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Validation of Systemic and Local Tumour Immune Response to Eribulin Chemotherapy in the Treatment of Breast Cancer

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Abstract. *Background/Aim: In addition to its cytocidal effects as a microtubule dynamics inhibitor, eribulin mesylate (eribulin) regulates the tumour microenvironment. We examined the clinical significance of tumour infiltrating lymphocytes (TILs) and transforming growth factor-β (TGF-β), which are local markers of host immunity, and of the neutrophil-lymphocyte ratio (NLR) and absolute lymphocyte count (ALC), which are systemic markers. Patients and Methods: We administered eribulin chemotherapy to 106 patients with locally advanced or metastatic breast cancer. Of these, 21 had their lesions resected. Results: The response to eribulin was significantly associated with ALC (p=0.007). The expression of pSmad2 (an indicator of activation of TGF-β downstream signaling) was significantly decreased before and after eribulin chemotherapy (p<0.001). Moreover, a baseline ALC ≥ 1,500 /μl was observed in a significantly high number of patients with pSmad2 negative conversion (p<0.001). Conclusion: Eribulin improved the tumour immune microenvironment by decreasing TGF-β expression. This demonstrated that local change can be evaluated based on ALC.*

In addition to its cytocidal effects as a microtubule dynamics inhibitor, eribulin mesylate (eribulin) regulates the tumour microenvironment (TME) by promoting vascular remodelling and inhibiting epithelial-mesenchymal transition (EMT) (1-5). The TME refers to the dynamic changes and malignant traits that occur in the microenvironment of cancer cells, such as EMT, tumour hypoxia, and tumour immune response. These changes are induced by cytokines such as transforming growth factor-β (TGF-β) and cell growth factors (6-8). In one international phase III study on locally advanced or metastatic breast cancer (MBC) (EMBRACE, Study 305), the TME-improving effect of eribulin treatment resulted in significantly prolonged overall survival (OS) (9). Moreover, in immunotherapies such as immune checkpoint inhibitor therapy, the survival curve follows a characteristic pattern called a delayed separation curve; in this regard, the effect of eribulin on the tumour immune microenvironment (TIME) is also drawing attention.

In previous studies, we used clinical samples to show that the TME changes after eribulin treatment (1, 2). We also confirmed that eribulin treatment led to EMT suppression and hypoxia-induced release in patients with MBC (1). Regarding tumour immune response, programmed cell death protein (PD)-1, programmed death ligand-1 (PD-L1), and forkhead box P3 (FOXP3) expression decreased, while CD8 expression increased in responders. This suggests that the TIME improved after eribulin treatment (2). Tumour infiltrating lymphocytes (TILs) can be used as an index to monitor TIME and likely predict prognosis and therapeutic effect in breast cancer (10-13). In another study, we reported that, among patients with triple-negative breast cancer (TNBC) treated with eribulin, those with high TIL values had significantly better prognosis than those with low TIL values (14). It follows that, in patients with MBC, if local TIME is good, eribulin should be effective.

Several recent studies have focused on the clinical significance of inflammatory markers as indices of systemic tumour immune response (15-17). The *in vivo* inflammatory reaction contributes to cancer progression. In this regard, the peripheral blood neutrophil-lymphocyte ratio (NLR), lymphocyte-monocyte ratio (LMR), and platelet-lymphocyte ratio (PLR) of tumour-bearing patients have been proposed

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as indicators of systemic inflammatory response. Moreover, several studies have reported that these factors may predict prognosis in various carcinomas (18-20). These inflammatory markers are suggested to reflect the systemic tumour immune response. In our own studies, we have shown that NLR, LMR, and PLR could be used as prognostic predictors in patients with TNBC treated with neoadjuvant chemotherapy (21-23). In other words, in highly immunogenic TNBC, prognosis can be predicted based on systemic tumour immune response, as indicated by inflammatory markers. In several other studies involving patients treated with eribulin chemotherapy, low NLR and high absolute lymphocyte count (ALC) also resulted in a good prognosis (24-26). In the present study involving patients with MBC undergoing eribulin chemotherapy, we examined the clinical significance of TILs and TGF-β, which are local markers of host immunity, and of NLR and ALC, which are systemic markers.

