Activation of Src Kinase in Primary Colorectal Carcinoma

An Indicator of Poor Clinical Prognosis

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BACKGROUND. The specific activity of the non-receptor protein tyrosine kinase, Src, is increased in the majority of colon and rectal adenocarcinomas compared to normal mucosa. However, the prognostic significance of this difference is unknown. The objective of the current study was to determine if Src activity is a marker for poor clinical prognosis in colon carcinoma patients. As Src activation leads to expression of urokinase/plasminogen activator receptor (u-PAR), expression of Src and u-PAR were correlated with patient survival.

METHODS. Tumors and adjacent normal colonic mucosae from 45 patients with colorectal carcinoma were screened for Src activity by the immune complex kinase assay. Expression of u-PAR was determined by enzyme linked immunoabsorbent assay. The primary tumor-to-normal mucosa ratios of activity were compared following classification and regression tree (CART) analysis to determine the prognostic significance of elevated specific Src activity. Expression of u-PAR was correlated with Src activity.

RESULTS. By CART analysis, Src activity in tumors elevated more than twofold over normal mucosa was significant. Increased Src activity significantly correlated with Dukes stage, pT and pN classification, and increased u-PAR levels ($P < 0.001$). Kaplan Meier analysis showed a significant association between elevated Src activity and shorter overall survival of all patients ($P = 0.0004$) and of Dukes Stage A-C patients ($P = 0.0037$). In patients who underwent curative resection, a significant correlation with a decreased disease-free survival rate was found ($P < 0.0001$). Multivariate analysis revealed that elevated Src activity was a prognostic parameter independent of M classification ($P = 0.0125$, relative risk 3.54, 95% confidence interval 1.31 – 9.76).

CONCLUSIONS. Src activity is an independent indicator of poor clinical prognosis in all stages of human colon carcinoma. These data suggest that Src-specific inhibitors may have a therapeutic role in inhibiting tumor progression and metastasis, and that measurement of Src activity may aid in selection of early stage patients for adjuvant therapy. ***Cancer* 2002;94:344–51.** © *2002 American Cancer Society.*

KEYWORDS: Src, urokinase-type plasminogen activator receptor, colorectal carcinoma, protein tyrosine kinase.

The non-receptor protein tyrosine kinase, Src, is the archetype of a nine-member family of highly related protein tyrosine kinases (PTKs). Src family PTKs function in signaling pathways regulating diverse cellular functions including proliferation, migration, cytoskeletal reorganization, and cellular survival by interacting with and/or phosphorylating specific substrates (reviewed by Thomas and Brugge).[1] The retroviral form of Src (v-Src) has long been studied for its ability to induce malignant transformation of mammalian cells in

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tissue culture and to induce metastatic tumors in animals (reviewed by Bishop).[2] Additionally, in animal models, considerable evidence has implicated activation of "normal" c-Src in tumorgenicity. For example, association of c-Src with polyoma middle T antigen leads to increased specific activity of Src, and is required for malignant transformation by polyoma virus.[3,4] Increased specific activity of c-Src has been observed in a number of human tumors, most commonly occurring in colon,[5–11] breast,[12] and ovarian[13] carcinomas. When Src expression and activity is decreased in specific colon[14] and ovarian[13] carcinoma cell lines, corresponding decreases in tumorgenicity in nude mice are observed.

Deregulation of multiple pathways that contribute to tumorgenicity may be affected by Src activation. Ellis et al.[15] showed that Src activity regulates constitutive as well as hypoxia-induced vascular endothelial growth factor expression in colon adenocarcinoma cells. Pories et al.[16] directly showed that transfection of a colonic cell line with Src conferred a higher invasive capacity to these cells. Proteolytic enzymes, which in part mediate invasion such as MMP-9,[17] cathepsin-L,[18] or u-PA,[19,20] are induced by v-Src. Furthermore, expression of activated Src in colon carcinoma cell lines leads directly to increased transcription and expression of the urokinase-type plasminogen activator receptor gene,[21] resulting in increased ability to proteolytically cleave laminin, a component of basement membranes which is degraded in the process of metastasis.

