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Clinicopathologic characteristics of STAT1 positive/interleukin-8 negative subgroup in triple negative breast cancer defined by surrogate immunohistochemistry

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Summary. Background: The aim of this study was to define immune-related triple negative breast cancer (TNBC) using immunohistochemistry for STAT1, CD20, CD3, IL-8, and IFN-γ and to assess its clinicopathologic characteristics.

Material and methods: Tissues from 133 cases of TNBC were used for a tissue microarray. Expression of STAT1, CD20, CD3, IL-8, and IFN-γ were evaluated by immunohistochemical staining of the tissue microarrays. Immune-related type was defined as TNBC which was positive for STAT1 and negative for IL-8. A separate assessment of IL-8 and STAT1 status in tumor and stroma compartment was used to further classify immune-related type into tumor-based and stroma-based immune-related TNBC.

Results: Stroma-based, immune-related TNBC showed a significantly smaller central acellular zone ($p=0.043$), more lymphocytic infiltration ($p<0.001$), higher CD20 index ($p=0.001$), and higher CD3 index ($p=0.018$) than stroma-based, non-immune-related TNBC. IL-8 was independently associated with shorter disease-free survival (Hazard ratio: 3.804, 95% CI: 1.234-11.729, $p=0.020$) and shorter overall survival (Hazard ratio: 3.434, 95% CI: 1.132-10.414, $p=0.029$).

Conclusions: Immune-related proteins such as STAT1, IFN-γ, IL-8, and CD20 were variably expressed in TNBCs. Stroma-based, immune-related TNBC (when positive for stromal STAT1 and negative for stromal IL-8) showed significantly higher lymphocytic infiltration including both CD3 positive T cell and CD20 positive B cell.

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Introduction

Breast cancer is heterogeneous in its clinical behavior, histological characteristics, and genetic signature. In order to classify heterogeneous breast cancer into subtypes with clinical implications, a molecular classification based upon gene expression profile has been established (Perou et al., 2000; Sorlie et al., 2001). Aside from the five subtypes, luminal A, luminal B, HER-2, normal breast-like, and basal-like type, defined by gene expression profiles, breast cancers not expressing estrogen receptor (ER), progesterone receptor (PR), or HER2 is clinically referred to as triple negative breast cancer (TNBC). TNBC accounts for 10-17% of all breast cancers (Haffty et al., 2006; Harris et al., 2006; Bauer et al., 2007; Carey et al., 2007; Dent et al., 2007; Rakha et al., 2009) and there is significant biological heterogeneity within the TNBC group. TNBC has been classified into basal-like type, molecular-apocrine type, and claudin-low type by molecular stratification, which account for 39-54%, 25-39%, and 7-14% of all TNBCs, respectively (van de Vijver et al., 2002; van't Veer et al., 2002; Hess et al., 2006; Prat et al., 2010). Distinct molecular characteristics and clinicopathologic features of each subtype can lead to different therapeutic approaches.

In order to find the gene expression modules involved in the carcinogenic process of breast cancer, a comprehensive meta-analysis encompassing the heterogeneous gene expression of breast cancer and clinicopathologic correlation studies was performed. The identified genes were associated with tumor invasion/

metastasis, impairment of immune response, sustained angiogenesis, evasion of apoptosis, self-sufficiency in growth signals, and ER and HER-2 signaling (Desmedt et al., 2008). Among them, the immune response has dual opposite effects on the tumor in that it contributes to not only host protection but also tumor progression (Dunn et al., 2002). There have been many studies using microarray analysis of bulk breast cancer tissue to investigate the association between immune-related genes and patient prognosis. As a result, several genes, such as the interferon-related gene (Huang et al., 2003; Perou et al., 1999), B lymphocyte marker (Perou et al., 1999), and T-lymphocyte associated genes (Huang et al., 2003) were proposed as significant immune-related genes. Interferon (IFN)-γ and its transcriptional regulator, STAT1, are well known for their involvement in the immune response (Darnell et al., 1994; Ihle, 1996; Desmedt et al., 2008). A high infiltration of CD20 positive B lymphocyte was reported as a favorable prognostic factor of TNBC, and the ratio of CD20 positive B cells to IL-8 also had an important impact on prognosis (Rody et al., 2011). IL-8 was reported to be an important mediator of inflammatory response in tumor cells (Ning et al., 2011). Accordingly, STAT1, IFN-γ, IL-8, CD20 positive B lymphocyte, CD3 positive T lymphocyte can be considered important indicators of the immune response in breast cancer.

As a possible novel subgroup of TNBC, an immune-related type has been proposed. The immune-related type has been reported to highly express inflammatory cells and/or interferon pathway-related genes and have a considerably better prognosis (Rody et al., 2011; Teschendorff et al., 2007). However, very little is known about immune-related TNBC.

The aim of this study is to assess the expression of immune-related markers including STAT1, CD20, CD3, IL-8 and IFN-γ in TNBC, and to understand the clinocopathologic characteristics of the immune related subgroup of TNBC.

Materials and methods

Patient selection

Patients diagnosed with TNBC who underwent surgical excision at Severance Hospital between January 2000 and December 2006 were included in the study group. This study was approved by the Institutional Review Board of Severance hospital. All tumors were diagnosed as invasive ductal carcinoma, not otherwise specified (NOS). TNBC was defined by negativity for ER, PR, and HER2 assessed by immunohistochemistry and by fluorescence *in situ* hybridization (FISH).

