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A tumor metastasis-associated molecule TWIST1 is a favorable target for cancer immunotherapy due to its immunogenicity

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Abstract

Although neoantigens are one of the most favorable targets in cancer immunotherapy, it is less versatile and costly to apply neoantigen-derived cancer vaccines to patients due to individual variation. It is, therefore, important to find highly immunogenic antigens between tumor-specific or associated antigens that are shared among patients. Considering the cancer immunoediting theory, immunogenic tumor cells cannot survive in the early phase of tumor progression including two processes: elimination and equilibrium. We hypothesized that highly immunogenic molecules are allowed to be expressed in tumor cells after an immune suppressive tumor microenvironment was established, if these molecules contribute to tumor survival. In the current study, we focused on TWIST1 as a candidate for highly immunogenic antigens because it is upregulated in tumor cells under hypoxia and promotes tumor metastasis, which is observed in the late phase of tumor progression. We demonstrated that TWIST1 had an immunogenic peptide sequence $TWIST1_{140-162}$, which effectively activated TWIST1-specific $CD4^+$ T-cells. In a short-term culture system, we detected more TWIST1-specific responses in breast cancer patients compared with in healthy donors. Vaccination with the TWIST1 peptide also showed efficient expansion of TWIST1-reactive HTLs in humanized mice. These findings indicate that TWIST1 is a highly immunogenic shared antigen and a favorable target for cancer immunotherapy.

KEYWORDS
cancer immunoediting, cancer immunotherapy, cancer vaccine, metastasis-associated molecules, shared, tumor antigens, tumor antigens, vaccination therapy

Abbreviations: DCs, dendritic cells; ELISPOT, enzyme-linked immunospot; EMT, epithelial-to-mesenchymal transition; GM-CSF, granulocyte–macrophage colony-stimulating factor; HIF, hypoxia-inducible factor; HLA, human leukocyte antigen; HTLs, $CD4^+$ helper T-cells; ICIs, immune checkpoint inhibitors; IFN, interferon; IHC, immunohistochemistry; IL, interleukin; TAAs, tumor-associated antigens; TCR, T-cell receptor; TNF, tumor necrosis factor.

1 | INTRODUCTION

As immune checkpoint inhibitors show marked antitumor effects in clinical settings, immunotherapy has been in the spotlight in cancer research.[1] It is important to choose what types of tumor antigens make the immune system target tumor cells. Tumor antigens are categorized into several families[2]: (1) shared antigens, which are also expressed in normal cells such as MART-1 and HER2[3,4]; (2) tumor-associated antigens, which are upregulated in tumor cells and weakly expressed in normal cells such as Survivin[5]; (3) stealth antigens, which are epigenetically silenced in tumor cells such as SPESP1[6]; and (4) neoantigens, which are encoded by mutated genes in tumor cells.[7] Because of their high immunogenicity, neoantigens are the most favorable targets in cancer immunotherapy. However, it is less versatile and costly to apply neoantigen-derived cancer vaccines to patients, because epitope sequences vary in individuals. This problem would be resolved if we had highly immunogenic shared or tumor-associated antigens and applied them clinically.

Based on cancer immunoediting theory,[8] tumor cells cannot survive with high immunogenicity under active immunosurveillance, especially in the early phase of tumor progression, in which there would be few immunosuppressive cells such as Tregs and myeloid-derived suppressor cells at the tumor site. Conversely, tumor cells in the late phase could upregulate even highly immunogenic molecules if they played a role in promoting tumor progression, because they have already been protected by immunosuppressive cells. From this point of view, we hypothesized that cancer metastasis-related molecules are favorable target antigens, because cancer metastasis generally happens in the late phase of tumor progression.

Twist is evolutionarily conserved from invertebrates to humans and two Twist genes exist in vertebrates, *Twist1* and *Twist2*.[9] In the late phase of tumor development, tissue oxygen tension is reduced (hypoxia) in the tumor microenvironment,[10] resulting in ectopic TWIST1 expression in a hypoxia-inducible factor (HIF) transcription factor, HIF-2α-dependent manner.[11] Ectopic Twist 1 expression is found in various types of tumors including breast cancer,[12] non–small-cell lung cancer,[13] prostate cancer,[14] gastric cancer,[15] melanoma,[16] osteosarcoma,[17] and hepatocellular carcinoma,[18] and esophageal squamous cell carcinoma.[19] Twist1 promotes transcriptional activity to upregulate N-cadherin and suppress E-cadherin expression and induces epithelial-to-mesenchymal transition (EMT) in cancer cells.[20,21] Therefore, its enhanced expression predicts a poor prognosis in cervical cancer,[22] gastric cancer,[23] ovarian cancer,[24] esophageal squamous cell carcinoma,[25] and chronic kidney disease.[26] Based on those characteristics of TWIST1 showing ectopic expression in late phase of tumor progression, we hypothesized that Twist1 has a high immunogenicity and could be a favorable target for cancer immunotherapy.

In the current study, we found that a TWIST1-derived peptide efficiently expanded TWIST1-specific $CD4^+$ helper T-cells (HTLs), which also responded to TWIST1-expressing tumor cell lines. TWIST1-specific HTLs were detected more frequently in patients with breast cancer who were positive for TWIST1 compared with healthy donors. Furthermore, vaccination with the TWIST1 peptide also showed efficient expansion of TWIST1-reactive HTLs in humanized mice. These findings suggested that TWIST1 is a highly immunogenic shared antigen and a favorable target for cancer immunotherapy.

