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

Yuki Yajima1 | Akemi Kosaka2 | Kei Ishibashi3 | Shunsuke Yasuda3 |
Hiroki Komatsuda2 | Toshihiro Nagato2 | Kensuke Oikawa2 | Masahiro Kitada3 |
Masanori Takekawa1 | Takumi Kumai4 | Kenzo Ohara4 | Takayuki Ohkuri2 |
Hiroya Kobayashi2

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 TWIST1140–162, 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
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;\(^2,4\) (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 SYFPEITHI (http://www.syfpeithi.de), Immune Epitope (http://tools.iedb.org/mhcii/), and NetMHCIIpan-4.0 (https://services.healthtech.dtu.dk/services.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
peptide TWIST1 Scripture (SDKLSKIQLAARYIDFLYQV) and TWIST1 Scripture (KMASCYSYVAHERLSYAFSVWMEG), 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. 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 PrimerScript 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 Scripture (Hs00498991_s1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Hs02758991_g1). Gapdh was used as an internal control and to

### 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               | II                | 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 ductal carcinoma; ILC, invasive lobular carcinoma, NE, not evaluated.

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

| Candidates | Allele       | SYFPEITI Score (Larger is better) | IMMUNE EPITOPE Adjusted rank (Smaller is better) | NetMHCII-pan 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.
normalize each mRNA expression level, which was calculated in each experiment using the ΔΔCt method.

2.6 | Addressing TWIST1-specific responses with established HTLs

HTLs (1–1.5 \times 10^5) were co-cultured with irradiated autologous PBMCs (1.5 \times 10^5), HLA-DR-expressing L-cells (3 \times 10^5), tumor cell lines (3 \times 10^5), or DCs (5 \times 10^5) in some experiments. Human tumor cell lines were treated with 500 U/ml IFN-γ for 48h 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-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 \times 10^5) of healthy donors and patients with breast cancer were stimulated with the TWIST1\textsubscript{140-162} peptide (10 μg/ml) in the presence of IL-2 (10 IU/ml) in 24-well plates as described previously.\textsuperscript{28} Seven days after peptide stimulation, counts of IFN-γ-producing cells were assessed using ELISPOT assay.

2.8 | Vaccination with TWIST1\textsubscript{140-162} peptide into humanized mice

HHDII-DR1 mice were intradermally administrated with the TWIST1\textsubscript{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) at 100 μg in 200 μl of PBS/shot) with the peptide vaccine.

2.9 | ELISPOT assay

Enzyme-linked immunospot assay was performed using a ELSpot kit (Mabtech) according to the manufacturer’s instructions as described previously.\textsuperscript{29} Peptide-stimulated human PBMCs (2 \times 10^5) or mouse lymphocytes (5 \times 10^5) were cultured in the presence of peptide (3 μg/ml) in a MAHAS4510 plate (Millipore) for 24h, 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 \times 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.\textsuperscript{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\textsubscript{140-162} (SDKLSKIQTLEKLAARYIDFLYQV), whose sequence is shared with TWIST2\textsubscript{126-130} and TWIST1\textsubscript{171-194} (KMCASCSYVAHERLSYAFSVWRMEG) 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\textsubscript{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\textsubscript{171-194+}-peptide-specific responses, we analyzed the immunogenicity of TWIST1\textsubscript{140-162} peptide in further 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 TWIST1140–162-specific HTLs to autologous PBMCs in the presence of TWIST1140–162 peptide using anti-HLA-DR or anti-HLA class I mAbs. The IFN-γ production of all TWIST1140–162-specific HTLs was inhibited by the antibody for HLA-DR, but not HLA class I, indicating that their recognition of the TWIST1140–162 peptide was restricted to HLA-DR (Figure 1B). Furthermore, we addressed the reactivity of TWIST1140–162-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 TWIST1140–162 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 TWIST1140–162-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 TWIST1140–162-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
Jurkat cells, which were negative for HLA class II (Figure 2B). These results suggested that the defined TWIST1\textsubscript{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\textsubscript{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\textsuperscript{+} T-cells derived from a patient with breast cancer expressing TWIST1. In vitro stimulations with the TWIST1\textsubscript{140–162} peptide easily expanded HLA-DR-restricted TWIST1\textsubscript{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 this, 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\textsubscript{140–162} peptide once and assessed their specific T-cell responses to the TWIST1\textsubscript{140–162} peptide in ELISA or ELISPOT assay. As expected, we detected a TWIST1\textsubscript{140–162} peptide-specific T-cell response in CD4\textsuperscript{+} T-cells derived from a patient with breast cancer expressing TWIST1. Although we did not detect TWIST1 expression in tumor tissues of BC2, 3, and 7 in spite of high frequencies of TWIST1-specific CD4\textsuperscript{+} 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.\textsuperscript{30} In some breast cancer patients BC8 and BC9, the TWIST1\textsubscript{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\textsubscript{140–162} peptide-specific production of not only IFN-\textgamma but also TNF-\alpha 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 granyme B-producing HTLs directly killed tumor cells,\textsuperscript{31,32} these TWIST1-specific HTLs would show antitumor cytotoxicity. These results suggested that the TWIST1\textsubscript{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\textsubscript{140–162} peptide in vivo

