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Molecular Characteristics of Lymphocyte-predominant Triple-negative Breast Cancer

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Abstract. *Background/Aim: Tumor-infiltrating lymphocytes (TILs) are considered a prognostic marker for triple-negative breast cancer (TNBC). Immune checkpoint inhibitor (ICI)-based treatments are more effective for tumors with PD-L1-positive TILs, suggesting crucial roles of TILs in the local tumor immunity. However, factors attracting TILs are still largely unknown. Focusing on tumor antigenicity, we examined TNBC samples to identify the characteristics of TIL-high tumors. Patients and Methods: Nine treatment-naïve TNBCs (TIL-high: five, TIL-low: four) were subjected to next-generation sequencing (NGS). Loss of heterozygosity (LOH) of PTEN was also analyzed. Results: A variety of copy number variations were observed, and no genes differed significantly between TIL-high and -low groups. However, PTEN loss was more frequently observed in the TIL-high group: 60% compared to 25% in TIL-low tumors. NGS correlated well with LOH analysis in identifying PTEN loss. All three tumors with PTEN loss in the TIL-high group showed high PD-L1. All nine samples were microsatellite-stable. Conclusion: Frequent PTEN loss and high expression of PD-L1 in TIL-high TNBC suggest that PTEN mutation may be a biomarker for ICIs.*

TILs have been identified as a prognostic marker in some populations with breast cancer. Patients with TIL-high triple-negative breast cancer (TNBC) reportedly have better outcome, suggesting that the presence of TILs reflects host immune response to the tumor (1-4). Moreover, identifying the TIL status before systemic treatments might serve as a predictive marker for chemotherapies (5). Meanwhile, some immune checkpoint inhibitors (ICIs) have recently been introduced for breast cancer, after being used successfully in other cancers. For instance, atezolizumab has been approved for patients with metastatic TNBC in combination with nab-paclitaxel (6, 7). This regimen was particularly effective for tumors with PD-L1-positive TILs (7). While most ICIs basically target the PD-L1/PD-1 axis, the amount of TILs is also crucial as it is reportedly a predictive factor for ICI treatments according to a meta-analysis, suggesting crucial roles of TILs in local tumor immunity (8).

Attractants of TILs in breast cancer. Factors attracting TILs have not been fully established, despite numerous studies on the topic (9, 10). Tumor mutation burden (TMB) might exert a strong influence, but breast cancer has a lower TMB compared to other cancers, such as lung cancer, which respond well to ICI (9). Microsatellite instability (MSI) is another indicator for ICI for solid cancers. However, MSI-high tumors are almost non-existent in breast cancer, including TNBC (10, 11). We recently examined 79 TNBCs with a high density of TILs, and all were microsatellite-stable (12). In hormone receptor-positive breast cancer, density of TILs is considered unrelated to patient outcomes (1, 13). Moreover, TILs are also frequently observed in non-invasive ductal carcinoma. Taken together, attractants of TILs in breast cancer are still largely unknown.

Therefore, focusing on tumor antigenicity, we investigated TNBC tumor samples by next-generation sequencing (NGS) in order to reveal the characteristics of tumors with high levels of TILs.

Patients and Methods

Patients. We examined surgical specimens of treatment-naïve TNBC primary tumors from patients who underwent curative surgery at our hospital from 2011 to 2016. We excluded patients who had received pre-operative systemic chemotherapy, to avoid any effect of these treatments on TIL expression. Only invasive breast carcinoma of no special type was examined in the current study. Following TIL

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Table I. *Clinicopathological features of the nine patients.*

	TIL-high					TIL-low			
Case	1	2	3	4	5	6	7	8	9
Age	28	42	56	68	70	65	67	77	80
Tumor size (mm)	13	18	50	80	30	22	27	15	30
Lymph node involvement	No	No	Yes	No	Yes	No	No	Yes	No
Tumor grade	High	Intermediate	High	High	High	Intermediate	High	High	High
Ki-67 labeling index (%)	95	90	70	90	20	40	70	65	80
TIL (% stromal TILs)	70	90	90	90	60	30	20	30	10

evaluation (details shown below), five samples for each TIL-high and TIL-low tumor were randomly chosen. NGS could not be completed in one TIL-low tumor due to poor DNA quality. Consequently, nine tumors were investigated in the current study. Clinicopathological features for these patients are shown in Table I. All participants were Japanese women. This study was carried out with approval from the ethics committee of Juntendo University (No. 2016106), and the research plan is presented on the homepage of our hospital. All patients were offered the choice to opt out of the study at any time.

