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Calretinin expression in high-grade invasive ductal carcinoma of the breast is associated with basal-like subtype and unfavorable prognosis

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Abstract

Calretinin, a calcium-binding protein, is a widely utilized marker for mesothelial differentiation. There is accumulating evidence of calretinin expression in epithelial and mesenchymal malignancies as well. The objectives of this study were 1) further delineate the expression of calretinin in grade 3 breast carcinomas in the context of molecular subtypes and 2) identify the impact of calretinin expression on overall- and disease-free survival. On the basis of immunohistochemical expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor-2 (HER2), CK5/6 and epidermal growth factor receptor (EGFR), 214 grade 3 invasive ductal carcinomas were stratified into 36 luminal A, 63 luminal B, 24 HER2-positive, 81 basal-like (including 13 metaplastic carcinomas), and 10 unclassified. Tissue microarrays were analyzed for immunohistochemical expression of calretinin. High-level calretinin expression was identified in a significant proportion of basal-like (54.3%), HER2 (33.3%) and unclassified (30%) tumors. In contrast, luminal A and B subtypes demonstrated high-level calretinin expression in only 11.1% and 12.7%, respectively (P<0.0001). Within the basal-like group, 38.5% of the metaplastic carcinomas demonstrated high-level expression, associated predominantly with the epithelial component and squamous metaplasia. High-level calretinin expression was strongly associated with decreased overall survival in the entire cohort of grade 3 cancer (P=0.0096) and in the basal-like group (P=0.039). Multivariate analysis revealed that both tumor stage and high-level calretinin expression were independent predictors of overall survival (P=0.0002 and P=0.0023, respectively). In conclusion, high-level calretinin expression is most common in grade 3 tumors with a basal-like phenotype and is associated with poor overall survival.

Keywords

Breast; Carcinoma; Basal-like; Immunohistochemisty; Calretinin

1. Introduction

Current management of breast carcinoma is based primarily on a relatively long-standing set of clinicopathologic features including tumor morphology (tumor size, grade, etc.), lymph

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node status, assessment of expression of hormone receptors (estrogen receptor [ER], progesterone receptor [PR]) and, more recently, assessment of human epidermal growth factor receptor-2 (HER2). Despite these advances, many tumors within these existing coarse subcategories often behave very differently and those patients with so-called "triple-negative" (ER-/PR-/HER2-) have more limited treatment options. As a result, clinicians and researchers alike continue to struggle to identify tumor characteristics that will better define prognostic and predictive factors.

With the application of array-based genomic analysis, the unique, but heterogeneous nature of triple-negative tumors has become clearer. Employing a cDNA expression array, five main intrinsic molecular subtypes of breast cancer have been identified: luminal A, luminal B, HER2, basal-like, and "normal breast-like"[1]. The basal-like group was characterized by low expression of ER, HER2 and genes expressed by luminal breast epithelial cells, but high expression of genes of associated with basal-type cells, including CK5, CK17, and epidermal growth factor receptor (EGFR) [1]. The expression of basal-type keratins has long been associated with more aggressive clinical behavior, early recurrence, and, more recently, with *BRCA1* mutation or BRCA1 pathway dysfunction [2].

With the exception of limited-panel commercial tests such as Oncotype Dx and MammaPrint that are aimed primarily at risk stratification of ER+, lymph node-negative tumors, genomic assays remain impractical and limited to the research setting. As a result, there has been substantial interest in the use of immunohistochemistry as a surrogate method for the identification of breast cancer subtypes, particularly those of the basal-like phenotype. Using an immunohistochemical panel of five markers (ER, PR, HER2, epidermal growth factor receptor (EGFR), and cytokeratin 5/6), and Ki-67 proliferation index, breast cancers may be classified as luminal (A or B), HER2, basal-like or unclassifiable [3, 4]. Immunohistochemical expression of a number of additional markers has also been identified in basal-like breast carcinomas, including cytokeratins 14 and 17, vimentin, laminin, caveolins 1 and 2, integrin β4, c-kit and calretinin [5-10]. Rationale for a more complete understanding of this breast cancer subtype is twofold. Firstly, this subgroup of tumors frequently exhibits atypical metastatic patterns, notably a significantly higher incidence of early visceral metastases [11]. As these tumors are triple negative, additional diagnostic markers may be needed to confirm/exclude breast origin. Thus familiarity with the immunohistochemical characteristics of basal-like breast cancer is essential if accurate, punctual diagnoses are to be rendered. Secondly, the elucidation of the molecular mechanisms of pathogenesis in this subgroup may lead to the development of novel therapies.

Calretinin is a 29-kD, intracellular, vitamin D-dependent calcium binding protein that likely has multiple functional roles including intracellular calcium buffering and message targeting [12]. First identified in the central nervous system, it has also been identified in a wide variety of non-neural cells, both neoplastic and non-neoplastic [12, 13]. In the oncologic arena calretinin is most commonly used as part of a panel in the separation of pleural mesothelioma from poorly differentiated pulmonary adenocarcinomas [14]. While in most cases highly sensitive and specific for mesothelial origin, calretinin positivity has been reported in carcinomas arising in a myriad of other tissues including ovary, testis, adrenal cortex, colon, breast, sinonasal tract, thymus, skin and even soft tissue [10, 13, 15-18].

In this study, we examined calretinin expression in a cohort of patients with grade 3 invasive ductal carcinoma representing the different molecular subtypes of breast cancer and demonstrated significant association between strong calretinin expression and poor patient survival within this aggressive subgroup of breast carcinomas.

