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RESEARCH ARTICLE

Immunohistochemical Evaluation of COL11A1 and FGD3 Expression in Invasive Breast Cancer

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Abstract

Background: Collagen type XI, alpha 1 (COL11A1) is a minor component of extracellular matrix and its overexpression is associated with tumoral progression and poorer outcome in several human cancers; data on breast cancer are promising but scarce. FGD3 expression has been shown to be a strong independent prognostic factor in breast cancer. The aim of our study was to investigate whether COL11A1 expression correlates with other classic pathologic prognostic factors including FGD3 expression, as well as with clinical outcome, to evaluate its potential use as prognostic factor in breast cancer patients.

Methods: We evaluated by immunohistochemistry COL11A1 expression and we studied the relationship between this protein expression and traditional breast cancer prognostic factors, FGD3 expression, as well as with patients' outcome.

Results: We found that higher stromal COL11A1 expression was associated with higher tumour grade (G3) ($p = 0.001$), higher Ki67 proliferation index ($p = 0.006$), more advanced AJCC stage ($p = 0.031$) and lower FGD3 expression ($p = 0.039$). In a case-control analysis, we observed that patients with high-COL11A1 had a higher risk of recurrence (OR = 2.0) and of dying of the disease (OR = 2.0). Patients with high-COL11A1-expressing tumours had shorter disease-free survival and overall survival (difference not significant). There was a linear positive correlation between COL11A1 expression on epithelial tumoral cells and surrounding stromal cells ($r = 0.247$, $p = 0.04$).

Conclusion: Our findings suggest that COL11A1 may represent a marker of aggressiveness in invasive breast cancer and that its detection warrants further study on larger series to evaluate its possible use in clinical practice.

Keywords: breast cancer; collagen type XI, alpha 1; outcome; immunohistochemistry; biopsy tissue blocks

Introduction

Breast cancer is the most common cancer in women, with decreasing mortality rates worldwide, thanks to earlier detection and progress in cancer treatment, despite its increasing incidence¹. Several researches on molecular signatures and cancer gene expression such as Oncotype DX² or MammaPrint³ have been developed to identify new prognostic and predictive factors for breast cancer and to better stratify affected women. Despite of these advances, the mRNA-based signatures do not consider the post-transcriptional and post-translational changes in tumoral lesions and the potential role of microenvironment on tumour development and progression. Extracellular matrix and its constitutional proteins, such as collagen, plays a fundamental role on normal cells behaviour, as well as on tumour cells development⁴: collagen is aberrantly produced by cancer-associated fibroblasts, with a negative impact on proliferation, migration and differentiation⁵.

Collagen type XI, alpha 1 (COL11A1), a fibrillary collagen encoded by COL11A1 gene, is a minor component of extracellular matrix, which contributes to its tensile strength⁶. COL11A1 expression has been correlated to poor prognosis in non-small cells lung carcinoma⁷, ovarian cancer⁸, squamous cells carcinoma of head and neck⁹, pancreatic¹⁰, gastric¹¹, colorectal carcinoma¹² and some sarcomas¹³.

A recent study by Zhu et al.¹⁴ investigated the origin of a particular type of cancer-associated

fibroblast (CAFs), characterized by a strong presence of COL11A1, using computational analysis of rich single-cells datasets: they demonstrated that COL11A1 served as a proxy of the full signature and that there is a natural progression from normal tissue, which presents a set of identical attractors corresponding to adipose-derived stromal/stem cells, to tumoral tissue, which presents a major amount of COL11A1-based signatures. There is wide agreement that CAFs may represent promising targets for cancer therapies. In breast cancer the expression of COL11A1 seems to be regulated by miR-139-5p: hence, overexpression of miR-139-5p inhibits COL11A1 expression, promoting apoptosis and inhibiting proliferation¹⁵. In addition, in breast cancer, COL11A1 expression was associated with metastasis^{16,17}, with invasive cancer compared to in situ ones¹⁸, with a major risk of malignant relapse of intraductal papilloma¹⁹ and with recurrence after first diagnosis²⁰. A study by Toss et al. demonstrated the association between high COL11A1 expression and hormonal receptor negative, HER2 positive and triple negative molecular subtypes, high proliferation index and dense tumour infiltrating lymphocytes, DCIS with invasive component, shorter recurrence-free interval and poor outcome also with radiotherapy treatment²¹. In two recent studies the correlation between COL11A1 gene expression and prognosis was assessed: Luo et al.²² demonstrated that higher COL11A1 gene and mRNA expression correlated with shorter overall survival; Shi et al.²³ showed that higher COL11A1 gene expression was associated with shorter overall