ER and PR were considered positive when expressed in more than 1% of invasive tumor cells (Hammond et al., 2010). HER-2 staining was scored according to the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) guidelines using the following categories: 0, no immunostaining; 1+, weak incomplete membranous staining in any proportion of tumor cells; 2+, complete membranous staining, either non-uniform, or weak in at least 10% of tumor cells; and 3+, uniform intense membranous staining in >30% of tumor cells (Wolff et al., 2007). Cases with 0 to 1+ were regarded as negative, and cases with 3+ were considered as positive. Cases with HER2 2+ were investigated using FISH (Vysis Pathvision HER-2 kit) for HER-2 gene status. As proposed by the ASCO/CAP guidelines, an absolute HER2 gene copy number lower than 4 or a HER2 gene/chromosome 17 copy number ratio (HER2/Chr17 ratio) of less than 1.8 was considered HER2 negative; an absolute HER-2 copy number between 4 and 6 or a HER2/Chr17 ratio between 1.8 and 2.2 was considered HER2 equivocal; and an absolute HER2 copy number greater than 6 or a HER2/Chr17 ratio higher than 2.2 was considered HER2 positive.

Formalin-fixed and paraffin-embedded tissue specimens from 133 cases of primary breast cancer were included. All archival hematoxylin and eosin (H&E)-stained slides for each case were reviewed by two pathologists (Koo JS, and Kim S). The histological grade was accessed using the Nottingham grading system (Elston and Ellis, 1991). Tumor staging was based on the 7th American Joint Committee on Cancer (AJCC) criteria. Disease-free survival (DFS) time was measured from the date of the first curative surgery to the date of the first locoregional or systemic relapse or to the date of death without any type of relapse. Overall survival (OS) time was calculated from the date of the first curative operation to the date of the last follow-up or death from any cause. Histologic parameters were evaluated from the H&E-stained slides. Based upon the histologic findings, we confirmed the following: tumor margins (infiltrative or expanding), central acellular zone, central necrotic zone, central fibrotic zone, lymphocytic infiltration, tumor cell discohesiveness, apocrine differentiation and tumor cell necrosis. Tumor cell discohesiveness was defined when at least 50% of the tumor cell population showed loss of cell to cell cohesiveness. Apocrine differentiation was defined when at least 10% of the tumor cell population showed abundant granular eosinophilic cytoplasm, cytoplasmic vacuolization and vesicular nuclei with prominent nucleoli. Clinical parameters evaluated in each tumor included patient age at initial diagnosis, lymph node status, local recurrence, systemic recurrence, and patient survival.

Tissue microarray

On H&E-stained slides of tumors, a representative area was selected, and a corresponding spot was marked on the surface of the paraffin block. Using a punch machine, the selected area was punched out, and a 3-mm tissue core was placed into a 6x5 recipient block. Two tissue cores were extracted to minimize extraction bias. Each tissue core was assigned with a unique tissue

microarray location number that was linked to a database containing other clinicopathologic data.

Immunohistochemistry

The antibodies and dilution used for immunohistochemistry (IHC) are shown in Table 1. Formalin-fixed, paraffin-embedded tissue sections from the tissue microarray were prepared for IHC. The 3-mm sections were deparaffinized in xylene and rehydrated through a graded alcohol series to distilled water. The slides were subjected to antigen retrieval by microwave irradiation and then incubated with primary antibodies. Binding was detected with biotinylated anti-mouse immunoglobulin, followed by peroxidase-labeled streptavidin with 3,3'-diaminobenzidine chromogen as the substrate. Optimal primary antibody incubation times and concentrations were determined by serial dilution for each immunohistochemical assay using a tissue block fixed and embedded exactly as for the experiments. Slides were counterstained with Harris hematoxylin. Two pathologists (Kim S, Koo JS) interpreted the staining using a multi-view microscope.

Interpretation of immunohistochemical staining

All immunohistochemical markers were accessed by light microscopy. Scoring of immunostained slides was done according to the percentage of tumor cells exhibiting cytoplasmic (IL-8, STAT1, IFN-γ, and CK5/6), and membranous (CD20, CD3 and EGFR) staining. Immunohistochemical stain results for CK5/6 and EGFR were considered positive when expressed at least 1% of tumor cells (Rakha et al., 2009). For IL-8, STAT1 and IFN-γ, expression in 10% or more of tumor cells was considered positive. The CD20 and CD3 index were defined by the percentage of the area with CD20/CD3-positive lymphocyte infiltration to entire area including both tumor and surrounding stroma. We examined entire TMA core field when evaluating the CD20 and CD3 index. Immunohistochemical stain results of Ki-67 were scored by counting the number of positively stained nuclei and were expressed as a percentage of total tumor cells [Ki-67 labeling index (LI)].

Table 1. Clone, dilution, and source of antibodies used.

Antibody	Clone	Dilution	Company
Immune related			
CD20	L26	1:100	Abcam, Cambridge, UK
CD3	F 7.2.38	1:1000	DAKO, Glostrup, Denmark
Interleukin-8	807	1:50	Abcam, Cambridge, UK
STAT1	Polyclonal	1:100	Abcam, Cambridge, UK
Interferon-γ	Polyclonal	1:150	Abcam, Cambridge, UK
Basal-like related			
Cytokeratin 5/6	D5/16B4	1:50	DAKO, Glostrup, Denmark
EGFR	EGFR.25	1:50	Novocastra, Newcastle, UK
Proliferation related			
Ki-67	MIB-1	1:150	DAKO, Glostrup, Denmark

EGFR: epidermal growth factor receptor.

