*American Journal of Pathology, Vol. 152, No. 1, January 1998*


# The Expression of p120ctn Protein in Breast Cancer Is Independent of α- and β-Catenin and E-Cadherin

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**Several studies have reported loss or alteration of expression of E-cadherin in breast cancer and more recently changes in levels of expression of the catenins. We used immunofluorescence to examine E-cadherin, α-catenin, β-catenin, and p120ctn (formerly p120CAS) expression in 91 cases of invasive ductal carcinoma. As expected, all four proteins co-localize to the junctional regions of the cells. Although nuclear localization has been described for β-catenin in colonic polyps, no examples were found in these breast cancer cases. We found that, although alteration is common in the catenins and E-cadherin, complete loss, as exemplified by E-cadherin in lobular carcinoma (where E-cadherin is frequently mutated), is rarely seen. In contrast, the catenin-related protein p120ctn shows an expression pattern that is significantly unrelated to the other catenins (or E-cadherin), including complete loss of expression in approximately 10% of the cases. No statistically significant correlations with traditional prognostic indicators were observed with any of these proteins. We conclude 1) that expression of E-cadherin and α- and β-catenin are generally retained at the membrane although frequently reduced or altered, 2) that complete loss of p120ctn expression is seen in approximately 10% of the cases, and 3) that there is a significant correlation in the expression of E-cadherin and the catenins but no correlation between these molecules and p120ctn, suggesting an absence of coordinate regulation.** ***(Am J Pathol 1998, 152:75–82)***

Many studies have been done examining levels of expression of adhesion-associated markers with the goal of identifying an association with metastasis. Epithelial cell-cell adhesion is primarily mediated by E-cadherin (see Ref. 1 for review) and its associated cytoplasmic proteins, the catenins (see Ref. 2 for review). E-cadherin expression is frequently altered in both ductal and lobular carcinomas[3–7] and E-cadherin gene mutations have been detected in lobular carcinoma.[8–10] Alterations in expression of α-catenin[11,12] and also β-catenin and plakoglobin[7,13] have been reported in breast cancer and breast cancer cell lines.[14] Although no correlation with survival has yet been shown for the catenins, one study has recently suggested that E-cadherin alterations may be correlated with decreased disease-free survival.[6] Other studies have shown no correlation.[15–17]

Expression of the catenin-related protein p120ctn (formerly p120CAS) has not yet been examined in human breast tissue. This protein was first discovered as a major substrate of the src tyrosine kinase[18] and several receptor tyrosine kinases, including the epidermal growth factor, colony-stimulating factor-1, and platelet-derived growth factor receptors.[19,20] More recently, p120ctn has been defined as a member of the cadherin-based cell-cell adhesion complex.[21–23] p120ctn contains a series of 42 amino acid *armadillo* repeats, placing it in the *arm* family with the other cadherin-associated proteins β-catenin and plakoglobin.[24] Additional studies showed p120ctn binds directly to E-cadherin, but unlike plakoglobin and β-catenin, it does not bind to either α-catenin or the adenomatous polyposis coli protein.[25] Biochemical studies suggest that p120ctn interacts with E-cadherin at a different site from the β-catenin/plakoglobin binding site.[21] In addition, p120ctn co-precipitates with other members of the classical cadherin family (such as N- and P-cadherin), suggesting that the interaction is broadly applicable to cadherin biology. Although its exact function is still unknown, it may play a role in modulation of adhesion as the association of the tyrosine-phosphorylated p120ctn with E-cadherin is elevated in *ras*-transformed breast epithelial cell lines.[26]

As p120ctn plays a role in modulation of adhesion, it may be another candidate for assessment of the metastatic potential of tumors, as down-regulation of adhesion is a primary event in metastasis.[27,28] Unlike other adhesion proteins where there is loss of expression in tumor cell lines,[14,29] p120ctn shows heterogeneous expression patterns, with isoform variability but, as yet, no evidence of complete loss of expression.[30] Several murine isoforms of p120ctn have been defined,[30] and at least six

Supported by grants from the William and Catherine Weldon Donaghue Foundation for Medical Research and the U.S. Army (DAMD17-94-J4366) to D. L. Rimm and E. R. Fearon and NIH grant (CA-55724) to A. B. Reynolds.

Accepted for publication October 24, 1997.