2. Materials and methods

2.1. Tissue selection

Tissue samples of grade 3 invasive ductal carcinomas from 214 consecutive patients aged 27 to 96 years were collected between the years 1996 and 2009 from the archives of the Departments of Pathology at the Rhode Island Hospital and The Miriam Hospital. From this same archival database 51 grade 1 tumors including 26 ductal of no special type (NST), 6 lobular, 3 mixed ductal and lobular, 4 tubular, 3 cribriform, 6 mixed tubular and cribriform, and 3 mucinous, and 112 grade 2 breast carcinomas, including 58 ductal NST, 38 lobular, 7 mixed ductal and lobular, 4 mucinous, and 5 micropapillary were also collected for comparison. The study was approved by the Institutional Review Boards of The Miriam and Rhode Island Hospitals. Only patients who did not receive preoperative neoadjuvant chemotherapy or radiotherapy were included in the study. One hundred forty patients (65%) with grade 3 carcinoma underwent mastectomy and 74 (35%) lumpectomy. After surgery, 130 (63%) of patients with grade 3 breast cancer received chemotherapy, 53 (26%) received hormonal therapy, and 111 (53%) received radiotherapy. One hundred sixty eight (82%) patients received at least one type of adjuvant therapy. The histologic grade was previously determined according to the Nottingham modification of the Bloom-Richardson scoring system and confirmed independently by 2 pathologists (E. Y. and K. S.) [19]. Stage of disease was defined according to the American Joint Committee on Cancer [20]. Based on the immunohistochemical expression of ER, PR, HER2, EGFR, cytokeratin 5/6, and Ki-67 proliferation index, grade 3 tumors were stratified into 36 luminal A (ER+ and/or PR+, HER2-, any EGFR and/or cytokeratin 5/6, Ki-67<14%), 63 luminal B, including 26 luminal B/HER2 negative (ER+ and/or PR+, HER2-, any EGFR and/or cytokeratin 5/6, Ki-67≥14%) and 37 luminal B/HER2 positive (ER+ and/or PR+, HER2+, any EGFR and/or cytokeratin 5/6, any Ki-67), 24 HER2 enriched (HER2+ and ER-/PR-), 81 basal like (ER-, PR-, HER2-, CK5/6+, and/or EGFR+), and 10 unclassified (ER^-, PR-, $HER2^-$, $EGFR^-$, cytokeratin $5/6^-$) [3, 4]. The basal-like group included 13 metaplastic carcinomas, of which 6 had a squamous component, 5 a spindled/sarcomatous component and 7 heterologous elements (5 chondroid and 2 osseous). Four metaplastic carcinomas contained two or more components.

2.2. Tissue microarray construction

Paraffin blocks containing representative tumor areas were identified on corresponding hematoxylin-eosin–stained sections. Areas of interest were identified and marked on the source block. The source block was cored, and a 1-mm core was transferred to the recipient “master block” using the Beecher Tissue Microarrayer (Beecher Instruments, Silver Spring, MD). Five representative cores of tumor and 2 cores of normal breast tissue were arrayed per specimen.

2.3. Immunohistochemical staining

Immunohistochemical staining was performed according to the following protocol. Sections from paraffin-embedded tissue microarrays were cut at 4 μm, deparaffinized, and rehydrated with xylene and graded alcohols. Microwave epitope retrieval was performed in target retrieval pH 6.0 (DAKO, Carpinteria, CA) for ER, PR, HER2, Ki-67 and calretinin; high pH target retrieval for cytokeratin 5/6. The following primary antibodies were used: clone ER1D5 against ER (1:300 dilution; DAKO), clone 1A6 against PR (1:100 dilution; Vector Laboratories), clone CB11 against HER2 (1:150 dilution; Vector Laboratories), clone D5/16B4 against cytokeratin 5/6 (1:40 dilution; Cell Marque, Rocklin, CA), clone MIB-1 against Ki-67 (1:20 dilution; DAKO), clone Z11-E3 against calretinin (1:100 dilution; Invitrogen, Grand Island, NY). Appropriate positive and negative controls were used simultaneously with test slides.

Immunohistochemical staining for ER, PR, HER2, CK5/6, Ki-67 and calretinin was performed using the DAKO Autostainer Plus and EnVision Dual Link detection reagent (DAKO) with DAB (DAKO). EGFR was stained using the PharmDX kit (DAKO) according to manufacturer's instructions. For HER2 fluorescent in situ hybridization assay, slides were hybridized with probes to LSI *HER2* and CEP 17 with the PathVysion HER-2 DNA Probe Kit (Abbott Molecular, Inc, Des Plaines, IL, USA) according to the manufacturer's instructions. Slides were counterstained with 4′,6-diamidino-2-phenylindole and visualized on a Zeiss Axioplan epifluorescent microscope (Zeiss, Baden-Wurttemberg, Germany).

