ANTICANCER RESEARCH *43*: 4045-4053 (2023)
doi:10.21873/anticanres.16593

Ganglioside GD2 Expression Is Associated With Unfavorable Prognosis in Early Triple-negative Breast Cancer

CHIHIRO HIGASHI[1], KANAKO SAITO[2], YUJI KOZUKA[3], HIROTO YUASA[3], KAHO NAKAMURA[1], MAKOTO ISHITOBI[1], MIKIYA ISHIHARA[2], TOSHIRO MIZUNO[2], ISAO TAWARA[4], HIROSHI FUJIWARA[5] and TOMOKO OGAWA[1]

[1]*Department of Breast Surgery, Mie University Graduate School of Medicine, Mie, Japan;*
[2]*Department of Medical Oncology, Mie University Hospital, Mie, Japan;*
[3]*Department of Pathology, Mie University Hospital, Mie, Japan;*
[4]*Department of Hematology and Oncology, Mie University Graduate School of Medicine, Mie, Japan;*
[5]*Department of Personalized Cancer Immunotherapy, Mie University Graduate School of Medicine, Mie, Japan*

Abstract. *Background/Aim: Gangliosides (acidic glycosphingolipids) have crucial regulatory roles in normal physiological processes, as well as in pathological conditions, including tumor onset and progression. GD2 is highly expressed in triple-negative breast cancer (TNBC), particularly in cancer stem cells. However, little is known on the clinical impact of GD2 expression on the prognosis of TNBC. Consequently, we aimed to investigate the association between GD2 expression in TNBC and the prognosis of TNBC. Patients and Methods: We assessed GD2 expression in 76 patients with primary TNBC who had undergone surgery at our Institute between 2012 and 2015 using immunohistochemical analysis with a tissue microarray technique. We investigated the relationship between GD2 expression and clinicopathological factors in TNBC, recurrence-free survival (RFS), and overall survival (OS). Results: Increased GD2 expression was observed in 45% of TNBC patients. There was no significant association between GD2 expression and clinicopathological factors in TNBC. The 5-year RFS rate among patients with GD2-positive TNBCs was significantly worse than that among patients with GD2-negative TNBCs (75.4% and 94.9%; HR=4.931; 95%CI=1.024-23.752; p=0.027). The OS in patients with GD2-positive TNBCs tended to be inferior to that of patients with GD2-negative TNBCs (HR=5.357; 95%CI=0.599-47.939; p=0.092). Interestingly, in patients with GD2-positive TNBCs, a higher grade of tumor-infiltrating lymphocytes (TILs) displayed a significantly better impact on OS (TILs-high vs. TILs-low; p=0.04). Both univariate and multivariate analyses showed that GD2 expression negatively affected RFS (p=0.027, p=0.021, respectively). Conclusion: GD2 expression is an independent unfavorable prognostic factor for TNBC.*

Globally, breast cancer is one of the most prevalent cancers among women (1). Although the mortality rate of early breast cancer is improving, metastatic and recurrent breast cancers continue to be a treatment challenge. Triple-negative breast cancer (TNBC), which lacks estrogen and progesterone receptors and does not overexpress human epidermal growth factor receptor 2 (HER2), has the worst prognosis among all breast cancer subtypes (2, 3).

Recent evidence revealed that TNBC tends to have higher immunogenicity due to genomic instability and a higher mutational load than other subtypes, leading to immunologically inflamed environments (4-6). Tumor-infiltrating lymphocytes (TILs) are more abundant in the tumor microenvironment of TNBC than in other subtypes, and patients with early TNBC and high levels of TILs typically have a favorable prognosis (7, 8). In addition, the expression of programmed death-ligand 1 (PD-L1) is possibly increased not only in tumor cells but also in immune cells within the tumor microenvironment compared to other subtypes (9, 10). Combination of immune checkpoint inhibitors with chemotherapy in patients with TNBC has become a new standard treatment (11, 12). However, owing

Correspondence to: Kanako Saito, Department of Medical Oncology, Mie University Hospital, 2-174, Edobashi, Tsu, Mie, 514-8507, Japan. Tel: +81 592315016, Fax: +81 592315200, e-mail: kana-s@med.mie-u.ac.jp and Hiroshi Fujiwara, Department of Personalized Cancer Immunotherapy, Mie University Graduate School of Medicine, 1577, Kurimamachiya-cho, Tsu, Mie 514-8507, Japan. Tel: +81 592315187, Fax: +81 592315276, e-mail: rieyunahiroshi@med.mie-u.ac.jp

Key Words: GD2, triple-negative breast cancer, tumor infiltrating lymphocytes (TILs), prognosis, early-stage.

Table I. *Association between GD2 expression and clinicopathological factors of patients included in the study.*

Factors		Total n=76	GD2		*p*-Value
			Positive n=34 (%)	Negative n=42 (%)	
Median age (range)		56 (31-84)	56 (31-84)	55.5 (31-83)	0.329
Surgery	Mastectomy	52 (68.4)	24 (70.6)	28 (66.7)	0.715
	Breast conserving surgery	24 (31.6)	10 (29.4)	14 (33.3)	
Histology	IDC	62 (81.6)	28 (82.4)	34 (81.0)	0.706
	ILC	2 (2.6)	0 (0.0)	2 (4.8)	
	Apocrine	5 (6.6)	2 (5.9)	3 (7.1)	
	Metaplastic	2 (2.6)	1 (2.9)	1 (2.4)	
	Others	5 (6.6)	3 (8.8)	1 (4.8)	
Tumor status	pT1	40 (52.6)	20 (58.8)	20 (47.6)	0.126
	pT2	34 (44.7)	12 (35.3)	22 (52.4)	
	pT3-4	2 (2.6)	2 (5.9)	0 (0.0)	
Nodal status	pN0	51 (67.1)	23 (67.6)	28 (66.7)	0.103
	pN1	19 (25.0)	8 (23.5)	11 (26.2)	
	pN2	3 (3.9)	0 (0.0)	3 (7.1)	
	pN3	3 (3.9)	3 (8.8)	0 (0.0)	
Nuclear grade	1-2	15 (19.7)	8 (23.5)	7 (16.7)	0.739
	3	59 (77.6)	25 (73.5)	34 (81.0)	
	Unknown	2 (2.6)	1 (2.9)	1 (2.4)	
pStage	I	33 (43.4)	17 (50.0)	16 (38.1)	0.344
	II	36 (47.4)	13 (38.2)	23 (54.8)	
	III	7 (9.2)	4 (11.8)	3 (7.1)	
Adjuvant chemotherapy	Yes	54 (71.1)	22 (64.7)	32 (76.2)	0.272
	No	22 (28.9)	12(35.3)	10 (23.8)	
Radiotherapy	Yes	31 (40.8)	12 (35.3)	19 (45.2)	0.380
	No	45 (59.2)	22 (64.7)	23 (54.8)	
PD-L1	Positive	34 (44.7)	15 (44.1)	19 (45.2)	0.986
	Negative	40 (52.6)	18 (52.9)	22 (52.4)	
	Unknown	2 (2.6)	1 (2.9)	1 (2.4)	
TILs	≤10%	45 (71.1)	20 (58.8)	25 (59.5)	0.951
	>10%	31 (40.8)	14 (41.2)	17 (40.5)	

