ColXα1 is a stromal component that colocalizes with elastin in the breast tumor extracellular matrix

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Abstract

The tumor microenvironment regulates tissue development and homeostasis, and its dysregulation contributes to neoplastic progression. Increased expression of type X collagen α-1 (ColXα1) in tumor-associated stroma correlates with poor pathologic response to neoadjuvant chemotherapy in estrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2)-positive breast cancers. Evaluation of ColXα1 expression patterns suggests a potential connection with elastin fibers. To investigate the possible interaction between ColXα1 and elastin, we evaluated the expression of ColXα1 in relation to elastin fibers in normal breast tissue, ductal carcinoma in situ, and invasive breast carcinomas at cellular and subcellular levels. Our findings demonstrate that ColXα1 colocalizes with elastin in invasive breast cancer-associated stroma by immunohistochemistry, immunofluorescence, and electron microscopy. In 212 invasive breast carcinomas, this complex was aberrantly and selectively expressed in tumor extracellular matrix in 79% of ER+/HER2−, 80% of ER+/HER2+, 76% of ER−/HER2+, and 58% of triple negative breast cancers. In contrast, ColXα1 was generally absent, while elastin was present perivascularly in normal breast tissue. ColXα1 and elastin were coexpressed in 58% of ductal carcinoma in situ (DCIS) in periductal areas. In mass-forming DCIS with desmoplastic stroma, the complex was intensely expressed in periductal areas as well as within the tumor-associated stroma in all cases. Our data suggest that the breast carcinoma neoplastic process may involve aberrant expression of ColXα1 and elastin in the tumor microenvironment emerging early at the DCIS stage. Enrichment of these complexes in tumor-associated stroma may represent a stromal signature indicative of intrinsic differences between breast cancers. These findings shed light on investigation into the role of aberrant collagen complex expression in tumorigenesis and tumor progression which may be leveraged in therapeutic and theranostic applications.

Keywords: tumor microenvironment; type X collagen α-1; ColXα1; elastin; extracellular matrix; breast cancer; neoadjuvant

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Conflict of interest statement: YW, ASB and MBR declare that a patent application has been approved 7/2017 titled as Collagens as Markers For Breast Cancer Treatment to ASB, YW, and MBR of Rhode Island Hospital, A Lifespan-Partner (Application No. 15/187279). SL, JX, KS, YH, CZ, DY, GJ, MO, CS, and RAD have nothing to disclose.

Introduction

Neoplastic progression is attributed to the accumulation of somatic mutations in epithelial cells. Neoplastic behavior is also influenced by the tumor microenvironment that includes the extracellular matrix (ECM), blood and lymphatic vasculature, inflammatory cells, and fibroblasts [1]. The ECM contributes to diverse functions. ECM dynamics are tightly regulated to ensure normal development, physiology, and robustness of organ systems. When such control mechanisms are disrupted, the resultant deregulation contributes to the initiation and progression of cancer [2].
ColXα1 and elastin colocalize in the breast tumor stroma

The ECM is composed of a collection of biochemically and structurally diverse components, each with diverse subcategories contributing to various physical and biochemical properties. Some ECM proteins, including fibrillar collagens and elastin, form fibrils from protein monomers contributing to tensile strength and viscoelasticity of the tissue [3,4]. In tumors including head and neck, cervical, esophageal and prostate cancer, stromal properties have been shown to affect disease progression and patient prognosis [5–9]. Although the specific pathophysiologic processes driving collagen reorganization in breast cancer remain unclear, the cross-linked and orientated collagen in cancer tissue is a reliable marker associated with poor survival, regardless of tumor grade, size, subtype, estrogen receptor (ER) expression, progesterone receptor (PR) expression, and nodal status [10]. High stromal content predicts poor survival in triple negative breast cancer [11]. Increased collagen VI deposition stimulates cancer cell proliferation [10,12]. ColVα2 and ColXα1 are highly expressed in invasive ductal carcinoma compared to ductal carcinoma in situ (DCIS) and are involved in triggering cancer cells to disseminate [13,14]. Together, these studies lend credence to the influence of collagen deposition in cancer growth and metastasis.