Classification of TNBC according to IHC

According to the immunohistochemical stain result of CK5/6 and EGFR, TNBCs were classified into basal-like type (CK5/6 positive and/or EGFR positive group) and non-basal-like type (CK5/6 and EGFR negative group). According to the immunohistochemical stain result of IL-8 and STAT1, TNBCs were classified into immune-related type (IL-8 negative and STAT1 positive group) and non-immune-related type (IL-8 positive and/or STAT1 negative group). As the assessment of IL-8 and STAT1 status was performed separately in tumor and stroma compartment, immune related type was further classified into tumor-based, immune-related type (when tumor cells were negative for IL-8 and positive for STAT1) and stroma-based, immune-related type (when stromal cells were negative for IL-8 and positive for STAT1).

Statistical analyses

Data were analyzed using SPSS for Windows, version 12.0 (SPSS Inc., Chicago, IL, USA). For determination of statistical significance, Student's t and Fisher's exact tests were used for continuous and categorical variables, respectively. Statistical significance was determined when $p<0.05$. Kaplan-Meier survival curves and log-rank statistics were employed to evaluate time to tumor recurrence and overall survival. Multivariate regression analysis was performed using a Cox proportional hazards model.

Results

The clinicopathologic characteristics of the 133 TNBC patients of the study group are shown in Table 2. Based upon the immunohistochemical stain results, patients were categorized into the basal-like type (n=82, 61.7%) or non-basal-like type (n=51, 38.3%) group. A central acellular zone was significantly more common in basal-like type than in non-basal-like type tumors (p=0.036). Tumor cell discohesiveness was significantly more common in non-basal-like type than in basal-like type (p=0.044) tumors. In the 133 TNBCs, lymphocytic infiltration and apocrine differentiation were observed in 33 (24.8%) and 24 (18.0%) cases, respectively. The mean clinical follow-up period was 56.3±21.8 months. Of 133 patients, 47 (35.3%) underwent breast conserving surgery and 86 (64.7%) underwent total mastectomy. After surgery, 92 (69.2%) received adjuvant chemotherapy and 52 (39.1%) received adjuvant radiation therapy.

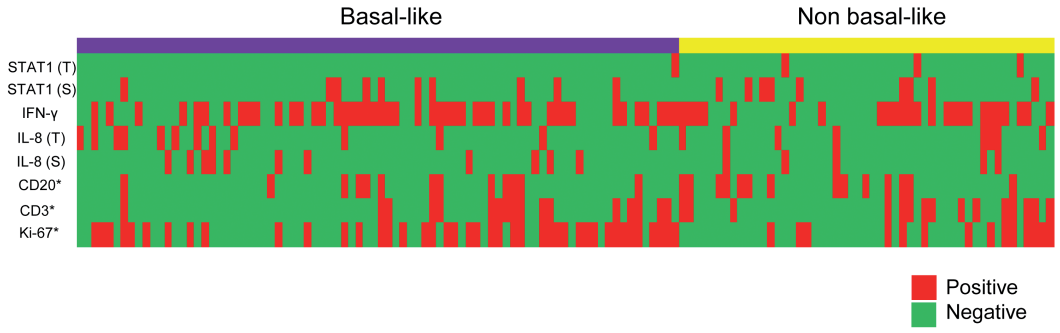

Fig. 1. A heatmap of immune-related protein expression in triple negative breast cancer. T: tumor; S: stroma; *: the mean value was used as the positive cut-off value.

Table 2. Clinicopathologic characteristics of TNBC.

Parameters	Number of patients (n=133) (%)	Basal-like type (n=82) (%)	Non basal-like type (n=51) (%)	P-value
Age (years, mean±SD)	48.2±12.4	47.3±11.9	49.6±13.3	0.310
Histologic grade				0.338
I/II	42 (31.6)	23 (28.0)	19 (37.3)	
III	91 (68.4)	59 (72.0)	32 (62.7)	
Tumor stage				0.366
T1	51 (38.3)	34 (41.5)	17 (33.3)	
T2/T3	82 (61.7)	48 (58.5)	34 (66.7)	
Nodal stage				0.134
N0	87 (65.4)	58 (70.7)	29 (56.9)	
N1/N2/N3	46 (34.6)	24 (29.3)	22 (43.1)	
Central acellular zone				0.036
No	101 (75.9)	57 (69.5)	44 (86.3)	
Yes	32 (24.1)	25 (30.5)	7 (13.7)	
Central necrotic zone				0.741
No	123 (92.5)	75 (91.5)	48 (94.1)	
Yes	10 (7.5)	7 (8.5)	3 (5.9)	
Central fibrotic zone				0.075
No	106 (79.7)	61 (74.4)	45 (88.2)	
Yes	27 (20.3)	21 (25.6)	6 (11.8)	
Lymphocytic infiltration				0.541
Absent	100 (75.2)	60 (73.2)	40 (78.5)	
Present	33 (24.8)	22 (26.8)	11 (21.6)	
Tumor cell discohesiveness				0.044
No	123 (92.5)	73 (96.3)	44 (86.3)	
Yes	10 (7.5)	3 (3.7)	7 (13.7)	
Tumor margin				0.134
Expanding	113 (85.0)	73 (89.0)	40 (78.4)	
Infiltrative	20 (7.5)	9 (11.0)	11 (21.6)	
Apocrine differentiation				0.247
No	109 (82.0)	70 (85.4)	39 (76.5)	
Yes	24 (18.0)	12 (14.6)	12 (23.5)	
Tumor cell necrosis (%, mean±SD)	9.7±14.2	10.4±14.8	8.5±13.2	0.442
Tumor recurrence	14 (10.5)	8 (9.8)	6 (11.8)	0.775
Patient death	14 (10.5)	7 (8.5)	7 (13.7)	0.391
Duration of clinical follow-up (months, mean±SD)	56.3±21.8	55.3±21.8	58.0±22.0	0.495
Surgical procedures				0.191
Conserving surgery	47 (35.3)	25 (30.5)	22 (43.1)	
Total mastectomy	86 (64.7)	57 (69.5)	29 (56.9)	
Chemotherapy	92 (69.2)	61 (74.4)	31 (60.8)	0.123
Radiation therapy	52 (39.1)	31 (37.8)	21 (41.2)	0.718