IDC, Invasive ductal carcinoma; ILC, invasive lobular carcinoma ; PD-L1, programmed cell death-ligand 1; TILs, tumor infiltrating lymphocyte.

to the paucity of targeted therapies for TNBC, patients are still commonly treated with conventional chemotherapy and radiotherapy without any particular specificity for TNBC, resulting in development of resistance. Therefore, development of new treatments for TNBC is urgently needed.

Gangliosides (acidic glycosphingolipids) have crucial regulatory roles in normal physiological processes, such as embryogenesis, as well as in pathological conditions, including tumor onset and progression (13). Physiologically, ganglioside GD2 is exclusively and modestly expressed in the neural tissues of the central and peripheral nervous systems, as well as in skin melanocytes (14). In contrast, GD2 is overexpressed in neuroectodermal tumors such as neuroblastoma and melanoma, soft tissue sarcoma, small lung cell cancer, and breast cancer (14-18).

Furthermore, GD2 has been reported to be abundant in $CD44^{high}CD24^{low}$ cancer stem cells (CSCs), that are important for cancer chemoresistance (19). In breast cancer, it has been reported that GD2 expression was associated with aggressive features such as the triple negative phenotype, enhanced invasiveness, and a CSC-like phenotype (18).

Taken together, owing to the exclusive overexpression of GD2 in malignant tumor cells and its close correlation with CSCs, GD2 seems to have potential as a promising target for TNBC. However, little is known on the clinical impact of GD2 expression on the prognosis of TNBC. Consequently, in this study, we first attempted to clarify the impact of GD2 on the prognosis of patients with TNBC.

Patients and Methods

Patients. Seventy-six patients with triple-negative invasive BC, who underwent surgery for stage I to III tumors, between January 2012 and December 2015, at the Mie University Hospital (Tsu, Japan) were included in the study. Patients with ductal carcinoma *in situ* (DCIS), small tumors (<5 mm), tumors that had received neoadjuvant chemotherapy, or those with recurrent tumors were

Table II. *Antibodies and immunohistochemical assays.*

Marker	Source	Type	Clone	Procedure	Dilution	Antigen retrieval	Visualization	Manufacturer
ER	Mouse	Monoclonal	6F11/2	Autostainer	1:320	Heat (95˚C, 30 min.), CC1: EDTA buffer (pH 8.5)	iVIEW DAB Detection Kit	Novocastra
PgR	Rabbit	Monoclonal	1E2	Autostainer	Ready-to-use	Heat (95˚C, 60 min.), CC1: EDTA buffer (pH 8.5)	iVIEW DAB Detection Kit	Roche
HER2	Rabbit	Monoclonal	4B5	Autostainer	Ready-to-use	Heat (95˚C, 60 min.), CC1: EDTA buffer (pH 8.5)	iVIEW DAB Detection Kit	Roche
Ki-67	Mouse	Monoclonal	MIB-1	Autostainer	1:80	Heat (95˚C, 30 min.), CC1: EDTA buffer (pH 8.5)	iVIEW DAB Detection Kit	DAKO/ Agilent
PD-L1	Rabbit	Monoclonal	SP142	Autostainer	Ready-to-use	Heat (95˚C, 48 min.), CC1: EDTA buffer (pH 8.5)	OptiView Universal kit	Ventana
GD2	Mouse	Monoclonal	14G2a	Autostainer	1:50	Pressure cooker (121˚C, 5 min)	iVIEW DAB Detection Kit	Santa Cruz Biotechnology

excluded. Table I shows the clinical and pathological characteristics of the patients enrolled in this study. This study was conducted in accordance with ethical principles and as per the tenets of the Declaration of Helsinki, and it was approved by the Institutional Review Board of Mie University Hospital (No. H2021-021). The requirement for written informed consent was waived because of the retrospective nature of the study.

Histological evaluation. All the patients underwent surgical intervention for primary breast tumors. The specimens were formalin-fixed, paraffin-embedded (FFPE), and cut into 4-μm-thick sections for hematoxylin and eosin (HE) staining. Nuclear grades (NG) were assigned according to the histological classification of breast tumors in the General Rules for Clinical and Pathological Recording of Breast Cancer (Japanese Breast Cancer Society) using surgical specimens.

Immunohistochemistry (IHC) for GD2 and PD-L1 expression was performed using tissue microarrays (TMAs). As described in our previous report (10), TMAs were constructed from tumor blocks of surgical specimens, using 2.0-mm (diameter) tumor cores from selected blocks. These cores were assembled in a TMA format, and the paraffin-embedded TMA blocks were then sectioned at 4-μm thickness and subjected to IHC analysis.

IHC analysis. Estrogen receptor (ER) and progesterone receptor (PgR) positivity was evaluated against a cut-off of 1%, and HER2 overexpression was evaluated in accordance with the American Society of Clinical Oncology/College of American Pathologists 2013 guidelines. Fluorescence *in situ* hybridization to assess HER2 amplification was performed whenever equivocal results (2+) were obtained. Ki-67 expression was assessed by IHC using MIB-1 monoclonal antibody (mAb) (Table II). The HER2 protein expression status was determined by immunohistochemical analysis using an anti-HER2 mAb assay. IHC staining was performed using an automatic immunostainer (BenchMark XT; Ventana Medical Systems, Tucson, AZ, USA).

