Prognostic significance of the expression levels of T-cell immunoglobulin mucin-3 and its ligand galectin-9 for relapse-free survival in triple-negative breast cancer

KATSUHIRO YOSHIKAWA[1,2], MITSUAKI ISHIDA[1], HIROTSUGU YANAI[2],
KOJI TSUTA[1], MITSUGU SEKIMOTO[2] and TOMOHARU SUGIE[2]

[1]Department of Pathology and Division of Diagnostic Pathology; [2]Department of Surgery,
Kansai Medical University, Hirakata, Osaka 573-1191, Japan

Abstract. T-cell immunoglobulin mucin-3 (TIM-3) expressed at the T-cell surface acts as an immune checkpoint when bound by its ligand galectin-9. Blockade of immunosuppression by the TIM3/galectin-9 signalling pathway may offer novel therapeutic approaches for cancer immunotherapy. Consistent with this, TIM-3 expression is associated with poorer prognosis in several different types of cancer, possibly as a result of suppression of anticancer immunosurveillance. A number of studies have now documented some effectiveness of immune checkpoint blockade even in triple-negative breast cancer (TNBC), which is highly aggressive. However, clinical responses are relatively weak, suggesting that several different pathways may be involved. In this context, the role of the TIM-3/galectin-9 checkpoint in TNBC is not clear. The present study aimed to determine the clinicopathological significance of TIM-3 and galectin-9 expression in this cancer. To this end, 62 patients with TNBC undergoing surgery at Kansai Medical University Hospital (Hirakata, Japan), but not given neoadjuvant chemotherapy, were examined. Tissue microarrays were employed for immunohistochemistry to analyse associations of TIM-3 and galectin-9 expression and their impact on relapse-free survival relative to other poor prognostic risk factors. Galectin-9 expression was detected in 49 of 62 patient samples (79%), and TIM-3 in 30 of them (48.4%). Tumour cell galectin-9 expression was associated with a more favourable prognosis (P=0.027) as was TIM-3 expression on tumour-infiltrating lymphocytes (P=0.007). Multivariate analysis indicated that galectin-9- and TIM-3-double-positivity was significantly associated with a more favourable prognosis compared with galectin-9 and/or TIM-3 negativity (P=0.044). Thus, the TIM-3/galectin-9 signalling pathway may impact anticancer immune reactions in the tumour microenvironment of patients with TNBC. Further investigation will be necessary to determine the molecular mechanisms underlying these relationships.

Introduction

Female breast cancer is a common malignancy globally (1). Triple-negative breast cancer (TNBC), making up 12-17% of cancers of the breast, is defined by the absence of receptors for oestrogen, progesterone, and human epidermal growth factor receptor 2 (HER2) on the tumour cells (2-4). TNBC is the most aggressive form of breast cancer, and rates of recurrence, distant metastasis, and mortality are significantly higher than for other types of breast cancer (2,3). Part of the reason for this may be a more limited range of treatment options for TNBC than for these other types of breast cancer.

Over the last decade, cancer immunotherapy has become established as a highly effective treatment modality for certain cancers (5). Immune checkpoint blockade (ICB) has achieved remarkable clinical results in some patients with malignant melanoma, renal cancer, non-small cell lung cancer, and other solid tumours (6). Currently, treatment with antibodies to programmed death-ligand 1 (PD-L1) or programmed death-1 (PD-1) is the mainstay of ICB, and has also been investigated for TNBC, with some degree of success. Thus, the IMpassion 130 trial (NCT02425891) used anti-PD-L1 (atezolizumab) together with nab-paclitaxel as first-line treatment for advanced or metastatic TNBC and reported that this combination was superior to nab-paclitaxel alone (7). Additionally, KEYNOTE-355 investigated the efficacy of anti-PD-1 (pembrolizumab) combined with chemotherapy (nab-paclitaxel; paclitaxel; or gemcitabine plus carboplatin) and reported increased progression-free survival of TNBC patients relative to chemotherapy alone (8). Nonetheless, only 20-58% of TNBC tumours express PD-L1, making it likely that many TNBC patients will not experience any clinical benefit from ICB directed to this molecule (9-15). Also, repetitive administration can result in resistance to ICB, as with chemotherapy (16). Hence, there is an urgent unmet medical need for novel treatment targets in TNBC.

Correspondence to: Dr Mitsuaki Ishida, Department of Pathology and Division of Diagnostic Pathology, Kansai Medical University, 2-3-1, Shinmachi, Hirakata, Osaka 573-1191, Japan
E-mail: ishidamt@hirakata.kmu.ac.jp

Potential ICB targets other than PD-L1 and PD-1 may also be considered for application in TNBC. One of these, immunoglobulin superfamily member, T-cell immunoglobulin mucin-3 (TIM-3), is a checkpoint molecule present on many different immune cells, including dendritic cells, macrophages, and T cells (17-20). TIM-3 mediates suppressive activity after binding a variety of different ligands, including phosphatidylserine, CEACAM-1, and galectin-9 (17,21,22). The latter is one of the family of h-galactoside-binding proteins which is over-expressed by many tumours; its binding to TIM-3 on T cell surface results in cytotoxic T cell suppression via an autocrine pathway (23-29). It has therefore been hypothesized that either or both TIM-3 and galectin-9 could represent novel therapeutic targets (30-32). However, the prognostic significance of these two molecules has not been unequivocally established, because their high expression has been reported to associate with either a better or worse prognosis, depending on the specific tumour entity (33-38). In the case of TNBC, TIM-3 or galectin-9 expression has been associated with certain clinicopathological features and with prognostic significance (34,38) but to the best of our knowledge, no studies to date have examined the relationship between TIM-3 in combination with one of its ligands, galectin-9. Therefore, we explored correlations between TIM-3 and galectin-9 expression in TNBC by immunohistochemistry, and investigated their impact on patient prognosis and clinicopathological features.