TNBC: triple negative breast carcinoma.

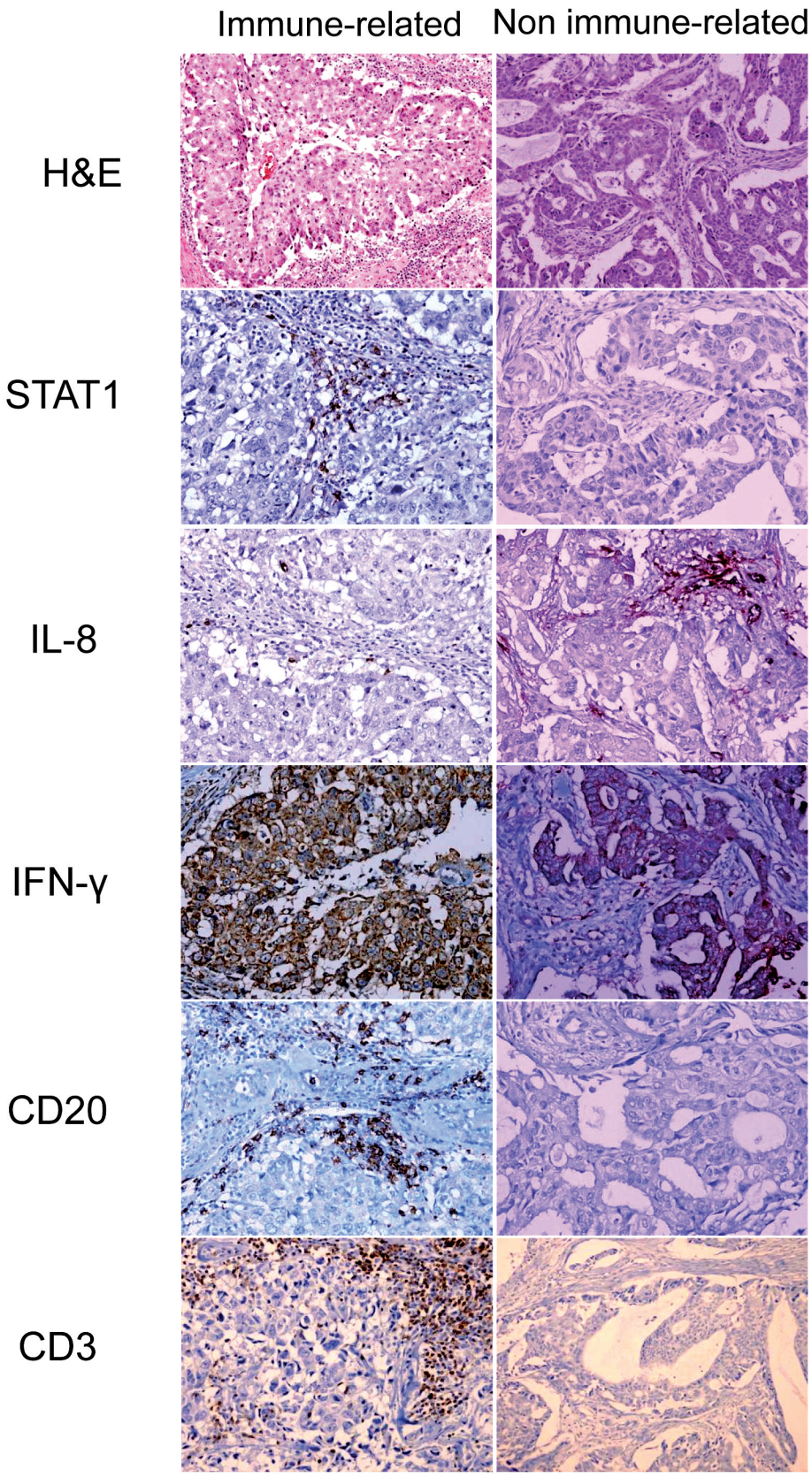

Fig. 2. Expression of immune-related proteins in triple negative breast cancer. Stroma-based, immune-related TNBC (stromal cells are positive for STAT1 and negative for IL-8) shows marked CD3 positive T cell and CD20 positive B cell infiltration on Hematoxylin and eosin stain. x 200

Immune-related proteins expression in triple negative breast cancer

Expression of immune-related proteins including STAT1, IFN-γ, IL-8, and CD20 are shown in Table 3 and figure 1. STAT1 was more frequently expressed in stromal cells than in cancer cells. IL-8 expression rate was similar between cancer cells and stromal cells. CD20 index was significantly higher in non-basal-like type than in basal-like type (p=0.010), but Ki-67 L.I was significantly higher in basal-like type than in non-basal-like type (p=0.004).

