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Ductal carcinoma in situ of the breast: immune cell composition according to subtype

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Abstract

Ductal carcinoma in situ of the breast includes several subtypes with a divergent biological behavior. Data regarding the composition of ductal carcinoma in situ-associated immune cells and their potential role in progression is limited. We studied ductal carcinoma in situ-associated immune response by characterizing immune cell subsets according to ductal carcinoma in situ subtypes. Ductal carcinoma in situ-associated tumor infiltrating lymphocyte (TIL) density was evaluated based on hematoxylin and eosin (H&E)-stained sections from 473 patients. Cases were subtyped based on ER, PR, and HER2. Patients were categorized as TIL-high or low. Ductal carcinoma in situ-associated immune cells of TIL-high cases were immunostained on whole slides with CD4, CD8, CD20, CD68, FOXP3, and PD-L1 (SP142 and SP263). In total, 131/473 patients (28.0%) were considered as TIL-high. The percentage of TIL-high cases was significantly higher in HER2+ and triple-negative ductal carcinoma in situ ($P < 0.0001$). Overall, no statistical difference in immune cell composition according to subtypes was found. However, individual subtype comparison showed that ER+ HER2+ cases had a significantly higher proportion of CD8+ T cells compared with triple-negative cases ($P = 0.047$). In TIL-high cases, PD-L1-SP142 expression on tumor cells was associated with subtype ($P = 0.037$); the lowest number of positive cases was observed in the HER2+ subtype (independent of ER). However, in TIL-high ductal carcinoma in situ, PD-L1 expression by both clones was limited. In conclusion, high numbers of TILs are predominantly observed in HER+ and triple negative ductal carcinoma in situ. The ER+ HER2+ subtype seems to attract a higher proportion of CD8+ T cells compared with the triple negative subtype. Among TIL-high cases, the HER2+ subgroup had the lowest PD-L1-SP142 expression on tumor cells. This suggests a more pronounced antitumor immunity in HER2+ ductal carcinoma in situ, which could play a role in its biological behavior.

Introduction

Invasive breast cancer is a heterogeneous disease that can be divided in several molecular subtypes with distinct clinical outcome [1]. Each molecular subtype has an immunohistochemical surrogate: luminal (ER+ with or without

HER2+), basal (ER-, PR-, and HER2-, known as triple negative), and HER2-driven (ER−, HER2+) [2]. Invasive breast cancer subtypes trigger the immune system differently; high numbers of tumor infiltrating lymphocytes (TILs) are associated with triple-negative and HER2+ invasive breast cancer [3, 4]. Numerous reports illustrate a prognostic effect of TILs in invasive breast cancer [5–7].

Ductal carcinoma in situ is a nonobligate precursor of invasive breast cancer [8, 9]. The detection rate of ductal carcinoma in situ has exponentially increased with the implementation of population screening by mammography [10–12]. Since the majority of patients are treated, data with respect to its treatment-naive behavior are limited. However, cases of regressive ductal carcinoma in situ have been described and an estimated 40–50% remains in situ carcinoma when left untreated [13–18]. Consequently, a substantial proportion of patients with ductal carcinoma in situ

might be overtreated, resulting in unnecessary morbidity and health care costs. On the other hand, although a substantial proportion of breast cancer patients are detected in an early in situ stage, the incidence and morbidity of invasive breast cancer remains high [11]. Novel therapeutic and preventive strategies are therefore needed in order to optimize early risk assessment and intervention. In recent years, immunotherapy is regarded as one of the most promising approaches in cancer therapy [19]. However, the failure rate is substantial, potentially due to treatment at late stages and due to incomplete understanding of interactions between cancer cells and immune cells [20, 21]. Increased knowledge regarding the role of the immune response in an early disease stage as ductal carcinoma in situ could have major clinical consequences, as it could contribute to future immune modulation and potential prevention of progression.

Ductal carcinoma in situ has a heterogeneous biological behavior [22]. In studies restricted to patients with pure ductal carcinoma in situ, the frequency of HER2+ cases is relatively high (22–76%) compared with invasive breast cancer studies, which report HER2 positivity in about 11–23% of cases [18, 23–28]. In contrast, the frequency of triple negative ductal carcinoma in situ is relatively low in pure ductal carcinoma in situ studies (4–7.5%) compared with invasive breast cancer studies, which report that 10–13% of all invasive breast cancers are triple negative [23–27]. In line with this, there is a high frequency of extensive ductal carcinoma in situ adjacent to HER2+ invasive breast cancer, whereas the ductal carcinoma in situ component in triple negative invasive breast cancer, if present, is rather limited [27, 29, 30]. These data suggest a different biological behavior according to ductal carcinoma in situ subtype: HER2+ cases seems to either remain in situ for a longer period of time or has a rapid in situ growth rate. Triple negative ductal carcinoma in situ on the other hand seems to have a relatively rapid progression to invasion. These differences could be related to characteristics of the tumor cells and/or the microenvironment, i.e., the ductal carcinoma in situ-associated immune response.

