Altered Localization of p120 Catenin During Epithelial to Mesenchymal Transition of Colon Carcinoma Is Prognostic for Aggressive Disease


Abstract

We examined the expression and localization of p120 catenin (p120ctn) as a consequence of the epithelial to mesenchymal transition (EMT) of highly differentiated colon carcinoma cells (LIM1863 cells). This unique line grows in suspension as spheroids and undergoes an EMT within 24 hours following stimulation with transforming growth factor-β and tumor necrosis factor-α. Although p120ctn expression remains stable during the EMT, its localization shifts from cell-cell junctions to the cytoplasm. Interestingly, a marked decrease in RhoA activation coincident with E-cadherin loss occurs during the EMT and correlates with the formation of a p120ctn/RhoA complex. Use of RNA interference showed that p120ctn reduction results in increased RhoA activity and a significant decrease in the motility of post-EMT cells. To determine the relevance of these findings to colorectal cancer progression, we assessed p120ctn expression by immunohistochemistry in 557 primary tumors. Of note, we observed that 53% of tumors presented cytoplasmic staining for p120ctn, and statistical analysis revealed that this localization is predictive of poor patient outcome. Cytoplasmic p120ctn correlated with later-stage tumors, significantly reduced 5- and 10-year survival times and a greater propensity for metastasis to lymph nodes compared with junctional p120ctn. We also confirmed that altered localization of p120ctn corresponded with loss or cytoplasmic localization of E-cadherin. These alterations in E-cadherin are also associated with a significant reduction in patient survival time and an increase in tumor stage and lymph node metastasis. These data provide a compelling argument for the importance of both p120ctn and the EMT itself in the progression of colorectal carcinoma. (Cancer Res 2005; 65(23): 10938-45)

Introduction

Embryonic development, tissue remodeling, and wound repair involve the conversion of epithelial cells to a mesenchymal more conducive to cell migration, a process referred to as the epithelial to mesenchymal transition (EMT; refs. 1, 2). The EMT is also considered to be a powerful model for studying the mechanisms of carcinoma progression because highly invasive carcinoma cells show a loss of epithelial characteristics and acquisition of a mesenchymal phenotype (3, 4). This transition occurs via two distinct biological events: the breaking of cell-cell adhesions and a consequent increase in cell motility. A defining characteristic of EMT is the loss of E-cadherin. This cell surface protein, involved in Ca2+-mediated intercellular adhesion, has been implicated both as a tumor suppressor as well as a regulator of invasion, and these roles are commonly attributed to the functions of its cytoplasmic binding partners called catenins (5, 6).

p120 catenin (p120ctn), a relative of β-catenin, interacts with the cytoplasmic tail of classical cadherins to mediate strong cell-cell adhesions by facilitating cadherin clustering (7, 8). Recent data also indicate that expression of E-cadherin itself is dependent upon p120ctn function (9). The most extensively studied role of p120ctn, however, is in the regulation of the Rho family of small guanine nucleotide GTases, including RhoA, Rac1, and Cdc42 (10). Overexpression of p120ctn has been reported to alter cellular morphology and enhance migration in fibroblasts by activating Rac1 and Cdc42 and inhibiting RhoA activity (11). Although it is believed that binding to the guanine nucleotide exchange factor, Vav2, accounts for the ability of p120ctn to activate Rac1 and Cdc4, the mechanism of suppression of RhoA activity by p120ctn is still unclear. Interestingly, it has been reported that p120ctn may function as a RhoA guanine nucleotide dissociation inhibitor, as shown in Drosophila melanogaster (12).

Considerable insight into carcinoma progression has been obtained from studies on the behavior of E-cadherin and catenins (5, 6). Indeed, it is established for many carcinomas that loss of E-cadherin expression is associated with more aggressive tumors. The role of p120ctn in epithelial carcinogenesis, however, is not as well understood. The prevailing notion is that p120ctn expression is lost in many types of tumors, possibly as an early event in tumorigenesis (13), but definitive evidence is lacking. The behavior of p120ctn has not been evaluated rigorously within the context of a defined model of progression, and studies on its expression and localization in a large cohort of tumors with defined clinical outcomes have not been done.

