

M34 Actin Regulatory Protein Is a Sensitive Diagnostic Marker for Early- and Late-Stage Mammary Carcinomas

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ABSTRACT

Purpose: At present, there is no available molecular marker that reliably detects the earliest stages of epithelial transformation in the majority of patients affected with incipient breast carcinoma. Here we introduce M34 protein, a mammalian actin filament regulatory protein, as a highly sensitive and easily detected positive cellular marker for both early and late stages of breast carcinoma.

Experimental Design: In this study, 24 human lactation duct neoplasms from postmenopausal women, including fibroadenoma, ductal carcinoma *in situ*, intraductal lobular papilloma, and metastatic adenocarcinoma, were analyzed for the presence of M34 protein by histochemical staining of paraffin and fresh-frozen sections.

Results: All 24 neoplasias tested positive for M34, whereas none of the 4 normal breast tissues stained for the protein. M34 identification was strongly positive for transformed epithelium in all tumor types tested. Twelve precancerous lesions of fibroadenoma ($n = 4$), intraductal papilloma ($n = 4$), and incipient ductal carcinoma *in situ* ($n = 4$) all showed high levels of M34 staining, suggesting that precancerous tumors, as well as the earliest stages of mammary carcinoma, can be sensitively detected. Furthermore, anti-M34 antibody selectively stained all 12 advanced-stage metastatic adenocarcinoma cell masses and micrometastases in axillary lymph nodes tested. Single-cell micrometastases embedded in connective tissue or lymph node parenchyma could be clearly resolved by M34 with horseradish peroxidase staining. Lymphocytes, normal ductal endothelium, and vascular endothelial cells were M34-negative, as were muscle, nerve, and adipose tissues. Low-level M34 staining

was detected in connective tissue fibroblasts, macrophages, and neutrophils.

Conclusions: To our knowledge, no previously reported markers have shown high sensitivity of detection for both the earliest and most advanced stages of breast carcinoma. Consequently, M34 appears uniquely suited for diagnosis of the earliest stages of lactation duct transformation as well as for advanced-stage mammary carcinoma metastases in surgical margins and axillary lymph nodes.

INTRODUCTION

Breast cancer affects ~250,000 patients/year in the United States, with an accompanying mortality of 40,000 women in 2002 (1). The increase in annual mortality from 31,000 in 1971 (2) to 40,000 in 2002 reflects an increased population over the past 30 years but also points out a failure to significantly reduce breast cancer deaths over the same period. Because patients with stage I or II disease in which tumors are not associated with metastases to lymph nodes or distant sites have survival rates ranging from 66% (stage II) to 85% (stage I; Refs. 3–5), it is clear that the earliest possible detection of ductal neoplasias remains the key to successful therapeutic intervention. To date there has been no report of a marker that unequivocally detects the earliest stages of ductal carcinoma (6). Typically, protein markers for late-stage adenocarcinoma do not sensitively detect early stages, such as fibroadenoma and ductal carcinoma *in situ* (DCIS), and consequently are not specific enough for routine screening (7). Breast cancer protein markers, including cytokeratins, CA15-3, carcinoembryonic antigen, and CA125, are reserved for use in monitoring treatment response or disease progression (6, 8, 9). Genomic and proteomic approaches to diagnosis that appear promising are under heavy development; however, complexities of interpretation due to multiple mutations and individualized patient gene mutation and expression profiles have presented difficulties for assessing a broad patient population (6, 10, 11). Consequently, markers capable of detecting ductal neoplasias before tumor formation remain the goal for significantly improving survival rates in the general population. Economically feasible and clearly interpretable diagnostic markers with high sensitivity and specificity are also still needed to unequivocally assess disease progression and effective therapy (6).

Although advanced-stage adenocarcinomas characterized by cell degeneration and morphological transformations are easily recognized by conventional histological staining, detection of the earliest stages of epithelial cell transformation, as well as single cancer cells within normal tissue stroma, pose unresolved difficulties. Detection requires highly sensitive and specific immunohistochemical staining of tissue aspirates, needle biopsies, or surgical sections. For later-stage cancers, it is estimated that the 15 and 34% mortality rates associated with stage I and II diagnosis, respectively, are likely to indicate the

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presence of micrometastases in axillary nodes and distant organ sites that have gone undetected by routine examination (3). As many as 30% of patients presenting with negative lymph nodes by conventional histopathology, with or without current marker analysis, develop a recurrence within 10 years (3, 4). The 34% mortality rate accompanying stage II disease has been attributed to inadequate detection of occult metastases in axillary nodes (5) and to incomplete removal of undetected micrometastases during surgery (5, 7). Consequently, improvements in marker sensitivity and broad application to the patient population with metastatic breast adenocarcinoma are clearly needed to unequivocally detect occult micrometastases.

