Caveolin 1 Is Overexpressed and Amplified in a Subset of Basal-like and Metaplastic Breast Carcinomas: A Morphologic, Ultrastructural, Immunohistochemical, and *In situ* Hybridization Analysis

Kay Savage,[1] Maryou B.K. Lambros,[1] David Robertson,[1] Robin L. Jones,[1] Chris Jones,[2]
Alan Mackay,[1] Michelle James,[1] Jason L. Hornick,[3] Emilio M. Pereira,[4] Fernanda Milanezi,[5,6]
Christopher D.M. Fletcher,[3] Fernando C. Schmitt,[5] Alan Ashworth,[1] and Jorge S. Reis-Filho[1]

Abstract

Purpose: The distribution and significance of caveolin 1 (CAV1) expression in different breast cell types and role in breast carcinogenesis remain poorly understood. Both tumor-suppressive and oncogenic roles have been proposed for this protein. The aims of this study were to characterize the distribution of CAV1 in normal breast, benign breast lesions, breast cancer precursors, and metaplastic breast carcinomas; to assess the prognostic significance of CAV1 expression in invasive breast carcinomas; and to define whether *CAV1* gene amplification is the underlying genetic mechanism driving CAV1 overexpression in breast carcinomas.

Experimental Design: CAV1 distribution in frozen and paraffin-embedded whole tissue sections of normal breast was evaluated using immunohistochemistry, immunofluorescence, and immunoelectron microscopy. CAV1 expression was immunohistochemically analyzed in benign lesions, breast cancer precursors, and metaplastic breast carcinomas and in a cohort of 245 invasive breast carcinomas from patients treated with surgery followed by anthracycline-based chemotherapy. In 25 cases, *CAV1* gene amplification was assessed by chromogenic *in situ* hybridization.

Results: In normal breast, CAV1 was expressed in myoepithelial cells, endothelial cells, and a subset of fibroblasts. Luminal epithelial cells showed negligible staining. CAV1 was expressed in 90% of 39 metaplastic breast carcinomas and in 9.4% of 245 invasive breast cancers. In the later cohort, CAV1 expression was significantly associated with 'basal-like' immunophenotype and with shorter disease-free and overall survival on univariate analysis. *CAV1* gene amplification was found in 13% of cases with strong CAV1 expression.

Conclusions: The concurrent *CAV1* amplification and overexpression call into question its tumor-suppressive effects in basal-like breast carcinomas.

The *Caveolin 1* (*CAV1*) gene maps to 7q31.1 and encodes a 21- to 24-kDa integral membrane protein (1, 2). This protein is the main component of caveolar membranes, which are special invaginated microdomains of the plasma membrane found in the majority of mammalian cells (1). CAV1 is expressed in several types of human cells, including adipocytes, endothelial cells, fibroblasts, some types of epithelial cells, and myoepithelial cells (MEC). Owing to its subcellular localization and ubiquitous distribution, CAV1 has been reported to play a major role in lipid transport, membrane trafficking, gene regulation, and signal transduction (1, 2).

Conflicting results on the role of CAV1 in human cancers have been reported (1–16). Based on the high frequency of deletions of 7q31 (a fragile site known as *FRA7G*) in human cancers (17–19), the arguable presence of *CAV1* gene promoter methylation (3, 4) and inactivating gene mutations (3, 5), and the apparent reduction of CAV1 expression in breast carcinomas (3, 9), it has been suggested that *CAV1* is a tumor suppressor gene (1, 2, 20). However, *CAV1*-null mice are no more prone to mammary tumor development than are the wild-type animals (20, 21). Furthermore, there is only indirect evidence to suggest that CAV1 abrogation induces premalignant alterations in mammary epithelia (22), and a proposed inactivating mutation at codon 132 (P132L) could not be independently validated by two groups (3, 19) and was

reported to be restricted to estrogen receptor (ER)–positive (luminal) breast carcinomas in a study done with DNA extracted from formalin-fixed, paraffin-embedded tissue sections where multiple concurrent *CAV1* gene mutations were found in the same cases (23). In addition, studies showing CAV1 down-regulation in breast cancer cell lines used unsorted human mammary epithelial cells as reference (4) and there are several lines of evidence to suggest that human mammary epithelial cells, such as those used by Engelman et al. (4), show a myoepithelial phenotype (6, 15, 24). Hence, the comparison carried out in that study would identify genes preferentially expressed by MECs. In fact, at least two other putative tumor suppressor genes identified by a similar approach, maspin and 14-3-3σ, have both been proven to show a preferential myoepithelial distribution and are unlikely to be bona fide tumor suppressor genes (16, 25–27). By contrast, there are several lines of evidence to suggest that *CAV1* may have oncogenic properties in non–small cell lung (28), prostate (13, 29), bladder (30), esophageal (31), thyroid (32), pancreatic (33), and breast cancer (10, 12, 13, 16). Interestingly, amplification of *FRA7G* site in cancer cell lines (19) and gains of genomic material on 7q are frequently found in high-grade breast cancer (34).

The distribution of CAV1 in normal breast, benign breast lesions, and breast cancer precursors is controversial (3, 6, 9–11, 13, 15, 16). In a previous study comparing the expression profiles of normal luminal epithelial and MECs of the breast, we showed that *CAV1* is one of the genes preferentially expressed in normal MECs (6) and this has been confirmed with *in situ* studies at the protein level (10, 11, 15, 16). Pinilla et al. (10) have shown recently that CAV1 is expressed in ∼4% of all invasive breast carcinomas, being particularly prevalent in tumors with basal-like/myoepithelial differentiation (10). On the other hand, Park et al. (9) described CAV1 membrane and cytoplasmic expression in normal luminal epithelial cells and in 57% of invasive breast cancers (9). In this report (9), CAV1 expression was inversely correlated with human epidermal growth factor receptor (EGFR) 2 (HER2) or EGFR overexpression; however, the antibody and immunohistochemical methods used were not validated (9).

Given the controversy about the distribution of CAV1 in breast cell types and its significance in breast carcinogenesis, we set out to characterize the distribution of CAV1 in normal breast samples using a combination of immunohistochemistry, immunofluorescence, and immunoelectron microscopy. We observed that CAV1 is preferentially expressed in MECs compared with luminal epithelial cells. We then analyzed its distribution in a large series of benign breast lesions, breast cancer precursors, metaplastic breast carcinomas (tumors with known basal/myoepithelial differentiation; refs. 35, 36), and invasive breast carcinomas of different histologic types, grades, and immunophenotypic profiles to determine whether CAV1 was of any diagnostic usefulness as a myoepithelial marker or in the identification of tumors with basal-like phenotype. We also investigated whether *CAV1* gene copy number gains could be one of the underlying genetic mechanisms driving CAV1 overexpression in breast cancers. Finally, we analyzed the prognostic effect of CAV1 expression on the survival of 245 breast cancer patients treated with adjuvant anthracycline-based chemotherapy.

Materials and Methods

Samples

Samples of normal breast tissue as well as benign and malignant breast lesions were retrieved from the archives of Royal Marsden Hospital (London, United Kingdom), with appropriate Local Ethical Committee approval. All cases were reviewed by experienced pathologists (J.S.R-F., F.M., and F.C.S.) and graded according to a modified version of the Scarff-Bloom-Richardson system (37).

