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Expression of T cell-related proteins in breast ductal carcinoma *in situ*

Eunah Shin, Hye Min Kim and Ja Seung Koo

Department of Pathology, Yonsei University College of Medicine, Seoul, South Korea

Summary. This study aims to explore the expression of T cell subtype markers within the immune cells constituting the tumor microenvironment of ductal carcinoma *in situ* (DCIS) and to assess its implications. A tissue microarray comprising 191 cases of breast DCIS was created, and immunohistochemistry staining for T cell subtype markers (STAT3, STAT4, STAT-6, and FOXP3) was conducted. The DCIS cases were categorized into luminal, HER-2, and TNBC (Triple-negative breast cancer) types based on ER, PR, HER-2, and Ki-67 results. Additionally, they were classified as low-TIL (tumor-infiltrating lymphocytes) (<10%) or high-TIL (≥10%) types according to stromal TIL. Results revealed that 54.6% were luminal, 39.5% HER-2, and 5.9% TNBC. STAT3 exhibited a high positivity rate in luminal-type tumor cells, while STAT3, STAT4, STAT6, and FOXP3 showed elevated positivity rates in TNBC immune cells (p<0.05). Furthermore, a higher positivity rate was observed in high-TIL immune cells compared with low-TIL (p<0.001). The strongest agreement between T cell subtype markers in immune cells was found between STAT3 and STAT4 (OA=83.7%, κ=0.658), whereas the lowest was between STAT4 and FOXP3 (OA=71.7%, κ=0.370). In immune cells, STAT3 and STAT4 positivity correlated with necrosis (p<0.001), and the absence of positivity in all immune cell-related proteins in DCIS with necrosis was associated with poor prognosis (p=0.013). In conclusion, the immune cells in DCIS exhibit positivity for diverse T cell subtype markers, with TNBC and high-TIL DCIS displaying heightened positivity.

Key words: Breast, Ductal carcinoma *in situ*, Immune cells

Corresponding Author: Ja Seung Koo, MD, PhD, Department of Pathology, Yonsei University College of Medicine, Severance Hospital, 50 Yonsei-ro, Seodaemun-gu, Seoul, 120-752, South Korea. e-mail: kjs1976@yuhs.ac

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Introduction

Ductal carcinoma *in situ* (DCIS) is a pre-invasive lesion of breast cancer. Traditionally, breast cancer is known to progress from normal epithelium to atypical ductal hyperplasia (ADH), DCIS, and then to invasive carcinoma. Therefore, DCIS can be considered a direct precursor of invasive carcinoma. Additionally, approximately half of invasive carcinomas are accompanied by DCIS (Doebar et al., 2016), and the genetic changes in ADH, DCIS, and invasive carcinoma are quite similar (Gao et al., 2009), supporting the theory that DCIS is a direct precursor of invasive carcinoma. However, it has been reported that only 20-50% of untreated DCIS progress to invasive carcinoma, suggesting that additional events are required for DCIS to progress to invasive carcinoma (Page et al., 1982, 1995; Sanders et al., 2005). The factors contributing to the progression of DCIS to invasive carcinoma include intrinsic factors, such as genetic alterations in the tumor cells that make up DCIS, and extrinsic factors, such as the influence of the tumor microenvironment (TME) or tumor stroma.

The TME refers to non-transformed elements located in the area surrounding a tumor, which include immune system elements (such as macrophages and lymphocytes), blood vessel cells, fibroblasts, myofibroblasts, mesenchymal stem cells, adipocytes, and extracellular matrix (ECM). Among these, infiltrating immune cells form a diverse group of cells in the TME of breast cancer, including dendritic cells, T-cells, macrophages, myeloid-derived suppressor cells (MDSCs), and natural killer (NK) cells (Amer et al., 2022). These infiltrating immune cells play a dual role in inhibiting and promoting the progression of tumor cells by exerting their unique functions according to each cell type. Previous studies have reported that the characteristics of immune cells in the TME of DCIS are associated with the molecular subtype (Kim et al., 2016; Thompson et al., 2016; Miligy et al., 2017) and tumor recurrence (Kim et al., 2016; Campbell et al., 2017), indicating that immune cells influence the tumor biology of DCIS. Previous studies on tumor-infiltrating lymphocytes (TILs) in breast cancer have mainly

focused on research based on CD markers such as CD4, CD8 (Kim et al., 2013; Miyan et al., 2016), CD19, CD20, and CD138 (Miligy et al., 2017). Effector T cells, which are thought to be mainly located in the surrounding tissue of the tumor, are composed of Th1, Th2, Th17, and Treg cells (Zhou et al., 2009). However, studies on T-cell subtypes in DCIS are lacking. Representative markers of Th1, Th2, Th17, and Treg cells include STAT3 (Th17) (Han et al., 2019), STAT4 (Th1) (Nakayamada et al., 2011), STAT6 (Th2) (Karpathiou et al., 2021), and FOXP3 (Treg) (Wang et al., 2023).

This study aims to investigate the expression of T cell subtype markers in immune cells that make up the TME of DCIS and examine their implications.