survival and disease-free survival. Both studies also analysed COL11A1 protein expression by immunohistochemistry (IHC) in tumor samples and normal breast tissue, reporting an increased expression in tumoral tissue, but this figure was not correlated with outcome. An interesting finding of these two studies was the association between COL11A1 gene expression and tumor immune infiltration, suggesting that COL11A1 could play a role in the tumor immune evasion process and may be a potential target for the immune checkpoint inhibitors (ICI) therapies.

So far, studies on COL11A1 in breast cancer are scant, but COL11A1 expression appears a promising marker of aggressiveness. No previous study investigated by immunohistochemistry (IHC) the role of COL11A1 expression as prognostic factor in invasive breast cancer.

The aim of the current study was to investigate COL11A1 expression in invasive breast cancer and to assess whether its evaluation correlates with the classic clinical pathologic parameters and with FGD3 expression, a recently identified, independent, strong prognostic factor. For this purpose, we analysed COL11A1 expression by IHC on Core Needle Biopsy samples in a population of breast cancer patients already evaluated for FGD3 expression and compared the prognostic significance of this IHC marker with that of traditional prognostic factors and FGD3.

Materials and Methods

Patients Selection and Data Collection

We retrospectively analysed 69 patients with breast cancer, surgically treated and followed between 2006 and 2019 at the Breast Unit, Gynaecology section, Department of Health Sciences, Careggi Hospital, University of Florence. Patients were selected from a cohort previously studied for FGD3 expression. The inclusion criteria were: availability of tissue blocks from preoperative core needle biopsy; selection of a group of patients with unfavourable outcome (recurrence and/or death of disease); selection of a larger control group of patients with comparable clinical-pathologic characteristics but without recurrence or death. Patients' data were collected from medical records, including surgical treatment of primary tumour, clinical, pathological and molecular characteristics of tumours, disease-free survival and overall survival. The post-surgical follow-up consisted in clinical examination every 6 months during the first 5 years, and once a year thereafter; mammogram and breast ultrasound were performed every year. Average follow up interval was 50.9 months (range 12.7 – 137.9 months). All patients gave their written informed consent to the use of tissue blocks for study purposes at the time of the surgical procedure.

Institutional Review Board Statement

The study complied with the Ethical Principles for Medical Research Involving Human Subjects according to the World Medical Association Declaration of Helsinki and was

approved by the local ethics committee (Regione Toscana, Area Vasta Centro, # 15.018_AOUC).

COL11A1 expression - Immunohistochemistry

Expression of COL11A1 protein was assessed by immunohistochemistry using the Oncomatrix Biopharma (Code: CI001, clone 1E8.33, Spain). Sections (4 μ m) were stained with mouse monoclonal COL11A1 (dilution 1:100) according to a set protocol on Discovery ULTRA (Roche-Ventana, Tucson, AZ, USA).

Hyaline cartilage tissue section in bronchi was used as positive control while the negative control was obtained using Isotype control mouse IgG1 kappa, with the staining run.

Scoring of COL11A1 expression

Cytoplasmic expression of COL11A1 in tumor epithelial cells and the surrounding stromal fibroblasts was assessed. The tumor epithelial cells and surrounding stromal fibroblasts were assessed and scored separately (Figure 1).