Normal breast, benign breast lesions, and breast cancer precursors

Representative tissue sections of a series of benign and preinvasive breast lesions comprised 10 normal breast tissue samples obtained from mammoplasties, 7 apocrine changes (1 apocrine hyperplasia and 6 apocrine metaplasia), 17 fibroadenomas, 8 benign phyllodes tumors, 11 papillomas, 7 sclerosing adenosis, 8 radial scars, 9 hyperplasias of usual type, 7 columnar cell lesions/flat atypia, and 5 pseudoangiomatous stromal hyperplasias.

Preinvasive lesions and invasive breast carcinomas

Malignant and premalignant lesions included 2 malignant phyllodes tumors, 15 ductal carcinomas *in situ* (DCIS), and a series of 245 invasive breast carcinomas (186 invasive ductal carcinomas, 27 invasive lobular carcinomas, 24 invasive mixed carcinomas, and 8 invasive breast carcinomas of other special types). All of the 245 patients with invasive breast cancer were treated with therapeutic surgery (69 mastectomy and 155 wide local excision) and adjuvant anthracycline-based chemotherapy, and those with ER-positive tumors also received endocrine therapy. Follow-up was available for 245 patients, ranging from 0.5 to 135.3 months (median, 67 months; mean, 67 months). CAV1 expression was correlated with various clinicopathologic variables, including tumor size, tumor grade, presence of vascular invasion, presence of lymph node metastasis, and disease-free survival (DFS) and overall survival (OS). CAV1 expression was also correlated with that of the following immunohistochemical markers: ER, progesterone receptor (PgR), HER2, EGFR, cytokeratin 14, cytokeratins 5/6, cytokeratin 17, cyclin D1 expression, and *CCND1* gene amplification. All cases were classified into luminal, HER2, basal-like, and undetermined groups according to the immunohistochemical panel as described by Nielsen et al. (38).

Metaplastic breast carcinomas

To define the frequency of CAV1 expression in breast carcinomas with myoepithelial phenotype, 39 cases of metaplastic breast carcinomas, malignant tumors with known basal/myoepithelial differentiation (35, 36), were retrieved from the pathology files of the authors' institutions. In all participating institutions, Local Ethics Committee approval was obtained. Contributing authors reviewed all cases of metaplastic breast carcinoma and did further immunohistochemical analysis to corroborate the diagnosis. Cases were centrally reviewed by three of the authors (J.S.R-F., F.M., and F.C.S.) on a multiheaded microscope and classified into four categories according to the previously described and widely accepted criteria (35, 39).

Immunofluorescence on frozen sections of normal breast

To accurately define the distribution of CAV1 in normal breast samples, 10-μm frozen sections of normal human breast [cut onto polylysine-coated slides (VWR, Poole, United Kingdom) and stored at −70°C] were used. When the required slides were thawed, the sections were marked using a slide marker pen and fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. Sections were rinsed twice in PBS and then once in PBS plus 1% BSA plus 2% FCS (IFF). Primary antibodies used were the following: cytokeratins 8/18 (1:100; Novocastra), α-smooth muscle actin (1:5,000; Sigma, St. Louis, MO), caveolin (Santa Cruz Biotechnology, Santa Cruz, CA and Transduction Laboratories, Lexington, KY), and p63 (4A4; 1:200; Santa Cruz Biotechnology). Antibodies were diluted in IFF and incubated for

40 min in a moist chamber followed by 3 × 5–min washes in PBS and then 40 min in conjugates diluted 1:1,000 in IFF. The following conjugates were used in various combinations: anti-mouse IgG1–Alexa 488, anti-mouse IgG2a–Alexa 555, anti-rabbit IgG1–Alexa 488, and anti-rabbit IgG1–Alexa 555 (Molecular Probes, Invitrogen, Paisley, United Kingdom). Nuclei were counterstained by 3 × 5–min washes in 100 nmol/L Topro-3 iodide, which is a 1:10,000 dilution of stock solution (Molecular Probes). Sections were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and sealed with nail varnish. Slides were examined and photographed in a Leica Microsystems (Wetzlar, Germany) TCS-SP2 confocal microscope.

Ultrastructural analysis

Postembedding immunogold labeling of normal breast samples. Small pieces of normal human breast were fixed for 1 h at room temperature in 2% paraformaldehyde + 0.05% glutaraldehyde in PBS. The pieces were then embedded in Lowicryl HM20 resin using the progressive lowering of temperature technique as described previously (40). For immunogold labeling, 80-nm sections were cut onto naked nickel grids. Antibody incubation was overnight at 4°C in a moist chamber followed by a 90-min incubation with 5- or 10-nm gold conjugates (Aurion, Wageningen, the Netherlands). For low-power work, sections were silver enhanced with IntenSE (GE Healthcare, Amersham, United Kingdom). In double labeling experiments, no enhancement was used. Sections were counterstained with uranyl acetate and lead citrate and examined in an FEI (Hillsboro, OR) CM100 Biotwin electron microscope at 80 Kv accelerating voltage.

Immunohistochemistry

Owing to the heterogeneous distribution of CAV1 staining, whole tissue sections of benign breast lesions, breast cancer precursors, and invasive breast carcinomas were subjected to immunohistochemical analysis. Representative formalin-fixed, paraffin-embedded tissue sections were subjected to immunohistochemical analysis as described previously (41), with the mouse monoclonal antibody 2297 (Transduction Laboratories; ref. 10) at 1:150 dilution following heat-induced antigen retrieval [18 min, microwave oven, DAKO antigen retrieval solution (pH 6.0)]. Detection was achieved with the Vector avidin-biotin complex (ABC) system (Vector Laboratories). Positive controls (capillaries of normal breast and skin sections) and negative (omission of the primary antibody- and IgG-matched serum) controls were done for each immunohistochemical run. Furthermore, all sections had internal positive controls for CAV1 (nonneoplastic endothelial cells).

The distribution of CAV1 in tissue sections was assessed by two of the authors (K.S. and J.S.R-F.) on a multiheaded microscope. A consensus score was assigned for each case. The distribution and intensity of CAV1 staining were evaluated semiquantitatively: distribution: 0, <1% of cells stained; 1, 1% to <10%; 2, ≥10% to <25%; 3, ≥25% to <50%; and 4, ≥50%. The staining intensity in neoplastic cells was determined by a comparison with the expression of CAV1 in normal endothelial cells: 0, no staining in neoplastic cells; 1, weak staining; 2, moderate staining (slightly weaker than that seen in endothelial cells); and 3, strong staining (of similar intensity to that seen in endothelial cells). Invasive breast carcinomas with final scores (sum of distribution and intensity scores) ≥4 were considered positive. Only membranous with or without cytoplasmic staining was considered specific.

Data on the expression of ER, PgR, HER2, EGFR, cytokeratin 14, cytokeratins 5/6, and cytokeratin 17 in the invasive tumors and metaplastic breast carcinomas were described in detail elsewhere (35, 41).

