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Original article

Expression of proteoglycan versican in *in situ* breast lesions: Relations between stromal changes, histotype, and invasion

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ABSTRACT

The role of the stromal constituents in the natural history of breast cancers is still poorly defined. The aim of the present study was to evaluate the expression of proteoglycan versican, a constituent of desmoplastic stroma of invasive carcinomas, in preinvasive breast lesions. We selected 41 cases of breast carcinoma: 28 pure *in situ* lesions and 13 invasive lesions with *in situ*-associated lesions.

The study provided evidence that versican is strongly expressed in the perilesional stroma of a subclass of ductal *in situ* carcinomas, and that the extension of versican immunostaining is statistically related to the high grade (G3) category (54% of diffuse expressors; $p = 0.01$), and with a comedo pattern (67% of diffuse expressors, $p = 0.003$). On the other hand, the expression of versican in the cases of classic lobular *in situ* carcinomas that we selected for the study was confined to the anatomical structures that usually contain the proteoglycan in adult breast tissues. In our cohort, versican synthesis was found to be associated with spindle-shaped elements with myofibroblastic phenotype, as in the stroma of invasive carcinoma. These data, taken together with evidence from previous studies on proteins strongly related to versican, suggest that various histotypes of breast *in situ* carcinomas could follow different pathways of epithelial stromal interactions. In particular a category of *in situ* lesions shows constituents of desmoplastic stroma before the manifestation of the morphological signs of invasion. Study of the connective tissue modifications that trigger the pivotal phase of invasion could provide new prospects in oncology.

Introduction

The new screening programs and the enhancing prevention practice in breast oncology have led scientists to focus their research on preinvasive breast lesions representing a significant and increasing portion of the lesions that are nowadays target of treatment. While a considerable body of literature dealing with the morphological and molecular characteristics of the epithelial component of preinvasive breast lesions is available, data are scarce regarding the constituents of the stromal meshwork surrounding *in situ* lesions. Essential features of cellular stromal interactions are managed by the extracellular matrix proteoglycans that represent a complex dynamic system interacting with epithelial structures in development processes, in tissue modifications, and in pathological conditions.

A significant member of the matrix proteoglycan family involved in stromal modifications due to neoplastic signals or repair processes is versican, a hyaluronan-binding protein, the gene and protein of which are organized in a domain template. The G1 domain codes for the aminoterminal region of the protein, a binding site for the hyaluronan, while the G3 domain codes for the COOH terminal region of the protein that comprehends an EGF-like domain and a complement regulatory region [1]. Between these two domains, two large exons code for the middle part of the core protein with sites of attachment to the chondroitin sulfate (GAG) chains. This protein has various and complex roles in cell–stroma communications and in stromal constituent remodeling. Versican regulates the cell adhesion mechanisms via anti adhesive activity of G1 domain and pro adhesive activities of G3 domain. The function of matrix stroma remodeling during histogenesis and reparative processes is realized with interactions with other proteins, such as tenascin, elastic fiber-related proteins fibuline and fibrilline, and hyaluronan [2]. In adult tissues, versican is distributed in the elastic fibers and laminae of connective structures. In the normal breast, it defines a thin rim around lobules and ducts [3].

The expression of versican and other related matrix proteoglycans, such as tenascin, fibronectin, and integrins, has been assessed in breast pathology, and was detected in the desmoplastic stroma surrounding invasive and preinvasive breast lesions [4–7]. Versican and tenascin detected in peritumoral stroma with immunohis-

Abbreviation: SMA, smooth muscle actin-positive.

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tochemical staining were found to have prognostic value [8,9]. In particular, a study dealing with human breast carcinoma cell cultures demonstrated the effect of the versican G3 domain on aggressive behavior [10]. A recent paper evaluated the expression of versican isoforms in breast carcinomas, and faces the hypothesis of targeting the isoforms for therapeutic purposes [11]. Interestingly, the detection of these proteins in periductal fibroblasts and residual myoepithelial elements suggests the involvement of this cell population in protein synthesis [6].

The present work aims at investigating the expression of versican in perilesional stroma of situ breast lesions in order to extend the knowledge on the modifications of stromal matrix proteoglycans in neoplastic lesions still lacking the traditional histological signs of invasion. The available studies on the expression of matrix proteoglycans in breast *in situ* lesions are restricted mainly to tenascin, the expression of which has been correlated with nuclear grade, comedo type, and early invasion [12,13].

In our study, we enrolled cases of *in situ* foci surrounding invasive lesions in order to evaluate the expression of the protein in areas of transition between the invasive and preinvasive stage of the neoplastic process. Another purpose of the study was the evaluation of the cellular component of stroma with versican-positive matrix.

