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Tumor-extracellular matrix interactions: Identification of tools associated with breast cancer progression

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ABSTRACT

Several evidences support the concept that cancer development and progression are not entirely cancer cell-autonomous processes, but may be influenced, and possibly driven, by cross-talk between cancer cells and the surrounding microenvironment in which, besides immune cells, stromal cells and extracellular matrix (ECM) play a major role in regulating distinct biologic processes. Stroma and ECM-related signatures proved to influence breast cancer progression, and to contribute to the identification of tumor phenotypes resistant to cytotoxic and hormonal treatments. The possible clinical implications of the interplay between tumor cells and the microenvironment, with special reference to ECM remodelling, will be discussed in this review.

1. Introduction

Although cancer has been considered as a progression of genetic mutations in an aberrant tissue mass, tumors are increasingly viewed as tissues functionally interconnected with the surrounding microenvironment [1]. Recent genome sequencing and single cell-based analyses have revealed substantial genetic heterogeneity within tumors, with subclones that differ in driver mutations. In a breast carcinoma model [2], growth was sustained by a minor cell subpopulation that facilitated the proliferation of all tumor cells. Interestingly, this subpopulation stimulated tumor growth through microenvironmental changes related to re-organization of the collagen pattern and induction of intratumoral vascularization, suggesting that progression of a tumor relies on its ability to overcome microenvironment constraints.

The tumor microenvironment consists of an insoluble extracellular matrix (ECM), a stroma composed of fibroblasts, adipocytes, endothelial and resident immune cells, and a multitude of growth factors and cytokines. The ECM itself is composed by a complex mixture of components, including proteins, glycoproteins, proteoglycans and polysaccharides [3,4]. In addition to elucidating the role of single ECM components in development and homeostasis of normal breast, many studies have revealed abnormal changes in the amount and organization of such molecules during breast carcinoma development. These changes lead to altered biochemical and physical properties of tumor-associated ECM that contribute to tumor progression and resistance to therapy. Moreover, deregulation of ECM architecture impacts on tumor surrounding stroma cells, including endothelial, immune and other stromal cells which may come to favor tumor development. Although many single ECM components, reviewed in [5], have been identified as relevant markers in breast carcinoma progression, evaluation and targeting of a single molecule appears to have limited usefulness in predicting disease outcome or improving therapeutic benefit. A possible explanation might rest in the large number of ECM components, which, even if likely redundant, collectively contribute to distinctive physical, biochemical and biomechanical properties of the tumor microenvironment.

To address the complexity of the tumor ECM and elucidate the stromal properties relevant for breast carcinoma progression and response to therapy, cancer research in the last decade has shifted to gene expression studies focused on the tumor stromal compo-

Abbreviations: ER, estrogen receptor; IDC, invasive ductal carcinoma; DCIS, ductal carcinoma *in situ*; FEC, 5-fluorouracil+doxorubicin+cyclophosphamide; NKI, Netherland Cancer Institute; ECM, extracellular matrix; SFT, solitary fibrous tumors; DTF, desmoid-type fibromatosis.