Patients and Methods

Patient background. From August 2011 to April 2019, we administered eribulin chemotherapy to 106 patients with MBC. Of these, 21 had their lesions resected and were recruited as subjects in the present study. To assess the antitumor effect of eribulin, we stratified the cytoreductive effect of the treatment into complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD) groups using the Response Evaluation Criteria in Solid Tumors diagnostic criteria. The following outcome measures were used: objective response rate (ORR), defined as CR + PR, clinical benefit rate (CBR), defined as CR + PR + SD for >24 weeks, and disease control rate (DCR), defined as (CR + PR + SD) (27, 28). The chemotherapy regimen was eribulin mesylate administered intravenously once daily at 1.4 mg/m^2 (9, 28-30, 31) over 2 consecutive weeks, and was discontinued in week 3; this was regarded as one course. The treatment was repeated until discontinuation due to PD or adverse events. All patients received this chemotherapy at the outpatient clinic.

Blood sample analysis. To measure NLR and ALC, peripheral blood samples collected before eribulin treatment were used. The percentages of white blood cells were measured using a Coulter LH 750 Hematology Analyzer (Beckman Coulter, Brea, CA, USA). NLR was calculated as the ratio of ALC to the absolute neutrophil count. The cut-off values for ALC and NLR were set at 1,500 /μl and 3.0, respectively, based on previous studies (21, 24-26). An ALC value ≥1,500 /μl was defined as high, while one below 1,500 /μl was defined as low. An NLR value ≥3.0 was high while one <3.0 was low.

Evaluation of tumour morphology and TILs. To evaluate tumour morphology and TILs, needle biopsy specimens collected before eribulin chemotherapy were used. If biopsy was not possible, a resected specimen of primary breast cancer was used. Morphological assessments were carried out using conventional haematoxylin and eosin staining. Breast cancer was classified into subtypes according to the immunohistochemical expression of oestrogen receptor (OR), progesterone receptor (PgR), human epidermal growth factor receptor 2 (HER2), and Ki67, yielding the following immunophenotypes: luminal A (OR$^+$ and/or PgR$^+$, HER2$^-$, Ki67^{low}), luminal B (OR$^+$ and/or PgR$^+$, HER2$^-$, Ki67^{high}), luminal HER2 (OR$^+$ and/or PgR$^+$, HER2$^+$), HER2-enriched (OR$^-$, PgR$^-$, and HER2$^+$), and TNBC (OR$^-$, PgR$^-$, and HER2$^-$) (32).

TILs were evaluated on biopsy specimens by measuring the percentage area occupied by lymphocytes on the haematoxylin and eosin-stained tumour sections (33). The area of stromal TILs surrounding the stained cancer cells was quantified in each field of view (×400) (14, 34-36). The area of the stroma with lymphoplasmacytic infiltration around the invasive tumour cell nests was classified as >50%, 10%-50%, ≤10%, or absent, with corresponding scores of 3, 2, 1, or 0, respectively (Figure 1). The TIL count was defined as 'high' at scores of ≥2, and 'low' at scores of 1 or 0. Histopathological evaluation of TILs was performed by two professional breast cancer pathologists (S.T. and M.O.).

Immunohistochemistry of pSmad2. TGF-β was evaluated in terms of pSmad2 expression, which is an indicator of TGF-β downstream signalling, in biopsy specimens taken before eribulin chemotherapy and surgical specimens obtained after administration. If biopsy was not possible, a resected specimen of primary breast cancer was used. Tumour specimens were fixed in 10% formaldehyde solution and embedded in paraffin; 4-μm sections were then mounted onto glass slides, which were then deparaffinised in xylene and heated in an autoclave for 20 min at 105˚C and 0.4 kg/m^2 in Target Retrieval Solution (Dako, Carpinteria, CA, USA). Specimens were then incubated in 3% hydrogen peroxide in methanol for 15 min to block endogenous peroxidase activity, and then in 10% normal goat serum to block non-specific reactions. Primary monoclonal antibody directed against phospho-smad2 [(Ser465/467) (138D4) Rabbit mAb #3108, 1:1,000 dilution, Cell Signaling Technology, MA, USA] was then applied. Tissue sections were incubated in each antibody for 70 min at room temperature or overnight at 4˚C; they were then incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse Ig secondary antibodies [HISTOFINE (PO)™ Kit; Nichirei, Tokyo, Japan]. The slides were subsequently treated using streptavidin peroxidase reagent and incubated in phosphate-buffered saline-diaminobenzidine and 1% hydrogen peroxide (v/v). They were then counterstained using Mayer's haematoxylin. Positive and negative controls for each marker were used according to the manufacturer's data sheets.