Tumor associated immune cells are aggregated in heterogeneous infiltrates, of which TILs make up the largest group [31]. Data regarding the composition and role of TILs in ductal carcinoma in situ are limited, although this is an emerging field of research [32]. High TIL infiltration has been associated with high grade, triple negative and/or HER2+ ductal carcinoma in situ, which is in line with invasive breast cancer studies [25, 33–35]. Further analyses according to the composition of the immune cells showed an association between high numbers of CD8+ T cells, ductal carcinoma in situ regression, and low ipsilateral recurrence risk [18, 36, 37]. On the other hand, CD19/CD20+ B cells and CD68+Mac387+ macrophages

were associated with a larger ductal carcinoma in situ diameter, high grade, presence of comedonecrosis, and shorter recurrence free survival [36, 38]. In addition, two recent studies reported on PD-L1 expression in ductal carcinoma in situ [34, 39]. Hendry et al. reported PD-L1 (clone: SP263, Ventana) expression on 11% of ductal carcinoma in situ cells and 25% of ductal carcinoma in situ-associated TILs ($n = 138$ cases). On the other hand, Thompson et al. reported no PD-L1 (clone: 5H1, Abcam) expression in ductal carcinoma in situ cells, but 81% of ductal carcinoma in situ-associated TILs ($n = 23$ cases).

These studies described above have some limitations, including study size and the use of tissue micro arrays for analysis of ductal carcinoma in situ-associated immune cells. Larger series were restricted to the analysis of one immune cell subset (such as CD20 + B cells or CD8+ T cells) or did not include ER, PR, and HER2 status [18, 25, 38]. Therefore, a more complete knowledge on how different immune subsets are associated with ductal carcinoma in situ subtype is lacking. Based on the biological behavior of ductal carcinoma in situ subtypes, as described above, we hypothesize that there is an association between subtype and the composition of the immune infiltrate. The extensive growth pattern of HER2+ cases suggests a potential protective/antiinvasion effect of the immune cells. The biological behavior of triple negative ductal carcinoma in situ on the other hand, which is also associated with dense immune infiltrates like HER2+ ductal carcinoma in situ, rather suggests immune evasion. The objective of this study was therefore to characterize the presence and composition of ductal carcinoma in situ-associated TILs in a large series of patients, in order to allow subgroup analyses according to subtype.

Patients and methods

Patient selection and clinical data collection

For this retrospective study, we included patients with a primary diagnosis of ductal carcinoma in situ, diagnosed at the Erasmus Medical Center Cancer Institute Rotterdam or the Laboratory for Pathology Dordrecht between 2000 and 2016. Clinical data collection included age, type of surgery (breast-conserving surgery or mastectomy), and follow-up. Patients with bilateral ductal carcinoma in situ were included as two cases. Patients with an ipsilateral invasive breast cancer within 6 months after the initial diagnosis of ductal carcinoma in situ were excluded. Other exclusion criteria were microinvasion and insufficient tissue. Recurrences were defined as ipsilateral histologically proven ductal carcinoma in situ, invasive breast cancer or regional/distant breast cancer metastases without a contralateral invasive

breast cancer, occurring ≥6 months after the initial ductal carcinoma in situ diagnosis. For this study, we used encoded leftover patient material, and therefore did not need an informed consent or approval from an ethical committee according to the Code of Conduct of the Federation of Medical Scientific Societies in The Netherlands [40].

Pathological assessment of DCIS characteristics

According to Dutch guidelines, ductal carcinoma in situ lesions <4 cm are embedded completely since 2005. In lesions with a diameter >4 cm, at least ten blocks of the lesion are embedded in order to exclude an invasive component. All hematoxin and eosin (H&E)-stained whole sections of excision specimen were centrally reviewed by two observers to assess histological grade (based on nuclear atypia, according to the WHO classification), the predominant growth pattern, presence of comedonecrosis, microcalcifications, and TILs. In case of a disagreement, consensus was reached. Density of TILs was semi-quantified as minimal/absent, mild, moderate, or severe. Ductal carcinoma in situ-associated stroma was defined as the stroma within 1 mm of the duct, as described by Toss et al. [41]. Cut-offs for TIL density were defined as 0–5, 5–30, 30–50, and >50% of the ductal carcinoma in situ-associated stroma occupied by TILs, respectively, adapted from Beguinot et al. [35] and