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nent. In this context, different experimental approaches have been applied, including profiling of: (a) stromal cells isolated from carcinomas or modified by cultured conditions; (b) soft tissue tumors as surrogates for different stromal responses; (c) laser-captured tumor microenvironment or whole tumor samples followed by analysis of a gene set restricted to connective tissue-related genes. The first gene expression portrait of the breast cancer microenvironment came from a study [6] in which different stromal cell types composing normal and neoplastic tissues were profiled using serial analysis of gene expression upon separation by magnetic beads armed against cell type-specific surface markers. Significant changes in gene expression profile were detected in all cell types during tumor progression, indicating that the microenvironment actively participates in cancer growth and invasion. Focusing on myoepithelial cells as constituents relevant in controlling breast cancer cell growth, the authors detected upregulated expression of several proteases (cathepsin F, K and L, MMP2 and PRSS19), protease inhibitors (thrombospondin2, SERPING1, cytostatin C and TIMP3) and different collagens (COL1A1, COL3A1, COL6A1) in DCIS myoepithelial cells [6] implying ECM remodeling during cancer development. These results were later confirmed through the gene expression portrait of fibroblasts derived from invasive and benign breast diseases [7]. While analysis of gene expression profiles of stromal tissue isolated using laser capture microdissection (LCMD) showed no significant differences between adjacent tumor and reduction mammoplasty-derived stroma [8], genes involved in ECM-receptor interaction and focal adhesion were significantly up-modulated in tumor versus normal cells microdissected from mastectomy specimens of invasive ductal or lobular carcinomas [9]. Taking advantage of LCMD, gene expression profiles of patient-matched normal stroma and tumor-associated stroma specimens showed that the highest regulated genes in the tumor-associated stroma were those encoding ECM constituents and matrix metalloproteinases, including COL10A1, COL11A1, fibronectin, collagen triple helix repeat containing 1, COL12A1, COL8A1, MMP11, and MMP2 [10]. Thus, whereas early changes involved in cancer initiation *per se* do not appear to derive from the microenvironment, the cross-talk between tumor and stromal cells mediated though the ECM is one of the first events upon mammary cell transformation. As such, there is continuing scientific interest in the role of cross-talk in neoplastic progression as well as in the promise of ECM features as biomarkers able to predict risk of progression and treatment benefit in breast carcinoma patients. Moreover, recent findings on the role of specific microRNAs regulating a network of genes involved in ECM changes by tumor microenvironment or directly targeting ECM molecules mRNAs [11,12] open new perspectives for investigating whether any of such stroma/ECM variations are associated to release/modulation of signaling molecules relevant for tumor progression which can be easily detected in body fluids for a relatively non-invasive early diagnosis/risk assessment.

Here, we provide an overview of "microenviromental signatures" proved to be relevant as markers for progression and treatment of breast cancers. Since abnormal characteristics of breast carcinoma ECM induce changes in tumor tissue architecture and rigidity, we also discuss the role of stromal stiffness on tumor behavior.

2. The role of ECM in the transition from ductal carcinoma *in situ* to invasive breast cancer

Before the introduction of mammary screening, ductal carcinoma *in situ* (DCIS) represented only 2–5% of symptomatic breast cancers while, nowadays, it accounts for more than 20% of newly diagnosed symptomatic cases and up to half of screen-detected breast cancer, but uncertainties still remain on its biological behavior and the appropriate clinical management [13–15]. Thus, the identification of biomolecular markers associated to the risk of *in situ* recurrence rather than of invasive cancer to complement standard clinical and pathological factors represents an area of intense research [16,17] for the possibility to provide patients with a more appropriate use of local and systemic treatments.

At present, the progression of DCIS to invasive breast cancer may be explained by mainly two distinct mechanisms, as the results of the accumulation of additional genetic aberrations and inherited transcriptome alterations consequent to methylation modifications or, alternatively, as a genetic-independent process. Many studies focused on the molecular and genetic alterations in neoplastic cells, but evidence is also emerging on the fact that the transition from DCIS to invasive ductal carcinoma (IDC) is strongly dependent upon alterations in the microenvironment with a particular reference to the role of myoepithelial cells and stromal-epithelial interactions.

Initial studies which focused on epithelial cells only, barely found any gene expression differences among distinct stages of progression [10,16], especially when the comparison was done between *in situ* and invasive tumors of the same grade. Indeed at the epithelial cell level, the more dramatic changes were reported in the transition from normal epithelium to DCIS, with minor changes between atypical ductal hyperplasia, DCIS and IDC. With an increasing awareness on the role of the microenvironment in tumor biology [18], studies were undertaken to molecularly dissect the contribution of single microenvironmental components, and the first systemic study [6] on pure stromal cell subpopulations, isolated from few samples of DCISs, IDCs and normal mammary glands, demonstrated alterations in all the microenvironment components across the progression to *in situ* and invasive growth, with major changes occurring in the myofibroblasts and myoepithelial cells. Such alterations involved increased secretion of chemokines (*e.g.*, CXCL14, CXCL12), which stimulate in a paracrine fashion proliferation, invasion and migration of tumor cells.

The transition from DCIS to IDC was also studied integrating genomic alterations with transcriptomic profiles and again no similarities could be observed also at the genomic level [19] suggesting that these changes are shared between DCIS and IDC. Some caution should however be used in the interpretation of data since in the absence of a common mechanism accounting for DCIS progression, individual tumor alterations could represent confounding factors in patient-matched studies of DCIS/IDC as already observed when the analysis was done at single cell level [20,21].