Materials and methods

Patient selection. Patients with TNBC undergoing surgical resection at the Department of Surgery of the Kansai Medical University Hospital between January 2006 and December 2018 were enrolled. Patients receiving neoadjuvant chemotherapy, known to influence TIM-3 and galectin-9 expression, were excluded. Inclusion criteria were invasive breast carcinoma of no special type according to the recent World Health Organization Classification of Breast Tumors (39), but those with special types were excluded because each of these has different clinicopathological features. Finally, 62 TNBC patients were included. This cohort is essentially identical to that described in our previous studies (40-43). To date, in this cohort, we have analysed associations between adipophilin expression and prognosis (40), as well as the prognostic impact of PD-L1 expression by cancer-associated fibroblasts (41), and relationships between PD-L1 and the expression of the immune checkpoint protein CD155 (42). We have also compared three different PD-L1 assays in patients with TNBC using immunohistochemistry (43). The focus of the current study was to determine the prognostic significance of TIM3 and galectin-9 expression in this same cohort of TNBC patients.

This is a retrospective single-centre study conducted in accordance with the principles of the Declaration of Helsinki. The study protocol was approved by the Institutional Review Board of Kansai Medical University Hospital (Approval #2019041). Because of the retrospective study design, informed consent was obtained using the opt-out method, there being no risk to the participants. Information on the study, including the inclusion criteria and the opportunity to opt-out, was made available on the institutional website (https://www.kmu.ac.jp/hirakata/hospital/2671t800000136cd-att/a1565060399005.pdf).

Histopathology. All histopathological diagnoses were independently evaluated by at least two experienced diagnostic pathologists, using the TNM Classification of Malignant Tumors, 8th Ed. Grading followed the Nottingham scale (44). Dichotomization of the Ki-67 labelling index (LI) was set as high at ≥40% and low at <40%, following a meta-analysis of patients with TNBC (45). Stromal tumour-infiltrating lymphocytes (TILs) were identified using haematoxylin and eosin staining and were considered lymphocyte-predominant breast cancer (LPBC) at ≥60% and non-LPBC at <59%, according to TIL Working Group guidelines (46,47).

Tissue microarrays. Regions most morphologically representative of carcinoma were selected by H&E staining of the slides, and for every patient, three 2 mm-diameter tissue cores were punched out of the paraffin-embedded blocks.

Immunohistochemistry. Immunohistochemistry used the Discovery ULTRA System (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. Antibodies were as follows: TIM-3 (rabbit monoclonal antibody, D5D5R, Cell Signaling Technology, Danvers, MA, USA; diluted 1:200); galectin-9 (mouse monoclonal antibody, ab153673, 1G3, Abcam plc, Cambridge, UK; diluted 1:200). At least of two researchers independently evaluated the immunohistochemistry results. TIM-3-positivity was defined as membrane staining of any intensity on ≥1% of TILs (36). Galectin-9-positivity was defined as membrane staining of any intensity on ≥1% of tumour cells (48). The patient was classified as having TIM-3- or galectin-9-positive tumour when one or more cores from the same individual were positive according to this definition.

Statistical analysis. We used SPSS Statistics 27.0 (IBM, Armonk, NY, USA) for all analyses. Correlations between two groups were calculated using Fisher's exact test for categorical variables and Mann-Whitney U testing for continuous variables. Relapse-free survival (RFS) was determined using the Kaplan-Meier method, with log-rank testing. Cox proportional hazards modelling was used to estimate relationships between clinicopathological parameters and survival. Statistical significance was set at $P < 0.05$.

Results

Patients. The cohort of 62 women with TNBC studied here is the same as described earlier (43). Their clinicopathological characteristics were presented in the previous publication (43). Briefly, median age at initial diagnosis was 68 years (range, 31-93); the diagnosis of TNBC relied on biopsy results. Patients with invasive carcinoma of no special type were selected (see Materials and Methods). Median tumour diameter was 21 mm (range, 2-55 mm). Median follow-up was 58 months (range, 11-173 months). Eleven (17.7%) patients relapsed, all with distant metastases. There were no local recurrences. Nine patients (14.5%) died of their disease.

Correlations between galectin-9 or TIM-3 expression and clinicopathological factors. Of the 62 patients, 49 (79.0%) were classified as galectin-9-positive (Fig. 1). Table I

Table I. Association between clinicopathological factors and galectin-9 expression.

Factors	Galectin-9-positive (n=49)	Galectin-9-negative (n=13)	P-value
Median age ± SD, years	64±15	72±13	0.115
Menopausal status, n			
Premenopausal	9	0	0.184
Postmenopausal	39	13	
Unknown	1	0	
Tumour size, n			
≤20 mm	26	5	0.534
>20 mm	23	8	
Pathological stage, n			
I+II	45	9	0.052
III	4	4	
Lymph node status, n			
Positive	9	7	0.075
Negative	26	5	
Not tested	14	1	
Lymphatic invasion, n			
Positive	41	12	0.670
Negative	8	1	
Venous invasion, n			
Positive	27	10	0.210
Negative	22	3	
Nottingham histological grade, n			
1+2	22	8	0.357
3	27	5	
Ki-67 labeling index, n			
High (≥40%)	28	9	0.506
Low (<40%)	18	3	
Not tested	3	1	
Stromal TILs, n			
LPBC	16	3	0.737
Non-LPBC	33	10	
Adjuvant chemotherapy, n			
Performed	31	3	0.040
Not performed	17	8	
Undetermined	1	2	

LPBC, lymphocyte predominant breast cancer; TILs, tumour-infiltrating lymphocytes.

presents associations between galectin-9-positivity and clinicopathological factors. The use of adjuvant chemotherapy was associated with galectin-9 expression (P=0.040), but not with any other factors, including age and menopausal status. There were also no associations between galectin-9-positivity and the clinicopathological factors staging, Nottingham histological grade, lymph node status, lymphovascular invasion, Ki-67 LI, or stromal TILs.

Regarding TIM-3 expression, 30 patients (48.4%) were classified as TIM-3-positive (Fig. 2). Table II depicts associations between TIM-3 expression and clinicopathological factors in this cohort. Larger tumour size was associated with TIM-3-negativity (P<0.001), whereas LPBC correlated with TIM-3-positivity (P=0.013). However, there were no associations between TIM-3 expression and the clinicopathological factors, including age, menopausal status, administration of adjuvant chemotherapy, staging, Nottingham histological grade, lymph node status, lymphovascular invasion, or Ki-67 LI.