illustrated in Fig. 1. Distribution of TILs was scored as focal, patchy, or diffuse, defined as <10, 10–50, and >50% of all ductal carcinoma in situ ducts surrounded by TILs, respectively. Based on the density and distribution, cases were classified as either TIL-high or TIL-low. TIL-low ductal carcinoma in situ was defined as a low density of TILs (<30% of the ductal carcinoma in situ-associated stroma occupied by TILs) or a focal TIL distribution. TIL-high DCIS was defined as a moderate or high TIL density (>30% of the ductal carcinoma in situ-associated stroma occupied by TILs) and a patchy or diffuse TIL distribution. Ductal carcinoma in situ subtypes were determined by ER, PR, and HER2 immunohistochemistry (Table 1). ER and PR were defined as positive when at least 10% of the tumor cell was positive, which is extracted from the Dutch guidelines for IBC scoring [42]. HER2 was scored according to international guidelines [43]. HER2 In situ hybridization was performed in cases with equivocal HER2 immunohistochemistry. Ductal carcinoma in situ was classified as ER+PR±HER2−, ER+PR±HER2+, ER−PR−HER2+, or triple negative.

Pathological assessment of ductal carcinoma in situ-associated TILs

To determine ductal carcinoma in situ-associated immune cell composition, we immunostained the DCIS-associated

Fig. 1 H&E images of ductal carcinoma in situ with absent/minimal (A), mild (B), moderate (C), or severe (D) TIL density (×10 magnification)
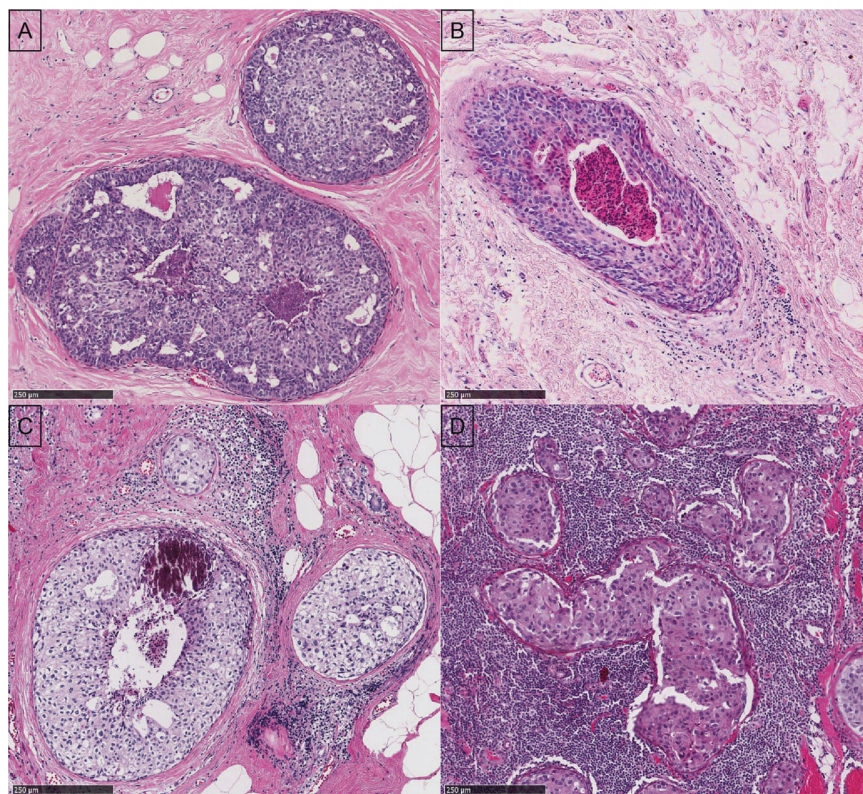
Table 1 Antibody characteristics and used protocol for whole tissue IHC

Antibody*	Cell type	Type	Concentration	Company	Clone	Lot number	Procedure	Incubation time
ER	n.a.	Anti-rabbit	1 µg/ml	Ventana	SP1	F02583	Ultraview CC1 64′	32 min
PR	n.a.	Anti-rabbit	1 µg/ml	Ventana	1.00E+02	Y08684	Ultraview CC1 36′	12 min
HER2neu	n.a.	Anti-rabbit	6 µg/ml	Ventana	4B5	E06192	Ultraview CC1 36′	32 min
CD4	T-helper cell	Anti-rabbit	2.5 µg/ml	Ventana	SP35	G07304	Ultraview CC1 64′	8 min
CD8	Cytotoxic T cell	Anti-rabbit	0.35 µg/ml	Ventana	SP57	Y04591	Optiview CC1 16′	32 min
CD20	B cell	Anti-mouse	0.3 µg/ml	Ventana	L2G	Y19660	Ultraview CC1 64′	44 min
CD68	Macrophage	Anti-mouse	0.4 µg/ml	Ventana	KP1	G01685	Optiview CC1 16′	8 min
FOXP3	Regulatory T-cell	Anti-mouse	0.5 mg/ml	Thermofisher	236A/E7	4339062	Optiview CC1 32′	32 min
PDL1**	n.a.	Anti-rabbit	7 µg/ml	Ventana	SP142	Y03641	Optiview CC1 64′	16 min
PDL1**	n.a.	Anti-rabbit	1.61 µg/ml	Ventana	SP263	Y2898U	Optiview CC1 64′	16 min