On the front of the non-genomic hypothesis of the transition from DCIS to IDC, Ma et al. [10] performed the molecular characterization of 14 patient-matched normal and tumor breast frozen samples in which gene expression profiles were analyzed in LCMD-isolated cells from normal breast epithelium, DCIS, IDC, normal stroma compartment, DCIS- and IDC-associated stroma. The progression from *in situ* to invasive growth proved to be associated to an extensive change in gene expression mainly in tumor-associated stroma, with an up-regulation of matrix metalloproteinases genes (MMP11, MMP2, MMP14 and MMP13) associated with invasion and with ECM remodeling. Conversely, only three typical stromal genes (*POSTN, SPARC, SPARCL1*) were up-modulated in the tumor epithelium of IDC compared to DCIS.

Later studies addressed the issue of DCIS progression in a similar way, comparing synchronous DCIS and IDC lesions, with some differences in study design (not necessarily patient-matched) and technical approaches (manually performed microdissection on FFPE samples at a distance up to 3 mm from the tumor lesion). At difference to the previous studies, Vargas et al. [22] observed major changes only in genes related to ECM remodeling in the epithelial compartment, including an increased expression

of COL11A1, COL5A2 and MMP13 in IDC samples. Altogether, these results highlight the level of complexity in investigating tumor-microenvironment interactions, in terms of importance of the relative proximity of stromal and epithelial tumor cell compartments, and of stromal contamination that could act as a confounder in evaluating the modulation of ECM genes in the epithelial compartment. Finally, it is also well known that tumor cells, and not only fibroblasts, produce ECM components and enzymes involved in its remodeling. However, independently of the specific cellular types expressing ECM structural components or remodeling enzymes, the transition to invasive disease appears convincingly associated with ECM modifications, in keeping with data reported by Lee et al. [23] who defined gene signatures able to classify DCIS and IDC, enriched by genes involved in ECM synthesis, organization and response to wounding. In a subgroup of patients, separate epithelial and stromal gene expression data were also available, suggesting that ECM-related ontologies significantly enriched in IDC compared to DCIS were restricted to epithelial cells [23]. Stromal genes supported instead a restricted angiogenesis and response to glucocorticoids in invasive tumors.

Other suggestions on the role of microenvironment and ECM derive from epidemiological observations regarding the postpartum increase in aggressive breast tumors linked to mammary gland involution [24]. The stromal compartment of the mammary gland changes during women life-span, in particular mammary gland involution occurring post-pregnancy and post-weaning (or at the onset of the menopause) is associated with an increased incidence of tumors [25]. During such an involution, the microenvironment presents features common to inflammation and wound-healing, and for a long time epidemiologist have noticed that breast tumors diagnosed post-pregnancy were characterized by a grim prognosis not understandable simply on the basis of young age and/or rise in hormone levels. In such a condition, the massive remodeling of the ECM favors the progression of pre-invasive lesions [26] as demonstrated in animal models where MCF10ADCIS human cells, inoculated into a mammary involuting environment, formed larger tumors characterized by abundant production of fibrillary collagen and by an invasive behavior. Those cells frequently spread through the mammary stroma and circulating tumor cells were identified in peripheral blood in a very short time. Fibrillary collagene and activation of COX-2 were instrumental for the acquisition of the invasive phenotype: collagen fibers in the involuting gland were radially aligned to ducts and the increased deposition of fibrillary collagen during involution increased COX-2 expression, which was required for cell migration. These data further underline the role of ECM in tumor progression and should indicate the rational of administering NSAIDs (able to block production and radial organization of fibrillar collagen) to women at high-risk for local and invasive recurrence, [26].

Additional indications on the role of ECM-remodeling under specific conditions in promoting tumor progression derive from studies on the gene coding for LOXL2, which promotes tissue invasion by regulating the TIMP1 and MMP9 activity, whose expression is co-regulated during mammary involution [27].

A long-time known barrier to the transition from *in situ* to invasive growth is given by the presence of myoepithelial cells which not only constitute a physical fence, but have a molecular active role in regulating ECM remodeling since secrete both ECM components and protease inhibitors (*e.g.*, maspin) that prevent the conversion to invasive phenotype [28]. DCIS animal models support such an hypothesis suggesting that progression towards IDC is not due to intrinsic or eventually acquired properties of epithelial cells, but rather determined by the complex interplay among all cell components of the microenvironment, where stromal cells promote, and myoepithelial cells inhibit DCIS to IDC transition [29].