To address whether the TWIST1\textsubscript{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\textsubscript{140–162} peptide on days 0 and 7, and then sacrificed to evaluate TWIST1\textsubscript{140–162} peptide-specific T-cell responses in draining lymph nodes on day 10. We detected TWIST1\textsubscript{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\textsubscript{140–162} peptide vaccination. As expected, treatment with the anti-PD-L1 mAb significantly expanded TWIST1-specific HTLs in mice that received the TWIST1\textsubscript{140–162} peptide vaccine (Figure 5C), suggesting that the combination therapy of the TWIST1\textsubscript{140–162} peptide vaccine and ICIs had antitumor effects.
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. These observations suggest that cancer vaccines may result in favorable effects when combined with ICIs, even if the target antigens are tumor-associated antigens.
(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

**Figure 4** Th1 phenotype of TWIST1-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 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 peptide. Supernatants were collected after 24 h to assess 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 peptide in vivo using humanized mice. HHDII-DR1 mice (N = 4 in each group in all experiments) were intradermally administrated with the TWIST1 peptide (100 μg) with cGAMP (10 μg) on days 0 and 7. Their draining lymph node cells were collected for evaluating TWIST1 peptide-specific T-cell responses by ELISPOT assay. (A) Their draining lymph node cells were stimulated with an irrelevant peptide (Control), TWIST1 peptide, TWIST1 peptide plus anti-mouse CD4 (αCD4) mAb, or TWIST1 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 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 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.
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, but also is involved in resistance to chemotherapy. 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.

ACKNOWLEDGMENTS
The authors thank Dr. François A Lemonnier the inventor of the HHDII-DR1 mice (Paris University) and Ms. Rie Matsumoto (Asahikawa Medical University) for devotedly maintaining the mice.

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.

ORCID
Hiroki Komatsuda https://orcid.org/0000-0002-3783-2746
Takumi Kumai https://orcid.org/0000-0002-3411-671X
Takayuki Ohkuri https://orcid.org/0000-0002-6889-7809