*All antibodies were purchased from Ventana Roche, **PD-L1 antibodies were supplied by ROCHE

immune cells of TIL-high cases by automated immunohistochemistry using the Ventana Benchmark ULTRA (Ventana Medical System Inc.). Since TIL-low ductal carcinoma in situ has little to no TILs to characterize, characterization of TILs was restricted to TIL-high cases. Sequential 4-µm-thick formal fixed paraffin-embedded whole tissue sections were stained for CD4 (T-helpers), CD8 (Cytotoxic T cells), CD20 (B cells), CD68, (Macrophages) and FOXP3 (Regulatory T cells). Table 1 provides an overview of used antibodies and protocols. In addition, PD-L1 (Programmed cell death ligand) was assessed using two clones (SP263 and SP142). Briefly, following deparaffinization and heat-induced antigen retrieval, tissue samples were incubated according to their optimized time and protocol with the antibody of interest. Incubation was followed by hematoxylin II counter stain for 8 min and then a blue coloring reagent for 8 min according to the manufacture's instructions (Ventana). The percentages of CD4+, CD8+, CD20+, and CD68+ immune cells were assessed manually by eyeballing, relatively to one another, with a combined score of 100%. Next, the percentage of FOXP3+ cells was determined as a proportion of all immune cells. PD-L1 expression in IBC is yet to be standardized; we therefore used two clones, which were scored individually. Membranous PD-L1 expression was scored for the ductal carcinoma in situ cells and the immune cells, as the percentage of positive cells. The cut-off for PD-L1 positivity was set at 1%, both for ductal carcinoma in situ cells and immune cells. Two observers, blinded for ER, PR, and HER2 status, assessed all immunohistochemical staining.

Statistical analysis

IBM SPSS statistics 21.0 was used to perform statistical analysis. The Chi-square test was used to test for associations between clinicopathological characteristics, ductal carcinoma in situ subtypes based on immunohistochemistry, TILs, and PD-L1 expression. After testing for normal distribution, the Kruskal–Wallis test was used to analyze difference in the proportion immune subset density across the ductal carcinoma in situ subtypes. Differences in the proportion immune subsets between individual IHC-subtypes were analyzed using the Mann–Whitney U test. Differences between PD-L1 expressions of both clones were assessed using the Wilcoxon Signed Ranks Test. A log-rank Mantel–Cox test was used to analyze the effect of TILs and PD-L1 expression on ipsilateral recurrence. Results were considered significant with a P-value < 0.05.

Results

General clinicopathologic characteristics

In total, 501 cases were reviewed for this study. After central revision, 28 samples were excluded because of an ipsilateral IBC within 6 months ($n = 22$) or at deeper sectioning for additional immunohistochemical staining ($n = 6$). We therefore included 473 patients with pure ductal carcinoma in situ for further characterization of TILs. Table 2 provides an overview of clinicopathologic characteristics of this series. The median age at diagnosis was 58 years (range: 27–84), with a median follow-up time of 98 months (range: 24–218). Most patients (62%) were treated with breast-conserving surgery. The majority of patients (52%) were diagnosed with high-grade, and most cases were associated with calcifications (72%). Ductal carcinoma in situ was subtyped as ER+PR±HER2− ($n = 225$; 54%), ER+PR ±HER2+($n = 80$; 17%), ER−PR−HER2+ ($n = 85$; 18%), TN ($n = 22$; 5%) or missing ($n = 31$).