Immunohistochemical staining was evaluated by two pathologists specialised in mammary gland pathology and blinded to the patient treatments. The cut-offs for positive staining were >30% of cells with phospho-smad2 cytoplasmic staining (Figure 2) (37, 38).

Ethics statement. The research reported herein conformed to the provisions of the Declaration of Helsinki (64th WMA General Assembly, Fortaleza, Brazil, October 2013). The study comprised a retrospective chart review. At the time of treatment, patients provided written informed consent for the use of their data in later research studies. The study protocol was approved by the Ethics Committee of Osaka City University (#926).

Statistical analysis. Statistical analysis was performed using SPSS® Statistics version 25 statistical software (IBM, Armonk, NY, USA). The chi-squared or Fisher's exact test was used to analyse whether clinical parameters were associated with NLR, ALC, TIL count, and

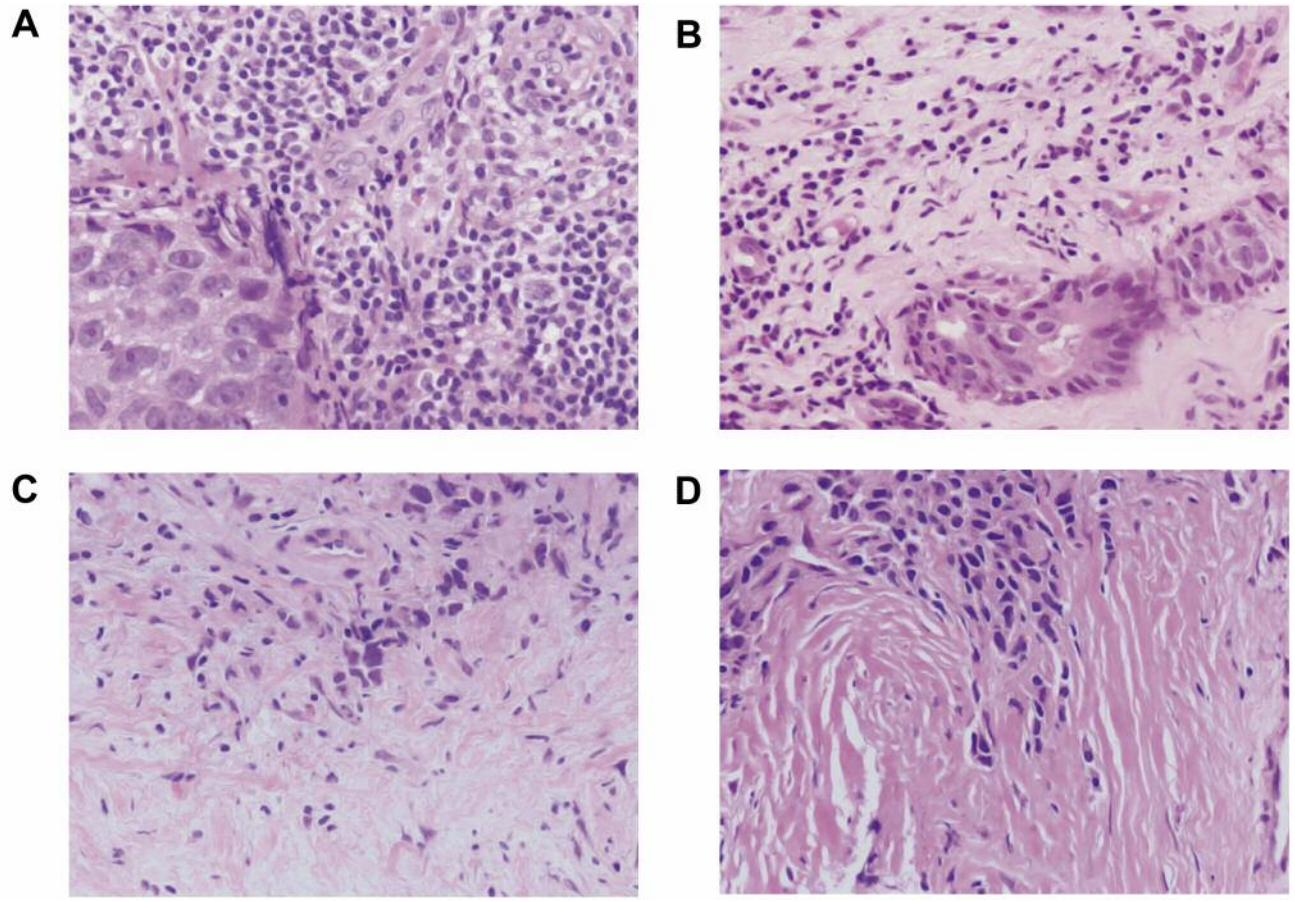