In this study, we used the LIM1863 colon carcinoma cell line to examine the expression and function of p120ctn during the EMT. This line forms three-dimensional spheroids in culture and will undergo EMT upon costimulation with transforming growth factor-β1 (TGF-β1) and tumor necrosis factor-α (TNF-α; ref. 14). The data obtained indicate that p120ctn expression persists subsequent to E-cadherin loss and that its localization shifts from...
cell-cell junctions to the cytoplasm. Moreover, the EMT results in a reduction of RhoA activity in a p120ctn-dependent manner that serves to enhance cell migration. Our in vitro observation that p120ctn expression is maintained and localizes to the cytoplasm during carcinoma progression is recapitulated in the majority of the 557 colorectal tumors analyzed. Importantly, statistical analysis indicated that cytoplasmic p120ctn correlates with advanced tumor stage, a propensity to metastasize, and reduced patient survival. Additionally, E-cadherin expression in tumors correlated with p120ctn localization and disease progression. These data provide a compelling argument for the importance of both p120ctn and the EMT itself in the progression of colorectal carcinoma.

Materials and Methods

LIM1863 cells and epithelial to mesenchymal transition. The LIM1863 human colon carcinoma cell line was maintained in RPMI 1640 supplemented with 5% FCS, 1-glutamine, penicillin, and streptomycin. These cells, which grow as nonadherent spheroids, were induced to undergo EMT as described previously (14). Briefly, the cells were treated with TGF-β1 and TNF-α (R&D Systems, Minneapolis, MN) at 2 and 10 ng/mL, respectively, and were incubated at 37°C. At defined times after cytokine stimulation, cells were harvested and assessed by immunoblot for E-cadherin expression (see below) to monitor the EMT.

Biochemical analyses. To evaluate the expression of specific proteins as a function of the EMT, LIM1863 cells were stimulated with cytokine for the times indicated, and extracted in a Triton X-100 buffer [1% Triton X-100, 50 mmol/L Tris (pH 7.6), 150 mmol/L NaCl] with protease inhibitors (pepsatin, phenylmethylsulfonyl fluoride, aprotinin, and leupeptin). Aliquots of these extracts were separated on either 6% or 12% SDS-PAGE gels and transferred to nitrocellulose by electrophoresis. Immunoblotting was done at 4°C overnight in 2.5% milk in TBS-Tween using anti-p120ctn, or anti-E-cadherin monoclonal (BD Pharmingen, San Diego, CA) or anti-RhoA (Santa Cruz, Santa Cruz, CA) or anti-actin (Sigma, St. Louis, MO) polyclonal antibodies. Blots were then incubated for 1 hour at room temperature with horseradish peroxidase-conjugated antimouse or antirabbit secondary antibody (Jackson ImmunolResearch, West Grove, PA) and were developed using enhanced chemiluminescence (Pierce, Rockford, IL).

Immunoprecipitations were done using the same extracts. These samples (300 μg protein/extract) were “precleared” twice by incubation with protein G-sepharose beads (Amersham Biosciences, Piscataway, NJ) at 4°C overnight in 2.5% milk in TBS-Tween using anti-p120ctn, or anti-E-cadherin monoclonal (BD Pharmingen, San Diego, CA) or anti-RhoA (Santa Cruz, Santa Cruz, CA) or anti-actin (Sigma, St. Louis, MO) polyclonal antibodies. Blots were then incubated for 1 hour at room temperature with horseradish peroxidase-conjugated antimouse or antirabbit secondary antibody (Jackson ImmunolResearch, West Grove, PA) and were developed using enhanced chemiluminescence (Pierce, Rockford, IL).

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RhoA activity. RhoA activity was assessed using the Rho-binding domain (RBD) of Rhotekin as previously described (15). Briefly, LIM1863 spheroids were stimulated with cytokine as described above and extracted in a combined detergent buffer [50 mmol/L Tris (pH 7.2), 500 mmol/L NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.08% SDS, and 10 mmol/L MgCl2]. Extracts were clarified by centrifugation (12,000 rpm), and the supernatants were then incubated with 2 μg of antibody for 1 hour, protein G-sepharose beads were added for 1 hour, and the samples were washed five times with the extraction buffer. Samples were boiled for 10 minutes in nonreducing Laemmli sample buffer.