Pathological assessment and TIL evaluation. Pathological examinations were carried out by two experienced pathologists at our hospital, based on the 5th Edition of the WHO classification of Tumors of the Breast. Invasive breast carcinoma of no special type was chosen in the current study. Tumor grade was judged based on the modified Bloom-Richardson histological grading system. For Ki67 labeling index, a hotspot was chosen under ×200 magnification, and cells positive for nuclear Ki67 were evaluated semi-quantitatively. Estrogen and progesterone receptor status were assessed semi-quantitatively by immunohistochemistry (IHC) and reported as positive when 1% or more of the nuclei of the cancer cells showed staining. Human epidermal growth factor receptor 2 (HER2) was judged to be positive if more than 10% of tumor cells showed strong staining across the entire cell membrane, or *HER2/neu* gene amplification was confirmed by fluorescence *in situ* hybridization. Therefore, a TNBC was defined as negative for estrogen and progesterone receptors as well as for HER2. TIL amounts were determined using hematoxylin and eosin-stained tumor surgical sections, based on recommendations made by an International TILs Working Group (14). Briefly, TILs in the stromal compartment (% stromal TILs), using the area of stromal tissue as a denominator, were determined semi-quantitatively in 10% increments. TILs were examined within the borders of the invasive tumor, and a full assessment of average TIL numbers in the tumor area, not focusing on hotspots, was conducted. TILs were judged to be present at a high level (TIL-high) if they comprised at least 50% of the stroma.

NGS and MSI test. Nine tumors, including five TIL-high and four TIL-low tumors, were subjected to NGS using the Ion Ampliseq Cancer Hotspot Panel v2, targeting 50 genes and 2,790 sites (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The details of this NGS analysis have been described previously (15, 16). From paraffin blocks of surgical tissue specimen, 10 μm sections were cut from the same areas as those used for TIL evaluation, and DNA was extracted using a QIAamp DNA FFPE Tissue kit (Qiagen Inc., Venlo, the Netherlands). Tissues were sectioned using macro-dissection to obtain a high tumor cell content. Nontumorous tissue from each patient was used as the control.

MSI testing was outsourced to TaKaRa Bio Inc. (Shiga, Japan) as previously described (12). BAT-25, BAT-26, NR-21, NR-24 and MONO-27 were employed as microsatellite markers. A tumor was determined to have high levels of MSI if instability was detected in two or more of the five markers, as recommended by the revised Bethesda Guidelines (17).

Loss of heterozygosity (LOH) analysis for PTEN. LOH analysis at the *PTEN* locus was performed in all nine cases. Three polymorphic microsatellite markers at the *PTEN* locus (D10S215, D10S541 and D10S583) were used (18, 19). The primers used for the amplification of the loci were as follows: 5'-TGGCATCATTCTGGGGA-3' forward and 5'-GCTTTACGTTTCTTCACATGGT-3' reverse primer for D10S215, 5'-AAGCAAGTGAAGTCTTAGAACCACC-3' forward and 5'-CCACAAGTAACAGAAAGCCTGTCTC-3' reverse primer for D10S541, 5'-TCTGACCAAATACCAAAAGAAC-3' forward and 5'-AGAGCATCCAGATGTTTGATGA-3' reverse primer for D10S583. The polymerase chain reaction (PCR) was performed in Platinum™ Green Hot Start PCR Master Mix (Invitrogen™). The amplified PCR products were evaluated with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). LOH was determined as previously described (20). Briefly, cases showing an allelic imbalance factor greater than 1.5 or less than 0.5 for at least one marker were considered to show LOH.

IHC. PTEN and PD-L1 protein expression was examined by IHC. PTEN expression both in the nuclei and cytoplasm of cancer cells was assessed semi-quantitatively in 10% increments, and an expression exceeding 10% was defined as positive. PTEN might have different roles according to intracellular localization (21). PTEN in the cytoplasm is involved in the down-regulation of AKT, leading to apoptosis. In contrast, nuclear PTEN has a variety of functions,

including cell cycle arrest and upregulation of DNA repair. Thus, we evaluated protein localization. For PD-L1, membrane staining of stromal immune cells (ICs) was determined. ICs consist of lymphocytes, macrophages, dendritic cells, and granulocytes, as assessed based on the guidelines from Roche Diagnostics for IHC assessment (SP142). Scoring for PD-L1 was assessed as 0: <1%, 1: ≥1% to <5%, 2: ≥5% to <10%, and 3: ≥10%, based on criteria applied clinically for breast cancer (7). Antibodies used were anti-PTEN mAb, 6H2.1 (Dako, Glostrup, Denmark), and anti-PD-L1 mAb, SP142 (Spring Bioscience, Pleasanton, CA, USA).