Clinicopathologic characteristics according to the immune subtype of TNBC

The clinicopathologic characteristics of immune-related TNBC are in Table 4. Clinicopathological

Table 3. Immune-related proteins expression according to molecular subtype of triple negative breast cancer.

Parameters	Total (n=133) (%)	Basal-like type (n=82) (%)	Non basal-like type (n=51) (%)	P-value
STAT1				0.371
Negative	128 (96.2)	80 (97.6)	48 (94.1)	
Positive	5 (3.8)	2 (2.4)	3 (5.9)	
Stromal STAT1				0.448
Negative	114 (85.7)	72 (87.8)	42 (82.4)	
Positive	19 (14.3)	10 (12.2)	9 (17.6)	
IFN-γ				0.481
Negative	60 (45.1)	35 (42.7)	25 (49.0)	
Positive	73 (54.9)	47 (57.3)	26 (51.0)	
IL-8			1.000	
Negative	112 (84.2)	69 (84.1)	43 (84.3)	
Positive	21 (15.8)	13 (15.9)	8 (15.7)	
Stromal IL-8				0.595
Negative	117 (88.0)	71 (86.6)	46 (90.2)	
Positive	16 (12.0)	11 (13.4)	5 (9.8)	
CD 20 index (mean ± SD)	2.4±6.3	1.3±2.7	4.2±9.3	0.010
CD 3 index (mean ± SD)	2.6±5.0	2.5±5.0	2.8±5.0	0.684
Ki-67 LI (%, mean ± SD)	27.5±23.4	32.0±24.0	20.2±20.5	0.004

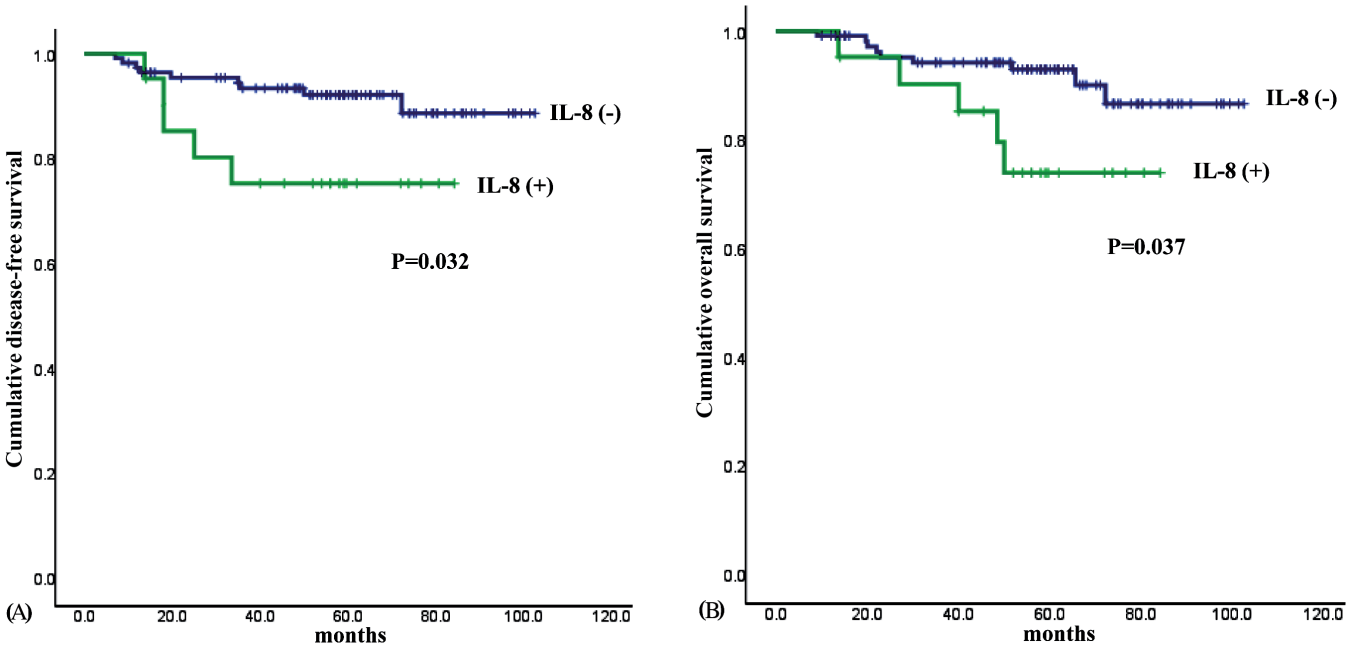

Fig. 3. Disease-free survival **(A)** and overall survival curves **(B)** according to interleukin-8 expression in TNBC.

Table 4. Clinicopathologic characteristics according to the immune subtype of triple negative breast cancer.