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RNA interference. Generation of retrovirus was done as previously described (16). Briefly, the pSuper.Retro vector containing human-specific p120 small interfering RNA (siRNA; ref. 17), GC CAG AGG TGG TTC GGA TA, or a nonspecific, scrambled control, GG TAG AGA GAC AGT CTC GC, was transfected with a viral envelope and packaging vector into 293T cells using LipofectAMINE reagent. Retroviral supernatant was collected 72 hours later, passed twice though a 0.2-μm filter and added to recipient LIM1863 cells in the presence of 5 μg/mL polybrene. After 72 hours of incubation with virus, cells were placed in low-calcium RPMI 1640 supplemented with 5% FCS, 1-glutamine, penicillin, and streptomycin to generate a single-cell suspension, and 5 μg/mL puromycin was added for 72 hours. Selected cells were maintained in normal medium containing 5 μg/mL of puromycin.

Migration assays. The lower compartment of Transwell chambers (6.5 mm diameter, 0.22 μm pore size; Corning, Corning, NY) was coated with Laminin-1 (20 μg/mL; Roche Diagnostics, Indianapolis, IN) at 4°C overnight. Conditioned 3T3 medium was added to the lower chamber. Cytokine-treated LIM1863 cells were added to the upper chamber (1 × 105/ chamber). After incubating for 72 hours at 37°C, cells were removed from the upper chamber with a cotton swab, and cells that had migrated to the lower surface of the membrane were fixed in 100% methanol. Each Transwell was mounted on a glass slide in Vectashield medium with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA), and the number of cells that had migrated was quantified by immunofluorescence on a Nikon Diaphot microscope using IP Lab Spectrum.

Immunofluorescence microscopy. LIM1863 cells were plated in suspension on untreated glass coverslips and stimulated with cytokine as described above. During the course of the EMT, these cells adhere to the coverslips. The coverslips were washed with PBS, fixed in 2% paraformaldehyde and permeabilized with 0.2% Triton X-100. Coverslips were blocked [1% bovine serum albumin, 50 mmol/L Tris (pH 7.5), 500 mmol/L NaCl] for 30 minutes and then incubated with anti-p120 antibody (5 μg/mL) for 1 hour. Following washes with PBS, Cy3-conjugated antiomouse IgG (Jackson ImmunolResearch) was applied for 1 hour at a 1:400 dilution. Coverslips were mounted in Vectashield medium with 4′,6-diamidino-2-phenylindole and sealed. To analyze LIM1863 spheroids prior to the EMT, cells were treated as above in suspension, and, following the final wash, stained cells were resuspended in Vectashield medium, a coverslip was applied and the samples were sealed. Images were acquired on a Nikon E800 light/fluorescence microscope with Bio-Rad (Hercules, CA) MRC 1020 confocal laser system, and red pseudocolor was added using IP Lab Spectrum.

Tissue microarray and immunohistochemistry. Tissue microarray slides were generated as described (18). Briefly, formalin-fixed, paraffin-embedded tissue blocks containing carcinoma specimens were retrieved from the archives of the Yale University Department of Pathology. Areas of invasive carcinoma were identified on corresponding H&E-stained slides, and the tissue blocks were cored and transferred to a recipient master block using a Tissue Microarrayer (Beecher Instruments, Sun Prairie, WI). Each core measured 0.6 mm at its greatest dimension, and cores were spaced 0.8 mm apart. After cutting the recipient block, and transfer with an adhesive tape to coated slides for subsequent UV cross-linkage (Insumedics, Inc., Hackensack, NJ), the slides were dipped in a layer of paraffin to prevent oxidation. The specimens were resected between 1962 and 1980, with a follow-up that ranged between 4 months and 53.8 years, with a mean follow-up of 15.6 years, and a median follow-up of 14.3 years.

For immunohistochemistry, the slides were deparaffinized with xylene and transferred through changes of ethanol. Antigen retrieval was done by boiling the slides in a sodium citrate buffer (pH 6.0), and endogenous peroxidase activity was blocked by 30 minutes of incubation in a 2.5% hydrogen peroxide/methanol buffer. The slides were then blocked with avidin and biotin for 10 minutes each (DakoCytomation, Carpinteria, CA) followed by 0.3% BSA in TBS for 30 minutes at room temperature, and primary antibody diluted in 0.3% BSA/TBS was applied at 1 μg/mL overnight at 4°C. After rinsing in TBS, the slides were incubated with Envision anti-mouse horseradish peroxidase solution (DakoCytomation) for 1 hour at room temperature. The slides were rinsed and visualized with a 5-minute incubation of 3,3′-diaminobenzidine+ solution, counterstained with hematoxylin, dehydrated and mounted with Permount (Sigma). An image corresponding to each of the usable 557 tumor spots was generated on an Axioshot microscope. Each spot was then analyzed for p120ctn staining and...
protein localization was scored by eye as junctional, cytoplasmic, or lost. Images for E-cadherin were collected and scored by eye for expression and localization as junctional, cytoplasmic, or lost.