Materials and methods

Patient selection and histologic evaluation

This study was conducted on patients diagnosed with DCIS who underwent surgery at Severance Hospital from January 2000 to December 2006. Patients who received chemotherapy or hormone therapy before surgery were excluded. The study was approved by the Institutional Review Board of Yonsei University Severance Hospital, and informed consent was waived (IRB number: 4-2023-1481). All cases were reviewed by a breast pathologist (Koo JS) using Hematoxylin & Eosin (H&E)-stained slides. Clinicopathologic parameters evaluated in each case included age at initial diagnosis, lymph node metastasis, tumor recurrence, distant metastasis, and patient survival. Stromal TILs in DCIS were observed and measured using a microscope, and cases with less than 10% TILs were defined as low TIL, and cases with 10% or more were defined as high TIL (Beguinot et al., 2018).

Tissue microarray

After reviewing the H&E-stained slides, the most appropriate formalin-fixed paraffin-embedded (FFPE) tumor tissue sample was retrospectively collected. The most representative tumor area was marked on the FFPE and a punch machine was used to extract the selected area, inserting a 3 mm tissue core into a 6x5 recipient block. All cases were made into tissue microarrays (TMAs) with two tissue cores each.

Table 1. Source, clone, and antibody dilution.

Antibody	Company	Clone	Dilution
Immune cell-related proteins			
STAT3	Cell Signaling Technology, Danvers, MA, USA	124H6	1:300
STAT4	Abcam, Cambridge, UK	EP1900Y	1:100
STAT6	Abcam, Cambridge, UK	YE361	1:50
FOXP3	Abcam, Cambridge, UK	Polyclonal	1:200
Molecular subtype-related proteins			
ER	Thermo Scientific, San Diego, CA, USA	SP1	1:100
PR	DAKO, Glostrup, Denmark	PgR	1:50
HER-2	DAKO, Glostrup, Denmark	Polyclonal	1:1500

Immunohistochemistry

The antibodies used for immunohistochemistry (IHC) in this study are shown in Table 1. IHC was performed using FFPE tissue sections. Three-μm-thick tissue sections were deparaffinized and rehydrated using xylene and alcohol solutions, respectively. Antigen retrieval was performed using CC1 buffer (Cell Conditioning 1; citrate buffer pH 6.0, Ventan Medical System). IHC was performed using the Ventana Discovery XT automated stainer (Ventana Medical System, Tucson, AZ, USA), including appropriate positive and negative controls.

Interpretation of immunohistochemical staining

All IHC markers were accessed by light microscopy. A cut-off value of 1% or more positively stained nuclei was used to define ER and PR positivity (Hammond et al., 2010). HER-2 staining was analyzed according to the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines using the following categories: 0 = no immunostaining; 1+ = weak incomplete membranous staining, less than 10% of tumor cells; 2+ = complete membranous staining, either uniform or weak in at least 10% of tumor cells; and 3+ = uniform intense membranous staining in at least 10% of tumor cells (Wolff et al., 2007). HER-2 immunostaining was considered positive when strong (3+) membranous staining was observed, whereas cases with 0 to 1+ were regarded as negative. To evaluate the expression of STAT3, STAT4, STAT-6, and FOXP3, IHC staining was performed using FFPE tissue sections. The staining was assessed by light microscopy and evaluated in both tumor cells and TILs in the adjacent stroma. We measured the proportion of tumor cells within the TMA core that exhibited expression of T cell subtype markers and the proportion of tumor cells showing expression of T cell subtype markers among the immune cells comprising stromal TIL, both at high magnification (x400). A positive result was defined as staining in at least 10% of tumor cells (Beguinot et al., 2018) or TILs (Agahozo et al., 2020).

Tumor phenotype classification

In this study, we classified DCIS phenotypes according to the IHC results for ER, PR, and HER-2. Results for HER-2 were as follows: *luminal type*, ER and/or PR positive, any HER-2 status; HER-2 positive type, ER and PR negative and HER-2 overexpressed and/or amplified; *TNBC type*: ER, PR, and HER-2 negative.

Statistical analysis

Data were analyzed using SPSS for Windows, Version 22.0 (SPSS Inc., Chicago, IL, USA). For the determination of statistical significance, Student's t and Fisher's exact tests were used for continuous and categorical variables, respectively. In the case of analyzing data with multiple comparisons, a corrected p-value with the application of the Bonferroni multiple comparison procedure was used. Cohen's kappa coefficient was used to assess the agreement between any two immune cell-related proteins antibody for each scoring method and was interpreted as: <0, no agreement; 0.0-0.20, slight agreement; 0.21-0.40, fair agreement; 0.41-0.60, moderate agreement; 0.61-0.80, substantial agreement; 0.81-1.00, almost perfect agreement (Huang et al., 2021). Statistical significance was set at $p<0.05$. Kaplan-Meier survival curves and log-rank statistics were employed to evaluate time to tumor recurrence and overall survival.