Parameters	Total (n=133) (%)	Tumor			Stroma		
		Immune related type (n=5) (%)	Non-immune related type (n=128) (%)	P-value	Immune related type (n=19) (%)	Non-immune related type (n=114) (%)	P-value
Age (years, mean ± SD)	48.2±12.4	54.0±2.3	48.0±12.6	0.296	45.7±13.6	48.6±12.3	0.346
Histologic grade				1.000			1.000
I/II	42 (31.6)	1 (20.0)	41 (32.0)		6 (31.6)	36 (31.6)	
III	91 (68.4)	4 (80.0)	87 (68.0)		13 (68.4)	78 (68.4)	
Tumor stage				0.649			0.800
T1	51 (38.3)	1 (20.0)	50 (39.1)		8 (42.1)	43 (37.7)	
T2/T3	82 (61.7)	4 (80.0)	78 (60.6)		11 (57.9)	71 (62.3)	
Nodal stage				0.340			0.603
N0	87 (65.4)	2 (40.0)	85 (66.4)		14 (73.7)	73 (64.0)	
N1/N2/N3	46 (34.6)	3 (60.0)	43 (33.6)		5 (26.3)	41 (36.0)	
Central acellular zone				0.336			0.043
No	101 (75.9)	5 (100.0)	96 (75.0)		18 (94.7)	83 (72.8)	
Yes	32 (24.1)	0 (0.0)	32 (25.0)		1 (5.3)	31 (27.2)	
Central necrotic zone				1.000			0.356
No	123 (92.5)	5 (100.0)	118 (92.2)		19 (100.0)	104 (91.2)	
Yes	10 (7.5)	0 (0.0)	10 (7.8)		0 (0.0)	10 (8.8)	
Central fibrotic zone				0.583			0.121
No	106 (79.7)	5 (100.0)	101 (78.9)		18 (94.7)	88 (77.2)	
Yes	27 (20.3)	0 (0.0)	27 (21.1)		1 (5.3)	26 (22.8)	
Lymphocytic infiltration				0.332			<0.001
Absent	100	5 (100.0)	95 (74.2)		7 (36.6)	93 (81.6)	
Present	33	0 (0.0)	33 (25.8)		12 (63.2)	21 (18.4)	
Tumor cell discohesiveness				1.000			0.356
No	123 (92.5)	5 (100.0)	118 (92.2)		19 (100.0)	104 (91.2)	
Yes	10 (7.5)	0 (0.0)	10 (7.8)		0 (0.0)	10 (8.8)	
Tumor margin				1.000			0.076
Expanding	113 (85.0)	5 (100.0)	108 (84.4)		19 (100.0)	94 (82.5)	
Infiltrative	20 (7.5)	0 (0.0)	20 (15.6)		0 (0.0)	20 (17.5)	
Apocrine differentiation				0.221			0.311
No	109 (82.0)	3 (60.0)	106 (82.8)		14 (73.7)	95 (83.3)	
Yes	24 (18.0)	2 (40.0)	22 (17.2)		5 (26.3)	19 (16.7)	
Tumor cell necrosis (%, mean ± SD)	9.7±14.2	14.0±15.1	9.5±14.2	0.497	6.8±11.5	10.2±14.6	0.340
CD 20 index (mean ± SD)	2.4±6.3	0.2±0.4	2.5±6.4	0.419	4.1±4.4	2.1±6.5	0.201
Ki-67 LI (%, mean ± SD)	27.5±23.4	21.6±23.9	27.7±23.4	0.566	34.5±26.0	26.3±22.8	0.157
CD20 index				0.583			0.001
<2.5	106	5 (100.0)	101 (78.9)		9 (47.5)	97 (85.1)	
≥2.5	27	0 (0.0)	27 (21.1)		10 (52.6)	17 (14.9)	
CD3 index				0.336			0.018
<2.5	101 (75.9)	5 (100.0)	96 (75.0)		10 (52.6)	91 (79.8)	
≥2.5	32 (24.1)	0 (0.0)	32 (25.0)		9 (47.4)	23 (20.2)	
IFN-γ				0.378			0.467
Negative	60 (45.1)	1 (20.0)	59 (46.1)		7 (36.8)	53 (46.5)	
Positive	73 (54.9)	4 (80.0)	69 (53.9)		12 (63.2)	73 (54.9)	
CK5/6				0.653			0.442
Negative	85 (63.9)	4 (80.0)	81 (63.3)		14 (73.7)	71 (62.3)	
Positive	48 (36.1)	1 (20.0)	47 (36.7)		5 (26.3)	43 (37.7)	
EGFR				0.386			0.321
Negative	75 (56.4)	4 (80.0)	71 (55.5)		13 (68.4)	62 (54.4)	
Positive	58 (43.6)	1 (20.0)	57 (44.5)		6 (31.6)	52 (45.6)	
Molecular subtype				0.371			0.448
Basal-like	82 (61.7)	2 (40.0)	80 (62.5)		10 (52.6)	72 (63.2)	
Non basal-like	51 (38.3)	3 (60.0)	48 (37.5)		9 (47.4)	42 (36.8)	

EGFR: epidermal growth factor receptor.

parameters did not differ between tumor-based, immune-related type and non-immune-related TNBCs. However, stromal-based, immune-related type and non-immune-related TNBCs showed some significant clinicopathologic differences. Stroma-based, immune-related TNBCs showed a significantly smaller central acellular zone (p=0.043), more lymphocytic infiltration (p<0.001), higher CD20 index (p=0.001), and higher CD3 index (p=0.018) than stroma-based, non-immune-related TNBC (Fig. 2). Although the differences were not statistically significant, a central necrotic zone, tumor cell discohesiveness, and infiltrative tumor margin were not observed in stroma-based, immune-related TNBC.