Correlations between galectin-9 or TIM-3 expression and relapse-free survival after surgery. RFS was superior for galectin-9-positive relative to -negative patients (60-vs.-44 months, P=0.027) as shown in Fig. 3A. For
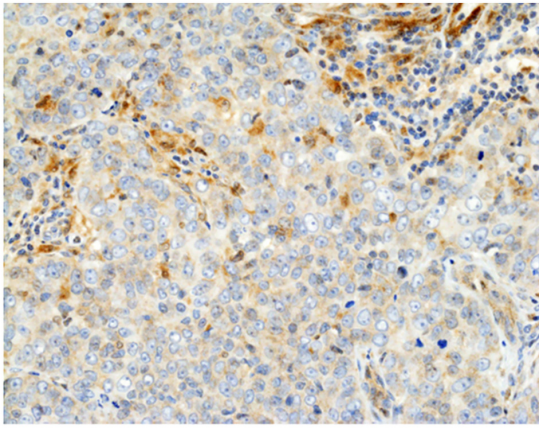
Figure 1. Immunohistochemical staining for galectin-9. Positive staining is seen for the neoplastic cells (magnification, x400).
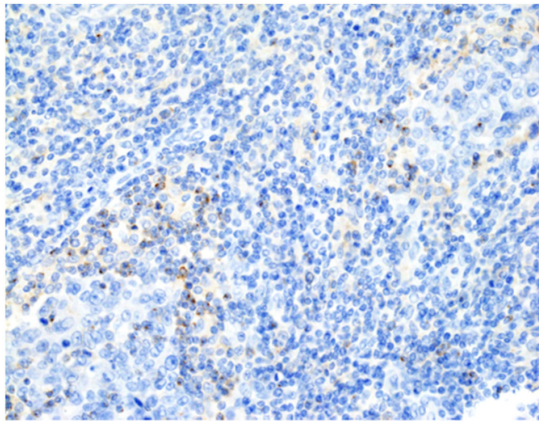

Figure 2. Immunohistochemical staining for T-cell immunoglobulin mucin-3. Positive staining is seen for lymphocytes (magnification, x400).

TIM-3-positive-vs.-negative patients, these values were 63 and 54 months (Fig. 3B, P=0.007).

Impact of positivity for both galectin-9 and TIM-3 on clinicopathological features. Correlations between galectin-9- and TIM-3-positivity are shown in Table III, indicating a lack of association between galectin-9 and TIM-3 expression (P=0.548). Eight patients (12.9%) were both galectin-9- and TIM-3-negative (double negative), and 25 (40.3%) were positive for both (double-positive). The remaining 29 patients were either galectin-9- or TIM-3-single-positive.

Table IV summarizes correlations between the galectin-9/TIM-3 double-negative group and clinicopathological factors in the present cohort. Only larger tumour size and higher Ki-67 LI correlated with double-negative status (P=0.029 and 0.020, respectively) but there were no associations with age, menopausal status, presence of adjuvant chemotherapy, staging, Nottingham histological grade, lymph node status, lymphovascular invasion, or stromal TILs.

Correlation between galectin-9 and TIM-3 combined expression and relapse-free survival after surgery. Fig. 3C shows RFS for double-negative, double-positive or single-positive patients.
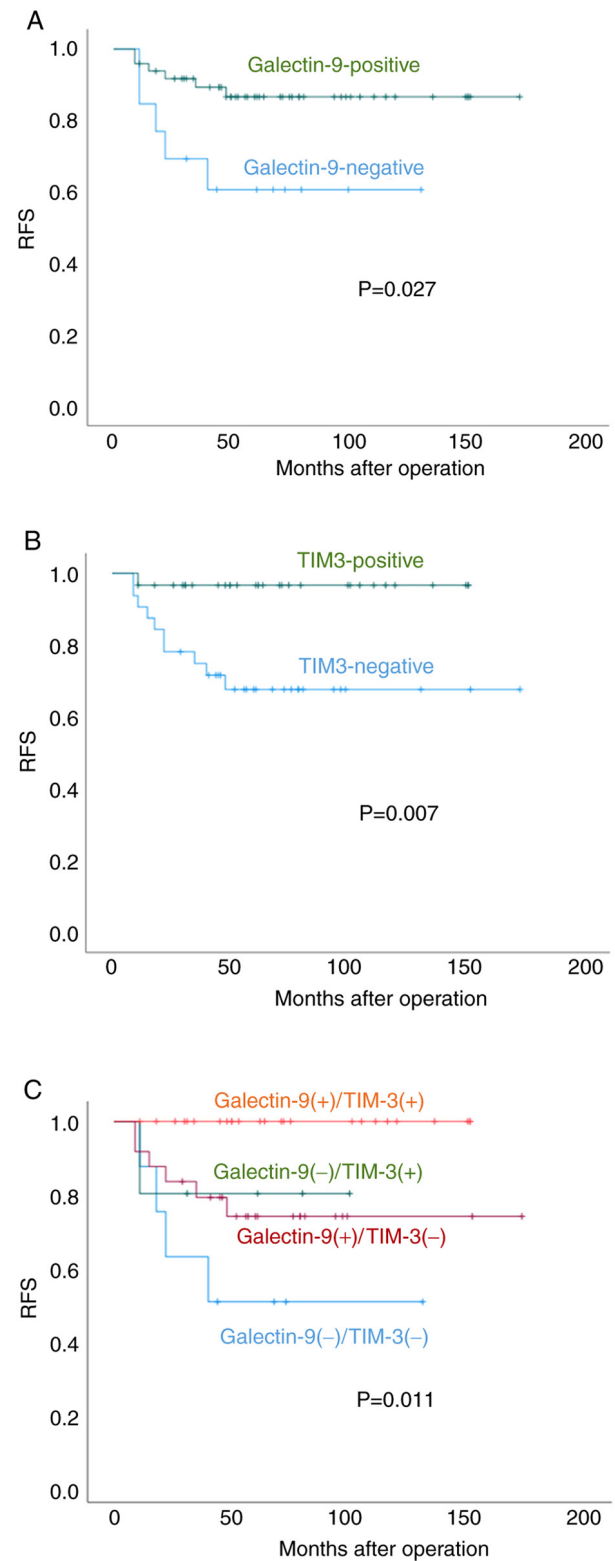