Statistical analysis. Statistical analyses were performed using JMP14.2 statistical software (SAS Institute, Inc., Cary, NC, USA). Associations between two parameters were evaluated using the Fisher's exact test. A *p*-value <0.05 was considered to indicate a statistically significant difference.

Results

Differences in mutation signature between TIL-high and -low TNBC. A summary of copy number variation (CNV) is shown in Table II (details of the results are indicated in Table III). A variety of CNVs were observed, and no gene differed statistically significantly between TIL-high and -low groups. However, *PTEN* loss was more frequently observed in the TIL-high group (60% or three out of five cases compared to 25% or one in four cases in TIL-low tumors). Similarly, *APC* loss was more frequently observed in the TIL-high group (60% or three out of five cases), while none of the TIL-low tumors had such mutation. None of the nine patients had a familial history of familial adenomatous polyposis (FAP). The c.333_335delGAT non-fs mutation in *PIK3CA* was observed in the TIL-high group. The details of the mutations in *TP53* were as follows: single nucleotide variant (SNV) at splice site (T>A) and deletions at c.686_687delGT-fs and c.311_318delAGGGCAG-fs were found in the TIL-high group. SNV of missense (c.581T>G) and deletions of c.626_627delG/GA, p.Arg209fs and c.626_626delG, p.Arg209fs were identified in the TIL-low group.

Relationships between PTEN loss and LOH, protein expression, and PD-L1 in ICs. Since *PTEN* loss was more frequently observed in the TIL-high group by NGS, we next examined LOH at the *PTEN* locus, and PTEN protein expression, along with PD-L1 (Table IV and Figure 1). As to polymorphic microsatellite markers, only D10S215 worked, as the majority of tumors showed homozygosity with other markers. LOH at the *PTEN* locus was observed in six out of eight informative cases (75%). LOH status in case 8 was not available because this tumor showed homozygosity. *PTEN* loss determined by NGS correlated well with that of LOH analysis (four out of four cases).

Representative IHC images are shown in Figure 2. By IHC, all four cases with *PTEN* loss were negative for PTEN protein expression in the nucleus, while some tumors without *PTEN* loss were also negative for this protein. Meanwhile, PTEN protein expression was observed in the cytoplasm in all TIL-low tumors, but did not seem to correspond to *PTEN* copy number variation status.

As for PD-L1, all three tumors with *PTEN* loss by CNV analysis in the TIL-high group showed high PD-L1 in ICs.

MSI status and TIL amounts. Finally, we examined MSI status. All nine samples were microsatellite-stable, regardless of TIL amounts. All five microsatellite markers assessed were negative.

Table II. *Mutation signatures according to TIL amounts*.

	TIL-high (n=5)	TIL-low (n=4)	*p*-Value
Amplification			
FGFR3	2/5	2/4	1.00
AKT1	2/5	2/4	1.00
NOTCH1	2/5	1/4	1.00
HRAS	1/5	2/4	0.52
SMO	1/5	1/4	1.00
JAK3	1/5	1/4	1.00
HNF1A	1/5	0/4	1.00
VHL	1/5	0/4	1.00
CDKN2A	0/5	1/4	1.00
RET	0/5	1/4	1.00
Deletion			
RB1	3/5	2/4	0.64
PTEN	3/5	1/4	0.52
KRAS	1/5	1/4	1.00
MET	1/5	1/4	1.00
APC	3/5	0/4	0.17
ATM	2/5	2/4	1.00
SMAD4	2/5	1/4	1.00
KIT	0/5	1/4	1.00
Mutation (SNV or indel)			
PIK3CA	1/5	0/4	1.00
TP53	3/5	3/4	0.60

SNV: Single nucleotide variant.