Despite the fact that data are being accumulated on the possible effectors of DCIS-IDC transition and of ECM involvement in this process, few translational studies so far published provided clinically validated biomarkers/signatures to solve the clinical dilemma of DCIS treatment. It has recently been shown that the expression of HER2 [30] and of COX2 can predict DCIS recurrence and that, in a clinical trial on women with ER+ pure DCIS, treatment with the COX-2 inhibitor colecoxib in addition to aromatase inhibitors was biologically effective in reducing Ki67 and Cox-2 expression [31]. Future studies directly comparing validated biomarkers for specific stromal cells activation and ECM remodeling and possibly taking into account mammary density [32] would probably provide hints for a more personalized therapy of patients with *in situ* disease at risk of progression

3. ECM molecular signatures and disease recurrence

Evidence of molecular changes in the ECM accompanying breast cancer progression has led to challenge as predictors of clinical outcome stroma/ECM-related signatures, developed in pilot studies on clinical samples or in preclinical studies in which microenvironment cells were isolated and profiled or manipulated *in vitro* to simulate a specific process. Nine translational studies were addressed to investigate whether ECM signatures might be associated to clinical progression in stage I-III breast cancers and/or to validate such findings on independent datasets even taking advantage of publicly available datasets (Table 1). Consistent with the theory that the molecular program of tumor-associated fibroblasts dictates breast cancer aggressiveness, the transcriptional response of normal fibroblasts to serum [33] showed that both distant metastasis-free and overall survival were significantly decreased in patients with tumors overexpressing genes significantly up-modulated in fibroblasts by serum stimulation [34]. Moreover, the fibroblast-activated signature stratified breast carcinomas according to risk of relapse regardless of previously established biomolecular markers such as molecular subtypes and 70-gene prognosis signature in the independent dataset of 295 early breast carcinomas of the Netherlands Cancer Institute (NKI) [35]. In addition to cell cycle genes, the genomic response of fibroblasts to serum includes ECM remodeling molecules (*e.g.*, LOXL2, PLOD2 and PLAUR), further suggesting that the composition and architecture of insoluble stroma can serve as a biomarker for breast cancer progression. However, it remains unclear why the same microenvironment is not present in every tumor, and further investigations are needed to define whether and how intrinsic tumor or host characteristics may account for that observation.

The NKI dataset was also challenged in the study by West et al. [36] by signatures developed from a set of 786 genes with an expression pattern that distinguishes soft tissue tumors [solitary fibrous tumors (SFT) and desmoid-type fibromatosis (DTF] mirroring different activation states of fibroblastic cells. Two main gene groups were apparent, one composed almost entirely of DTF genes such as collagens involved in fibrosis (*e.g.*, COL1A1 and COL3A1), and metalloproteinases including ADAMs and MMPs, and the second group comprising two further clusters characterized by high or low expression of SFT genes (*e.g.*, COL4A5 and COL17A1) involved in basal membrane formation. The two signatures had significantly different behavior, with better clinical outcome in patients with DTF-like breast cancer. These findings revealed distinct patterns of stroma reaction, and also showed for the first time that the ECM molecular milieu correlates with differences in the biology of tumors and consequently in clinical outcome. Support for the robustness of the DTF expression signature for breast cancer prognosis derives from the analysis of DTF-associated genes in four independent breast cancer datasets [37]. The DTF signature has also

Table 1
ECM signatures and clinical outcome of breast cancer patients.