Intense research has succeeded in revealing numerous cellular features characteristic of epithelial neoplasias, including activation of oncogenes, inhibition of tumor-suppressor gene products, and expression of steroid receptors, growth factors, and cell surface glycoproteins (reviewed in Ref. 6). However, no single marker is available at present that confidently identifies early transformation in lactation duct epithelium (6, 12). Furthermore, micrometastases require identification with confidence (13–16). Here we present M34 actin regulatory protein as a candidate for high-fidelity detection of both early- and late-stage breast cancers and their attendant metastases.

M34 actin-bundling protein is a mammalian cytoskeleton regulatory protein that is expressed in dividing and motile cells (17). M34 protein, like its ancestral homolog, *Dictyostelium* 30-kDa protein, is found concentrated in the cell cortex and cleavage furrow of migratory or proliferating cells (17, 18), where actin bundles constitute the contractile ring of dividing cells and are a component of cortical actin filament networks required for lamellipod extensions during migration. In the present study, we investigated the occurrence of M34 in normal human breast tissue, breast tumors, and axillary lymph nodes (ALNs) in postmenopausal women by use of a M34-specific antibody. M34 protein was completely absent from normal mammary duct endothelium and ALNs, but was abundantly expressed in lactation duct endothelial cells, from early-stage hyperplasias to metastatic mammary carcinoma.

MATERIALS AND METHODS

Biopsy Specimens. Biopsy tissue from patients with a histologically confirmed diagnosis of fibroadenoma, DCIS, lobular papilloma, or invasive adenocarcinoma with metastasis to ALNs were examined for the expression of M34 protein. Both frozen and paraffin-embedded tissues were analyzed, including fibroadenomas, DCIS, and human breast adenocarcinomas with matched ALNs, normal breast biopsies, and normal ALNs.

Immunoblots. M34 protein was detected by Western immunoblot analysis of human breast and ALN tissues. Tissue proteins were resolved by SDS-PAGE on 8% acrylamide gels (19). Electrophoretic transfer to nitrocellulose paper was performed according to Towbin *et al.* (20). Transferred blots were blocked with PBS (137 mM NaCl–8 mM Na₂HPO₄–1.5 mM KH₂PO₄–3 mM KCl) containing 5% nonfat dry milk (Carnation) and a 1:4000 dilution of normal rabbit serum for 4 h at 25°C. M34 was identified with anti-M34 polyclonal antibody raised against purified *Dictyostelium* 30-kDa actin-bundling protein (17). The primary antibody serum was diluted 1:4000 in PBS

containing 5% milk for subsequent detection by chemiluminescence. Blots were incubated for 1 h at room temperature with primary antibody and washed three times (5 min each) in PBS. The secondary antibody, horseradish peroxidase-conjugated goat antirabbit F(ab')₂ (Jackson ImmunoResearch), was diluted 1:2500 or 1:5000 in PBS and incubated with the blot for 30 min at room temperature. Blots were washed three times (5 min each) in PBS, followed by a 2-min development in Western Lightning chemiluminescent reagent (Perkin-Elmer). Blots were exposed to X-ray film from 30 s to 10 min to visualize.