Type X collagen α-1 (ColXα1) is a short-chain collagen, typically found underlying endothelial cells and in the hypertrophic zone of cartilage during endochondral ossification where it participates in calcifying cartilage formation [15]. ColXα1 is encoded by the COL10A1 gene, which is expressed by hypertrophic chondrocytes. Mutations in COL10A1 are associated with Schmid-type metaphyseal chondrodysplasia and Japanese-type spondylometaphyseal dysplasia [16]. We previously found that increased expression of ColXα1 was predictive of poor pathologic response in neoadjuvant-treated ER+/HER2+ breast tumors [17]. Although increased stromal collagen content has been clinically documented in breast cancers, its specific pattern of distribution and relationship to the malignant epithelial component and other ECM components is poorly understood.

Elastin is normally expressed in significant quantities in skin, lung, cartilage, and large arteries. Elastin fibers provide recoil to tissues subjected to repeated stretching motions. Importantly, elastin stretching is limited by tight association with collagen fibrils [18]. Together, collagen, elastin, and other ECM proteins such as fibronectin and tenascin influence cellular behavior including the promotion of fibroblast migration during wound healing, tumor growth, and metastasis [19,20].

The ECM associated with breast carcinoma is comprised of large aggregates of elastin fibers, known as elastosis [21–23]. Elastin can be cleaved into small peptide fragments, which can affect cellular processes including apoptosis, chemotaxis, and metastasis [24,25]. ColXα1 exhibits a patchy pattern of expression in breast tumors which is reminiscent of the elastosis patterns. We hypothesized that elastin and ColXα1 colocalize. To test this hypothesis, publically available data were collected and analyzed using Oncomine (Thermo Fisher Scientific, Waltham, MA, USA) for COL10A1 and elastin. Normal breast tissue, DCIS, and breast tumors were then examined through immunohistochemical, immunofluorescent, and electron microscopic techniques to assess ColXα1 and elastin expression and localization.

Materials and methods

Case selection

With institutional review board approval at Rhode Island Hospital (467617-9) and Women Infants Hospital (797108-3), human tissues from 2009 to 2017 were obtained for study. We evaluated 52 normal breast specimens from 26 reduction mammoplasties, 51 DCIS, and 212 breast cancer specimens (Table 1). The DCIS group included low, intermediate, and high nuclear grade lesions. Forty-three cases were DCIS with associated calcifications with some showing necrosis and normal appearing stroma. Eight were mass-forming exhibiting stromal changes resembling desmoplasia akin to those found in invasive cancer. The invasive tumors included breast cancers of all four molecular subtypes.

Pathological evaluation

For patients treated with neoadjuvant chemotherapy (NAC), pathologic response was assessed by the AJCC cancer staging and residual cancer burden (RCB) score [26,27]. The RCB system stratifies patients into classes I, II, and III (RCB class 0 is synonymous with having achieved a pathological complete response (pCR); on-line calculator is available at http://www.mdanderson.org/breastcancer_RCB). Patients who achieved a pCR or minimal residual disease (RCB class I) were considered good responders, while RCB class II and III were considered poor responders. For patients treated with primary surgery followed by adjuvant treatment, the outcome measure in this analysis was progression-free survival, defined as time from
surgery to documented progression or death. Patients who had no recurrence or death were considered good responders and patients who had recurrence or death were defined as poor responders. Overall survival (OS) was not analyzed due to the low overall death rate.

Bioinformatics

COL10A1 and elastin gene expression in breast cancer or cancer stroma was interrogated through Oncomine (www.oncomine.com, December 2017, Thermo Fisher Scientific) using filters including Gene name, ‘Cancer versus Normal Analysis’, and ‘Breast Cancer’. Curated breast cancer studies in Oncomine were selected. Analyses were focused on studies with normal tissue, with or without DCIS, and invasive cancer. Both whole tumor tissue extract and stroma-only studies were included.