Author (reference)	Discovery	Test settings					Outcomes		
	Source of DE genes/Platform for expression analysis	Investigated specimens/datasets	No. of cases	Tumor stage	Median follow-up (years)	Treatment	Predictive signature (no. of genes)	Selected ECM-related candidate genes*	Relation with
Chang [33,34]	50 fibroblast cultures isolated from 10 different anatomical sites/cDNA	Frozen primary tumors Public NKI dataset [35]	58 295	III I-II	6.5 7.8	Systemic treatments Surgery ± systemic treatments			Distant metastasis & death by uni/multivariable analyses, with a worse prognosis for patients with tumors enriched for serum response program (=wound-like phenotype) fibromatosis.
West [36] Beck [37]	Tumors with fibroblastic features/cDNA	Frozen primary tumors Publicly available datasets[33]	295 561	I-II	7.8	Surgery ± systemic treatments	2 fibroblast-related (483)	**COL1A1** COL5A1, **COL3A1** **COL6A1** COL18A1 FBN1, TPM1 **ADAM12**, ADAM19 **MMP1, MMP11** MMP19 MMP23b, COL4A5 COL17A1, MYL9, *etc.*	Distant metastasis & death by uni/multivariable analyses, with a better prognosis for patients with tumors enriched for desmoid-type fibromatosis genes.
Finak [8]	LCMD-IDC stroma/Agilent	Frozen primary tumors	53	I-II	3.6	Surgery	stroma-derived-prognostic predictor [26]	CXCL14 **MMP1,** MMP12 **ITGBL1**, SPP1	Relapse, validated by uni/multi-variable analyses in publicly available datasets [35,39].
Bergamaschi [40] Triulzi [41]	Tumor tissues/Agilent	Frozen primary tumors Nowegian dataset Publicly available datasets [39,42]	28 114 192	I-II I-II I	Not specified Not specified 5	Surgery ± systemic treatments Surgery ± systemic treatments Surgery	ECM-related genes initially derived from the literature (282), and then extended to 738 [39]	Structural ECM proteins (encoded by ECM-3 genes and including **COL1A1** and COL5A2) MMP2, **ADAM12**, ADAMTS2 ADAMTS5 CTSK, **MMP11** **MMP14**, **TIMP3** SERPINF1, SERPINH1 CDH11, SGCD, CNTN1, ITGB5 **ITGBL1,** MARCO, PUNC, SPARC LAMA4	Distant metastasis & death, with a prognosis better for patients with ECM-4 and worse for those with ECM-1 tumors Worse prognosis for patients with undifferentiated ECM-3 tumors, in uni/multivariable analyses
Planche [44]	LCMD-IDC breast stroma/Affymetrix	Public NKI dataset [35]	295	I-II	7.8	Surgery ± systemic treatments	Stromal signature [36]	**COL10A1** COL11A1, FN1, COMP, CADM1, P4HA3, **MMP11**, MFAP2	Distant metastasis & death by univariate analysis
Jansen [53]	Tumor tissue/cDNA	Frozen ER+ primary tumors	112	IV	8 from primary surgery; 4.5 from start of systemic therapy	Tamoxifen (1st line)	44-gene signature (from the initial set of 81 genes)	**TIMP3**, FN1, LOX, **COL1A1** **SPARC**, TNC	Disease progression and resistance to anti-estrogen therapy by ECM-related genes
Helleman [43]	Tumor tissue/qRT-PCR	Frozen primary tumors	1286 *680* *139* *240*	I-IV *I* *II,ER+* *IV,ER+*	8.5	Miscellanea surgery Tamoxifen Tamoxifen (1st line)	5-gene signature (from [53])	**TIMP3**, FN1, LOX, **COL1A1,** **SPARC**, TNC	Distant metastasis occurrence in stage I associated with TIMP3, FN1, LOX, SPARC expression levels Resistance to anti-estrogen therapy by TNC expression levels
Farmer [56]	Mixture of tumor and stromal cells/affymetrix	Frozen ER-primary tumors Public NKI dataset [35]	63 295	II-III I-II	Not specified 7.8	FEC (neo-adjuvant) Surgery ± systemic treatments	Stromal signature [50]	Metagene including DCN, COL1A2, **COL10A1**, **COL3A1**, COL6A3, **COL6A1, COL10A1**, MMP2, **MMP11, MMP14,** **SPARC**, LOXL1, FBLN1, **ADAM12**, ECM2	Resistance to pre-operative FEC chemotherapy associated with high expression of the stroma metagene No association with clinical outcome for patients not receiving systemic therapy

* In bold, genes present in >1 signature, with a consistent association with the different clinical end-points.

been described recently as a feature in other types of carcinomas (ovarian, colon and lung) [38], suggesting that up-modulation of genes encoding ECM components is a common stromal response to cancer development.

An additional stroma-derived signature, which has been developed by comparing the gene expression profile of LCMD-tumor and matched normal stroma from 53 primary IDCs, proved to be associated with relapse [8], and these results have been validated in external independent datasets [35,39].