Discussion

We found that *PTEN* loss was frequently observed in the TIL-high group, although the difference was not statistically significant. TNBC is well known for having the highest TMB among breast cancer subtypes (22). Somatic *PTEN* mutations are observed in 8% of TNBCs, the third most frequent following *TP53* and *PIK3CA* (23, 24). Loss or mutation of *PTEN* leads to activating phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR signals and may predict sensitivity to inhibitors of this pathway (25). In a randomized

Table III. *Details of copy number variation in the nine cases.*

TIL-high				
Case 1	Case 2	Case 3	Case 4	Case 5
NOTCH1 **JAK3** *MLH1|CTNNB1|PIK3CA* *APC* *PTEN* *ATM* *FLT3|RB1*	*IDH1|ERBB4* *CTNNB1|PIK3CA* *APC* *MET* *PTEN* *KRAS|PTPN11* *RB1* *SMAD4*		**VHL** **FGFR3** **HNF1A** AKT1 *PDGFRA|KIT|KDR|FBXW7|DEAR* *APC* *KRAS* *RB1*	**FGFR3** **SMO** **NOTCH1** **HRAS** **AKT1** *CTNNB1|PIK3CA* *PTEN* *ATM* *RB1* *SMAD4*
TIL-low				
Case 6	Case 7	Case 8	Case 9	
HRAS	*VHL|MLH1|* *CTNNB1|PIK3CA* *PDGFRA|KIT|KDR|* *FBXW7|DEAR*	**FGFR3** **CDKN2A** **RET** **AKT1** **JAK3** *KIT* *KDR|FBXW7|DEAR* *ATM* *RB1*	**FGFR3** **SMO** **NOTCH1** **HRAS** **AKT1** **STK11|GNA11|JAK3** *MLH1|CTNNB1|PIK3CA* *PDGFRA|KIT* *KDR|FBXW7|DEAR* *MET* *PTEN* *ATM* *KRAS* *RB1* *SMAD4*	

Highlighted in bold: Amplification; italics: deletion.

Table IV. *Relationships between PTEN loss, LOH, and protein expression.*

Case	TIL	Loss of *PTEN* (CNV)	*LOH* at *PTEN* (D10S215)	PTEN IHC (N)	PTEN IHC (C)	PD-L1 (IC)
1	High	+	+	–	-	3
2	High	+	+	–	+	2
3	High	–	-	+	+	0
4	High	+	+	–	-	3
5	High	–	+	–	+	0
6	Low	–	-	–	++	0
7	Low	–	+	–	++	0
8	Low	–	N.E.	+	+	2
9	Low	+	+	–	++	0

CNV: Copy number variation, IHC: immunohistochemistry, N: nucleus, C: cytoplasm, IC: immune cells; LOH: loss of heterozygosity.

phase II trial, improved progression-free-survival was seen in patients with metastatic PTEN-low TNBC treated with ipatasertib, a selective AKT inhibitor (26). Another AKT inhibitor, capivasertib, has also shown significant additional treatment effects in combination with paclitaxel in patients with *PTEN*-altered TNBC, along with *PIK3CA* and *AKT1* mutations (27). Nevertheless, associations of TIL amount and *PTEN* mutations have not been well studied (28).

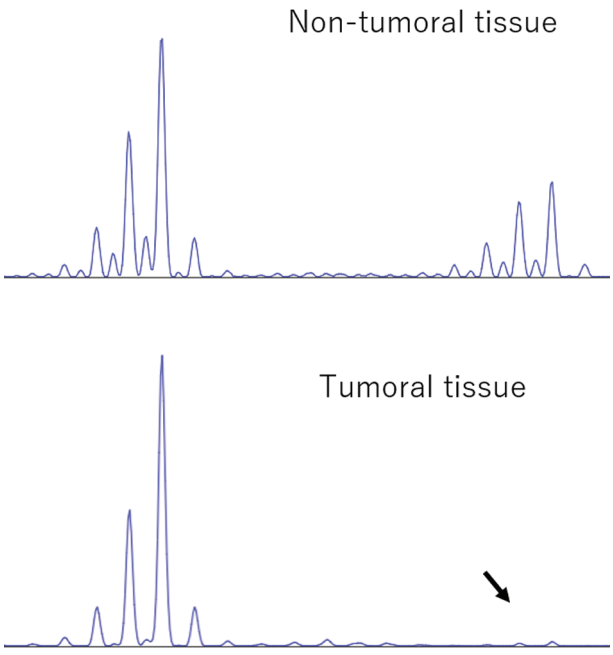

Figure 1. *Loss of heterozygosity (LOH) analysis of the PTEN locus. Loss of the long allele was observed in DNA from a tumor sample (lower) compared to a DNA sample from the corresponding normal tissue (upper), indicating LOH (D10S215).*

