Cadherins as Predictive Markers of Nodal Metastasis in Breast Cancer

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Adhesion molecules, particularly cadherins play a pivotal role in cancer invasion and metastasis. Because the therapeutic management of tumors with and without nodal metastasis differs considerably, our idea was to identify tumors with metastatic potential. We studied the expression of E-cadherin and P-cadherin immunohistochemically in 51 cases of breast cancer that included 29 node-negative and 22 node-positive cases. Expression of the cadherins was mainly membranous, with cytoplasmic staining in a few lesions. Both E-cadherin and P-cadherin showed significant down-regulation of their expression in node-positive tumors in comparison to node-negative tumors. Logistic regression analysis revealed that the positive expression of E-cadherin and P-cadherin showed low odds ratios of 0.1 and 0.2, respectively, and were statistically significant. On multivariate analysis, both the cadherins were found to be of independent prognostic value. This suggests that cadherin expression could be a marker of nodal metastasis. An observation of interest was that the expression of E-cadherin and P-cadherin were highly correlated (correlation coefficient = 0.5873), which requires further evaluation for confirmation of a common regulatory pathway that could be activated in the early onset of nodal metastasis.

KEY WORDS: E-cadherin, Immunohistochemistry Nodal metastasis, P-cadherin.

Metastasis poses the most serious threat to clinicians in the management of breast cancers. The problem attains even greater dimension because of the different therapeutic modalities adopted in cases with and without nodal metastasis. The process of metastasis, either locoregionally into lymph nodes or systemically into distant organs, remains intriguing, despite various studies in this field. As an initial step in metastasis, tumor cells should detach from the primary site and enter the circulation. This is thought to be due to changes in the cell–cell adhesive properties. Hence, adhesion molecules are supposed to play an important part in cancer invasion.

Cadherins deserve particular attention among the recently identified group of invasion suppressor genes (1). Cadherins are Ca^{2+}-dependent adhesion molecules that mediate homophilic cell–cell adhesion (2). The cadherin gene family encompasses E-cadherin, P-cadherin, and *N*-cadherin. A number of studies have found an inverse correlation between the expression of E-cadherin and the tumor stage in carcinomas of breast (3) and prostate (4), squamous cell carcinoma of head and neck (5), and so on. Graff *et al.* (6) found that the reduced expression of E-cadherin is due to hypermethylation of the CpG island of the promoter region of the E-cadherin gene. In the present study, we attempted to correlate the expression of cadherins with nodal metastasis in carcinomas of the breast.

MATERIALS AND METHODS

Patients and Tissue Specimens

Formalin-fixed, paraffin-embedded blocks of 51 randomly selected tumor specimens from primary breast cancer patients previously classified as node negative or node positive were retrieved from the files. The study group included 29 node-negative and 22 node-positive patients. The medical records of these patients were reviewed, and clinical details were collected.

Antibodies

Goat polyclonal antibodies against E-cadherin (N-20) and P-cadherin (N-19) of Santa Cruz Biotechnology (Santa Cruz, CA) were used at 1:20 dilution. All the dilutions were done in $1\times$ phosphate buffered saline (PBS).

Immunohistochemistry

The standard avidin-biotin indirect immunoperoxidase method (Santa Cruz Biotechnology kit) was used for immunohistochemistry. Briefly, 4-μm sections were cut from the paraffin embedded blocks using a microtome. The glass slides were previously coated with poly-L-lysine. The sections were then incubated at 37°C overnight. Thereafter, the sections were deparaffinised in xylene (30 minutes, twice), sequentially dehydrated by incubating in 1:1 xylene-alcohol mixture, 100% alcohol, 90% alcohol, 70% alcohol, 50% alcohol, 30% alcohol and $1\times$ PBS (10 minutes each). Antigen unmasking by autoclaving was done in 0.01 M citrate buffer (pH 6.0) for 5 min. After cooling and washing, the endogenous peroxidase of erythrocytes was blocked by incubating in 30% H_2O_2 for 30 minutes to prevent nonspecific binding of antibodies, the sections were incubated with 3% BSA for 30 minutes. Thence, the sections were incubated overnight with the primary antibodies, washed, incubated with biotinylated secondary antibodies (30 minutes), washed, and incubated with avidin-peroxidase complex (30 minutes). The peroxidase reaction was visualized by incubating with AEC for 10 minutes in the dark. All washings were done in $1\times$ PBS.