Chi-square analyses were used to evaluate the correlation of ColXα1 and elastin expression with patient outcome. Statistical analyses were performed using JMP 13.0 (SAS, Cary, NC, USA).

Immunohistochemistry and expression scoring

Four-micron sections were cut from formalin-fixed paraffin-embedded (FFPE) tissue blocks, heated at 60 °C for 30 min, deparaffinized and rehydrated. These were then subjected to antigen retrieval by heating in epitope retrieval buffer in a 95 °C water bath for 45 min. The slides were incubated with either mouse monoclonal antibodies or rabbit polyclonal antibodies for 30 min at room temperature, then with 2% bovine serum albumin (BSA) for 30 min rinsed, 1+ for <5% stroma tissue, 2+ for 5–10% of stroma tissue, and 3+ for >10% of stroma tissue. All scoring was performed blinded to the diagnosis. Developing cartilage and cartilaginous tumors were used as positive controls while normal bone and breast tissue were used as negative controls. Cases with a ColXα1 score 0 and elastin score 1–3+ or vice versa were not considered as coexpression or discordant expression patterns.

Electron microscopy

Post-embedding double labeling immunogold (colloidal-gold) electron microscopy was performed as follows. Specimens were fixed in 0.1% glutaraldehyde in sodium cacodylate (0.1 M: pH 7.4) at 37 °C for 3 h, washed in the same buffer, then dehydrated in 70–100% ethyl alcohol, placed in LR White resin (hard grade) for 2–3 h with at least two changes of resin, and then placed in a 50 °C oven overnight.

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Semi-thin sections (1 μm) were cut and examined under a light microscope for areas of interest. Pale gold ultra-thin sections were then cut on a Reichert-Jung Ultracut E ultramicrotome (Reichert, Depew, NY, USA) and collected on nickel grids. Sections were blocked with 0.4% BSA in PBS with 2% normal goat serum for 45 min, incubated overnight at 37 °C in polyclonal anti-elastin and anti-ColXα1 antibodies at 1:400 and 1:50 dilutions respectively. They were subsequently washed and placed in their respective secondary antibodies: IgG conjugated to 10 nm and 25 nm gold labels diluted 1:30 with 0.2% BSA in PBS for 90 min. Staining with uranyl acetate was performed and the tissue was examined under a Philips CM10 electron microscope. Digital images were acquired using a Gatan Erlangshen ES 1000 W camera (Gatan, Pleasanton, CA, USA).

Results

COL10A1 gene expression is increased in DCIS and invasive breast cancer

COL10A1 gene expression data were extracted from five studies examining whole tumor and three studies that assayed tumor cell and stroma separately [23,28–34]. Concurrently, elastin expression levels were studied. The data are summarized in Table 2. All eight studies showed significantly increased COL10A1 gene expression in DCIS and higher expression in invasive cancer compared to normal control breast tissue. In contrast, elastin gene expression levels were similar between normal breast tissue, DCIS, and invasive cancer. When whole extracts of tumor tissue were examined, COL10A1 expression levels increased 4.37–6.79-fold and 5.87–43.5-fold in DCIS and invasive cancers respectively. In studies evaluating only stroma, COL10A1 expression level increased 82.6-fold and 10.8–132-fold in DCIS and invasive tumors respectively.

ColXα1 and elastin expression in normal breast tissue

Since ColXα1 is expressed in developing cartilage and chondrogenic tumors [16], ColXα1, elastin IHC, and elastic special stains (Verhoff’s elastic stain) were first evaluated in normal mature bone and cartilage as well as in cartilaginous tumors including one cystic teratoma with cartilage, one enchondroma, and three well-differentiated chondrosarcomas. ColXα1 showed strong positive staining in portions of developing cartilage, enchondroma, and well-differentiated components of chondrosarcomas. Mature cartilage and the dedifferentiated component of chondrosarcoma lacked expression. In contrast, elastin was absent in cartilaginous tumors and present in the mature cartilage matrix as well as the cystic teratoma with developing cartilage.

