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Original Article

Expression of CD151/Tspan24 and integrin alpha 3 complex in aid of prognostication of HER2-negative high-grade ductal carcinoma in situ

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Abstract: The pro-tumorigenic and pro-metastatic functions of the tetraspanin protein CD151 (Tspan24) are thought to be dependent on its ability to form complexes with laminin-binding integrin receptors (i.e. alpha6beta1, alpha3beta1, alpha6beta4). We have previously reported that in invasive ductal carcinoma (IDC), CD151/alpha3beta1 complex was of prognostic value in patients with HER2-negative tumors. Extrapolating these findings to the pre-invasive setting, we aimed to make an assessment of a potential relationship between expression of the CD151/alpha3beta1 complex in DCIS and Van Nuys prognostic index (VNPI) in high-grade ductal carcinoma in situ (DCIS) in relation to the HER2 status. Protein distributions were analyzed in 49 samples of pure DCIS using immunohistochemistry. For each case immunoreactivity was assessed in at least 5 ducts (325 ducts in total) and an average score was taken for statistical analyses. When analyzed in the whole cohort, there was no statistical association between the VNPI and any of the proteins scored either separately or in combination. When stratified according to the HER2 status, in the HER2-negative subgroup, CD151 assessed in combination with alpha3beta1 was significantly correlated with VNPI ($P = 0.044$), while neither protein analyzed individually showed any significant link with the prognostic index. Expression of the CD151/alpha3beta1 complex in HER2-negative DCIS might reflect tumor behavior relevant to the patient outcome and thus might aid prognostication of the disease.

Keywords: CD151/tspan24, integrin alpha3, DCIS, HER2-negative, invasive progression

Introduction

Traditionally considered as the non-obligate precursor of invasive breast cancer (BCa), DCIS is characterized by proliferation of neoplastic cells within the duct lumen. However, increasing evidence indicates that, contrary to the generally accepted paradigm of a stepwise evolution of BCa, the molecular program conferring invasive growth is already switched on at the BCa pre-invasive stages, i.e., at the inception of the DCIS→IDC transition [1, 2]. This implicates that application of the knowledge of IDC biology to the DCIS setting is likely to unravel several aspects of DCIS pathophysiology relevant to the clinic. In practical terms, molecular markers identifying subsets of cells driving disease progression might be shared by IDC and DCIS [3].

The tetraspanin protein CD151 (Tspan24) has recently emerged as a new candidate indicator of tumor cell invasiveness [4-6]. Up-regulation of CD151 expression and its involvement in tumor progression have been found in many both human and murine adenocarcinomas [7]. In breast cancer, in particular, we and others have demonstrated that expression of the tetraspanin CD151 was elevated in IDC [8-10] and correlated with poor prognosis in breast cancer patients [9, 10]. The pro-tumorigenic and pro-metastatic functions of CD151 are thought to be dependent on its ability to form complexes with laminin-binding integrin receptors (i.e. alpha6beta1, alpha3beta1, alpha6beta4) and

Table 1. Patient characteristics

Number of patients	49
Age	
< 50	14
≥ 50	35
ER receptor status	
Negative	26
Positive	20
Unknown	3
PR receptor status	
Negative	28
Positive	16
Unknown	5
HER2 status	
Negative	27
Positive	22

coordinate integrin-dependent signalling networks [11]. Our finding that in IDC expression of CD151 in combination with integrin alpha3beta1 represents a more stringent indicator of poor survival than CD151 alone seems to reflect this interaction [12].

Results of our recent study indicate that CD151 may play an important role also in the development of pre-invasive lesions in the mammary gland. CD151 was found elevated in human DCIS and was shown to correlate with a higher tumor grade [13]. There was no association with disease pattern and the frequency of positivity for CD151 was similar in the pure cases to those with established invasion [13]. In a model of DCIS based on the HB2 non-tumorigenic mammary epithelial cell line, it was demonstrated that CD151 promoted proliferation of cells both *in vivo* (mouse xenografts) and in a 3-D in vitro set-ups. Although under experimental conditions proliferative activity of CD151 appeared independent of its association integrin alpha3beta1, the presence of fully functional CD151 was not sufficient to allow proliferation of α3β1-negative cells in 3D ECM [13]. Interdependence between CD151 and α3β1 in the intricate environment of developing human DCIS and its impact on disease prognosis has not been studied.