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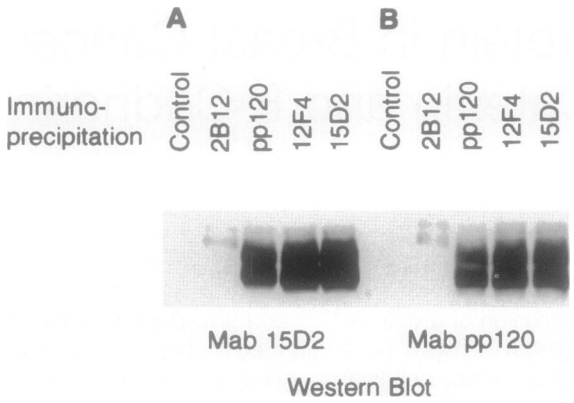


**Figure 1.** Specificity of p120ctn-specific MAbs. MDCK cell lysates were immunoprecipitated using the control MAb 12CA5 (control) or the p120ctn-specific MAbs listed across the top. Immunoprecipitates were separated on 8% polyacrylamide gels and then Western blotted with either MAb 15D2 (**A**) or MAb pp120 (Transduction Labs; **B**).

human isoforms are currently in the process of characterization (Frans Van Roy, personal communication). This isoform diversity is more complex than other cadherin-associated proteins of the adhesion complex, but alternative splice forms have been described for both β-catenin[31] and α-catenin.[32]

In this study we compared expression of p120ctn to E-cadherin and the conventional catenins. Tissue sections were labeled with Cy3-conjugated fluorescent antibodies rather than conventional enzyme-conjugated techniques to increase the signal-to-noise ratio to the point at which loss can be reliably distinguished from alteration, or decreased intensity, as is frequently seen for these adhesion-related proteins. To calibrate our assay, we use 10 cases of lobular carcinoma as a reference for true loss of E-cadherin expression. Confirming the more recent literature, we found alteration of E-cadherin and α- and β-catenin common but true loss extremely rare. This pattern is different from that seen for p120ctn where there is true loss in approximately 10% of the cases (as defined by loss of reactivity of multiple monoclonal antibodies). Furthermore, we find co-localization but no correlation in level of expression of p120ctn and the other adhesion proteins examined, suggesting independent regulation.

## *Materials and Methods*

### *Tissue Acquisition and Study Population*

The cohort included 91 patients who have had breast resections at Yale-New Haven Hospital for invasive ductal breast cancer. Approximately one-half of them presented with metastases to regional lymph nodes. The age at diagnosis ranged between 26 and 88 (average, 58.1) years, and survival time ranged from 39 days to 14.1 years (average, 5.4 years; median, 5.1 years). The cohort showed an 88% 10-year survival of node-negative cases and 45% 10-year survival of node-positive cases, which compares well with the literature[33] and suggests that this