2 | MATERIALS AND METHODS

2.1 | Cell lines and mice

Human cell lines HSC-3 (tongue squamous cell carcinoma [SCC], HLA-DR15/15), HSC-4 (tongue SCC, HLA-DR1/4), Sa-3 (gingival SCC, HLA-DR9/10), 5637 (urinary bladder carcinoma, HLA-DR1/9), and Jurkat cells were supplied by RIKEN BioResource Center (Tsukuba, Ibaraki, Japan). The tumor cell line SAS (tongue SCC, HLA-DR9/15) was purchased from the ATCC (Manassas, VA, USA). L-cells (mouse fibroblasts) expressing transfected HLA class II molecules were kindly donated by Dr. R. Karr (Karr Pharma, St. Louis, MO, USA) and Dr. T. Sasazuki (Kyushu University, Fukuoka, Japan). All cell lines were maintained in tissue culture as recommended by the supplier. All cell lines were meticulously cultured and used within 6 months, although no authentication assay was performed for any cell lines used. The humanized mice expressing HLA-A2.01 and HLA-DR1 (HHDII-DR1 mice)[27] were provided by the Institut Pasteur (France) based on an material transfer agreement (MTA) and maintained and handled according to the protocols approved by the Asahikawa Medical University Institutional Animal Care and Use Committee.

2.2 | Clinical samples

Tumor tissue samples were obtained from patients with breast cancer by surgical resection at Asahikawa Medical University Hospital. The characteristics of the nine breast cancer patients tested in the current study are summarized in Table 1. This study was approved by the Research Ethics Committee of Asahikawa Medical University and was performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all donors who provided samples.

2.3 | Synthetic peptides

We used the three computer-based algorithms SYFPEITI (http://www.syfpeithi.de), Immune Epitope (http://tools.iedb.org/mhcii/), and NetMHCIIpan-4.0 (https://services.healthtech.dtu.dk/service.php?NetMHCIIpan-4.0) for identifying potential HLA-DR (DRB1*0101, DRB1*0401, DRB1*0701, and DRB1*1501)-binding amino acid sequences of TWIST1. The peptides that showed high scores in those algorithms were selected as possible epitopes. Based on the results as shown in Table 2, we selected the TWIST1-derived

TABLE 1 Clinicopathological characteristics and TWIST1 expressions

BC No.	Gender	Age (y)	Primary site	ER	PR	HER2	Histological type	Histological grade	T/N/M	pStage	TWIST1
BC1	F	47	Rt Breast	+	+	−	IDC	I	1b/0/0	I	+
BC2	F	47	Rt Breast	+	+	−	IDC	I	1b/0/0	I	−
BC3	F	76	Rt Breast	+	+	−	IDC	II	1c/0/0	I	−
BC4	F	66	Rt Breast	+	+	−	IDC	II	1c/1/0	IIA	+
BC5	F	73	Lt Breast	+	+	−	ILC	−	3/0/0	IIB	NE
BC6	F	40	Rt Breast	+	+	+	IDC	II	1c/1/0	IIA	+
BC7	F	39	Lt Breast	+	+	−	IDC	I	1c/1/0	IIA	−
BC8	F	68	Lt Breast	−	−	+	IDC	III	1c/0/0	I	+
BC9	F	66	Lt Breast	+	+	−	IDC	II	1c/1/0	IIA	+

Abbreviations: IDC, invasive dactal carcinoma; ILC, invasive lobular carcinoma, NE, not evaluated.

TABLE 2 Prediction results for HLA class II alleles by several databases

		SYFPEITI	IMMUNE EPITOPE	NetMHCII-pan
Candidates	Allele	Score (Larger is better)	Adjusted rank (Smaller is better)	Rank (Smaller is better)
TWIST1 (140–162)	**DRB1*01:01**	31	4.3	3.48
	DRB1*04:01	26	6.8	8.07
	DRB1*07:01	30	2.6	1.83
	DRB1*15:01	24	6.4	2.06
TWIST1 (171–194)	DRB1*01:01	19	32	1.83
	DRB1*04:01	20	36	14.23
	DRB1*07:01	22	15	5.58
	DRB1*15:01	24	6.8	17.05

Bold indicates that its allele-restricted HTLs were generated.

peptide $TWIST1_{140–162}$ (SDKLSKIQTLKLAARYIDFLYQV) and $TWIST1_{171–194}$ (KMASCSYVAHERLSYAFSVWRMEG), and then commercially synthesized them (GenScript).

2.4 | In vitro generation of TWIST1-reactive HTL lines

The procedure for the expansion of peptide-specific HTLs has been described in detail previously.[6] Briefly, monocytes and $CD4^+$ T-cells were purified from PBMCs using MACS microbeads for CD14 and CD4, respectively (Miltenyi Biotech). Monocytes were differentiated into DCs using GM-CSF (50 ng/ml) and IL-4 (1000 IU/ml). DCs were pulsed with TWIST1 peptide (3 μg/ml for 3 h at room temperature) and then co-cultured with autologous $CD4^+$ T-cells in 96-well flat-bottomed culture plates. Seven days later, the $CD4^+$ T-cells were restimulated in individual microcultures with peptide-pulsed γ-irradiated autologous PBMCs (3 μg/ml), and 2 days later, recombinant human IL-2 (10 IU/ml) was added. After the two cycles of peptide stimulation, TWIST1-specific T-cell lines were restimulated weekly using irradiated autologous PBMCs pulsed with the peptide (3 μg/ml). Production levels of GM-CSF (BD Pharmingen), IL-5 (BD Pharmingen), TNFα (BD Pharmingen), IFN-γ (BD Pharmingen), and granzyme B (MABTECH) in culture supernatants were determined using ELISA kits according to the manufacturer's instructions. We measured the absorption at 450 nm using GloMax Discover Microplate Reader (Promega). AIM-V medium (Invitrogen) supplemented in 3% of human male AB serum (Innovative Research) was used as complete culture medium for all experiments. All blood materials were acquired after informed consent was appropriately obtained.