Paraffin Section Preparation and Staining. Tissues were fixed in 10% buffered formalin for 48 h, washed in tap water for 30 min, dehydrated in 95% ethanol with two changes of 1 h each and 100% ethanol with three changes of 1 h each, cleared by 100% xylene with two changes of 1 h each, and infiltrated with melted paraffin at 58°C with three changes of 1 h each. Ten- μ m sections were deparaffinized with xylene (three times, 5 min each time) and rehydrated sequentially in 100% ethanol (two times 3 min), 95% ethanol (two times 3 min), 70% ethanol (two times 1 min), water (once for 1 min), and PBS (once for 10 min). Slides were immersed in 0.01 M sodium citrate (pH 6), heated in a microwave oven at 700 W for twice for 5 min, cooled in a humidified box to room temperature, incubated for 20 min with 2% hydrogen peroxide in methanol, and washed in distilled H₂O (two times 5 min) and PBS (two times 5 min). Sections were blocked for 20 min in normal rabbit serum and incubated for 30 min with primary antiserum (anti-M30 rabbit polyclonal antibody; Ref. 17) at dilutions ranging from 1:200 to 1:1000, followed by washing in PBS (three times 10 min). Sections were incubated for 30 min with biotinylated secondary antibody using the Vectastain ABC Peroxidase (Rabbit IgG) Elite Series kit (Vector Labs), washed in PBS for 10 min, and stained for 2–7 min with ImmunoPure Metal Enhanced DAB Substrate Kit (Pierce Laboratories). Slides were washed for 5 min in tap water, counterstained with hematoxylin, and mounted in Permount.

Frozen Sections. Tissues flash frozen in liquid nitrogen were embedded in Tissue-Tek (Miles) and sectioned with an ultratome cryostat into 5–10- μ m sections. Thin sections were fixed in methanol at –20°C for 2 h, washed in PBS for 20 min, and stained as described for paraffin sections.

RESULTS

Expression of M34 Protein in Breast Cancer Epithelial Cells. M34 protein is selectively expressed in rapidly dividing and motile mammalian cells in culture, where it is a prominent component of the cortical actin network and the mitotic cell cleavage furrow (17, 18). This property of selective expression in actively mitotic and motile cell types extends to human tissues, where the protein is heavily expressed in breast tissue (Fig. 1, Lane d) and ALNs containing adenocarcinomas (Fig. 1, Lane b), but it is nearly undetectable in normal breast (Fig. 1, Lane c) or ALN (Fig. 1, Lane a). Antibody detection of M34 is highly sensitive and specific by Western blot criteria, lending credence to the potential use of anti-M34 as a marker for cell-specific expression of M34 protein in cancerous human breast tissue.

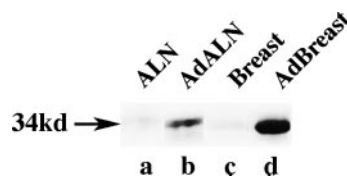


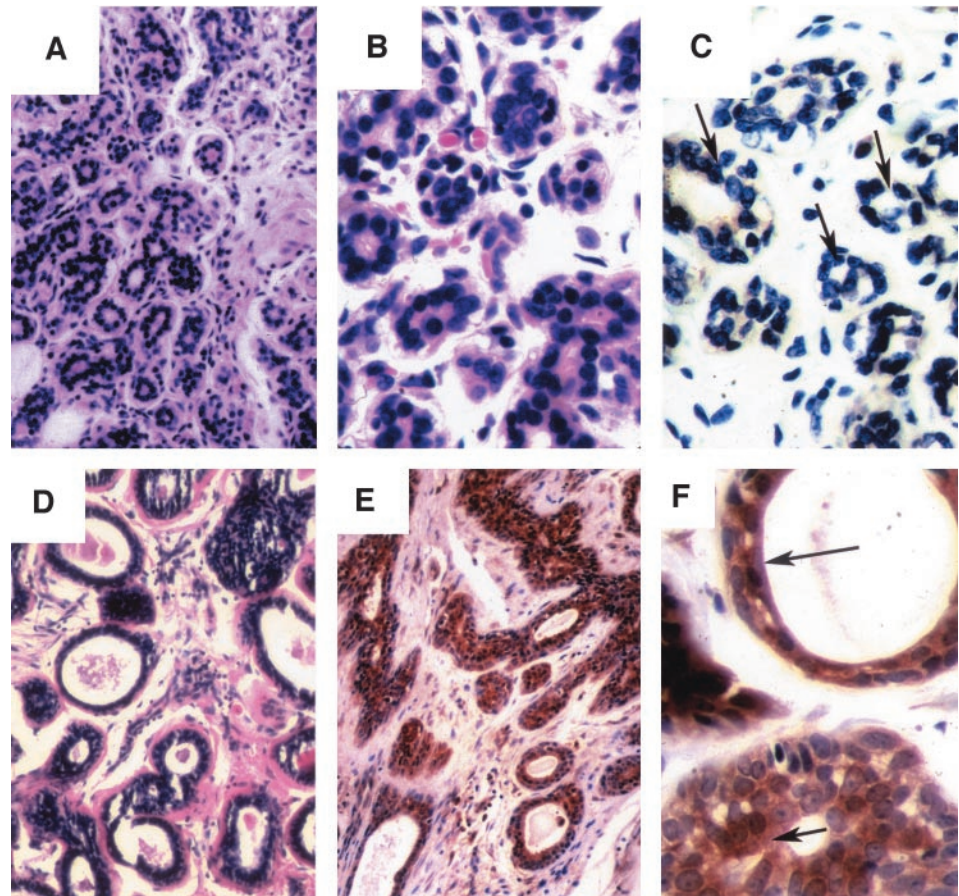
Fig. 1 Expression of M34 protein in breast and axillary lymph nodes (ALNs) with adenocarcinoma. Western blot detection of M34 protein in disease-free human ALN (Lane a), ALN adenocarcinoma (AdALN; Lane b), normal breast (Lane c), and breast adenocarcinoma (AdBreast; Lane d). Equivalent total protein loadings of 5 μ g were applied from each tissue. The 34-kDa M34 protein is lightly expressed in normal breast and ALNs, but becomes heavily expressed in breast or ALNs containing adenocarcinomas.