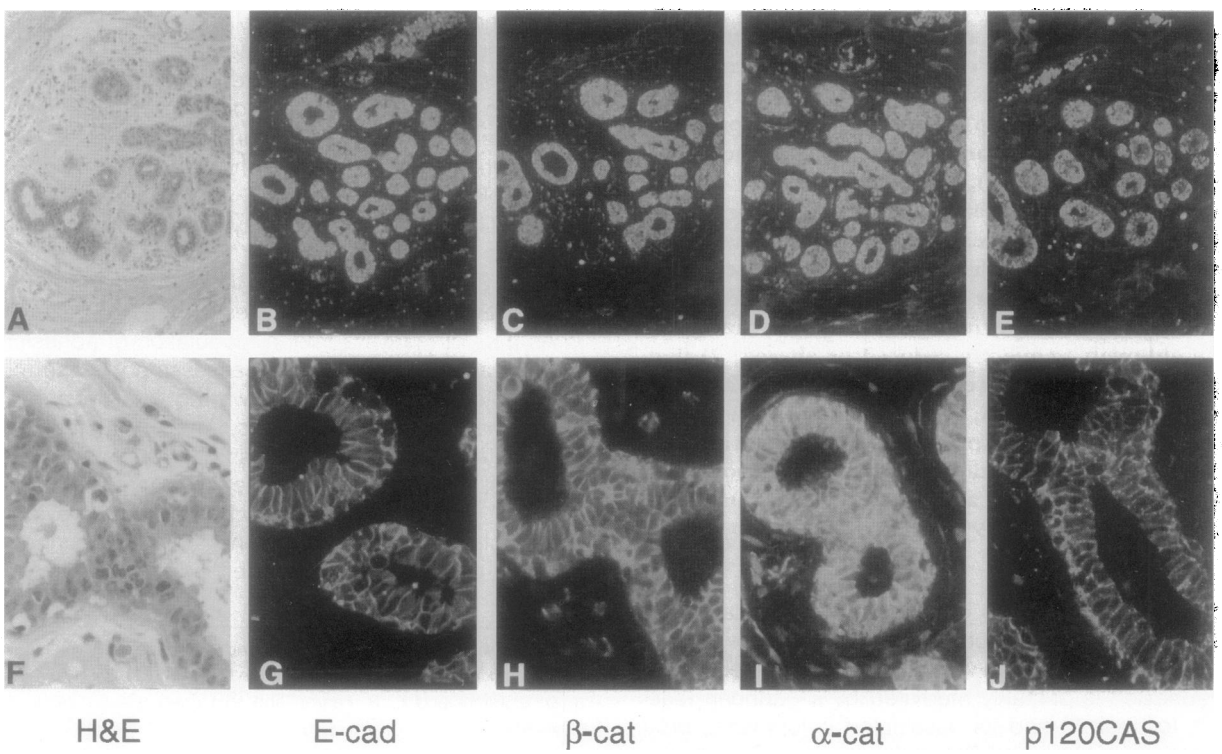


**Figure 2.** Normal staining patterns of all four proteins. The adhesion proteins E-cadherin, α-catenin, and β-catenin show classic basolateral membrane staining in normal breast ducts and lobules. As described in other tissues, the localization of p120ctn is essentially indistinguishable from E-cadherin and the catenins. Stains are as described in Materials and Methods and shown at low (×20 original magnification **A** to **E**) and high (×80; **F** to **J**) power. Stains, shown beneath each column, are H&E (**A** and **F**), E-cadherin (**B** and **G**), β-catenin (**C** and **H**), α-catenin (**D** and **I**), and p120ctn (**E** and **J**).

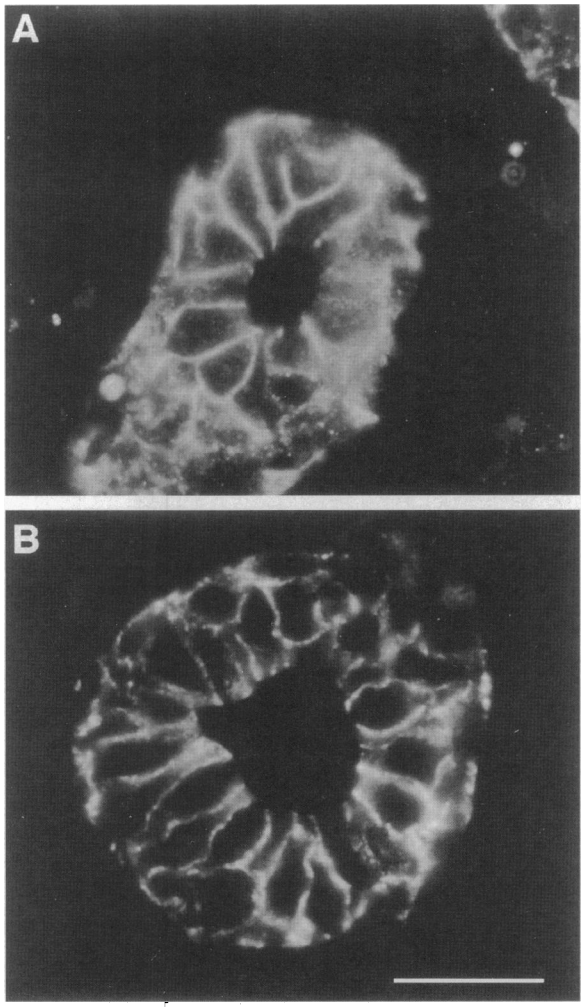


**Figure 3.** The subcellular localization of p120ctn is very similar to that seen for E-cadherin. High magnification views (×100 original magnification) shows bright membranous staining on the basolateral surfaces of the epithelial cells lining the small ducts for both E-cadherin (**A**) and p120ctn (**B**). Scale bar in **B**, 20 μm.

is a representative population. Clinicopathological parameters collected on each case included age, tumor size, tumor histological grade, estrogen and progesterone receptor status, lymph node involvement, and survival. This population is ethnically and racially diverse although, due to the nature of the disease, includes only females. All material was collected under the auspices of the Critical Technologies Program at Yale and in accordance with human investigation committee protocol 8219 to the principal investigator (D. L. Rimm). Ten cases of lobular carcinoma were also selected at random to use as controls for loss of cadherin staining. No clinical information was obtained on this group.