Evaluation of PD-L1 expression. PD-L1 staining was performed using anti-PD-L1 mAb (SP142) (Table II). PD-L1 status was evaluated based on the proportion of the tumor area occupied by PD-L1-expressing tumor-infiltrating immune cells (%IC) at any intensity. According to the results of a clinical trial that used SP142 for TNBCs (11), PD-L1 expression was assessed against a cut-off of 1%.

Evaluation of GD2 expression. GD2 was stained using an anti-GD2 mAb (14G2a) (Table II). GD2 expression was evaluated based on the proportion of GD2-positive tumor cells at any intensity, and scored as negative (<1%), low (1-10%), intermediate (11-50%), or high (>50%) by two pathologists independently that were blinded to clinical information. For statistical analysis, these categories were divided into two groups: negative (<1%) and positive (≥1%).

Evaluation of TILs. H&E-stained slides were reviewed by two pathologists in accordance with the criteria of the International TILs Working Group 2014 (20). As shown in our previous report (10), lymphoplasmacytic infiltration was evaluated in the stromal area around the invasive tumor, and the average of several tumor areas was determined, excluding lymphocyte infiltration around the DCIS and normal lobules. TILs were analyzed as a continuous parameter and categorized into two groups using 10% as the cutoff value, where <10% stromal TILs were defined as the low TILs group and >10% as the high TILs group.

Outcomes. Clinicopathological data, including age, histology, size, NG, Ki-67 index, lymph node status, type of surgery, ER/PgR/HER2 status, and use of adjuvant treatments (chemotherapy and radiation) were obtained from routine reports. Patients received anthracycline-and/or taxane-based regimens and radiation therapy according to their own risks as adjuvant treatments.

Statistical analysis. Continuous data were analyzed using the Mann-Whitney *U*-test. The χ^2-test was used to compare GD2-positive and GD2-negative groups. Kaplan-Meier analysis and log-rank tests

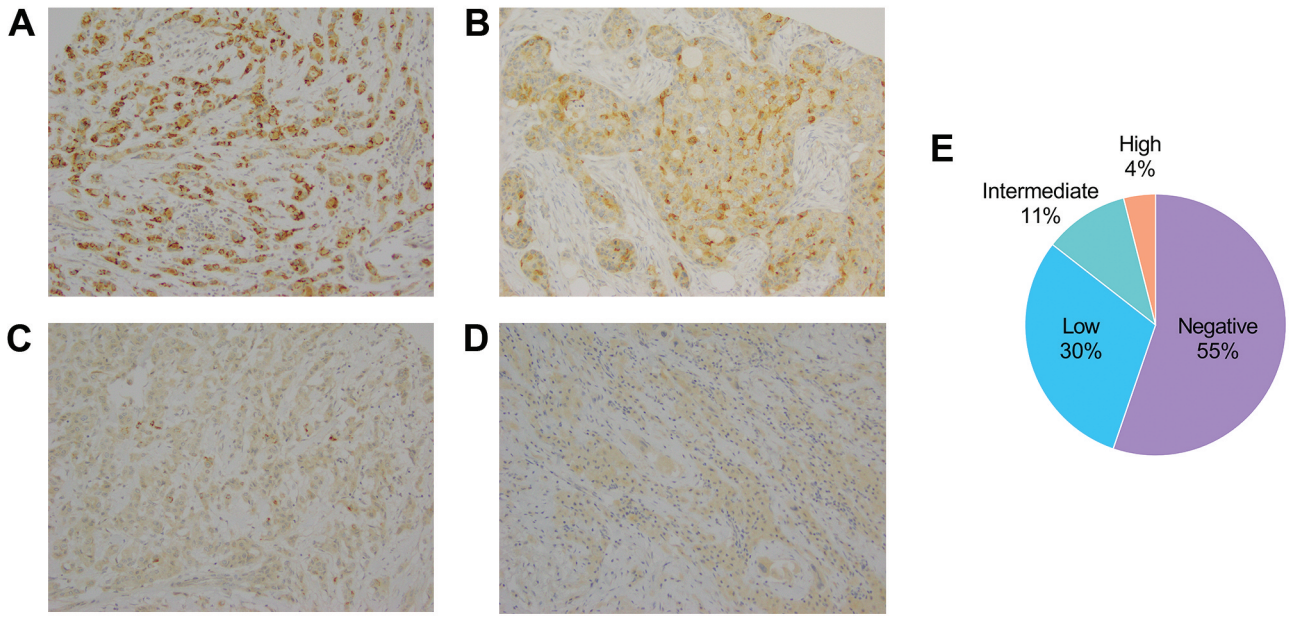

Figure 1. *GD2 staining of samples of triple negative breast cancer. (A) High (>50%), (B) intermediate (10-50%), (C) low (1-10%), and (D) negative (<1%) expression of GD2 are shown. (E) Pie charts presenting results of GD2 staining for all examined cases.*

were used to show differences in recurrence-free survival (RFS) and overall survival (OS) according to GD2 expression in combination with TIL infiltration. Cox regression proportional hazard models were used to estimate hazard ratios (HR) for RFS according to GD2 alone or in combination with TIL infiltration in both univariate and multivariate analyses. $p<0.05$ indicated statistically significant differences. Statistical analyses were performed using IBM SPSS Statistics 27.0 (SPSS Inc., Chicago, IL, USA).

Results

GD2 expression in TNBC. First, we assessed the expression of GD2 in TNBC on a 4-point scale (A-D). We found that 55% of the tumors (42/76) were GD2-negative, 30% (23/76) were GD2-low, 11% (8/76) were GD2-intermediate, and 4% (3/76) were GD2-high (Figure 1E). In this study, GD2-positive was defined as ≥1%, resulting in a GD2-positive rate of 45% (34/76) in patients with TNBC.