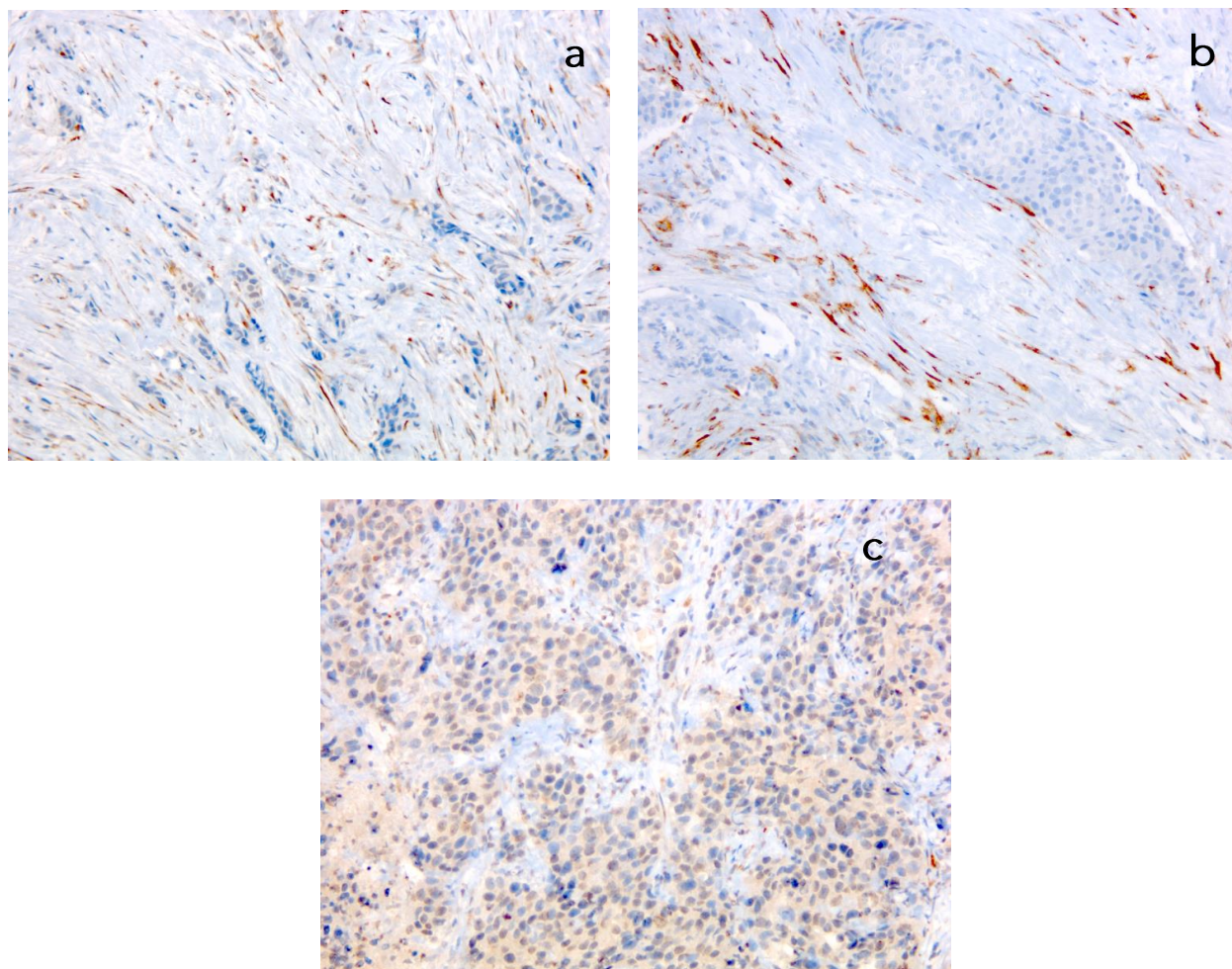


Figure 1 - Immunostaining for COL11A1 in Core Needle Biopsy tissue blocks. Note: a) and b) High expression of COL11A1 in stromal fibroblasts and negative expression in tumor epithelial cells(200x); c) Even distribution of mild COL11A1 cytoplasmic expression in tumor epithelial cells (200x)

Tumor epithelial cells showed no cytoplasmic expression of COL11A1 in the majority of cases; in positive cases, cytoplasmic expression showed an even distribution and the intensity of staining pattern was evaluated as “mild” or “moderate”.

The percentage of stromal fibroblasts showing cytoplasmic staining was estimated, as the intensity of staining within the scanty cytoplasm of the slender fibroblasts was difficult to assess consistently.

COL11A1 expression within stromal fibroblasts was considered “high” when more than or equal to 15% of cells were stained, “low” when less than 15% of cells were stained and undetectable when the staining was absent²¹. All scored cores showed representative areas of stroma (within two high power fields) surrounding the tumor epithelial cells. In addition, a few cases with malignant epithelial cells without surrounding stroma were excluded. Cases with multiple cores were scored and the average final score was used for the analysis. All cases were scored by two pathologists (SB, VV) using a multiheaded microscope.