ColXα1 and elastin expression in DCIS by IHC

The 51 DCIS cases studied included 43 non-mass-forming DCIS cases with normal-appearing stroma and 8 mass-forming DCIS cases with desmoplastic stromal reaction. Compared to normal breast tissue, there was a quantitatively significant increase in staining for ColXα1 and elastin in the DCIS cases (p < 0.001). In the 43 non-mass-forming DCIS cases with normal appearing stroma, there was no expression of ColXα1 in the stroma. Elastin in these cases was identified in perivascular and scant periductal patterns resembling those of normal breast stroma. However, in the expanding periductal areas of DCIS, ColXα1 was expressed in a periductal distribution in all cases. We noted that periductal ColXα1 staining was heterogeneous around DCIS. In some cases, staining was sparse, involving few periductal foci while in others staining was extensive involving all periductal areas. Among the 51 cases, 20 scored 1 (39.2%), 10 scored 2 (19.6%), and 3 scored 3 (17.6%). In contrast, stromal ColXα1 expression was present in all eight cases with mass-forming DCIS. In those cases, ColXα1 was not only present in periductal areas as seen in non-mass forming DCIS, but also in DCIS-associated reactive desmoplastic stroma in seven of eight cases. Elastin was coexpressed in 30 of 43 non-mass-forming DCIS cases and in all mass-forming DCIS cases. Elastin was coexpressed with ColXα1 in reactive desmoplastic stroma and periductal areas surrounding the expanding DCIS (Table 3 and Figure 1C–E).

ColXα1 and elastin are coexpressed in all molecular subtypes of invasive breast carcinoma

We evaluated 212 invasive breast cancer cases with both ColXα1 and elastin staining, including 56

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Table 2. Expression of COL10A1 and elastin genes in breast cancer and its stroma

| Study               | Index | Normal | DCIS  | Invasive lobular | Invasive ductal | DCIS | Invasive lobular | Invasive ductal |
|---------------------|-------|--------|-------|-----------------|-----------------|------|-----------------|-----------------|
| TCGA breast [28]    | Fold change | 1      | 15.9  | 43.5            | 1.59            | −1.01|
|                     | P value   |        | 7.22E−43 | 1.28E−52        | 6.52E−5         | 0.549|
|                     | Case number | 61     | 36    | 389             | 36              | 389  |
| Curtis et al, 2012 [29] | Fold change | 1      | 6.79  | 6.68            | 8.74            | 1.09 | 1.262           | 1.00            |
|                     | P value    |        | 2.63E−5 | 1.72E−57        | 3.32E−22        | 0.046| 1.75E−4         | 0.375           |
|                     | Case number | 144    | 10    | 148             | 1556            | 10   | 148             | 1556            |
| Richardson et al, 2006 [30] | Fold change | 1      | 13.6  | 1.05E−14        | 1.012           | 1.012|
|                     | P value    |        | 40    | 40              | 40              | 40   | 40              | 40              |
| Turashvili et al, 2007 [31] | Fold change | 1      | 19.4  | 12.9            | 2.84            | −1.10|
|                     | P value    |        | 0.006 | 0.005           | 0.098           | 0.583|
|                     | Case number | 10     | 10    | 10              | 10              | 10   | 10              | 10              |
| Radvanyi et al, 2005 [32] | Fold change | 1      | 4.37  | 5.87            | 7.16            | 3.71 | 2.88            | 1.22            |
|                     | P value    |        | 0.042 | 0.021           | 0.013           | 0.122| 0.107           | 0.393           |
|                     | Case number | 7      | 3     | 7               | 3               | 3    | 7               | 3               |
| Ma et al, 2009 [33]* | Fold change | 1      | 82.6  | 132             | 1.03            | 1.02 |
|                     | P value    |        | 1.43E−8 | 2.89E−6         | 0.298           | 0.379|
|                     | Case number | 14     | 11    | 9               | 11              | 9    | 9               | 9               |
| Finak et al, 2008 [23]* | Fold change | 1      | 16.8  | 4.35            | 6.55E−9         | 9.55E−9|
|                     | P value    |        | 1.25E−6 | 9.55E−9         | 9.55E−9         | 9.55E−9|
|                     | Case number | 6      | 53    | 53              | 53              | 53   | 53              | 53              |
| Karnoub et al, 2007 [34]* | Fold change | 1      | 10.8  | 1.30            | 0.225           | 0.225|
|                     | P value    |        | 5.80E−4 | 0.225          | 0.225           | 0.225|
|                     | Case number | 15     | 7     | 7               | 7               | 7    | 7               | 7               |