## *Antibody Preparation*

Recombinant fusion proteins were prepared from full-length human cDNA clones for both α-catenin and β-catenin by expression in glutathione-S-transferase (GST)-based expression vectors (Pharmacia, Piscataway, NJ). Each was purified on a glutathione affinity matrix, and antisera were raised in rabbits by injection in complete Freund's adjuvant. Antibodies were affinity purified in two steps. Anti-GST activity was depleted by passage over a column of Affi-gel linked to GST. The eluent was subsequently passed over an Affi-gel column with bound α-catenin or β-catenin. After washing, antibodies were eluted with 100 mmol/L glycine/HCl, pH 2.5. Fractions containing active antibodies as detected by enzyme-linked immunosorbent assay (ELISA) were pooled, dialyzed into phosphate-buffered saline containing 1 mmol/L sodium azide, and stored at −20°C. A commercial monoclonal antibody (MAb) to E-cadherin was used (Transduction Laboratories, Lexington, KY).

The p120ctn-specific MAbs 12F4 and 15D2 were chosen for their excellent reactivity with formaldehyde-fixed tissue sections from a panel of p120ctn-specific MAbs prepared previously (Wu and Reynolds, manuscript in preparation). These antibodies bind to different epitopes in the carboxyl-terminal 121 amino acids of p120ctn and recognize all known isoforms of p120ctn that can be distinguished by immunoprecipitation and Western blotting analysis. Both MAbs were affinity purified on protein-A Sepharose columns. For immunostaining experiments, MAbs 12F4 and 15D2 were used at 7 μg/ml and 2 μg/ml, respectively. Other p120ctn MAbs, including 9D5, 5A7, 6H11, 8D11, 9B8 (Wu and Reynolds, manuscript in preparation), and pp120 (Transduction Laboratories), were used on some cases for confirmation of the staining pattern.

## *Immunostaining*

Standard histological sections were cut from paraffin blocks and prepared for immunostaining using a pressure cooker antigen retrieval method.[34] Each section was baked at 60°C overnight and then deparaffinized and treated for antigen retrieval by immersion in 6.5 mmol/L sodium citrate (pH 6.0) for 5 minutes in a conventional pressure cooker (KMart). Sections were then blocked with 0.3% bovine serum albumin (BSA) in Tris-buffered saline (TBS) (150 mmol/L NaCl, 20 mmol/L Tris, pH 8). MAbs were diluted to 2 to 7 μg/ml and incubated in a humidity chamber overnight before washing seven times with TBS including 0.01% Triton X-100 in the sixth wash. For signal-to-noise ratio and better subcellular localization, Cy3-conjugated second antibodies were used instead of the conventional enzymatic reaction-based chromogens. Cy3-goat anti-mouse or rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) was diluted 1:500 in TBS with 0.3% BSA and placed in the sections for 1 hour before washing as above and coverslipping. Slides were stored at −20°C to maintain the fluorescent signal, which appears to be stable for over 1 year under these conditions.

## *Histological scoring and analysis*

Each slide was examined on at least two separate occasions by at least two individuals including two patholo-