Activation of c-Src occurs most frequently in human colon and rectal adenocarcinomas. Increased Src activity has been observed during both development and progression of the disease. Src activity increases in stages of tumor development as in dysplastic polyps with high malignant potential relative to more benign adenomas.[8] Patients with ulcerative colitis commonly display increased Src activity compared to normal or inflammatory colonic mucosa.[22] Nearly 80% of primary colon tumors express Src with abnormally high specific activity, and further increases in activity are common in distant metastases relative to the corresponding primary tumors.

To our knowledge, the potential prognostic significance of Src activation in human colon tumors has not yet been investigated. Therefore, in a series of colorectal carcinoma patients, we prospectively investigated the significance of elevated specific Src activity as an indicator of clinical prognosis. Moreover, since Src induces urokinase/plasminogen activator receptor (u-PAR) expression,[21] an important molecule in invasion of colon tumor cells[23–26] and a prognostic marker of patient survival,[27] a second study objective was to compare Src activity, u-PAR expression, and prognosis. The current study provides evidence that specific Src activity is an independent predictor of clinical prognosis for colorectal carcinoma patients and correlates with u-PAR-gene expression. These findings implicate Src as a new potential target for anti-metastatic cancer therapy.

TABLE 1
Characteristics of Colon Tumors Analyzed for Src Activity

Dukes stage	A	1[a]
	B	12
	C	9
	D	23
Pathologic T classification	T1	1
	T2	5
	T3	24
	T4	15
Pathologic N classification	N0	17
	N1	28
M classification	M0	22
	M1	23
Histologic grade	G1	4
	G2	34
	G3	7
Tumor location	Cecum/right hemicolon	12
	Transverse colon	1
	Left hemicolon	7
	Sigmoid colon	13
	Rectum	12

[a] Number of patients with indicated characteristic

MATERIALS AND METHODS

Patients and Tumors

Samples of colorectal adenocarcinomas and adjacent normal colonic mucosae were available from 45 patients who underwent primary tumor resection between 1990 and 1994 at The University of Texas M. D. Anderson Cancer Center. The mean patient age was 63.8 years (range 35 to 86 years); 30 patients were male, and 15 were female. Of these 45 patients, 28 (62%) underwent tumor resection with curative intent (R0 resection, 22 patients Dukes Stage A-C, 6 patients Dukes Stage D). The remaining 17 patients (38%) underwent palliative resection of the primary cancer; all patients in this group had macroscopic residual metastatic tumor (R2 resection, all 17 patients Stage D disease). Tumors were located in the colon ($n = 32$) or rectum ($n = 13$). Relevant tumor and stage characteristics are provided in Table 1.

Prospective follow-up was done 6, 12, 18, and 24 months after surgery and at one-year intervals thereafter in all patients. Follow-up included physical examination, abdominal and pelvic computed tomographic scans, colonoscopy, chest X-ray, hematology,

blood chemistry, and screening for the tumor markers carcinoembryonic antigen and CA 19-9. If tumor recurrence was suspected in Dukes Stage A-C patients, the patient was recommended for confirmation of diagnosis by biopsy or exploratory surgery. Imaging procedures and elevated serum tumor marker levels were accepted for the diagnosis of disease recurrence if pathologic evidence of recurrence was not feasible. Causes of death were evaluated clinically.