Evaluation of Immunostaining

Sections were examined under a light microscope. The staining pattern was assessed without the prior knowledge of histopathological diagnosis. The normal breast tissues adjacent to the tumor areas served as an internal control. The scoring was as follows: negative, 1; mild positive, 2; moderate positive, 3; intense positive, 4. The staining pattern was evaluated by an independent investigator (PB) and confirmed in consultation with the pathologist (EA). The staining score was expressed as mean value $\pm$ SE. The staining pattern in both E-cadherin and P-cadherin were almost similar and is shown in Figure 1, A-D.

Statistical Analysis

Association between the antigen expression and nodal status was evaluated by rank correlation using Mann-Whitney U Wilcoxon Rank Sum test for two independent samples. Using a logistic regression model, multivariate analysis was done, and odds ratios were estimated to assess whether cadherins could serve as a marker for developing node positivity. P values $<.05$ were considered statistically significant.

RESULTS

E-cadherin Expression

The expression of E-cadherin was localized uniformly to the cell membrane with intense staining at the intercellular junctions. A few samples showed loss of strictly membranous expression pattern but showed cytoplasmic expression. There was a significant difference in the expression of E-cadherin between those tumors with and without nodal metastases ($P = .009$; Table 1, Table 2). The expression of E-cadherin was significantly downregulated in higher grade tumors ($P = .028$; Table 3). The odds ratio was 0.1 for E-cadherin expression in node-positive patients compared with unity in node-negative patients, calculated at 95% confidence interval ($P = .001$; Table 4).

P-Cadherin Expression

The staining pattern of P-cadherin was almost similar to that of E-cadherin, with localization of expression to cell membrane. There was a significant down-regulation of P-cadherin in node-positive breast cancers, with a P value of .008 (Table 3). Though not significantly, the expression of P-cadherin was higher in low-grade tumors in comparison to high-grade tumors (Tables 1 and 2). The odds ratio was 0.2 for P-cadherin-positive patients, whereas it was unity for P-cadherin-negative patients ($P = .05$) (Table 4).

Using a logistic regression model, multivariate analysis was done using all the classical variables (stage, grade, tumor type, proliferation rate assessed by PCNA staining, menstrual status) and the cadherins. Of these variables, excluding tumor type and grade of tumor, all the other variables were found to be independent prognostic predictors of nodal positivity.

Looking at the bivariate correlations, we find that apart from the cadherins being significantly correlated to the nodal status, E-cadherin and P-cadherin also show a significant bivariate correlation between them (Table 3). On the whole, in 47% of the cases, both the cadherins were reduced or absent, whereas in 37% of the cases, both of them were expressed.