Figure 1. *The area of tumour infiltrating lymphocytes (TILs) in the stroma surrounding the stained cancer cells was quantitatively measured in each field under 400× magnification. The areas of in situ carcinomas and crush artefacts were not included. Proportional scores were defined as 3, 2, 1, and 0 if the area of stroma with lymphoplasmacytic infiltration around the invasive tumour cell nests was >50% (a); >10-50% (b); ≤10% (b); and absent (d), respectively.*

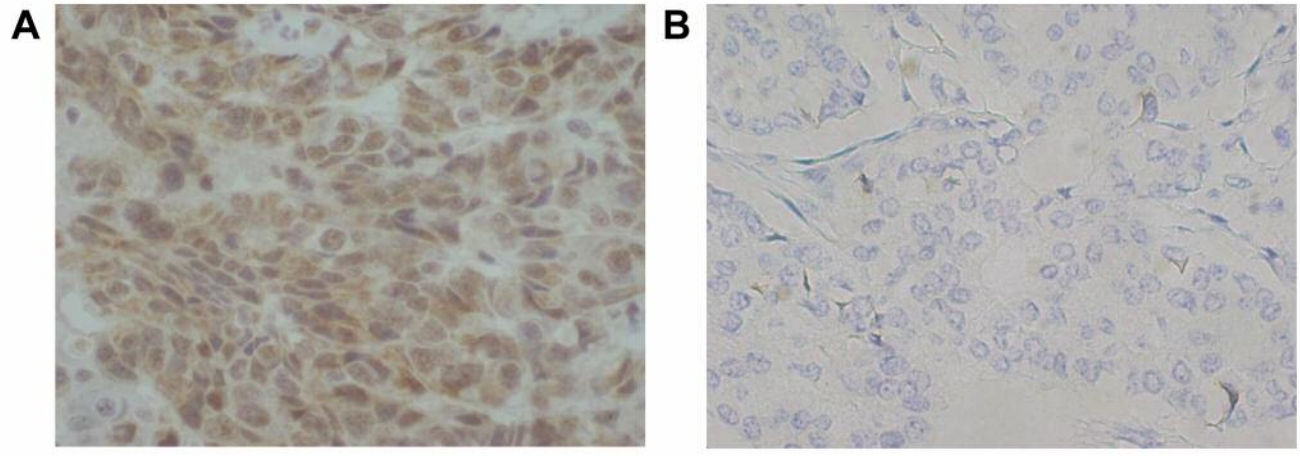

Figure 2. *Immunohistochemistry of pSmad2 expression. The cut-offs for positive staining were >30% of cells with Phospho-Smad2 cytoplasmic staining (positive: a, negative: b).*

Table I. *Patient characteristics*.

Variable	Total cohort (n=106)	Surgical cohort (n=21)
Age (years)	59.5±12.2	58.0±14.3
Progression		
Locally advanced	26 (24.5%)	11 (52.4%)
Visceral metastases	80 (75.5%)	10 (47.6%)
Site of metastases		
Lung	37 (34.9%)	4 (19.0%)
Bone	42 (39.6%)	7 (33.3%)
Liver	30 (28.3%)	1 (4.8%)
Life-threatening condition		
Life-threatening	23 (21.7%)	1 (4.8%)
Non-life-threatening	83 (78.3%)	20 (95.2%)