Population Subgroups based on COL11A1 expression

According to COL11A1 role in extracellular matrix and our immunohistochemistry results, we considered for our analysis the COL11A1 protein expression in stromal cells. We arbitrarily divided our population in low-COL11A1-expressing tumours (undetectable or low staining) and high-COL11A1-expressing tumours (high staining).

For explorative purposes, we also tested the association between COL11A1 expression in the epithelial cells and the other clinical pathologic parameters and clinical outcome, as well as using different subdivision categories of the stromal staining, but the level of statistical significance was not achieved (data not shown).

Statistical Analysis

The frequency distribution of parameters among the population was assessed by Fisher’s Exact Test or Chi-Square Test, as appropriate. Fisher’s Exact Test was used to evaluate the association between COL11A1 expression and other prognostic factors, as well as between COL11A1 expression and outcome (recurrence and death from disease). Disease-free survival and overall survival were calculated according to Kaplan-Meier method and evaluated by Log-Rank Test. A Spearman rank correlation test was used to determine the degree of association between COL11A1 epithelial and stromal expression. Data analysis was performed using IBM SPSS Statistics, version 28.0.

Results

We analysed 69 patients, with an average age of 54.8 years (range 22-86 years). Clinical and pathologic characteristics of patients are summarised in Table 1.

Table 1 - Clinical and pathologic characteristics of patients: overall and according to COL11A1 expression

Characteristic	All	%	COL11A1 -	%	COL11A1 +	%	<i>p</i>
Age, median (range)	54.8 (22-86)	-	54.8 (22-83)	-	55.5 (29-86)	-	-
Grade							
G1-2	31	44.9	26	60.5	5	19.2	0.001
G3	38	55.1	17	39.5	21	80.8	
Total	69	100.0	43	100.0	26	100.0	
LVSI							
Yes	27	39.1	17	39.5	10	38.5	ns
No	42	60.9	26	60.5	16	61.5	
Total	69	100.0	43	100.0	26	100.0	
Molecular Subtype							
ER/PgR +	54	78.3	36	83.7	18	69.2	ns
ER/PgR -	15	21.7	7	16.3	8	30.8	
Total	69	100.0	43	100.0	26	100.0	
Her2+	16	23.2	8	18.6	8	30.8	ns
Her2-	53	76.8	35	81.4	18	69.2	
Total	69	100.0	43	100.0	26	100.0	
Triple negative	8	11.6	3	7.0	5	19.2	ns
Not Triple Negative	61	88.4	40	93.0	21	80.8	
Total	69	100.0	43	100.0	26	100.0	
Ki67							
< 15 %	15	21.7	14	32.8	1	3.8	0.006
≥ 15 %	54	78.3	29	67.4	25	96.2	
Total	69	100.0	43	100.0	26	100.0	
AJCC stage							
I-II	43	62.3	31	72.1	12	46.2	0.031
III-IV	26	37.7	12	27.9	14	53.8	
Total	69	100.0	43	100.0	26	100.0	

Characteristic	All	%	COL11A1 -	%	COL11A1 +	%	<i>p</i>
Lymphonodes involvement							
N+	43	62.3	24	55.8	19	73.1	ns
N-	26	37.7	19	44.2	7	26.9	
Total	69	100.0	43	100.0	26	100.0	
FGD3 expression							
FGD3+	45	65.2	32	74.4	13	50.0	0.039
FGD3-	24	34.8	11	25.6	13	50.0	
Total	69	100.0	43	100.0	26	100.0	

Abbreviations: LVS, lympho-vascular space invasion; AJCC stage, The American Joint Committee on Cancer staging; N, lymphonodes involvement; FGD3, Facio-Genital Dysplasia 3 gene

Concerning COL11A1 expression, among 69 cases, 37.7% (n = 26) presented high COL11A1 expression and 62.3% (n = 43) presented low COL11A1 expression. We analysed the association between COL11A1 expression and standard breast cancer prognostic factors (histotype, grade of differentiation, molecular pattern, Ki67 proliferation index, tumour stage, lympho-vascular space invasion, lymph node involvement) using a Fisher's Exact Test. High COL11A1 expression was significantly associated with higher tumour grade and high proliferation index (Ki67 \geq 15%): 80.8% (n = 21/26) of high-COL11A1-expressing tumours had a G3 grading score vs 39.5% (n = 17/43) of low-COL11A1-expressing tumours (p = 0.001); 96.2% (n = 25/26) of high-COL11A1-expressing tumours presented a high Ki67 proliferation index vs 67.4% (n = 29/43) of low-COL11A1-expressing tumours (p = 0.006). We found no significant differences in COL11A1 expression according to histotype (ductal/lobular/other), molecular pattern

