8	Adenomas (27)	1.57 ± 0.45	<0.0001	–	0.3545
9, 10, 11	Invasive CRC (35)	1.77 ± 0.44	<0.0001	0.3545	–
12	Lymph node metastases (25)	2.26 ± 0.49	<0.0001	<0.0001	<0.0005

<sup>a</sup> Pak-1, p21-activated kinase 1; ScC, composite histoscore; CRC, colorectal cancer.

<sup>b</sup> Normal tissue from patients without inflammatory bowel disease, adenomas, or carcinomas (tissue code 1) was not available.

<sup>c</sup> Adjusted *P* = *P* with Tukey adjustment for multiple comparison from ANOVA.

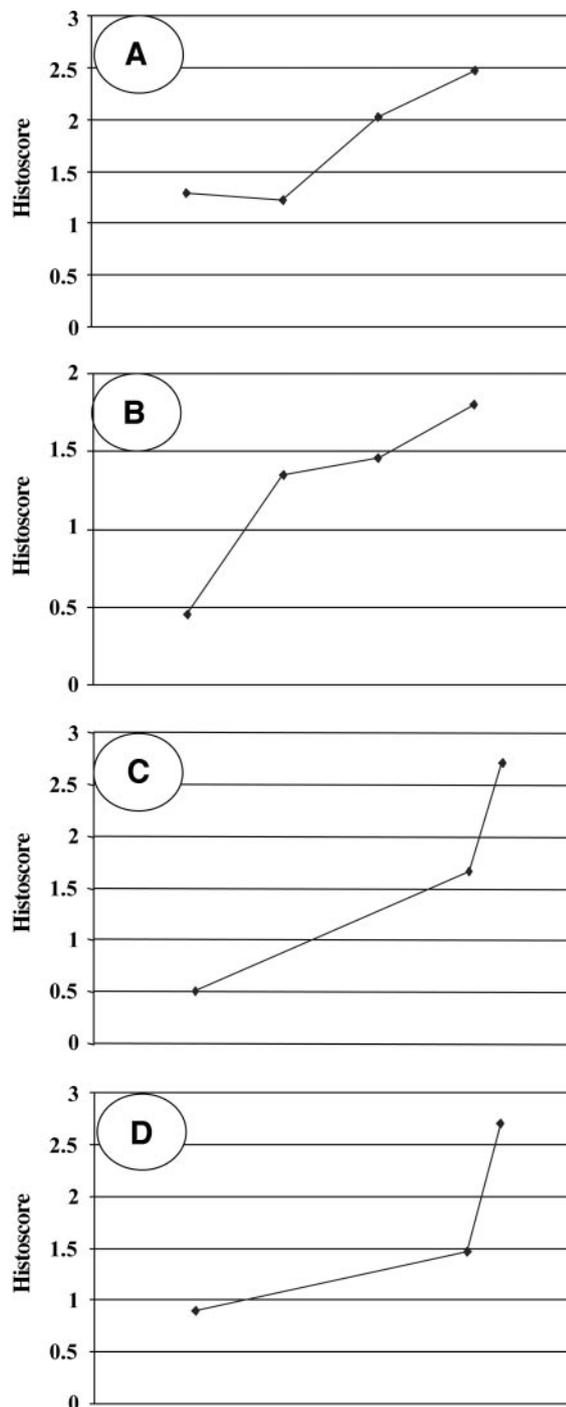


Fig. 4 p21-activated kinase-1 histoscores in multiple lesions from individual patients. The histoscores for p21-activated kinase-1 staining were plotted for representative individual patients. Each panel shows data from a single patient. The tissues represented are: (A) normal colon, adenoma, invasive colorectal carcinoma (CRC) stage I and invasive CRC stage III; (B) normal colon, adenoma, invasive CRC (mean histoscore of stage I and III) and lymph node metastasis; (C) normal colon, invasive CRC stage III and metastasis; (D) normal colon, invasive CRC stage III and metastasis.