Results

Clinicopathological characteristics of patients with ductal carcinoma in situ

Table 2 summarizes the basal characteristics of the 191 cases of DCIS included in this study. Among the 191 cases, 103 (53.9%) were of the luminal type, 79 (41.4%) were HER-2 positive, and 9 (4.7%) were triple-negative breast cancer (TNBC). According to the molecular subtype, there were statistically significant differences in age ($p=0.045$), architecture type ($p=0.001$), nuclear grade ($p=0.002$), necrosis ($p<0.001$), and stromal type ($p<0.001$). The HER-2 positive and TNBC subtypes had significantly higher proportions of patients aged 50 or older, comedo-type necrosis, and high-TIL types compared with the luminal subtype. The TNBC subtype had a higher proportion of high nuclear grade compared with other subtypes ($p<0.001$).

Expression of T cell-related proteins in DCIS according to surrogate molecular subtypes

Regarding T cell-related protein expression according to the DCIS surrogate molecular subtypes, significant differences were observed in the expression of STAT3 (T) ($p<0.001$), STAT3 (I) ($p<0.001$), STAT4 (I) ($p<0.001$), STAT6 (I) ($p<0.001$), and FOXP3 (I) ($p=0.001$), depending on the surrogate molecular subtype. STAT3 showed a high positivity rate in luminal-type tumor cells, while STAT3, STAT4, STAT6, and FOXP3 showed high positivity rates in TNBC-type immune cells (Table 3, Fig. 1).

Expression of T cell-related proteins in DCIS according to the stromal TIL status

The expression of T cell-related proteins according

Table 2. Basal characteristics of ductal carcinoma *in situ*.

Parameters	Total	Molecular subtype			*p*-value
	N=191 (%)	Luminal n=103 (%)	HER-2 n=79 (%)	TNBC n=9 (%)	
Age (years)					0.027
<35	15 (7.9)	9 (8.7)	6 (7.6)	0 (0.0)	
35-50	97 (50.8)	62 (60.2)	32 (40.5)	3 (33.3)	
≥51	79 (41.4)	32 (31.1)	41 (51.9)	6 (66.7)	
Architecture type					0.001
Cribriform	85 (44.5)	59 (57.3)	25 (31.6)	1 (11.1)	
Solid	78 (40.8)	34 (33.0)	37 (46.8)	7 (77.8)	
Micropapillary	17 (8.9)	4 (3.9)	12 (15.2)	1 (11.1)	
Papillary	8 (4.2)	6 (5.8)	2 (2.5)	0 (0.0)	
Apocrine	3 (1.6)	0 (0.0)	3 (3.8)	0 (0.0)	
Nuclear grade					0.002
Low	13 (6.8)	8 (7.8)	5 (6.3)	0 (0.0)	
Intermediate	101 (52.9)	62 (60.2)	39 (49.4)	0 (0.0)	
High	77 (40.3)	33 (32.0)	35 (44.3)	9 (100.0)	
Necrosis					<0.001
Absent	82 (42.9)	60 (58.3)	19 (24.1)	3 (33.3)	
Focal	31 (16.2)	19 (18.4)	11 (13.9)	1 (11.1)	
Comedo	78 (40.8)	24 (23.3)	49 (62.0)	5 (55.6)	
Calcification					0.884
Absent	162 (84.8)	88 (85.4)	66 (83.5)	8 (88.9)	
Present	29 (15.2)	15 (14.6)	13 (16.5)	1 (11.1)	
Stromal type					<0.001
Non-inflammatory	142 (74.3)	94 (91.3)	44 (55.7)	4 (44.4)	
Inflammatory	49 (25.7)	9 (8.7)	35 (44.3)	5 (55.6)	

Table 3. Expression of T cell-related proteins in ductal carcinoma *in situ* according to the surrogate molecular subtypes.

Parameters	Total	Molecular subtype			*p*-value
	N=191 (%)	Luminal n=103 (%)	HER-2 positive n=79 (%)	TNBC n=9 (%)	
STAT3 (T)					<0.001
Negative	82 (42.9)	30 (29.1)	47 (59.5)	5 (55.6)	
Positive	109 (57.1)	73 (70.9)	32 (40.5)	4 (44.4)	
STAT3 (I)					<0.001
Negative	127 (66.5)	82 (79.6)	43 (54.4)	2 (22.2)	
Positive	64 (33.5)	21 (20.4)	36 (45.6)	7 (77.8)	
STAT4 (T)					0.998
Negative	41 (21.5)	22 (21.4)	17 (21.5)	2 (22.2)	
Positive	150 (78.5)	81 (78.6)	62 (78.5)	7 (77.8)	
STAT4 (I)					<0.001
Negative	110 (57.6)	79 (76.7)	30 (38.0)	1 (11.1)	
Positive	81 (42.4)	24 (23.3)	49 (62.0)	8 (88.9)	
STAT6 (T)					0.835
Negative	139 (72.8)	74 (71.8)	59 (74.7)	6 (66.7)	
Positive	52 (27.2)	29 (28.2)	20 (25.3)	3 (33.3)	
STAT6 (I)					<0.001
Negative	142 (74.3)	91 (88.3)	49 (62.0)	2 (22.2)	
Positive	49 (25.7)	12 (11.7)	30 (38.0)	7 (77.8)	
FOXP3 (I)					0.001
Negative	160 (83.8)	95 (92.2)	60 (75.9)	5 (55.6)	
Positive	31 (16.2)	8 (7.8)	19 (24.1)	4 (44.4)	