**Statistical methods.** Kaplan-Meier method was used to estimate median survival, 5-year survival, and 10-year survival along with their 95% confidence intervals. Log-rank test was used to examine the differences in survival between groups. The proportional hazard ratio model was used in a multivariate setting to obtain more precise estimates of the effects of protein expression on survival by adjusting for the effects of other covariates, including age, gender, tumor stage, and lymph node status. $\chi^2$ test was used to determine the significance of association between protein expression and localization and other clinical categorical covariates, including tumor stage and lymph node status.

**Results**

**p120 Catenin expression persists but its localization is altered during epithelial to mesenchymal transition.** We have previously reported that the LIM1863 human colon carcinoma cell line can be induced to undergo irreversible EMT following stimulation with TGF-$\beta$ and TNF-$\alpha$ (14). Intrinsic to this transition is the loss of E-cadherin expression, which occurs 12 hours after cytokine stimulation (Fig. 1A), and the expression of mesenchymal markers such as N-cadherin (14). In contrast to E-cadherin, no discernable change in p120ctn protein expression is evident by immunoblotting during the course of the EMT (Fig. 1A). Although p120ctn expression does not change during the EMT, a marked alteration in its localization is evident by immunofluorescence microscopy. In intact organoids, p120ctn is maintained at cell junctions (Fig. 1B) where it colocalizes with E-cadherin (data not shown). Following EMT, however, distinct cytoplasmic staining of p120ctn is evident in addition to some residual reactivity at the cell periphery (Fig. 1C).

**p120 Catenin regulates the activity of RhoA during EMT and facilitates the migration of post-EMT cells.** The sustained expression of p120ctn observed during the EMT suggested that this catenin might contribute to the function of post-EMT cells. Given the ability of p120ctn to regulate the activity of Rho family GTPases and the potential contribution of these GTPases to the EMT, we focused on this aspect of p120ctn function. Initially, we examined the activity of RhoA during the EMT using the Rhotekin pulldown assay. Intact LIM1863 spheroids exhibit a relatively high basal activity of RhoA (Fig. 2A). Interestingly, this activity decreases markedly (~85%) between 12 and 24 hours of cytokine stimulation, a time frame subsequent to loss of E-cadherin (Fig. 1A). No change in the expression of RhoA was evident during this time course (Fig. 2A). We hypothesized that the translocation of p120ctn to the cytoplasm might provide a mechanism for the regulation of RhoA activity which we observed. To examine this possibility, extracts of cells obtained during the course of the EMT were immunoprecipitated for p120ctn and these were immunoblotted for RhoA. As shown in Fig. 2C, RhoA was evident in p120ctn immunoprecipitates 12 hours after induction of EMT, increasing further at 18 and 24 hours, a pattern coincident with the reduction in RhoA activity (Fig. 2A and B). A control isotype-specific IgG was unable to precipitate either p120ctn or RhoA (Fig. 2C). Hence, RhoA activity is dramatically reduced following loss of E-cadherin, and this decrease is concomitant with the formation of a p120ctn/RhoA protein complex.

LIM1863 cells acquire migratory potential upon induction of the EMT (14). Because RhoA activity decreases during this transition, we hypothesized that this change in GTPase activity by cytoplasmic p120ctn may facilitate post-EMT cell motility. To determine its contribution to post-EMT migration, we used a retroviral siRNA approach to reduce p120ctn by ~70% in LIM1863 cells. It is important to note that this reduction in expression is evident in all isoforms of p120 (Fig. 3A). Interestingly, treatment of LIM1863 with p120ctn-specific siRNA does not seem to noticeably affect the spheroid morphology (data not shown). These cells maintain three-dimensional structure comparable to cells expressing a scrambled sequence control siRNA. Furthermore, there is no significant reduction in E-cadherin expression in the p120ctn RNA interference (RNAi) pre-EMT cells (data not shown). Lastly, this decrease in the level of p120ctn seems to neither stimulate nor inhibit the EMT as determined by morphology and the timing of EMT in these cells.