GD2 expression in TNBC is not associated with known clinicopathological factors. Table I shows the clinical and pathological characteristics of the patients enrolled in this study. We attempted to characterize the clinicopathological features of patients with GD2-positive TNBC. However, there were no significant associations between GD2 expression and tumor histology, tumor size, nodal status, nuclear grade, Ki-67 index, pathological stage, PD-L1 expression, or the presence of TILs (Table I).

GD2 expression is significantly associated with worse recurrence-free survival of TNBC. Next, we assessed the effect of GD2 expression on the prognosis of TNBC. The median duration of follow-up was 73.5 months (range, 1 to 104). In 76 patients, the recurrence-free survival (RFS) of GD2-positive TNBCs was significantly worse than that of GD2-negative TNBCs (HR=4.931; 95%CI=1.024-23.752; p=0.027) (Figure 2A). The 5-year RFS rate of GD2-positive and GD2-negative TNBCs were 75.4% and 94.9%, respectively. GD2-positive TNBCs tended to have an inferior overall survival compared to GD2-negative TNBCs, but the difference was not significant (HR=5.357; 95%CI=0.599-47.939; p=0.092) (Figure 2B). The 5-year overall survival (OS) of GD2-positive and GD2-netative TNBCs were 87.4% and 97.4%, respectively. These results suggest that GD2-positive TNBC is associated with a poorer prognosis than GD2-negative TNBC.

Association between GD2 expression combined with TILs and prognosis of TNBC. Numerous studies have reported that tumor-infiltrating lymphocytes (TILs) are likely to accumulate in TNBC due to the high immunogenicity of these tumors, and high amounts of TILs in tumor areas are associated with a favorable prognosis. Therefore, we investigated whether the poor prognosis caused by GD2 expression in TNBC is affected by the presence of TILs. We divided the included cases in the study into four groups based on GD2 expression (positive/negative) and

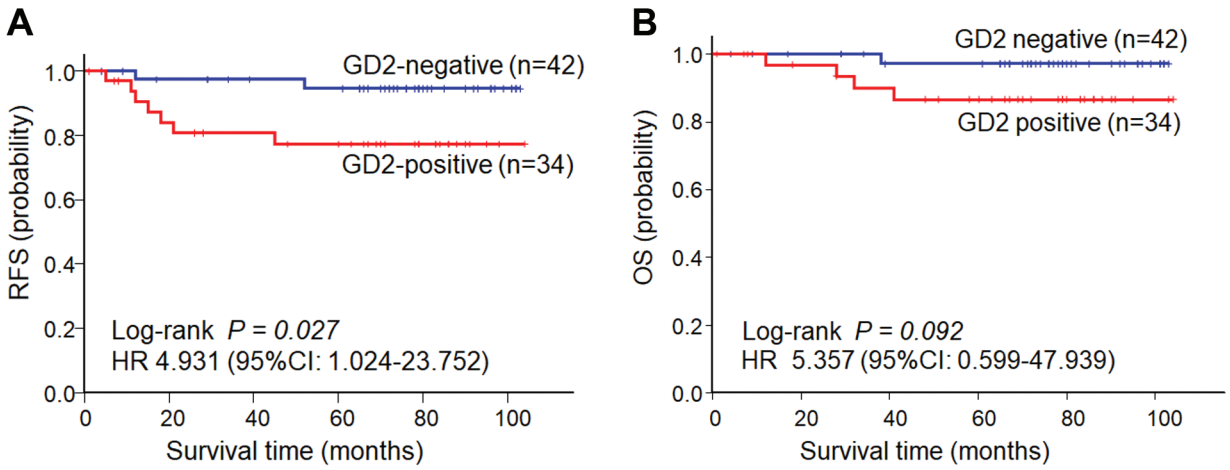

Figure 2. *Kaplan-Meier curves for recurrence-free survival (RFS) (A) and overall survival (OS) (B) in patients with GD2-positive (red line) and GD2-negative (blue line) TNBCs. TNBC: Triple negative breast cancer.*

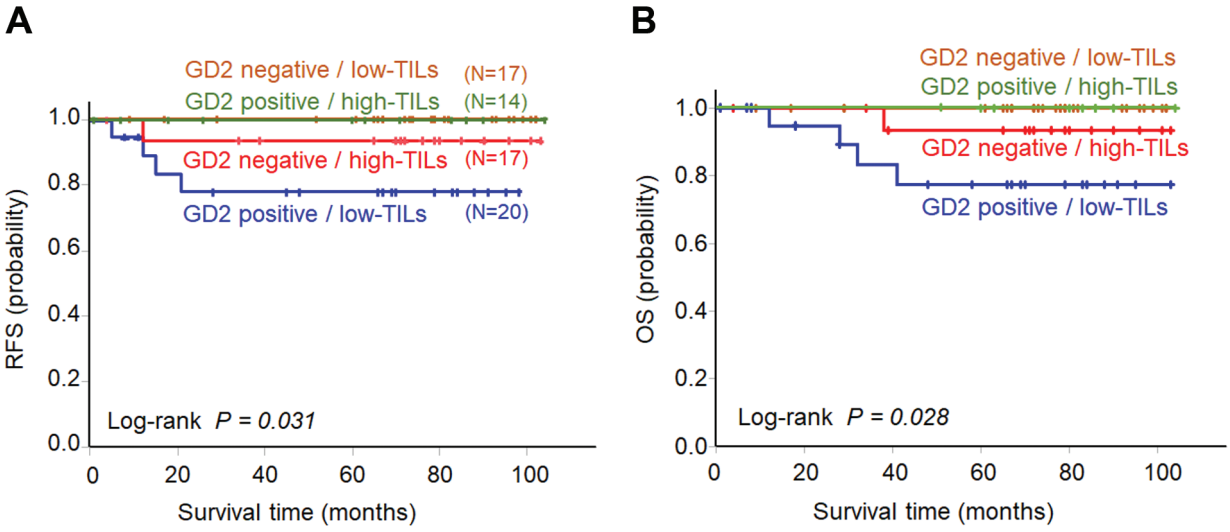

Figure 3. *Kaplan- Meier curves for RFS (A) and OS (B) in patients with GD2-positive and high-TILs (green line), GD2-positive and low-TILs (blue line), GD2-negative and high-TILs (red line), and GD2-negative and low TILs (brown line) TNBCs. TNBC: Triple negative breast cancer; RFS: recurrence-free survival; TIL: tumor-infiltrating lymphocyte; OS: overall survival.*

the number of TILs (high/low). As shown in Figure 3A and 3B, TNBC with GD2-positive and low TILs levels had the poorest RFS and OS among the four groups, with statistically significant differences (RFS, p=0.031; OS, p=0.028). These results suggest that high amounts of TILs can attenuate the unfavorable effects of GD2 expression in TNBC.