M34 Is a Sensitive Marker for Lactation Duct Epithelium Transformation. The efficacy of M34 as an early-stage reporter molecule in human breast carcinoma patients was assayed in progressive stages of breast cancer. In normal breast from four patients (Fig. 2, A–C), lactation duct endothelium was free of M34 protein (Fig. 2C, arrows). M34 staining was also negative in tissue extracellular matrix, muscle cells, and circ-

ulating blood and lymphatic cells. The null background of normal breast tissue stands in sharp contrast to the intense, positive staining of M34 in early breast hyperplasias (Fig. 2, D–F). Four patients with well-differentiated fibroadenoma showed distinctive M34 protein expression in transformed lactation duct epithelium [Fig. 2, E and F (arrows in F)]. Actively motile connective tissue cells such as fibroblasts, macrophages, and neutrophils also showed low levels of M34 expression. The unusual sensitivity of M34 expression for early transformation of ductal epithelium suggests its potential use as a high-fidelity probe for incipient breast carcinoma.

Detection of DCIS and Lobular Papilloma by M34 Protein. Intermediate-stage ductal hyperplasias were also readily detected with the M34 marker. In four patients presenting with DCIS (Fig. 3, A–D), lactation duct epithelium stained heavily for M34 protein in proliferating endothelial cells. Whereas advanced stages of DCIS are easily recognized by either standard histological H&E staining or M34 marker (Fig. 3, A and B), early-stage incipient disease is extremely difficult to diagnose on the basis of H&E staining alone. M34 protein marker analysis of early-stage DCIS clearly discriminated between disease-free (Fig. 3C) and early DCIS (Fig. 3D, arrow).

Fig. 2 M34 protein in normal breast and fibroadenoma. Paraffin biopsy sections from four disease-free patients and four patients with confirmed fibroadenoma of the breast were conventionally stained with H&E or for the presence of M34 protein by horseradish peroxidase immunostaining. M34-stained sections were counterstained with hematoxylin to detect nuclei. A, normal breast (H&E; magnification, $\times 100$). B, normal breast (H&E; magnification, $\times 400$). C, normal breast (M34 + hematoxylin; magnification, $\times 400$). M34 is not detected in normal lactation duct epithelium (arrows). D, fibroadenoma (H&E; magnification, $\times 200$). E, fibroadenoma (M34 + hematoxylin; magnification, $\times 100$). F, fibroadenoma (M34 + hematoxylin; magnification, $\times 400$). Heavy M34 staining is restricted to proliferating ductal epithelium (arrows).