To determine the effect of this siRNA on RhoA activity, a Rhotekin assay was done following induction of the EMT in these cells. RhoA activity increased by 115% in cells with reduced p120ctn expression compared with control cells (Fig. 3B). To test the effect of reduced expression of p120ctn in post-EMT cell motility, we did a migration assay using the stable RNAi cells. Following EMT, the migration of these cells was reduced by 63% compared with controls (Fig. 3C). In addition, the ability of these cells to spread from the spheroid body was dramatically stunted compared with controls (Fig. 3D). These data indicate that one function of p120ctn is to inhibit the activity of RhoA and facilitate the migration of post-EMT cells.

Cytoplasmic p120 catenin is prevalent in colorectal tumors and is associated with aberrant expression and localization of E-cadherin. If the EMT is a valid paradigm for carcinoma...
progression, we predict that p120ctn expression is sustained in colorectal tumors and that it exhibits a predominantly cytoplasmic localization correlating with progression of the disease. To test this postulate, p120ctn expression was assessed by immunohistochemistry in tissue microarrays of primary colorectal adenocarcinomas. Of the 557 tumors examined, only 232 (41.7%) showed the junctional localization of p120ctn that is associated with a well-differentiated epithelium (Fig. 4A). However, more than half of the tumors (52.9%) exhibited cytoplasmic localization of p120ctn (Fig. 4B and C). Interestingly, there were relatively few tumors that exhibited a loss of p120ctn expression (5.4%).

We also investigated the colocalization of p120ctn and E-cadherin. E-cadherin expression was restricted to the cell periphery in 89% of the tumors demonstrating junctional localization of p120ctn (Fig. 4D; Table 1). However, E-cadherin expression was not detectable by immunohistochemistry in 36% of the tumors presenting cytoplasmic p120ctn (Fig. 4E; Table 1) indicating that these cells had lost this cadherin protein without losing expression of p120ctn. In 62% of the samples exhibiting delocalization of p120ctn, E-cadherin expression was detectable, but it was localized in the cytoplasm and not at intercellular junctions (Fig. 4F; Table 1). In relatively few tumors (2%), E-cadherin exhibited a junctional localization when p120ctn

Figure 2. RhoA expression, activation, and association with p120ctn during the EMT. A, extracts from LIM1863 cells that had been stimulated with TGF-β and TNF-α for the times indicated were assayed for their ability to bind the Rho binding domain of Rhotekin to assess the level of GTP-RhoA (active RhoA) and for total RhoA expression. GTPγS was included as a positive control. B, the blots generated in (A) were analyzed by densitometry to obtain relative RhoA activation (active RhoA/total RhoA) as a function of time of cytokine stimulation. Data representative of multiple separate experiments. C, extracts from LIM1863 cells that had been stimulated for the times indicated were immunoprecipitated with a p120ctn antibody, and the immunoprecipitates were blotted with either a p120ctn or RhoA antibodies (top). In addition, aliquots of the same extracts were blotted with the same antibodies to assess the relative expression of these proteins in the extracts.

Figure 3. Evaluation of p120ctn expression in the migration of LIM1863 cells by RNAi and its effect on RhoA activation. A, immunoblot of extracts of LIM1863 cells that express either a siRNA specific for all p120ctn isoforms or a scrambled control. Expression of the p120ctn isoforms is reduced by ~70% in cells that express the p120 siRNA compared with control cells as assessed by densitometry. B, RhoA assay for RhoA activity. Rhotekin-bound RhoA (top) and total RhoA protein (bottom). The p120ctn RNAi cells exhibit an increase of ~15% in RhoA activity as assessed by densitometry. C, chemotactic migration assay. Cells that express the p120ctn siRNA had a significant reduction in migration (63% (P < 0.05)) in comparison to control cells. D, photomicrographs of control (Scr) and p120ctn siRNA cells after stimulation with TGF-β and TNF-α for 24 hours. In the control organoid, note the spread and individual cells at the periphery of the organoid that seem to be motile. Such cells are absent in the p120ctn siRNA sample; bar, 10 μm.
localized in the cytoplasm. However, all tumors with loss of p120ctn expression showed either lost expression or cytoplasmic localization of E-cadherin (Table 1).

