Histol Histopathol (2015) 30: 87-93
DOI: 10.14670/HH-30.87

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Overexpression of proCOL11A1 as a stromal marker of breast cancer

Nelson Fuentes-Martínez[1], Carmen García-Pravia[1,3], Marcos García-Ocaña[4], Primitiva Menendez-Rodríguez[1], Jokin Del Amo[7], Laura Suarez-Fernandez[3], José A. Galván[3,5], Juan R. De los Toyos[3,6] and Luis Barneo[2,3,5]

[1]Department of Pathology, University Hospital of Asturias, [2]Department of General Surgery, University Hospital of Asturias, [3]Oncology University Institute of the Principality of Asturias, [4]Biotechnological and Biomedical Assays Unit, Technical-Scientific Services; [5]Department of Surgery, School of Medicine and Health Sciences, University of Oviedo, Spain, [6]Department of Immunology, School of Medicine and Health Sciences, University of Oviedo, Spain and [7]Progenika Biopharma S.A., Science and Technologic Park, Zamudio, Derio, Spain

Summary. Background: Our previous studies demonstrated the expression of procollagen11A1 in fibroblasts of pancreatic cancer desmoplasia and the lack of expression in fibroblasts of pancreatitis by means of the polyclonal antibody (anti-proCOL11A1 pAb) we generated. In a similar way, we decided to compare the expression of procollagen11A1 in fibroblasts of infiltrating ductal carcinoma of the breast and fibroblasts of benign sclerosing lesions of the breast, in order to validate the anti-proCOL11A1 pAb in this setting and to study how proCOL11A1 expression relates to other prognostic and predictive factors, as well as to survival. Methods: 45 core biopsies of sclerosing adenosis and 50 core biopsies of infiltrating ductal carcinoma of the breast were stained with anti-proCOL11A1 pAb, a polyclonal antibody highly specific to the less homologous fraction of proCOL11A1 (in comparison with proCOL5A1 and proCOL11A2). In addition, the expression of the proCOL11A1 gene was measured by RT-qPCR. On the other hand, the expression of proCOL11A1 was compared to the expression of estrogenic receptors, progestagen receptors, the state of the epidermal growth factor receptor 2 (HER2), the histologic grade and the stage of the disease. We also compared the immunohistochemical expression of proCol11A1 to the disease-free interval, and to overall survival. Results: The immunohistochemical analysis showed that proCOL11A1 was expressed in 100% of infiltrating ductal carcinomas, but only focally expressed in 2.2% (1 case) of sclerosing adenosis, in agreement with RT-qPCR results. ProCOL11A1 expression did not prove to have a prognostic value in relation to the disease-free interval or to overall survival in infiltrating ductal carcinoma. Conclusion: The anti-proCOL11A1 pAb is a stromal marker for breast cancer and the expression of proCOL11A1 does not seem to have a prognostic value in infiltrating ductal carcinoma of the breast.

Key words: Collagen type XI, Breast carcinoma, Stromal marker, Immunhistochemistry, Polymerase, Chain reaction

Offprint requests to: Dr Nelson Fuentes-Martínez, Lloreda SN San Martín de Podes, Gozón, 33418, Asturias, Spain. e-mail: nelsonfuentesmartinez71@gmail.com

Introduction

Breast carcinoma ranks first in incidence and mortality among women, hence the importance of advancing in early diagnosis and treatment (Parkin et al., 2001). Several studies have pointed out different molecular targets localized in the epithelial component of these malignancies; however, to the best of our knowledge, there is not any molecular target in the fibroblasts of the desmoplasia of this type of cancer.

In a previous study on pancreatic cancer, our group identified the overexpression of the COL11A1 gene using DNA microarrays and RT-qPCR. Besides, in order to differentiate chronic pancreatitis from ductal pancreatic adenocarcinoma, we generated a rabbit polyclonal antibody anti-proCOL11A1 pAb which stains fibroblasts of pancreatic cancer desmoplasia with high specificity and sensitivity, but not chronic pancreatitis fibroblasts (Barneo et al., 2006). COL11A1 gene up-

regulation has been reported in some other scenarios, such as colon adenocarcinoma (Fischer et al., 2001a,b), non-small cell lung cancer (Chong et al., 2006), squamous cell carcinoma of head/neck (Sok et al., 2003) and infiltrating ductal carcinoma (IDC) of the breast (Feng et al., 2007; Goldstein, 2007). However, to the best of our knowledge, protein expression has only been characterized by Halsted et al (Halsted et al., 2008). They described a low COL11A1 expression in IDC compared to benign breast tissue, in contrast with our results.