DISCUSSION

Cadherins have been proposed to play an important role in activating the mechanism of tumor dissemination from the primary site. They are an important member of the recently identified group
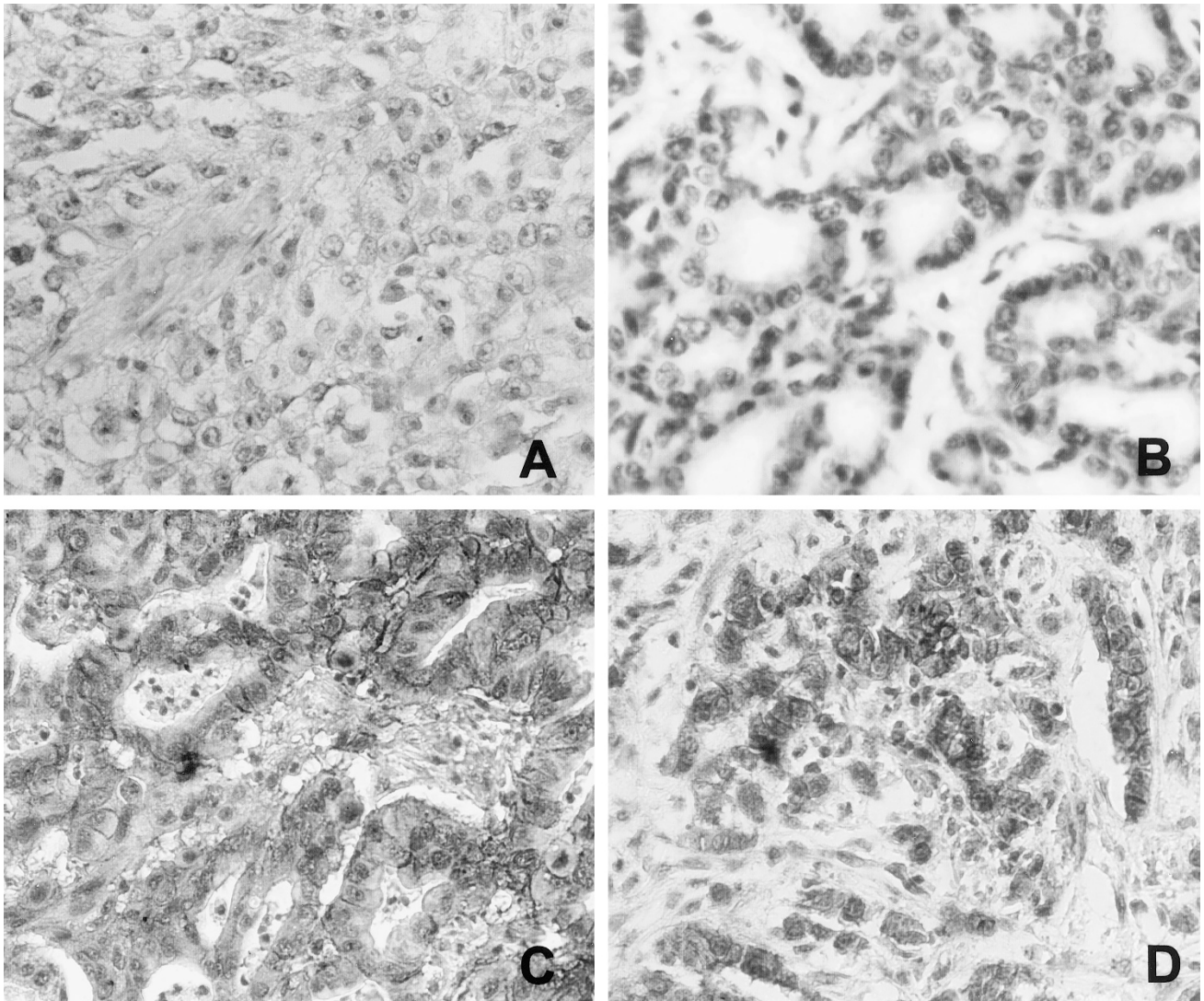
FIGURE 1. Microphotograph of breast cancer tissue showing staining pattern of cadherins (E-cadherin and P-cadherin). (**A**) Negative staining. (**B**) Mild membranous staining. (**C**) Moderate membranous staining. (**D**) Intense membranous staining.

TABLE 1. Expression of E-Cadherin and P-Cadherin in Breast Cancer

Protein	Mean Score ± SE		P Value
	Node −ve	Node +ve	
E-cadherin	28966 ± 0.167	1.2273 ± 0.091	.009
P-cadherin	2.6897 ± 0.173	1.4545 ± 0.171	.364

