Differential Activation of pp60c-src and pp62c-yes in Human Colorectal Carcinoma Liver Metastases

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ABSTRACT

pp60c-src and pp62c-yes are protein tyrosine kinases whose specific activities are increased in primary colorectal carcinomas. Activity of pp60c-src is further increased in colorectal liver metastases. This study was undertaken to compare pp60c-src and pp62c-yes expression and activity in human colorectal carcinoma liver metastases and to determine the potential prognostic significance of differences in activation of these two kinases. The pp60c-src and pp62c-yes tyrosine kinase activities and protein levels relative to those in normal colorectal mucosa were determined using an immune complex kinase assay and immunoblot analysis in tissue specimens from 22 patients with primary colorectal carcinoma and synchronous metastatic liver disease and from 9 patients with metachronous colorectal carcinoma liver metastases. Of the primary colon tumors, 64% of the tumors contained elevated activities of both pp60c-src and pp62c-yes. For liver metastases, however, only 10% had activation of both tyrosine kinases, 61% had elevated pp60c-src activity only, and 23% had elevated pp62c-yes activity only. Analysis of synchronous metastases from primary tumors with elevated activities in both kinase demonstrated that in 71% of these patients, the activity of either pp60c-src or pp62c-yes decreases relative to the primary tumor. Protein levels of pp60c-src and pp62c-yes in primary carcinomas and metastases remained unchanged from levels in normal colorectal mucosa. These results demonstrate that differential regulation of the activities of pp60c-src and pp62c-yes occurs during tumor progression. Patients with either synchronous or metachronous liver metastases and elevated pp62c-yes kinase activity have biologically more aggressive disease and a worse prognosis than patients without elevated pp62c-yes activity in their liver metastases (median survival, 13 months versus 30 months, P < 0.005, Wilcoxon signed rank test). Analysis of patients with synchronous liver metastases also demonstrated a worse prognosis for those with elevated pp62c-yes kinase activity (P < 0.05, Wilcoxon signed rank test).

INTRODUCTION

The Src family of nonreceptor protein tyrosine kinases is comprised of nine closely related members (src, yes, fyn, lck, hck, lyn, fgr, blk, and yrk) that function in signal transduction pathways controlling cellular proliferation and differentiation (1–3). Although many of the members are restricted in their expression to hematopoietic cells, c-src, c-yes, and c-fyn have a more ubiquitous pattern of expression. The c-src and c-yes gene products, pp60c-src and pp62c-yes, respectively, are consistently expressed in colonic epithelial cells (4, 5). Previous studies have implicated the c-src and c-yes proto-oncogenes in colonic carcinogenesis. In vitro protein tyrosine kinase activity of pp60c-src is significantly increased in many colon carcinomas when compared with adjacent normal colonic mucosa (5, 6). Examination of colonic polyps and colonic epithelia from ulcerative colitis patients demonstrated a progressive increase in pp60c-src activity from benign to malignant disease (6, 7). In benign polyps, a close correlation was found between increased pp60c-src activity and malignant potential as predicted by polyp size, histology, and degree of dysplasia (6). This activation of the protein tyrosine kinase of pp60c-src and/or pp62c-yes correlates with transition from benign to malignant disease.

Increases in pp60c-src activity also have been observed in colorectal carcinoma hepatic and extrahepatic metastases when compared to normal colonic mucosa and primary colon tumors (8, 9). These results suggest an important role for pp60c-src in tumor progression and metastases.

Recently, pp62c-yes activity was analyzed in colonic adenomas, and like pp60c-src activity was also found to be elevated in adenomas with the greatest malignant potential (10). Furthermore, similar to pp60c-src activity, analysis of pp62c-yes activity in colon carcinoma cell lines demonstrated a 10–20-fold increase in activity in 3 of 5 cell lines compared to normal fibroblasts and a 5-fold increase in 10 of 21 primary colon cancers compared to normal colonic mucosa (11).

In colon carcinoma cell lines, however, some differences in activity of pp60c-src and pp62c-yes have been described. In a recent study, the specific activity of pp60c-src was increased during mitosis of HT-29 cells (a human colon carcinoma cell line) whereas specific activity of pp62c-yes was decreased (12). Another example of differences between pp60c-src and pp62c-yes is the observation that pp60c-src activation (in >95% of primary colorectal carcinoma specimens) occurs more frequently than pp62c-yes (~50%) in primary colorectal carcinoma (5, 8, 11). These results suggest that expression and activity of these two related enzymes may differ throughout tumor progression. To examine this possibility, the relative levels and activities of pp60c-src and pp62c-yes in colorectal carcinoma liver metastases were determined. Differences in the activation of pp60c-src and pp62c-yes were observed, suggesting differences in the regula-
tion of these two closely related family members. Furthermore, patients with activation of pp62cYes had a shorter survival time when compared with the patients without activation of pp62cYes.