Staining was assessed by two pathologists (K.S. and E.Y.) in a blinded fashion. ER and PR stains were considered positive if expression was present in more than 1% of tumor nuclei. EGFR stains were considered positive if any (weak or strong) membranous expression in invasive carcinoma cell staining was observed. CK5/6 stains were scored positive if any (weak or strong) cytoplasmic and/or membranous staining was detected in the tumor cells. Ki-67 staining was interpreted as low or high using a 14% threshold [4]. For HER2 status, tumors were considered positive if scored as 3+ according to the guidelines of the American Society of Clinical Oncology/College of American Pathologists [21], and fluorescent in situ hybridization with amplification ratio 2.2 or more was used to segregate immunohistochemically equivocal (2+) results. For calretinin, both nuclear and cytoplasmic staining was required to be considered positive staining. The degree of immunoreactivity was assessed based on a combined score of the extent and intensity of staining. Scores 0–3 were assigned according to the percentage of positive tumor cells (0=0%; 1=<25%; 2=25–50%; 3=>51%) and the intensity of staining in tumor (0=0; 1=1+; 2=2+; 3=3+) as previously described [22]. The two scores were multiplied to give an overall score of 0–9, of which 0 was considered negative, 1–2 weak, 3–6 moderate, and 9 strong staining. Negative or weak staining and moderate or strong staining were characterized as low- and high-level expression, respectively. Any discordant scores were reviewed together by both pathologists to obtain a consensus score.

2.4 Statistical Methods

The χ^2 analysis was used to assess the associations between the expression of calretinin and molecular subtypes. Hierarchical clustering was used to detect the association between calretinin expression and ER, PR, HER2, EGFR, and CK5/6 expression by centroid method with data standardization. Overall survival (OS) time was calculated from the time of diagnosis until the time of death. The disease-free survival (DFS) was defined as the interval from the date of the primary surgery to the first locoregional recurrence or distant metastases. The method of Kaplan-Meier was used to generate OS or DFS curves, and curves were compared using a log-rank test. The prognostic significance of calretinin expression and the other clinical variables including tumor size, lymph node status, presence of metastasis, and American Joint Committee on Cancer (AJCC) stage was determined using a univariate Cox proportional hazards model. Multivariate analysis was done using a multivariate Cox proportional hazards model including the following variables: age, calretinin expression, and AJCC stage. All tests were 2 sided with 0.05 as the threshold to be considered statistically significant. All analyses were performed using SAS software, JMP Base version 8.0.0 (SAS, Cary, NC, USA).

3. Results

3.1 Clinicopathologic characteristics

The clinicopathological characteristics of the patients with grade 3 invasive ductal carcinomas are summarized in Table 1. Ninety-seven patients (45.3%) presented with T2 or larger tumors; 76 (35.5%), with nodal involvement; and 5 (2.3%), with metastatic disease.

Twenty-nine patients (13.6%) had advanced stage (3-4) disease at diagnosis (defined according to the AJCC) [20]. Clinical follow-up was available for all cases. Mean follow-up was 55 months (range, 1-211.6 months).

3.2 Calretinin expression in molecular subtypes of grade 3 invasive ductal carcinoma

No calretinin expression was identified in normal breast tissue (Figure 1A). In invasive ductal carcinomas calretinin exhibited both nuclear and cytoplasmic staining (Figure 1B). High-level calretinin expression (moderate or strong) was identified in 67 (31%) cases and low-level expression (negative or weak) in 147 (69%) of cases.

Hierarchical clustering analysis was performed to ascertain the distribution of high-level calretinin-expressing breast carcinomas amongst the various molecular subtypes (Figure 2). Cases with high expression of calretinin clustered in one group with EGFR and CK5/6. In contrast, cases with low-level calretinin expression clustered with ER+ and PR+ tumors.

While cluster analysis provided a comprehensive overview, correlation analysis examined the distribution of high- versus low-level calretinin expression amongst the various molecular subtypes and versus the individual surrogate markers (Table 2). High-level calretinin expression was observed in 11.1% of luminal A, 12.7% of luminal B, 33.3% of the HER2 enriched, 54.3% of basal-like, and 30% of the unclassified subtypes, respectively ($P<0.0001$). High-level calretinin expression was more frequently seen in association with CK5/6 or EGFR ($P<0.0001$) but only infrequently in tumors expressing ER ($P<0.0001$), PR ($P<0.0001$) or HER2 ($P=0.0018$). No significant association was found between high-level calretinin expression and other clinicopathological parameters including age, race, stage, tumor size, lymph node status, presence of metastases, and systemic adjuvant therapy ($P>0.05$).

3.3 Calretinin expression in metaplastic breast tumors

The basal-like group included 13 metaplastic carcinomas, of which six had a squamous component, five spindled/sarcomatous areas, and seven heterologous elements (five chondroid and two osseous). Four tumors contained two or more components. High-level calretinin expression was identified in five (38.5%) of these tumors; a smaller fraction than non-metaplastic basal-like carcinomas (57.4%). Four of these five cases were characterized by squamous differentiation (Figure. 1C). In cases with a spindle cell component, high-level staining was limited to epithelial areas; only low-level staining was present in the adjacent spindle cell component (Figure 1D).

3.4 Calretinin expression in grade 1 and 2 carcinomas

No high-level calretinin staining was identified in any of the grade 1 or grade 2 carcinomas. Low-level calretinin expression was identified in three (2.7%) of the grade 2 carcinomas (two ductal carcinomas, NST, and one mixed ductal and lobular carcinoma), but none of the grade carcinomas.

3.5 Calretinin expression and survival in grade 3 carcinoma

Univariate analysis of survival was performed in order to evaluate the impact of conventional prognostic predictors and calretinin expression on patient survival. Kaplan-Meier survival curves were constructed, followed by the log-rank test. Not unexpectedly, univariate analysis revealed that tumor stage significantly influenced overall patient survival ($P<0.0001$, Figure 3A). However, a significant correlation was also found between high-level calretinin expression and poor overall patient survival ($P=0.0096$, Figure 3B); 18% of patients with calretinin-high tumors died of disease compared to only 7% of patients with

calretinin-low tumors. No significant difference was found between high-level calretinin expression and disease-free survival (P=0.08).