Chromogenic *in situ* hybridization

To evaluate whether *CAV1* gene amplification might be the underlying genetic mechanism for CAV1 expression in metaplastic and basal-like invasive ductal breast carcinomas, we analyzed 25 cases, 15 with strong CAV1 expression and 10 devoid of CAV1 staining, by means of chromogenic *in situ* hybridization using an in-house generated probe made up of two contiguous, fluorescent *in situ*

hybridization mapped, bacterial artificial chromosome clones (RP11-691L23 and RP11-730H09), which map to 7q31.1 (115.6-115.8 Mb) and do not span the *MET* gene (115.9-116.0 Mb), according to Ensembl Genome Browser.[7] The in-house probe was generated, biotin labeled, and used in hybridizations according to a protocol described by Lambros et al. (42). Hybridizations were done as described previously. Chromogenic *in situ* hybridization experiments were analyzed by two of the authors (K.S. and J.S.R-F.) on a multiheaded microscope. Only unequivocal signals were counted at × 400 (chromogenic *in situ* hybridization) in 60 morphologically unequivocal neoplastic cells. Amplification was defined as more than five signals per nucleus in >50% of cancer cells or when large gene copy clusters were seen (42, 43). All chromogenic *in situ* hybridizations were evaluated with observers blinded to the immunohistochemical results.

Statistical analysis

The StatView 5.0 software package (SAS Institute, Inc., Cary, NC) was used for all calculations. Correlations between categorical variables were done using the χ^2 test and Fisher's exact test. Correlations between continuous and categorical variables were done with ANOVA. DFS and OS were expressed as the number of months from diagnosis to the occurrence of an event (local recurrence/metastasis and disease-related death, respectively). Cumulative survival probabilities were calculated using the Kaplan-Meier method. Differences between survival rates were tested with the log-rank test. All tests were two tailed, with a confidence interval of 95%.

Multivariate analysis was done using the Cox multiple hazard model. A *P* value of 0.05 in the univariate survival analysis was adapted as the limit for inclusion in the multivariate model. Cases with missing values were excluded in the multivariate analysis model.

Results

Normal breast tissue. CAV1 was expressed consistently in MECs arranged as a continuous layer around ducts and lobular units, whereas luminal epithelial cells were devoid of any staining (Fig. 1). Because antigen retrieval was required for immunohistochemical analysis of CAV1 on formalin-fixed paraffin-embedded tissue sections, we analyzed the distribution of this protein on frozen tissue sections using double and triple immunolabeling fluorescent microscopy and immunoelectron microscopy (44). The distribution of CAV1 in normal MECs proved to be similar to that of traditional myoepithelial markers, such as p63 and α-smooth muscle actin (Fig. 1). Interestingly, ductal MECs were consistently strongly decorated by CAV1, whereas MECs of the lobules occasionally showed moderate-to-strong staining. Luminal epithelial cells showed negligible expression of CAV1 in frozen tissue sections and lacked any CAV1 in formalin-fixed paraffin-embedded sections. At the ultrastructure level, abundant CAV1-positive caveolae were found in MECs, whereas CAV1-positive caveolae were exceedingly rare in luminal epithelial cells (Fig. 1).

The stromal compartments of the breast showed a differential distribution of CAV1: intralobular fibroblasts (i.e., fibroblasts of the modified stroma) showed strong membranous staining for CAV1, whereas interlobular fibroblasts and periductal fibroblasts were either negative or showed weak-to-moderate staining. Adipocytes and endothelial cells showed consistent, strong staining (Fig. 1), whereas perineurial cells displayed moderate intensity staining. Given that endothelial cells were consistently positive for CAV1, immunohistochemical staining

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[7] http://www.ensembl.org.
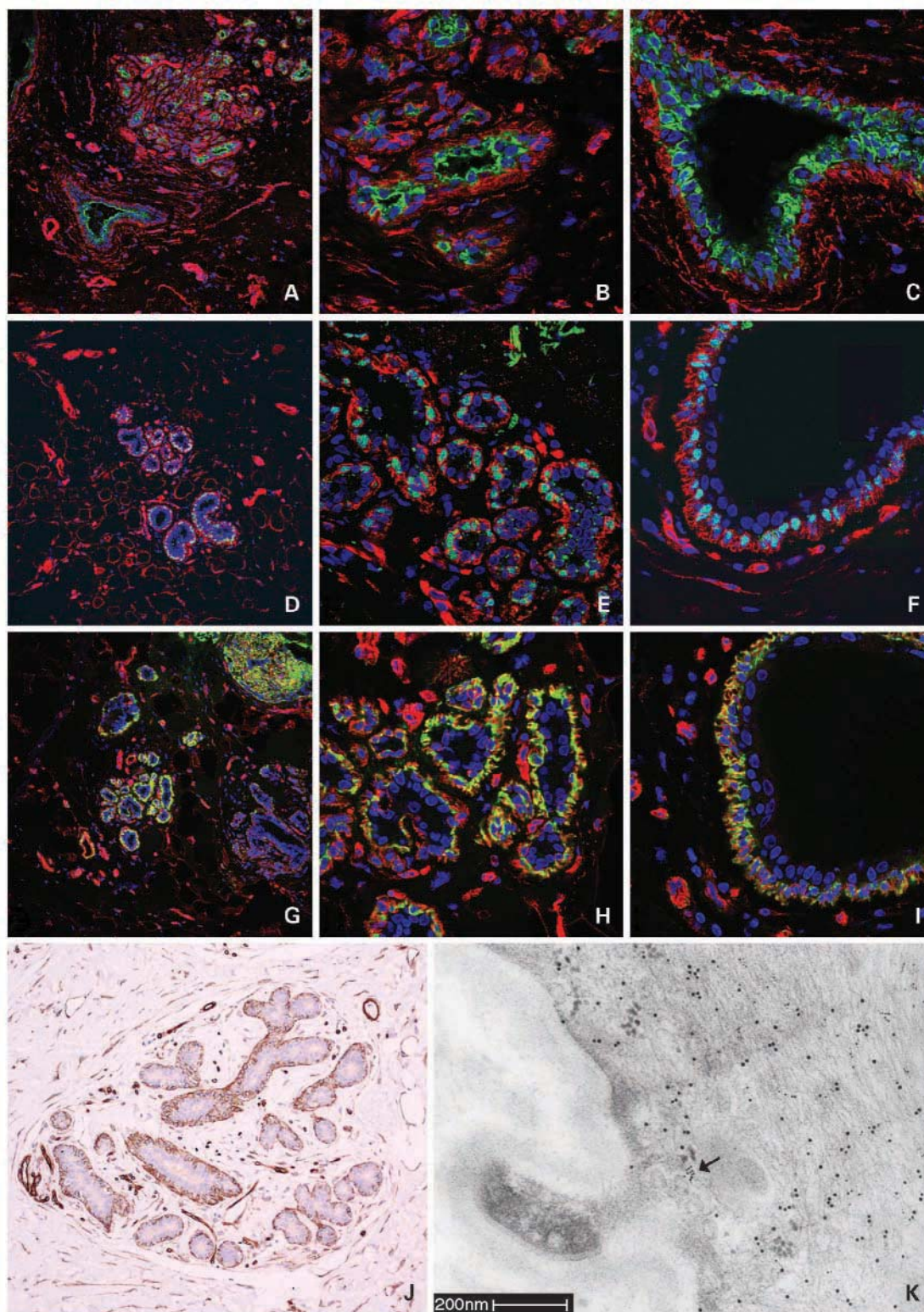
Fig. 1. CAV1 expression in normal breast. CAV1 was strongly expressed in capillaries, adipocytes, myofibroblasts, and MECs (*red*; *A, D, E*, and *G*). In normal breast acini (*B, E*, and *H*) and ducts (*C, F*, and *I*), CAV1 was preferentially expressed in MECs. Expression of CAV1 was consistently seen in p63-positive MECs (*green*; *D-F*) and not in cytokeratin 8/18 – positive luminal epithelial cells (*green*; *A-C*). Note the CAV1 and α-smooth muscle actin (*green*; *G-I*) coexpression in MECs and scattered fibroblasts (coexpression; *yellow*). Expression of CAV1 in a formalin-fixed paraffin-embedded section of normal breast (*J*). Note its similar distribution when compared with that seen in frozen sections. In both frozen and formalin-fixed tissue sections, luminal epithelial cells displayed negligible staining with CAV1 antibodies. Double immunoelectron microscopy for α-smooth muscle actin (10-nm gold particles) and CAV1 (5-nm gold particles) in a MEC (*K*). Note the presence of caveolae with positive staining for CAV1 (*arrow*). Original magnifications, ×40 (*A, D*, and *G*), ×200 (*B, C, E, F, H*, and *I*), and ×100 (*J*). Bar, 200 nm (*K*).