to stromal TIL status in DCIS was investigated, and significant differences in expression were observed in STAT3 (I) (p<0.001), STAT4 (I) (p<0.001), STAT6 (I) (p<0.001), and FOXP3 (I) (p<0.001) depending on stromal TIL status. In all cases, immune cells showed higher positivity in the high-TIL type compared with the low-TIL type (Table 4 and Fig. 1). In addition, the investigation into differences in T cell-related protein expression according to surrogate molecular subtypes in DCIS with high TIL revealed no significant differences between the subtypes, excluding the expression of STAT3 in tumor cells (p=0.045) (Table 5). However, the expression rate of STAT3 in tumor cells was significantly higher in the luminal type with high TIL compared with the HER-2-positive and TNBC types with high TIL.

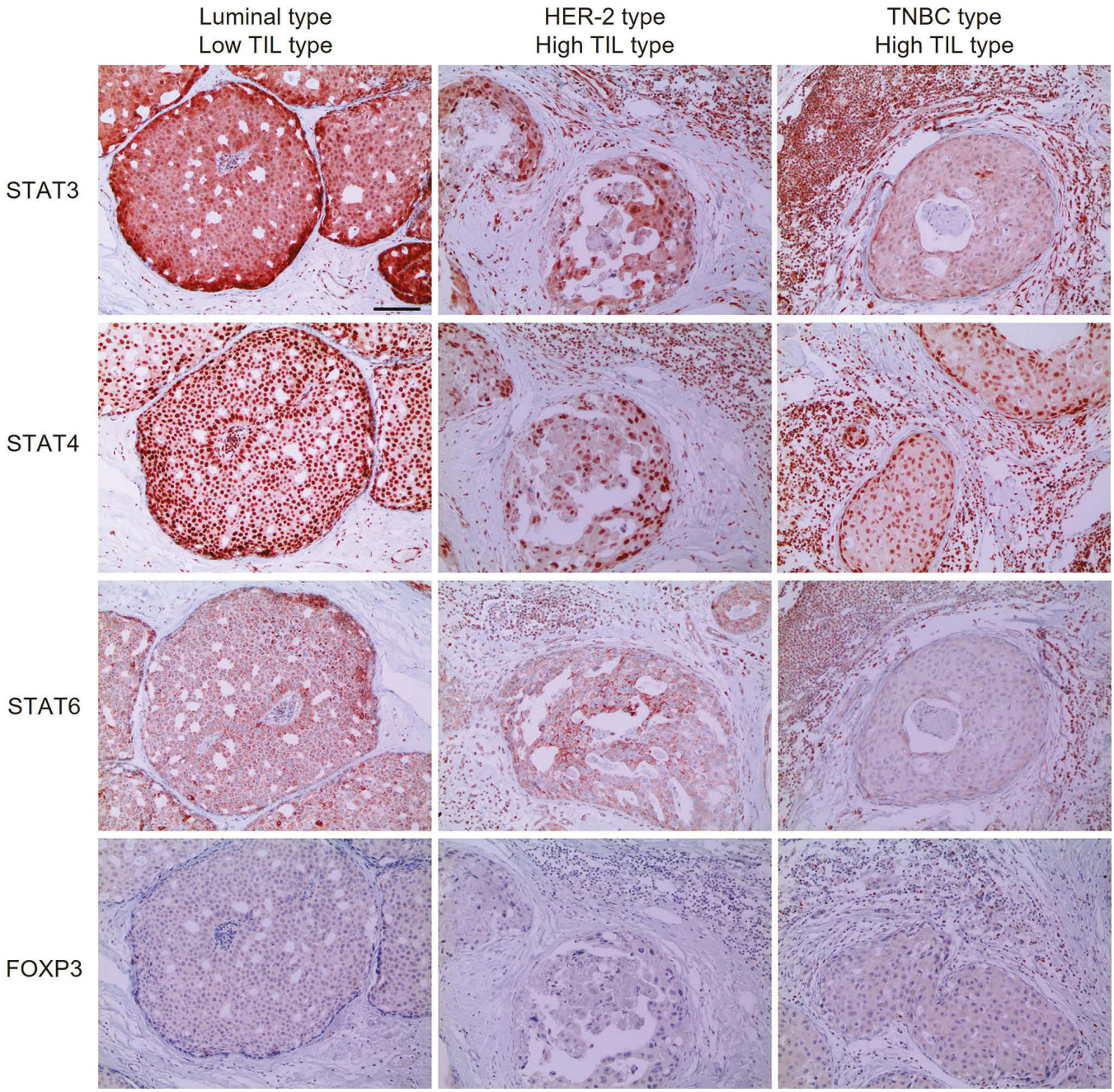

Fig. 1. Expression of T cell-related proteins in DCIS according to the molecular subtypes and stromal TIL type. STAT3 shows high positivity in luminal-type tumor cells, while STAT3, STAT4, STAT6, and FOXP3 show high positivity in immune cells in TNBC-type cells. Moreover, STAT3, STAT4, STAT6, and FOXP3 all show higher positivity in immune cells in the high-TIL type compared with the low-TIL type. Scale bar: 300 μm.