The findings that primary breast carcinomas can be classified based on ECM composition and that this classification is relevant for disease progression were confirmed by our analysis of 278 genes encoding ECM molecules in primary breast carcinoma samples profiled on an Agilent platform, wherein tumors were divided into four main groups (ECM1–4) that were significantly independent of intrinsic characteristics of neoplastic cells [40]. Using 4 different clustering algorithms to test the robustness of this breast carcinoma classification based on the expression profile of ECM-related genes in 6 additional independent datasets of 643 invasive breast tumors profiled by different platforms, ECM3 tumors were recognized as an independent subset including about 40% of breast carcinomas. ECM3-tumors identified across different datasets consistently showed overexpression of 58 genes, including 43 encoding structural ECM proteins (*e.g.*, different collagen chains, fibronectin, laminin, SPARC) coordinately overexpressed by both stromal and breast cancer cells [41]. Multivariate analysis of two joined datasets of node-negative, untreated primary breast carcinoma patients [39,42] evidenced a significant interaction between ECM3 and tumor differentiation status (genomic grade) and indicated that ECM3 was significantly associated with high risk of relapse in patients with undifferentiated (grade III) tumors, independent of intrinsic molecular subtypes. These findings indicate the significance of interaction between tumor and ECM features in controlling tumor progression and support not only the importance of cross-talk between transformed cells and the microenvironment in conditioning breast carcinoma evolution, but also the observation that the predictive value of the ECM depends on tumor cell-specific properties.

Consistent with these findings, an ECM gene cluster including COL1A1, TIMP3, FN1, LOX, TNC and SPARC was shown to be relevant for tumor prognosis as evaluated by quantitative real-time PCR in 1286 primary breast carcinomas [43]. Specifically, TIMP3, FN1, LOX and SPARC were associated with metastasis-free survival in lymph node-negative patients who received no adjuvant therapy.

Genes encoding ECM components (*e.g.*, COL11A1, COMP and FN) were significantly up-modulated in tumor versus normal stroma in a transcriptome analysis of LCMD-stromal cells derived from human invasive breast carcinomas [44]. Interestingly, hierarchical clustering of a publicly available early-stage breast carcinoma dataset [35] based only on the expression profile of detected stromal genes identified two groups of patients that differed significantly in overall survival.

With respect to ECM molecular modules associated with breast cancer outcome, SPARC (secreted protein acidic and rich in cysteine) was shared by different signatures due to its relevance in regulating ECM *via* interaction with different components as collagen, laminin, VEGF, PDGF [45–47], affecting tumor progression *via* regulation of integrin-linked kinases [48] and acting in the immune response and immunosurveillance against tumors [49]. A pooled analysis of two publicly available datasets [35,50] to evaluate SPARC expression according to clinical outcome in early breast cancer [51] showed that this matricellular protein as well as a metagene including genes highly correlated with SPARC was significantly associated with poor prognosis in patients with basal and HER2-positive breast tumors. Moreover, in neoadjuvantly treated patients with HER-positive tumors, SPARC signature was associated with resistance to chemotherapy, supporting the relevance of the ECM structural framework in determining patient outcome. Overall, the interaction between tumor and ECM features in tumor progression, as abundantly described in preclinical models, appears to be a key force affecting breast cancer evolution. The development of new therapeutic interventions targeting this tumor niche awaits further studies to define how biochemical and biophysical properties of ECM components differ according to ECM molecular signature and whether the signal from the matrix to the cells within depends primarily on ECM features or whether the tumor cell differentiation status contributes.

4. ECM molecular signature and response to systemic treatments

Tumor microenvironment can contribute to the occurrence of tumor resistance to chemical and physical agents through different mechanisms, which include (a) the presence of dense ECM or limited blood flow that may limit drug availability to tumor cells; (b) interactions of tumor cells with ECM constituents that may activate survival pathways allowing the acquirement of resistance through genetic changes; (c) secretion of pro-survival factors (*e.g.*, growth factors, cytokines) by stromal cells [52]. Such findings are supported even by translational studies within clinical trials, where stroma- and ECM-derived signatures proved to be associated with resistance to cytotoxic and anti-estrogen agents (Table 1).

Studies from the Erasmus Medical Center demonstrated the association of the ECM gene cluster including COL1A1, TIMP3, FN1, LOX, TNC and SPARC with tamoxifen resistance in patients with metastatic breast cancer [53], and confirmed in a successive study also including adjuvant settings the involvement of TNC with anti-estrogen resistance. The predictive relevance of TNC, which is a target of microRNA-355 [54], could be explained by two mechanisms: (1) high TNC expression may be indicative of a defective estrogen pathway in ER+ breast cancers; (2) TNC interacts with integrins, which are targets of microRNA-31 [55] and could activate growth factor signaling or act directly as a growth stimulus.