Table 1. CAV1 distribution in benign breast lesions, breast cancer precursors, and metaplastic breast carcinomas

| | n | Myoepithelial compartment* | | | | | | | Luminal compartment* | | | | | | |
| | | CAV1 scores, % cases | | | | | | | CAV1 scores, % cases | | | | | | |
		0	2	3	4	5	6	7	0	2	3	4	5	6	7
Benign lesions															
Apocrine change	7	0	0	0	42.9	28.6	14.3	14.3	100	0	0	0	0	0	0
Papilloma	11	0	0	0	9.1	9.1	36.4	45.5	54.5	0	36.4	9.1	0	0	0
Radial scar	8	0	0	0	12.5	25.0	37.5	25.0	100	0	0	0	0	0	0
Sclerosing adenosis	7	0	0	0	0	14.3	28.6	57.1	100	0	0	0	0	0	0
Fibroadenoma	17	0	11.8	5.9	23.5	35.3	17.6	5.9	70.6	11.8	5.9	11.8	0	0	0
Benign phyllodes tumor	8	0	0	12.5	12.5	37.5	37.5	0	50	0	37.5	12.5	0	0	0
Precursors															
HUT	9	0	0	0	0	44.4	44.4	11.1	0	33.3	22.2	22.2	22.2	0	0
CCL	7	0	0	0	0	42.9	42.9	14.2	71.4	0	14.3	0	14.3	0	0
DCIS	15	0	0	0	26.7	33.3	20	40	86.6	0	0	0	6.7	0	6.7

| | n | Neoplastic cells* | | | | | | |
| | | CAV1 scores, % cases | | | | | | |
		0	2	3	4	5	6	7
Metaplastic carcinomas	39	0	0	10.2	20.5	10.3	30.8	28.2
Spindle cell carcinomas	19	0	0	10.5	5.3	5.3	36.8	42.1
Carcinoma with squamous metaplasia	7	0	0	14.3	42.9	14.3	28.6	0.0
Matrix-producing carcinoma	10	0	0	10.0	30.0	20.0	30.0	10.0
Carcinoma with heterologous elements	3	0	0	0.0	33.3	0.0	0.0	66.7

Abbreviations: HUT, hyperplasia of usual type; CCL, columnar cell lesion.
*Semiquantitative scoring system: sum of the scores for distribution (distribution: 0, <1% of cells stained; 1, 1% to <10%; 2, ≥10% to <25%; 3, ≥25% to <50%; and 4, ≥50%) and intensity [0, no staining in neoplastic cells; 1, weak staining; 2, moderate staining (slightly weaker than that seen in endothelial cells); and 3, strong staining (of similar intensity to that seen in endothelial cells)].

for CAV1 in these structures served as reference staining and internal control for the experiments.

Benign breast lesions/fibrocystic changes. Apocrine metaplasia and hyperplasia (Table 1) displayed CAV1 expression in the form of a continuous layer of CAV1-positive MECs surrounding CAV1-negative apocrine cells; however, the intensity varied from moderate to high. This subtle decrease of CAV1 staining was also noticed in dilated ducts of specimens with fibrocystic change.

Intraductal papilloma. CAV1 was consistently positive in the MECs and in endothelial cells of capillaries found in the fibrovascular cores (Fig. 2A and B). Scattered luminal cells showed weak-to-moderate positivity in five cases. Fibroblasts of the fibrovascular cores were usually negative, unlike those of sclerotic zones, which expressed moderate-to-strong levels of CAV1.

Radial scar. CAV1 was positive in the outer MECs of ductal structures in all cases, whereas luminal epithelial cells consistently lacked this marker. Of note, ducts entrapped in the central elastotic areas showed a moderate-to-strong staining in MECs; however, adjacent fibroblasts were also positive, making the interpretation difficult at times (Fig. 2C and D). Capillaries and endothelial cells of medium-sized and large vessels showed consistent CAV1 expression.

CAV1 expression in breast cancer precursors. Data on CAV1 expression in breast cancer precursors are summarized in Table 1. All hyperplasias of usual type exhibited a continuous or near-continuous layer of CAV1-stained MECs; in these lesions, the solid areas of proliferating hyperplastic cells were weakly-to-moderately positive for CAV1 (Fig. 2E and F).

In columnar cell lesions and DCIS, CAV1 was consistently expressed in a continuous/near-continuous layer of MECs (Fig. 2G and H). However, the staining intensity of CAV1 was never as strong and consistent as that seen with p63 and SMM-HC (data not shown). Furthermore, a variable number of neoplastic cells also showed weak-to-moderate positivity in two columnar cell lesions and one DCIS. In five samples of DCIS, CAV1 was consistently positive in stromal cells (myofibroblasts) arranged in an onionskin pattern surrounding the affected ducts.

Metaplastic breast carcinomas consistently overexpress CAV1. All metaplastic breast carcinomas included in this study showed the typical immunohistochemical profile of basal-like breast carcinomas (i.e., ER and HER2 negative and positive for either cytokeratins 5/6 and/or EGFR; Fig. 3; ref. 38). Thirty-five (89.7%) of 39 samples were considered positive for CAV1 (Table 1).