As expression of calretinin was not restricted to tumors exhibiting a basal-like phenotype, analysis of overall and disease-free survival was also performed separately within the individual molecular subtypes. Only 11.1% of the tumors within the luminal A subgroup demonstrated high-level calretinin expression and none were associated with either death or disease recurrence. Within the luminal B subgroup, 12.5% of patients with calretinin-high tumors and 9.3% of patients with calretinin-low tumors died of disease; however, the difference did not reach statistical significance (P=0.89). None of luminal B tumors showed an association of calretinin expression with recurrent disease (P=0.49). Similarly, within the HER2-enriched subgroup, there was no statistically significant difference in either overall (P=0.83) or disease-free (P=0.29) survival between the calretinin-low or -high tumors. Amongst basal-like tumors, however, there was a significant difference in overall survival between calretinin subgroups with only 5% of patients with calretinin-low basal-like tumors succumbing to disease versus 23% of patients with calretinin-high tumors (P=0.039, Figure 3C). Despite the association with decreased overall survival, no significant difference in disease free survival between the calretinin subgroups was apparent in basal-like tumors (P=0.82). Multivariate analysis of survival revealed that the tumor stage (P=0.0002) and high calretinin expression (P=0.0023) were the only independent predictors of survival (Table 3).

4. Discussion

Calretinin is a widely used immunohistochemical maker of mesothelial cells and malignant mesothelioma. While relatively sensitive and specific for mesothelioma, as previously noted, expression of calretinin has been encountered in a wide variety of poorly differentiated carcinomas as well as tumors of mesenchymal origin. In this study we demonstrated that calretinin is strongly expressed in a cohort of patients with high-grade (grade 3) invasive ductal carcinoma and its expression is associated with a basal-like subtype of breast cancer.

To date, only a few studies have addressed the expression of calretinin in breast carcinoma. In a comprehensive immunohistochemical tissue microarray study involving more than five thousand tissue samples from 128 different tumor types, Lugli, *et al* identified calretinin expression in less than 10% of 158 breast carcinomas [13]. Strong calretinin expression was found in 44.4% of medullary carcinomas, 25% of apocrine carcinomas, 14.3% of papillary carcinomas, 1.9% of invasive ductal carcinoma and 4.4% of ductal carcinoma in-situ (DCIS). No calretinin immunoreactivity was detected in invasive lobular, cribriform or tubular carcinomas. Although the expression of calretinin in the study by Lugli *et al* was not analyzed in relationship to molecular subtype or hormone receptor status, the highest expression of calretinin was observed in medullary and apocrine breast carcinoma; tumor subtypes that frequently exhibit a basal-like phenotype [23, 24]. In contrast, tumors consistently negative for calretinin in the Lugli study included invasive lobular carcinoma, tubular and cribriform carcinomas; tumor subtypes that are usually low-grade and characteristically ER+ and only very rarely express basal markers [25]. Our findings of the lack of calretinin expression in special subtypes of breast carcinoma, including lobular, tubular, and cribriform are in complete agreement with the study by Lugli *et al* [13].

With 53% of basal-like tumors showing high-level expression of calretinin, our findings of the association of calretinin expression with basal-like tumors are in also in agreement with two recent, but much smaller studies by Powell *et al* and Duhig *et al* [10, 16]. Using a binary cutoff of 1%, Duhig *et al* observed calretinin expression in 28 of 53 (53%) cases of grade 2 and 3 breast carcinoma [16]. Their study contained 23 basal-like tumors, 17 (74%) of which

demonstrated calretinin expression, including eight cases with expression in >50% of tumor cells. Powell *et al* evaluated calretinin expression in 53 breast carcinomas, 16 (30%) of which were grade 3 [10]. Six of the grade 3 carcinomas in their study were categorized as basal-like, of which 4 (67%) expressed calretinin.

Our study also comprehensively analyzed calretinin expression in other molecular subtypes and addressed expression of calretinin in metaplastic carcinoma. Depending upon the immunohistochemical markers and inclusion criteria employed, expression of basal markers has been demonstrated in approximately 56 to 93% of metaplastic carcinomas, regardless of the type of metaplastic elements present [26, 27]. Neither of the aforementioned calretinin-focused studies addressed the distribution of calretinin positivity in the various epithelial and mesenchymal elements. The expression of calretinin in the epithelial and mesenchymal components of some of the metaplastic tumors is particularly interesting in light of the demonstration of variable calretinin expression in both the epithelial and sarcomatous components of synovial sarcoma [18]. Although in our study strong calretinin positivity was characteristic feature of epithelial component with predominant squamous metaplasia, we identified less positivity in mesenchymal spindle cell areas than might have been anticipated given the presence of calretinin expression in the spindle cell component of 55% of synovial sarcomas.

With the exception of a recent study by Kao, *et al* that identified increased calretinin expression as a poor prognostic indicator in patients undergoing extrapleural pneumonectomy for malignant mesothelioma, little has been reported about the prognostic implications of calretinin expression in human neoplasia [28]. Our study is the first to demonstrate a significant association between strong calretinin expression and poor overall survival in patients with basal-like subtype of breast cancer. As the expression of calretinin in the luminal and HER2 subtypes did not correlate with patient survival, significant correlation of strong calretinin expression with poor survival in the entire cohort of grade 3 cancers may be a function of the strong correlation seen in the basal-like group. The mechanism for reduced survival in calretinin-high basal-like breast tumors is unclear. A role for calretinin in the epithelial-mesenchymal transition may exist, as basal-like breast carcinomas have long been known to frequently express vimentin, and more recently epithelial to mesenchymal transition inducers and tumor initiating cells have been identified in metaplastic carcinomas, although low calretinin expression in mesenchymal component does not entirely support this hypothesis [29-30].