		Preoperative (n=21)	Postoperative (n=20)[a]
Nuclear grade			
1	46 (43.4%)	9 (42.9%)	8 (40.0%)
2	28 (26.4%)	4 (19.0%)	8 (40.0%)
3	32 (30.2%)	8 (38.1%)	5 (20.0%)
Estrogen receptor-positive	65 (61.3%)	12 (57.1%)	8 (40.0%)
Progesterone receptor-positive	44 (41.5%)	8 (38.1%)	6 (30.0%)
HER2-positive	11 (10.4%)	0 (0.0%)	0 (0.0%)
Ki67-high	48 (45.2%)	12 (57.1%)	8 (40.0%)
Intrinsic subtype			
Luminal A	38 (35.9%)	7 (33.3%)	2 (10.0%)
Luminal B	24 (22.6%)	4 (19.0%)	4 (20.0%)
Luminal HER2	2 (1.9%)	0 (0.0%)	0 (%)
HER2 enriched	5 (4.7%)	0 (0.0%)	0 (%)
Triple-negative	37 (34.9%)	10 (47.7%)	14 (70.0%)

[a]Immunohistochemistry was unavailable in 1 patient with pathological complete response. Values are expressed as the mean±standard deviation or number (%) of patients. HER2: Human epidermal growth factor receptor 2.

pSmad2 expression, as appropriate. In all statistical tests, a *p*-value of less than 0.05 was considered statistically significant.

Results

Eribulin-treated patients with investigated samples. Of the 106 patients who received eribulin chemotherapy to treat MBC, 21 (19.8%) had undergone lesion resection (Table I). With regards to disease progression, among all 106 patients, 26 (24.5%) showed locally advanced disease and 80 (75.5%) had visceral metastases. The intrinsic subtypes were luminal A in 38 patients (35.9%), luminal B in 24 patients (22.6%), luminal HER2 in two patients (1.9%), HER2-enriched in five patients (4.7%), and TNBC in 37 patients (34.9%). Regarding anti-tumour efficacy, no patients had CR (0%), 43 (40.5%) had PR (4.7%), five had long-term SD (4.7%), 13 had SD (12.3%), and 39 (36.8%) had PD. ORR was measured in 43 patients (40.6%), CBR in 48 patients (45.3%), and DCR in 61 patients (57.5%) (Table II). In the 21 patients who had undergone surgery (surgical cohort), the intrinsic subtype was luminal A in seven patients (33.3%), luminal B in four patients (19.0%), luminal HER2 and HER2-enriched in no patients (0.0%), and TNBC in 10 patients (47.7%). With regards to anti-tumour effect, no patients (0%) showed CR, 11 (52.4%) showed PR, two (9.5%) showed long SD, two (9.5%) showed SD, and six (28.6%) showed PD. ORR was measured in 11 of the 21 patients (52.4%), CBR in 13 of them (61.9%), and DCR in 15 of them (71.4%).

Table II. *Clinical effects of eribulin chemotherapy on locally advanced or metastatic breast cancer.*

Measure	Total cohort (n=106)	Surgical cohort (n=21)
ORR	43 (40.6%)	11 (52.4%)
CBR	48 (45.3%)	13 (61.9%)
DCR	61 (57.5%)	15 (71.4%)
CR	0 (0.0%)	0 (0.0%)
PR	43 (40.5%)	11 (52.4%)
LSD	5 (4.7%)	2 (9.5%)
SD	13 (12.3%)	2 (9.5%)
PD	39 (36.8%)	6 (28.6%)
NE	6 (5.7%)	0 (0.0%)

Values are expressed as the number (%) of patients. ORR: Objective response rate; CBR: clinical benefit rate; DCR: disease control rate; CR: complete response; PR: partial response; LSD: stable disease >24 weeks; SD: stable disease; PD: progressive disease; NE: not evaluable.

Table III. *Correlation between the response to eribulin and absolute lymphocyte count, neutrophil-lymphocyte ratio, tumor infiltrating lymphocytes, pSmad2 expression*.

	Responders (n=11, 52.4%)	Non-responders (n=10, 47.6%)	*p*-Value
NLR (baseline)			0.063
≥3	2 (81.8%)	6 (60%)	
<3	9 (18.2%)	4 (40%)	
ALC (baseline)			0.007
≥1500/μl	9 (18.2%)	2 (20%)	
<1500/μl	2 (81.8%)	8 (80%)	
TILs (baseline)			0.410
High	5 (45.5%)	6 (60%)	
Low	6 (54.5%)	4 (40%)	
pSmad2 (change)			<0.001
Negative conversion	10 (90.9%)	1 (10%)	
Other	1 (9.1%)	9 (90%)	

Values are expressed as the number (%) of patients. ALC: Absolute lymphocytes count; NLR: neutrophil-lymphocyte ratio; TILs: tumor infiltrating lymphocytes.