In clinical practice, morphological examination of DCIS samples using conventional histology and classification according to the Van Nuys scoring system remains the 'Gold Standard' for estimation of the anticipated biological behavior of the tumor and, consequently, the guideline for treatment options [14]. However, the biology and natural history of DCIS are still poorly understood. Numerous attempts to identify biological markers of a risk of invasive progression have not produced conclusive results and a value of HER2 as a sole prognostic indicator in DCIS remains controversial [15-19]. We have shown previously that an impact of CD151 on IDC patient's survival was inversely correlated with the level of HER2 expression [12]. The aim of the study was therefore to assess a potential clinical significance of the CD151/α3β1 complex in DCIS by evaluating its relationship with the Van Nuys prognostic index (VNPI) in relation to the HER2 status. As high-grade DCIS is considered to progress more rapidly to invasive disease [20], the study focused exclusively on grade 3 DCIS lesions.

Material and methods

Patient selection and samples

Specimens of high-grade pure DCIS were obtained from 49 patients treated at the Oncology Department of Copernicus Memorial Hospital in Łódź, Poland and the Holycross Cancer Center in Kielce, Poland, between 2011 and 2015. The characteristics of the population relevant to the study are summarized in the **Table 1**. The use of the samples was approved by the Local Research Ethics Committee (# RNN/284/13/KE).

Immunohistochemistry

The initial pathological diagnosis was confirmed on haematoxylin/eosin-stained sections. ER/PR/HER2 status was determined by routine histological assessment. Serial 5 μm paraffin sections of formalin-fixed blocks were processed for immunohistochemistry for CD151 (monoclonal mouse anti-human; 1:100; Novocastra, UK) and integrin alpha3beta1 (INTA3) (polyclonal goat anti-human; 1:200, Santa Cruz, UK) using protocols described previously [12]. As a negative control for the immunostaining, primary antibodies were replaced by non-immune sera.

Scoring of immunostaining for CD151 was based on the Guidelines for Scoring recommended by the manufacturer of the HerceptTest™ (Dako, Denmark) and modified as follows: i) 0/negative-no reactivity or only partially