Type XI Collagen is a type of fibrillar collagen that plays a key role in the fibrillar network regulation of the cartilage matrix. It is composed of three polypeptides alpha 1, alpha 2 and alpha 3, encoded by COL11A1, COL11A2, and COL11A3 genes, respectively. The alpha 1 chain is synthesized as proCOL11A1, to which our antibody binds. Anti-proCOL11A1 was designed to recognize the proCOL11A1 protein fraction less homologous to proCOL11A2 and proCOL5A1, which are the procollagens most similar to proCOL11A1 (Fichard et al., 1994).

Taking into account the reports to date pointing to COL11A1 gene overexpression in different malignancies, including breast carcinoma, we decided to validate the expression of proCOL11A1 by RT-qPCR and immunohistochemistry in IDC, and to study how it relates to classical prognostic and predictive factors in breast cancer, as well as to survival.

Materials and methods

Tissue samples

95 cases of paraffin-embedded core needle biopsies from the Pathology Department of Hospital Universitario Central de Asturias (HUCA) were analyzed: 45 cases of sclerosing adenosis (SA) and 50 cases of IDC. The study complies with the Helsinki Declaration and was approved by the HUCA ethics committee.

COL11A1 gene expression analysis by RT-qPCR

Total RNA was isolated from a subset of 14 tissue biopsies (7 IDC and 7 SA) using TRIzol reagent (Life Technologies), and cDNA was synthesized with the reverse transcriptase enzyme SuperScript II RNAse (Life Technologies). All PCR reactions were run in duplicate on a 2.0 LightCycler® (Roche Diagnostics) using the FastStart DNA master SYBR Green I kit (Roche) and following the manufacturer's instructions. Primer sequences were as follows: COL11A1 (target gene): forward 5' TGTCGCCAACAAAATTCTCA 3', reverse 5' TGTCGGCAGAGAAGAGTTGA 3'; PUM1 pumilio homolog 1 (reference gene) (Lyng et al., 2008): forward 5' CAGTCAAAAGGACGTGCAAA 3', reverse 5' TACAAAAGGGAAGGGCGATT 3'. The efficiency of the PCR reactions was calculated using reference curves with serial dilutions of cDNA, and Ct values were obtained using the Fit Points quantification method (LightCycler® Software 4.05, Roche). COL11A1 expression data were normalized to the reference gene and corrected for differences in PCR efficiency. Finally, gene expression data were compared between IDC and SA samples using the Mann-Whitney U test.

Generation of a rabbit polyclonal antiserum to the variable region of human procollagen 11A1 (anti-proCOL11A1pAb)

We have previously described the generation of this antiserum (Barneo et al., 2006). Briefly, the DNA sequence coding for the 133 amino acid stretch (E268 to E400), within the variable region of human proCOL11A1, was expressed in fusion with GST (COL11A1-T-GST). The recombinant truncated (T) fusion protein was purified by affinity chromatography on glutathione columns and used to immunize New Zealand white rabbits by intramuscular injection, at 2-week intervals. The resulting antisera were extensively depleted of the anti-GST reactivity by chromatography on GST-agarose columns. This anti-COL11A1-T purified rabbit IgG recognized COL11A1-T but not proCOL5A1.

Immunohistochemistry

The tissue obtained from the breast biopsies was fixed in 10% formaldehyde, paraffin embedded and cut at 3 μm thick and stained with H&E for histological examination. Antigen retrieval was done by heating in PTLink (DakoCytomation, Denmark) in buffer solution at high pH for 20 minutes. Endogenous peroxidase activity was blocked with Peroxidase Blocking Reagent (DakoCytomation, Denmark) for 5 minutes. Samples were incubated at 37°C with the primary antibodies : estrogen receptor (Clone 6F11 mAb, dilution 1:400, Novocastra Laboratories Ltd, United Kingdom), progesterone receptor (Clone PgR 636 mAb, prediluted, Dako, Denmark), HER2 (Kit 5206 mAb, dilution 1:100, Dako, Denmark) and Anti-proCol11A1 pAb (dilution 1:2000 in buffer S2022 (Dako)).