GD2 is an independent prognostic factor for RFS of TNBC. Finally, we performed both univariate and multivariate analyses to investigate the prognostic factors in 76 patients with TNBC. Univariate regression model analysis showed that RFS was significantly correlated with tumor size (pT1 *vs.* pT2-4, p=0.005) and GD2 expression (negative *vs.* positive, p=0.027) (Table III). When these parameters were subjected to multivariate analysis, tumor size (odds ratio=13.366; 95% CI=1.661-107.533; p=0.015) and GD2 expression (odds ratio=6.407; 95% CI=1.324-30.966; p=0.021) were significant independent factors for RFS (Table III).

Table III. *Univariate and multivariate analysis for recurrence-free survival.*

	Univariate analysis			Multivariate analysis		
Covariates	Hazard ratio	95%CI	*p*-Value	Hazard ratio	95%CI	*p*-Value
Age <50 *vs.* age≥50	3.486	0.436-27.875	0.209			
pT1 *vs.* pT2-4	10.734	1.341-85.929	0.005	13.366	1.6661-107.533	0.015
pN0 *vs.* pN1-3	1.896	0.508-7.068	0.333			
NG1-2 *vs.* NG3	2.041	0.255-16.326	0.492			
Ki67 <30 *vs.* Ki67 ≥30	2.301	0.288-18.417	0.432			
pStage1 *vs.* pStage2-3	7.057	0.882-56.468	0.032			
No-chemotherapy *vs.* chemotherapy	1.313	0.273-6.321	0.733			
GD2 negative *vs.* GD2 positive	4.931	1.024-23.752	0.027	6.407	1.324-30.996	0.021

Discussion

Herein we investigated the impact of GD2 on the prognosis of TNBC patients. In this study, GD2 was incrementally expressed in 45% of the patients with TNBC in our cohort. Furthermore, we found that the 5-year RFS of GD2-positive TNBC was significantly worse than that of GD2-negative TNBC (p=0.027) and that GD2 expression was an independent prognostic factor for RFS. Our data indicated that GD2-positivity is associated with an unfavorable prognosis, particularly, those patients with TNBC that were GD2-positive and lacking TILs displayed the poorest overall survival.

Previous studies using breast cancer cell lines showed that GD2 was more highly expressed in basal-like cell lines with TNBC features than in other subtypes (19). However, the data on FFPE samples of human breast cancer are not only limited but also controversial. In one report, GD2 was shown to be more frequently expressed in high-grade tumors, ER-negative tumors, and the elderly (18), and in another report, GD2 expression was observed more frequently in ER-positive tumors than in ER-negative tumors (21). GD2-positive cases of TNBC have been reported to be 67% (18) and 18% (21) in two previous studies and 45% in the present study, demonstrating considerable variation. These variations may be attributed to the different antibodies used for GD2-staining, the different cutoff values for IHC staining, and the evaluation criteria in each study. Since a standard GD2 evaluation method has not yet been established, we used the 14G2a clone of mouse antibody for our GD2 staining procedure. This antibody was produced in the 1980s and has been used in several clinical trials for neuroblastoma since the 2000s. We determined positivity as 1% of the cells are positive for the expression of the marker, as described in the Materials and Methods section. However, the appropriateness of the present criteria must be validated in a larger cohort. Especially, for clinical application of GD2-targeted immunotherapy, the establishment of GD2 evaluation methods required urgent attention.

GD2 has been reported to be associated with aggressive features and advanced stages of all subtypes (18, 22). Most TNBC tumors are highly aggressive, and none of the clinicopathological factors, including the presence of TILs and PD-L1 expression, correlated with GD2 expression. Nevertheless, in this study on patients with early-stage TNBC, we observed that patients with GD2-positive TNBCs showed significantly worse RFS than those with GD2-negative TNBCs, and GD2-positivity was found to be an independent unfavorable prognostic factor for RFS in TNBC. One possible reason is that GD2-positive cells may be resistant to conventional adjuvant chemotherapy and radiotherapy and persist durably, which strongly suggests the behavior of CSCs. Al-Hajj *et al.* reported that $CD44^{high}CD24^{low}$ cells had breast cancer stem cell characteristics that were capable of initiating tumors and differentiating into other subtypes (23). Battula *et al.* reported that most of the $CD44^{high}CD24^{low}$ cells in breast cancer cell lines expressed GD2 and had the characteristics of CSCs (19). Although we did not evaluate the expression of CD44/CD24 in tumor cells, our observations also suggest that GD2-positive cells may have cancer stem cell properties related to resistance to conventional chemoradiotherapy. We further examined RFS and OS only in patients who received postoperative adjuvant chemotherapy and observed that GD2-positive patients tended to have a worse prognosis, without statistical significance (data not shown). Unfortunately, the sample size was quite small, requiring further analyses with larger cohorts.

GD2 is a good antigen for immunotherapy of neuroblastoma. Anti-GD2 antibody has been clinically used for neuroblastoma and has shown prolonged survival when administered in combination with cytotoxic drugs (24). Additionally, although preclinical evaluation of dinutuximab (anti-GD2 antibody; ch14.18) against TNBC cells has been affirmatively reported (25), considering its efficacy and adverse events, this treatment strategy is unsatisfactory. Recently, advanced immunotherapy, such as GD2-targeting chimeric antigen receptor T (CAR-T) cell therapy, has been under development for refractory neuroblastoma (26).