Figure 3. RFS of patients with triple-negative breast cancer. (A) RFS of galectin-9-positive (green) or -negative (blue) patients. (B) RFS of TIM-3-positive (green) or -negative (blue) patients. (C) RFS of galectin-9(+)/TIM-3(+) (double positive) (orange), galectin-9(-)/TIM-3(+) (green), galectin-9(+)/TIM-3(-) (red) and galectin-9(-)/TIM-3(-) (double negative) (blue) patients. RFS, relapse-free survival; TIM-3, T-cell immunoglobulin mucin-3.

The median RFS time of galectin-9/TIM-3-double-positive, galectin-9-positive/TIM-3-negative, galectin-9-nega-tive/TIM-3-positive, and galectin-9/TIM3-double negative

Table II. Association between clinicopathological factors and TIM-3 expression.

Factors	TIM-3-positive (n=30)	TIM-3-negative (n=32)	P-value
Median age ± SD, years	63±15	67±14	0.313
Menopausal status, n			
Premenopausal	5	4	0.724
Postmenopausal	24	28	
Unknown	1	0	
Tumour size, n			
≤20 mm	22	9	<0.001
>20 mm	8	23	
Pathological stage, n			
I+II	29	25	0.054
III	1	7	
Lymph node status, n			
Positive	6	8	0.752
Negative	17	16	
Not tested	7	8	
Lymphatic invasion, n			
Positive	23	30	0.077
Negative	7	2	
Venous invasion, n			
Positive	15	22	0.195
Negative	15	10	
Nottingham histological grade, n			
1+2	15	15	>0.999
3	15	17	
Ki-67 labeling index, n			
High (≥40%)	16	9	0.062
Low (<40%)	12	21	
Not tested	2	2	
Stromal TILs, n			
LPBC	14	5	0.013
Non-LPBC	16	27	
Adjuvant chemotherapy, n			
Performed	19	15	0.295
Not performed	10	15	
Undetermined	1	2	

LPBC, lymphocyte predominant breast cancer; TILs, tumour-infiltrating lymphocytes; TIM-3, T-cell immunoglobulin mucin-3.

Table III. Association between galectin-9 and TIM-3 expression.

	TIM-3	
Galectin-9	Positive, n	Negative, n
Positive	25	24
Negative	5	8

P=0.548. TIM-3, T-cell immunoglobulin mucin-3.

patients was 64, 57, 61, and 42 months, respectively. Thus, double-positive patients had a better prognosis, and double-negative patients had the worst prognosis (P=0.011).

Prognostic significance of galectin-9 and TIM-3 expression. According to univariate analysis, the presence of lymph node metastasis (P=0.004), galectin-9 negativity (P=0.039), TIM-3 negativity (P=0.029), and galectin-9/TIM-3 double-negativity (P=0.020) were each significantly correlated with poor RFS (Table V). Multivariate Cox proportional hazards analyses showed that galectin-9/TIM-3 double-negativity was an

Table IV. Association between clinicopathological factors and galectin-9 and TIM-3 expression.

Factors	Galectin-9 and TIM-3-negative (n=8)	Galectin-9 and/or TIM-3-positive (n=54)	P-value
Median age ± SD, years	72±10	64±15	0.166
Menopausal status, n			
Premenopausal	0	9	0.590
Postmenopausal	8	44	
Unknown	0	1	
Tumour size, n			
≤20 mm	1	30	0.029
>20 mm	7	24	
Pathological stage, n			
I+II	5	49	0.059
III	3	5	
Lymph node status, n			
Positive	3	11	0.670
Negative	5	28	
Not tested	0	15	
Lymphatic invasion, n			
Positive	8	45	0.580
Negative	0	9	
Venous invasion, n			
Positive	7	30	0.128
Negative	1	24	
Nottingham histological grade, n			
1+2	4	26	>0.999
3	4	28	
Ki-67 labeling index, n			
High (≥40%)	7	30	0.020
Low (<40%)	1	20	
Not tested	0	4	
Stromal TILs, n			
LPBC	1	18	0.416
Non-LPBC	7	36	
Adjuvant chemotherapy, n			
Performed	3	31	0.691
Not performed	3	22	
Undetermined	2	1	

LPBC, lymphocyte predominant breast cancer; TILs, tumour-infiltrating lymphocytes; TIM-3, T-cell immunoglobulin mucin-3.

independent predictor of poor prognosis [hazard ratio (HR) 3.627, 95% confidence interval (CI) 1.037-12.68; P=0.044] (Table V). Additionally, lymph node metastasis was an independent risk factor for poor RFS (HR 5.925, 95% CI 1.555-22.58, P=0.009).

Discussion

Recently, the importance of TIM-3 in cancer immunology has been increasingly recognized due to its role as a checkpoint receptor inhibiting cytotoxic T cells (30-32). A previous meta-analysis implicated TIM-3 expression as an independent risk factor predicting poor overall survival (OS), but not cancer-specific and disease-free survival, in different malignant tumours (49). It was hypothesized that interactions of TIM-3 with its ligands, including galectin-9, results in the inhibition of both T cell responses and natural killer cell-mediated tumour cell cytotoxicity, resulting in the dampening of anti-tumour immunity and thence tumour escape (17). TIM-3 is believed to be expressed by exhausted T cells, the presence of which is associated with poor prognosis in several different cancers (49,50). In contrast, as mentioned above, TIM-3-positivity was

Table V. Univariate and multivariate analyses of relapse-free survival of patients with triple-negative breast cancer.