Table 2 Clinicopathological characteristics (*n* = 473)

Age at diagnosis (in years)	
- Median (range)	58 (27–84)
Surgery (%)	
- Breast-conserving surgery	293 (62)
- Mastectomy	180 (38)
Diameter (in cm)	
- Median (range)	2.00 (0.1–23.0)
- Missing (*n*)	65
Growth pattern (%)	
- Solid	239 (51)
- Cribriform	190 (40)
- Micropapillary	37 (8)
- Papillary	7 (1)
Grade (%)	
- Low	63 (13)
- Intermediare	166 (35)
- High	244 (52)
Calcification (%)	
- Absent	134 (28)
- Present	339 (72)
Comedonecrosis (%)	
- Absent	234 (49)
- Present	239 (51)
TILs density (%)	
- Minimal	212 (45)
- Mild	110 (23)
- Moderate	113 (24)
- Severe	38 (8)
TILs distribution (%)	
- Focal	71 (15)
- Patchy	133 (28)
- Diffuse	57 (12)
- N.A.	212 (45)
Immunohistochemical subtype (%)	
- ER+PR±HER2−	255 (54)
- ER+PR±HER2+	80 (17)
- ER−PR−HER2+	85 (18)
- ER−PR−HER2−	22 (5)
- Missing	31 (7)
Ipsilateral recurrence (%)	
- None	450 (95)
- Ductal carcinoma in situ	4 (1)
- Invasive breast cancer	5 (1)
- Invasive breast cancer and metastasis	5 (1)
- Metastasis	2 (0)
- Missing	7 (2)

Presence of TILs according to ductal carcinoma in situ characteristics

After stratification according to the TIL density and distribution, 131 out of 473 patients (28%) were classified as TIL-high. TIL density and distribution were strongly associated (*P* < 0.0001, Chi-square test, Supplementary Table 1).

TIL-high ductal carcinoma in situ was associated with high grade (*P* < 0.0001), a predominantly solid growth pattern (*P* = 0.006), presence of comedonecrosis (*P* < 0.0001), larger diameter (*P* < 0.0001), and subtype based on immunohistochemistry (*P* < 0.0001). In multivariate analysis, only the diameter, grade, and subtype remained significantly associated with TILs (Table 3).

Overall, the majority of the ER-PR-HER2+ and triple-negative subtype was TIL high (61% and 64%, respectively), whereas the opposite was true for the ER+PR+HER2- and ER+PR+HER2+ subtype (11% and 39%, respectively) (Fig. 2). Within the ER+HER2- and ER-PR-HER2+ subtypes, there was a significant association between the grade and presence of TILs; high-grade cases had significantly more TILs compared with low-grade cases (*P* = 0.001 for ER+HER2− ductal carcinoma in situ and *P* = 0.008 for ER−PR−HER2+ ductal carcinoma in situ).

Immune cell composition

Due to insufficient remaining tissue, 13 TIL-high cases were excluded for immune cell composition analysis. We therefore included 118 TIL-high cases to study CD4, CD8, CD20, CD68, FOXP3, and PD-L1 expression. Overall, the majority of the ductal carcinoma in situ-associated immune cells were CD4+, followed by CD20+, CD8+, and CD68+ with a median of 46.0% (range 4–80), 34.5% (range 2–90), 10.0% (range 2–40), and 5.0% (range 1–74), respectively (Fig. 3). The median proportion of FOXP3 expression was 3.0% (range 0–20).

In these TIL-high cases, PD-L1 expression was observed in both ductal carcinoma in situ cells and immune cells (Fig. 4). In total, 9 out of 119 cases (8%) were PD-L1-SP142 positive in ductal carcinoma in situ cells and 55 out of 119 cases (46%) were PD-L1-SP263 positive in ductal carcinoma in situ cells. Regarding TILs, 97 out of 119 cases (82%) were PD-L1-SP142 positive and 112 out of 119 cases (94%) were PD-L1-SP263 positive. However, the overall PD-L1 expression was limited in both components. The median % of PD-L1 positive ductal carcinoma in situ cells was 0 for both SP142 and SP263 (SP142 range 0–6 and SP263 range 0–52). The median % of PD-L1+ immune cells was 1.0% for both SP142 and SP263 (SP142 range 0–7 and SP263 range 0–10). However, the expression of the PD-L1-SP263 clone was higher compared with the PD-L1-SP142 clone, for both ductal carcinoma in situ and immune cells (*P* < 0.0001).

TIL composition according to ductal carcinoma in situ characteristics

Overall, there was no statistical difference in the composition of immune cell subsets according to ductal carcinoma