The table summarizes eight studies reanalyzed through Oncomine [23,28–34]. The first five studies are based on the entire tumor including both epithelial and stromal components. In the remaining three studies, stromal tissue was dissected out and the analyses were based solely on stroma. All the studies used normal tissue as control. The gene expression levels of COL10A1 and elastin in DCIS and/or invasive cancers were compared to the levels in normal tissue. Expression levels in tumor are presented as fold changes of normal tissue (whose fold change was set as one). P values are listed below the fold change to illustrate statistical significance.

*Only the expression in stroma was studied in these reports.

ER+/HER2−, 50 ER+/HER2+, 54 ER−/ HER2+, and 52 triple negative tumors.

In invasive carcinoma, a significant increase of ColXα1 and elastin in the tumor-associated stroma was readily apparent in all histological grades and molecular subtypes.

ColXα1 and elastin were coexpressed in the stroma of invasive carcinomas in 155 of the 212 (73.1%) cases. The distribution of expression among the different molecular subtypes is summarized in Table 3. Twenty-three tumors (23/212, 10.9%) lacked expression of either ColXα1 or elastin. Thirty-four of 212 (16.1%) cases were discordant in ColXα1 and elastin expression. Among these, 5 cases expressed only ColXα1 while 29 cases expressed only elastin. In 22 of the 34 discordant cases, the discrepancy between elastin and ColXα1 staining was minor with a difference in scores of 0 to 1. ColXα1 was expressed in a patchy pattern within the stroma (Figure 1F,G). In cases where DCIS was admixed with invasive tumor encased in dense stroma, ColXα1 was expressed in a periductal distribution similar to DCIS only cases. When elastin was coexpressed with ColXα1, it was present in a similar pattern as ColXα1 in tumor-associated stroma in the vast majority of the cases. In addition, elastin was also present in the intratumoral vasculature that demonstrated minimal to absent ColXα1 expression.

Outcome data were available in a subset of the invasive tumors (184 of 212). This included 92 neoadjuvant-treated cases and 92 primary surgery cases followed by adjuvant treatment and average follow up of 33 months (range 12–88 months). Tumors with different ColXα1 and elastin status correlated significantly with outcome, that is, high ColXα1/elastin expression correlated significantly with poor response in neoadjuvant-treated patients (p = 0.027, see supplementary material, Table S1). Both ColXα1 expression and ColXα1/elastin complex were significantly correlated with outcome, that is, high ColXα1 alone or ColXα1/elastin expression correlated significantly with poor response in neoadjuvant-treated cases by Chi-square analysis (p = 0.0127 and 0.0274, respectively). Similar correlations were not identified in the adjuvant setting. When separating the tumors into different molecular subtypes, high expression of ColXα1 and/or elastin correlated with poor outcome (p ranges from 0.0487 to 0.0735) in ER+/HER2− and...
ColXα1 and elastin colocalize in the breast tumor stroma