Secondly, increased expression of other calcium-binding proteins has been identified in breast neoplasia. For example, a number of the S100 calcium-binding protein family members have been identified in breast carcinoma, including S100A4, S100A7 and S100P, for which increased expression has been associated with biological aggressiveness [31-35]. As the potential contributory roles for the S100 protein family in neoplasia and biological aggressiveness are myriad, similar roles for other calcium-binding proteins such as calretinin in cellular processes such as peritumoral inflammation, cellular motility and modulation of cellular growth are not inconceivable. Further characterization of proteomic and molecular pathways involving calretinin may lead to novel therapeutic approaches.

In summary, we demonstrated a differential calretinin expression in the four molecular subtypes of grade 3 invasive ductal carcinoma. High-level calretinin expression appears to be a strong predictor of adverse prognosis.

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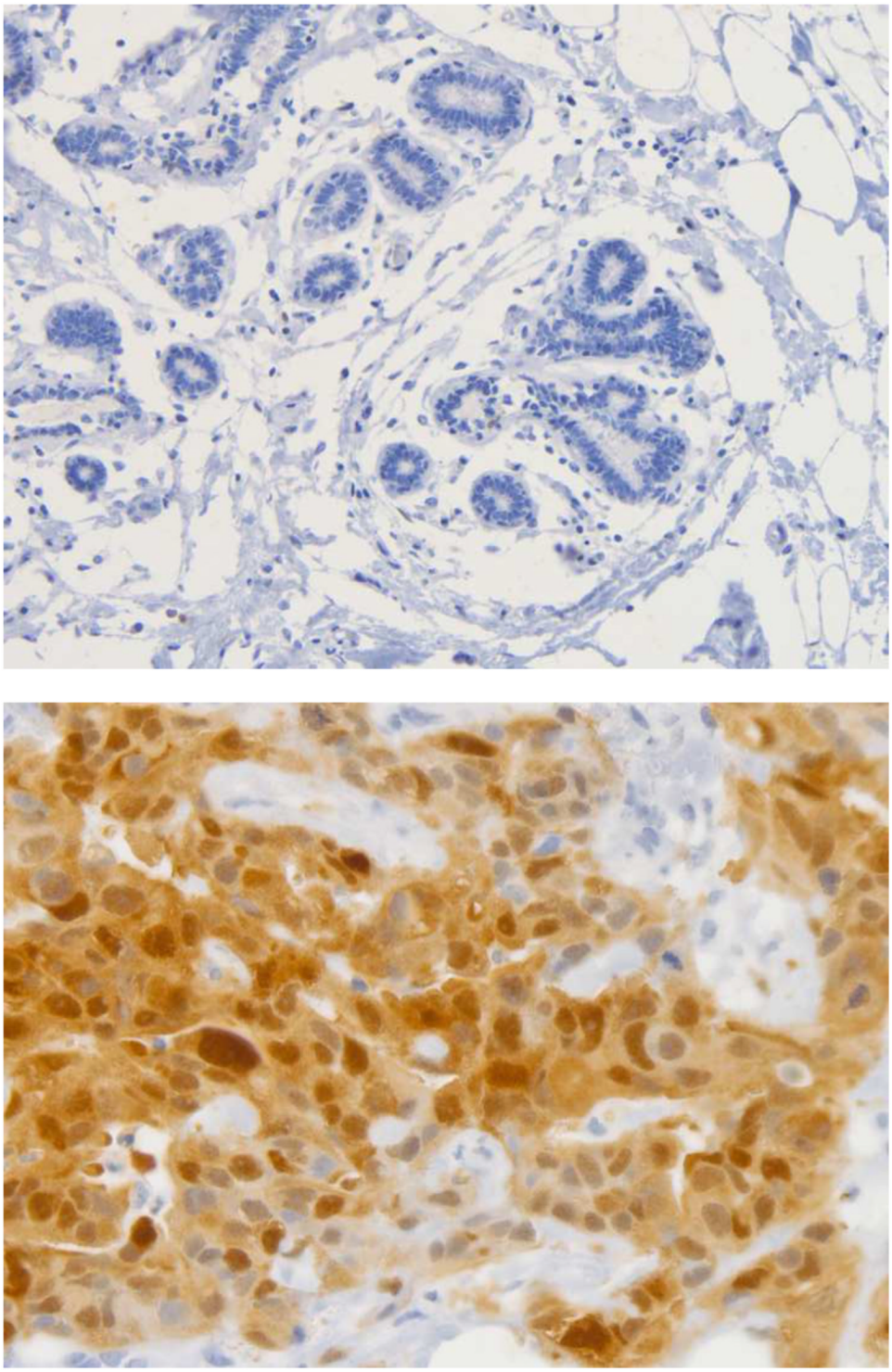