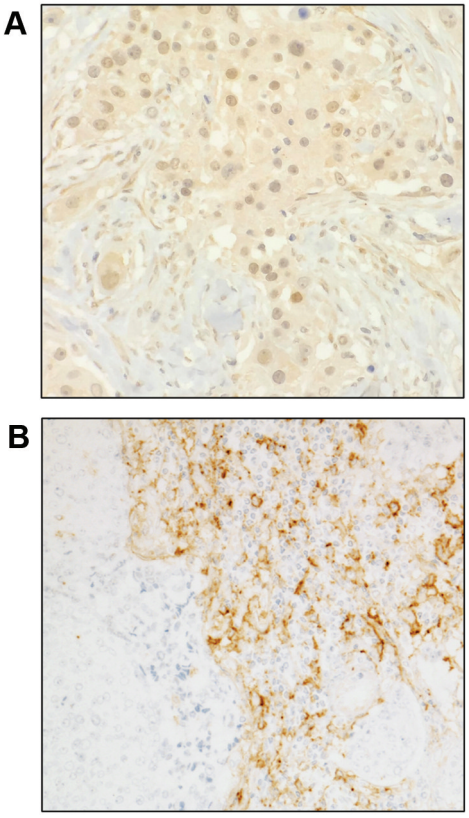

Figure 2. *Representative images of immunohistochemistry for PTEN and PD-L1. A: A case positive for PTEN, both in the nuclei and cytoplasm. B: A case with positive membrane staining for PD-L1 in stromal immune cells (IC score: 3).*

Interestingly, PD-L1 in immune cells was high in all three tumors with *PTEN* loss in the TIL-high group. The amount of TILs is one predictive marker for ICI (8) and PD-L1 is rightly a predictor for ICI (7, 29). However, neither predicts treatment success perfectly. Some researchers have tried to find genetic alterations relating to the ICI effect. For instance, *JAK2* amplification was frequently observed in PD-L1 over-expressed tumors (30). In our series, amplification of *JAK3,* but not *JAK2*, was observed in one tumor from each group; in Case 1 and 8 (Table II), and PD-L1 expression in ICs was positive in both cases. Our results indicate that *PTEN* loss might promote tumor antigenicity, thus we speculate that patients with such tumors will benefit from ICI. Direct relationships between *PTEN* mutation and PD-L1 protein expression or efficacy of ICI have been poorly investigated to date (31, 32). Barrett *et al*. examined surgical TNBC specimens and revealed that *PTEN* deletion was not associated with PD-1 and PD-L1 expression (31). However, there are a number of methodological differences between their study and ours, including patient cohorts, copy number analysis and PD-L1 assessments. In a recent retrospective study, Barroso-Sousa *et al*. reported *PTEN* alteration as a negative predictive marker for ICI-based treatments (32). However, it is too early to draw a definitive conclusion from their patient cohort in which a variety of chemotherapies were administered with ICI, such as eribulin. They observed that *PTEN* loss correlated with decreased TIL and PD-L1, but again, their patient cohort was completely different from ours. Possibly, *PTEN* alterations can be used as a biomarker for ICI, however, this suggestion warrants further investigation.

As to the MSI status, all samples were microsatellite-stable. Our results were consistent with previous studies, showing very low frequency of tumors with high levels of MSI in breast cancer (10-12). Considering the rarity, the significance of testing MSI status might be low in breast cancer.

The main limitations of this study were the sample size and the lack of validation set. To confirm our results, a validation study of *PTEN* loss in a larger number of TNBCs according to TIL numbers is necessary. The potential of *PTEN* alteration as a predictive marker for ICI in our cohort remains unclear as no patient received ICI-based treatments. Mechanisms elucidating how *PTEN* loss is involved in promoting tumor antigenicity might also merit further studies.

In conclusion, frequent *PTEN* loss was observed in TIL-high TNBC, and PD-L1 expression in ICs was high in such tumors, indicating the possibility of *PTEN* mutation as a biomarker for ICI-based treatments.

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

Authors' Contributions

YH and AKA, TS designed the current study. YH and TS wrote the manuscript. RS, YH, and KN acquired the clinical data. YH, AKA and AA conducted the pathological assessments. RS, YY and AKA performed experiments. MS reviewed and revised the manuscript. All Authors read and approved the final manuscript.

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