Figure 1. Elastin and ColXα1 immunohistochemistry staining of normal breast and breast tumors. (A,B) In normal breast stroma, ColXα1 was largely negative by IHC apart from occasional perivascular and periductal faint staining. Elastin was present around normal structures including periductal, lobular, and perivascular areas. Scattered elastin staining was also present in normal breast stroma. (C,D) Non-mass-forming DCIS, D at high magnification: ColXα1 and elastin expression in a periductal pattern. Elastin highlighted the vessels while ColXα1 was negative. (E) Mass-forming DCIS: ColXα1 and elastin coexpression in a periductal and stromal pattern of distribution. (F,G) ColXα1 and elastin were strongly expressed in a similar patchy distribution in an ER+/HER2+ breast cancer (F) and a triple negative breast cancer (G). (H) Invasive ductal carcinoma with DCIS. ColXα1 and elastin expression were present in a periductal pattern and within the stroma. Scale bars = 10 μm.
Figure 1. (Continued)
ER+/HER2+ groups, although the patient numbers in each group were small (49 and 46, respectively). Grouping the ER+ tumors irrespective of their HER2 status, ColXα1/elastin complex was associated with poor outcome ($p = 0.0466$ and $p = 0.0186$ respectively) (see supplementary material, Table S1).

ColXα1 and elastin colocalize in breast tumor-associated stroma by IF and immunoelectron microscopy

The distribution and colocalization of ColXα1 and elastin in the breast tumor ECM was determined using IF (Figure 2). ColXα1 and elastin was patchily distributed in the ECM similar to patterns seen by IHC. ColXα1 staining colocalized with elastin throughout the ECM. Elastin appeared to be expressed more extensively in ECM without ColXα1. Tumor stroma and perivascular areas were also replete with elastin. Stromal areas expressing ColXα1 and elastin were used for immunoelectron microscopy (IEM) to elucidate subcellular morphology and localization. With IEM, we identified irregular amorphous aggregates of material that stained with both anti-ColXα1 and anti-elastin antibodies (Figure 3). This revealed that elastin is not deposited in the ECM as fibrils. Rather, it forms amorphous aggregates of polymerized tropoelastin. ColXα1, a nonfibrillar collagen, was also

Table 3. Elastin and ColXα1 expression in DCIS and invasive carcinomas

| Subtypes | Elastin --/ColXα1-- (%) | Elastin +/ColXα1+ (%) | Elastin --/ColXα1+ (%) | Elastin +/ColXα1-- (%) |
|---|---|---|---|---|
| Normal (52 specimens in 26 patients) | 11 (21.2) | 0 | 0 | 41 (78.8) |
| DCIS (51) | 1 (2.0) | 30 (58.8) | 9 (17.7) | 11 (21.6) |
| Invasive (212) | | | | |
| ER+/HER2− (56) | 5 (8.9) | 44 (76.8) | 2 (3.6) | 5 (8.9) |
| ER+/HER2+ (50) | 3 (6.0) | 40 (80.0) | 3 (6.0) | 4 (8.0) |
| ER−/HER2+ (54) | 5 (9.3) | 41 (76.0) | 0 (0) | 8 (14.2) |
| ER−/HER2− (52) | 10 (19.2) | 30 (57.7) | 0 (0) | 12 (23.1) |
| All invasive subtypes (212) | 23 (10.9) | 155 (73.1) | 5 (2.4) | 29 (13.7) |

Figure 2. Immunofluorescence staining of elastin and ColXα1 in tumor-associated stroma. DAPI highlights the tumor; elastin and ColXα1 are distributed and colocalized in tumor-associated stroma. Intratumoral vessels stain with elastin but not ColXα1. Scale bar = 10 μM.
identified colocalizing with elastin in the amorphous material located near the fibroblast. Bundles of collagen fibrils consisting of type I collagen were also seen in the stroma.

Discussion

The tumor microenvironment plays an integral role in the neoplastic process. Tumorigenesis and progression are influenced by biochemical and biomechanical properties of the tumor microenvironment [35]. The ECM in the tumor microenvironment serves as the scaffold upon which tissue is organized. Along with vasculature and immune cells, it provides the framework and critical cues that direct cell growth, survival, differentiation and migration. Thus, a dynamically evolving tumor microenvironment modulates tumor cell behavior [36,37].