Factor	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
Tumor size (>20 vs. ≤20 mm)	2.660	0.706-10.03	0.148			
Lymph node status (positive vs. negative)	6.891	1.825-26.02	0.004	5.925	1.555-22.58	0.009
Nottingham histological grade (3 vs. 1+2)	1.829	0.535-6.256	0.336			
Ki-67 labeling index (high vs. low)	1.497	0.387-5.793	0.559			
Stromal TILs (LPBC vs. non-LPBC)	0.470	0.101-2.175	0.334			
Adjuvant chemotherapy (performed vs. not performed)	0.358	0.104-1.225	0.102			
Galectin-9 (negative vs. positive)	3.508	1.068-11.52	0.039	2.736	0.809-9.253	0.106
TIM-3 (negative vs. positive)	9.888	1.265-77.27	0.029	7.141	0.905-56.33	0.062
Galectin-9 and TIM-3 (double negative vs. others)	4.321	1.260-14.82	0.020	3.627	1.037-12.68	0.044

95% CI, 95% confidence interval; HR, hazard ratio; LPBC, lymphocyte predominant breast cancer; TILs, tumour-infiltrating lymphocytes; TIM-3, T-cell immunoglobulin mucin-3.

Table VI. Summary of the relationship between TIM-3 expression and prognosis of patients with triple-negative breast cancer.

First author/s, year	Patients, n	Prognosis	(Refs.)
Cabioglu et al, 2021	61	No prognostic significance was noted (using operative specimens after neoadjuvant chemotherapy), although TIM-3 expression was associated with a worse chemotherapy response	(36)
Burugu et al, 2018	387	TIM-3 expression was associated with good disease-free and overall survival	(37)
Byun et al, 2018	109	TIM-3 expression was associated with good cancer-specific survival	(38)
Present study	62	TIM-3 expression was associated with good relapse-free survival	-

TIM-3, T-cell immunoglobulin mucin-3.

associated with more favourable OS in patients with TNBC (38,49), although this conclusion is based only three studies which investigated whether TIM-3 expression predicted prognosis in TNBC. Table VI presents details of these previous studies, together with the current study (36-38). According to our results, TIM-3 expression on TILs from TNBC correlates with a more favourable prognosis. The reason for differences in the prognostic relevance of TIM-3 expression for TNBC as opposed to other cancer entities remains to be established. In this context, Burugu et al (37) suspected that it might reflect a more potent recognition of cancer cells by the immune system. The immune response of TILs expressing TIM-3 to tumour cells might be different in the tumour microenvironment of TNBC compared to that of other cancers. Therefore, additional studies examining the molecular mechanisms underlying the immune response of TILs expressing TIM-3 to carcinoma cells in TNBC are needed in order to explain this difference.

Additionally, some other associations between clinicopathological features of TIM-3 expression in TNBC patients have been reported by other investigators. One study included 30 TNBC patients, reporting that TIM-3 expression by TILs correlated significantly with the presence of lymph node metastasis and a higher Ki-67 LI, but its prognostic significance was not discussed (51). It may be important to note that

TIM-3 expression is more frequent in TNBC than in other forms of breast cancer (51,52) and is also associated with higher PD-L1/PD-1 expression (37,38). Furthermore, consistent with the results presented here, it has also been noted that TIM-3-positivity is associated with the presence of abundant TILs (38).

Here, we have also demonstrated that negativity for the TIM-3 ligand galectin-9 is a poor prognostic factor, but this association lost significance in the multivariate analysis. However, we did find that TIM-3/galectin-9-double-negativity remains significantly predictive of poor prognosis in such a multivariate analysis. Galectin-9 on tumour cells is also a key protein that negatively regulates T cell function, leading to suppression of anti-cancer immune surveillance (21,26,27,53). Using breast cancer cell lines it was found that galectin-9 expression was associated with the suppression of anti-cancer immune surveillance (53). However, similar to our findings, some studies reported that galectin-9 expression predicts a favourable prognosis in breast cancer (34,54). Nonetheless, it must be recognized that there is a discrepancy between the generally reported immunosuppressive function of galectin-9 and its opposite prognostic significance in TNBC. Although galectin-9 ligation of TIM-3 pathway induces dysfunction of TILs [for example, in hepatocellular carcinoma (55)], here we found that it was the double-negativity for

TIM-3 and galectin-9 that predicted a poor prognosis whereas positivity for both was associated with a more favourable course. The functional role of galectin-9 and TIM-3 in anti-tumour responses might be different in TNBC than in some other types of cancer. It is clear that the TIM-3/galectin-9 pathway can suppress cytotoxic T cells and NK cells and protect the tumour (16), but it is also known that the presence of galectin-9 on breast cancer cells increases the strength of cell-cell interactions. This could thus prevent metastasis or at least reduce the metastatic potential of the tumour (56). As such, the outcome might be more favourable when breast tumour cells express galectin-9. To resolve this issue, additional molecular studies are needed, especially for TIM-3/galectin-9 double-negativity. Better understanding of the oncoimmunology of TNBC will hopefully lead to improved prognosis.

Some limitations of the present study must be recognized, including a relatively small sample size. Thus, additional studies with a larger number of participants must be performed. Second, this study used tissue microarrays to evaluate immunohistochemical staining for TIM-3 and galectin-9. This might have led to a bias in evaluating the expression of these proteins, despite the fact that we selected the most morphologically representative regions from the patients. Third, TIM-3 can be glycosylated, and glycosylated TIM-3 has a weaker ability to bind galectin-9 (57). Moreover, galectin-9 has three isoforms (58). This study analysed TIM-3 and galectin-9 expression by immunohistochemical methods using monoclonal antibodies which may have different specificities. Thus, the galectin-9 antibody used in this study reacts with all three isoforms of galectin-9 (59), so positivity for galectin-9 may reflect different isoforms of galectin-9. Although this method is versatile, further studies using Western blotting conducted on fresh tumour samples could provide further information on the roles of glycosylated TIM-3 and/or the different galectin-9 isoforms. Fourth, chemotherapy and/or ICB might influence the expression of TIM-3 and/or galectin-9. Anthracycline and taxane upregulate galectin-9 expression in some types of cancer cells (60)-although there were no patients receiving neoadjuvant chemotherapy in the present study. Nonetheless, additional studies should determine whether these drugs influence the expression of TIM-3 and/or galectin-9 in TNBC.