Alterations in p120 catenin and E-cadherin correlate with poor prognosis and advanced disease. Statistical analysis was done to determine whether there is a significant difference in the survival of patients presenting tumors with altered p120ctn or E-cadherin. Utilizing univariate analysis, it was determined that there is a marked reduction in survival associated with delocalization of p120ctn. Specifically, the 5-year survival rate for patients that have a tumor demonstrating cytoplasmic localization is 54%, compared with 67% for tumors with normal, membranous localization. Ten-year survival rates are 45% for cytoplasmic and 62% for junctional p120ctn (Fig. 5A; Table 1). The difference in survival between these two groups is statistically significant \( (P = 0.0033) \). To confirm the validity of this approach, we also determined that, predictably, tumor stage and lymph node status are predictive for patient outcome.

E-cadherin expression and localization in these tumors are also prognostic for patient survival in a univariate analysis. The 5-year survival rates for patients with loss or delocalization of E-cadherin are 55% and 53%, respectively, compared with 67% for tumors with normal expression and localization of E-cadherin. The 10-year survival rates also show this trend with 49% and 43% for loss or delocalization, respectively, compared with 62% survival for membranous E-cadherin (Fig. 5B; Table 1). It is important to note that there is no significant difference in the survival between patients with either lost or delocalized expression of E-cadherin. However, the disparity between patients with tumors demonstrating either aberrant or junctional E-cadherin is statistically significant \( (P = 0.0127) \). These data show that the altered localization and expression of p120ctn and E-cadherin are prognostic for patient survival. Interestingly, whereas both p120ctn and E-cadherin are predictors of patient outcome in this univariate analysis, they do not seem to be significant in a multivariate setting, with lymph node status being the single best predictor using such an approach \( (P < 0.001) \). It is important to note that tumor stage is also not predictive using this method.

Our analysis of the tissue microarrays revealed a significant correlation between p120ctn localization and tumor stage. For tumors with junctional localization of p120ctn, the majority present as low stage (56% as stage 1 or 2) compared with 44% being described as high stage (3 or 4). Remarkably, of tumors with cytoplasmic p120ctn, there is a prominent shift towards advanced disease with only 43% at low stage whereas 57% are high stage (Fig. 6A). This association of delocalized p120ctn with increased tumor stage is statistically significant \( (P = 0.0078) \). As with p120ctn, aberrant E-cadherin expression or localization was detected

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cytoplasmic p120ctn in Colon Carcinoma

Figure 5. Kaplan-Meier survival analysis of patients with altered p120ctn and E-cadherin using a log-rank test. A, samples were grouped according to p120ctn membranous or cytoplasmic localization. B, samples were grouped according to membranous, cytoplasmic, or lost E-cadherin. The variations in survival as a function of p120ctn and E-cadherin are significant ($P = 0.0033$ and $P = 0.0127$, respectively).

Discussion

In this study, we show that the EMT provides an excellent model for examining the role of p120ctn in carcinoma progression and, importantly, for the identification of markers of aggressive carcinomas. Our key finding is that the translocation of p120ctn from adherens junctions to the cytoplasm following loss of E-cadherin is characteristic of aggressive colorectal tumors, and it is coincident with the ability of this catenin to suppress activation of the RhoA GTPase, an event that facilitates the migration of post-EMT cells. Moreover, cytoplasmic localization of p120ctn in tumors is associated with either loss of E-cadherin expression or altered localization, an observation that substantiates the hypothesis that adherens junction proteins have pivotal functions in carcinomas.

Although previous studies have ascribed a function for p120ctn in cell migration and the regulation of Rho GTPases, the significance of our data is that they assess these functions in the context of a defined pathophysiologic process that is associated with the acquisition of cell migration, the EMT of carcinoma. What is apparent from this approach is that the function of p120ctn differs markedly between epithelial and mesenchymal cells. Presumably, the major function of p120ctn in epithelial cells is to maintain the integrity of adherens junctions (7–9). It is interesting to note, however, that reduction of p120ctn in LIM1863 spheroids did not seem to significantly alter epithelial morphology or the expression of E-cadherin. This seemingly contradictory observation may simply reflect the fact that the retroviral infected cells were selected not only for the presence of the siRNA construct, but also for their ability to reform three-dimensional structures. Cells lacking E-cadherin would not be able to form spheroids and would undergo apoptosis as single cells in suspension (19). Nevertheless, it is apparent from our data that a reduction in p120ctn expression in post-EMT cells has a marked effect on their ability to migrate as well as to regulate the activity of RhoA, an observation that supports the duality of p120ctn function in epithelial and mesenchymal cells. Another important aspect of our results is that previous work implicating p120ctn in cell migration was based largely on its exogenous expression. Such an