During our previous study of ColXα1 expression, we observed a possible overlap with putative elastin fibers. Oncomine data analysis demonstrated that COL10A1 RNA was significantly increased in both the tumor and tumor-associated stroma. We then systematically combined traditional pathologic review with immunohistochemical, immunofluorescent, and IEM techniques to investigate stromal ColXα1 and elastin organization in normal and neoplastic breast tissue. These observations endorse our hypothesis that most ColXα1 expressed in breast tumors is associated with elastin. However, not all of the elastin is associated with ColXα1. Overlapping ColXα1 and elastin expression was only observed in breast cancers, not in normal breast or normal tissues that exclusively express elastin, suggesting that this colocalization is specific for neoplastic transformation.

ColXα1 is a collagen type specific to the chondrocyte lineage. Functional studies predicate its importance in maintaining the stem cell pool and driving differentiation [38]. This study found that ColXα1 is virtually absent in normal breast tissue but is expressed in a variable periductal distribution pattern surrounding areas of DCIS. In 58% of the cases, ColXα1 expression was accompanied by elastin. In non-mass-forming DCIS cases, ColXα1 is recruited to

Figure 3. Immunoelectron microscopy of ColXα1 and elastin localization in the tumor microenvironment using double labeling. The micrograph shows a ColXα1 and elastin complex (CE) as patchy amorphous material in the extracellular space near a fibroblast nucleus which is double stained with gold conjugated anti-ColXα1 antibody (10-nm gold particles) and gold conjugated anti-elastin antibody (25-nm gold particles). The field also contains collagen fibrils (C) and fine cytoplasmic extension of the fibroblasts (F) responsible for elaboration of the extracellular constituents. Original magnification (×25 000). Low (A), intermediate (B), and high (C) magnifications are shown. Larger particles – elastin (long arrows) and smaller particles – ColXα1 (short arrows) are identified in (C).
the periductal region of DCIS despite the absence of histologically apparent stromal reaction. In mass-forming DCIS with reactive stroma [39], ColXα1/elastin complex is expressed in a pattern similar to that of the stroma associated with invasive cancer. This aberrant expression of ColXα1 raises the possibility that it may herald the initiation of neoplastic stromal transformation. Predicting tumor progression is an unresolved issue and a clinical concern. While histologic factors such as nuclear grade and hormone receptor expression have been extensively scrutinized, less is understood regarding the impact of ECM alterations. Our observations endorse the hypothesis that ColXα1 in the tumor microenvironment of preinvasive lesions is an indicator of neoplastic progression. In pancreatic adenocarcinoma, Shi et al demonstrated that low-grade pancreatic intraepithelial neoplasms show a marked increase in periductal collagen deposition [40]. It is possible that increased activation of fibroblasts in tumor-associated stroma may result in the collagen remodeling within the tumor microenvironment that we observed. Prior investigations have adduced that cancer-associated fibroblasts are the principal source of fibrillar collagens, other ECM proteins, and soluble factors promoting growth, invasion, metastasis, and survival of cancer cells [41–43].

As a component of vasculature and ECM in normal stroma, elastin exhibited changes associated with the neoplastic process. Uchiyama et al identified ‘breast cancer elastosis’ as condensed accumulations of irregularly arranged small amorphous elastin components associated with only a few microfibrils [44]. These amorphous components were ill-defined and occasionally associated with spiraling collagen fibrils and cellular debris. We demonstrated that ColXα1 and elastin are both patchily distributed and colocalized in the invasive breast cancer-associated stroma. Ultrastructurally, the colocalized ColXα1/elastin complexes appeared as amorphous irregular clumps that are comparable to the elastosis described by Uchiyama et al. Using radioactive labeling and HPLC methodology, Kao et al found that the synthesis of collagen and elastin increased by 50 and 70% in desmoplastic breast cancer stroma on a per-cell basis [45]. However, unlike COL10A1, elastin RNA expression levels were not increased in DCIS or invasive carcinoma compared to normal tissue when analyzed through Oncomine. This may be attributable to the presence of elastin in normal breast tissue as a component of vascular and periductal structures. Accruing evidence suggests that focally aligned collagen at the stroma–cancer interface guides the migration of cancer cells away from the tumor and toward the vasculature during the metastatic cascade [46]. An increase in collagen deposition or ECM stiffness, alone or in combination, up-regulates integrin signaling and thus promotes cell survival and proliferation [47,48]. The quantitative changes in elastin within breast carcinoma stroma may be negligible. However, given its colocalization with ColXα1, elastin may undergo redistribution thus contributing to the neoplastic process.