One of the new findings in this study was that GD2-positive TNBCs with low amounts of infiltrating TILs showed worse prognosis than that of GD2-positive TNBCs with high TILs. This observation suggests that GD2-positive tumor cells exhibit aggressive behavior in the absence of tumor-reactive T cells, leading to the early recurrence of TNBC or short survival. Conversely, the infusion of GD2-positive tumor cell-reactive effector lymphocytes may be a promising treatment for GD2-positive TNBCs. However, it remains unclear whether the same strategy can be applied to refractory TNBCs. For examples, whereas GD2 expression in glioblastoma (GB) is preserved in both primary and metastatic tumors without antigen loss, which provides the advantage of GD2-targeted immunotherapy against GB (27), there are no data on GD2 expression in metastatic breast cancer. This point should be clarified in future studies to extend the clinical application of GD2-CAR-T cell therapy for the treatment of advanced-stage TNBC. In addition, although the recent report showed the repression of natural killer cell activity mediated by GD2-sialic acid immunoglobulin-like lectin (SIGLEC) interaction (28), until now, the machinery of GD2 mediated suppression of host anti-cancer immunity has not been fully elucidated. The mechanisms of potential targeted for GD2 therapy in TNBCs need to be better clarified.

This study had several limitations. One is a small sample size from a single Institute. Further analyses with larger cohorts are required to confirm that GD2 is a prognostic factor in TNBCs. The next limitation is the lack of standardized evaluation methods for GD2 using FFPE primary samples. Since GD2 is a glycolipid, not a protein, GD2 expression could be attenuated during the deparaffinization, leading to the underestimation of GD2 expression compared to immunohistochemistry using frozen primary tumor tissues. In addition, because our Institute actively treats patients with stages I to III (most cases are early breast cancer), the presented data are largely exclusive to patients with early-stage TNBC. Conversely, our observations demonstrated that GD2 expression has a negative impact on the prognosis, even in early-stage TNBC, and a novel treatment strategy should be applied in this early phase to improve the prognosis of patients with TNBC.

Conclusion

In summary, we are the first to report the clinical significance of GD2 expression in Japanese patients with TNBC, particularly those with early-phase TNBC. We showed that GD2 is an independent poor prognostic factor for TNBC. Moreover, we also indicated the potential advantages of cellular immunotherapy targeting GD2, such as GD2-CAR-T cell therapy, towards treatment of TNBC. In this context, we are currently preparing for a clinical trial using our novel GD2 CAR-T cell therapy.

Funding

This study was supported in part by research grants from JSPS (KAKENHI, No. 21K08593), OKASAN-KATO FOUNDATION (Mie, Japan), and Ekiden for Life (Charity Load Marathon for Cancer Patients in Mie, Japan).

Conflicts of Interest

Kanako Saito received honoraria from Daiichi-Sankyo, Chugai, Kyowa Kirin, Eli Lilly, Novartis, Eisai, and Pfizer outside the submitted work. Mikiya Ishihara received honoraria from Chugai, Eisai, MSD, Ono, Daiichi-Sankyo, Otsuka, and Eli Lilly outside the submitted work. Hiroshi Fujiwara is a member of the department funded by T Cell Nouveau Inc. The other Authors have no conflicts of interest.

Author's Contributions

KS contributed to the conception, design, and writing of the article. CH contributed to the analysis, interpretation, and writing of the manuscript. KN contributed to the analysis and interpretation. YK and HY contributed to the pathological analysis. M. Ishitobi, M. Ishihara, and TM contributed to data collection. TO and IT supervised this study. HF contributed to the conception, and supervised the study, and reviewed and edited the manuscript.

Acknowledgements

We thank Dr. Hiroshi Shiku (Department of Personalized Cancer Immunotherapy Center for Comprehensive Cancer Immunotherapy, Mie University Graduate School of Medicine, Japan; d, September 4, 2022) for the conception of the study and for the fruitful discussions. We would also like to thank Ms. Masako Ichishi (Department of Pathology, Mie University Hospital, Japan) for immunohistochemical staining.

References

1 Ghoncheh M, Pournamdar Z, Salehiniya H: Incidence and mortality and epidemiology of breast cancer in the world. Asian Pac J Cancer Prev 17(sup3): 43-46, 2016. DOI: 10.7314/apjcp.2016.17.s3.43

2 Howlader N, Cronin KA, Kurian AW, Andridge R: Differences in breast cancer survival by molecular subtypes in the united states. Cancer Epidemiol Biomarkers Prev 27(6): 619-626, 2018. DOI: 10.1158/1055-9965.Epi-17-0627

3 Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, Lickley LA, Rawlinson E, Sun P, Narod SA: Triple-negative breast cancer: Clinical features and patterns of recurrence. Clinical Cancer Research 13(15): 4429-4434, 2007. DOI: 10.1158/1078-0432.Ccr-06-3045

4 Barroso-Sousa R, Jain E, Cohen O, Kim D, Buendia-Buendia J, Winer E, Lin N, Tolaney S, Wagle N: Prevalence and mutational determinants of high tumor mutation burden in breast cancer. Ann Oncol 31(3): 387-394, 2020. DOI: 10.1016/j.annonc.2019.11.010

5 Stephens PJ, McBride DJ, Lin ML, Varela I, Pleasance ED, Simpson JT, Stebbings LA, Leroy C, Edkins S, Mudie LJ,

Greenman CD, Jia M, Latimer C, Teague JW, Lau KW, Burton J, Quail MA, Swerdlow H, Churcher C, Natrajan R, Sieuwerts AM, Martens JW, Silver DP, Langerød A, Russnes HE, Foekens JA, Reis-Filho JS, van 't Veer L, Richardson AL, Børresen-Dale AL, Campbell PJ, Futreal PA, Stratton MR: Complex landscapes of somatic rearrangement in human breast cancer genomes. Nature 462(7276): 1005-1010, 2009. DOI: 10.1038/nature08645

6 Bareche Y, Buisseret L, Gruosso T, Girard E, Venet D, Dupont F, Desmedt C, Larsimont D, Park M, Rothé F, Stagg J, Sotiriou C: Unraveling triple-negative breast cancer tumor microenvironment heterogeneity: Towards an optimized treatment approach. J Natl Cancer Inst 112(7): 708-719, 2020. DOI: 10.1093/jnci/djz208