reflected increases in both the area (percent) of epithelial cells stained and in the intensity of stain ( $P < 0.001$ ; Fig. 3B). After Tukey adjustment for multiple samples, the difference between the percentage of epithelium stained in normal *versus* adenomas was not significant ( $P = 0.0612$ ). However, the difference in the percentage of epithelium stained between normal and invasive cancer and between normal and lymph node metastases was highly significant ( $P < 0.0001$ ). The difference in area of epithelium stained in metastatic cancer was also significantly higher than in adenomas ( $P = 0.0311$ ). The difference in stain intensity was also highly significant between normal and either adenomas, invasive cancer, or lymph node metastases ( $P < 0.0001$ ; Fig. 3B). The difference in stain intensity was also highly significant between lymph node metastases and adenomas ( $P = 0.0009$ ) and between lymph node metastases and invasive cancers ( $P = 0.0007$ ; Fig. 3B).

**Inter- and Intraindividual Variation in Pak-1 Expression.** The CRC progression-related increases in cytoplasmic Pak-1 expression (Fig. 3) were independent of region of the colon (proximal *versus* distal) or of sex (female *versus* male). Interindividual variation in cytoplasmic expression of Pak-1 decreased with tumor progression as indicated by CV. The CV in normal = 44.5%, adenomas = 28.8%, invasive cancers = 25.0%, and lymph node metastases = 21.5%, suggesting that tumors become more uniformly Pak-1 positive with progression.

To determine intraindividual variation within multiple specimens of normal colon from the same patient and within multiple specimens from the same neoplasm, the CVs for percentage of area stained and stain intensity were calculated. Intraindividual variation between multiple specimens of normal colon from the same patient was greater than between multiple specimens from the same neoplasm in both percentage of epithelium stained and staining intensity (for normal *versus* neoplasm, CV area = 22.2% *versus* 2.6%, and CV intensity 30.6% *versus* 12.4%), suggesting that Pak-1 expression is uniformly elevated throughout tumors.

**Pak-1 Expression during Neoplastic Progression in Individual Patients.** Nearly 50% of patients with one adenoma develop others, and multiple adenomas are a risk factor for synchronous and future CRC (2, 9). By evaluating numerous lesions from different stages of disease progression from multiple individuals, we have shown that Pak-1 expression is increased significantly in more advanced lesions, which suggests that Pak-1 expression increases with disease progression. To better assess whether Pak-1 expression increases with disease progression, we chose to evaluate lesions at different pathological stages from individual patients. If Pak-1 expression is increasing with disease progression, the more advanced lesions of a particular patient should show higher Pak-1 expression than the less advanced, earlier stage lesions from that same patient. As represented in Fig. 4, the more advanced lesions progressively showed higher Pak-1 expression than earlier lesions, supporting the notion that Pak-1 expression increases with malignant progression of CRC.

**Pak-1 Expression in Hyperplastic Polyps.** Although some hyperplastic polyps have been shown recently to have malignant potential and some have activated ras, hyperplastic polyps are not generally considered as part of the adenoma to carcinoma sequence (34, 35). Consequently, these lesions were

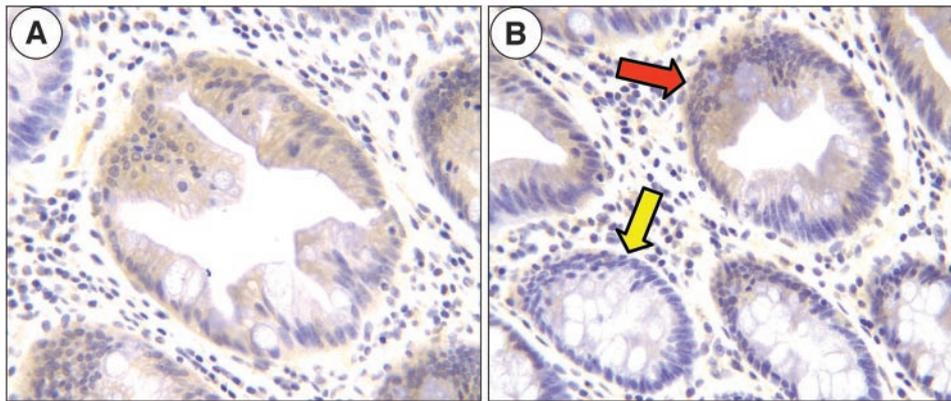


Fig. 5 Immunohistochemical staining for p21-activated kinase-1 in hyperplastic polyps. A, moderate staining in a hyperplastic polyp. B, moderate staining in a hyperplastic polyp (red arrow) adjacent to a lightly stained normal colonic crypt (yellow arrow). Photomicrographs shown with original magnification,  $\times 400$ .