of metastasis suppressor genes. In the present study, we used immunohistochemistry because it facilitates the identification of the protein localization and a semiquantitation of the protein. The results of the study demonstrate that the expression of cadherins is lower in carcinomas of the breast that are node positive than in those that are node negative. E-cadherin has been used as an immuno-histochemical tool for the diagnosis and prognosis of various carcinomas (7). Umbas *et al.* (8) reported a decreased expression of E-cadherin as indicative of a poor clinical course of the disease. The transi-tion from well-differentiated adenoma to invasive carcinoma in mouse pancreatic β-cell carcinogen-esis has been found to be due to loss of E-cadherin expression (9). The potential for E-cadherin to function as an invasion suppressor in epithelial tu-morigenesis has been supported by *in vitro* studies. E-cadherin-negative epithelial cells grow invasive with a mesenchymal phenotype. After transfection with E-cadherin cDNA, epithelial structure is re-stored (10). The loss of membranous staining pat-tern in some of our samples could mean the pres-ence of a nonfunctional E-cadherin, as suggested by Gagliardi et al. (11). Diminution of cadherin expression is postulated to remove the regulatory control of contact inhibition on proliferation, thus allowing escape from growth control signals (12). Pece *et al.* (13) have shown that E-cadherin can initiate an outside-in signal-transducing pathway that regulates the activity of phosphoinositide-3-kinase and Akt, thus providing a novel mechanism

TABLE 2. Distribution of Staining Intensity of Cadherins According to Nodal Status

Nodal Status	Distribution of Staining Intensity (%)							
	E-cadherin				P-cadherin			
	Negative	Mild	Mode rate	Intense	Negative	Mild	Mode rate	Intense
Negative	17.4	13	56.5	13	17.4	17.4	34.8	30.4
Positive	50	29.2	20.8	0	48	32	8	12

TABLE 3. Relation of Cadherin Expression with Nodal Status and Grade of Tumor

Variables	Rank Correlation Coefficient	P Value
E-cadherin/node	−0.5436	.001
P-cadherin/node	−0.3701	.008
E-cadherin/grade	−0.3670	.028
P-cadherin/grade	−0.2301	.177
E-cadherin/P-cadherin	0.5873	.001

TABLE 4. Analysis of Cadherins and Other Variables as Risk Factors for Node Positivity

Variable	Regression Coefficient	Odds Ratio	P Value
Stage	1.4267	4.1648	.001
Proliferation rate	1.7578	5.7998	.006
Menstrual status	0.6951	2.000	.040
E-cadherin	−2.2254	0.1000	.001
P-cadherin	−1.6864	0.2000	.001

whereby the intercellular adhesion may control the fate of the epithelial cells. This study shows a down-regulation of the cadherins in node-positive tumors, and at multivariate analysis, cadherins were found to be of independent prognostic value in predicting the nodal status. We found that alterations in the expression of cadherins offer a useful adjunct to current prognostic indicators. This is also evidence for the significant inverse correlation of E-cadherin with grade of the tumor. Thus, measurement of cadherin expression, in concert with other markers of differentiation, may provide an additional prognostic tool.

What leads to down-regulation of cadherins upon metastasis, be it locoregional or distant, is still not clearly defined. It is proven that calcium influx-induced proteolysis of E-cadherin not only disrupts the cell–cell adhesion but also activates intracellular signaling pathway, potentially leading to alterations in motility and proliferation activity of cells. Calcium influx activates a membrane-bound metalloprotease that induces degradation of the ectodomain of E-cadherin (14). Graff *et al.* (15) reported that the cadherin that was downregulated in the initial stage of metastasis should get reexpressed in the metastatic deposits.

Analysis of odds ratio of the cadherins showed that their down-regulation can serve as predictive markers of nodal metastasis in breast cancers. Taken together, the cadherins could be better indi-

cators of nodal metastasis. This suggests a crucial role for the expression of cadherins in demarcating node-positive from node-negative cases. This is very significant in patients presenting with a primary tumor and histologically undiagnosable micrometastasis. At the same time, subjecting node-negative patients with palpable nodes to higher courses of treatment can be avoided. We suggest that development and use of cadherins as diagnostic markers could solve this problem and could be an additional tool for physicians in the effective therapeutic management of such tumors.

Another important result is a significant correlation between the cadherins. This could mean a common regulatory pathway for the cadherins that gets activated upon initiation of metastasis. This putative pathway could be crucial in deciding the metastatic fate of a tumor. Elucidation of this pathway may help in the therapeutic design of anti-metastatic drugs. Further investigations are necessary to examine the molecular mechanism underlying the regulation of cadherins.

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