Src Expression and Activity Assays

Tumors and corresponding normal mucosae were snap-frozen at the time of resection and stored in liquid nitrogen. Samples were prepared and assayed as described previously.[10] Briefly, normal colonic mucosal and adenocarcinoma tissues were separated from underlying muscle by blunt dissection. Light microscopy evaluation showed that the separation occurred at the level of muscularis mucosa. Histopathologic examination was performed on all tumors to confirm that the primary tumor origin was colonic. Samples were homogenized in radioimmunoprecipitation assay (RIPA) buffer A (1% triton X-100, 0.1% sodium dodecyl sulfate [SDS], 0.5% deoxycholate, 150 mM disodium chloride, 5 mM sodium pyrophosphate, 20 mM sodium phosphate, pH 7.4) using a Polytron homogenizer (Brinkman Instruments, Westbury, NY). Protein in clarified lysates was estimated by the bicinchoninic (BCA) protein assay (Pierce Chemical Co., Rockford, IL). For determination of relative Src activity, the immune complex kinase assay was employed. Aliquots of lysate containing 200 μg of protein were incubated with mAb 327 (Oncogene Sciences, Mineola, NY) for 2hours at 4 °C, followed by a similar incubation with 6 μg rabbit anti mouse immunoglobulin G, then 50 μL 10% (vol/vol) Pansorbin (Calbiochem, La Jolla, CA) as described previously.[10] Immunoprecipitates were washed three times and resuspended in reaction buffer containing 10 mM magnesium chloride, 20 mM Hepes, 100 mM sodium vanadate, 10 μCi [γ-^{32}P ATP] and 5 μg of acid-denatured rabbit muscle enolase (Sigma Chemical Co., St. Louis, MO). Reactions were allowed to proceed for 10 minutes at 25 °C and terminated using a final buffer consisting of 2% SDS, 5% β-mercaptoethanol, 0.125 M Tris (pH6.8), 1 mM ethylenediamine tetraacetic acid, 10% glycerol, and 0.02% bromophenol blue. Proteins were resolved on 8% SDS-polyacrylamide gels, and radiolabeled proteins were detected and relative protein intensities were determined by densitometric scanning as described previously.[10]

Determination of U-PAR Expression

The expression of u-PAR protein was determined by enzyme linked immunoabsorbent assay (ELISA). Lysates of the resected tissue were prepared and assayed as described by the manufacturer (American Diagnostica, Greenwich, CT).

Statistical Analyses

For determination of cutoff points for the definition of elevated Src activity and for the u-PAR amounts as measured by ELISA, classification and regression tree analysis (CART) was performed.[28] CART is a structured classification method allowing the determination of a cut point by recursive partitioning of a learning sample.[28] Chi-square analysis was performed to determine the correlations between expected and detected frequencies, considering the parameters as follows: Src activity (dichotomized at 2.1 or higher according to CART analysis), gender (male vs. female), age (dichotomized at median), tumor location (colon vs. rectum), surgical curability (R0 vs. R2), and pT, pN, M classification and Dukes stage. Group-oriented life-table curves were calculated with Kaplan-Meier analysis and compared with the Mantel-Cox log rank statistics.[29] To correct the univariate prognostic relevance of Src activity for its correlation with established risk factors in colon carcinoma, multivariate analysis was performed using the Cox proportional hazard model.[30] Parameters for multivariate analysis were considered as stated for chi-square analysis. The parameters were entered into the multivariate model after a significant univariate P value had been calculated. All statistics were two-sided at a significance level of $P = 0.05$, using the BMDP statistical software (Statistical Solutions, Saugus, MA).[31]

RESULTS

The 45 colorectal carcinoma patients were followed prospectively for a median of 36 months (range 1-96 months) after primary tumor resection. Of those patients, 28 underwent curative (R0) resection. During the study time, 31 of 45 patients died, 30 from tumor-related causes. In the 28 curatively resected patients, 21 recurrences occurred, 11 of which were liver metastases only and 10 of which were multiple metastases (nine in the liver and lung, one a local recurrence and lung metastisis). An overview of the tumor characteristics for all 45 patients is provided in Table 1. All resected tumors were adenocarcinomas.

Correlation of Elevated Src Activity with Established Tumor Characteristics

To determine the optimal cutoff point for Src activation, CART analysis was performed (log rank test, P

TABLE 2
Comparison of Established Patient and Tumor Prognostic Factors with Two-fold or Greater Src-Activity (Chi-Square)

Parameter	Significance of 2-fold or greater Src-activity[a]
Gender	NS
Age (dichotomized at median)	NS
Dukes stage	0.0468
Pathologic T classification	0.0071
Pathologic N classification	0.0137
M classification	NS
Pathologic grade	NS
Tumor location (colon vs. rectum)	NS
R (surgical curability)	NS

[a] All significant correlations are positive correlations.
NS: not significant.