2.5 | Quantitative real-time PCR

Total RNA was purified from human tumor cell lines using an RNeasy Micro Kit (Qiagen Inc.), reverse-transcribed using a PrimeScript 1st strand cDNA synthesis kit (TaKaRa Bio Inc.), and then amplified with a LightCycler 480 Probes Master system (Roche Life Science) for each probe according to the manufacturer's instructions. The following probes were obtained from Applied Biosystems (Life Technologies): *TWIST1* (Hs004989912_s1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Hs02758991_g1). *Gapdh* was used as an internal control and to

normalize each mRNA expression level, which was calculated in each experiment using the $\Delta\Delta C_t$ method.

2.6 | Addressing TWIST1-specific responses with established HTLs

HTLs ($1–1.5\times10^5$) were co-cultured with irradiated autologous PBMCs (1.5×10^5), HLA-DR-expressing L-cells (3×10^4), tumor cell lines (3×10^4), or DCs (5×10^3) in some experiments. Human tumor cell lines were treated with 500 U/ml IFN-γ for 48 h to upregulate HLA-DR expression, and then IFN-γ was removed before the assay. To determine antigen specificity and HLA class II restriction, anti-HLA-DP mAb BRAFB6 (Santa Cruz), anti-HLA-DQ mAb SPV-L3 (NOVUS Biologicals), anti-HLA-DR mAb L243 (IgG2a, prepared from the supernatants of hybridoma HB-55 obtained from ATCC), and anti-HLA-A/B/C mAb W6/32 (IgG2a; ATCC) were added to the culture at 10 μg/ml for a 48-h incubation period. T-cell responses were evaluated by ELISA or ELISPOT assay.

2.7 | Evaluating frequency of TWIST1-specific T-cells in a short-term culture system

PBMCs ($1.5–2\times10^6$) of healthy donors and patients with breast cancer were stimulated with the $TWIST1_{140–162}$ peptide (10 μg/ml) in the presence of IL-2 (10 IU/ml) in 24-well plates as described previously.[28] Seven days after peptide stimulation, counts of IFN-γ-producing cells were assessed using ELISPOT assay.

2.8 | Vaccination with $TWIST1_{140–162}$ peptide into humanized mice

HHDII-DR1 mice were intradermally administrated with the $TWIST1_{140–162}$ peptide (100 μg/shot) with cGAMP (Invivogen, 10 μg/shot) on days 0 and 7. After 3 days, mice were sacrificed, and their draining lymph nodes were collected to evaluate the frequency of TWIST1 peptide-specific T-cells by ELISA or ELISPOT assay. In some experiments, mice simultaneously received an intraperitoneal injection of anti-PD-L1 mAb (10F.9G2, Bio X Cell, 100 μg in 200 μl of PBS/shot) with the peptide vaccine.

2.9 | ELISPOT assay

Enzyme-linked immunospot assay was performed using a ELISpot kit (Mabtech) according to the manufacturer's instructions as described previously.[29] Peptide-stimulated human PBMCs (2×10^5) or mouse lymphocytes (5×10^3) were cultured in the presence of peptide (3 μg/ml) in a MAHAS4510 plate (Millipore) for 24 h, and the number of IFN-γ-producing cells in the culture was measured using an ELISpot kit (Mabtech) according to the manufacturer's instructions. BCIP/NBT plus substrate (Mabtech) was used for detection. Plates were scanned using an automated ELISpot plate reader (Autoimmun Diagnostika GmbH). Spots were counted and analyzed using AID ELISPOT plate reader software (Autoimmun Diagnostika GmbH). For mouse lymphocytes, splenocytes (3×10^5) were used as APC.

2.10 | Immunohistochemistry

Immunohistochemistry (IHC) analysis of breast cancer specimens was performed using the EnVision™ HRP System (K5361, Dako) as described previously.[29] Formalin-fixed sections were obtained from breast cancer patients. Samples were boiled in EDTA buffer (pH 9.0) for antigen retrieval, and endogenous peroxidase activity was inhibited according to the manufacturer's instructions. Sections were then incubated with mouse anti-human TWIST polyclonal antibody (C-17, Santa Cruz 1:300) overnight at 4°C, followed by incubation with an HRP-conjugated secondary antibody and substrate. Images were acquired using a BZ-X700 microscope (Keyence).

2.11 | Statistical analysis

Statistical analysis was performed using GraphPad Prism 9.3.1 (GraphPad Software). Differences between two groups and among multiple groups were analyzed using unpaired *t*-tests and one-way ANOVA with Tukey's multiple comparison test, respectively. Data are presented as mean ± SD or SE, and a p-value <0.05 was considered statistically significant.

3 | RESULTS

3.1 | Identification of a TWIST-derived helper peptide epitope and generation of TWIST-specific helper T-cell lines

To address whether the peptide $TWIST1_{140–162}$ (SDKLSKIQTL KLAARYIDFLYQV), whose sequence is shared with $TWIST2_{98–120}$ and $TWIST1_{171–194}$ (KMASCSYVAHERLSYAFSVWRMEG) was capable of inducing antigen-specific helper T-cell responses, $CD4^+$ T-cells were purified from PBMCs of healthy donors and stimulated with these peptides onto autologous $CD14^+$ monocyte-derived DCs and restimulated weekly with peptide-pulsed γ-irradiated autologous PBMCs. We generated three lines of $TWIST1_{140–162}$-reactive HTLs from two healthy donors (TW-1 and TW-2 from HLA-DR1/DR15 and TW-3 from HLA-DR4/DR53). Because we could not detect any $TWIST1_{171–194}$ peptide-specific responses, we analyzed the immunogenicity of $TWIST1_{140–162}$ peptide in further experiments.