Table 4. Expression of T cell-related proteins in ductal carcinoma *in situ* according to the stromal TIL type.

Parameters	Total	Stromal TIL type		*p*-value
	N=191 (%)	low TIL n=142 (%)	high TIL n=49 (%)	
STAT3 (T)				0.511
Negative	82 (42.9)	59 (41.5)	23 (46.9)	
Positive	109 (57.1)	83 (58.5)	26 (53.1)	
STAT3 (I)				<0.001
Negative	127 (66.5)	112 (78.9)	15 (30.6)	
Positive	64 (33.5)	30 (21.1)	34 (69.4)	
STAT4 (T)				0.540
Negative	41 (21.5)	32 (22.5)	9 (18.4)	
Positive	150 (78.5)	110 (77.5)	40 (81.6)	
STAT4 (I)				<0.001
Negative	110 (57.6)	105 (73.9)	5 (10.2)	
Positive	81 (42.4)	37 (26.1)	44 (89.8)	
STAT6 (T)				0.899
Negative	139 (72.8)	103 (72.5)	36 (73.5)	
Positive	52 (27.2)	39 (27.5)	13 (26.5)	
STAT6 (I)				<0.001
Negative	142 (74.3)	120 (84.5)	22 (44.9)	
Positive	49 (25.7)	22 (15.5)	27 (55.1)	
FOXP3 (I)				<0.001
Negative	160 (83.8)	129 (90.8)	31 (63.3)	
Positive	31 (16.2)	13 (9.2)	18 (36.7)	

Table 5. Expression of T cell-related proteins in high-TIL ductal carcinoma *in situ* according to the surrogate molecular subtypes.

Parameters	Total	Surrogate molecular type			*p*-value
	N=49 (%)	Luminal n=9 (%)	HER-2 positive n=35 (%)	TNBC n=5 (%)	
STAT3 (T)					0.045
Negative	23 (46.9)	1 (11.1)	20 (57.1)	2 (40.0)	
Positive	26 (53.1)	8 (88.9)	15 (42.9)	3 (60.0)	
STAT3 (I)					0.201
Negative	15 (30.6)	2 (22.2)	13 (37.1)	0 (0.0)	
Positive	34 (69.4)	7 (77.8)	22 (62.9)	5 (100.0)	
STAT4 (T)					0.286
Negative	9 (18.4)	3 (33.3)	6 (17.1)	0 (0.0)	
Positive	40 (81.9)	6 (66.7)	29 (82.9)	5 (100.0)	
STAT4 (I)					0.328
Negative	5 (10.2)	0 (0.0)	5 (14.3)	0 (0.0)	
Positive	44 (89.8)	9 (100.0)	30 (85.7)	5 (100.0)	
STAT6 (T)					0.874
Negative	36 (73.5)	7 (77.8)	25 (71.4)	4 (80.0)	
Positive	13 (26.5)	2 (22.2)	10 (28.6)	1 (20.0)	
STAT6 (I)					0.096
Negative	22 (44.9)	4 (44.4)	18 (51.4)	0 (0.0)	
Positive	27 (55.1)	5 (55.6)	17 (48.6)	5 (100.0)	
FOXP3 (I)					0.403
Negative	31 (63.3)	4 (44.4)	24 (68.6)	3 (60.0)	
Positive	18 (36.7)	5 (55.6)	11 (31.4)	2 (40.0)	