In conclusion, this study documented that TIM-3 or galectin-9 positivity predicts a more favourable prognosis in TNBC patients, in particular when the TILs are TIM-3-positive and the tumour is galectin-9-positive. Generally, the TIM-3/galectin-9 pathway is thought to suppress anti-cancer immunosurveillance, but here we reveal a positive influence on TNBC prognosis. However, the molecular mechanisms underlying the difference between TNBC and other cancers in this respect remain unclear and further analyses are needed to resolve this issue. This could contribute to improved therapy for patients with TNBC.

Acknowledgements

Not applicable.

Funding

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Authors' contributions

KY and MI conceived and designed the study. KY and MI were involved in immunohistochemical analyses. KY, MI, HY, KT, MS and TS acquired and analysed data. KY and MI drafted the manuscript, tables and figures. KY and MI confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was conducted in accordance with the Declaration of Helsinki, and the study protocol was approved by the Institutional Review Board of the Kansai Medical University Hospital (protocol no. 2019041; Hirakata, Japan). All data are completely anonymized. The Institutional Review Board waived the requirement of informed consent due to the retrospective design of the study, with no risk of identity exposure for the patients. The present study did not include any minors.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 68: 394-424, 2018.
2. Cleator S, Heller W and Coombes RC: Triple-negative breast cancer: Therapeutic options. Lancet Oncol 8: 235-244, 2007.
3. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, Lickley LA, Rawlinson E, Sun P and Narod SA: Triple-negative breast cancer: Clinical features and patterns of recurrence. Clin Cancer Res 13: 4429-4434, 2007.
4. Foulkes WD, Smith IE and Reis-Filho JS: Triple-negative breast cancer. N Engl J Med 363: 1938-1948, 2010.
5. Kakimi K, Karasaki T, Matsushita H and Sugie T: Advances in personalized cancer immunotherapy. Breast Cancer 24: 16-24, 2017.
6. Sharma P and Allison JP: The future of immune checkpoint therapy. Science 348: 56-61, 2015.
7. Schmid P, Adams S, Rugo HS, Schneeweiss A, Barrios CH, Iwata H, Diéras V, Hegg R, Im SA, Shaw Wright G, *et al*: Atezolizumab and nab-paclitaxel in advanced triple-negative breast cancer. N Engl J Med 379: 2108-2121, 2018.
8. Cortes J, Cescon DW, Rugo HS, Nowecki Z, Im SA, Yusof MM, Gallardo C, Lipatov O, Barrios CH, Holgado E, *et al*: Pembrolizumab plus chemotherapy versus placebo plus chemotherapy for previously untreated locally recurrent inoperable or metastatic triple-negative breast cancer (KEYNOTE-355): A randomised, placebo-controlled, double-blind, phase 3 clinical trial. Lancet 396: 1817-1828, 2020.

9. Mittendorf EA, Philips AV, Meric-Bernstam F, Qiao N, Wu Y, Harrington S, Su X, Wang Y, Gonzalez-Angulo AM, Akcakanat A, et al: PD-L1 expression in triple-negative breast cancer. Cancer Immunol Res 2: 361-370, 2014.

10. Tung N, Garber JE, Hacker MR, Torous V, Freeman GJ, Poles E, Rodig S, Alexander B, Lee L, Collins LC and Schnitt SJ: Prevalence and predictors of androgen receptor and programmed death-ligand 1 in BRCA1-associated and sporadic triple-negative breast cancer. NPJ Breast Cancer 2: 16002, 2016.

11. Ali HR, Glont SE, Blows FM, Provenzano E, Dawson SJ, Liu B, Hiller L, Dunn J, Poole CJ, Bowden S, et al: PD-L1 protein expression in breast cancer is rare, enriched in basal-like tumours and associated with infiltrating lymphocytes. Ann Oncol 26: 1488-1493, 2015.

12. Wang C, Zhu H, Zhou Y, Mao F, Lin Y, Pan B, Zhang X, Xu Q, Huang X and Sun Q: Prognostic value of PD-L1 in breast cancer: A meta-analysis. Breast J 23: 436-443, 2017.

13. Dill EA, Gru AA, Atkins KA, Friedman LA, Moore ME, Bullock TN, Cross JV, Dillon PM and Mills AM: PD-L1 expression and intratumoral heterogeneity across breast cancer subtypes and stages: An assessment of 245 primary and 40 metastatic tumors. Am J Surg Pathol 41: 334-342, 2017.

14. Mori H, Kubo M, Yamaguchi R, Nishimura R, Osako T, Arima N, Okumura Y, Okido M, Yamada M, Kai M, et al: The combination of PD-L1 expression and decreased tumor-infiltrating lymphocytes is associated with a poor prognosis in triple-negative breast cancer. Oncotarget 8: 15584-15592, 2017.

15. Li Z, Dong P, Ren M, Song Y, Qian X, Yang Y, Li S, Zhang X and Liu F: PD-L1 expression is associated with tumor FOXP3 (+) regulatory T-cell infiltration of breast cancer and poor prognosis of patient. J Cancer 7: 784-793, 2016.

16. Gong B, Kiyotani K, Sakata S, Nagano S, Kumehara S, Baba S, Besse B, Yanagitani N, Friboulet L, Nishio M, et al: Secreted PD-L1 variants mediate resistance to PD-L1 blockade therapy in non-small cell lung cancer. J Exp Med 216: 982-1000, 2019.

17. Wolf Y, Anderson AC and Kuchroo VK: TIM3 comes of age as an inhibitory receptor. Nat Rev Immunol 20: 173-185, 2020.

18. Monney L, Sabatos CA, Gaglia JL, Ryu A, Waldner H, Chernova T, Manning S, Greenfield EA, Coyle AJ, Sobel RA, et al: Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. Nature 415: 536-541, 2002.