(luminal A/luminal B/HER2+/Triple Negative), lympho-vascular space invasion and lymph node involvement. As regards the AJCC tumour stage, we stratified our population in patients with early AJCC stage (I-II, n = 43) and advanced AJCC stage (III-IV, n = 26); 53.8% of patients with high-COL11A1-expression (n = 14/26) was affected by a III-IV stage tumour vs 27.9% of patients with low-COL11A1-expression (n = 14/43), whereas 72.1% (n = 31/43) of low-COL11A1-expressing tumours presented a I-II AJCC stage vs 46.2% (n = 12/26) high-COL11A1-expressing ones (p = 0.031).

Then, we analysed the association between COL11A1 expression and the expression of a recently detected marker of good prognosis, FGD3, using a Fisher's Exact Test. We found an inverse correlation between these two markers. In fact, a higher COL11A1-expression correlated with a lower FGD3-expression (p = 0.039): the 74.4% (n = 32/43) of low-COL11A1-expressing tumours

presented a high FGD3-expression, whereas the 50.0% (n = 13/26) of high-COL11A1-expressing tumours presented a low FGD3-expression.

The results are shown in detail in Table 2.

Table 2 – Case-control analysis on the risk of death from disease and recurrence depending on COL11A1 expression

Patient Status	All	%	COL11A1-	%	COL11A1+	%	OR	CI 95%	p
Died of disease	18	26.1	9	50.0	9	50.0	2.0	0.67-5.96	ns
Alive	51	73.9	34	66.7	17	33.3			
Total	69	100.0	43	100.0	26	100.0			
Recurrence									
Yes	18	26.1	9	50.0	9	50.0	2.0	0.67-5.96	ns
No	51	73.9	34	66.7	17	33.3			
Total	69	100.0	43	100.0	26	100.0			

Furthermore, in order to investigate the association of COL11A1 expression with patients' outcome, we performed a case-control analysis dividing our population in patients who died from the disease (n = 18, 26.1%) and alive patients, presenting or not recurrence (n = 51, 73.9%), assessing the COL11A1 expression in these two subgroups: the 50.0 % (n = 9/18) of patients who died of the disease had a high-COL11A1-expressing tumour, compared to the 33.3% (n = 17/51) of alive patients, with an OR of 2.0 (CI 95% 0.67-5.96). The level of significance was not achieved, even though a tendency of presenting higher COL11A1 expression was shown in patients with poorer outcome.

A similar case-control analysis was carried out dividing the population in patients who presented recurrence in the follow-up period (n = 18, 26.1%) and patients without recurrence (n = 51, 73.9%): the 50.0% (n = 9/18) of patients who faced recurrence of the disease had a high-COL11A1-expressing tumour, compared to the 33.3% (n = 17/51) of patients without recurrence in their lives, with an OR of 2.0 (CI 95% 0.67-5.96, p = ns), with a tendency of higher-expressing-COL11A1 tumours in patients who faced recurrence, although the level of significance was not achieved.

Using the Kaplan-Meier method we evaluated the impact of COL11A1 expression on disease-free survival and overall survival (Figure 2). A trend of shorter DFS and OS for patients with high-COL11A1-expressing

tumours can be observed. However, the differences between the two groups according to COL11A1 expression did not achieve the level of statistical significance.