< 0.05).[28] The optimal Src activity cutoff point in the tumor was 2.1 times the level in normal mucosa; this level of increase is consistent with increases in Src previously considered biologically relevant.[10] Applying this criterion to the current patient sample, significant correlations (chi-square) were observed between increased Src-kinase activity and primary tumor classification pT, pN, and Dukes stage (Table 2). Median specific Src activity in the tumor tissues was 2.9 times the activity in the corresponding normal mucosa (range 0.3 - 57.0 times normal mucosa activity).

Correlation of Elevated Src Activity with Endogenous Amounts of u-PAR Protein

In a previous study, we showed that Src activity was proportional to the expression of u-PAR, an invasion-related molecule shown to be of functional and prognostic relevance in colon carcinoma.[27] We therefore tested for a correlation of endogenous Src activity with u-PAR levels in the current patient series. In 17 samples, an additional ELISA analysis for endogenous amounts of urokinase receptor was performed. Consistent with previous results, a high level of u-PAR (3.2 ng/mg tissue, according to CART analysis) in the primary tumor was predictive of a significantly shorter overall survival as determined by Kaplan-Meier analysis and Mantel-Cox log rank-statistics (Fig. 1). In linear regression analysis, the specific Src activity in these samples showed a significant correlation with endogenous u-PAR-amounts ($P < 0.001$, Pearson's correlation coefficient: 0.8487, Figure 2).

Analysis of Univariate Prognostic Impact of Increased Src Activity

Because of the ability of Src activation to lead to increased expression of u-PAR, we next determined if

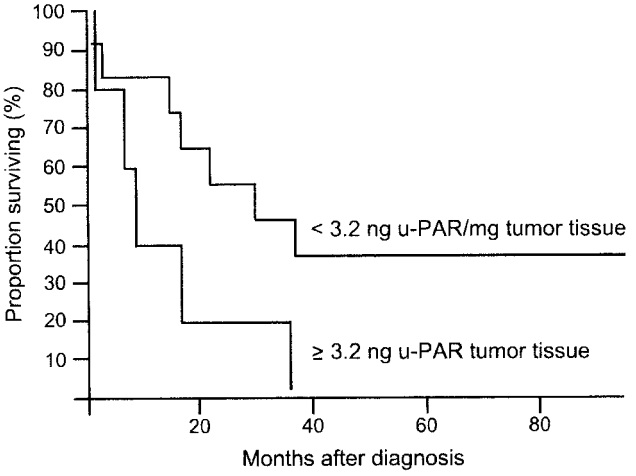

FIGURE 1. Survival of patients with and without elevated levels of urokinase/plasminogen activator receptor (u-PAR). Relative u-PAR levels in tumor tissues were determined by enzyme linked immunoabsorbent assay as described. In the graph, the survival times of 12 patients with less than 3.2 ng u-PAR/mg tumor tissue (upper curve) are compared to 5 patients with greater than 3.2 ng u-PAR/mg tumor tissue (lower curve). For the 12 patients with less than 3.2 ng u-PAR/mg tissue, the mean survival time was 46.8 months (standard deviation [SD], 11.5). For the 5 patients with greater than 3.2 ng u-PAR/mg tumor, the mean survival time was 14.2 months (SD, 6.0). The differences in survival are significant ($P = 0.05$, Mantel-Cox log rank). The 3.2 ng u-PAR/mg tumor tissue as an estimate of significantly increased u-PAR expression was determined by classification and regression tree analysis.