Recent studies have described the presence of SMA+ fibroblastic elements in the stroma associated with invasive and preinvasive breast lesions, replacing the CD31+ fibroblasts of the stroma encircling the acinar structures of the breast normal parenchyma [14–16]. Considering these data, we planned to evaluate the distribution of SMA+ cells with fibroblastic features in the stroma of the lesions enrolled for the study.

Materials and methods

The study was conducted considering the histological material obtained from 491 breast resections performed in the breast surgery department of our structure between January 2006 and December 2008. Forty-one resections (32 lumpectomies (78%) and 9 skin-sparing or nipple-sparing mastectomies (22%)) containing foci of *in situ* carcinomas with tissue available for immunohistochemical studies were selected for the study. Thirteen of the 41 lesions (31%) were invasive lesions with peritumoral foci *in situ* (9 lobular carcinomas, 3 ductal carcinomas, and 1 mixed carcinoma). All the remaining cases were pure *in situ* lesions. The histological data concerning the selected lesions are resumed in Table 1.

Immunohistochemistry

Primary antibodies: monoclonal anti-human versican clone 2B1 (Seikagaku Corporation code 270428) working solution 1:50; anti-smooth muscle actin clone 1A4 (Zymed code 18-0106) working solution 1:50; anti-human cytokeratin clone MNF116 (Dako code M0821) dilution 1:50.

The material was fixed in buffered formaldehyde (4%) and paraffin-embedded. Sections (4 μm) were obtained, fixed on superfrost microscope slides, and dried at 45 °C for 30′. Sections for anticytokeratin and antiversican Mab staining were treated with antigen retrieval solution (DAKO code K8004) for 15′ at 97 °C and then rinsed in distilled water. Sections for actin Mab were deparaffinized and rinsed in distilled water. All the sections were then treated with H_2O_2 3% for 10′, rinsed with PBS buffer solution, and processed in DAKO immunostainer for 30′, rinsed with PBS buffer solution, incubated with Envision + System-Hrp for mouse primary antibody (Dako code K4007) for 30′, rinsed with PBS buffer solution, incubated with DAB for 5′, rinsed in distilled water, and counterstained with hematoxylin for 1′. Slides were then glass-mounted.

Staining evaluation

As, to our knowledge, a scale for the quantification of immunostaining in the connective tissue surrounding *in situ* lesions is not available in the literature, we adopted an original semiquantitative method after an initial assessment of the immunostaining in all the cases studied. The staining with versican around *in situ* lesions was classified as follows: (1) linear – a thin rim of staining around ducts corresponding to the distribution of the protein in normal breast ductulolobular structures (see Introduction). The staining pattern reproduces the distribution of the elastic fibers of the basal lamina, as shown with orcein staining; (2) mean – stain extended beyond the rim of the linear pattern, but without filling the connective tissue between ductulolobular structures with neoplastic colonization; (3) intense – filling spaces among ductulolobular structures, and creating a halo around the lobular structures, unrelated to the presence of elastic fibers (Fig. 4). The evaluation is referred to the maximal extension of the staining within the single lesion. Staining intensity was not quantified since in all cases, staining intensity was roughly comparable to the staining of elastic fibers of vascular and ductal structures that we use as an indicator of staining adequacy.

The distribution of SMA+ elements was evaluated in versican-positive areas of cases with mean and diffuse expression, using the following criteria: (1) absent – when no actin element was detected in versican-positive stroma surrounding *in situ* lesions; (2) scarce – when only scattered elements were evident; and (3) present – when the elements were easily detectable in the stroma. We performed keratin staining to differentiate fibroblastic spindle-shaped cells from myoepithelial cells.

Histochemistry

Orcein staining was performed to define the distribution of elastic fibers in tissues on which immunohistochemical staining was performed. Deparaffinized sections were treated with orcein elastic fiber kit (BioOptica code 04-0558029).

Statistics

Statistical analysis was performed using the X square test.

Results

Expression of versican in in situ carcinomas

The distribution of versican in normal breast tissues was limited to the basal lamina of lobular structures and to the wall of vascular channels as a constituent of the elastic fibers, as defined in previous studies [2,3]. In this study, the staining pattern in normal parenchyma was used as an indicator of staining adequacy. As already stated in other articles, no staining of epithelial elements was detected in our cases.

As described in the "Materials and methods" section, we defined a semiquantitative scale to quantify the extension of versican staining in the stroma around the lesions that we selected for this study. The scale ranged from the staining that was previously described around the normal breast lobule (linear) to the diffuse staining assessed in desmoplastic stroma of invasive breast carcinomas, with two grades of extension around epithelial structures (mean and diffuse, see "Materials and methods").

Immunostaining with versican was evaluated around the *in situ* foci of 41 carcinomas (28 pure *in situ* carcinomas and 13 invasive lesions with a peritumoral *in situ* component). The results are listed in Table 1.

We observed mean or diffuse (desmoplastic) expression of versican in 26/27 ductal *in situ* carcinomas, with diffuse