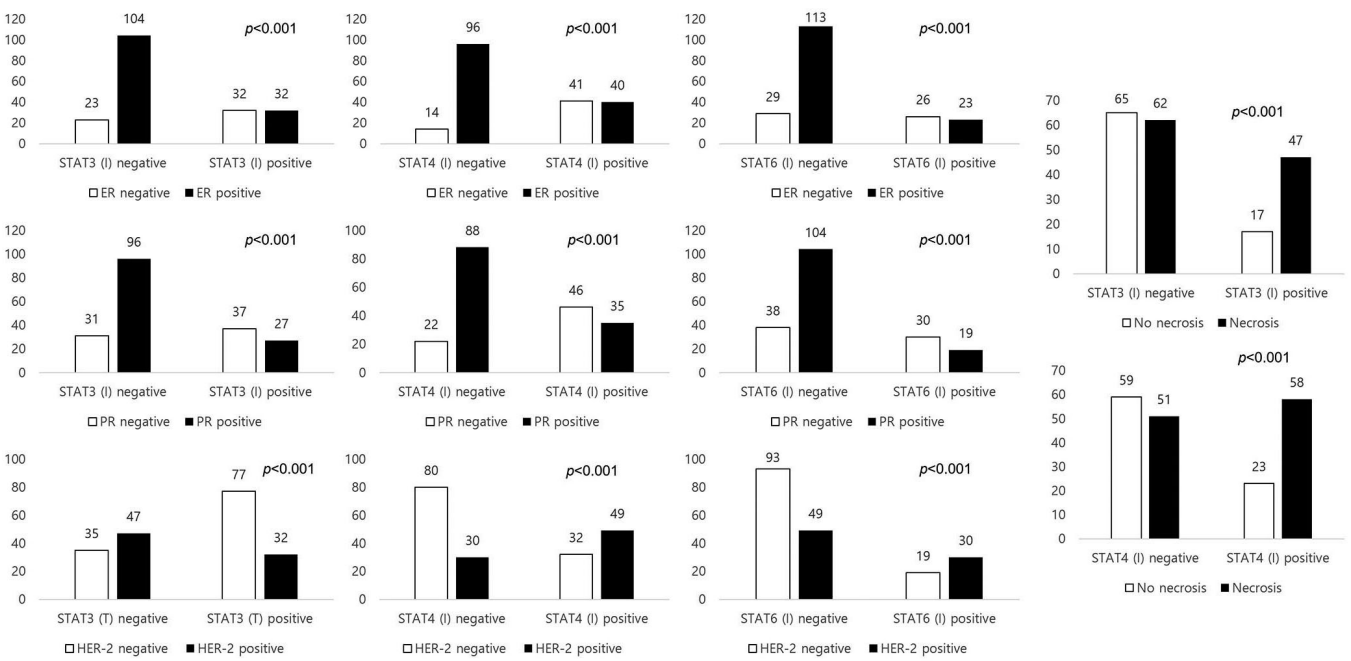

Fig. 2. Correlation between clinicopathological factors and expression of T cell-related proteins. The expression of STAT3, STAT4, and STAT6 in immune cells shows an association with ER negativity, PR negativity, and HER-2 negativity (p<0.001), while the expression of STAT3 and STAT4 in immune cells shows an association with necrosis (p<0.001).

Difference and concordance of the expression of T cell-related proteins in DCIS according to tumor compartment

As regards T cell-related protein expression in DCIS based on the tumor compartment, the expression agreement was slightly consistent among T cell-related proteins in the tumor cell compartment, with the highest agreement observed between STAT3 and STAT4 (OA=61.8%, Kappa coefficient=0.169) and the lowest between STAT4 and STAT6 (OA=40.3%, Kappa coefficient=0.053). The expression agreement was fair or higher among T cell-related proteins in the immune cell compartment, with the highest agreement observed between STAT3 and STAT4 (OA=83.7%, Kappa coefficient=0.658) and the lowest between STAT4 and FOXP3 (OA=71.7%, Kappa coefficient=0.370) (Table 6).

Correlation between clinicopathological factors and expression of T cell-related proteins

The study investigated the association between clinicopathological factors and the expression of T cell-related proteins in DCIS. The results showed that STAT3 (I), STAT4 (I), and STAT6 (I) were associated with ER, PR, and HER-2 status; the positive expression of STAT3 (I), STAT4 (I), and STAT6 (I) being associated with ER negativity, PR negativity, and HER-2 negativity ($p<0.001$). STAT3 (I) and STAT4 (I) were also associated with necrosis, with positive expression of STAT3 (I) and STAT4 (I) being associated with necrosis ($p<0.001$, Fig. 2).

Table 6. Pairwise comparisons for concordance and kappa statistics among T cell-related proteins.

Immune cell-related proteins	Overall agreement (OA) (%)	Kappa coefficient (95%CI)	Category of agreement
Tumor cell compartment			
STAT3 vs. STAT4	61.8	0.169-0.065	Slight
STAT3 vs. STAT6	54.5	0.144-0.058	Slight
STAT4 vs. STAT6	40.3	0.053-0.039	Slight
Immune cell compartment			
STAT3 vs. STAT4	83.7	0.658-0.055	Substantial
STAT3 vs. STAT6	76.4	0.439-0.070	Moderate
STAT3 vs. FOXP3	75.4	0.367-0.069	Fair
STAT4 vs. STAT6	75.9	0.480-0.062	Moderate
STAT4 vs. FOXP3	71.7	0.370-0.059	Fair
STAT6 vs. FOXP3	79.1	0.376-0.078	Fair

The impact of the expression of T cell-related proteins on patient prognosis

The univariate analysis showed no significant association between the expression of T cell-related proteins and prognosis (Data not shown). However, in a subgroup analysis, in DCIS with necrosis, the absence of immune cells in all T cell-related proteins was associated with shorter disease-free survival (DFS) ($p=0.069$) and shorter overall survival (OS) (p=0.013) (Fig. 3).