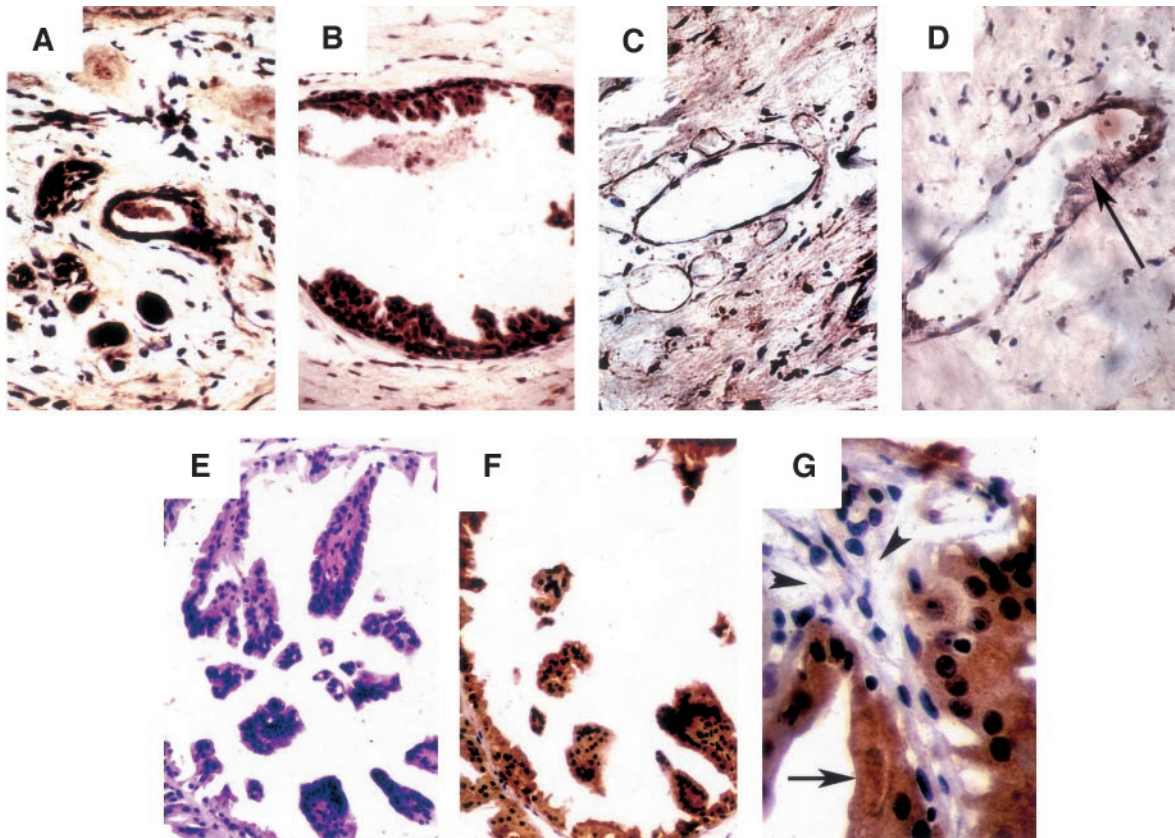


Fig. 3 Detection of ductal carcinoma *in situ* (DCIS) and lobular papilloma by M34 protein. Paraffin biopsy sections from patients with confirmed DCIS and lobular papilloma of the breast were stained either with H&E, or with M34 protein + hematoxylin (to detect nuclei). **A**, DCIS (M34 + hematoxylin; magnification, $\times 100$). **B**, DCIS (M34 + hematoxylin; magnification, $\times 400$). **C**, normal lactation duct (M34 + hematoxylin; magnification, $\times 400$). M34 is heavily expressed in DCIS ductal epithelium (**A** and **B**) but is not detected in the simple epithelium of a normal lactation duct (**C**). **D**, early-stage DCIS (M34 + hematoxylin; magnification, $\times 400$). M34 detects the earliest stage neoplasia in DCIS (*arrow*). **E**, lobular papilloma (H&E; magnification, $\times 400$). **F**, lobular papilloma (M34 + hematoxylin; magnification, $\times 400$). **G**, lobular papilloma (M34 + hematoxylin; magnification, $\times 630$). M34 protein staining is restricted to proliferating ductal epithelium (*arrow*). Connective tissue stroma and cells do not stain for M34 (*arrowheads*).

Four cases of lobular papilloma were also detected by M34 (Fig. 3, *E–G*). M34 staining was strongly correlated with proliferating lactation duct epithelium (Fig. 3*G*, *arrow*) in stark contrast to negative connective tissue cells surrounding the ducts (Fig. 3*G*, *arrowhead*). Invasive cells were not observed in either DCIS or papilloma biopsy tissues.

Detection of Adenocarcinoma of the Breast and ALNs.