The samples were incubated with the EnVision HRP Flexsystem (DakoCytomation, Denmark) for 30 minutes at room temperature and stained with DAB (3-3'-Diaminobenzidine) (Dako, Denmark) for 10 minutes. Finally, the samples were counterstained for 10 minutes with hematoxylin.

For those HER2 cases which showed uncertain immunohistochemical positivity (2+), chromogenic in situ hybridation (CISH) was performed using HER2 CISH kit (pharmDx™ Kit, DakoCytomation, Denmark).

Immunohistochemical assessment

Anti-proCol11A1 pAb

Staining was separately evaluated by two pathologists (N.F-M/C.G-P), taking into account the two

following parameters

1-Number of positive fields 10X (NPF)

A maximum of four fields of 10X were analyzed, selecting the largest area and the most intensely stained field. This decision was based on the focal staining pattern of anti-proCOL11A1 pAb. Cases which presented 4 positive fields were assigned the maximum score of 1, while negative cases were assigned the minimum score of 0. The score was obtained by dividing the number of positive fields into the number of assessed fields.

2-Percentage of stained cells in relation to total stromal surface (%C/SS)

A field of 20X with the largest area and the most intense staining was chosen. Cases with less than 10% positive cells in relation to the stromal surface were assigned a score of 1, cases with between 10% and 50% were assigned a score of 2, and cases with more than 50% positive cells were assigned a score of 3 (Fig. 1). Negative cases were scored with 0.

Estrogen receptor (ER), progesterone receptor (PR) and epidermal growth factor receptor 2 (HER2):

ER and PR staining was assessed using the Allred score system (Allred et al., 1998) which considers the percentage of stained cells and the intensity of stain, on a scale from 0 to 8. HER2 staining was assessed according to the recommendations of the College of American Pathologists guidelines 2007 (Wolff et al., 2007).

Statistical method

Statistical analysis was carried out using the freely available software package R2.10 (www.r-project.org). Inter-observer correlation was assessed using the Kappa index. The χ^2 (chi-square) test was used for the comparison of IDC and SA groups, as well as to analyze how anti-proCOL11A1 pAb expression related to the other variables. A receiver operating characteristics (ROC) curve was drawn, with the aim of studying the sensitivity and specificity of anti-proCOL11A1 pAb as a marker to discriminate between IDC and SA. The analysis of survival data was performed using the Kaplan-Meier survival curve. To compare the two curves, the logRank test was used. Besides, Cox proportional hazards model was used to measure the differences between the two curves. P-values under 0.05 were considered statistically significant.