not included in our statistical analyses for disease progression. We did, however, evaluate Pak-1 expression in hyperplastic polyps (Fig. 5A). Relative to normal colon, hyperplastic polyps had increased cytoplasmic Pak-1 expression (Fig. 5B). The composite cytoplasmic histoscore in hyperplastic polyps was  $1.65 \pm 0.48$ , significantly higher than in normal colonic epithelium  $0.91 \pm 0.41$  ( $P < 0.0001$ ). Hyperplastic polyps were statistically similar to adenomas and invasive cancers in Pak-1 expression. However, these tissues had significantly less Pak-1 than CRC lymph node metastases ( $P = 0.0238$ ).

**Patient Survival and Pak-1 Expression.** Clinical follow-up was available for all but 3 cancer cases in this study and indicated that death from CRC is related to stage of disease at diagnosis, local recurrence, distant metastases, and additional new primary tumors (Table 3). Patients in this study that died from CRC survived  $22 \pm 17$  months, whereas those that survived  $>5$  years were followed or lived  $147 \pm 45$  months. Pak-1 expression in the tumor (primary or metastatic) with the highest Pak-1 composite histoscore from patients who died from CRC in  $\leq 5$  years was significantly higher ( $2.38 \pm 0.43$ ) than that from patients who survived  $>5$  years ( $1.99 \pm 0.45$ ;  $P = 0.0363$ ) suggesting that increased Pak-1 expression is related to reduced patient survival. Because death from CRC was usually attributable to distant metastases, we next chose to evaluate whether the expression pattern of Pak-1 in primary tumors alone (*i.e.*, staining in metastases was excluded) could be related to patient survival. The average Pak-1 histoscore of stage II and III/IV primary tumors in both groups was compared and found to be similar ( $1.92 \pm 0.29$ , survived, *versus*  $1.84 \pm 0.40$ , died;  $P = 0.6138$ ) indicating that examining Pak-1 expression exclusively

in primary tumors (*i.e.*, excluding metastases) was not predictive of patient survival. These data indicate that Pak-1 expression is related to the progression to metastasis, and thereby to patient survival, but may not itself be indicative of disease course when evaluated in earlier stage, primary tumors.

## DISCUSSION

The evolution of the metastatic phenotype is complex but clearly requires enhanced cell motility and invasiveness to enable the tumor cell to break from the primary site, enter and exit the circulation, and successfully establish a metastatic colony (10, 11). Cell motility and invasiveness require cytoskeletal reorganization (12), a process that is centrally regulated by the activity of Pak-1 (13–21). Kinase-dead mutants of Pak-1 can suppress cellular transformation by Ras, Rac, and Rho (27, 28), heregulin-induced breast epithelial cell migration (26) and invasiveness of human breast cancer cell lines (25), emphasizing the role for Pak-1 in tumor cell migration and invasion. Indeed, increased Pak-1 expression has been implicated recently in breast cancer progression. Pak-1 expression was significantly increased in 55% of human breast cancer tissues examined (22). Moreover, Pak-1 expression is increased with neoplastic progression in the breast [normal breast  $<$  benign breast disease  $<$  atypical hyperplasia  $<$  intraductal carcinoma  $<$  invasive carcinoma  $<$  metastatic carcinoma ( $P$  for the trend  $< 0.0001$ ); Ref. 36]. Finally, overexpression of active Pak-1 in transgenic mice induced mammary hyperplasia (22). The data in this report now implicate increased Pak-1 expression in the malignant progression of human CRC.

Table 3 Survival in CRC<sup>a</sup> patients in this study as related to tumor stage and recurrence

Patients in this study that died of CRC survived  $22 \pm 17$  months whereas those that survived  $>5$  years were followed or lived  $147 \pm 45$  months. All of the deaths in patients living  $\leq 5$  years were cancer-related (*i.e.*, local recurrences, growth of primary tumor, resistance to therapy, metastases, or new primary cancers). Clinical follow-up information was available for 30 of 33 total cancer cases evaluated in this study. These 30 cases are represented in this table. See "Materials and Methods" for pathological staging.