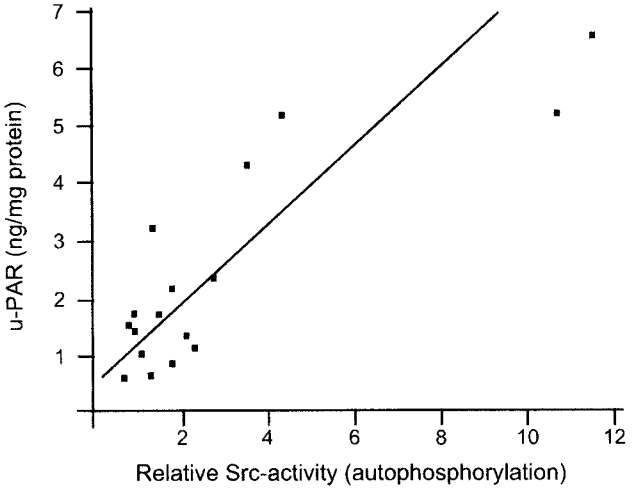

FIGURE 2. Linear regression analysis demonstrating the correlation between endogenous urokinase/plasminogen activator receptor amounts (ng/mg tissue, enzyme linked immunoabsorbent assay) and specific Src activity in resected colon cancers. The correlation is highly significant with $P < 0.001$ (Pearson correlation coefficient: 0.8487).

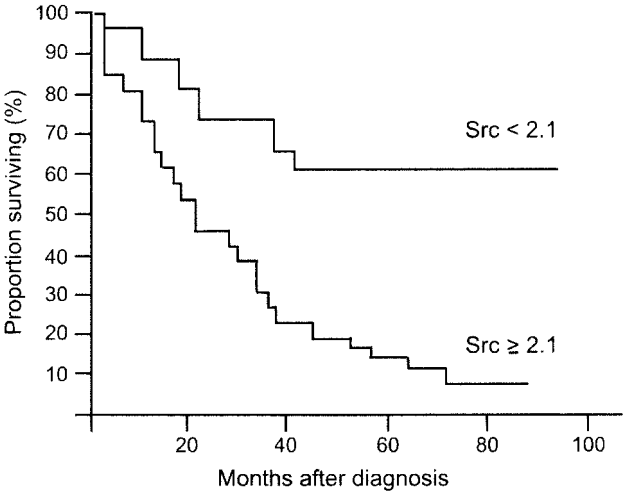

FIGURE 3. Survival of all colon carcinoma patients with or without Src activation. The relative Src activity was determined as the ratio of activity in tumors to adjacent normal mucosa, as described. In the graph, the survival times of 17 patients whose tumors had Src activities less than 2.1 fold relative to normal mucosa (upper curve) are compared to those of 28 patients with a greater than 2.1 fold increase in Src activity (lower curve). For the 17 patients expressing Src activity at a level less than 2.1 times that of normal colonic mucosa, mean survival time was 68.6 months, standard deviation (SD) = 9.1 months. For the 28 patients with tumors expressing Src activity increased by greater than 2.1 fold, the mean survival time was 27.8 months, SD = 4.5 months. The survival difference between the groups is significant ($P = 0.0004$, Mantel-Cox analysis). The significance of a 2.1 fold increase in Src activity was determined by classification and regression tree analysis.