The contribution of stromal genes signatures to drug sensitivity clearly emerges in a correlative study with a high level of evidence since carried out in the context of the EORTC 10994 neoadjuvant trial of chemotherapy on patients with ER-negative tumors [56], Gene expression profiles obtained on pre-treatment biopsies allowed the identification of a stroma metagene, composed by the expression average of 50 genes, with a prominent exhibition of ECM proteins, whose high expression was associated with resistance to 5-fluorouracil, epirubicin, and cyclophosphamide (FEC) treatment (Table 1). This metagene includes genes encoding for different chains of collagene (COL1A2, COL10A1, COL3A1, COL6A3, COL6A1, COL10A1), metalloproteases (MMP2, MMP11, MMP14) and other genes involved in the regulation of ECM (SPARC, LOXL1, FBLN1, ADAM12, ECM2). This same stromal metagene proved to be associated with resistance to chemotherapy even in an independent cohort of ER-negative tumors, included in a study of neoadjuvant chemotherapy with paclitaxel, 5-fluorouracil, doxorubicin and ciclophosphamide [57], at the MD Anderson Medical Center.

The dynamic change of ECM, in addition to have a predictive role on tumor progression, also demonstrated to be relevant in mediating resistance to chemo-, hormone and radio-treatment through different mechanisms [58], including low vascular density, which causes an insufficient blood flow determining a poor drug delivery [59], the expression of matricellular proteins SPP1 and THBS1 able to induce resistance to apoptosis mediated by cyclophosphamide and doxorubicin, respectively [60,61], the increase of collagene I that in response to drugs [62] induces ECM to become stiffer and

thereby further reduces the ability of the drugs to penetrate the tumor.

Weigelt and colleagues showed that the sensitivity to trastuzumab, pertuzumab and lapatinib of non-resistant HER2+ cells grown on laminin-rich ECM in 3D cultures, could be increase by adding anti-integrin β1 antibody [63]. The explanation of this data is that the major ECM proteins fibronectin, collagen and laminin are ligands for integrins, and this binding changes profoundly the behavior of tumor cells, through the activation of different molecular pathways, which lead the drug resistance [64], through a mechanism called "cell adhesion-mediated drug resistant".

Much efforts are needed to clearly define function and composition of different stroma components, in order to define predictive signatures for specific treatments and to design new targeted therapies.

5. ECM stiffness in tumor progression and resistance to therapy

It is generally recognized that breast cancer is characterized by increasing stiffness of tissue, helping to detect disease by palpation or elastography [65]. Indeed, it is well-documented that the healthy mammary gland is highly compliant, while the average tumor is more than one order of magnitude stiffer [66,67]. Epithelial cancers are characterized by an altered tissue tension homeostasis that reflects increased cell-generated force in the transformed cells, increased compression force due to the solid state pressure exerted by the expanding tumor mass, and matrix stiffening due to the desmoplastic response [68,69]. ECM changes dynamically in composition and orientation [70], inducing mechanical perturbations that may also have a causal role in tumor progression [71]. Provenzano et al. [72], using an *in vivo* model of bi-transgenic mice, provided the first evidence of a causal link between increased stroma collagen deposition and enhanced tumorigenesis, local invasion and metastases due to this augmented density. Since then, the role of stiffness in driving tumor progression has been widely studied. Using the MMTV-Neu transgenic mouse model, Levental et al. [73] demonstrated that breast cancer development is accompanied by progressive collagen deposition and crosslinking, LOX expression and increased ECM stiffness, with consequent enhanced focal adhesion and PI3K activity in tumor cells. Consistent with these findings, increased matrix stiffness was found to enhance adhesion signals and a chronic activation of the FAK-Rho-ERK network [74]. More recently, Mouw et al. [75] demonstrated microRNA involvement in integrin-dependent cell-matrix interaction. Specifically, their findings in *in vivo* models identified miR-18a as a mechanically regulated tumor enhancer that represses PTEN and promotes PI3K, inducing tumor progression after FAK- and B-catenin-mediated induction. In human breast carcinoma samples, the authors found a positive correlation between miR-18a expression levels and tumor increased stiffness, and an inverse correlation between miR-18a expression and time to distant relapse-free survival regardless of tumor molecular subtypes. Very recently, Wei et al. [76] showed that increasing matrix stiffness activates epithelial–mesenchimal transition (EMT) and tumor invasion through the EMT-inducing transcription factor TWIST1. Mechanistically, their study revealed that stiffness induces the release of TWIST1 from its anchor G3BP2 to enter the nucleus and trascriptionally promotes EMT through clustering and activation of integrins. Notably, patients whose breast cancers showed organized collagen (evaluated by second armonic generation imaging and considered as a surrogate marker for matrix rigidity), and reduced G3BP2 had a markedly unfavorable outcome compared to disorganized/G3BP2-high cases. Overall, current data indicate that a variety of molecular characteristics of ECM lead to different mechanical properties of stiffness which, in turn, can differentially affect breast cancer progression.