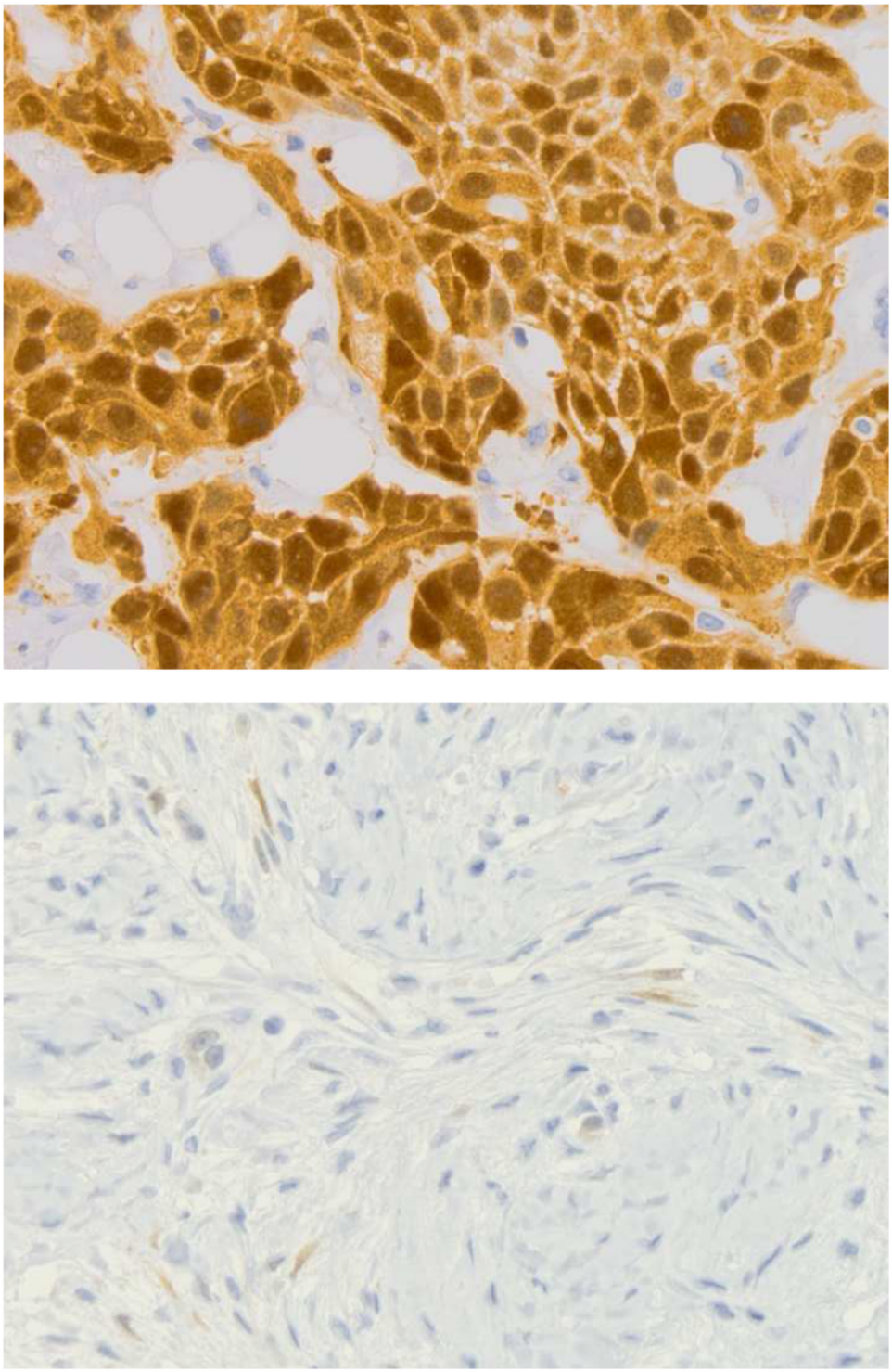


Figure 1.

Calretinin expression in non-neoplastic and malignant breast tissue. Non-neoplastic breast tissue is negative for calretinin staining (A, ×200). Strong nuclear and cytoplasmic immunoreactivity in basal-like breast carcinoma (B, ×400). Strong nuclear and cytoplasmic calretinin immunostaining in squamous component of metaplastic carcinoma. Note

intercellular bridges indicative of squamous differentiation (C, ×400). Weak focal calretinin immunoreactivity in mesenchymal spindle cell component of metaplastic carcinoma (D, ×400).