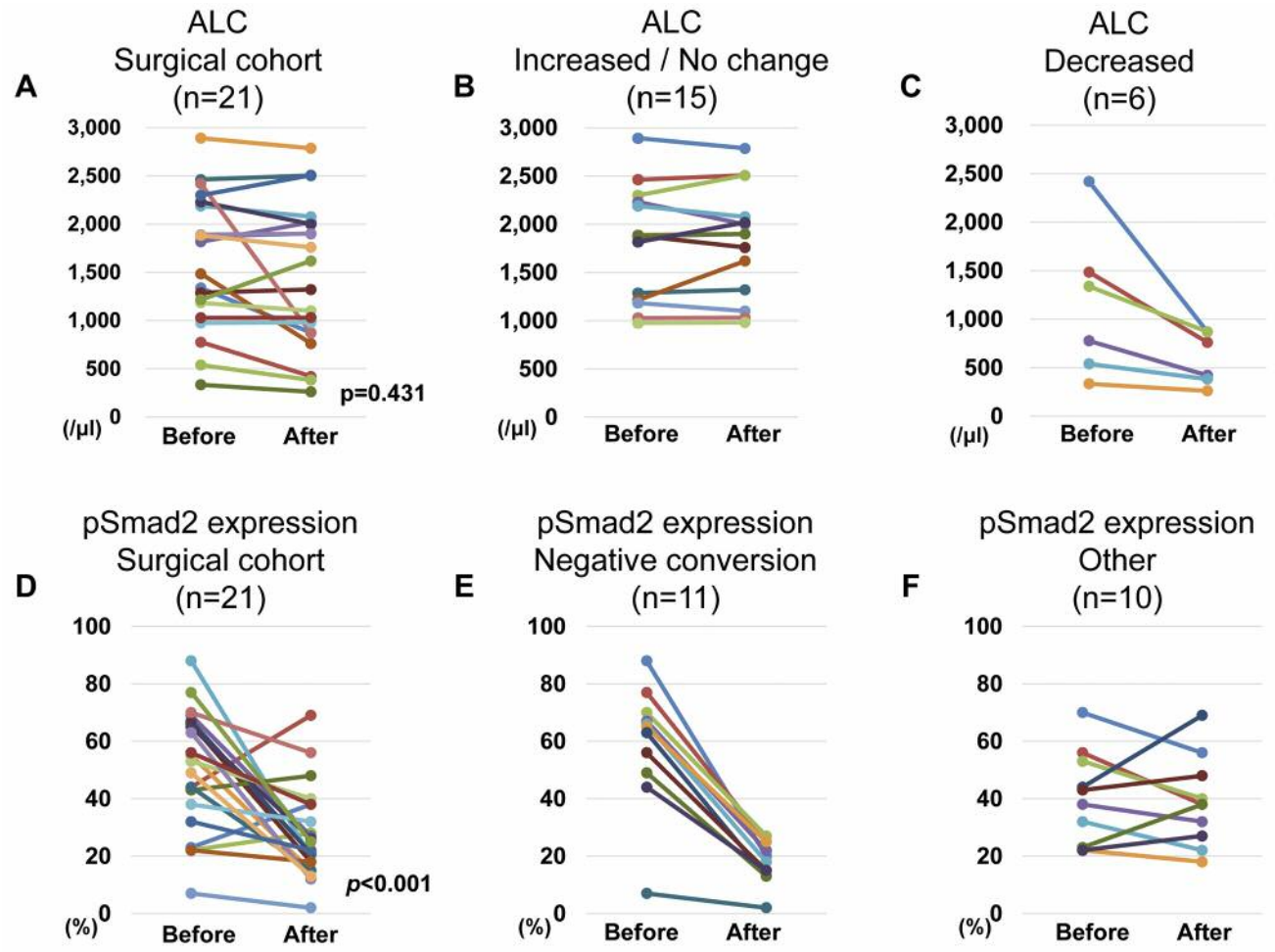

Figure 3. *Changes in absolute lymphocyte count (ALC) and pSmad2 expression in eribulin chemotherapy. ALC didn't change significantly before and after eribulin chemotherapy (p=0.431) (a). However, in 71.4% (15/21) of cases, ALC increased or remained unchanged (b, c). The expression of pSmad2 was significantly decreased before and after eribulin chemotherapy (p<0.001) (d). Also, in 52.3% (11/21) of cases, pSmad2 expression was negative conversion (e, f).*

Clinical significance of systemic indicators (NLR, ALC) in eribulin chemotherapy. Blood samples were obtained at the start of eribulin treatment in 21 patients: 11 (52.4%) with PR and 10 (47.6%) non-responders (Table III). There was no correlation between NLR and response to eribulin chemotherapy in the 21 patients of the surgical cohort (p=0.063). Among all patients, the response to eribulin was significantly associated with ALC (p=0.007), and ALC did not change significantly after eribulin chemotherapy (p=0.431) (Figure 3). However, in 15 of the 21 patients in the surgical cohort (71.4%), ALC increased or remained unchanged.