Results

Sample description

Table 1 contains the description of the sample. 95

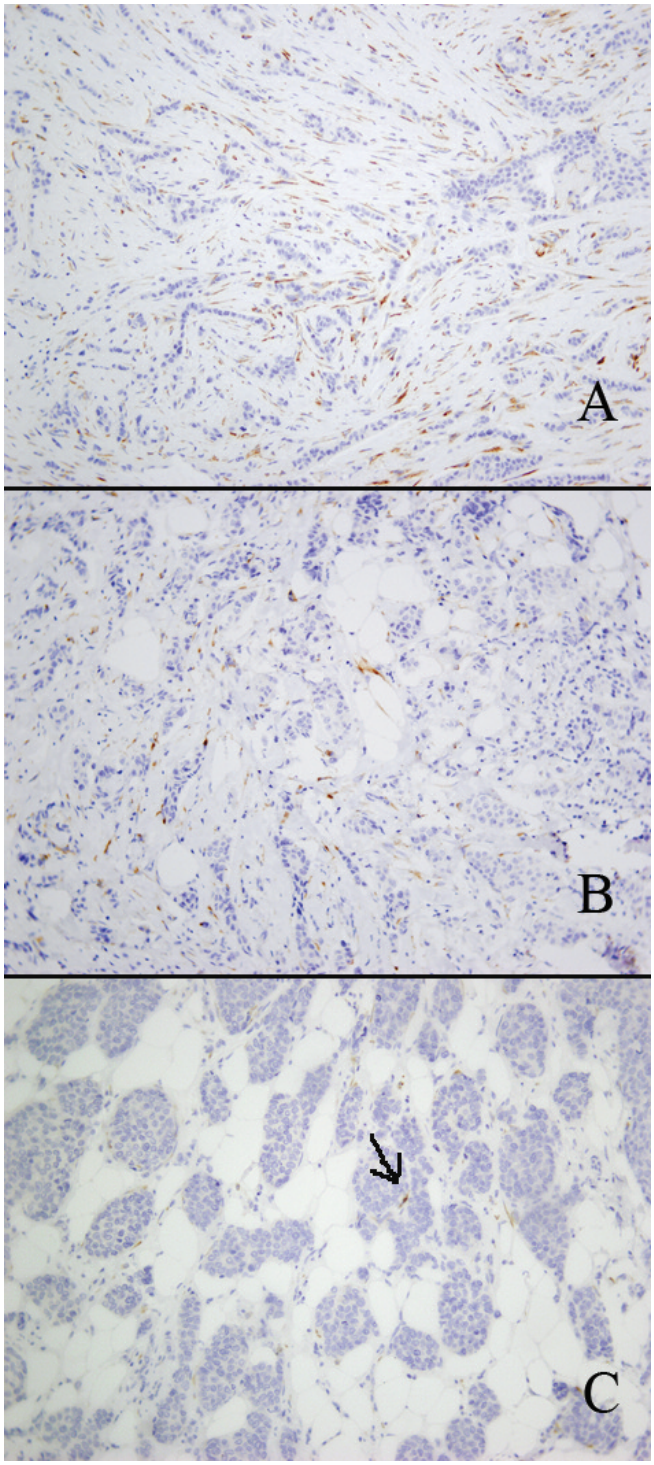

Fig. 1. Immunohistochemical staining with anti-proCOL11A1 pAb. **A.** Infiltrating ductal carcinoma (IDC) score 3 (more than 50% cells/SS). **B.** IDC score 2, (positive cells between 10-50%/SS). **C.** IDC score 1 (positive cells under 10%/SS), arrow. x 20

cases were analyzed, 45 of which were SA and 50 were IDC cases. As regards hormone receptors expression and HER2 in IDC, 38 cases were positive for hormone receptors and 5 were positive for HER2. Out of 12 cases which were negative for ER and PR, 11 were negative for HER2 (triple-negatives). Out of 5 cases which were positive for HER2, 3 were confirmed by immunohistochemistry and 2 by CISH.

Table 1. Patient's Description.

		SA n(%)	IDC n(%)
Gender	Female	45 (100)	49 (98)
	Male	0	1 (2)
Age (years)	Average(range)	46 (33-66)	61 (36-93)
Menopause	Yes	10 (22,2)	35 (70)
	No	35 (77,8)	14 (28)
Histology Grade	1		10 (20)
	2		23 (46)
	3		17 (34)
Stage	1		16 (32)
	2		19 (38)
	3		9 (18)
	4		6 (12)
NPF	Score= 0	44 (97,8)	0
	Score>0	1 (2,2)	50 (100)
%C/SS	Score=0	44 (97,8)	0
	Score>0	1 (2,2)	50 (100)
Her-2	Positive		5 (10)
	Negative		45 (90)
Estrogen Receptors	Score=0		12 (24)
	Score>0		38 (76)
Progesterone Receptors	Score=0		19 (38)
	Score>0		31 (62)
Survival time(60 months)	Dead	0	12 (24)
	Alive	45 (100)	38 (76)

n, number of cases; SA, sclerosing adenosis; IDC, infiltrating ductal carcinoma; NPF, number of positive fields; %C/SS, percentage of stained cells in relation to total stromal surface.