Table 3 Ductal carcinoma in situ characteristics according to TILs

	Overall TILs in ductal carcinoma in situ		P-value univariate analysis	P-value multivariate analysis
	TILs low (n = 342)	TILs high (n = 131)		
Age at diagnosis (years)			0.366	0.660
- Median (range)	58.00 (29.00–84.00)	57.00 (25.00–82.00)		
Surgery				
- Breast-conserving therapy	222 (76)	71 (24)	**0.032**	0.513
- Mastectomy	120 (67)	60 (33)		
Diameter (missing n = 53) (in cm)			**<0.0001**	**0.044**
- Median (range)	1.65 (0.1–23.0)	2.90 (0.1–16.0)		
Growth pattern (%)				
- Solid	160 (67)	79 (33)		
- Cribriform	153 (81)	37 (19)	**0.006**	0.350
- Micropapillary	23 (62)	14 (38)		
- Papillary	6 (86)	1 (14)		
Grade (%)				
- Low (G1)	60 (90)	3 (5)		
- Intermediate (G2)	149 (90)	17 (10)	**<0.0001**	**<0.0001**
- High (G3)	133 (55)	111 (45)		
Calcification (%)				
- Absent	104 (78)	30 (22)	0.105	0.796
- Present	238 (70)	101 (30)		
Comedonecrosis (%)				
- Absent	191 (82)	43 (18)	**<0.0001**	0.208
- Present	151 (63)	88 (37)		
Immunohistochemical subtype (missing n = 31) (%)				
- ER+PR±HER2−	226 (89)	29 (11)		
- ER+PR±HER2+	49 (61)	31 (39)	**<0.0001**	**<0.0001**
- ER−PR−HER2+	33 (39)	52 (61)		
- ER−PR−HER2−	8 (36)	14 (64)		
Ipsilateral recurrence				
- No	330 (73)	120 (27)	0.338	0.175
- Yes	10 (63)	6 (38)		

Significant values (P < 0.05) are indicated as bold

in situ subtypes. However, since HER2+ and triple-negative cases have a distinct biological behavior, we performed subgroup analyses restricted to these subtypes. This showed a significantly higher proportion of CD8+ T cells in the ER+HER2+ subtype compared with the triple negative subtype, although the absolute difference was limited (14 versus 10%; P-value = 0.047). The ER−HER2+ subtype was associated with a higher proportion of CD4+ T cells compared with the triple-negative group, although significance was not reached (P-value = 0.061). In addition, among TIL-high ductal carcinoma in situ, PD-L1-SP142 expression on tumor cells was associated with immunohistochemical subtype (P = 0.037); the lowest number of positive cases was observed in the HER2+ subgroups (both ER+HER2+ and ER-HER2+). There was no significant association between PD-L1 status (positive versus negative), and other features of neither the ductal carcinoma in situ component nor other immune cell parameters.

TILs and ipsilateral recurrence

In total, follow-up was available for 466 cases. The median follow-up time was 98 months (range 24–218 months). We

Fig. 2 The proportion of TILs (TIL-low versus high cases) according to ductal carcinoma in situ (DCIS) subtype. The *X*-axis shows the ductal carcinoma in situ subtypes and the *Y*-axis illustrates the proportion of patients
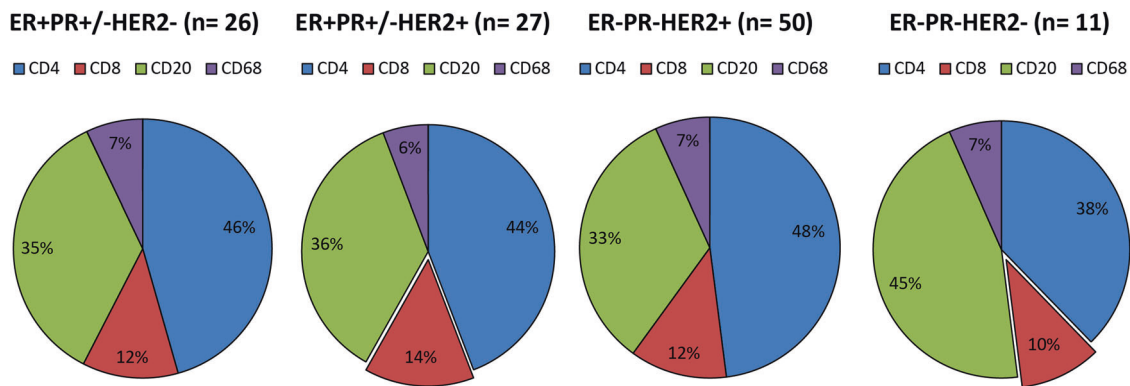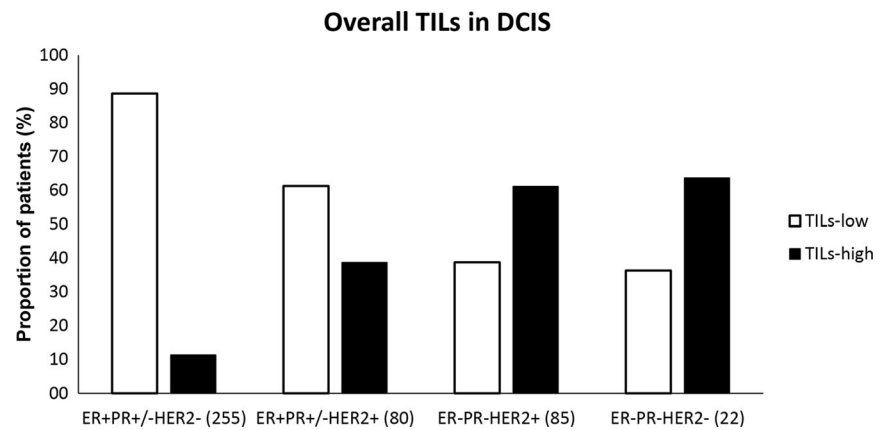