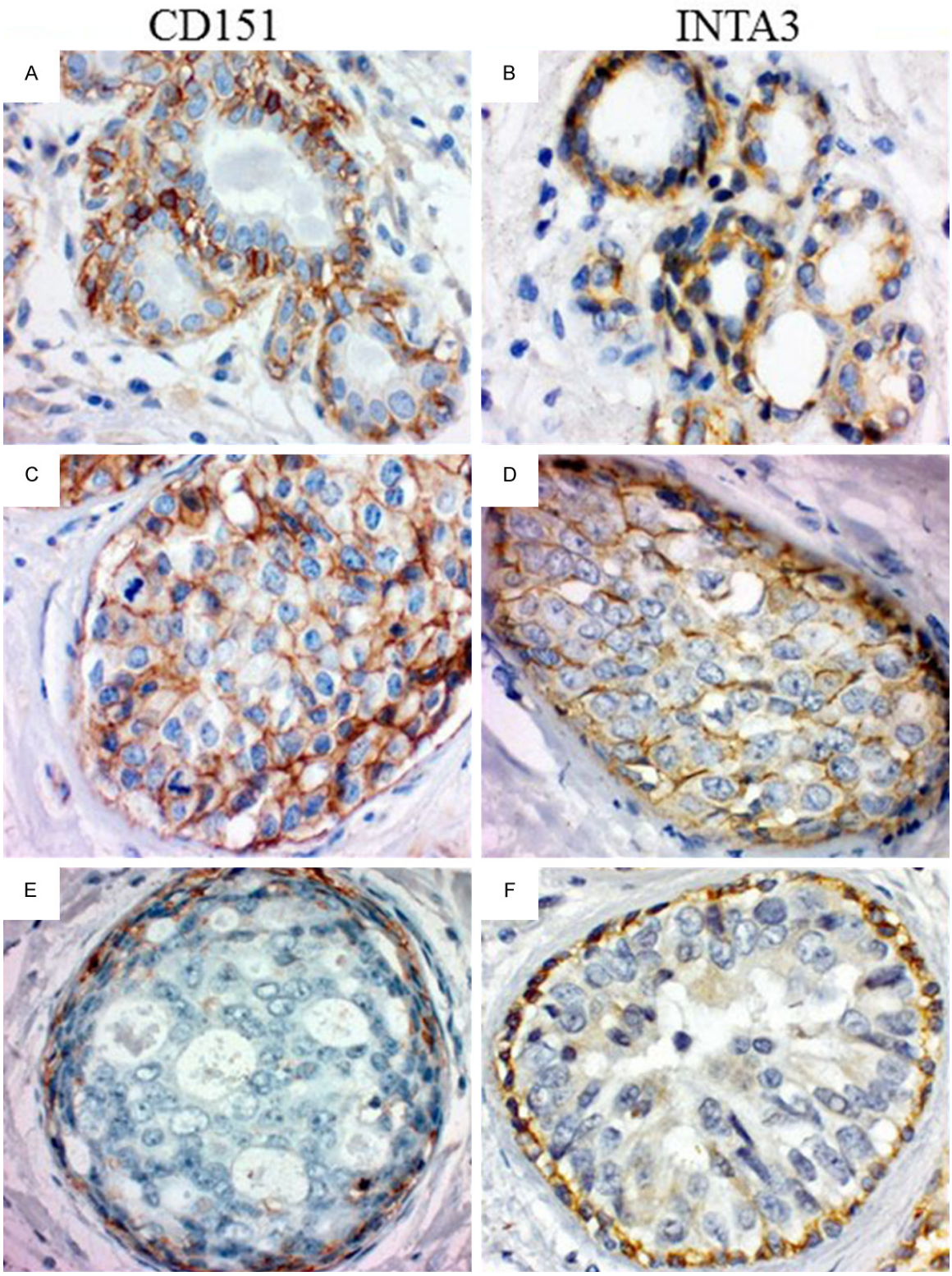

Figure 1. Expression profiles of CD151/Tspan24, INTA3. (A, B) Normal mammary gland. CD151 and INTA3 showed moderate to strong, predominantly, membranous immunoreactivity, confined to the basal and lateral surfaces of myoepithelial cells, with no or very weak staining in luminal epithelial cells. (C-F) DCIS. Two patterns of CD151 and INTA3 expression: membranous/cytoplasmic present in all cells of the lesion (C, D); restricted to the cells at the tumor-stroma interface (E, F).

Table 2. Association between CD151 and/or INTA3 expression and tumor phenotypic characteristics

Feature	*P* value		
	INTA3 (high: n = 33)	CD151 (high: n = 25)	CD151/INTA3 (high: n = 16)
		0.610	
CD151	0.610		
HER2 (-)	0.468	0.064	0.468
ER (-)	0.065	0,041	0.334
PR	0.421	0.075	0.317
ER/PR	0.065	0.041	0.334

Table 3. Association between CD151 and/or INTA3 expression and VNPI

A. All cases			
Feature	*P* value		
	INTA3 (high: n = 33)	CD151 (high: n = 25)	CD151/INTA3 (high: n = 16)
VNPI	0.597	0.059	0.070
B. HER2-negative cases			
Feature	*P* value		
	INTA3 (high: n = 17)	CD151 (high: n = 17)	CD151/INTA3 (high: n = 10)
VNPI	0.631	0.248	0.044
C. HER2-positive cases			
Feature	*P* value		
	INTA3 (high: n = 16)	CD151 (high: n = 8)	CD151/INTA3 (high: n = 6)
VNPI	0.171	0.647	0.832

membranous reactivity in ≤ 10% of tumor cells; ii) 1+/negative-faint membranous or partially membranous in ≥ 10% of tumor cells; iii) 2+/positive-weak to moderate complete membranous in ≥ 10% of tumor cells; iv) 3+/positive-strong complete membranous in ≥ 30% of the tumor cells. Taking into account well recognized heterogeneity of DCIS lesions within an individual case, for each specimen, immunoreactivity was assessed in at least five the largest ducts (325 ducts in total) and an average score was taken for statistical analyses. Scoring of immunoreactivity for INTA3 was carried out as follows: i) 0/negative-no reactivity, ii) 1+/positive-weak to moderate membranous and/ or cytoplasmic staining in ≤ 10% of tumor cells; iii) 2+/positive-moderate membranous and/or cytoplasmic staining in ≥ 10% of tumor cells; iv) 3+/positive-strong membranous and/or cytoplasmic staining in ≥ 30% of the tumor cells. Immunohistochemical staining was evaluated and scored independently by two observers (HR, RK*). The agreement on staining intensity was > 90%. Where there was disagreement, intensity was determined by consensus.