MATERIALS AND METHODS

Human Tissue Specimens. Tissue specimens from 31 patients undergoing operations at The University of Texas M. D. Anderson Cancer Center were collected and then stored at −135°C within 20 min after resection. Primary colorectal carcinomas and synchronous liver metastases were collected from 22 patients during their initial operations. Additionally, metachronous colorectal liver metastases were collected from nine patients. All cancerous lesions from the liver and colon were separated from the surrounding normal parenchyma by sharp dissection prior to storage. Diagnosis of colorectal adenocarcinoma and colorectal carcinoma liver metastases were confirmed by pathological examination of permanent tumor sections. In addition, viable tumor cell content versus necrosis and inflammatory cell infiltration were estimated in representative sections of tumor tissue. Most of the tumor tissues analyzed contained at least 40% viable tumor cells and minimal inflammatory cell infiltration. Normal colon specimens at least 2 cm from the primary tumor were resected, and the normal colonic mucosa was then sharply dissected away from the underlying muscularis mucosa prior to storage. Whenever possible, normal liver tissue was also collected and analyzed.

Tissue Lysates. Tissue specimens (~0.5 g) were homogenized in 1 ml of lysis buffer (1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 2% aprotinin, 5 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 1 mM sodium vanadate, and 20 mM sodium phosphate, pH 7.4), using a Brinkman polytron homogenizer. HT-29 (a human colon cancer cell line with known pp60csrc and pp62cYes activities) was used as a positive control for kinase assay and immunoblot analysis, and HT-29 lysates were prepared using identical lysis conditions as for tissue specimens. Lysates were then clarified by centrifugation at 10,000 rpm for 10 min at 4°C. Protein standardization of the lysates was obtained using the bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL).

Immunocomplex Kinase Assay. pp60cSrc and pp62cYes were immunoprecipitated from aliquots of tissue lysates containing 250 μg cellular protein. Identical tissue lysates for each of the different tissue types were used for both the pp60cSrc and pp62cYes kinase assays. In brief, lysates were incubated with either 0.6 μg mAbAb 327 (Oncogene Science, Inc., Mineola, NY) specific for pp60cSrc, or 1.2 μg mAb 1B7 (Wako Chemicals, Richmond, VA) specific for pp62cYes, for 1 h on ice. Further descriptions of the immune complex kinase assay can be found in a previous publication (8). Protein specimens were resolved on 8% SDS-polyacrylamide gels. Radiolabeled proteins were detected with Kodak XAR film with intensifying screens at −85°C. Incorporation of 32P into proteins was quantified by scanning densitometry using a DU-70 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). Results for each specimen were expressed as changes relative to adjacent normal colonic mucosa from the same patient whenever possible. Unpaired liver metastasis specimens were compared with a randomized pool of normal colonic mucosa specimens. Variation among the normal colonic mucosal specimens was <20%.

Immunoblot Analysis of pp60cSrc and pp62cYes. Aliquots of identical lysates used in the immune complex kinase assay containing 250 μg cellular protein were resolved on 8% SDS-polyacrylamide gels. Proteins were then transferred to nitrocellulose filters (0.1-μm pore size; Schleicher & Schuell, Inc., Keene, NH) at 100 V for 3 h at 4°C in transfer buffer (200 mM glycine, 25 mM Tris, and 20% methanol). Ponceau red staining was used to confirm transfer of protein. The nitrocellulose filters were then incubated in blocking buffer containing PBS (140 mM NaCl, 1 mM KH2PO4, 8 mM Na2HPO4·7 H2O, and 3 mM KCl, pH 7.4) and 15% skim milk for 3 h at room temperature followed by incubation with either 0.6 μg mAb 327/lane or 1.2 μg mAb 1B7/lane in blocking buffer for 10 h at 4°C. The filters were then washed three times with blocking buffer before the addition of horseradish peroxidase-conjugated sheep anti-mouse IgG and incubated for 1 h at room temperature. Proteins were detected using enhanced chemiluminescence (Amersham, Arlington Heights, IL) on filters exposed to Kodak XAR with intensifying screens. Protein levels were quantified using scanning densitometry. Results for each specimen were expressed as changes relative to adjacent normal colonic mucosa.