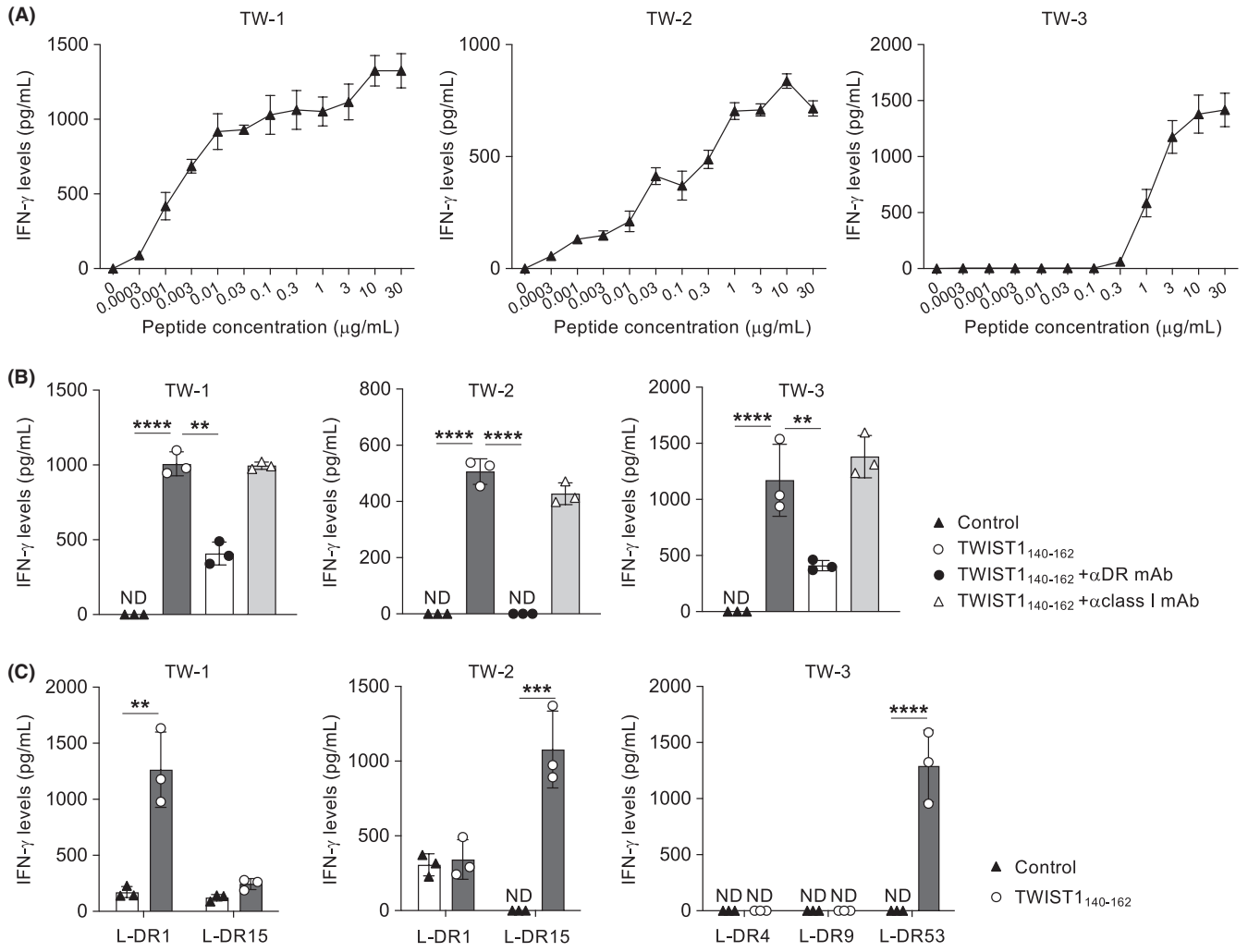

FIGURE 1 An immunogenic $TWIST1_{140-162}$ peptide promiscuously presented to HTLs. (A) $TWIST1_{140-162}$-specific HTL lines (TW-1, TW-2, and TW-3) were co-cultured with autologous PBMCs in the presence of various concentrations (0–30 μg/ml) of $TWIST1_{140-162}$ peptide. (B) $TWIST1_{140-162}$-specific HTL lines (TW-1, TW-2, and TW-3) were stimulated with autologous PBMCs in the presence of an irrelevant peptide (Control), $TWIST1_{140-162}$ peptide, $TWIST1_{140-162}$ peptide plus anti-HLA-DR (αDR) mAb, or $TWIST1_{140-162}$ peptide plus anti-HLA class I (αclass I) mAb. (C) HLA-restriction of the $TWIST1_{140-162}$-specific HTL lines (TW-1, TW-2, and TW-3) was assessed using irrelevant (Control) or $TWIST1_{140-162}$ peptide-pulsed L-cells expressing individual HLA-DR allele. Supernatants were collected after 24 h to assess IFN-γ production by ELISA in all experiments. Data are shown as mean ± SE. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; one-way ANOVA with interaction followed by Tukey's multiple comparisons test (B) and unpaired *t*-test (C). ND, not detected. Experiments were performed with at least three biological replicates and are representative of at least two independent experiments

These three HTLs released IFN-γ in a dose-dependent manner (Figure 1A). To define their HLA-DR restriction, we evaluated the reactivity of $TWIST1_{140-162}$-specific HTLs to autologous PBMCs in the presence of $TWIST1_{140-162}$ peptide using anti-HLA-DR or anti-HLA class I mAbs. The IFN-γ production of all $TWIST1_{140-162}$-specific HTLs was inhibited by the antibody for HLA-DR, but not HLA class I, indicating that their recognition of the $TWIST1_{140-162}$ peptide was restricted to HLA-DR (Figure 1B). Furthermore, we addressed the reactivity of $TWIST1_{140-162}$-specific HTLs using L-cells transfected with the HLA-DR allele gene and found that TW-1, TW-2, and TW-3 responded to L-DR1, L-DR15, and L-DR53 cells, respectively, indicating that the $TWIST1_{140-162}$ peptide bound to HLA-DR1, DR15, and DR53, respectively, to activate TWIST1-reactive HTLs (Figure 1C).