There is emerging interest in stratifying breast carcinoma patients on the basis of stromal characteristics. Studies of invasive breast cancer-associated stroma have furnished conflicting prognostic results between the molecular subtypes of breast cancer. Downey et al reported that high stromal contents are associated with improved outcomes in ER-positive breast cancers [49]. However, these portend an adverse prognosis in triple negative tumors [11]. Dennison et al recently discovered that a particular stromal protein signature in breast carcinoma is associated with a highly differentiated phenotype [50]. The outcome analysis in our study identified significant correlation between ColXα1/elastin expression in the neoadjuvant-treated tumor but not in the adjuvant setting. Compared to neoadjuvant-treated cases, patients with early stage small tumors are treated with primary surgery and often have a good prognosis. Neoadjuvant-treated tumors are often of higher stage and histologic grade. Therefore, studying the predictive value of ColXα1 and/or elastin expression may be more useful in patients undergoing neoadjuvant treatment. We tried repeating the analysis in each molecular subtype. Our case numbers with available outcome data were relatively small for each subtype, but ER+ tumors (ER+/HER2+ and ER+/HER2−) showed a steady trend of correlation between higher ColXα1 and/or elastin expression and poor outcome. Further studies with larger patient numbers in each subtype and a longer follow-up period are needed.

Increased deposition of ECM components has also been discussed to enhance tumor progression. Overall, breast tumors exhibiting certain stromal biomarkers have a greater propensity for metastasis [11,51]. At the molecular level, Wnt7a was found to potentiate TGFβ, which correlates with stromal desmplasia and poor prognosis [42]. Expression of matrix remodeling genes such as matrix-metalloproteinases and collagen cross-linkers is also predictive of a poor prognosis [52]. Therefore, expression patterns of factors within tumor-associated stroma may have therapeutic and theranostic applications. However, barriers to identifying effective anticancer therapies include the inability of in vitro cell culture models to recapitulate the complex three-dimensional (3D) tumor microenvironment for screening platforms to identify therapeutic candidates [53,54]. Novel complex 3D multicellular tumor models have shown promise in this application [55–58].
In summary, we demonstrate elastin and ColXα1 colocalization and plausible complexing as suggested by immunofluorescent and IEM ultrastructural assays. Our data support a model where ColXα1 and elastin colocalization is specific to the neoplastic process. Progression of DCIS to invasive carcinoma may involve aberrant expression of ColXα1 and elastin in the tumor microenvironment. Enrichment of these complexes in tumor-associated stroma may represent a stromal signature indicative of fundamental biological and intrinsic differences between breast cancers. Accordingly, the success of cancer prevention and therapy may require an intimate understanding of reciprocal feedback processes between tumor cells and their associated stroma. These findings establish a foundation for investigation into the role of aberrant collagen complex expression in tumorigenesis and tumor progression that may be leveraged in future therapeutic and theranostic applications.

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Author contributions statement

YW and MBR conceived the study and prepared the manuscript. SL performed the Oncomine data analysis and statistical analyses. YW, JX, KS, CZ, and MO collected and reviewed all the cases in the study including morphological and immunohistochemical evaluation. DY and CS performed the molecular experiments. GJ performed electron microscopy experiments. RD, ASB, and HY participated in data reviewing and revising the manuscript. All authors read and approved the final manuscript.

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**SUPPLEMENTARY MATERIAL ONLINE**

**Table S1.** Differential tumor expression of ColXα1 and elastin