COL11A1 gene overexpression

Real-time quantitative RT-qPCR data showed that COL11A1 is strongly overexpressed in IDC samples compared to samples of SA, with a significant mean fold change of 18.7 (p=0.025, two-tailed Mann-Whitney U-

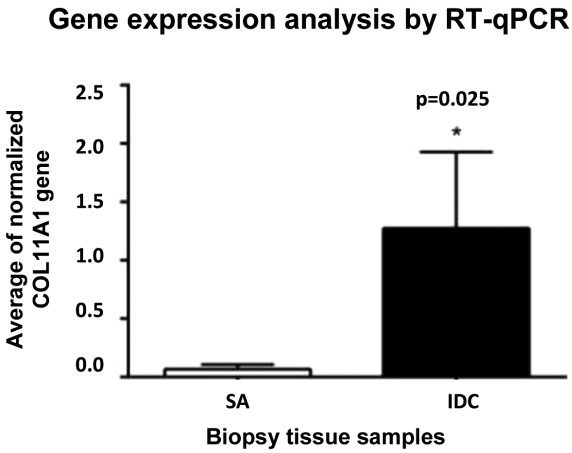

Fig. 2. Normalized gene expression levels of COL11A1 in infiltrating ductal carcinoma (IDC) samples compared to sclerosing adenosis (SA): (p<0.025), Mann-Whitney test.

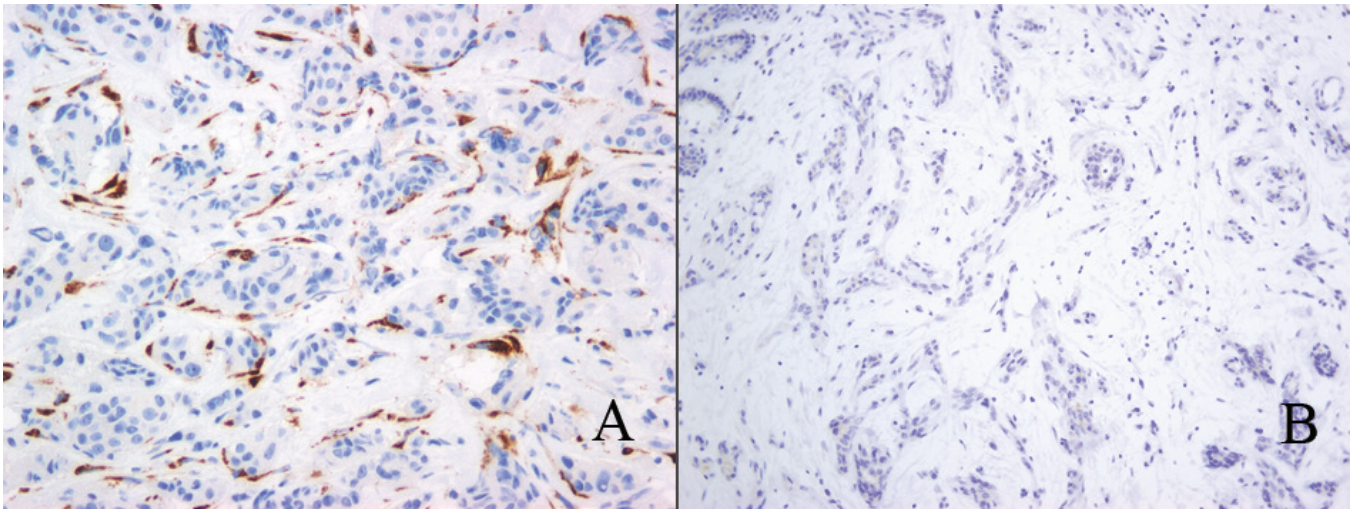

Fig. 3. Immunohistochemical staining with anti-proCOL11A1 pAb in sclerosing adenosis (SA) and infiltrating ductal carcinoma (IDC). **A.** Diffuse positivity for anti-proCOL11A1 pAb in IDC desmoplasia. Negativity of epithelial cells and absence of background staining. **B.** Negativity of stromal cells in SA. x 20

test) (Fig. 2).

Immunohistochemical evaluation of anti-proCol11A1 pAb

All cancer samples tested with anti-proCOL11A1 pAb showed intracytoplasmatic labeling of tumor-surrounding desmoplastic stromal fibroblasts (Fig. 3A). In some cases the staining was focal with uneven distribution. Extracellular staining was never observed. In contrast, the expression of proCOL11A1 in SA was either absent (Fig. 3B) or low and restricted to a few fibroblasts.