3.2 | Direct tumor recognition by TWIST1-specific HTLs

To assess whether $TWIST1_{140-162}$-specific HTLs would directly target TWIST1-expressing tumor cells, we addressed TWIST1 gene expression in several human tumor cell lines (HSC-4, 5637, HSC-3, SAS, Sa-3, and Jurkat) using qPCR and found that all cell lines tested expressed TWIST1 (Figure 2A). Therefore, we stimulated the $TWIST1_{140-162}$-specific HTL lines with the HLA-DR-matched tumor cell lines. DR1-restricted TW-1, DR15-restricted TW-2, and DR53-restricted TW-3 HTLs produced IFN-γ against HLA-DR1-(HSC-4 and 5637), HLA-DR15-(HSC3 and SAS), and HLA-DR53-(HSC-4 and Sa-3) expressing tumor cells, respectively. These responses were abrogated by an antibody for HLA-DR. All HTL lines did not respond to

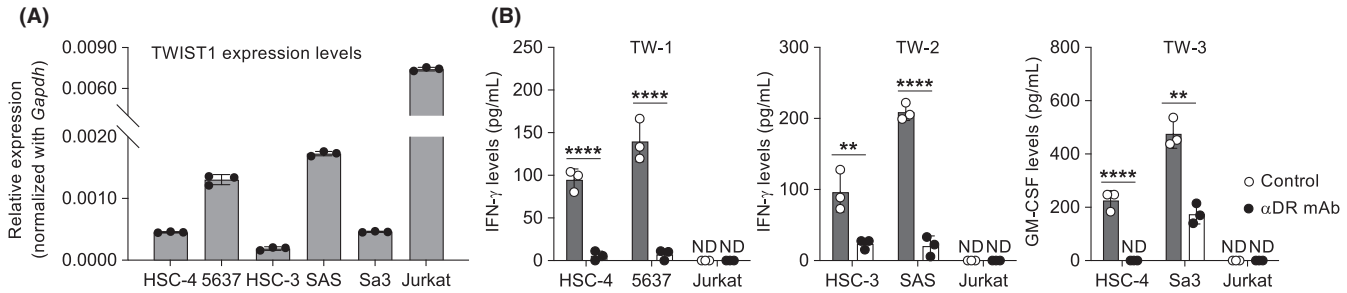

FIGURE 2 Effective responses of $TWIST1_{140–162}$-specific HTLs to tumor cells expressing TWIST1. (A) Expression levels of *Twist1* in human tumor cell lines HSC-4, 5637, HSC-3, SAS, Sa3, and Jurkat cells were evaluated by qPCR. (B) $TWIST1_{140–162}$-specific HTL lines (TW-1, TW-2, and TW-3) were co-cultured with tumor cell lines that were matched for HLA-DR subtype in the absence or presence of αDR mAb. Jurkat cells were used as a negative control due to its loss of cell surface HLA class II. Supernatants were collected after 24 h and analyzed by ELISA for production of IFN-γ or GM-CSF. Data are shown as mean ± SE. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; unpaired *t*-test (B). ND, not detected. Experiments were performed with at least three biological replicates and are representative of at least two independent experiments

Jurkat cells, which were negative for HLA class II (Figure 2B). These results suggested that the defined $TWIST1_{140–162}$ peptide could efficiently activate HTLs reactive to TWIST1-positive tumor cells, and TWIST1 would be a highly immunogenic antigen favorable as an immunological target.

3.3 | High frequency of TWIST1-specific HTLs in the periphery of patients with breast cancer expressing TWIST1

Because we found that $TWIST1_{140–162}$-specific HTL lines responded to endogenous TWIST1-expressing tumor cell lines, we addressed whether TWIST1 was highly immunogenic enough to spontaneously stimulate TWIST1-specific T-cells in the body. Therefore, we stimulated purified $CD4^+$ T-cells derived from a patient with breast cancer expressing TWIST1. In vitro stimulations with the $TWIST1_{140–162}$ peptide easily expanded HLA-DR-restricted $TWIST1_{140–162}$-reactive HTLs from a patient (BC1) with breast cancer expressing TWIST1 (Figure 3A). Based on these data, we hypothesized that there was a higher frequency of TWIST1-specific HTLs in the periphery of breast cancer patients compared with that of healthy donors. To address that, we stimulated PBMCs of five healthy donors (HD1, 2, 3, 4, and 5) and eight breast cancer patients (BC2, 3, 4, 5, 6, 7, 8, and 9) with the $TWIST1_{140–162}$ peptide once and assessed their specific T-cell responses to the $TWIST1_{140–162}$ peptide in ELISA or ELISPOT assay. As expected, we detected a $TWIST1_{140–162}$ peptide-specific T-cell response in $CD4^+$ T-cells of breast cancer patients, but not in those of the healthy donors (Figure 3B). Although we did not detect TWIST1 expression in tumor tissues of BC2, 3, and 7 in spite of high frequencies of TWIST1-specific $CD4^+$T-cells in their periphery, this might reflect incomplete coverage of TWIST1 expression due to the limited tumor mass available for this study (Table 1). Cesson and colleagues also experienced a similar situation when they analyzed the immunogenicity of MAGE-A3 in cancer patients.[30] In some breast cancer patients BC8 and BC9, the $TWIST1_{140–162}$ peptide-specific T-cell response was restricted to HLA-DR, even though there were no specific responses in healthy donors even in the ELISPOT assay (Figure 3C). These findings suggested that TWIST1 is highly immunogenic and naturally activates TWIST1-specific T-cells in breast cancer patients.