As epithelial cells of the normal gland displayed strong immunoreactivty for CD151 and much weaker for ITNA3, final scores were dichotomized into: a) 'negative' and b) 'positive' for CD151/0-2; INTA3/0 and CD151/3 and INTA3/1-3, respectively.

Statistical analysis

The data were assessed by unpaired t test and chi-square or Fisher exact test using the StatsDirect software (StatsDirect Ltd, Altrincham, UK). Two-sided *P* value < 0.05 was considered as significant.

All cases were reviewed and stained for ErbB2/HER2 using HercepTest™ (Dako). Immunohistochemical staining was recorded using a semiquantitative scoring system recommended by the manufacturer.

Follow-up data were available only in 6 cases precluding a DFS analysis.

Results

Expression of CD151 and INTA3 in normal mammary gland

Both CD151 and INTA3 showed moderate to strong, predominantly, membranous immunoreactivity, confined to the basal and lateral surfaces of the basal layer cells, with no or very weak staining in luminal epithelial cells (**Figure 1A**, **1B**).

Expression of CD151 and INTA3 in DCIS

In 51% of cases (25/49) there was no expression of CD151. In the remaining samples, CD151 was localized predominantly in the cell membrane and present in the majority of tumor cells. Moderate to strong complete membranous staining homogenously distributed within the lesions was seen in 23 cases (**Figure 1C**); in 1, confined to the outer layer cells (**Figure 1E**).

Immunoreactivity for INTA3 was seen in 67% of cases (33/49). As for CD151, two patterns of INTA3 expression, membranous/cytoplasmic

present in all cells of the lesion (**Figure 1D**) and restricted to the cells at the tumor-stroma interface (**Figure 1F**), were observed in 17 and 16 cases, respectively.

No apparent histological differences between DCIS specimens positive and negative for CD151 and/or INTA3 were noticed.

Expression of CD151 was inversely associated with ER ($P = 0.041$) and hormone receptor status (ER and/or PR) ($P = 0.041$) and there was a trend towards statistical significance of an inverse relationship with HER2 ($P = 0.064$). Level of INTA3 expression did not correlate with any of the tumor phenotypic characteristics (**Table 2**). Expression of neither CD151 nor INTA3 when assessed alone or in combination, correlated with the VNPI.

As reported previously in IDC, CD151/integrin alpha3 was of prognostic value only in patients with HER2-negative tumors [12]. Assuming that the traits of cell invasiveness are maintained throughout the BCa development, we looked at the relationship between expression of CD151/INTA3 and an equivalent of prognostic indicator in DCIS, the VNPI, in the subpopulation of HER2-negatvie patients. Indeed, levels of CD151/INTA3 expression correlated with VNPI in HER2-negative patients ($P = 0.044$), whereas there was no significant relationship with either CD151 ($P = 0.248$) or INTA3 ($P = 0.631$) when assessed individually (**Table 3**). Respective values in HER2-positive cases were for: i) CD151/INTA3 $P = 0.834$, ii) CD151, $P= 0.647$ and iii) INTA3 $P = 0.171$.

Discussion

Following our report implicating the tetraspanin CD151 in the development of mammary carcinoma *in situ* [13], here we have undertaken an evaluation of a relationship between CD151 in complex with its principal molecular partner, the integrin alpha3beta1 and a prognostic indicator in high grade DCIS. Due to low availability of pure high grade DCIS, the number of specimens was relatively low. However, results of our statistical analyses clearly demonstrate that in a subgroup of HER2-negative tumors, CD151 and integrin apha3 assessed in combination, were significantly correlated with VNPI, while neither protein analyzed individually showed any significant link with the prognostic index.