Correlation between CAV1 and clinicopathologic variables and immunohistochemical markers. CAV1 expression was found in 9.4% of 245 cases (Fig. 3) of invasive breast carcinomas. Of the positive cases, seven (30.4%) were of score 4, nine (39.1%) were of score 5, five (21.7%) were of score 6, and two (8.7%) were of score 7. CAV1 expression showed a strong correlation with histologic grade and lack of lymph node metastasis. When compared with the expression of other immunohistochemical markers, an inverse correlation between CAV1 and ER, PgR, HER2, and cyclin D1 expression was observed. In addition, CAV1 expression showed a strong

statistical association with the expression of EGFR, cytokeratins 5/6, cytokeratin 14, cytokeratin 17, and 'basal markers'; high MIB-1 proliferation index; and p53 immunoexpression. When CAV1 was correlated with the immunohistochemical groups defined by Nielsen et al. (38), all but one of CAV1-positive cases were classified as basal-like carcinomas. These results are displayed in Table 2.

In this cohort of patients, univariate analysis revealed grade, presence of lymph node metastasis, ER, PgR, and cytokeratin 17; and proliferation index assessed by MIB-1 were statistically significant predictors of DFS, whereas presence of lymph node metastasis, ER, PgR, cytokeratins 5/6, cytokeratin 14, and cytokeratin 17; proliferation index assessed by MIB-1; p53 immunoexpression; and cyclin D1 expression were significantly

Fig. 2. CAV1 expression in papilloma (*A* and *B*), radial scar (*C* and *D*), hyperplasia of usual type (*E* and *F*), and ductal carcinoma *in situ* (*G* and *H*). Low-power magnification of a papilloma (*A,* H&E). Note the specific staining in the continuous layer of MECs and endothelial cells of vessels in the papillary cores (*arrowhead*) and lack of staining in luminal cells [*B*, ABC/3,3′-diaminobenzidine (DAB)]. Low-power magnification of the central area of a radial scar (*C*, H&E). Note the expression of CAV1 in MECs, fibroblasts, and capillaries (*D*, ABC/DAB). Inset, note the presence of CAV1 expression in the MECs surrounding a duct in a sclerotic zone. CAV1 expression in hyperplasia of usual type (*E*, H&E). Note the presence of CAV1-positive MECs surrounding ductal structures and admixed with the hyperplastic population (*F*, ABC/DAB). CAV1 expression in a grade 3, comedo DCIS (*G,* H&E; *H*, ABC/DAB). Note the expression of CAV1 in MECs, endothelial cells, and scattered fibroblasts. Original magnifications, ×100 (*A, B, C, D, G,* and *H*) and ×200 (*E* and *F*).
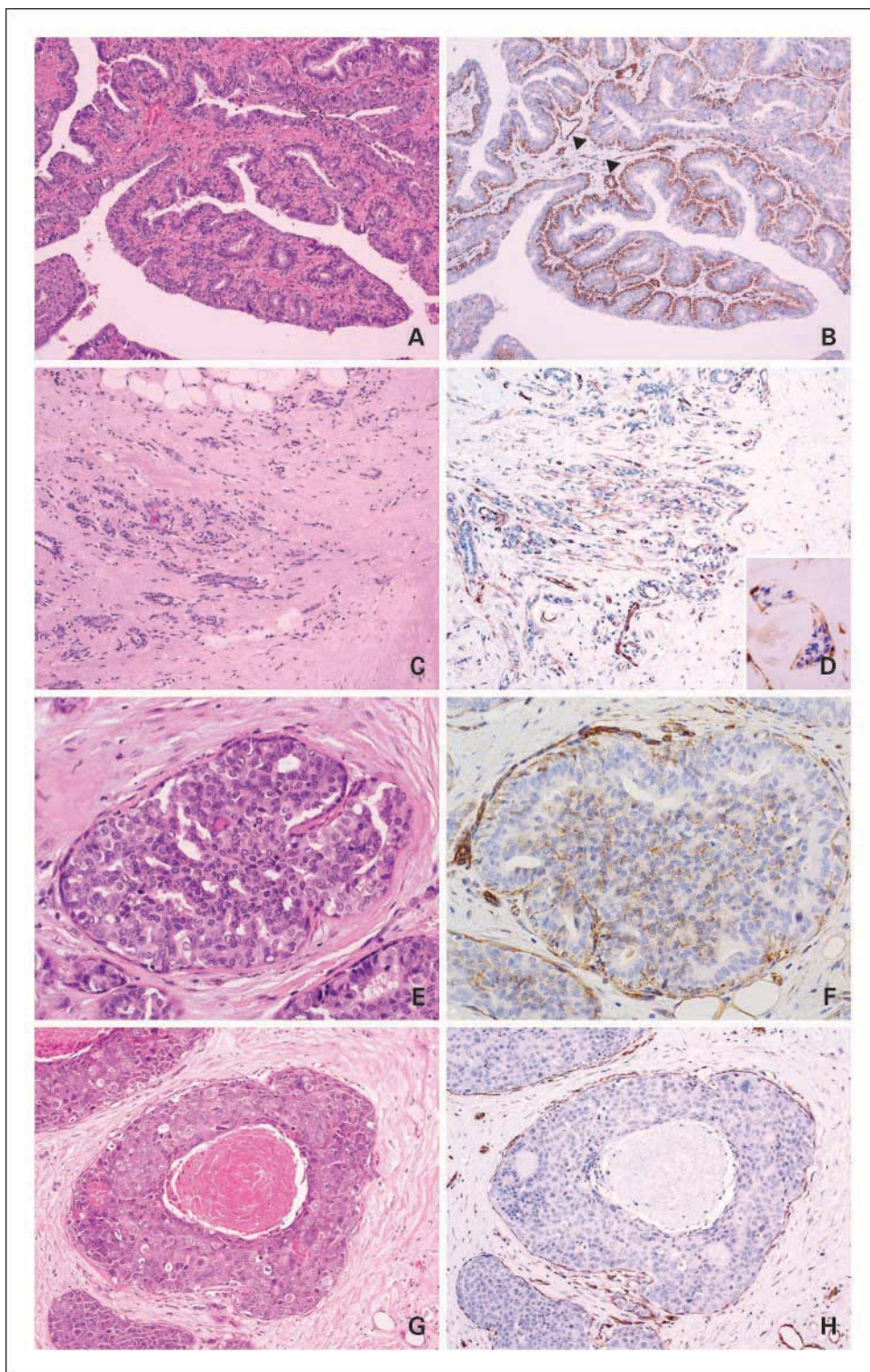
associated with OS (Table 3). In univariate analysis, tumors expressing CAV1 showed a significantly shorter DFS and OS (Table 3). These associations were also significant when considering only lymph node metastasis-positive patients and the OS of lymph node metastasis-negative patients (data not shown).

On multivariate analysis, only size, grade, and presence of lymph node metastasis were independent prognostic factors for

DFS, whereas lymph node metastasis, cytokeratin 17 expression, and p53 expression were independent prognostic factors for OS (data not shown).