As regards the impact of ECM composition on response to therapy, matrix stiffness has also been documented as an important parameter in drug resistance. *In vitro* studies [77,78] have demonstrated the low response to chemotherapeutic agents by different cancer cell lines, including breast, when cultured on stiff substrates. In this context, JNK was found to be a key mediator of sorafenib resistance [77]. Consistent with these preclinical data, Hayashi et al. [79] demonstrated the potential value of tumor stiffness, as evaluated by elastography, in predicting response to neoadjuvant chemotherapy; indeed, patients with soft breast carcinomas were highly responsive to therapy and more frequently displayed pathological complete response than did patients with stiff tumors. Such a relationship was also confirmed using shear-wave elastography to measure stiffness [80].

In addition, a study performed on *in vivo* xenograft mouse models indicated that albumin and immunoglobulins diffusion within tumor was impaired by tumor rigidity [81], suggesting that efficacy of therapies with biological drugs as monoclonal antibodies may be profoundly affected by tissue architecture and rigidity. A possible explanation may be that mechanical forces generated by stiffed microenvironment impair perfusion due to vessel compression decreasing drug delivery and efficacy as clearly demonstrated in pancreatic cancer models [82,83].

It is worthy of note that tumor stiffness decreases when patients respond to therapy, indicating the importance of using ultrasound elastography as a measure of tumor stiffness to monitor neoadjuvant treatment response in locally advanced breast cancer patients [84].

While current data clearly identify ECM stiffness as an important parameter to consider when studying the ECM role in breast carcinomas, the variety of ECM signatures with prognostic and predictive significance and the different effects of mechanical cues depending on ECM components and consequent cognate receptors engaged by tumor cells [85] call for further studies to elucidate the relationship between ECM composition and mechanical cues before translating knowledge of tumor microenviromental signals to clinical practice.

6. Conclusions

An aggressive phenotype could be the result of an efficient interaction between *in situ* or invasive lesions and the surrounding stroma in two possible ways: (i) stromal changes in the organ might create an environment permissive for cancer growth and/or (ii) early (pre)neoplastic lesions might establish a productive crosstalk with the surrounding stroma in a process even mediated by ECM components and dependent on the genetic alterations of the epithelial cells and on the microenvironmental responses of the host (also possibly dependent on constitutive genetic variations). In both scenarios the study of stromal and ECM alterations is instrumental for providing novel information to change our understanding of the aggressive and lethal form of cancer. Predicted outcomes could be (i) the identification of individuals at risk of developing aggressive disease from signatures of circulating biomarkers of stromal origin and (ii) the definition of stromal changes indicative of high risk of invasive evolution or recurrence for individual neoplastic lesions.

Transcriptional and even microRNA signatures of target tissues may represent a clue of early changes in stromal microenvironment that could be related to the predisposition to develop malignant lesions characterized by distinct clinical behaviors. Nevertheless, in breast cancer gene expression profiles of tumor fibroblasts iden-

tify distinct stroma patterns indicative of clinical outcome, and the expression profile of some extracellular matrix genes provide clinically relevant information to identify patients at high risk to progress and/or to become resistant to chemical and physical agents. However, all these findings, although promising and relatively robust if considering the overlapping of ECM-related candidate genes across the different signatures, need to be validated and corroborated by further structural and functional studies to define function and composition of the distinct stroma components, and integrated by proteomic studies to compose and clarify the complex frame of interactions between tumor cells and their surrounding microenvironment.

Conflict of interest statement

The authors declare that there are no conflict of interest.

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