To date, HER2/ErbB2 is one of the most extensively studied biological prognostic factors in IDC, but available data on its importance in DCIS is scarce and contradicting [15-19]. While there have been several reports on lack of significant association between HER2 and the risk of recurrence after a DCIS [16, 18, 19], a recent study by Zhou et al. demonstrated that a high level of HER2 overexpression was highly predictive of disease relapse [21]. Furthermore, HER2 status determined the type of recurrence. HER2+DCISs were more likely to recur as new *in situ* lesions, while HER2-tumors were related to recurrences being invasive. This is consistent with the observation by Lu et al., that although HER2 robustly promotes growth factor-independent cell proliferation, it is unable to induce basement membrane breakdown and subsequent invasive growth [22]. Activation of additional mechanisms acting in cooperation with HER2-mediated signaling are required to induce invasive potential in HER2-positive cells, conferring a high risk of progression to IDC [22].

We have previously reported that CD151 supported proliferation of HB2 cells, a non-tumorigenic HER2-positive breast epithelial cell line, in 3-D extracellular matrices (ECM) and in Matrigel-based xenografts and that its pro-proliferative activity did not require the direct interaction with integrins [13]. On the other hand, results of our older work demonstrated that depletion of CD151 attenuated transforming growth factor beta1 (TGFbeta1)-induced scattering and proliferation of MDA-MB 231 cells, a HER2-negative breast cancer cell line, in 3D Matrigel. CD151-dependent cell scattering of these cells proved to be dependent on its association with either alpha3 or alpha6 integrins [23]. While a functional link between HER2 and CD151 has already been documented [12, 24], molecular basis of an impact of this tetraspanin on cellular behaviour of HER2-negative cells is unknown. It seems, however, to bestow invasive phenotype to these cells, as indicated by clinical analysis of a large cohort of breast cancer patients demonstrating a correlation between the elevated expression of CD151 and poor overall survival only of patients with HER2-negative (luminal A and quintuple-negative) tumors [10]. Although the mechanisms governing the DCIS-IDC progression are poorly understood, it is well recognized that, in addition of

the intrinsic changes associated with genetic mutations, other events, and in particular, interaction with the stroma may play a critical role in the progression to invasion [25]. Like its invasive counterpart, DCIS is characterized by a high degree of both inter-and intra-tumor heterogeneity in terms of conventional histological grades, prognostic biomarkers and patterns of growth [26]. It is likely that in certain biological contexts, this evolution is due to the competition for dominance among multiple cell subclones co-existing within an individual tumor [26]. The selective pressure of dominant HER2-negative cells might contribute to the development of IDC and provide an explanation for the low incidence of HER2-positive invasive tumors (20-25%), as compared to a high frequency of HER2 expression seen in DCIS (50-60%) [20]. In light of previous reports and current findings, it is tempting to speculate that the presence of CD151 on the membrane of HER2-negative cell might be a common trait to pre-invasive and malignant breast cancer cells, that makes them more responsive to the stimuli released by the environment, thus conferring their invasive phenotype.

The Van Nuys prognostic index (VNPI), described as a numerical representation of measurable prognostic factors (tumor size, margin width, nuclear grade, the presence or absence of comedonecrosis and, recently added age), was introduced in 1996 as an aid to be used in conjunction with clinical experience in the treatment decision-making process [14]. A multivariate regression analysis shown that, unlike its individual components, the VNPI treated as whole is a good prognostic indicator of a risk of local recurrence [27, 28]. VNPI has been based on recurrence data from large series of DCIS patients and shown a good correlation with disease outcome but a biological meaning of this empirically developed indicator in terms of a relationship to molecular mechanisms underlying DCIS progression remains poorly understood. Results of our study demonstrating a significant correlation between VNPI and CD151 seem to shed some light on pathophysiology of a subtype of DCIS tumors and suggest that CD151 expression might be a trait, common to IDC and DICS, identifying a subpopulation of DCIS HER2-negative cells likely to drive an invasive progression.

In summary, our results suggest that acting in complex with the integrin alpha3beta1 CD151 might play a role in the pathophysiology of DCIS and contribute to the process of invasive progression of HER2-negative lesions. Further analysis of a large cohort of DCIS patients with a long follow-up as well as mechanistic studies using experimental models are required to assess the emerging possibility for identification of DCIS lesions with invasive characteristics, potentially to be guiding clinical evaluation and management.

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Disclosure of conflict of interest

None.

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