REFERENCES
1. Brahmer J, Reckamp KL, Baas P, et al. Nivolumab versus docetaxel in advanced squamous-cell non–small-cell lung cancer. N Engl J Med. 2015;373(2):123-135. doi:10.1056/NEJMoa1504627
2. Hacohen N, Fritsch EF, Carter TA, Lander ES, Wu CJ. Getting personal with neoantigen-based therapeutic cancer vaccines. Cancer Immunol Res. 2013 Jul;1(1):11-15. doi:10.1158/2326-6066.CIR-13-0022
3. Kawakami Y, Eliyahu S, Delgado CH, et al. Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. Proc Natl Acad Sci U S A. 1994;91(9):3515-3519. doi:10.1073/pnas.91.9.3515
4. Peoples GE, Goedegebureue PS, Smith R, Linehan DC, Yoshino I, Eberlein TJ. Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide. Proc Natl Acad Sci U S A. 1995;92(2):432-436. doi:10.1073/pnas.92.2.432
5. Ohtake J, Ohkuri T, Togashi Y, Kitamura H, Okuno K, Nishimura T. Identification of novel helper epitope peptides of Survivin cancer-associated antigen applicable to developing helper/killer-hybrid epitope long peptide cancer vaccine. Immunol Lett. 2014;161(1):20-30. doi:10.1016/j.imlet.2014.04.010
6. Kosaka A, Yajima Y, Hatayama M, et al. A stealth antigen SPESP1, which is epigenetically silenced in tumors, is a suitable target for cancer immunotherapy. Cancer Sci. 2021;112(7):2705-2713. doi:10.1111/cas.14973
7. Rooij N van, Buuren MM van, Philips D, et al. Tumor exome analysis reveals neoantigen-specific T-cell reactivity in an ipilimumab-responsive melanoma. J Clin Oncol. 2020:38(31):e439-442. 10.1200/JCO.2020.20.1040
8. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol. 2002;3(11):991-998. doi:10.1038/niumm022
9. Chang AT, Liu Y, Ayyanathan K, et al. An evolutionarily conserved DNA architecture determines target specificity of the TWIST family bHLH transcription factors. Genes Dev. 2015;29(6):603-616. doi:10.1101/gad.242842.114
10. Vaupel P, Kelleher DK, Höckel M. Oxygenation status of malignant tumors: pathogenesis of hypoxia and significance for tumor therapy. Semin Oncol. 2001;28:29-35. doi:10.1056/s0993-7754(01)90210-6
11. Gort EH, van Haafken G, Verlaan I, et al. The TWIST1 oncogene is a direct target of hypoxia-inducible factor-2α. Oncogene. 2008;27(11):1501-1510. doi:10.1038/sj.onc.1210795
12. Martin TA, Goyal A, Watkins G, Jiang WG. Expression of the transcription factors snail, slug, and Twist and their clinical significance of hypoxia inducible factor-1 in human breast cancer. Ann Surg Oncol. 2005;12(8):488-496. doi:10.1145/91.24824.114
13. Martin TA, Goyal A, Watkins G, Jiang WG. Expression of the transcription factors snail, slug, and Twist and their clinical significance of hypoxia inducible factor-1 in human breast cancer. Ann Surg Oncol. 2005;12(8):488-496. doi:10.1145/91.24824.114
14. Alexander NR, Tran NL, Rekapalli H, Summers CE, Glackin C, Heimark RL. N-cadherin gene expression in prostate carcinoma is modulated by integrin-dependent nuclear translocation of Twist1. Cancer Res. 2006;66(7):3365-3369. doi:10.1158/0008-5472.CAN-05-3401
15. Luo G-Q, Li J-H, Wen J-F, Zhou Y-H, Hu Y-B, Zhou J-H. Effect and mechanism of the Twist gene on invasion and metastasis of gastric carcinoma cells. World J Gastroenterol. 2008;14(16):2487-2493. doi:10.3748/wjg.v14.i16.2487
16. Hoek K, Rimml DL, Williams KR, et al. Expression profiling reveals novel pathways in the transformation of melanocytes to melanomas. Cancer Res. 2004;64(15):5270-5282. doi:10.1158/0008-5472.CAN-04-0731
17. Entz-Werlé N, Stoetzel C, Berard-Marec P, et al. Frequent genomic abnormalities at TWIST in human pediatric osteosarcomas. *Int J Cancer*. 2005;117(3):349-355. doi:10.1002/ijc.21068

18. Lee TK, Poon RTP, Yuen AP, et al. Twist overexpression correlates with hepatocellular carcinoma metastasis through induction of epithelial-mesenchymal transition. *Clin Cancer Res*. 2006;12(18):5369-5376. doi:10.1186/1756-9966-28-158

19. Sasaki K, Natsugoe S, Ishigami S, et al. Significance of Twist expression and its association with E-cadherin in esophageal squamous cell carcinoma. *J Exp Clin Cancer Res*. 2009;28(1):158. doi:10.1186/1756-9966-28-158

20. Yang J, Mani SA, Donaher JL, et al. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell*. 2004;117(7):927-939. doi:10.1016/j.cell.2004.06.006