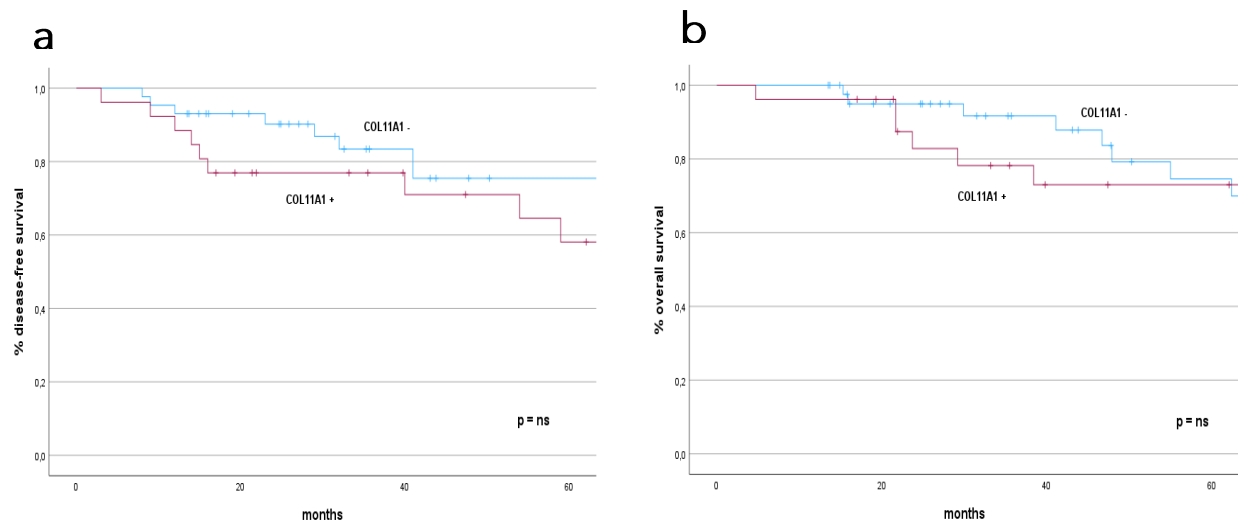


Figure 2 - Disease-free survival and overall survival according to COL11A1 expression: (a) Disease-free survival; (b) Overall survival

Finally, a Spearman rank correlation test was used to determine the degree of association between COL11A1 epithelial and stromal expression in the tumoral tissues analysed: a positive linear correlation was found (Spearman's correlation coefficient $r = 0.247$, $p = 0.04$) (data not shown).

Discussion

In the genomic and transcriptomic era, breast cancer management depends more and more on molecular and genetic tumor patterns. For this reason, researches on new molecular signatures and gene expression in tumours are constantly ongoing to better define tumours' profile and patients' risks. In this panorama, several pancancer prognostic

factors have been identified, defining their role in each neoplasm. COL11A1, a minor fibrillary collagen expressed in normal cartilage and overexpressed in many pathological conditions, has been correlated with tumour progression and poorer prognosis in several cancers, including in situ breast cancer. Liu et al.²⁴ recently identified COL11A1 as a potential target of therapy in ER-positive breast cancer patients, showing in their study on mice, that gelatine nanospheres delivering miRNA can knockdown COL11A1 gene, inhibiting cell proliferation and migration in the subgroup of ER-positive breast tumors.

Another recently identified prognostic factor is the expression of FGD3 gene (Facio-Genital

Dysplasia 3 gene), localised on long arm of chromosome 9, codifying for FYVE, RhoGEF and PH-Domain containing protein 3, which plays an inhibiting role on cell migration in both normal and neoplastic cells²⁵. Two studies by Yang et al. in 2015²⁶ and Willis et al. in 2017²⁷ demonstrated its prognostic role in breast cancer patients. Two previous studies from our group demonstrated that FGD3, evaluated by immunostaining, was an independent prognostic factor in young breast cancer patients as well as in a large series of breast cancer patients not selected by age, confirming that its low expression was associated with a poorer outcome^{28,29}. The expression of both these proteins (COL11A1 and FGD3) on tumoral tissue may be assessed by immunohistochemistry (IHC), a relatively inexpensive and feasible method, which could be used in clinical practice to add useful information on cancer's behaviour in patients with diseases belonging to the same AJCC stage or to the same molecular subtype.