Fig. 3 The immune cell distribution according to subtype. The proportion of CD4 (blue), CD8 (red), CD20 (green), and CD68 (purple) expression is displayed as a percentage (%) according to subtype
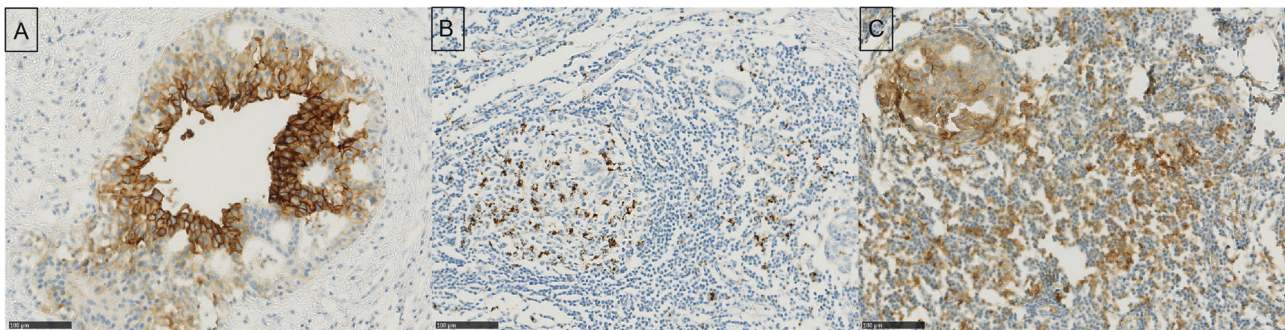

Fig. 4 Images of immunohistochemistry of PD-L1 expression in ductal carcinoma in situ cells (clone SP263) (**A**), ductal carcinoma in situ-associated immune cells (clone SP142) (**B**) and in both (clone SP263) (**C**)

observed an ipsilateral recurrence in 16 patients (Table 2). The majority of these recurrences were invasive (10 out of 16); the remaining cases had a ductal carcinoma in situ recurrence. Half of the patients with an invasive recurrence (5 out of 10) were associated with regional or distant metastasis. Two of these patients had a histologically proven distant breast cancer metastasis (liver and bone, respectively), in the absence of a proven primary invasive breast cancer. Overall, there were no significant associations between general ductal carcinoma in situ features and ipsilateral recurrence. The majority of patients with an ipsilateral recurrence had a primary diagnosis of ER+PR+HER2- ductal carcinoma in situ ($n = 10$), remaining cases were HER2+. No recurrences were observed in those patients with a triple-negative ductal carcinoma in situ. In total, 6 out of 16 patients with a recurrence had TIL-high ductal carcinoma in situ in the original specimen; the remaining ten cases were TIL low. There was no significant

association between TILs (TIL high versus low cases) and ipsilateral recurrence.

We compared the proportion of immune cell subsets and PD-L1 expression of TIL-high cases with ipsilateral recurrences to those without ipsilateral recurrence. There was no association between immune cell subset composition and ipsilateral recurrence. However, cases with an ipsilateral recurrence were associated with PD-L1-SP263 expression in ductal carcinoma in situ cells ($P = 0.007$), whereas there was no association between expression in TILs and recurrence ($P = 0.522$) by comparing PD-L1 positive versus negative cases (Supplementary table 2). Regarding PD-L1 expression as a continuous variable, however, expression levels in both ductal carcinoma in situ and TILs were significantly higher in the recurrent group versus the non-recurrent group (P = 0.002 and P = 0.022 respectively).

There was no significant association between PD-L1-SP142 expression and ipsilateral recurrence ($P = 0.408$ for ductal carcinoma in situ and $P = 0.221$ for TILs) when comparing PD-L1 positive versus negative cases. However, the level of PD-L1-SP142 expression as a continuous variable in TILs was higher in the recurrent group compared with the nonrecurrent group ($P = 0.045$).