19. De Mingo Pulido A, Gardner A, Hiebler S, Soliman H, Rugo HS, Krummel MF, Coussens LM and Ruffell B: TIM-3 regulates CD103⁺ dendritic cell function and response to chemotherapy in breast cancer. Cancer Cell 33: 60-74.e6, 2018.

20. Yan W, Liu X, Ma H, Zhang H, Song X, Gao L, Liang X and Ma C: Tim-3 fosters HCC development by enhancing TGF-β-mediated alternative activation of macrophages. Gut 64: 1593-1604, 2015.

21. Zhu C, Anderson AC, Schubart A, Xiong H, Imitola J, Khoury SJ, Zheng XX, Strom TB and Kuchroo VK: The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. Nat Immunol 6: 1245-1252, 2005.

22. Sabatos-Peyton CA, Nevin J, Brock A, Venable JD, Tan DJ, Kassam N, Xu F, Taraszka J, Wesemann L, Pertel T, et al: Blockade of Tim-3 binding to phosphatidylserine and CEACAM1 is a shared feature of anti-Tim-3 antibodies that have functional efficacy. Oncoimmunology 7: e1385690, 2018.

23. Gitt MA and Barondes SH: Evidence that a human soluble beta-galactoside-binding lectin is encoded by a family of genes. Proc Natl Acad Sci USA 83: 7603-7607, 1986.

24. Paroutaud P, Levi G, Teichberg VI and Strosberg AD: Extensive amino acid sequence homologies between animal lectins. Proc Natl Acad Sci USA 84: 6345-6348, 1987.

25. Caron M, Bladier D and Joubert R: Soluble galactoside-binding vertebrate lectins: A protein family with common properties. Int J Biochem 22: 1379-1385, 1990.

26. Kikushige Y, Miyamoto T, Yuda J, Jabbarzadeh-Tabrizi S, Shima T, Takayanagi S, Niiro H, Yurino A, Miyawaki K, Takenaka K, et al: A TIM-3/Gal-9 autocrine stimulatory loop drives self-renewal of human myeloid leukemia stem cells and leukemic progression. Cell Stem Cell 17: 341-352, 2015.

27. Gonçalves Silva I, Yasinska IM, Sakhnevych SS, Fiedler W, Wellbrock J, Bardelli M, Varani L, Hussain R, Siligardi G, Ceccone G, et al: The Tim-3-galectin-9 secretory pathway is involved in the immune escape of human acute myeloid leukemia cells. EBioMedicine 22: 44-57, 2017.

28. Sakhnevych SS, Yasinska IM, Bratt AM, Benlaouer O, Gonçalves Silva I, Hussain R, Siligardi G, Fiedler W, Wellbrock J, Gibbs BF, et al: Cortisol facilitates the immune escape of human acute myeloid leukemia cells by inducing latrophilin 1 expression. Cell Mol Immunol 15: 994-997, 2018.

29. Gonçalves Silva I, Rüegg L, Gibbs BF, Bardelli M, Fruehwirth A, Varani L, Berger SM, Fasler-Kan E and Sumbayev VV: The immune receptor Tim-3 acts as a trafficker in a Tim-3/galectin-9 autocrine loop in human myeloid leukaemia cells. Oncoimmunology 5: e1195535, 2016.

30. Sharma P, Hu-Lieskovan S, Wargo JA and Ribas A: Primary adaptive and acquired resistance to cancer immunotherapy. Cell 168: 707-723, 2017.

31. Yang R, Sun L, Li CF, Wang YH, Yao J, Li H, Yan M, Chang WC, Hsu JM, Cha JH, et al: Galectin-9 interacts with PD-1 and TIM-3 to regulate T cell death and is a target for cancer immunotherapy. Nat Commun 12: 832, 2021.

32. Cong Y, Liu J, Chen G and Qiao G: The emerging role of T-cell immunoglobulin Mucin-3 in breast cancer: A promising target for immunotherapy. Front Oncol 11: 723238, 2021.

33. Jikuya R, Kishida T, Sakaguchi M, Yokose T, Yasui M, Hashizume A, Tatenuma T, Mizuno N, Muraoka K, Umemoto S, et al: Galectin-9 expression as a poor prognostic factor in patients with renal cell carcinoma. Cancer Immunol Immunother 69: 2041-205, 2015.

34. Irie A, Yamauchi A, Kontani K, Kihara M, Liu D, Shirato Y, Seki M, Nishi N, Nakamura T, Yokomise H, et al: Galectin-9 as a prognostic factor with antimetastatic potential in breast cancer. Clin Cancer Res 11: 2962-2968, 2005.

35. Chen H, Wang M, Weng T, Wei Y, Liu C, Yang L, Ren K, Tang Y, Tang Z and Gou X: The prognostic and clinicopathological significance of Tim-3 and PD-1 expression in the prognosis of upper urinary tract urothelial carcinoma. Urol Oncol 39: 743-753, 2021.

36. Cabioglu N, Onder S, Oner G, Karatay H, Tukenmez M, Muslumanoglu M, İgci A, Eralp Y, Aydiner A, Saip P, et al: TIM3 expression on TILs is associated with poor response to neoadjuvant chemotherapy in patients with locally advanced triple-negative breast cancer. BMC Cancer 21: 357, 2021.

37. Burugu S, Gao D, Leung S, Chia SK and Nielsen TO: TIM-3 expression in breast cancer. Oncoimmunology 7: e1502128, 2018.

38. Byun KD, Hwang HJ, Park KJ, Kim MC, Cho SH, Ju MH, Lee JH and Jeong JS: T-cell immunoglobulin mucin 3 expression on tumor infiltrating lymphocytes as a positive prognosticator in triple-negative breast cancer. J Breast Cancer 21: 406-414, 2018.

39. Rakha EA, Allison KH, Bu H, Ellis IO, Foschini MP, Horii R, et al: Invasive breast carcinoma of no special type. In: WHO classification of tumours: Breast tumours. 5th edition. Volume 2. IARC, Lyon, pp102-109, 2019.