Statistical Analysis. Clinical characteristics (Table 1) and survival information were collected for each patient after reviewing inpatient and outpatient hospital records and through direct communications with patients, family members, and physicians. Statistical tests were performed using Statview (Abacus Concepts). The Wilcoxon signed rank method was used to analyze the statistical significance of survival differences between patients with or without increased pp62cYes tyrosine kinase activity in colorectal carcinoma liver metastases, and also between patients with synchronous colorectal liver metastases with either increased pp60cSrc activity only or increased pp62cYes activity only. In addition, cumulative survival curves were calculated using the Kaplan-Meier method. The Fisher exact test was used to determine relationships between pp60cSrc or pp62cYes activation and the patient’s clinical characteristics. One patient was excluded from survival analysis because of death from a noncancer-related cause, and one patient’s tumor differentiation status was unavailable for review.

RESULTS

Both pp60cSrc and pp62cYes Are Activated in Primary Colorectal Carcinomas. Tyrosine kinase activities of pp60cSrc and pp62cYes were analyzed simultaneously in four tissue types (normal colonic mucosa, primary colorectal cancer, normal liver parenchyma, and colorectal liver metastasis) in 22 patients who underwent resections of primary colorectal carcinomas and synchronous liver metastases during the same operation. Results of the immune complex kinase assays and immunoblot analyses for two patients with primary tumors and synchronous metastases are shown in Figs. 1 and 2. For the primary tumors, the average increase in pp60cSrc kinase activity was 7-fold by autophosphorylation and 5-fold by enolase phos-
phorylation. In the same specimens, pp62<sup>vr</sup> tyrosine kinase activity was increased 5-fold by autophosphorylation and 3-fold by enolase phosphorylation relative to pp62<sup>vr</sup> in normal mucosa. In our analysis, 21 of 22 (95%) cancerous specimens demonstrated increased pp60<sup>vr</sup> activity, defined as a >2-fold increase in either autophosphorylation or enolase phosphorylation with respect to adjacent normal mucosa. In contrast, pp62<sup>vr</sup> activity was increased in 14 of 22 (64%) cancerous specimens. These results are in close agreement with previous reports (8, 11). All 14 specimens with increased pp62<sup>vr</sup> activity also demonstrated elevated pp60<sup>vr</sup> activity. No specimens had decreased pp60<sup>vr</sup> activity with increased pp62<sup>vr</sup> activity. Protein levels of pp60<sup>vr</sup> and pp62<sup>vr</sup> activity were similar to levels found in normal colonic mucosa.

**pp62<sup>vr</sup> Is Less Frequent in Liver Metastases.** Levels and activities were determined from 22 synchronous and 9 metachronous liver metastases. Analysis of pp60<sup>rvr</sup> activity revealed 22 (71%) specimens with elevated activity. Of these 22 specimens, 19 (61%) had elevation in pp60<sup>rvr</sup> activity only. Only 3 of 31 (10%) liver metastases had elevations in both pp60<sup>rvr</sup> and pp62<sup>vr</sup>. An example of one such patient is shown in Fig. 1a. The pp62<sup>vr</sup> activity was increased in a total of 10 of 31 (32%) specimens, 7 (23%) of which had increased pp62<sup>vr</sup> activity only. An example of one such patient is shown in Fig. 2a. Analysis of 14 synchronous metastases from primary tumors with increased activities in both tyrosine kinases demonstrated that in 10 (71%) of these patients, the activity of either pp60<sup>rvr</sup> or pp62<sup>vr</sup> decreases relative to the primary tumor (see Fig. 2a, A). Protein levels of pp60<sup>rvr</sup> and pp62<sup>vr</sup> in most of the primary carcinomas and metastases remained unchanged from levels in normal colonic mucosa (Figs. 1b and 2b). In two liver metastases, neither kinase was activated (data not shown).

In the nine metachronous metastases, eight had increased pp60<sup>rvr</sup> activity with decreased or unchanged pp62<sup>vr</sup> activity. Fig. 3 shows an immune complex kinase assay and immunoblot of four patients with metachronous colorectal liver metastases. In three specimens, increased pp60<sup>rvr</sup> activity was observed with decreased or unchanged expression and activity of pp62<sup>vr</sup>. Fig. 4 summarizes pp60<sup>rvr</sup> and pp62<sup>vr</sup> total kinase activities in the primary colorectal carcinomas and liver metastases analyzed.