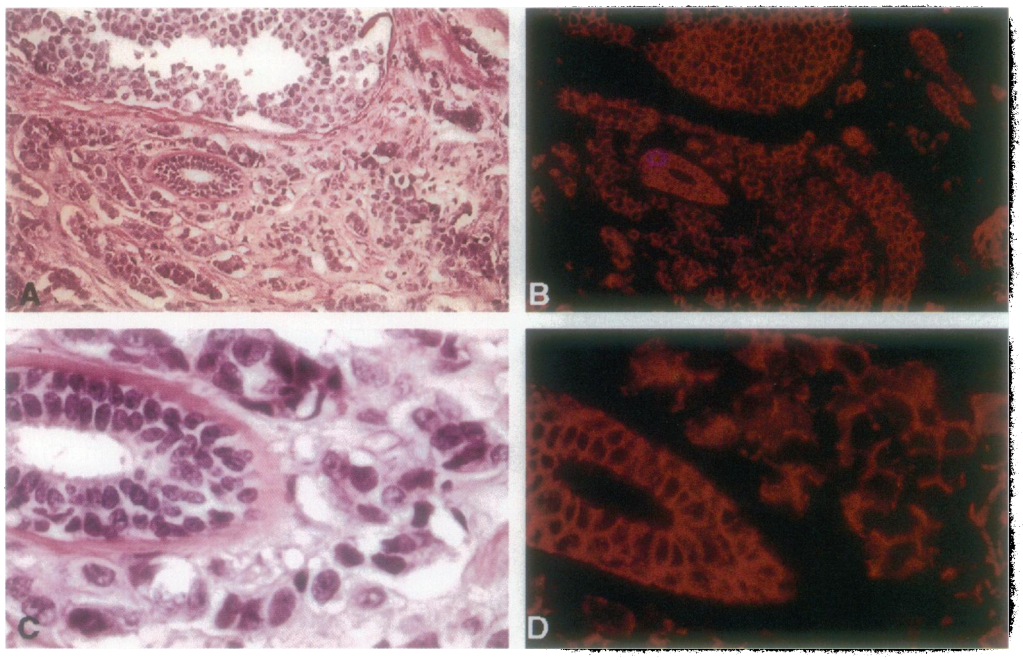


**Figure 4.** Example of altered staining of α-catenin in the tumor surrounding a normal duct. H&E stains (**A** and **C**) at ×20 and ×80, respectively, show a normal duct surrounded by a poorly differentiated ductal carcinoma. The staining pattern showing α-catenin expression (**B** and **D**) at similar magnifications shows normal staining surrounded by a pattern designated altered but not lost.

gists and a technologist using either a Zeiss epifluorescence microscope or an Olympus AX-70 epifluorescence photomicroscope. The expression of each antigen was scored as one of three final groupings. The final grouping used were 1) normal, cases not reproducibly distinguishable from normal, in either pattern or intensity; 2) altered, a broken or discontinuous staining pattern, or a patchy staining pattern, with or without a decrease in intensity; and 3) loss, a complete loss of staining as the predominant pattern in the section examined, comparable to that seen for E-cadherin in cases of lobular carcinoma, where E-cadherin is frequently mutated. In each case, a serial hematoxylin and eosin (H&E) section was examined for orientation and confirmation of the histological diagnosis. Each case was scored blindly with respect to patient history, presentation, and previous scoring. Patient follow-up information was obtained from Dr. Diane Fisher in conjunction with the Yale Comprehensive Cancer Center and the Connecticut Tumor Registry. Data analysis was done using StatView 4.5 for Macintosh.

## Results

The specificities of p120ctn MAbs 12F4 and 15D2 were compared by immunoprecipitation and Western blotting (from MDCK cell lysates) with the previously characterized MAbs 2B12 and pp120 (from Transduction Laboratories) (Figure 1). MAb 2B12 specifically recognized the so-called CAS1 isoforms due to the location of its epitope in the amino-terminal 100 amino acids of p120ctn.[21,30] This amino-terminal sequence is spliced out in many cell types, resulting in the faster migrating CAS2 isoforms, which are more abundant than CAS1 isoforms in this cell type. Both CAS1 and CAS2 isoforms were recognized by MAbs 12F4 and 15D2, and their staining patterns were similar to those of MAb pp120. In addition to the similar immunoprecipitation and Western blotting patterns illustrated here, the properties of MAb 15D2 are nearly identical to that of MAb pp120 in every parameter tested to date, including immunofluorescence, species cross-reactivity, and isoform specificity. MAb 12F4 was chosen for the bulk of the experiments as it showed the best signal-to-noise characteristics in formaldehyde-fixed tissues. A selection of 30 cases were examined with 15D2 to confirm the staining pattern. Cases showing true loss of expression were re-examined with multiple MAbs to confirm the loss of expression.

The adhesion protein p120ctn is associated with E-cadherin and shows some homology to β-catenin, but its role in stabilization or regulation of adhesion has yet to be defined. Like the catenins and cadherins, the protein localizes to lateral membranes. In breast tissue, it co-localizes exactly with E-cadherin and α- and β-catenin (Figure 2). At the resolution of immunofluorescence, the distribution of expression is predominantly basolateral and membranous with pale cytoplasmic expression (Figure 3). In some ductal cells there are increased membrane densities near the apex, presumably correlating with the location of the adherens junction. This finding is more prominent in colonic epithelium than in breast tissue.

Alteration of adhesion molecules in breast cancer has been described frequently in the literature.[35] Alteration or discontinuity of staining is grouped with loss in some studies, but that grouping may overlook a biological distinction. We have observed numerous cases where there are discontinuous or altered staining patterns, frequently with reduced intensity of adhesion protein staining, but