With an eye toward detecting occult invasive and metastatic adenocarcinomas in breast and ALNs, we used M34 protein to analyze 12 patients with confirmed metastatic adenocarcinoma (Fig. 4, *A–C*). All invasive breast adenocarcinoma tumors tested stained heavily for M34 protein (Fig. 4*A*), and the marker was sufficiently sensitive to routinely detect small nests of metastatic cells (Fig. 4*B*, *arrow*). Moreover, the highly challenging objective of detecting single-cell metastases was accomplished by M34 immunostaining. Single adenocarcinoma cells could be routinely detected with high fidelity at distant sites from the primary lesion (Fig. 4*C*, *arrows*). Stromal cells did not stain for M34, making unambiguous identification of single carcinoma cells possible.

The same high-level sensitivity and specificity of M34

staining for metastatic adenocarcinoma was noted in ALNs (Fig. 4, *D–G*). Resident cells of normal lymph nodes, including all lymphocytes, did not stain for M34 (Fig. 4*E*). Metastatic adenocarcinoma cells stained by M34 were therefore clearly discernible against the null lymph node background (Fig. 4*D*). Extremely sensitive detection could be obtained when lymph node sections were stained solely with M34 without hematoxylin counterstaining (Fig. 4*F*). Single cells and nests of metastatic adenocarcinoma cells were easily visualized by M34 in the absence of counterstaining. As in breast tissue, occult micrometastases containing either single cells or cell clusters were clearly detected by M34 staining (Fig. 4*G*, *arrows*). ALN metastases were easily visualized by M34 in all patients with confirmed ALN metastases, suggesting the possibility of reliable, high-fidelity detection of occult micrometastases in axillary or sentinel lymph nodes by M34.

DISCUSSION

It is now clear that highly complex gene expression patterns characterize successive stages of carcinoma and individual