7 Loi S, Sirtaine N, Piette F, Salgado R, Viale G, Van Eenoo F, Rouas G, Francis P, Crown JP, Hitre E, De Azambuja E, Quinaux E, Di Leo A, Michiels S, Piccart MJ, Sotiriou C: Prognostic and predictive value of tumor-infiltrating lymphocytes in a phase III randomized adjuvant breast cancer trial in node-positive breast cancer comparing the addition of docetaxel to doxorubicin with doxorubicin-based chemotherapy: BIG 02-98. J Clin Oncol 31(7): 860-867, 2013. DOI: 10.1200/jco.2011.41.0902

8 Adams S, Gray RJ, Demaria S, Goldstein L, Perez EA, Shulman LN, Martino S, Wang M, Jones VE, Saphner TJ, Wolff AC, Wood WC, Davidson NE, Sledge GW, Sparano JA, Badve SS: Prognostic value of tumor-infiltrating lymphocytes in triple-negative breast cancers from two phase III randomized adjuvant breast cancer trials: ECOG 2197 and ECOG 1199. J Clin Oncol 32(27): 2959-2966, 2014. DOI: 10.1200/JCO.2013.55.0491

9 Huang W, Ran R, Shao B, Li H: Prognostic and clinicopathological value of PD-L1 expression in primary breast cancer: a meta-analysis. Breast Cancer Res Treat 178(1): 17-33, 2019. DOI: 10.1007/s10549-019-05371-0

10 Nagano M, Saito K, Kozuka Y, Ichishi M, Yuasa H, Noro A, Imai N, Shibusawa M, Kimoto M, Ishitobi M, Tono Y, Oda H, Ishihara M, Mizuno T, Ogawa T, Katayama N: CD204-positive macrophages accumulate in breast cancer tumors with high levels of infiltrating lymphocytes and programmed death ligand-1 expression. Oncol Lett 21(1): 36, 2021. DOI: 10.3892/ol.2020.12297

11 Schmid P, Adams S, Rugo HS, Schneeweiss A, Barrios CH, Iwata H, Diéras V, Hegg R, Im S, Shaw Wright G, Henschel V, Molinero L, Chui SY, Funke R, Husain A, Winer EP, Loi S, Emens LA: Atezolizumab and nab-paclitaxel in advanced triple-negative breast cancer. N Engl J Med 379(22): 2108-2121, 2018. DOI: 10.1056/NEJMoa1809615

12 Cortes J, Cescon DW, Rugo HS, Nowecki Z, Im S, Yusof MM, Gallardo C, Lipatov O, Barrios CH, Holgado E, Iwata H, Masuda N, Otero MT, Gokmen E, Loi S, Guo Z, Zhao J, Aktan G, Karantza V, Schmid P, Luis F, Gonzalo GA, Diego K, Ruben K, Matias M, Mirta V, Sally B, Stephen B, Philip C, Sherene L, Dhanusha S, Andrea G, Donatienne T, Carlos B, Leandro B, Fabiano C, Ruffo DFJ, Roberto H, Domicio Carvalho L, Fernando Cezar Toniazzi L, Roberto Odebrecht R, Antonio Orlando SN, Felipe S, David C, Danielle C, Cristiano F, Xinni S, Joanne Y, Alejandro A, Carlos G, Claudio S, Cesar S, Eduardo Y, Alvaro GD, Jesus S, Petra H, Zdenek K, Bohuslav M, Katarina P, Jana P, Vesna G, Erik J, Jeanette J, Soren L, Tamas L, Herve B, Isabelle D, Anthony G, Anne-claire H, Luis T, Jens-uwe B, Peter F, Dirk F, Nadia H, Jens H, Anna KFDS, Christian K, Sibylle L, Diana L, Tjoung-won P, Raquel Von S, Pauline W, Louis C, Ava K, Kai Cheong Roger N, Peter A, Tibor C, Zsuzsanna K, Laszlo L, Karoly M, Gabor R, John C, Catherine K, Seamus O, Saverio C, Antonietta D, Enrico R, Tomoyuki A, Takaaki F, Kenichi I, Takashi I, Yoshinori I, Tsutomu I, Hiroji I, Yoshimasa K, Koji M, Yasuo M, Hirofumi M, Seigo N, Naoki N, Shoichiro O, Akihiko O, Yasuaki S, Eiji S, Masato T, Yuko T, Kenji T, Koichiro T, Junichiro W, Naohito Y, Yutaka Y, Teruo Y, Anita B, Mastura MY, Angel GV, Alejandro JR, Jorge MR, Flavia M, Jessica RC, Karin B, Vivianne T, David P, Ewa C, Ewa N, Zbigniew N, Barbara R, Joanna S, Cezary S, Rafal T, Bogdan Z, Alexander A, Natalia F, Oleg L, Andrey M, Vladimir M, Guzel M, Jin Hee A, Seock-ah I, Keun Seok L, Kwong Hwa P, Yeon Hee P, Begona BDLH, Javier C, Josefina CJ, Luis DLCM, Jose GS, Maria G, Esther H, Esther ZA, Chien-ting L, Mei-ching L, Chiun-sheng H, Chao-jung T, Ling-ming T, Cagatay A, Gul B, Irfan C, Erhan G, Seyda G, Nil MM, Mustafa O, Ozgur O, Sinan Y, Steve C, Janine G, Iain M, Peter S, Nicholas T, Mark T, Christopher T, Duncan W, Hryhoriy A, Oleksandr B, Igor B, Oleksii K, Olena K, Hanna K, Anna K, Iurii L, Alla N, Natalya O, Olga P, Andrii R, Sergii S, Yaroslav S, Dmytro T, Grygorii U, Ihor V, Sibel B, Madhu C, Michael C, Patrick C, Scott C, Jennifer D, Keerthi G, Jeffrey H, Kent H, William I, Randa L, Janice L, Raul M, Susan M, Rita N, Ira O, Coral O, Timothy P, Amit P, Brian P, Hope R, Irina R, Michael S, Robert S, Michael S, Laura S, Bradley S, Michaela T, Frances V: 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(10265): 1817-1828, 2020. DOI: 10.1016/s0140-6736(20)32531-9

13 Birklé S, Zeng G, Gao L, Yu R, Aubry J: Role of tumor-associated gangliosides in cancer progression. Biochimie 85(3-4): 455-463, 2003. DOI: 10.1016/s0300-9084(03)00006-3