Survival	Stage 0 (n) (%)	Stage I (n) (%)	Stage II (n) (%)	Stage III (n) (%)	Stage IV (n) (%)
Living $> 5$ years	3/3 (100%)	5/5 (100%)	7/8 (87.5%)	3/10 (30%)	
Living $\leq 5$ years			1/8 (12.5%)	7/10 (70%)	4/4 (100%)

<sup>a</sup> CRC, colorectal cancer.

Our data show that Pak-1 expression is markedly increased with disease progression in colorectal carcinoma. Pak-1 expression was significantly increased in adenomas and invasive cancers relative to normal colonic epithelium and was additionally increased in lymph node metastases. These data indicate that Pak-1 expression is specifically increased in the most advanced lesions, thereby suggesting that Pak-1 is increased with disease progression. The CVs for Pak-1 immunostaining decreased markedly in the most advanced lesions when compared with normal colonic epithelia, which further suggests a selection for increased Pak-1 expression during malignant progression. The evaluation of multiple lesions from individual patients representing different stages of disease revealed increased Pak-1 expression in the more advanced lesions further supporting the notion that Pak-1 expression is increased with disease progression. Finally, our data reveal that 5-year survival is inversely related to increased Pak-1 expression as CRCs that were lethal, primarily due to metastases, had higher Pak-1 expression than CRCs that did not kill the patient. Together, these data demonstrate that Pak-1 expression is increased with malignant progression of human colorectal carcinomas.

The mechanisms that may contribute to increased Pak-1 expression are currently unclear. Recent reports have implicated DNA amplification of the *Pak-1* locus in both human breast and ovarian carcinomas (23, 24). Whether such DNA amplification plays a role in increased Pak-1 expression in colorectal carcinomas remains to be explored. *Pak-1* transcription may also be up-regulated directly as a downstream consequence of the signaling pathways involved in CRC progression. Certainly, the  $\beta$ -catenin/T-cell factor transcription complex, which is frequently activated in CRCs, can up-regulate the transcription of many key genes known to be involved in malignancy such as *cyclin D1* and *matrilysin* (37). However, there have been no studies to date that reveal the regulatory mechanisms governing Pak-1 expression.

CRC has four mechanisms of spread including: (a) local extension from the primary tumor; (b) lymphatic invasion resulting in lymph node metastases; (c) venous invasion resulting in visceral metastases; and (d) direct implantation on abdominal peritoneal surfaces (9). Although only local extension and lymphatic invasion have been considered here, taken together, the data strongly suggest that Pak-1 overexpression in CRC cells is associated with an increased ability of the tumor cells to spread. First, CRC cells spreading into the pericolonic fat, into smooth muscle, and into tissue spaces resembling lymphatics, have increased Pak-1 expression (Fig. 2). Second, semiquantitative evaluation of stain intensity and area of epithelium stained indicate a significant increase in the Pak-1 expression in lymph node metastases relative to intraepithelial and invasive neoplasms or normal colon (Figs. 1 and 3; Tables 1 and 2). Finally, death from CRC was associated with local recurrence, distant metastases, and additional new cancers (Table 3). Patients who died from CRC usually had multiple tumors (both primaries and lymph node metastases). The highest Pak-1 histoscore of these multiple lesions from a single patient were then evaluated in relation to 5-year patient survival. This analysis revealed that patients who died from CRC had a lesion or lesions with significantly higher Pak-1 expression than those from patients who lived. Pak-1 expression was consistently highest in meta-

static lesions and consequently may be associated with "metastatic fitness" (38). As such, the relationship between Pak-1 expression and 5-year patient survival may reflect the relationship with Pak-1 and metastases.

In summary, the data presented in this report are the first to demonstrate that Pak-1 expression is increased with progression of human CRC. Most striking, these data reveal that Pak-1 expression is highest in CRC lymph node metastases. These data, coupled to numerous functional studies demonstrating a central role for Pak-1 activity in tumor invasiveness and motility, implicate Pak-1 as an exciting potential target for therapy of colorectal carcinoma.

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