The Kappa index showed an inter-observer correlation of 0.754 for NPF and of 0.805 for %C/SS. The difference in proCOL11A1 expression when comparing IDC and SA groups was statistically significant (p<0.001). Only 1 SA case (2.2%) was positive, compared to 100% of IDC cases that were positive (Table 2). Sensitivity and specificity of anti-proCOL11A1 pAb to discriminate between IDC and SA were 100% and 97.8%, respectively, with a cut-off point of 0 (Fig. 4).

The relationship of proCOL11A1 to other predictive and prognostic variables, the disease-free interval, and overall survival

The expression of proCol11A1 did not have a statistically significant relationship with the histologic grade of IDC (p=0.450 for NPF; p=0.621 for %C/SS), nor with the stage of the disease (p=0.292 for NPF; p=0.480 for %C/SS), although cases with a higher score in NPF and %C/SS had a histologic grade of 2 or 3 (40 cases) compared to cases with a lower score, which had a histologic grade of 1 (10 cases).

Likewise, the earlier stages of the disease showed higher scores than the later stages. The relationship to HER2 expression was not statistically significant (p=0.497 for NPF; p=0.239 for %C/SS). The 5 positive

Table 2. Comparison of anti-proCol11A1 pAb between sclerosing adenosis and infiltrating ductal carcinoma.

		SA	IDC
NPF P<0,001	0	44 (97,8%)	0
	0,25	0	3 (6%)
	0,50	0	4 (8%)
	0,75	1 (2,2%)	9 (18%)
	1	0	34 (68%)
%C/SS P<0,001	0	44 (97,8%)	0
	1	0	6 (12%)
	2	1 (2,2%)	11 (22%)
	3	0	33 (66%)

SA, sclerosing adenosis; IDC, infiltrating ductal carcinoma; NPF, number of positive fields; %C/SS, percentage of stained cells in relation to total stromal surface.

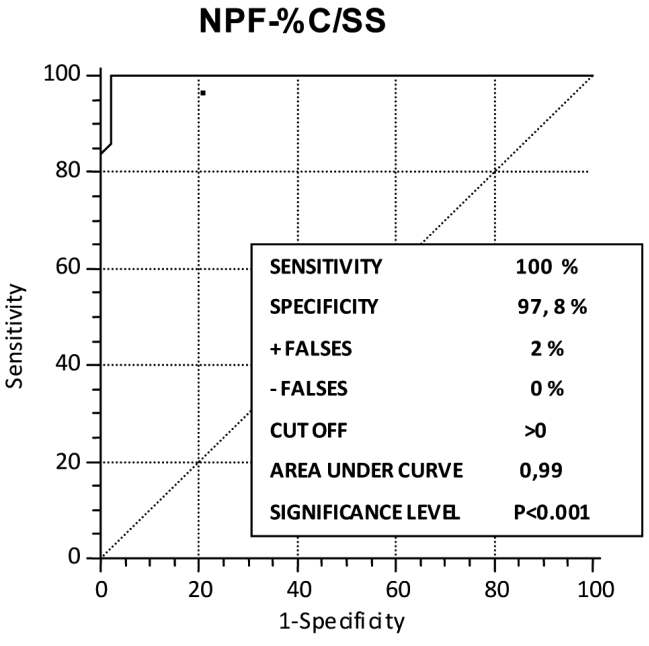

Fig. 4. ROC curves of infiltrating ductal carcinoma (IDC) vs. sclerosing adenosis (SA). The number of positive fields (NPF) ROC curve and the percentage of stained cells in relation to total stromal surface (%C/SS) ROC curve show a sensitivity of 100% and a specificity of 97.8% (cut off=0).