CAV1 gene amplification is the underlying genetic cause for caveolin expression in a subset of invasive breast cancers. Given that the locus of *CAV1* is reported to be frequently gained in basal-like breast cancer (34), we investigated whether *CAV1* amplification would be the underlying genetic mechanism
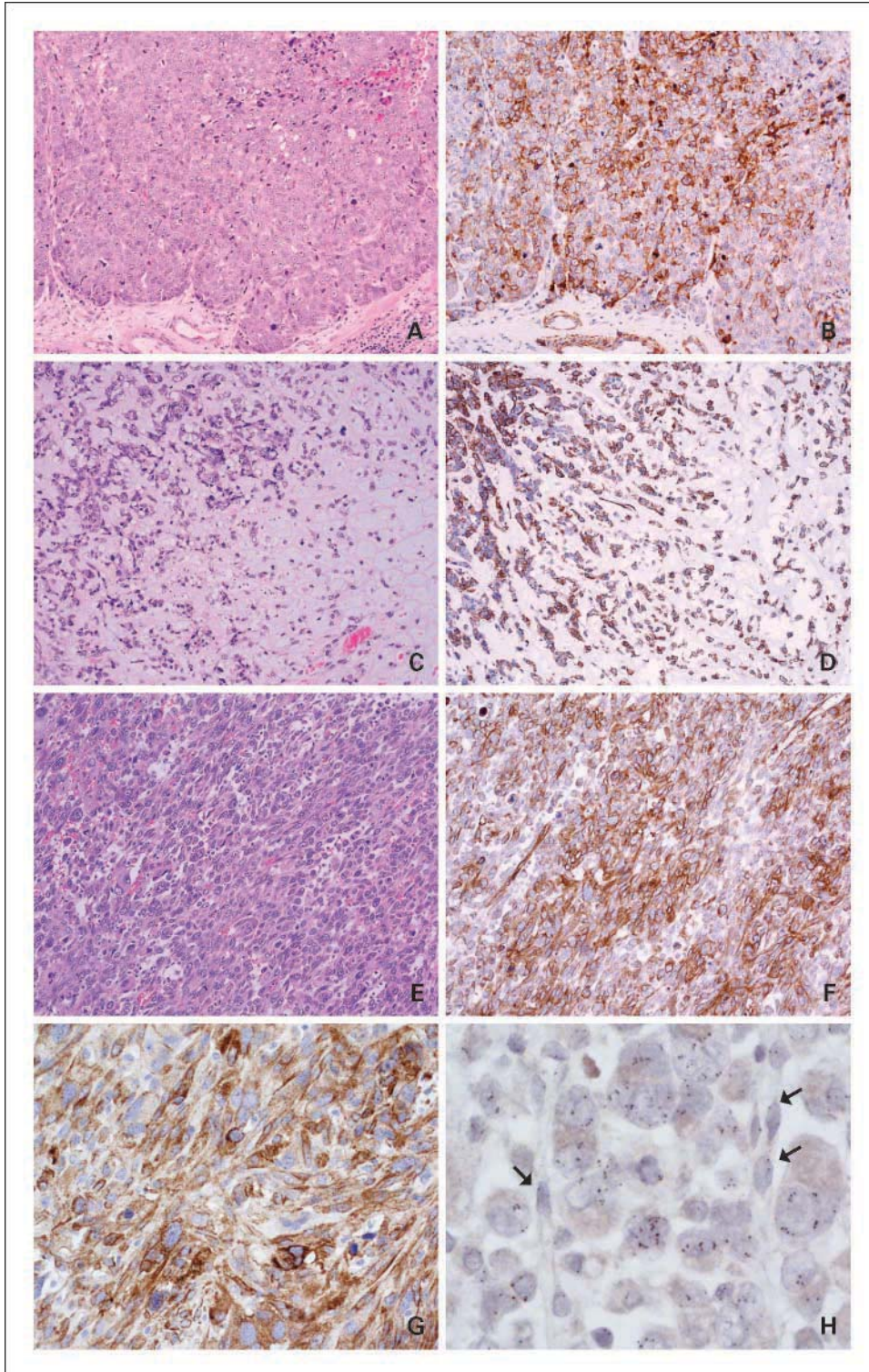
Fig. 3. CAV1 expression and *CAV1* gene amplification in invasive carcinomas. *A* and *B,* grade 3 ER-negative basal-like invasive ductal carcinoma (*A*, H&E; *B,* ABC/DAB) displaying positivity for CAV1. *C* and *D,* matrix-producing metaplastic breast carcinoma (*C*, H&E; *D,* ABC/DAB) with strong and diffuse staining. *E* and *F,* metaplastic spindle cell carcinoma (*E*, H&E; *F,* ABC/DAB) with diffuse and strong CAV1 expression. *G* and *H,* metaplastic spindle cell carcinoma harboring strong CAV1 expression (*G*, ABC/DAB) and gene amplification (*H*, DAB) in the form of more than five signals in the nuclei of neoplastic cells. Note stromal cells with one or two copies of *CAV1* (*arrows*). Original magnifications, ×100 (*A-F*), ×400 (*G*), and ×630 (*H*).

Table 2. Correlations between CAV1 expression, clinicopathologic variables, and immunohistochemical markers in 245 invasive breast carcinomas

Variable	N	NA	CAV1 − (%)	CAV1 + (%)	P
Size	243	2			0.8930*
T_1			115 (90.6)	12 (9.4)	
T_2			90 (90)	10 (10)	
T_3			15 (93.8)	1 (6.3)	
Grade	240	5			0.0028*
1			23 (100)	0 (0)	
2			68 (98.6)	1 (1.4)	
3			127 (85.8)	21 (14.2)	
Type	245	0			0.2370*
IDC			167 (89.8)	19 (10.2)	
ILC			26 (96.3)	1 (3.7)	
Mixed			23 (95.8)	1 (4.2)	
Other			6 (75)	2 (25)	
Lymphovascular invasion	243	2			0.3550†
−			72 (87.8)	10 (12.2)	
+			148 (91.9)	13 (8.1)	
Lymph node metastasis	237	8			0.0023†
−			68 (81.9)	15 (18.1)	
+			146 (94.8)	8 (5.2)	
ER	241	4			<0.0001†
−			26 (54.2)	22 (45.8)	
+			192 (99.5)	1 (0.5)	
PgR	241	4			<0.0001†
−			45 (69.2)	20 (30.8)	
+			173 (98.3)	3 (1.7)	
HER2	238	7			0.0302†
−			179 (88.6)	23 (11.4)	
+			36 (100)	0 (0)	
EGFR	245	0			<0.0001†
−			215 (96.4)	8 (3.6)	
+			7 (31.8)	15 (68.2)	
Ck-14	243	2			<0.0001†
−			213 (96.4)	8 (3.6)	
+			7 (31.8)	15 (68.2)	
Ck-5/6	235	10			<0.0001†
−			203 (96.7)	7 (3.3)	
+			10 (40)	15 (60)	
Ck-17	241	4			<0.0001†
−			204 (95.8)	9 (4.2)	
+			14 (50)	14 (50)	
Basal markers	235	10			<0.0001†
−			193 (99)	2 (1)	
+			19 (47.5)	21 (52.5)	
Nielsen groups	232	13			<0.0001*
Basal			9 (30)	21 (70)	
Luminal			165 (99.4)	1 (0.6)	
HER2			36 (100)	0 (0)	
p53	226	19			0.0002†
−			151 (95)	8 (5)	
+			52 (77.6)	15 (22.4)	
MIB-1 (%)	227	18			<0.0001*
<10			97 (100)	0 (0)	
10-30			89 (91.8)	8 (8.2)	
>30			18 (54.5)	15 (45.5)	
Cyclin D1					<0.0001*
Negative	224	21	15 (57.7)	11 (42.3)	
Moderate			39 (83)	8 (17)	
Strong			147 (97.4)	4 (2.6)	
CCND1 amplification	206	39			0.3238†
No amplification			156 (88.6)	20 (11.4)	
Amplification			29 (96.7)	1 (3.3)	