21. Shibata K, Kajiyama H, Ino K, et al. Twist expression in patients with cervical cancer is associated with poor disease outcome. *Ann Oncol*. 2008;19(1):81-85. doi:10.1093/annonc/mdm344

22. Zhi D-Y, Guo Q-S, Li Y-L, et al. Twist1 correlates with poor differentiation and progression in gastric adenocarcinoma via elevation of FGFR2 expression. *World J Gastroenterol*. 2014;20(48):18306-18315. doi:10.3748/wjg.v20.i48.18306

23. Hosono S, Kajiyama H, Terauchi M, et al. Expression of Twist increases the risk for recurrence and for poor survival in epithelial ovarian carcinoma patients. *Br J Cancer*. 2007;96(2):314-320. doi:10.1038/sj.bjc.6603533

24. Lee K-W, Kim JH, Han S, et al. Twist1 is an Independent Prognostic Factor of Esophageal Squamous Cell Carcinoma and Associated with Its Epithelial–Mesenchymal Transition. *Ann Surg Oncol*. 2012;19(1):326-335. doi:10.1245/s10434-011-1867-0

25. Sun S, Du R, Xia L, et al. Twist is a new prognostic marker for renal survival in patients with chronic kidney disease. *Am J Nephrol*. 2012;35(2):141-151. doi:10.1159/000335191

26. Pajot A, Michel M-L, Fayolleau N, et al. A mouse model of human adaptive immune functions: HLA-A2.1+/HLA-DR1-transgenic H-2 class I−/class II-knockout mice. *Eur J Immunol*. 2004;34(11):3060-3069. doi:10.1002/eji.200425463

27. Ohkuri T, Wakita D, Chamoto K, Togashi Y, Kitamura H, Nishimura T. Identification of novel helper epitopes of MAGE-A4 tumour antigen: useful tool for the propagation of Th1 cells. *Br J Cancer*. 2009;100(7):1135-1143. doi:10.1038/sj.bjc.6604966

28. Kosaka A, Ishibashi K, Nagato T, et al. CD47 blockade enhances the efficacy of intratumoral STING-targeting therapy by activating phagocytes. *J Exp Med*. 2021;218(11):e20200792. doi:10.1084/jem.20200792

29. Cesson V, Rivals JP, Escher A, et al. MAGE-A3 and MAGE-A4 specific CD4+ T cells in head and neck cancer patients: detection of naturally acquired responses and identification of new epitopes. *Cancer Immunol Immunother*. 2011;60(1):23-35. doi:10.1007/s00262-010-0916-z

30. Hayashi R, Nagato T, Kumai T, et al. Expression of placenta-specific 1 and its potential for eliciting anti-tumor helper T-cell responses in head and neck squamous cell carcinoma. *Onco Targets Ther*. 2020;10(1):1856455. doi:10.1080/2162402X.2020.1856545

31. Ohara M, Ohara K, Kumai T, et al. Phosphorylated vimentin as an immunotherapeutic target against metastatic colorectal cancer. *Cancer Immunol Immunother*. 2020;69(6):989-999. doi:10.1007/s00262-020-02524-9

32. Salvatori E, Lion L, Compagnone M, et al. Neoantigen cancer vaccine augments anti-CTLA-4 efficacy. *Npj Vaccines*. 2022;7(1):1-10. doi:10.1038/s41541-022-00433-9

33. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest*. 2009;119(6):1420-1428. doi:10.1172/JCI39104

34. Cheng GZ, Chan J, Wang Q, Zhang W, Sun CD, Wang L-H. Twist transcriptionally up-regulates AKT2 in breast cancer cells leading to increased migration, invasion, and resistance to paclitaxel. *Cancer Res*. 2007;67(5):1979-1987. doi:10.1158/0008-5472.CAN-06-1479

How to cite this article: Yajima Y, Kosaka A, Ishibashi K, et al. A tumor metastasis-associated molecule TWIST1 is a favorable target for cancer immunotherapy due to its immunogenicity. *Cancer Sci*. 2022;113:2526-2535. doi:10.1111/cas.15429