predominantly in late stage tumors. Of tumors with normal localization, 36% presented as early stage, with 44% described as late stage. This is in contrast with tumors demonstrating cytoplasmic E-cadherin, in which 48% were low stage and 52% were high stage. Tumors with loss of E-cadherin were skewed even more towards advanced disease with only 36% presenting as low stage and 64% as late stage (Fig. 6B). This difference is statistically significant ($P = 0.0047$). Importantly, the correlation with prognosis is independent of this association with tumor stage as shown by a significant reduction in survival in stages 3 and 4 tumors with cytoplasmic p120ctn or aberrant E-cadherin.

Further analysis revealed that the expression and localization of these proteins is also correlated with the extent to which the primary tumors metastasize. For tumors with normal localization of p120ctn, nearly 62% show no metastases ($N_o$), with 25.6% metastasizing to three or less ($N_1$), and only 12.6% metastasizing to four or more local lymph nodes ($N_2$). However, for cytoplasmic p120ctn, the proportion of nonmetastatic tumors was reduced to 50%, with an increase observed in both $N_1$ and $N_2$ populations to 31% and 19%, respectively (Fig. 6C). This correlation between p120ctn localization and metastasis is statistically significant ($P = 0.0356$). Similarly, E-cadherin expression and localization are also correlated with local lymph node metastases. Tumors with normal E-cadherin expression and localization were predominantly nonmetastatic with 61.8% $N_o$ whereas 25.6% $N_1$ and 12.6% $N_2$ were observed. The presence of E-cadherin in the cytoplasm is associated with an increase in metastases with proportions of 53.6% $N_o$, 30.3% $N_1$, and 16.1% $N_2$. More than half the tumors with loss of E-cadherin expression metastasize, with only 43.7% being nonmetastatic, whereas 32.2% spread to three or less lymph nodes and 24.1% show infiltration of more than four (Fig. 6D). This increase in lymph node metastases associated with abnormal E-cadherin is statistically significant ($P = 0.0415$). These data show that cytoplasmic p120ctn and cytoplasmic or delocalized E-cadherin in colorectal tumors are indicative of cancer progression as evidenced by its correlation with advanced tumor stage and increased metastases.
An intriguing finding is that the EMT of colon carcinoma is associated with a significant decrease in the activity of RhoA. Intact LIM1863 spheroids possess a relatively high basal activation of RhoA that may reflect the involvement of its GTPase in the maintenance of three-dimensional epithelial structures. The reduction in RhoA activity that we observed during the course of the EMT is coincident with loss of E-cadherin and the cytoplasmic localization of p120ctn. Moreover, the fact that we can detect an association between p120ctn and RhoA by immunoprecipitation suggests an intimate functional relationship between these two proteins. This relationship is supported further by the fact that a reduction in p120ctn expression by RNAi mitigated the decrease in RhoA activity during the EMT. The regulation of RhoA activity by p120ctn was first described by Reynolds and colleagues who suggested that p120ctn may function as a Rho guanine nucleotide dissociation inhibitor, a possibility that has been substantiated in Drosophila embryos in which a complex between p120ctn and RhoA has also been detected (10, 20). This putative function for p120ctn as a Rho guanine nucleotide dissociation inhibitor provides an attractive mechanism for p120ctn to regulate the migration of post-EMT cells, and it is consistent with recent data from our lab that suppression of RhoA facilitates the migration of carcinoma cells (21).