Discussion

In this study, we aimed to investigate the expression of T cell subtype markers in DCIS. Firstly, STAT3 was found to be highly expressed in tumor cells of luminal type DCIS. Although there has been no previous study on the expression of STAT in DCIS, a previous study on the expression of STAT3 in invasive breast cancer reported positivity in 69.2% of tumors, which was not associated with clinical parameters (Dolled-Filhart et al., 2003). STAT3 plays a crucial role in the differentiation of T cells (Th17 helper T cell) (Yang et al., 2007) but it also has an important function in tumorigenesis. STAT3 regulates the expression of various genes, especially *cyclin D1* and *c-Myc*, to increase cancer cell growth, and suppress cancer cell death by regulating BCL-2, BCL-XL, survivin, and Mcl-2, contributing to tumor growth (Banerjee and Resat, 2016). Among them, the expression of cyclin D in the breast was examined, reporting that cyclin D expression increased from 18% in benign lesions and ADH to 76% in low-grade DCIS, 87% in

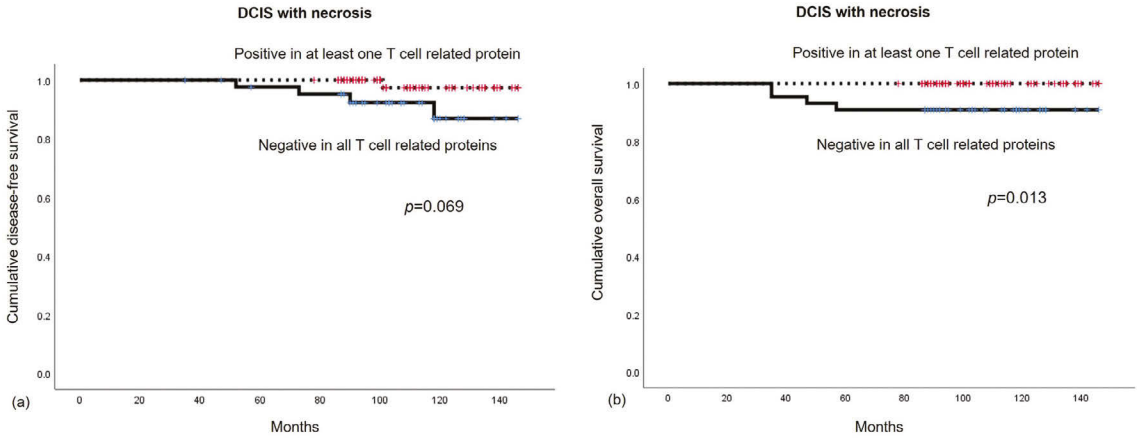

Fig. 3. The impact of the expression of T cell-related proteins on patient prognosis. In DCIS with necrosis, the absence of immune cells in all T cell-related proteins is associated with shorter DFS $p=0.069$) and shorter OS ($p=0.013$).

high-grade DCIS, and 83% in Invasive ductal carcinoma (IDC) (Weinstat-Saslow et al., 1995). Cyclin D1 overexpression was observed in about 20% of DCIS, and Cyclin D1 overexpression was found to be associated with ER positivity (Vos et al., 1999). Cyclin D is not only a regulatory subunit of cyclin-dependent kinases but also mediates ER transactivation in breast cancer (Bindels et al., 2002). Therefore, since cross-talk exists between STAT3, cyclin D, and ER, the mechanism underlying the high expression of STAT3 in tumor cells of luminal-type DCIS could be elucidated by this study, and further research is needed.