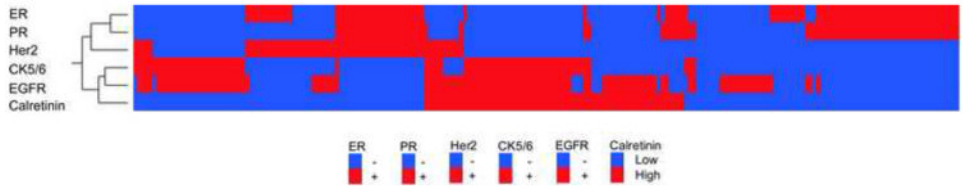
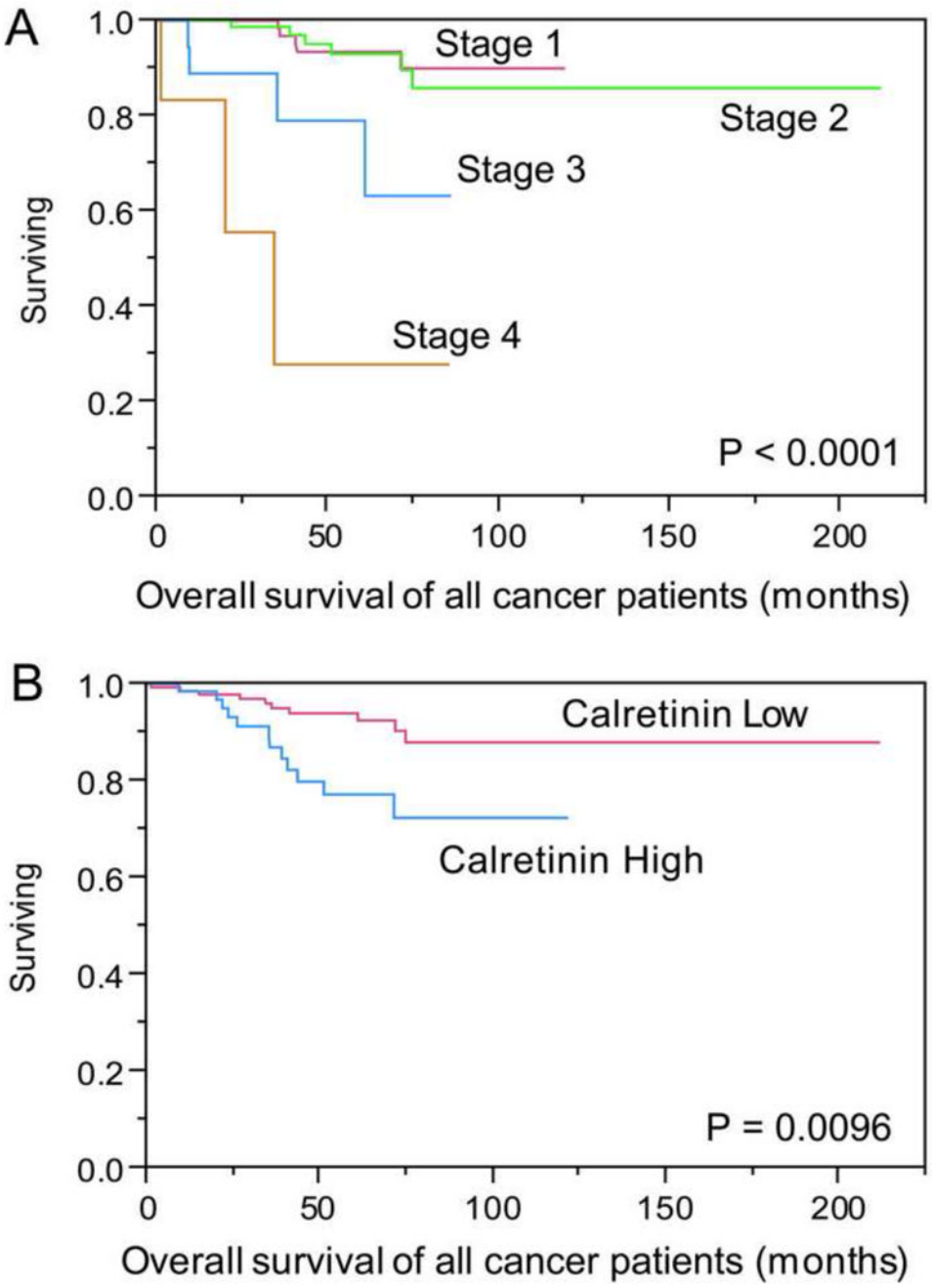


Figure 2.

Hierarchical clustering analysis (Complete method with data standardization) of the expression of calretinin, estrogen receptor (ER), progesterone receptor (PR), HER2, CK5/6, and epidermal growth factor receptor (EGFR) in grade 3 carcinoma. Each column represents a different tumor, and each row a marker. Red: highest expression; blue: lowest expression. The analysis shows that calretinin-high cases are clustered predominantly with cases positive for basal markers CK5/6 and EGFR, as indicated by short dendrogram branches linking these markers. Calretinin-low cases are clustered with ER and PR positive tumors.



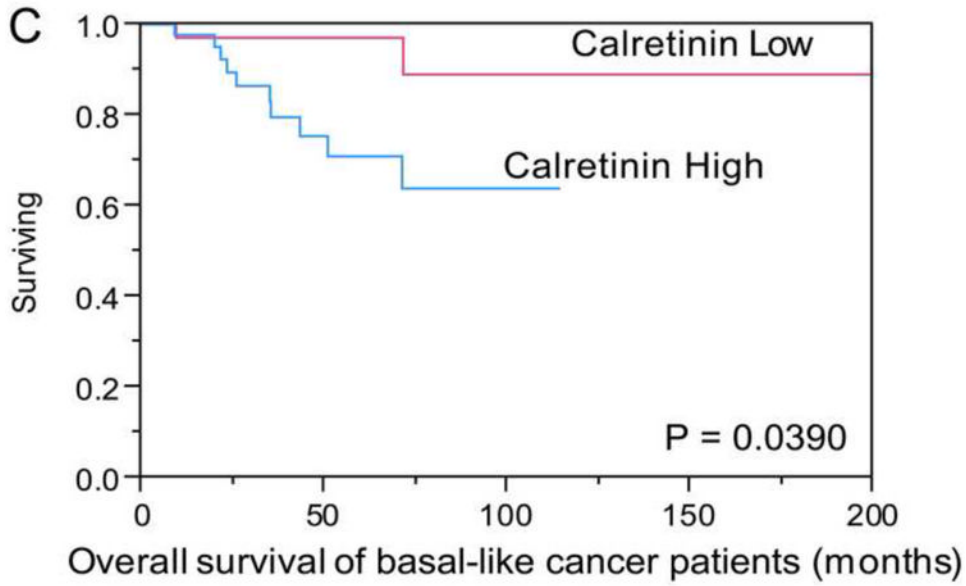


Figure 3.