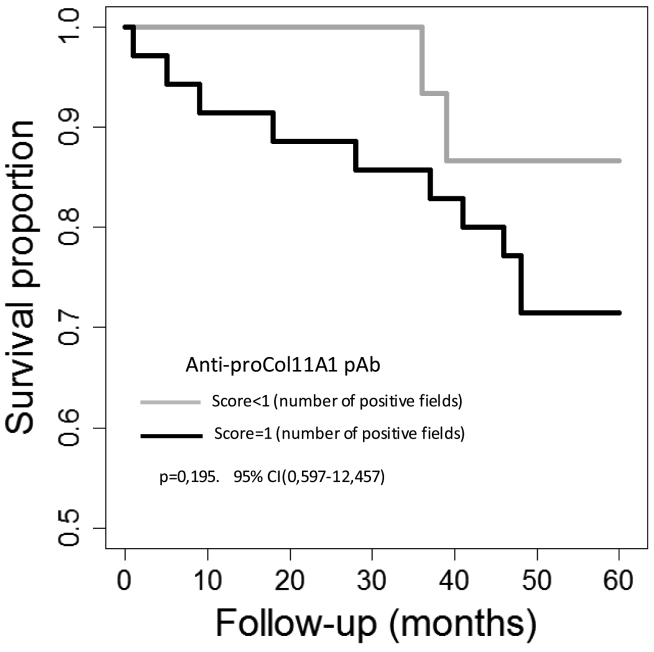

Fig. 5. The curve shows higher mortality for cases with score 1 for NPF (10 deceased cases with score 1 and only 2 deceased cases with score <1). This relationship was not statistically significant (p=0.195, 95% CI[0.597-12.457]). Kaplan Meier.

cases for HER2 showed a score of 1 for NPF and a score of 3 for %C/SS. Cases which showed more expression for hormone receptors, showed lower proCOL11A1 expression. As regards ER, this relationship was not statistically significant (p=0.416 for NPF; p=0.351 for %C/SS); however, for PR, a statistically significant relationship with NPF (p=0.008) was observed, and a non-significant one with %C/SS (p=0.430). On the other hand, the relationship between the disease-free interval and the expression of proCOL11A1 was not statistically significant (p=0.161); nevertheless, the 5 relapsed cases showed score 1 for NPF. Lastly, although the relationship between survival and proCOL11A1 expression was not significant, out of the 12 deceased cases, 10 presented a score 1 for NPF (p=0.176) (Fig. 5). It is worth noting that the hazard ratio (HR) observed was 2.73 [95% CI of (0.597-12.457)], somewhat lower when adjusted for age and stage: 2.38 (0.46-12.22).

Discussion

In a previous study on pancreatic cancer our group demonstrated the overexpression of COL11A1 gene using DNA microarrays and RT-qPCR (Barneo et al., 2006). Also, in order to differentiate chronic pancreatitis from ductal pancreatic adenocarcinoma, we generated an antibody anti-proCOL11A1pAb which we proposed as a potential desmoplastic stromal marker (García-Ocaña et al., 2012). Taking those results into account, we decided to study the expression of -proCOL11A1 in IDC desmoplasia.

A recent computational analysis of gene expression from multiple cancers, including breast cancer, reveals that overexpression of COL11A1 and other genes is a high specificity biomarker of cancer invasion and predicts the response to neoadjuvant therapy. In this analysis, the authors describe a sub-type of COL11A1-producing fibroblasts which they call metastasis-associated fibroblasts (MAFs), and which belong to the cancer-associated fibroblasts class (CAFs) (Kim et al., 2010). The presence of the core genes of the MAF signature in breast cancer would be a good indicator of resistance to neoadjuvant chemotherapy.

As the staining with anti-proCOL11A1pAb is negative in fibroblasts of normal breast and almost absent in a benign inflammatory process, this is suggestive that this staining could be a specific marker of CAFs. Work is in progress to demonstrate by immunohistochemical methods whether fibroblasts with anti-proCOL11A1 pAb staining also have the MAF signature. In any case, the origin of the fibroblasts expressing proCOL11A1 falls beyond the aims of this study. Nevertheless, we can affirm that this fibroblast subpopulation is present in 100% of IDC, but only in very few cases of SA.

From the genetic point of view, Vargas et al demonstrated that the COL11A1 gene is up regulated in epithelial and stromal cells of IDC in comparison with in situ carcinoma (Vargas et al., 2012). We proved an up regulation of COL11A1 in IDC in comparison with SA; however, since that was not an objective of our study, we did not analyze whether those expressions came from epithelial or from mesenchymal cells.