Clinical significance of local indicators (TILs, pSmad2) in eribulin chemotherapy. Tissue samples were obtained at two points in the 21 patients of the surgical cohort: 1) at diagnosis; 2) after surgical resection and eribulin treatment. In general, eribulin was administered within 1 month of diagnosis, and the resections were performed within 1 month of determining the effect of eribulin. There was no correlation between TIL density and response to eribulin chemotherapy in the 21 patients of the surgical cohort (p=0.410). In 11 of these 21 cases (52.4%), pSmad2 negative conversion was observed. The response to eribulin was significantly associated with pSmad2 negative conversion (p<0.001). Using the immunohistochemical data of these 21 patients, the expression rate of pSmad2 in each tissue specimen before and after eribulin treatment was plotted (Figure 3). The expression of pSmad2 was significantly decreased after eribulin chemotherapy (p<0.001). Moreover, in 11 of the 21 patients (52.3%), pSmad2 expression showed negative conversion.

Table IV. *Correlation between tumor infiltrating lymphocytes, pSmad2 expression, and absolute lymphocyte count and neutrophil-lymphocyte ratio.*

	Baseline NLR (n=21)		*p*-Value	Baseline ALC (n=21)		*p*-Value
	≥3 (n=8, 50%)	≥3 (n=8, 50%)		≥1500/μl (n=11, 52.4%)	<1500/μl (n=10, 47.6%)	
TILs (baseline)			0.392			0.835
High (n=11)	5 (Responder 1/5)	6 (Responder 4/6)		6 (Responder 4/6)	5 (Responder 1/5)	
Low (n=10)	3 (Responder 1/3)	7 (Responder 5/7)		5 (Responder 5/5)	5 (Responder 1/5)	
pSmad2 (change)			0.063			<0.001
Negative conversion (n=11)	2 (Responder 2/2)	9 (Responder 8/9)		10 (Responder 9/10)	1 (Responder 1/1)	
Other (n=10)	6 (Responder 0/6)	4 (Responder 1/4)		1 (Responder 1/1)	9 (Responder 0/9)	

Values are expressed as the number (%) of patients. ALC: Absolute lymphocytes count; NLR: neutrophil-lymphocyte ratio; TILs: tumor infiltrating lymphocytes.

Correlation between systemic indicators (NLR, ALC) and local indicators (TILs, pSmad2). We examined whether TIL density and pSmad2 expression, which are local indicators of host cancers, were correlated with NLR and ALC, which are systemic indices (Table IV). Baseline NLR showed no correlation with TILs or pSmad2 expression (p=0.392 and p=0.063, respectively). However, a baseline ALC ≥ 1,500 /μl was observed in a significantly high number of patients with pSmad2 negative conversion (p<0.001), although no correlation was seen with TIL concentration (p=0.835). Relatedly, a high response rate was obtained (9/10 patients) among patients with a baseline ALC ≥1,500 /μl and in whom pSmad2 negative conversion was found.

Discussion

The prolongation of OS due to chemotherapy is challenging in individuals with MBC because of the therapy's relative biological mildness. For example, in several studies (E2100, AVADO, RIBBON-1), bevacizumab combination therapies improved progression-free survival, with a high response rate. However, the treatment had no effect on OS (39-42). Therefore, to prolong OS in patients undergoing chemotherapy to treat MBC, treatment must improve TIME and block the signalling pathways and pharmacological action of cytotoxicity. Even in the EMBRACE study (Study 305) (9), which showed that eribulin improves OS in patients with MBC, the authors presumed that the tumour immune response was involved. In the same study, the prognosis of patients with high ALC, which is a systemic index of immunity, was reported to be good (24). In addition, real world data of MBC patients treated with eribulin occasionally report NLR, ALC prognosis, and predictors of therapeutic efficacy (25, 26). In the present study, ALC value ≥1,500/μl was correlated with response to eribulin chemotherapy.