Of the seven specimens that demonstrated increased pp62<sup>vr</sup> activity without activation of pp60<sup>rvr</sup>, most expressed less pp60<sup>rvr</sup> than the corresponding normal mucosa; however, a 2-fold decrease was measured in only three of seven specimens. In one of nine specimens, an increase in the pp62<sup>vr</sup> protein level of >2-fold observed. Therefore, the protein expressions of pp60<sup>rvr</sup> and pp62<sup>vr</sup> could not account

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Table 1  Selected characteristics of patients with colorectal carcinoma liver metastases

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<th>Patient</th>
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<th>Differentiation</th>
<th>Chemotherapy</th>
<th>Liver metastases</th>
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*a* ELEV, elevated; DEC, decreased; UNCH, unchanged; 5FU, 5-fluorouracil; FUDR, 2'-deoxy-5-fluorouridine; BILOBAR, metastases in both lobes of liver; UNILBAR, metastases in one lobe of liver.

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"— unavailable for review.

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Clinical Cancer Research 1399

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for their decreased activities in liver metastases relative to primary tumors.

Clinical Characteristics of Patients and Tumors. Patient clinical characteristics are summarized in Table I and include differentiation status of the tumors, postoperative chemotherapy regimen, extent of liver metastases, whether the patient presented with extrahepatic metastases, Karnofsky's performance status (13), and previous treatments. The male:female ratio was 22:9, and the range of ages for the 31 patients was 29–82 years, with a median of 57 years. There were eight patients (26%) who received treatment for their cancer prior to evaluation at M. D. Anderson. None of the treatment regimens had an obvious effect on pp60c-src or pp62c-yes expression and/or activity. The Fisher exact test demonstrated no correlation between pp60c-src or pp62c-yes activation and the clinical characteristics analyzed. Survival time was calculated from the time of initial diagnosis of colorectal carcinoma. Patients with metastatic liver metastases frequently had prolonged disease-free intervals between diagnosis of primary colorectal carcinoma and liver metastases. The median disease-free interval was 12 months. Our analysis of all patients in the study showed that patients with elevated pp62c-yes activity had shorter survival times from diagnosis than patients without pp62c-yes activation ($P < 0.005$, Wilcoxon signed rank test). Median survival for patients with activation of pp62c-yes was 13 months versus 30 months for patients without activation of pp62c-yes in their liver metastases (Fig. 5). In the subgroup with decreased pp62c-yes activity, eight patients were still alive with potentially prolonged survival versus only two patients in the elevated pp62c-yes activity subgroup. Since all but one of the metachronous colorectal liver metastasis specimens had decreased pp62c-yes activity, this could partly account for the prolonged survival observed. Therefore, further analysis was performed only on patients with synchronous colorectal carcinoma liver metastases with either increased pp62c-yes activity only (pp60c-src decreased) or increased pp60c-src activity only (pp62c-yes decreased). Although the subgroups contained a small number of patients, a trend toward differences in survival between the two subgroups was noted, as shown in Fig. 6. In the increased pp60c-src-only subgroup, two patients were still alive versus only one patient still alive in the increased pp62c-yes-only subgroup. Statistical signifi-
cance was again demonstrated using the Wilcoxon signed rank method ($P < 0.05$).

**DISCUSSION**

As many as 25% of the estimated 150,000 new cases of colorectal carcinoma will present with liver metastases, and another 50% of patients will develop recurrent disease within the liver (14). Yet, despite recent improvements in early diagnosis, surgical techniques, and adjuvant chemotherapy, one third of the patients who undergo curative operative resections will die of recurrent disease or metastases resistant to conventional therapies (15, 16). Therefore, a better understanding of the biology of colorectal metastases may lead to the identification of new targets for the development of novel chemotherapeutic agents.