To the best of our knowledge, only Halsted et al. used anticollagen11 antibodies to differentiate protein expression in IDC and normal breast tissue. They used 5 different types of antibodies against different fractions of COL11A1. Their results were in contradiction to ours, because they saw a low expression of COL11A1 in breast cancer in comparison with normal tissue (Halsted et al., 2008). In this regard, we took into account the similarities between COL11A1 and other types of collagen, designing a very specific antibody against the less homologous fraction of proCOL11A1 in comparison with COL5A1 and COL11A2, avoiding a cross reaction between those similar collagens. Furthermore, the evaluation parameters we used results in a low interobserver variability, which is patent in the high correlation between the assessments of the two pathologists. Finally the results of genetic analysis by RT-qPCR support our immunohistochemical findings.

On the other hand, the relationship observed between proCOL11A1 expression and ER, and with PR, has been similar to that reported by Halsted et al. It is an inverse relation, where proCOL11A1 is less expressed in cases which show a more intense and widespread staining with hormone receptors (Halsted et al., 2008). These are interesting results although we cannot assert that proCOL11A1 is a prognostic marker in IDC, since this relationship was not statistically significant in our study. The fact that the relationship between PR and NPF is significant and that it is not in the case of %C/SS, might suggest that this relationship may be stronger when considering the area of stain in the sample and not the focal percentage of stained cells.

On the other hand, the expression of proCOL11A1 does not have a prognostic value in our study, since it has no relation with the disease-free interval nor with overall survival. Nonetheless, some observations should be taken into account for future studies, such as the fact that this marker seems to have more expression in HER2-positive tumors, in those with a higher histologic grade and in the earliest stages of disease. It is also worth noting that the cases which presented relapses of the disease, and most of the deceased cases, were among the ones where proCOL11A1 was most expressed. Maybe if the sample was increased, a statistically significant relation might exist between the disease-free interval/survival and this marker's expression.

Although it wasn't an objective of our study, the data exposed demonstrated that antiproCol11A1 is useful to differentiate between SA and IDC. The differential diagnosis between both entities is usually easy, although the anti-proCOL11A1 pAb antibody could be used in difficult cases, taking into account the lack of other specific markers of stromal desmoplastic fibroblasts.

We conclude that proCOL11A1 is overexpressed in IDC of breast and anti-proCol11A1 pAb is a stromal

marker of IDC desmoplasia. We have not found a prognostic value for its immunohistochemical expression, nor a correlation with other prognostic or predictive factors.

Acknowledgements. The authors thank Marta Montserrat Bravo for her technical assistance and Pablo Martínez Camblor, from the HUCA Biomedical Research Unit (OIB-FICYT), for his help with the statistical analysis of this study. This work has been financed by Progenika Biopharma, and by Instituto de Salud Carlos III (FIS PS09/01911) and INNPACTO (IPT-010000-2010-31).

Conflict of interest disclosure. This work has been supported by Progenika Biopharma, S.A. (www.progenika.com), Science and Technology Park of Zamudio, 48160 Derio, Spain. Jokin Del Amo-Iribarren works for Progenika Biopharma, S.A. This does not alter the authors' adherence to all the Histology and Histopathology policies on sharing data and materials. For the remainingof authors none were declared.

Funding. This research has been cofinanced by FEDER Funds from the European Union; Proyecto INNPACTO-ONCOPAN IPT-010000-2010-31; by Proyecto FISS-09-PS09/01911, Ministerio de Ciencia e Innovación, Spain; by Proyecto FC-11-PC10-23, FICYT, Eje 1 del Programa Marco Operativo FEDER del Principado de Asturias 2007-2013, Spain; and by Progenika Biopharma, S.A. and Oncomatrix, S.L. Derio, Spain. The proCOL11A1 mAb has been patented by Oncomatrix, S.L. (PCT/ES2012/070616; WO 2013/021088 A2).

Meetings. Some results of this study were presented at the 22nd European Congress of Pathology in Florence, Italy, and published in an abstract form in Virchows Arch 2009;455(suppl 1): S32.

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Accepted July 9, 2014