Conversely, with regards to the TME, excessive cell proliferation and insufficient oxygen supply due to long distances from blood vessels cause tumour hypoxia (43, 44), which induces EMT by activating the hypoxia inducible factor (45). Under hypoxia, immune cells that should attack cancer cells do not function adequately, resulting in an immunosuppressed state (46-48). Moreover, tumour hypoxia leads to overproduction of TGF-β and VEGF, which inhibit vascular remodelling that is a characteristic of normal angiogenesis. In this regard, several preclinical studies have reported that eribulin is correlated with the remodelling effect of tumour blood vessels (4, 5, 49, 50). Furthermore, in tissue specimens collected after eribulin treatment, TME improvement, such as reduced tumour hypoxia and EMT suppression, was observed in the responders (1). In addition, it has been reported that eribulin suppresses the development of new metastases through EMT suppression (28, 51). Eribulin also improves oxygen saturation in tumour tissues and significantly reduces plasma TGF-β concentration through vascular remodelling in MBC (5). In the same report, no change in TGF-β concentration was seen when patients were administered the VEGF inhibitor, bevacizumab. This effect on TME is likely the main mechanism by which eribulin prolongs OS. In the present study, changes in pSmad2 expression in tissue samples were evaluated after eribulin treatment, and decreased expression was observed in approximately 70% of patients. Thus, eribulin decreases TGF-β in the TME as well as in the plasma (5).

In our previous study, we reported that eribulin has a therapeutic effect in patients with high TIL concentrations, which are local immune indices in highly immunogenic TNBC (14). Similarly, several reports, including the EMBRACE study, have stated that ALC and NLR, which are indicators of a systemic tumour immune response, may be predictive factors of the prognosis and therapeutic effect of eribulin (24-26). The present results suggest that, if a patient shows good systemic and local immune status before eribulin treatment, a therapeutic effect can be expected. However, the effect of eribulin on tumour immunity itself remains unclear.

In our previous study, the eribulin-resistant TNBC cell line MDA-MB-231 showed lower CD274 (PD-L1) expression than the parental cell line (52). In tissue samples taken before and after eribulin treatment, decreased expression of PD-1, PD-L1, and FOXP3, as well as increased expression of CD8, were confirmed in responders. These results suggest that eribulin improves tumour immune response (2).

TGF-β is a cytokine that regulates the proliferation, differentiation, and function of a wide range of cell types, and has a wide range of biological activities, affecting glucose metabolism and fibrosis (7, 53). In addition, TGF-β definitively enhances immune homeostasis and tolerance, while inhibiting the expansion and function of many immune system components (54, 55). TGF-β is the centre of immunosuppression in the TIME (56), and local immune response can be monitored by confirming TGF-β expression before and after eribulin treatment. In the present study, we evaluated TGF-β expression by examining pSmad2 expression, which is located downstream of the TGF-β signal. We also evaluated TILs, which are a useful TIME monitoring index. In the surgical cohort of the present study, response was not correlated with TIL density. However, significantly more patients that became pSmad2 negative were responders. Furthermore, when a combination of local and systemic indices host immunity was used, pSmad2 (TGF-β) negative conversion was seen in many patients with an ALC ≥1,500 /μl before eribulin treatment (baseline), indicating that the patients responded to the treatment.

The present study had several limitations. For example, it included a small number of retrospective visual analyses and used the downstream signalling molecule pSmad2 to evaluate TGF-β. However, it was the first report to link the trends of systemic and local immune responses to eribulin. The TIME was also improved by eribulin, and clinical benefits leading to OS extension were obtained. Moreover, the evaluation could be performed using baseline ALC.

In patients with MBC, eribulin improved the tumour immune microenvironment by decreasing TGF-β expression. This demonstrated that local change can be evaluated based on ALC.

Conflicts of Interest

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

Authors' Contributions

All Authors were involved in the preparation of this manuscript. SK participated in the design of the study and drafted the manuscript. YA, WG, KT, TM, RK, AY, and TT helped with study data collection and manuscript preparation. ST and MOhs helped with pathological diagnosis. KH and MOhi conceived the study, and participated in its design and coordination and helped to draft the manuscript. All Authors have read and approved the final manuscript.

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