Activated tyrosine kinases of the src family may represent one such target. Both c-src and c-yes are expressed in normal colonic mucosa (4, 5), with relatively high activities of the enzymes observed in cytoskeletal fractions of rapidly dividing chicken crypt cells (2). Targeted disruption of the src gene, leading to the generation of src knockout mice, has demonstrated no defects in the development or function of the colon (17), suggesting that the functions of these two related protein tyrosine kinases are redundant in normal colonic epithelial cells. Previous studies have demonstrated that pp60$c-src$ and pp62$c-yes$ activities are greatly increased in most primary tumors, with further increases in pp60$c-src$ activity observed in metastases. The present work was undertaken to determine whether expression and activity of pp62$c-yes$ was similar to that of pp60$c-src$ at late stages of colorectal carcinoma. For these studies, synchronous primary and hepatic metastases, as well as metachronous metastases, were investigated.

The results demonstrated distinct patterns of activity of the two related kinases. As previously reported, pp60$c-src$ activity is elevated in >90% of primary tumors and remains activated in 71% of liver metastases. In contrast, pp62$c-yes$ is activated in 64% of primary tumors but only 32% of liver metastases. Furthermore, in many of the patients with pp62$c-yes$ activation in the primary tumors, activity of the enzyme decreases in hepatic metastases. These data would suggest that pp60$c-src$ might be more critical to tumorigenicity and/or progression than pp62$c-yes$. Several types of experiments support this conclusion. Recent studies from this laboratory have demonstrated that
Fig. 3  

a, tyrosine kinase activities of pp60c-src (A) and pp62c-yes (B) in four patients with metachronous colorectal carcinoma liver metastases. b, corresponding immunoblot analysis of identical tissue lysates. Kinase activities and protein levels of pp60c-src and pp62c-yes relative to a pool of normal colonic mucosa specimens were quantified using scanspec densitometry. NC, normal colonic mucosa; LM1–4, four different colorectal liver metastases from four different patients.

Fig. 4  

Summary of pp60c-src or pp62c-yes total kinase activity in patients with primary colon cancer (22 total) and patients with synchronous or metachronous liver metastases (31 total). Columns 7, 0, 14, and 19, primary carcinomas analyzed; columns 19, 7, 3, and 2, metastases analyzed. The pp60c-src and pp62c-yes were defined as activated if kinase activities were >2-fold increased relative to normal colonic mucosa. Columns, patients with activation of pp60c-src only, pp62c-yes only, activation of both pp60c-src and pp62c-yes, or neither kinases activated.

decreased expression of pp60c-src alone by transfection of c-src antisense expression vectors in colon tumor cell lines greatly reduces their tumorigenicity in nude mice.4

In the development of mammary tumors in mice, pp60c-src activation also appears to be more important than pp62c-yes activation. In transgenic mice expressing the polyoma virus-encoded middle I oncogene in mammary epithelium, mammary tumors develop universally (18). When these mice are crossed with src knockout mice, the progeny do not develop mammary tumors. In contrast, when the mice are crossed with yes knockout mice, mammary tumors develop consistently (19). These results suggest that, in the development of metastases, selection for increased pp60c-src expression and activity may be sufficient.

However, our studies demonstrated a small but distinct subset of colon metastases in which pp62c-yes activity was increased and pp60c-src activity was decreased relative to that of the primary tumor. Patients with increased pp62c-yes activity in

Kinase activities elevated, either pp60c or pp62c activity is of synchronous metastases from primary tumors with both development of metastasis appears to coincide with selection metastases that are distinct from pp60c activation. Interestingly, against activation of one or the other Src family enzymes. In activation may have important biological consequences in increased pp62c activity. These results suggest that pp62c src activation resulted in a better prognosis cannot be determined from this small sample size. Alternatively, activation of these enzymes may be correlative with an as yet undefined prognostic variable.

Currently, considerable effort has been devoted to development of tyrosine kinase inhibitors as potential antineoplastic agents. Previous work with herbimycin A, an ansamycin anti-biotic that inhibits several tyrosine kinases including pp60c-src (20), has demonstrated a dose-dependent reversible inhibition of cell growth and a concomitant decrease in pp60c-src kinase activity in human colon carcinoma cell lines (21). Since herbimycin A inhibits many different tyrosine kinases, tissue toxicity in humans prohibits its use as a systemic therapeutic. Nevertheless, the use of inhibitors that can specifically target either pp60c-src or pp62c-src tyrosine kinases may be an effective and novel approach to the treatment of metastatic colorectal carcinoma. This study demonstrates that the activation status of both pp60c-src and pp62c-src tyrosine kinases may require therapeutic consideration.

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Differential activation of pp60(c-src) and pp62(c-yes) in human colorectal carcinoma liver metastases.

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