40. Yoshikawa K, Ishida M, Yanai H, Tsuta K, Sekimoto M and Sugie T: Adipophilin expression is an independent marker for poor prognosis of patients with triple-negative breast cancer: An immunohistochemical study. PLoS One 15: e0242563, 2020.

41. Yoshikawa K, Ishida M, Yanai H, Tsuta K, Sekimoto M and Sugie T: Prognostic significance of PD-L1-positive cancer-associated fibroblasts in patients with triple-negative breast cancer. BMC Cancer 21: 239, 2021.

42. Yoshikawa K, Ishida M, Yanai H, Tsuta K, Sekimoto M and Sugie T: Immunohistochemical analysis of CD155 expression in triple-negative breast cancer patients. PLoS One 16: e0253176, 2021.

43. Yoshikawa K, Ishida M, Yanai H, Tsuta K, Sekimoto M and Sugie T: Immunohistochemical comparison of three programmed death-ligand 1 (PD-L1) assays in triple-negative breast cancer. PLoS One 16: e0257860, 2021.

44. Elston CW and Ellis IO: Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: Experience from a large study with long-term follow-up. Histopathology 19: 403-410, 1991.

45. Wu Q, Ma G, Deng Y, Luo W, Zhao Y, Li W and Zhou Q: Prognostic value of Ki-67 in patients with resected triple-negative breast cancer: A meta-analysis. Front Oncol 9: 1068, 2019.

46. Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, Wienert S, Van den Eynden G, Baehner FL, Penault-Llorca F, et al: The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: Recommendations by an International TILs Working Group 2014. Ann Oncol 26: 259-271, 2015.

47. Denkert C, von Minckwitz G, Darb-Esfahani S, Lederer B, Heppner BI, Weber KE, Budczies J, Huober J, Klauschen F, Furlanetto J, et al: Tumour-infiltrating lymphocytes and prognosis in different subtypes of breast cancer: A pooled analysis of 3771 patients treated with neoadjuvant therapy. Lancet Oncol 19: 40-50, 2018.

48. Sideras K, Biermann K, Verheij J, Takkenberg BR, Mancham S, Hansen BE, Schutz HM, de Man RA, Sprengers D, Buschow SI, *et al*: PD-L1, galectin-9 and CD8$^+$ tumor-infiltrating lymphocytes are associated with survival in hepatocellular carcinoma. Oncoimmunology 6: e1273309, 2017.
49. Zang K, Hui L, Wang M, Huang Y, Zhu X and Yao B: TIM-3 as a Prognostic marker and a potential immunotherapy target in human malignant tumors: A meta-analysis and bioinformatics validation. Front Oncol 11: 579351, 2021.
50. Saleh R, Toor SM and Elkord E: Targeting TIM-3 in solid tumors: Innovations in the preclinical and translational realm and therapeutic potential. Expert Opin Ther Targets 24: 1251-1262, 2020.
51. Zhang H, Xiang R, Wu B, Li J and Luo G: T-cell immunoglobulin mucin-3 expression in invasive ductal breast carcinoma: Clinicopathological correlations and association with tumor infiltration by cytotoxic lymphocytes. Mol Clin Oncol 7: 557-563, 2017.
52. Solinas C, Garaud S, De Silva P, Boisson A, Van den Eynden G, de Wind A, Risso P, Rodrigues Vitória J, Richard F, Migliori E, *et al*: Immune checkpoint molecules on tumor-infiltrating lymphocytes and their association with tertiary lymphoid structures in human breast cancer. Front Immunol 8: 1412, 2017.
53. Yasinska IM, Sakhnevych SS, Pavlova L, Teo Hansen Selnø A, Teuscher Abeleira AM, Benlaouer O, Gonçalves Silva I, Mosimann M, Varani L, Bardelli M, *et al*: The Tim-3-Galectin-9 pathway and its regulatory mechanisms in human breast cancer. Front Immunol 10: 1594, 2019.
54. Yamauchi A, Kontani K, Kihara M, Nishi N, Yokomise H and Hirashima M: Galectin-9, a novel prognostic factor with antimetastatic potential in breast cancer. Breast J 12 (5 Suppl 2): S196-S200, 2006.
55. Li H, Wu K, Tao K, Chen L, Zheng Q, Lu X, Liu J, Shi L, Liu C, Wang G and Zou W: Tim-3/galectin-9 signaling pathway mediates T-cell dysfunction and predicts poor prognosis in patients with hepatitis B virus-associated hepatocellular carcinoma. Hepatology 56: 1342-1351, 2012.
56. Zhang ZY, Dong JH, Chen YW, Wang XQ, Li CH, Wang J, Wang GQ, Li HL and Wang XD: Galectin-9 acts as a prognostic factor with antimetastatic potential in hepatocellular carcinoma. Asian Pac J Cancer Prev 13: 2503-2509, 2012.
57. Lee MJ, Heo YM, Hong SH, Kim K and Park S: The binding properties of glycosylated and non-glycosylated Tim-3 molecules on CD4CD25 T cells. Immune Netw 9: 58-63, 2009.
58. Sato M, Nishi N, Shoji H, Seki M, Hashidate T, Hirabayashi J, Kasai Ki K, Hata Y, Suzuki S, Hirashima M and Nakamura T: Functional analysis of the carbohydrate recognition domains and a linker peptide of galectin-9 as to eosinophil chemoattractant activity. Glycobiology 12: 191-197, 2002.
59. Barjon C, Niki T, Vérillaud B, Opolon P, Bedossa P, Hirashima M, Blanchin S, Wassef M, Rosen HR, Jimenez AS, *et al*: A novel monoclonal antibody for detection of galectin-9 in tissue sections: Application to human tissues infected by oncogenic viruses. Infect Agent Cancer 7: 16, 2012.
60. Yoon HK, Kim TH, Park S, Jung H, Quan X, Park SJ, Han J and Lee A: Effect of anthracycline and taxane on the expression of programmed cell death ligand-1 and galectin-9 in triple-negative breast cancer. Pathol Res Pract 214: 1626-1631, 2018.