Interestingly, we detected $TWIST1_{140–162}$ peptide-specific production of not only IFN-γ but also TNFα and granzyme B in the samples from BC5, BC6, and BC7, indicating that they showed a Th1 cell phenotype (Figure 4). As we had previously demonstrated that granzyme B-producing HTLs directly killed tumor cells,[31,32] these TWIST1-specific HTLs would show antitumor cytotoxicity. These results suggested that the $TWIST1_{140–162}$ peptide could effectively activate TWIST1-specific HTLs to enhance cell-mediated immunity against tumors expressing TWIST1/2.

3.4 | Highly immunogenic activity of the $TWIST1_{140–162}$ peptide in vivo

To address whether the $TWIST1_{140–162}$ peptide effectively expands TWIST1-specific HTLs in vivo, we administrated the peptide to HLA-DR1-transgenic mice. The mice were vaccinated with the $TWIST1_{140–162}$ peptide on days 0 and 7, and then sacrificed to evaluate $TWIST1_{140–162}$ peptide-specific T-cell responses in draining lymph nodes on day 10. We detected $TWIST1_{140–162}$ peptide-specific HTL responses, which were restricted to HLA-DR1, but not DR9 (Figure 5A,B). As immune checkpoint inhibitors (ICIs) such as anti-PD-L1 mAb promote T-cell activity by blocking inhibitory signals to T-cells, we simultaneously introduced an anti-PD-L1 mAb with $TWIST1_{140–162}$ peptide vaccination. As expected, treatment with the anti-PD-L1 mAb significantly expanded TWIST1-specific HTLs in mice that received the $TWIST1_{140–162}$ peptide vaccine (Figure 5C), suggesting that the combination therapy of the $TWIST1_{140–162}$ peptide vaccine and ICIs had antitumor effects.