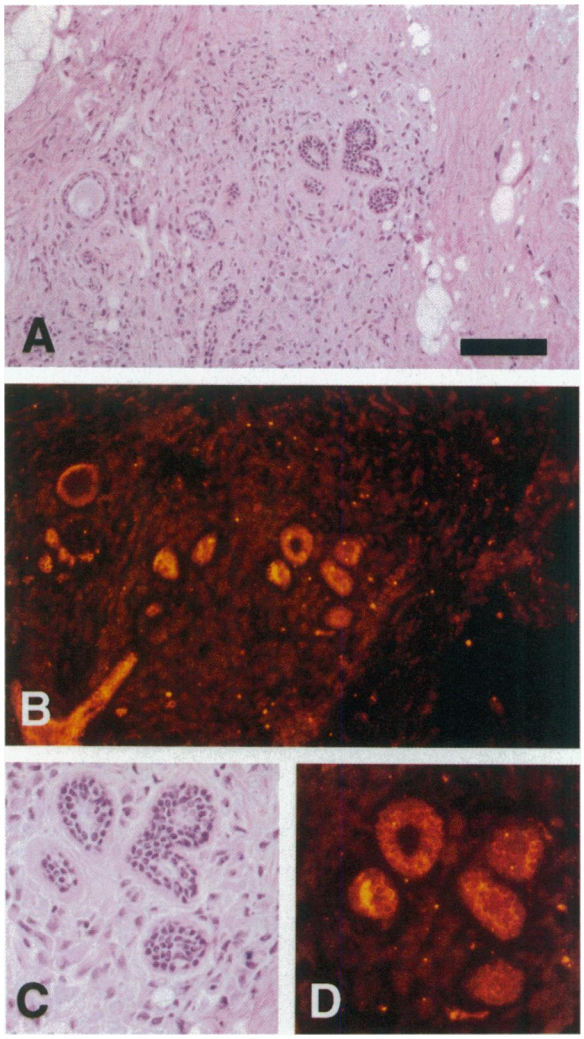


**Figure 5.** E-cadherin staining pattern in lobular carcinoma as an example of true loss of expression. Matched serial sections stained with H&E (**A** and **C**) and anti-E-cadherin visualized with Cy3-conjugated secondary antibodies (**B** and **D**) show low- and high-magnification views of normal small ducts surrounded by malignant cells of a lobular carcinoma. The immunofluorescent frames (**B** and **D**) are overexposed to show shadows of the malignant cells with complete absence of E-cadherin expression. Scale bar in **A**, 100 μm.

true loss is rare. In this study, we define a category called altered for all cases with a staining pattern that is reproducibly and easily distinguished from normal but does not meet the criteria for true loss of expression. Generally, normal ducts are present in these cases and provide excellent internal calibration by which to make the judgment of alteration or loss. An example of alteration is shown in Figure 4, where tumor surrounds a normal duct. The staining pattern, defined as altered, shows broken and discontinuous but predominantly membranous staining. In this case the discontinuous α-catenin staining pattern is contrasted with the normal staining in the adjacent duct. The intensity may be reduced compared with normal ducts but is still easily seen. No attempt was made to quantify intensity.

True loss of expression is defined as a complete absence of antibody reactivity, similar to that seen in the absence of the primary antibody. As a reference for this pattern we examined expression of E-cadherin in 10 lobular carcinoma specimens as mutations in E-cadherin occur in greater than 50% of the cases.[9] We found 7 of 10 selected cases showed complete loss of expression of E-cadherin, as illustrated in Figure 5. Again, in each case enveloped normal ducts were used as an internal reference for normal antibody reactivity. Complete loss in the lobular carcinoma cells shows a staining pattern and intensity indistinguishable from the negative control.

Using these criteria for expression, we examined over 100 cases of ductal carcinoma of the breast. Each was scored on the basis of the consensus of at least two individuals. The distribution of expression pattern is shown in Figure 6. It is notable that complete loss of expression was never seen for α- or β-catenin and seen in only one case for E-cadherin. All cases showed membranous localization. Nuclear staining of β-catenin as reported in colonic polyps[36] was not seen in any case. In contrast, approximately 10% of the cases showed loss of p120ctn. To examine correlation of level of expression between proteins as a first assessment of coordinate regulation, the cases were analyzed using the $\chi^2$ test. We found highly significant correlations between E-cadherin and α- and β-catenin but no correlation between p120ctn expression level and any of these adhesion proteins (Table 1); that is, frequently cases with normal E-cadherin and catenins showed altered p120ctn or *vice versa*.

The lack of correlation between p120ctn expression levels and the other proteins led to re-examination of these cases. True loss, as seen in 10% of the cases, was similar to loss of E-cadherin expression observed in lobular carcinoma. Normal-staining benign ducts were seen surrounded by nests of tumor cells with complete absence of p120ctn reactivity (Figure 7). These cases were recut for examination with other MAbs to confirm loss. The MAbs, including 5A7, 6H11, 8D11, and 9B8, showed a pattern of loss indistinguishable from 12F4. The 15D2 antibody showed a pattern of high background, with cytoplasmic staining but no true membranous staining as seen in the adjacent non-neoplastic ducts. A similar pattern was seen with 9D5 and in some cases with the pp120 antibody from Transduction Laboratories.