The present study, to the best of our knowledge, is the first to assess COL11A1 protein expression in a cohort of surgically treated invasive breast cancer patients and analyse its association with other clinical features and prognosis. We investigated whether there was an association between the COL11A1 protein expression and traditional and more recent prognostic factors, as well as patient's outcome. We evaluated COL11A1 expression by IHC on breast Core Needle Biopsy tissues: a similar approach was carried out by Freire et al.¹⁸ to evaluate the relationship between COL11A1 expression and tumour's invasiveness. In the current

study we explored the hypothesis that COL11A1 expression may give additional advice to the clinicians, before surgical treatment, also on tumour's aggressiveness. No previous study investigated the role of COL11A1 expression in invasive breast cancer by IHC. We found an association between COL11A1 expression and several traditional breast cancer prognostic factors. In fact, high COL11A1 expression significantly correlated with high tumour grade (G3), higher proliferation index (Ki67 \geq 15%) and high AJCC stage (III-IV), in accordance with the known role of COL11A1 expression in other tumors, suggesting a poorer prognosis for patients affected by this type of breast tumors.

Another interesting finding of our study was the reverse association between COL11A1 expression and FGD3 expression, a well-recognized strong prognostic factor. In our series, the higher COL11A1 expression was found, the lower FGD3 expression was observed. This result is in line with the known role of the two proteins on tumours' behaviour. In fact, COL11A1 expression is a marker of aggressiveness and progression, whereas high FGD3 expression protects from tumoral spread and lymph node involvement and correlates with better prognosis. No previous study assessed simultaneously the expression of COL11A1 and FGD3 in invasive breast cancer.

When evaluating the clinical outcome according to COL11A1 expression, we found a tendency to have a shorter DFS and OS for patients with high-COL11A1-expressing

tumours. However, the difference did not reach the level of statistical significance. This could be possibly explained by the low number of cases of our series, as the correlation between COL11A1 gene and mRNA expression and outcome in wider series was demonstrated in previous studies^{22,23}. On the other hand, no previous study evaluated the role of COL11A1 protein expression by IHC in invasive breast cancer. However, In a previous study on *in situ* breast cancer, it was reported a correlation between a higher COL11A1 expression and shorter DFS²¹.

By splitting our population in patients who presented or not a recurrence and carrying out a case-control analysis, we observed that patients with high-COL11A1-expressing tumours had an increased risk of relapse, with an OR of 2.0 (CI 95% 0.67-5.96) compared to those with low-COL11A1-expressing tumours. Similarly, dividing our population in patients who died from the disease and alive patients, patients with high-COL11A1-expressing tumours showed a double risk of dying from the disease (OR 2.0, CI 95% 0.67-5.96). However, again, the differences were not statistically significant. So, our results suggest that further studies on a larger sample of patients may be warranted to confirm the hypothesis that high COL11A1 expression evaluated by IHC, may represent a marker of tumor aggressiveness in invasive breast cancer, and may give advice on patients' risk of poorer outcome.

One last finding from our analysis was the linear correlation between COL11A1

expression on tumoral epithelial cells and surrounding stromal cells, in line with the Toss et al. study²¹, where the COL11A1 positivity and the related analysis were conducted with both the cellular lines.

Strengths and limitations

The main strength of our study is that this is the first to investigate the role of COL11A1 protein expression in invasive breast cancer as a potential prognostic factor. Another interesting aspect of this research is that we employed IHC, a simple and relatively inexpensive method, easily available in most pathologic laboratory. A third interesting aspect is the use of core needle biopsy material for the analysis, that may provide a chance to characterize tumor aggressiveness prior to surgery. Finally, the correlation between COL11A1 expression and FGD3 expression was made for the first time in the present study. FGD3 is another important prognostic factor in breast cancer investigated by our group in previous studies^{26,27} and the inverse correlation between these two proteins reinforce the hypothesis that COL11A1 may represent a prognostic marker of aggressiveness in invasive breast cancer. The main limitation of our research is obviously represented by the limited sample size.

As both proteins can be studied with a relatively simple and cheap technique (IHC), we could hypothesize that the investigation of COL11A1 expression may be added to routine analysis, providing more insights on the role of COL11A1 in determining breast cancer aggressiveness.

Conclusions

Our study supports the role of COL11A1 expression as a potential marker of aggressiveness in invasive breast cancer, in agreement with the current literature on other human tumors and in situ breast carcinoma. The lack of a significant impact in the

prediction of DFS and OS reported in the current series might be due to the limited sample size, but our findings should encourage further studies on this promising component of the tumor stroma on larger cohorts of invasive breast cancer patients.

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formal analysis, I.R. and T.S.; design, T.S., data
interpretation, T.S. and I.R.; writing—original
draft preparation, I.R.; writing—review and
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and agreed to the published version of the
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