The impact of pathologic parameters and immunohistochemical results on prognosis

IL-8 expression in tumor cell was significantly associated with tumor recurrence (p=0.047), shorter DFS (p=0.032), patient death (p=0.047), and shorter OS (p=0.037) by univariate analysis (Table 5 and Fig. 3). Multivariate Cox analysis demonstrated that a higher N stage (Hazard ratio: 6.859, 95% CI: 1.895-24.822, p=0.003) and IL-8 expression (Hazard ratio: 3.804, 95% CI: 1.234-11.729, p=0.020) were independent factors of shorter DFS. Higher N stage (Hazard ratio: 4.318, 95% CI: 1.341-13.907, p=0.014) and IL-8 expression (Hazard ratio: 3.434, 95% CI: 1.132-10.414, p=0.029) were also independent factors of shorter OS (Table 6).

Discussion

This study assessed the clinicopathologic characteristics of immune-related TNBC according to the expression of immune-related molecules. STAT1 expression was observed in 4% of cancer cells and 14% of stromal cells in TNBCs. A previous study reported

Table 5. The impact of immunohistochemical results on tumor recurrence and patient survival.

Parameters	Tumor recurrence			Disease free survival		Patient survival			Overall survival	
	Absent (n=119)	Present (n=14)	P-value	Mean survival (95% CI) months	P-value	Survival (n=119)	Death (n=14)	P-value	Mean survival (95% CI) months	P-value
Cytokeratin 5/6			0.571		0.418			1.000		0.794
Negative	77 (64.7)	8 (57.1)		94 (89-99)		76 (63.9)	9 (64.3)		93 (88-99)	
Positive	42 (35.3)	6 (42.9)		88 (80-96)		43 (36.1)	5 (35.7)		90 (83-97)	
EGFR			1.000		0.896			1.000		0.880
Negative	67 (56.3)	8 (57.1)		93 (86-99)		67 (56.3)	8 (57.1)		93 (86-99)	
Positive	52 (43.7)	6 (42.9)		90 (83-97)		52 (43.7)	6 (42.9)		91 (84-97)	
STAT1			1.000		n/a			1.000		n/a
Negative	114 (95.8)	14 (100.0)		n/a		114 (95.8)	14 (100.0)		n/a	
Positive	5 (4.2)	0 (0.0)		n/a		5 (4.2)	0 (0.0)		n/a	
Stromal STAT1			0.691		0.529			0.691		0.591
Negative	101 (84.9)	13 (92.9)		92 (87-97)		101 (84.9)	13 (92.9)		92 (87-97)	
Positive	18 (15.1)	1 (7.1)		75 (68-82)		18 (15.1)	1 (7.1)		75 (68-82)	
IFN-γ			0.574		0.289			1.000		0.482
Negative	55 (46.2)	5 (35.7)		95 (90-101)		54 (45.4)	6 (42.9)		94 (88-100)	
Positive	64 (53.8)	9 (64.3)		85 (78-93)		65 (54.6)	8 (57.1)		87 (80-94)	
IL-8			0.047		0.032			0.047		0.037
Negative	103 (86.6)	9 (64.3)		95 (90-99)		103 (86.6)	9 (64.3)		95 (90-99)	
Positive	16 (13.4)	5 (35.7)		68 (56-80)		16 (13.4)	5 (35.7)		71 (62-81)	
Stromal IL-8			0.067		0.064			0.067		0.096
Negative	107 (89.9)	10 (71.4)		94 (90-99)		107 (89.9)	10 (71.4)		94 (89-99)	
Positive	12 (10.1)	4 (28.6)		78 (60-96)		12 (10.1)	4 (28.6)		81 (65-97)	
CD20 index			0.734		0.619			0.734		0.724
<2.5	94 (79.0)	12 (85.7)		92 (87-97)		94 (79.0)	12 (85.7)		92 (87-98)	
≥2.5	25 (21.0)	2 (14.3)		94 (85-103)		25 (21.0)	2 (14.3)		94 (86-103)	
CD3 index			0.022		n/a			0.186		0.234
<2.5	87 (73.1)	14 (100)		n/a		88 (73.9)	13 (92.9)		92 (86-97)	
≥2.5	32 (26.9)	0 (0.0)		n/a		31 (26.1)	1 (7.1)		63 (60-66)	
TNBC tumor immune phenotype			1.000		n/a			1.000		n/a
Immune	5 (4.2)	0 (0.0)		n/a		5 (4.2)	0 (0.0)		n/a	
Non-immune	114 (95.8)	14 (100.0)		n/a		114 (95.8)	14 (100.0)		n/a	
TNBC stroma immune phenotype			0.691		0.529			0.691		0.591
Immune	18 (15.1)	1 (7.1)		75 (68-82)		18 (15.1)	1 (7.1)		75 (68-82)	
Non-immune	101 (84.9)	13 (92.9)		92 (87-97)		101 (84.9)	13 (92.9)		92 (87-97)	

EGFR: epidermal growth factor receptor; TNBC: triple negative breast carcinoma.

that immunohistochemical expression of STAT1 was observed in 82% of breast cancers (Sheen-Chen et al., 2007). However, because that study did not investigate STAT1 expression by the molecular subtypes of breast cancer, the STAT1 expression rate of TNBC we found cannot be directly compared with those previously reported results. We observed STAT1 expression in stromal cells as well as in tumor cells. There are only a few reports of STAT1 expression by stromal cells. A previous study identified molecular subclasses of ER negative breast cancer by microarray expression analysis. One of the subclasses was distinguished by overexpression of immune response genes, and STAT1 was suggested as a representative of the immune response genes (Teschendorff et al., 2007). Because the study was performed using microarray analysis of fresh tissue, it was impossible to differentiate whether STAT1 was expressed in the tumor cells or in the tumor microenvironment.