NOTE: Nielsen groups are immunophenotypic groups defined based on the expression of ER, HER2, cytokeratins 5/6, and EGFR.
Abbreviations: Ck, cytokeratin; *CCND1, cyclin D1* gene; NA, not accessible; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma.
*χ^2 test.
†Fisher's exact test.

driving the expression of CAV1. We analyzed 15 cases with strong CAV1 expression (5 metaplastic breast carcinomas and 10 grade 3 invasive ductal carcinomas) and 10 cases without CAV1 expression (10 invasive ductal carcinomas). Two (one metaplastic breast carcinoma and one grade III, ER, PgR, HER2, and basal keratin plus invasive ductal carcinoma) of 15 cases with strong CAV1 expression (13%) showed gene amplification (Fig. 3E and F), whereas none of the cases without CAV1

Table 3. Univariate survival analysis of 245 breast cancer patients treated with surgery followed by anthracycline-based adjuvant chemotherapy

Variable	*n* (%)	Events	DFS (mean ± SD)	*P*, log-rank test	Events	OS (mean ± SD)	*P*, log-rank test
Size				<0.005			>0.1
T$_1$	127 (52.3)	25	111.7 ± 4.33		20	115.4 ± 4.09	
T$_2$	100 (41.1)	31	99.2 ± 5.41		18	114.9 ± 4.35	
T$_3$	16 (6.6)	9	54.9 ± 7.45		4	76.4 ± 7.47	
Grade				<0.005			<0.1
1	23 (9.6)	1	116.8 ± 3.94		1	117 ± 3.64	
2	69 (28.7)	12	116.4 ± 4.98		8	121 ± 4.93	
3	148 (61.7)	50	95.9 ± 4.52		33	109 ± 4.00	
Lymph node metastasis				<0.0001			<0.0005
No	83 (35.0)	10	122.2 ± 3.96		5	129 ± 2.60	
Yes	154 (65.0)	54	93.5 ± 4.61		37	105 ± 4.32	
Lymphovascular invasion				>0.1			>0.1
No	82 (33.7)	19	109.9 ± 5.18		11	121 ± 4.15	
Yes	161 (66.3)	46	94.9 ± 4.04		31	104 ± 3.73	
ER				<0.05			0.0001
Negative	48 (20.1)	19	81.2 ± 6.94		17	86.8 ± 6.53	
Positive	191 (79.9)	44	107.9 ± 3.73		24	119.2 ± 3.17	
PgR				>0.1			<0.0005
Negative	64 (26.8)	21	89.3 ± 6.25		20	92.8 ± 5.84	
Positive	175 (73.2)	42	106.9 ± 3.92		21	119.7 ± 3.33	
HER2				>0.1			>0.1
Negative	200 (84.7)	52	104.3 ± 3.75		32	115 ± 3.43	
Positive	36 (15.3)	11	92.9 ± 7.71		9	102 ± 7.28	
EGFR				>0.1			<0.1
Negative	222 (91.0)	57	105 ± 3.55		35	115.5 ± 3.18	
Positive	22 (9.0)	8	86 ± 9.62		7	92.3 ± 8.79	
Ck-14				>0.1			<0.05
Negative	221 (90.9)	57	104.2 ± 3.65		34	116.0 ± 3.13	
Positive	22 (9.1)	8	84.5 ± 10.13		8	86.6 ± 9.47	
Ck-5/6				<0.1			<0.01
Negative	210 (89.4)	53	105.4 ± 3.63		32	116.2 ± 3.19	
Positive	25 (10.6)	10	80.4 ± 9.81		9	86.8 ± 8.95	
Ck-17				<0.05			<0.0001
Negative	213 (88.4)	51	106.5 ± 3.60		28	118.5 ± 3.06	
Positive	28 (11.6)	12	77.2 ± 9.41		12	80.3 ± 8.72	
Basal markers				<0.05			<0.001
Negative	204 (84.0)	49	106.8 ± 3.67		28	117.7 ± 3.19	
Positive	39 (16.0)	16	79.4 ± 7.85		14	87.1 ± 7.13	
Nielsen groups				>0.1			<0.005
Basal	30 (13.0)	12	81.3 ± 8.74		11	87.2 ± 8.01	
Luminal	164 (71.3)	11	92.9 ± 7.71		19	102.2 ± 7.28	
HER2	36 (15.7)	37	107.9 ± 4.13		9	119.1 ± 3.68	
p53				<0.05			<0.001
Negative	158 (70.2)	37	107.8 ± 3.99		18	120 ± 3.52	
Positive	67 (29.8)	23	94.9 ± 6.93		20	103 ± 6.06	
MIB-1 (%)				<0.05			<0.005
<10	96 (42.5)	18	112.2 ± 5.04		11	122.4 ± 3.66	
10-30	97 (42.9)	29	100.8 ± 5.47		16	111.4 ± 5.48	
>30	33 (14.6)	15	76.4 ± 8.38		13	88.8 ± 8.44	
Cyclin D1				<0.1			<0.05
Negative/Weak	26 (11.7)	11	70.8 ± 7.81		26	81.1 ± 7.64	
Moderate	46 (20.6)	12	86.8 ± 6.04		46	106.2 ± 6.29	
Strong	151 (67.7)	36	107.7 ± 4.08		151	118.2 ± 3.51	
CCND1				>0.1			>0.1
Nonamplified	211 (87.6)	56	103.6 ± 3.67		37	114 ± 3.25	
Amplified	30 (12.4)	9	94.8 ± 8.17		5	107 ± 6.81	
CAV1				<0.05			<0.05
Negative	221 (90.6)	55	160 ± 3.48		34	116.3 ± 3.08	
Positive	23 (9.4)	10	71.4 ± 8.17		8	82.2 ± 7.91	

NOTE: Nielsen groups are immunophenotypic groups.

expression showed abnormal *CAV1* gene copy numbers. In the two cases with *CAV1* amplification, normal chromosome 7 centromere copy numbers were observed and the ratios *CAV1*/chromosome 7 copy numbers were >2.0 (data not shown).

Discussion

Using a combination of immunofluorescence, ultrastructural analysis, and immunohistochemistry, we have shown that CAV1 is preferentially expressed in MECs, fibroblasts, and endothelial cells in normal breast tissue. Luminal epithelial cells showed a negligible expression of CAV1 in our immunofluorescence and immunoelectron microscopic analyses. At variance with previous studies, where CAV1 was reported to be expressed in normal luminal epithelial cells of the breast, our results provide definite data on the lack of CAV1 expression in normal luminal cells and consistent expression in MECs of the breast. These results not only corroborate but also expand the findings of our group and others (6, 10, 11, 15), who have described consistent expression of CAV1 in MECs at the mRNA and protein levels and lack of expression in luminal epithelial cells of normal breast.