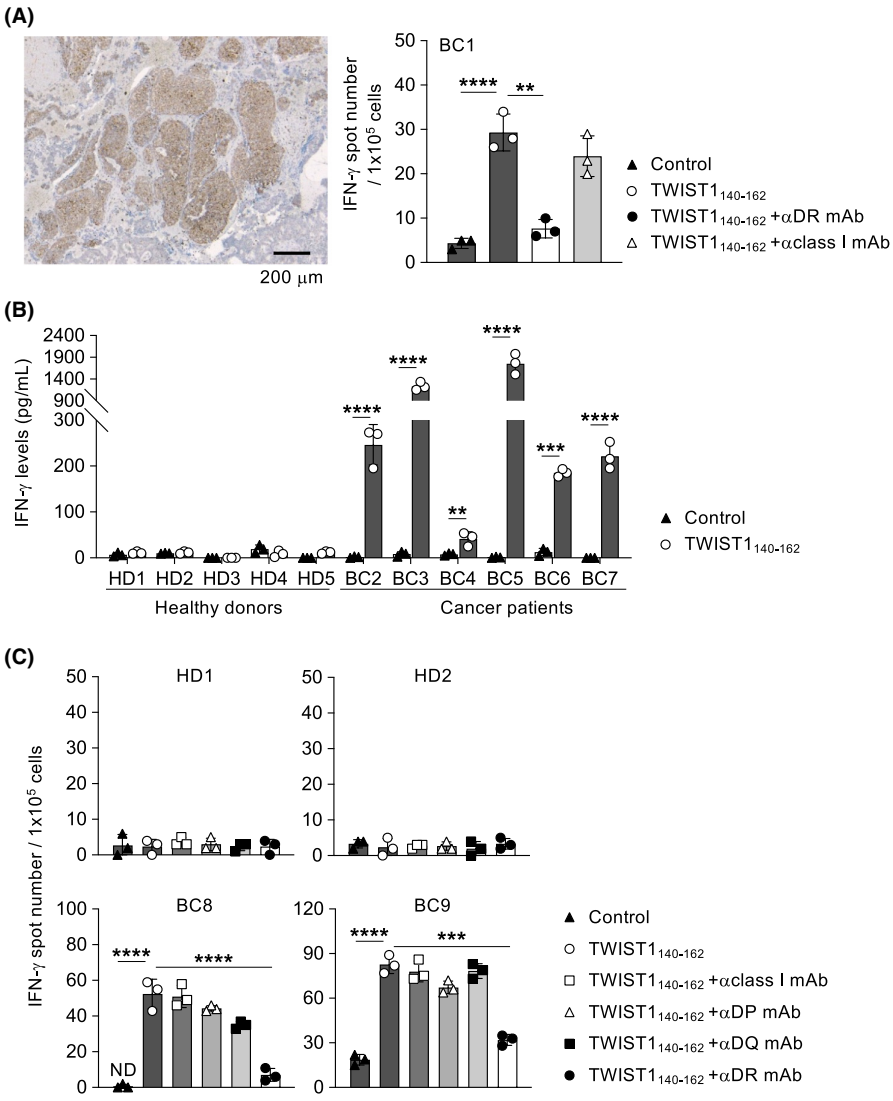

FIGURE 3 Pre-existence of $TWIST1_{140-162}$-specific HTLs in the periphery of patients with breast cancer. (A) Representative IHC image for TWIST1 in a breast cancer sample is shown (left panel). Scale bar indicates 200 μm. $CD4^+$ T-cells derived from a patient with breast cancer positive for TWIST1 (BC1) were stimulated with autologous PBMCs in the presence of $TWIST1_{140-162}$ peptide. After three stimulation cycles, the BC1-derived $CD4^+$ T-cell line was co-cultured with autologous PBMCs in the presence of irrelevant peptide (Control), $TWIST1_{140-162}$ peptide, $TWIST1_{140-162}$ peptide plus αDR mAb, or $TWIST1_{140-162}$ peptide plus αclass I mAb in an ELISPOT plate. IFN-γ-producing cell numbers were assessed by ELISPOT assay (right panel). (B) PBMC derived from five healthy donors (HD1, HD2, HD3, HD4, and HD5) and six breast cancer patients (BC2, BC3, BC4, BC5, BC6, and BC7) were stimulated with $TWIST1_{140-162}$ peptide in the presence of IL-2 (10 IU/ml). Seven days later, PBMCs were washed with PBS and then stimulated with irrelevant (Control) or $TWIST1_{140-162}$ peptide. Supernatants were collected after 24 h to assess IFN-γ production by ELISA. (C) Frequency of TWIST1-specific T-cells was addressed by ELISPOT assay. PBMCs derived from healthy donors (HD1 and HD2) and breast cancer patients (BC8 and BC9) were stimulated with irrelevant (Control) or $TWIST1_{140-162}$ peptide in the presence of antibody against HLA class I, DP, DQ, or DR, respectively. IFN-γ-producing cell numbers were measured by ELISPOT assay. Data are shown as mean ± SE. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; unpaired *t*-test (B) and one-way ANOVA with interaction followed by Tukey's multiple comparisons test (A, C). ND, not detected. Experiments were performed with at least three biological replicates

4 | DISCUSSION

The marked therapeutic efficacy of ICIs clearly demonstrated that the effector functions of T-cells are suppressed in cancer-bearing hosts beyond expectations, even though tumor cells express highly immunogenic antigens such as neoantigens. That is, the conventional strategy for therapeutic cancer vaccines, whose goal is just to increase tumor-specific T-cells, would not provide the expected antitumor effects. Indeed, a preclinical study showed that a neoantigen cancer vaccine did not result in significant tumor regression without ICIs, although the vaccine efficiently expanded neoantigen-specific T-cells.[33] These observations suggest that cancer vaccines may result in favorable effects when combined with ICIs, even if the target antigens are tumor-associated antigens

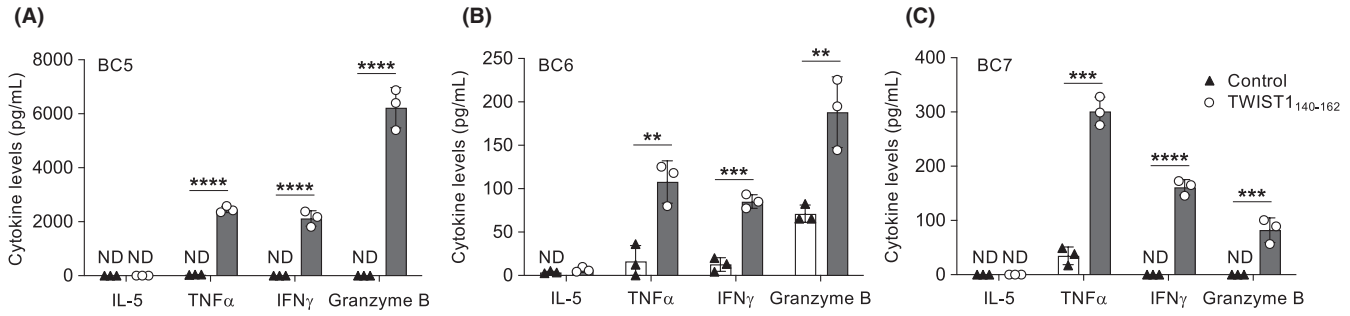

FIGURE 4 Th1 phenotype of $TWIST1_{140-162}$-specific HTLs in the patients with breast cancer. PBMCs derived from breast cancer patients BC5 (A), BC6 (B), and BC7 (C) were stimulated with $TWIST1_{140-162}$ peptide in the presence of IL-2 (10 IU/ml). Seven days later, PBMCs were washed with PBS and then stimulated with irrelevant (Control) or $TWIST1_{140-162}$ peptide. Supernatants were collected after 24 h to assess by ELISA the production of IL-5, TNFα, IFN-γ, and granzyme B. Data are shown as mean ± SE. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; unpaired *t*-test. ND, not detected. Experiments were performed with at least three biological replicates