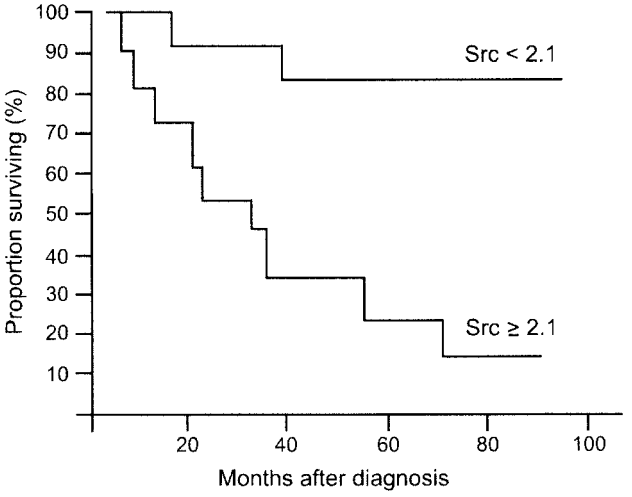

FIGURE 4. Survival of patients with Dukes Stage A-C tumors with or without Src activation. Relative Src activities were determined as described in Figure 3. In the graph, the survival of 11 patients with Src activity less than 2.1 fold greater than normal adjacent mucosa (upper curve) is compared to 9 patients with a greater than 2.1 fold increase in Src activity relative to adjacent normal mucosae (lower curve). None of these patients had evidence of distant metastasis (Dukes Stage D) at the time of resection of their primary colorectal carcinoma. For the 11 patients presenting with tumor Src activity less than 2.1 fold that of the normal mucosae, the mean survival time was 84.2 months (standard deviation [SD], 7.7 months). For the nine patients with increased Src activity, mean survival time was 43.7 months (SD, 7.7 months). The survival difference between the two groups is significant ($P = 0.0037$, Mantel-Cox analysis).

Src activation itself was a marker for clinical prognosis. The cohort of patients with a 2.1 fold or greater increase in Src activity in their tumors was found to have a poor overall survival (Kaplan-Meier survival analysis, Mantel-Cox log rank test, $P = 0.0004$, Figure 3). Further, the patients who underwent curative (R0) resection who had a 2.1 fold or greater increase in Src activity were found to have significantly decreased disease-free survival ($n = 28$, $P < 0.0001$). To determine if Src activation was a prognostic marker for patients presenting without distant metastases, we analyzed the results from Dukes Stage A-C tumors only. As shown in Figure 4, a 2.1 fold or greater increase in Src activity also was associated with a decreased overall survival for these patients ($P = 0.0037$). These results suggest that a high endogenous Src activity in the primary tumor as compared to normal mucosa is a significant prognostic marker in colorectal carcinoma.

Analysis of Multivariate Prognostic Impact of Elevated Src Activity

To correct the univariate results for correlations with established risk factors in colon carcinoma, multivariate analysis was performed for the overall survival of all 45 patients (Cox proportional hazard model) with intended surgical curability (R0 versus R2), Dukes stage, pT, pN, M classification and tumor location (colon/rectum), and covariates (Table 3). In this analysis, an elevated Src activity of more than 2.1 fold was an independent prognostic indicator of survival distinct from the presence of metastasis ($P = 0.0125$, relative risk as estimated by odds ratio 3.54, 95% confidence interval [CI] 1.31 - 9.76). Elevated Src activity was also an independent factor in Dukes' A-C patients for overall survival ($P = 0.0150$, relative risk 20.82, 95% CI 1.80 - 240.60) and in R0 patients for disease-free survival ($P = 0.0034$, relative risk 11.53, 95% CI 2.23 - 59.09). Taken together, these results strongly imply that Src activity in the primary tumor is a prognostic marker for patient survival.

DISCUSSION

Several studies from our laboratory[9,10] and others[6-8,11] have shown that Src activation is common throughout the development and progression of human colon carcinoma. Higher activities of Src are common in

TABLE 3
Multivariate Analysis of Survival of All 45 Patients Including All Parameters that were Significant in Univariate Analysis (Cox Proportional Hazard)

Parameter	Multivariate *P*-value	Relative risk (odds ratio)	95% confidence interval
Src-activity two-fold or higher in tumor tissue	0.0125	3.54	1.31–9.76
Dukes stage	NS	—	—
Pathologic T classification	NS	—	—
Pathologic N classification	NS	—	—
M classification	0.0151	3.78	1.27–9.39
Tumor location (colon vs. rectum)	NS	—	—
R (surgical curability)	NS	—	—

NS: not significant in multivariate analysis.

metastases relative to primary tumors.[10,32,33] These results suggest that increased Src activity confers a selective advantage in growth and/or survival of colon tumor cells. In the current analysis of 45 patients, we show that increased Src activity in primary tumors is associated with significant decreases both in disease-free intervals and in overall survival of colorectal carcinoma patients. Specifically, using CART analyses, Src activation was defined as a greater than twofold increase in activity in tumor tissue relative to adjacent normal tissue, similar to Src increases considered significant in previous studies.[10] By this criterion, Src activation was equally predictive of patient outcome irrespective of the presence of metastasis at the time of initial surgery. Thus, deregulation of Src activity may be one of critical events in the development of metastases from colon tumors.