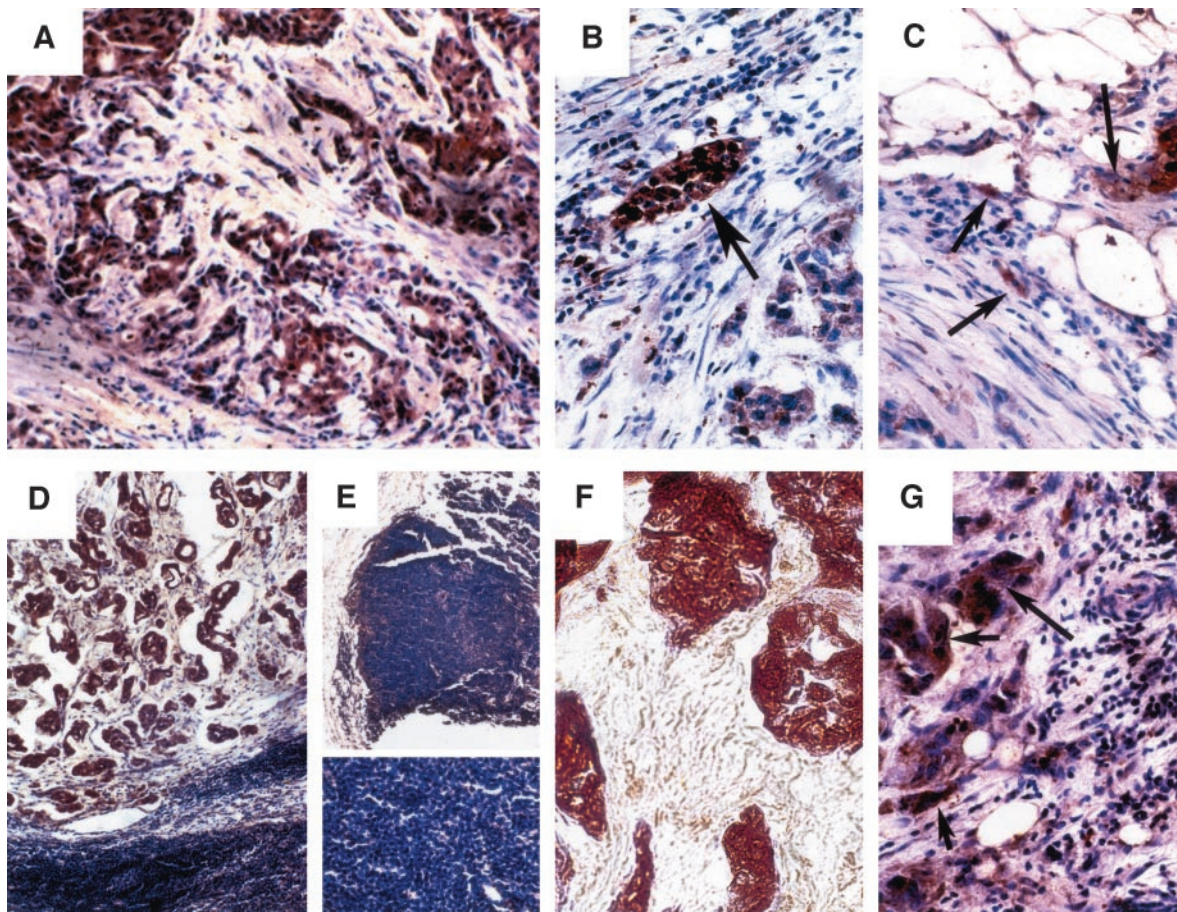


Fig. 4 Mammary adenocarcinoma and axillary lymph node (ALN) metastatic adenocarcinoma detection by M34. Matched surgical biopsy sections from breasts and ALNs from 12 patients diagnosed with adenocarcinoma of the breast with metastasis to ALNs were stained for M34 protein (to detect cancer cells) plus hematoxylin (for nucleus morphology). **A**, mammary adenocarcinoma (M34; magnification, $\times 100$). **B**, mammary adenocarcinoma (M34; magnification, $\times 400$). Small clusters of invasive cells could be detected with anti-M34 antibodies (arrow). **C**, biopsy margin (M34; magnification, $\times 400$). Isolated single cells and cell clusters were detected by M34 at biopsy margins. **D**, ALN metastatic adenocarcinoma (b protein; magnification, $\times 400$). M34 heavily stained metastatic breast adenocarcinoma in ALN. **E**, normal ALN (M34 + hematoxylin; magnification, $\times 100$). **F**, ALN metastatic adenocarcinoma (M34 only; magnification, $\times 400$). High-sensitivity detection of adenocarcinoma from M34 staining. **G**, ALN metastatic adenocarcinoma (M34 + hematoxylin; magnification, $\times 400$). Single cells and clustered carcinoma cells (arrow) are clearly identified in the ALN by M34.

patient genetic profiles (21, 22). In view of this genetic complexity, it is surprising that the singular expression of M34 protein during transformation suffices to sensitively mark all cells progressing through advancing stages of breast adenocarcinoma. These fortuitous events may be due to the conceptually simple notion that expression of M34 protein is essential for cancer cell proliferation by virtue of its role in forming a functional contractile ring required for cytokinesis. Consequently, M34 is abundantly expressed at every cancer cell stage, beginning with the earliest transformation event through metastasis and disseminated disease. Furthermore, it is not expressed in nondividing, normal epithelium, providing a striking positive marker on a null background for the detection of early-stage transformation of epithelium. Of the postmenopausal patients submitted for analysis with confirmed diagnoses of fibroadenoma ($n = 4$), intraductal papilloma ($n = 4$), DCIS ($n = 4$), or adenocarcinoma ($n = 12$), all tested positive with the M34

marker, whereas none of the normal tissues analyzed ($n = 7$) tested positive (Table 1). Consequently, false-positive and false-negative rates were 0%, with 100% sensitivity and 100% specificity for the population tested.

Regarding M34 marker reliability for diagnosis in premenopausal women, the question arises whether normally proliferating breast epithelial cells observed during the estrus cycle are positive for this protein. We do not know the specific answer to this question, although our research on a variety of tissue types is quite convincing that all proliferating cells observed to date express M34 protein. In Madin-Darby canine kidney (MDCK) epithelial cells, M34 protein is abundantly expressed in actively dividing subconfluent cultures. M34 expression is abruptly turned off as the cultures become confluent and nondividing. The regulation of M34 expression in epithelial cells and M34 localization to the cell cleavage furrow during mitosis is discussed in a separate report. In this report, we have delib-

Table 1 Patient profiles for M34 staining of fibroadenoma and adenocarcinoma of the breast

Postmenopausal patients with histologically confirmed diagnoses of noncancerous lactation duct tumors (fibroadenoma and lobular papilloma) or mammary adenocarcinoma with or without axillary lymph node involvement were examined.