Strategies that have been used to determine the tumor-suppressive functions of CAV1 in human breast cancer have included the following: (*a*) knockout of *CAV1* gene in cells with a luminal phenotype (22, 23) and (*b*) comparisons between breast cancer and cultured unsorted breast epithelial cells/human mammary epithelial cells (HMECs; ref. 4). However, as the majority of breast carcinomas harbor a luminal or HER2 immunophenotype and cultured unsorted breast epithelial cells/human mammary epithelial cells (HMECs) consistently harbor a basal/myoepithelial phenotype, the results of our study call into question these means of determining tumor-suppressive functions of CAV1.

Furthermore, our findings contradict those of Li et al. (23) proposing a 'novel pathway leading toward mammary 'tumorigenesis', involving down-regulation of CAV1 by loss of function or genetic ablation of *CAV1* gene expression in normal epithelial cells and thus driving tumorigenesis of estrogen-positive breast carcinomas by increasing ER levels (23). In fact, according to our results and those of other groups (10, 15), using comprehensively validated immunohistochemical methods, normal luminal epithelial cells of the human breast express negligible levels of CAV1. Therefore, we do not dispute that the pathway described Li et al. (23) may take place in engineered mouse models; however, it is unlikely to take place in human breast.

CAV1 showed consistent expression in MECs of radial scar, sclerosing adenosis, columnar cell lesion, and DCIS; however, its intensity was variable. That is not surprising given that different levels of CAV1 expression were seen in ductal and lobular MECs. Moreover, CAV1 was frequently expressed in myofibroblasts surrounding ducts affected by DCIS and in endothelial cells. Therefore, we would not advocate the use of CAV1 as a myoepithelial marker to differentiate between noninvasive and invasive breast lesions. On the other hand, CAV1 seems to be a useful marker to identify tumors with basal-like phenotype. In the present study, all metaplastic breast carcinomas, tumors with known basal-like/myoepithelial phenotype, displayed CAV1 expression, and 70% of invasive ductal carcinomas with basal-like phenotype (Table 2)

showed positivity for this marker. These results agree with those of previous studies that showed that CAV1 is preferentially expressed in tumors and breast cancer cell lines with basal-like immunophenotype as defined by cDNA microarrays or immunohistochemistry (10, 15).

In the present study, CAV1 expression was significantly associated with high histologic grade and lack of hormone receptors and HER2 expression and directly correlated with p53 immunoexpression and high proliferation rates, all features of basal-like breast carcinomas. CAV1 expression was significantly correlated with positivity for EGFR (i.e., CAV1 was expressed in 68% of EGFR-positive breast cancers, whereas only 3.6% of EGFR-negative breast carcinomas displayed CAV1 expression). Initial studies suggested that EGFR would be localized to caveolae and that CAV1 expression might modulate EGFR signaling activity by receptor sequestration and also played a role in controlling receptor trafficking (45, 46). However, there are several lines of evidence to suggest that interactions between CAV1 and EGFR are cell type and context dependent (45). On the other hand, it has been shown recently that EGFR and CAV1 are coexpressed in several tumor types (10, 45, 47) but do not necessarily colocalize to the same subcellular compartment (45, 48). More recent data, based on ultrastructural analysis, show that EGFR seems to be expressed in flat lipid rafts rather than caveolae (45, 48). There is compelling evidence to suggest that EGFR phosphorylation negatively modulates direct interactions between EGFR and CAV1 and that EGFR signaling activation down-regulates CAV1 levels (47, 49). In fact, in glioblastoma cells harboring *EGFR* gene amplification or mutation, overexpression of CAV1 does not abrogate EGFR activity when EGFR is phosphorylated (47). In addition, under oxidative stress, CAV1 is hyperphosphorylated and transports EGFR to a perinuclear location where it is not degraded and remains active (46). Recently, it has been shown that EGFR-driven Wnt pathway activation seems to be dependent on EGFR-induced CAV1 down-regulation (49). Given the conflicting information on the interactions between CAV1 and EGFR, the cell type–dependence of CAV1 functions, and the role played by the signaling pathways activated by EGFR phosphorylation (i.e., ERK, phosphatidylinositol 3-kinase, and Wnt pathways) in the biology of basal-like breast cancer, further studies analyzing the mechanistic interactions between CAV1 and EGFR signaling in basal-like breast carcinomas are warranted.

CAV1 expression was significantly associated with a shorter OS; however, it did not prove to be an independent prognostic factor in multivariate survival analysis. Our findings are at variance with those of other studies where nonmicrodissected breast cancer samples were subjected to real-time PCR analysis of CAV1 mRNA levels (11). Given that CAV1 is consistently expressed in myofibroblasts and endothelial cells, an accurate measurement of CAV1 mRNA levels in neoplastic cells is not possible without precise microdissection or by using *in situ* methods. Therefore, those results (11) should be interpreted with caution. In addition, due to the particular characteristics of our series (i.e., patients who received adjuvant anthracyclines), our results may not be applicable to the general population of early breast cancer patients.

Given that CAV1 is preferentially expressed in basal-like breast carcinomas, which are consistently of high histologic grade (10), and that gains of 7q are reported to be frequently found in high-grade breast carcinomas (34, 50), we sought to

define whether *CAV1* gene amplification would be the underlying genetic mechanism driving CAV1 expression. *CAV1* amplification was seen in 13% of the strong CAV1 expressers and in none of cases without CAV1 expression, suggesting that at least in a subgroup of basal-like breast carcinomas, CAV1 expression is driven by gene copy number gains. These findings agree with those of Jones et al. (34), where tumors with basal-like phenotype as defined by cytokeratin 14 showed copy number gains of 7q in 14% of cases and no deletions of this chromosomal arm (34). Although *CAV1*-specific probes were used, we cannot rule out that *MET* was also coamplified in the two cases analyzed in this study.

The underlying mechanism of CAV1 expression in the majority of cases remains to be elucidated. Given that CAV1 is consistently expressed in normal MECs (6, 10, 11) and tumors with basal and/or myoepithelial differentiation (10, 15), it is possible that CAV1 expression in basal-like breast carcinomas might constitute the maintenance of a myoepithelial phenotype or might be part of a transcriptomic program of myoepithelial/basal-like differentiation.

In summary, our findings and those of other recently reported studies show that CAV1 is consistently expressed in MECs and in a subgroup of breast carcinomas with basal-like phenotype. On univariate analysis, overexpression of CAV1 was associated with high histologic grade, high proliferation rates, and p53 immunoexpression and with shorter DFS and OS. However, on multivariate analysis, CAV1 was not a significant independent prognostic factor for DFS and OS. In up to 13% of the cases with CAV1 expression, *CAV1* gene amplification was found and is likely to be the driving mechanism of CAV1 overexpression in these cases. Taken together, these findings call into question the tumor-suppressive functions of CAV1 in breast cancer, particularly in the subgroup of basal-like breast carcinomas. Further studies to define the oncogenic properties of CAV1 in basal-like breast carcinomas are warranted.

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Caveolin 1 Is Overexpressed and Amplified in a Subset of Basal-like and Metaplastic Breast Carcinomas: A Morphologic, Ultrastructural, Immunohistochemical, and *In situ* Hybridization Analysis

Kay Savage, Maryou B.K. Lambros, David Robertson, et al.

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