Since the identification of p120ctn as a target of the nonreceptor tyrosine kinase Src, as well as a constituent of cadherin complexes, there has been considerable effort to assess its possible link to cancer progression. A prevailing notion that has emerged is that altered E-cadherin is associated with reduced patient survival time, as well as increased tumor stage and lymph node metastasis. Indeed, the inclusion of nodal metastasis in the multivariate setting suppresses the utility of tumor stage as a significant prognostic marker in a univariate setting, it is not as useful with multivariate analysis. This is most likely due to the tight correlation between p120ctn localization and tumor stage and lymph node status. The association of aberrant p120ctn and E-cadherin with increased tumor stage and lymph node metastases is statistically significant (25), or because of biased grouping of tumors (25). Our conclusion that p120ctn expression is sustained in the majority of colorectal carcinomas and that its cytoplasmic localization is a statistically significant indicator of advanced disease is based on the analysis of an exceptionally large cohort of tumors (557) with detailed clinical information for each. In concert with the functional data we provide on p120ctn, which expand upon previous findings, it seems that p120ctn can be an important determinant of colorectal cancer progression. In a univariate setting, it is not as useful with multivariate analysis. This is most likely due to the tight correlation between p120ctn localization and tumor stage and lymph node status. The inclusion of nodal metastasis in the multivariate setting suppresses the utility of tumor stage as a predictor of patient outcome as well.

Our data analysis revealed a tight correlation between cytoplasmic p120ctn localization and either the loss of E-cadherin expression or its altered localization. This finding is in agreement with smaller scale studies that noted a link between cytoplasmic p120ctn and altered E-cadherin (24, 26). Moreover, we observed that altered E-cadherin is associated with reduced patient survival time, as well as increased tumor stage and lymph node metastasis. Although this latter finding is consistent with the abundant literature on E-cadherin and human carcinomas, definitive data correlating E-cadherin alterations with the clinical end points for colorectal cancer has been lacking. The combined p120ctn and published data in colon carcinoma derive from studies that assessed p120ctn in a relatively small number of tumors, and detailed information on the clinical behavior of these tumors, including patient outcome, was lacking. Moreover, it was common to score p120ctn expression in a manner that was misleading by not considering cytoplasmic localization or by describing such tumors as demonstrating loss of membranous staining (25). In previous studies that did actually identify p120ctn in the cytoplasm of colon cancer cells, it was not possible to determine clinical significance due to the small sample size and lack of patient data (26), or because of biased grouping of tumors (25). Since the identification of p120ctn as a target of the nonreceptor tyrosine kinase Src, as well as a constituent of cadherin complexes, there has been considerable effort to assess its possible link to cancer progression. A prevailing notion that has emerged is that altered E-cadherin is associated with reduced patient survival time, as well as increased tumor stage and lymph node metastasis. Indeed, the inclusion of nodal metastasis in the multivariate setting suppresses the utility of tumor stage as a significant prognostic marker in a univariate setting, it is not as useful with multivariate analysis. This is most likely due to the tight correlation between p120ctn localization and tumor stage and lymph node status. The association of aberrant p120ctn and E-cadherin with increased tumor stage and lymph node metastases is statistically significant (25), or because of biased grouping of tumors (25). Our conclusion that p120ctn expression is sustained in the majority of colorectal carcinomas and that its cytoplasmic localization is a statistically significant indicator of advanced disease is based on the analysis of an exceptionally large cohort of tumors (557) with detailed clinical information for each. In concert with the functional data we provide on p120ctn, which expand upon previous findings, it seems that p120ctn can be an important determinant of colorectal cancer progression. In a univariate setting, it is not as useful with multivariate analysis. This is most likely due to the tight correlation between p120ctn localization and tumor stage and lymph node status. Indeed, the inclusion of nodal metastasis in the multivariate setting suppresses the utility of tumor stage as a predictor of patient outcome as well.

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![Figure 6](image_url)
E-cadherin tumor data, as well as the results from the EMT model, support the hypothesis that loss of junctional E-cadherin results in the cytoplastic localization of p120ctn and its ability to influence tumor cell migration. It should be noted, however, that evidence has been provided recently for the role of p120ctn in maintaining expression of E-cadherin in SW48 colon carcinoma cells (17). The implication of this finding is that tumors with a loss or reduction in p120ctn expression will have a concomitant loss of E-cadherin. Although we did identify tumors with loss of p120ctn and associated alteration in E-cadherin, we were unable to adequately assess any clinical relevance because only a small percentage of tumors in our study exhibited these characteristics (5%; Table 1).

In summary, our findings show that the EMT can be a powerful means of dissecting the mechanisms involved in the genesis of invasive and metastatic carcinomas, and for the identification of molecular markers that predict disease progression and patient mortality.

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References

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