As yet, the mechanism by which Src is activated in colon tumors and metastases is unclear. In a subset of hepatic colorectal carcinoma metastases, Irby et al.[34] reported a truncating mutation at codon 531 in 12% of the samples they analyzed. The truncation led to increased specific activity of Src, and expression of this mutated form of Src led to transformation of NIH 3T3 cells. However, this mutation was not observed in other studies of diverse and much larger patient populations,[35–37] suggesting that this mutation is rare and cannot account for the increased activity of Src found in the majority of metastases. In normal cells, Src is activated by association with other signaling molecules, including growth factor receptors such as c-Met, and focal adhesion kinase, both of which are overexpressed in human colon tumors.[38,39] Irrespective of the mechanism of Src activation, increases in total enzymatic activity are due largely to increased specific activity, not overexpression of Src in either colon tumors (reviewed by Jessup and Gallick).[40] Recently, similar results have been observed in bladder tumors in which Src is activated,[41] suggesting that activation of Src occurs most commonly through deregulation of signaling pathways as opposed to mutational activation. Thus, in the current study, each tumor was analyzed for activity of Src in the standard immune complex kinase assay. This technique has several limitations, including the requirement of approximately 200 μg of protein from tissue. For this reason, the sample size in the current study is smaller than in other studies that employ immunohistochemic techniques to detect overexpression of potential marker proteins. Immunohistochemic techniques have the potential advantage of visualizing individual cells; thus, questions of heterogeneity can be addressed. Therefore, we are currently investigating whether antibodies recognizing specific Src phosphorylation sites might be able to detect activated Src in individual cells.

The current study suggests further that specific substrates of Src might be candidate prognostic markers for colon carcinoma. One such substrate, p120(ctn), which by association with E-cadherin participates in cell-cell adhesion, was shown to be downregulated in 86% of 44 primary colorectal carcinomas, with downregulation correlating with advanced stage of disease and decreased survival.[42] Whether phosphorylation of this protein by Src decreases its half-life was not examined. Alternatively, enzymes involved in Src activation have potential as markers of tumor progression. In this regard, the expression of N-myristoyltransferase, an enzyme catalyzing myristoylation of Src (required for membrane association and activation of Src) is increased in gallbladder carcinomas, and this increase correlated with decreased mean survival times in these patients.[43]

Finally, an understanding of alterations in fundamental pathways regulating adhesion and migration as a result of Src activation may be important not only in defining the mechanism by which Src activation contributes to tumor progression, but may also serve as a potential prognostic marker. For example, Src activation leads to deregulation of adhesion molecules and associated pathways such as E-cadherin/beta-catenin[44] (reviewed by Noe et al.),[45] leading to rearrangement of cell-cell contacts resulting in increased migration of cells. Src activation has been shown to induce diverse proteases such as cathepsin L,[18,46] MMP-9,[17] u-PA,[19,20] MT1-MMP, and TIMP-1[47] in various tumors. In colon tumors, we previously showed that Src activation leads to increased u-PAR expression,[21] further corroborated by the current clinical study. For u-PAR, there is not only evidence for a transcriptional regulation by Src[21] but also for a recip-

rocal regulation of Src-family kinases by ligand-bound u-PAR at the protein level.[48,49] These results suggest the possibility of a positive-regulatory feedback between u-PAR and Src kinases that might augment a tumor cell's invasive capability.

The presentation in the current study that Src activation is associated with invasion and metastasis as well as poor clinical prognosis suggests that this protein tyrosine kinase is a promising target for development of inhibitors as potential therapeutic agents for colon carcinoma. Indeed, inhibition of Src has been shown to inhibit growth in nude mice of HT 29 colon tumor cells[14] and u-PAR-mediated proteolysis of SW480 cells expressing activated Src.[21] As other Src family members such as Fyn and Yes perform many of the normal functions of Src itself, selective inhibitors may also lead to less serious toxicity than traditional regimens of chemotherapy.

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