Finally, attempts were made to correlate alterations in expression with other known prognostic markers in breast cancer, including lymph node status, tumor size, steroid receptor expression, and age as well as overall survival. Although there were some general trends sug-

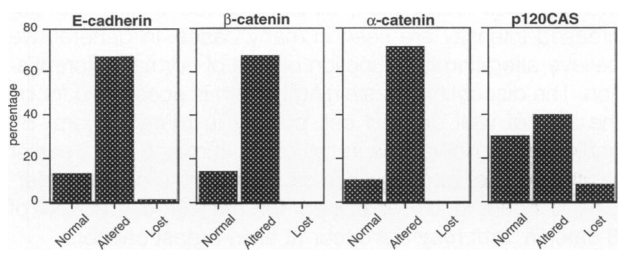


**Figure 6.** Distribution of expression of each antigen shows a different pattern for p120ctn as compared with the other adhesion molecules.

**Table 1.** High Correlation of Expression Levels Is Seen between E-Cadherin, α-Catenin, and β-Catenin but Not p120ctn

| | E-cadherin | α-Catenin | β-Catenin | p120ctn |
|---|---|---|---|---|
| E-cadherin | | 46.3 ($P < 0.0001$) | 29.2 ($P < 0.0001$) | 0.36 ($P = 0.547$) |
| α-catenin | 46.3 ($P < 0.0001$) | | 42.2 ($P < 0.0001$) | 0.68 ($P = 0.408$) |
| β-catenin | 29.2 ($P < 0.0001$) | 42.2 ($P < 0.0001$) | | 0.04 ($P = 0.835$) |
| p120ctn | 0.36 ($P = 0.547$) | 0.68 ($P = 0.408$) | 0.04 ($P = 0.835$) | |

Each cell shows the $\chi^2$ value and the corresponding $P$ value in parentheses.

gesting an association between alteration and poor prognostic factors, no statistically significant associations were found. Similarly, alteration of expression of any of these proteins was not associated with decreased survival. As only p120ctn showed complete loss of expression, we compared this with a combined group of altered and normal expression. We again found no correlation with conventional prognostic markers. As only eight patients fell into this group, it was difficult to meaningfully address survival.

## Discussion

Although this is one of the first studies assessing expression of p120ctn in tumors, many studies have examined the other adhesion molecules. Our findings with respect to those studies generally confirm that alteration of expression is a frequent finding. We find 1) alteration of expression is common in invasive carcinoma, but membranous localization is retained, even for β-catenin, in all cases; 2) true loss of expression of E-cadherin, α-catenin, and β-catenin is very rare in ductal carcinoma of the breast; 3) the pattern of expression of E-cadherin, α-catenin, and β-catenin are highly correlated within each case, but do not correlate with expression of p120ctn; 4) there appears to be complete loss of p120ctn in approximately 10% of the cases, although we do not yet know whether this is a function of genetic mutation or down-regulation; and 5) there are general trends correlating alteration of expression of these proteins with traditional poor prognostic markers, but none are statistically significant or independently predictive.

Although use of immunoperoxidase is an accepted standard approach, by using immunofluorescence we attempted to achieve an increased signal-to-noise ratio and more specific subcellular localization. As the role of β-catenin in signal transduction has become better defined,[37] localization to the nucleus is suggested and has been described in colonic polyps.[36] In invasive ductal carcinoma, nuclear localization is not seen; instead, each case shows membranous localization, although discontinuity and decreased intensity are seen in many cases. In general, we believe alteration is a function of loss of normal differentiation. The discontinuous staining pattern is accounted for by the loss of well defined cell polarity in many tumors. By analogy to observations in the colon, it may be that earlier lesions (ductal carcinoma *in situ* or atypical ductal hyperplasia) need to be examined for nuclear expression of β-catenin, or it may not occur at all in breast cancers.