Discussion

This is the largest study evaluating the presence, including the composition, of ductal carcinoma in situ-associated immune cells in relation to ductal carcinoma in situ subtype based on immunohistochemistry. In our series, we found an association between ductal carcinoma in situ subtype and the presence of TILs, whereby ER-PR-HER2+ and triple-negative cases had the highest numbers of TILs, which is in line with previous studies [25, 33, 34].

The majority of ductal carcinoma in situ-associated immune cells were CD4+ T cells, of which 3% were likely to be regulatory T cells (FOXP3 positive). Overall PD-L1 expression was rather low in both ductal carcinoma in situ cells and immune cells. Nonetheless, high CD4+ FOXP3 +T-cell infiltrations, which partly express PD-L1, suggest a suppressed tumor immune microenvironment. This is in line with our finding, whereby PD-L1-SP263 expression was associated with an ipsilateral recurrence. Besides, this was also previously suggested by other studies demonstrating that CD4+ FOXP3+ regulatory T-cell infiltration was associated with large, highly proliferative ductal carcinoma in situ and microinvasion [35, 36, 39, 44].

Our data regarding ductal carcinoma in situ subtype and immune cell composition of TIL-high cases shows that the HER2+ subtype (independent of ER) had significantly less PD-L1-SP142 expression in tumor cells compared with the other subtypes, which suggests less immune suppression.

This is in line with a previous study of Thompson et al., reporting that HER2+ ductal carcinoma in situ did not have PD-L1-high TILs, whereas all included triple negative cases had PD-L1-high TILs [39]. Additionally, the ER+PR ±HER2+ subtype was associated with a significantly higher proportion of CD8+ T cells compared with the triple negative subtype. This association was also suggested by Morita et al., who reported an association between HER2+ ductal carcinoma in situ and high numbers of CD8+ T cells [18]. Previous studies also reported an association between high infiltration of ductal carcinoma in situ-associated CD8 + T cells and a low recurrence risk [36, 37], which is in line with invasive breast cancer studies [45–47]. These findings suggest a more pronounced anti-tumorigenic immune response in HER2+ ductal carcinoma in situ cases, which could play a role in its biological behavior.

To our knowledge, our study is the first to characterize ductal carcinoma in situ-associated immune cells and their association with subtype at this scale. Previous cohorts either evaluated immune cells, included ductal carcinoma in situ with microinvasion, small cohorts, did not consider the ER, PR, and HER2 status or they only evaluated TILs based on H&E in relation to ER/PR/HER2 status [18, 25, 34–36, 38, 39, 41, 44]. Moreover, we are the first to have used two sets of PD-L1 clones (SP142 and SP263, Ventana) to assess PD-L1 expression in TIL-high ductal carcinoma in situ. Several studies reported about the clinical results of immunotherapy in invasive breast cancer, but the nonresponse rate is still high [20, 21]. There is currently no generally accepted PD-L1 clone or scoring system for invasive breast cancer or ductal carcinoma in situ. Increased knowledge regarding PD-L1 expression during breast carcinogenesis could contribute to future development of immune modulating therapies. Currently, immune modulation is mainly used for late stages of disease. However, if side effects and costs decrease during future drug development, it is likely that immune modulation will also be considered for earlier disease stages. The limited expression of PD-L1 expression in our DCIS cohort does however not support the use of immunotherapy in the in situ stage.

Nevertheless, our study also has some limitations. First, consensus for evaluation of TILs in ductal carcinoma in situ is yet to be reached, which results in different quantification methods, which restricts the comparison of results from several studies [41]. Besides, the quantification of TILs and immune cell subset composition is partly subjective, due to tissue selection bias and/or quantification methods, which could affect reproducibility between observers. We only analyzed PD-L1 expression of TIL-high cases, which limited our knowledge with respect to PD-L1 expression on tumor cells of TIL-low ductal carcinoma in situ. Besides, there are several limitations of the PD-L1 clones. The use of the SP142 antibody using the Ventana assay to assess

PD-L1 expression was reported to be less sensitive than other commercially available antibodies, even though this antibody itself is equally sensitive [48]. This difference in sensitivity due to the assay could have impacted our results. In addition, we started with a large series, but the numbers of ipsilateral recurrences were low during a relatively limited follow-up time, which restricted the correlation of several pathological features with outcome.

In conclusion, high numbers of TILs are mainly observed in HER+ and triple-negative ductal carcinoma in situ and the majority of these are CD4+ T cells. The ER+HER2+ subtype seems to attract a higher proportion of CD8+ T cells compared with the triple-negative subgroup. In addition, the TIL-high HER2+ subgroup (independent of ER) had the lowest PD-L1-SP142 expression on tumor cells. This suggests a more pronounced antitumorigenic immune response in HER2-positive ductal carcinoma in situ, which might play a role in its distinct biological behavior.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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