Analysis of overall survival in grade 3 invasive ductal carcinoma by stage (A), calretinin expression (high vs low) in the entire cohort (B), and calretinin expression (high vs low) in basal-like subtype only (C).

Table 1

Clinicopathologic characteristics of patients with different molecular subtypes of grade 3 breast carcinoma

Parameter	Total	Molecular subtype					
		Luminal A	Luminal B (Her2-)	Luminal B (HER2+)	HER2-enriched	Basal-like	Unclassified
Sample Size (%)	214	36 (16.8%)	26 (12.1%)	37 (17.3%)	24 (11.2%)	81 (37.9%)	10 (4.7%)
Mean age (years)+/-SD	214	65.3 +/- 15.9	61.0+/-14.9	59.9+/-14.7	61.1+/-16.0	58.4+/-15.2	63.9+/-12.4
Race							
White	193	34 (94.4%)	26 (100%)	35 (94.6%)	21 (87.5%)	68 (84.0%)	9 (90%)
Black	21	2 (5.6 %)	0	2 (5.4%)	3 (12.5%)	13 (16.0%)	1 (1%)
AJCC Stage							
Stage 1	88	16 (44.4%)	6 (23.1%)	16 (43.2%)	11 (45.8%)	33 (40.7%)	6 (60%)
Stage 2	97	17 (47.2%)	12 (46.2%)	18 (48.6%)	10 (41.6%)	37 (45.7%)	3 (30%)
Stage 3	22	2 (5.6%)	6 (23.1%)	3 (8.1%)	2 (8.3%)	8 (9.9%)	1 (1%)
Stage 4	7	1 (2.8%)	2 (7.7%)	0	1 (4.2%)	3 (3.8%)	0
Tumor status							
T1	117	18 (50%)	12(46.2%)	23 (62.2%)	12 (50%)	44 (54.3%)	8 (80%)
T2	80	15 (41.7%)	10 (38.5%)	11 (29.7%)	12 (50%)	31 (38.3%)	1 (10%)
T3	9	1 (2.8%)	1 (3.8%)	3 (11.1%)	0	3 (3.7%)	1 (10%)
T4	8	2 (5.6%)	3 (11.5%)	0	0	3 (3.7%)	0
Node status							
N0	114	18 (50%)	8 (30.8%)	21 (56.8%)	15 (62.5%)	45 (55.6%)	7 (70%)
N1	56	6 (16.7%)	11 (42.3%)	11 (29.7%)	6 (25%)	20 (24.7%)	2 (20%)
N2	15	2 (5.5%)	4 (15.4%)	0	2 (8.3%)	6 (7.4%)	1 (10%)
N3	5	1 (2.8%)	0	2 (5.4%)	1 (4.2%)	1 (1.2%)	0
Nx	24	9 (25%)	3 (11.5%)	3 (8.1%)	0 (3.1%)	9 (11.1%)	0
Metastasis status							
M0	156	21 (58.3%)	18 (69.2%)	29 (78.4%)	20 (83.3%)	60 (74.1%)	8 (80%)
M1	5	1 (2.8%)	1 (3.8%)	0	1 (4.2%)	2 (2.5%)	0
Mx	53	14 (38.9%)	7 (26.9%)	8 (21.6%)	3 (12.5%)	19 (23.5%)	2 (20%)

Table 2

Associations of calretinin expression with different molecular subtypes and other immunohistochemical markers in grade 3 breast cancer

	Calretinin		
	Low (0,1)	**High (2,3)**	**P-value**
Total	147 (69%)	67 (31%)	
Molecular subtype			< 0.0001
Luminal A	32 (88.9%)	4 (11.1%)	
Luminal B	55 (87.3%)	8 (12.7%)	
Luminal B (HER2-)	20 (76.9%)	6 (23.1%)	
Luminal B (HER2+)	35 (94.6%)	2 (5.4%)	
HER2-enriched	16 (66.7%)	8 (33.3%)	
Basal-like	37 (45.7%)	44 (54.3%)	
Unclassified	7 (70%)	3 (30%)	
ER			< 0.0001
ER-	65 (52.4%)	59 (47.6%)	
ER+	82 (91.1%)	8 (8.9%)	
PR			< 0.0001
PR-	81 (58.7%)	57 (41.3%)	
PR+	66 (86.8%)	10 (13.2%)	
Her2			0.0018
Her2-	96 (62.7%)	57 (37.3%)	
Her2+	51 (83.6%)	10 (16.4%)	
CK5/6			< 0.0001
CK5/6-	113 (79.6%)	29 (20.4%)	
CK5/6+	34 (47.2%)	38 (52.8%)	
EGFR			< 0.0001
EGFR-	92 (89.3%)	11 (10.7%)	
EGFR+	55 (49.6%)	56 (50.5%)	

Table 3

Multivariate survival analysis (Cox regression model) of grade 3 breast cancer patients

Risk factors	RR[a]	95% CI[b] of RR	P-value
Calretinin high vs low	4.76	1.76-13.64	0.0023
Age	0.98	0.94-1.03	0.1196
Cancer Stage			0.0002
Stage 2 vs 1	0.86	0.32-4.05	0.86
Stage 3 vs 2	6.69	1.65-24.5	0.0099
Stage 4 vs 3	4.35	0.91-24.1	0.0632

[a]RR, relative risk;

[b]CI, confidence interval