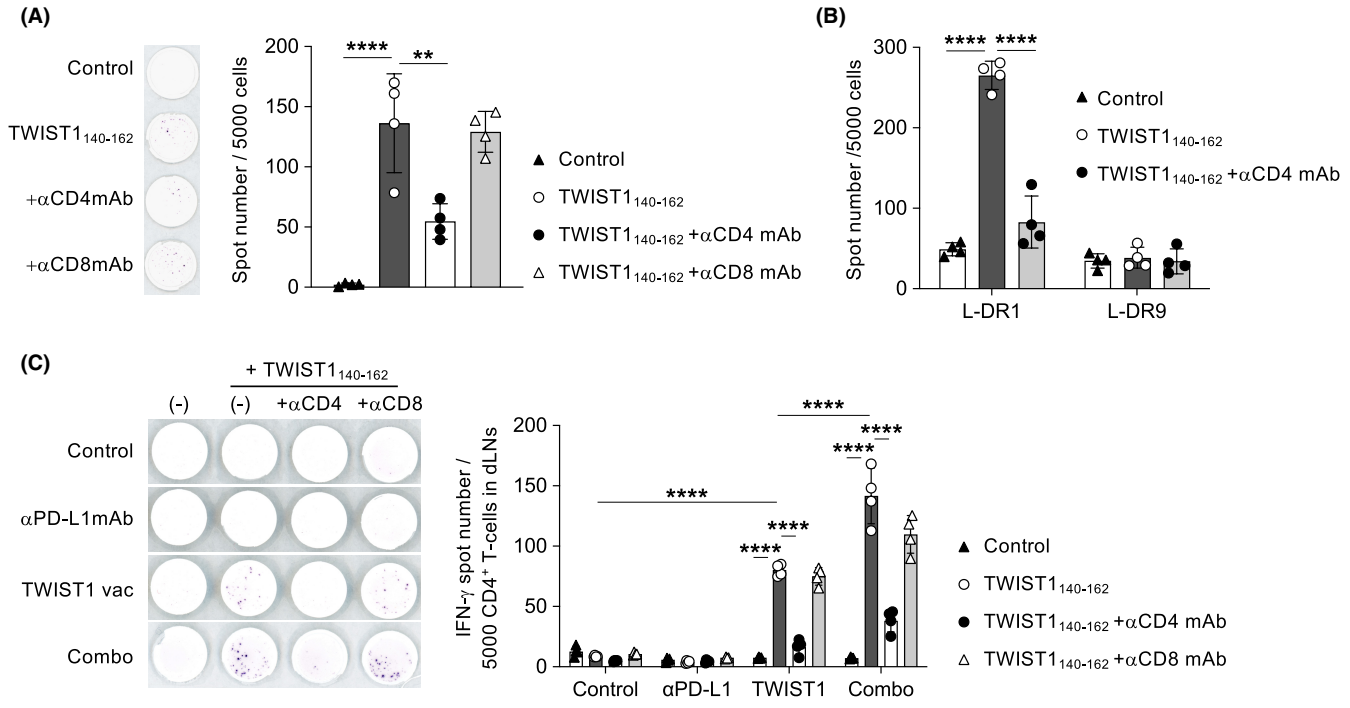

FIGURE 5 Immunogenicity of $TWIST1_{140-162}$ peptide in vivo using humanized mice. HHDII-DR1 mice ($N = 4$ in each group in all experiments) were intradermally administrated with the $TWIST1_{140-162}$ peptide (100 μg) with cGAMP (10 μg) on days 0 and 7. After 3 days, their draining lymph nodes were collected for evaluating $TWIST1_{140-162}$ peptide-specific T-cell responses by ELISPOT assay. (A) Their draining lymph node cells were stimulated with an irrelevant peptide (Control), $TWIST1_{140-162}$ peptide, $TWIST1_{140-162}$ peptide plus anti-mouse CD4 (αCD4) mAb, or $TWIST1_{140-162}$ peptide plus anti-mouse CD8 (αCD8) mAb for 24 h. Representative images for IFN-γ spots are shown on the left. (B) Their lymphocytes were co-cultured with $TWIST1_{140-162}$ peptide-pulsed L-cells expressing HLA-DR1 or DR9 (as a negative control) for 24 h. (C) Mice received intraperitoneal anti-PD-L1 (αPD-L1) mAb (100 μg) injection simultaneously with $TWIST1_{140-162}$ peptide vaccination on days 0 and 7, and then their T-cell responses were addressed in the same way as in (A). Representative images for IFN-γ spots are shown on the left. Data are shown as mean ± SD. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; one-way ANOVA with interaction followed by Tukey's multiple comparisons test. Experiments were performed with at least three biological replicates and are representative of at least two independent experiments

(TAAs) such as shared tumor antigens. Moreover, if the therapeutic outcome of TAA-targeting vaccine therapy is comparable with neoantigen-targeting when ICIs are combined, it would reduce the cost of cancer therapy more efficiently and be more economical compared with using neoantigens.

We observed strong expression of TWIST1 in tumor cells but not normal cells in an IHC analysis using breast cancer specimens, suggesting that tumor cells expressed much higher levels of TWIST1 compared with normal cells, in which TWIST1 was also expressed at a low level. Due to the TWIST1 expression pattern, TWIST1 is categorized as a TAA, which had the potential to be expressed in normal cells at a low level. These seem to be undesirable target antigens for cancer immunotherapy because the T-cells that have high affinity T-cell receptors (TCR) for MHC-presenting

peptides derived from normal tissues are eliminated during T-cell development in the thymus. However, if transformed tumor cells have upregulated expression of TAAs in the late phase of tumor progression, some TAA peptides would be presented on the MHC of tumor cells. This increased MHC/peptide complex could change the immunogenicity of the tumor cells from low to high, and highly immunogenic tumor cells could be easily targeted even by T-cells with low affinity TCRs. Indeed, we also generated with high efficiency TWIST1-specific T-cell lines at higher peptide concentrations in vitro (data not shown), and this is important in terms of preventing effector T-cells activated by cancer vaccines from targeting normal tissues. That means that TWIST1-specific T-cells would recognize and kill only tumor cells with high expression of TWIST1, but not normal cells, because TWIST1-specific T-cells would not respond to cells expressing low levels of TWIST1. Taken together, TAAs seem to be beneficial target antigens for cancer immunotherapy.

The function of TWIST1 is still controversial. Recent studies have shown that TWIST1 not only contributes to metastasis of tumor cells through EMT,[34] but also is involved in resistance to chemotherapy.[35] However, in either case, TWIST1 is highly expressed in metastatic tumor cells and therefore is a potential therapeutic target for inhibiting tumor metastasis and treating metastatic tumors. Especially in patients with chemotherapy-resistant tumors, cancer immunotherapy targeting TWIST1 would provide an alternative treatment strategy.

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DISCLOSURE

The authors have no conflict of interest.

ETHICS STATEMENT

- Approval of the research protocol by an Institutional Reviewer Board: This study was approved by the Research Ethics Committee of Asahikawa Medical University and was performed in accordance with the Declaration of Helsinki.
- Informed Consent: Written informed consent was obtained from all donors who provided samples.
- Registry and the Registration No. of the study/trial: N/A.
- Animal Studies: The protocols for animal studies were approved by the Asahikawa Medical University Institutional Animal Care and Use Committee.

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