In this study, the antibodies of unphosphorylated STAT1 (uSTAT1) were used to evaluate STAT1. It is generally known that cytoplasmic uSTAT1 is switched to phosphorylated STAT1 (pSTAT1) under the stimulation of IFN-γ, then pSTAT1 is translocated from cytoplasm to nucleus to perform its function. Therefore, estimation of phosphorylated STAT1 might be considered a more reliable method to examine the level of immune response-related STAT1. However, estimation of phosphorylated proteins by immunohistochemistry can lead to inaccurate results due to various constraints (Baker et al., 2005; Burns et al., 2009). In addition, it was reported that STAT1 is rapidly phosphorylated in response to IFN-γ and lasts for only a few hours, while unphosphorylated STAT1 is synthesized by pSTAT1-activated transcription and lasts for several days (Lehtonen et al., 1997). Furthermore, unphosphorylated STAT1 was considered as the latent form of STAT1; however, it later became known that unphosphorylated STAT1 also worked as transcription factor without stimulation of IFN-γ (Kumar et al., 1997; Chatterjee-Kishore et al., 1998). Taken together, using the unphosphorylated STAT1 antibody was the appropriate method for estimation of STAT1 in paraffin embedded tissue in this study.

Immune-related TNBC was reported to be associated with low activity of IL-8 and increased infiltration of CD20-positive B cells (Rody et al., 2011). However, in the current study, we found that cliniopathologic parameters did not differ between the tumor-based, immune-related type and the non-immune related type. Stroma-based, immune-related type and non-immune-related type showed some significant clinicopathologic differences. Immune-related type revealed significantly more lymphocytic infiltration ($p<0.001$), higher CD20 index ($p=0.001$), and higher CD3 index ($p=0.018$). Thus, the results of stroma-based, immune-related type were more consistent with previously reported findings than that of tumor-based, immune-related type. However, the main source of the IL-8 is not the stroma but the cancer cells, as evidenced in a previous study by immunohistochemical analysis and gene expression data of cancer cell lines (Rody et al., 2011).

In this study, tumor-based, immune-related TNBC was defined when tumor cells were positive for STAT1 and negative for IL-8, which was consistent with the stroma-based, immune-related TNBC, except for one case. Further studies are required to clarify how to define immune-related TNBC using immunohistochemical staining. In this study, stroma-based, immune-related TNBC showed distinct lymphocytic infiltration. This finding was consistent with prior studies, which reported that strong lymphocytic infiltration is associated with breast cancers with higher expression of immune related genes (Teschendorff et al., 2007; Rody et al., 2011).

IL-8 expression was an independent and poor prognostic factor of TNBC in this study. IL-8 is an inflammatory and immune-related cytokine that also has tumorigenic and pro-angiogenic properties (Waugh and Wilson, 2008; Ning et al., 2011). Originally, it was known to be secreted by monocytes and endothelial cells

Table 6. Multivariate analysis for survival in TNBC.

Parameters	Disease free survival			Overall survival		
	Hazardratio	95% CI	P-value	Hazard ratio	95% CI	P-value
T stage			0.069			0.078
T1 vs T2-3	6.673	0.861-51.741		6.325	0.813-49.241	
N stage			0.003			0.014
N0 vs N1-3	6.859	1.895-24.822		4.318	1.341-13.907	
Histologic grade			0.823			0.194
I/II vs III	0.875	0.273-2.803		0.477	0.156-1.459	
Molecular subtype			0.615			0.942
Basal-like vs Non basal-like	1.334	0.434-4.104		1.043	0.339-3.206	
IL-8			0.020			0.029
Negative vs Positive	3.804	1.234-11.729		3.434	1.132-10.414	

TNBC: triple negative breast carcinoma.

(Yoshimura et al., 1987), but cancer cells, infiltrating neutrophils, and tumor-associated macrophages are also sources of IL-8, suggesting that IL-8 is involved in regulatory functions of the tumor microenvironment, such as angiogenesis, tumor progression and cancer cell invasion (Lin et al., 2005; Chen et al., 2011; Ju et al., 2012). A previous study reported that IL-8 expression in the breast was a poor prognostic factor (Yao et al., 2006), while another study showed an inverse correlation with metastasis and local recurrence (Zuccari et al., 2012). Thus, there is controversy about whether IL-8 expression in breast cancer is a prognostic predictor. However, prognostic factors used in conventional breast cancer are not useful in TNBC. IL-8 was a top ranked gene associated with poor prognosis in TNBC in a previous study (Rody et al., 2011), which was consistent with the results of our study. Thus, it is likely that IL-8 is a possible prognostic factor in TNBC. Because genomic prognostic signatures such as 70-gene profiles, recurrence score, and genomic grading index predicted poor prognosis for all TNBCs (Finn et al., 2007; Reyal et al., 2008), more studies are needed to determine the prognostic predictors among TNBCs.

In this study, we assessed the expression of immune related proteins, including STAT1, IFN-γ, IL-8, and CD3, CD20 in TNBC and determined that stroma-based, immune-related type shows significant CD3 positive T cell and CD20 positive B cell infiltration. Given that expression of immune-related molecules varied among TNBCs, the possibility of targeted therapy using these molecules should be discussed. Studies on STAT1 blocker and IL-8 pathway blocker are now in progress (Ginestier et al., 2010; Yao et al., 2006).

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