Patient	Age (yrs)	Pathology finding	M34 staining
197293	57	Breast; normal	–
196828	63	Breast; normal	–
173586	58	Breast; normal	–
198420	70	Breast; normal	–
92-93033	65	ALN; ^a normal	–
92-13424	63	ALN; normal	–
98-93735	55	Adc; breast	+
92-20304	61	Adc; breast	+
98-64890	66	Adc; breast	+
92-16390	65	Adc; breast	+
92-15247	71	Adc; ALN mets	+
92-14695	66	Adc; ALN mets	+
98-99134	60	Adc; ALN mets	+
92-13424	65	Adc; ALN mets	+
92-13410	55	Adc; ALN mets	+
140996	57	Adc; ALN mets	+
92-15247	61	Adc; ALN mets	+
166023	56	Adc; ALN mets	+
152135	73	Fibroadenoma	+
198428	53	Fibroadenoma	+
146218	61	Fibroadenoma	+
139642	59	Fibroadenoma	+
122100	61	Intraduct papill	+
125309	53	Intraduct papill	+
01-69825	55	Intraduct papill	+
97-38796	58	Intraduct papill	+
173588	63	DCIS	+
00-79683	60	DCIS	+
99-93662	58	DCIS	+
185972	55	DCIS	+

^aALN, axillary lymph node; Adc, adenocarcinoma; mets, metastasis; Intraduct papill, intraductal papilloma; DCIS, ductal carcinoma *in situ*.

erately focused on cancers in postmenopausal women to define M34 protein as a reliable marker for both early- and late-stage tumors.

Although several histological markers, such as antibodies to cancer antigens CA15-3, carcinoembryonic antigen, CA125, and cytokeratins, as well as the hMAM and MAGE genes have been used to detect advanced-stage adenocarcinoma of breast and ALN (6, 8–11, 13–15, 22), at present there is no marker that unequivocally detects early-stage neoplasias in the population at large (reviewed in Ref. 6). Pancytokeratins are the histological markers most often used for detection of metastatic disease in ALNs (3, 5, 7), but they do not detect early-stage breast cancer (12, 16). There are also reports that the keratins may not reliably detect occult metastases in 20–30% of patients with metastatic disease to the ALN (3–5, 7, 13).

The expression or repression of several actin cytoskeleton-associated proteins has been shown to accompany neoplastic transformation. Transcriptional repression of gelsolin, a tumor repressor, is frequently observed in breast cancers in humans, rats, and mice (23). Loss of gelsolin provides a potential negative marker for neoplasia, but its usefulness has been limited by residual low levels in transformed cells and the inherent diffi-

culty in detecting the disappearance of a marker signal in tissue sections. Gelsolin appears to be replaced by a related actin-filament-severing protein, M-severin, in transformed epithelium, opening the possibility for M-severin as a useful positive marker for transformation (24). Fascin, an actin-bundling protein that induces membrane protrusions and enhanced cell motility in epithelial cells (25), has been shown to undergo altered expression in germinal center dendritic cells in neoplastic follicles of B-cell lymphomas (26). Fascin expression is diminished or eliminated in dendritic cells in follicular lymphomas but appears normal or increased in follicular dendritic cells in cases of follicular hyperplasia (26). On the basis of these results, fascin may also be classified as a negative marker for transformation in lymphocytes, but expression of fascin has not been sufficiently documented in epithelial cell carcinomas to determine its usefulness as a carcinoma marker. α -Actinin, an actin-cross-linking protein, has tumor-suppressor properties when expressed in BALB/c 3T3 cells (27). Positive correlations between expression and transformation have been found for L-plastin, an actin-binding protein involved in leukocyte adhesion (28). A role for L-plastin in tumorigenicity has been postulated because low-level expression occurs in several human cancer cell lines (29). To date, no actin-associated protein has been determined to be required for transformation or shown to be an unequivocal positive marker for epithelial neoplasias. The exceptional ability of M34 to reliably detect the earliest stages of carcinogenesis in mammary duct epithelium as well as advanced disseminated disease appears unprecedented compared with other established markers. Although M34 holds promise as a reliable marker for confidently assessing occult metastases in breast and ALN biopsies, it remains of utmost interest to determine whether M34-bearing cells, M34 protein, or M34 mRNA can be detected in serum or mammary duct aspirates for purposes of early evaluation of mammary neoplasias in the general population.

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