In this study, STAT3, STAT4, STAT6, and FOXP3 showed high positivity rates in immune cells in TNBC and high-TIL types ($p \leq 0.001$). Since high levels of stromal TILs were observed in TNBC (Althobiti et al., 2018; Thike et al., 2020), which is also present in invasive carcinoma and DCIS, it is expected that the expression of T cell subtype markers such as STAT3 (Th17), STAT4 (Th1), STAT6 (Th2), and FOXP3 (Treg) would be higher in immune cells in TNBC and high-TIL DCIS, which have many stromal immune cells. Previous studies on breast cancer TILs have shown that the number of CD3+, CD8+, FOXP3+, CD20+, and CD68+ cells in TILs has a positive correlation with TIL level (Althobiti et al., 2018), which is consistent with the findings of this study. Previous studies investigating the TIL components of breast cancer (invasive carcinoma) reported that CD3+ cells were the main component of TILs, while CD20+ cells were the smallest component (Althobiti et al., 2018). In DCIS, CD3+ cells were also reported to be the main component of TILs in staining for CD3, CD4, CD8, FoxP3, and CD20 (Thompson et al., 2016), indicating that T cells occupy the main component of TILs. In a study investigating DCIS TIL using CD4, CD8, CD20, CD68, and FOXP3, CD4+ cells were identified as the main component of TILs, followed by CD20+, CD8+, CD68+, and FOXP3+ cells (Agahozo et al., 2020). However, a study comparing the immune microenvironment between invasive carcinoma and DCIS showed differences in the T-cell component, with more CD4+ T cells in DCIS than CD8+ T cells, while invasive carcinoma had more CD8+ T cells. The number of CD4+, CD8+, and FOXP3+ T cells was higher in invasive carcinoma than in DCIS. When comparing pure DCIS, DCIS with microinvasion, and DCIS associated with invasive carcinoma, CD4+-TIL infiltration gradually increased in the hormone-negative group, while FOXP3+-TIL infiltration was significantly higher in DCIS associated with invasive carcinoma than in pure DCIS in the hormone-positive group (Kim et al., 2020). Furthermore, it has been reported that, similar to T cells, the number of B-cell components in TILs is higher in pure DCIS compared with DCIS with invasive components (Miligy et al., 2017). Therefore, there may be several limitations in applying the results of studies on invasive carcinoma to DCIS. However, studies on the expression of various effector T cell subgroup markers in TILs through IHC staining in invasive carcinoma and/or DCIS are still lacking, making it difficult to compare with previous studies. In a study investigating immune cell populations through staining for CD4, CD8, CD20, FoxP3, CD68, CD115, Mac387, MRC1, and HLA-DR in DCIS, there was a difference between high-grade DCIS and non-high-grade DCIS. High-grade DCIS had significantly higher numbers of FoxP3+ cells, CD68+ macrophages, HLA-DR+ cells, CD4+ T cells, CD20+ B cells, and total TILs (Campbell et al., 2017), which is similar to the results of this study showing high expression of various T-cell subgroup markers in TNBC TILs, which are high-grade DCIS. Although there are obstacles, such as intratumoral and spatial heterogeneity in TME research such as TILs, the development of single-cell RNA sequencing, multiplexed imaging methods, and spatial transcriptomics has shed light on the complexity and diversity of the breast cancer TME. These research methods have shown that the T-cell compartment of breast cancer is not a limited subtype that is differentiated at a specific stage but a continuum of diverse cellular states that are influenced by T-cell receptor diversity and the local niche within the TME (Azizi et al., 2018; Wu et al., 2021). Therefore, in this study, cells expressing T cell subtype markers can be considered dynamic cells with a specific functional status rather than differentiated cells at a specific stage. Thus, it is possible for a single cell to express various T cell subtype markers, which warrants further research on the intratumoral and spatial heterogeneity of cells expressing T cell subtype markers in DCIS TIL. In previous studies targeting IDC, methods such as multiplex IHC (Sun et al., 2021; Lin et al., 2023), multiplexed immunofluorescence (Liang et al., 2020; Taube et al., 2021), and single-cell RNA-seq (Chung et al., 2017; Guo et al., 2023) were employed to investigate the intratumoral and spatial heterogeneity of immune cells. In future studies, it is necessary to utilize these research methods to examine the intratumoral and spatial heterogeneity of immune cells in DCIS.

In this study, the expression of STAT3, STAT4, and STAT6 in TILs was shown to be associated with ER negativity, PR negativity, and HER-2 negativity, and the expression of STAT3 and STAT4 in TILs was found to be associated with necrosis. Previous studies reported that immune markers are associated with clinicopathologic characteristics in DCIS, and high levels of CD4+ T cells, CD20+ B cells, and total TILs (CD4+, CD8+, and CD20+ cells) are associated with high-risk features, such as large tumor size, high grade, comedo-necrosis, high Ki67, HER2 positivity, and HR negativity (Campbell et al., 2017). High CD4+ T cell density has been reported to be associated with high nuclear grade, microinvasion, ER negativity, PR negativity, HER2 positivity, and triple negativity (Thike et al., 2020), similar to the results of this study. In this study, in DCIS showing necrosis, cases in which immune cells were negative for all T cell-related proteins were found to be associated with poor prognosis, and previous studies have reported that the

risk of recurrence in DCIS varies depending on the characteristics of the immune cell population (Campbell et al., 2017; Thike et al., 2020), indicating a need for further study on the prognostic implications of T cell subtype populations in TILs.

This study has several limitations. Firstly, it utilized TMA containing limited tissue samples. Given the occurrence of tumor heterogeneity, particularly in breast cancer, there may be doubts about whether TMAs represent the entire tumor. However, previous studies comparing the results of IHC staining of various molecules between TMAs and tissue whole slides have shown similar results, suggesting that the representativeness of results obtained using TMAs may not be a significant concern (Kyndi et al., 2008; Tramm et al., 2018). Secondly, the limited number of cases is another constraint, especially considering that there were only nine cases (4.7%) of the TNBC type among the DCIS cases, which could pose significant limitations during analysis. Previous studies reported a very low proportion of TNBC type in DCIS, ranging from 6-7% (Tamimi et al., 2008; Vincent-Salomon et al., 2008), which is consistent with our findings. Therefore, to include a sufficient number of TNBC-type DCIS cases, large-scale studies involving multiple institutions are likely necessary. Hence, single-center studies like this one may have inherent limitations in this regard. In conclusion, immune cells in DCIS show positive expression of various T cell subtype markers, with TNBC and high-TIL DCIS showing higher levels of positivity.

Competing interest. The authors declare no conflicts of interest.

Authors' Contributions. ES and HMK participated in the design of the study, performed the statistical analysis, and carried out the immunoassays. JSK conceived the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

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