Our finding that true loss of expression is rare or absent in ductal carcinoma has been observed by others.[4,9] Although loss of expression is reported in the literature, it is often grouped with decreased expression,[5,16] possibly because the methods used are unable to distinguish reduction from true loss.[6] Molecular analysis to detect mutations in these genes also suggests that loss due to mutation will be rare as mutations in the E-cadherin gene

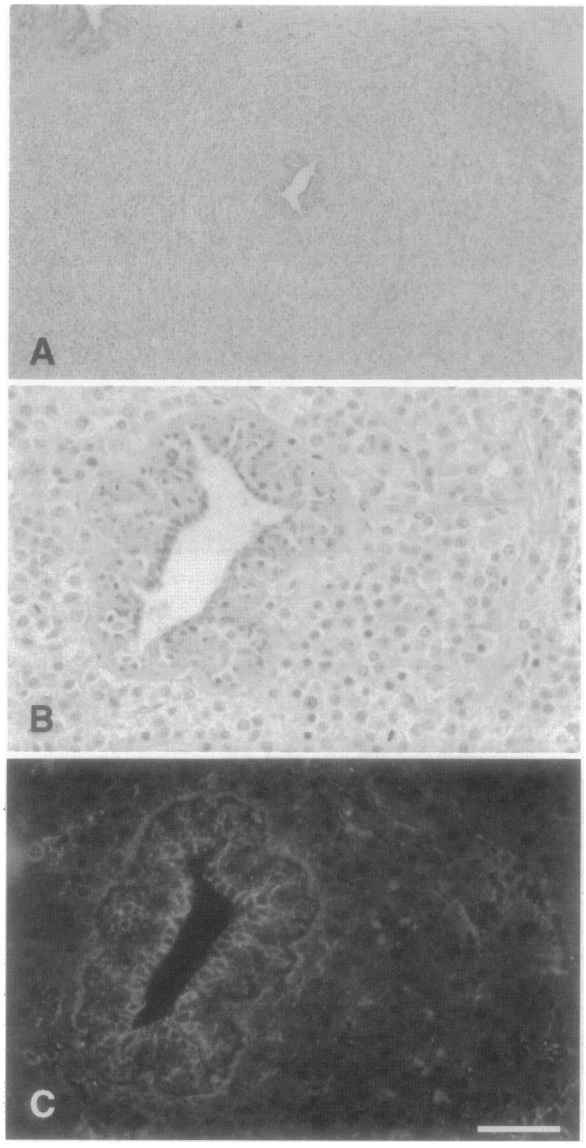


**Figure 7.** An example of p120ctn staining showing true loss of expression. H&E sections (**A** and **B**) show low (×20) and high (×80) magnification views of a small duct lined by benign epithelium and surrounded by the cells of an invasive ductal carcinoma. **C** shows a complete absence of expression of p120ctn in a serial section, with good membranous staining of the entrapped normal duct. Scale bar in **C**, 50 μm.

have never been found in ductal carcinoma.[9] The catenins are less well studied, although preliminary evidence suggests that their loss due to mutation will also be rare in human tumors,[10] even though loss of expression has been described in a number of cell lines.[14]

In a previous study using a panel of breast cancer cell lines, there were no examples of loss of expression of p120ctn.[30] In contrast, this study shows some clear examples of complete loss of p120ctn in breast cancer tissue sections. One explanation of this finding could be an isoform switch resulting in loss of many epitopes. This seems unlikely because previous studies indicate that MAbs 15D2 and 12F4 recognize all known isoforms of p120ctn.[30] An alternative explanation is that the biological processes that are occurring in the tumors with loss of expression represent a stage in the multi-step process of carcinogenesis that is not recapitulated by any cell line yet tested; it is possible that cell lines will eventually be found that show complete loss of p120ctn.

The expression pattern and potential loss of p120ctn has not been previously studied in human breast cancer tissues. Decreased levels of p120ctn expression have been reported in colonic carcinoma[38] (preliminary data in our laboratory), bladder cancer,[39] and more recently in adenomatous polyps.[36] Our study of breast tissue shows reduction or alteration, similar to the colonic cases, but also true loss of expression is seen in a small percentage of the cases. It is unknown whether this loss represents mutation or simply transiently extinguished expression. The fact that some of our cases show areas of loss and other areas with reduction or alteration suggests that the absence of expression may be a result of down-regulation. As the factors that regulate expression of p120ctn are not well understood, we cannot test these cases to distinguish these possibilities. However, consistent with the study in bladder cancers[39] and studies on transformed L-cell lines,[40] it appears that p120ctn is not coordinately regulated with E-cadherin and $\alpha$- and $\beta$-catenin. Future studies will attempt to address the possibility of down-regulation *versus* mutation in these cases.

## Acknowledgments

This work is dedicated to patients with breast cancer and their families, including relatives of an author (D. L. Rimm), Ruthy and Robin Brown, Barbara Rimm, and Mary Ruth Reitz.

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