14 Hersey P, Jamal O, Henderson C, Zardawi I, D'alessandro G: Expression of the gangliosides GM3, GD3 and GD2 in tissue sections of normal skin, naevi, primary and metastatic melanoma. Int J Cancer 41(3): 336-343, 1988. DOI: 10.1002/ijc.2910410303

15 Roth M, Linkowski M, Tarim J, Piperdi S, Sowers R, Geller D, Gill J, Gorlick R: Ganglioside GD2 as a therapeutic target for antibody-mediated therapy in patients with osteosarcoma. Cancer 120(4): 548-554, 2014. DOI: 10.1002/cncr.28461

16 Furukawa K, Hamamura K, Ohkawa Y, Ohmi Y, Furukawa K: Disialyl gangliosides enhance tumor phenotypes with differential modalities. Glycoconj J 29(8-9): 579-584, 2012. DOI: 10.1007/s10719-012-9423-0

17 Yoshida S, Fukumoto S, Kawaguchi H, Sato S, Ueda R, Furukawa K: Ganglioside g(D2) in small cell lung cancer cell lines: Enhancement of cell proliferation and mediation of apoptosis. Cancer Res 61(10): 4244-4252, 2001. DOI:

18 Orsi G, Barbolini M, Ficarra G, Tazzioli G, Manni P, Petrachi T, Mastrolia I, Orvieto E, Spano C, Prapa M, Kaleci S, D'Amico R, Guarneri V, Dieci MV, Cascinu S, Conte P, Piacentini F, Dominici M: GD2 expression in breast cancer. Oncotarget 8(19): 31592-31600, 2017. DOI: 10.18632/oncotarget.16363

19 Battula VL, Shi Y, Evans KW, Wang RY, Spaeth EL, Jacamo RO, Guerra R, Sahin AA, Marini FC, Hortobagyi G, Mani SA, Andreeff M: Ganglioside GD2 identifies breast cancer stem cells and promotes tumorigenesis. J Clin Invest 122(6): 2066-2078, 2012. DOI: 10.1172/JCI59735

20 Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, Wienert S, Van den Eynden G, Baehner FL, Penault-Llorca F, Perez EA, Thompson EA, Symmans WF, Richardson AL, Brock J, Criscitiello C, Bailey H, Ignatiadis M, Floris G, Sparano J, Kos Z, Nielsen T, Rimm DL, Allison KH, Reis-Filho JS, Loibl S, Sotiriou C, Viale G, Badve S, Adams S, Willard-Gallo K, Loi S, International TILs Working Group 2014: The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. Ann Oncol 26(2): 259-271, 2015. DOI: 10.1093/annonc/mdu450

21 Erber R, Kailayangiri S, Huebner H, Ruebner M, Hartmann A, Häberle L, Meyer J, Völkl S, Mackensen A, Landgraf L, Geppert CI, Schulz-Wendtland R, Beckmann MW, Fasching PA, Farwick N, Rossig C, Gass P: Variable Expression of the Disialoganglioside GD2 in Breast Cancer Molecular Subtypes. Cancers (Basel) 13(21): 5577, 2021. DOI: 10.3390/cancers13215577

22 Mansoori M, Roudi R, Abbasi A, Abolhasani M, Abdi Rad I, Shariftabrizi A, Madjd Z: High GD2 expression defines breast cancer cells with enhanced invasiveness. Exp Mol Pathol 109: 25-35, 2019. DOI: 10.1016/j.yexmp.2019.05.001

23 Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF: Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 100(7): 3983-3988, 2003. DOI: 10.1073/pnas.0530291100

24 Mody R, Naranjo A, Van Ryn C, Yu AL, London WB, Shulkin BL, Parisi MT, Servaes SE, Diccianni MB, Sondel PM, Bender JG, Maris JM, Park JR, Bagatell R: Irinotecan-temozolomide with temsirolimus or dinutuximab in children with refractory or relapsed neuroblastoma (COG ANBL1221): an open-label, randomised, phase 2 trial. Lancet Oncol 18(7): 946-957, 2017. DOI: 10.1016/S1470-2045(17)30355-8

25 Ly S, Anand V, El-Dana F, Nguyen K, Cai Y, Cai S, Piwnica-Worms H, Tripathy D, Sahin AA, Andreeff M, Battula VL: Anti-GD2 antibody dinutuximab inhibits triple-negative breast tumor growth by targeting GD2(+) breast cancer stem-like cells. J Immunother Cancer 9(3): , 2021. DOI: 10.1136/jitc-2020-001197

26 Yu L, Huang L, Lin D, Lai X, Wu L, Liao X, Liu J, Zeng Y, Liang L, Zhang G, Wang B, Wu Z, Tao S, Liu Y, Jiao C, Chang LJ, Yang L: GD2-specific chimeric antigen receptor-modified T cells for the treatment of refractory and/or recurrent neuroblastoma in pediatric patients. J Cancer Res Clin Oncol 148(10): 2643-2652, 2022. DOI: 10.1007/s00432-021-03839-5

27 Park JA, Cheung NV: Targets and antibody formats for immunotherapy of neuroblastoma. J Clin Oncol 38(16): 1836-1848, 2020. DOI: 10.1200/JCO.19.01410

28 Theruvath J, Menard M, Smith BAH, Linde MH, Coles GL, Dalton GN, Wu W, Kiru L, Delaidelli A, Sotillo E, Silberstein JL, Geraghty AC, Banuelos A, Radosevich MT, Dhingra S, Heitzeneder S, Tousley A, Lattin J, Xu P, Huang J, Nasholm N, He A, Kuo TC, Sangalang ERB, Pons J, Barkal A, Brewer RE, Marjon KD, Vilches-Moure JG, Marshall PL, Fernandes R, Monje M, Cochran JR, Sorensen PH, Daldrup-Link HE, Weissman IL, Sage J, Majeti R, Bertozzi CR, Weiss WA, Mackall CL, Majzner RG: Anti-GD2 synergizes with CD47 blockade to mediate tumor eradication. Nat Med 28(2): 333-344, 2022. DOI: 10.1038/s41591-021-01625-x

Received June 14, 2023
Revised July 16, 2023
Accepted July 19, 2023