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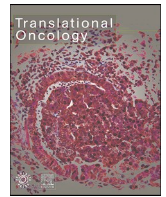

Effectiveness of 4-1BB-costimulated HER2-targeted chimeric antigen receptor T cell therapy for synovial sarcoma

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A R T I C L E I N F O

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A B S T R A C T

Background: Synovial sarcoma is a rare malignant soft-tissue tumor that is prevalent in adolescents and young adults, and poor prognosis has been reported in patients with metastatic lesions. Chimeric antigen receptor (CAR) T-cell therapy is an emerging novel therapy for solid tumors; however, its application in synovial sarcoma has not yet been explored.

Methods: A novel human epidermal growth factor receptor 2 (HER2)-targeted CAR containing scFv-FRP5, CD8α hinge and transmembrane domains as well as 4-1BB costimulatory and CD3ζ signaling domains was developed. Three synovial sarcoma cell lines that expressed the fusion transcript SS18-SSX1/2/4 were used in the study. Cytokine secretion assay, cytotoxicity assay, and real-time cell analysis experiments were conducted to confirm the function of T cells transduced with the CAR gene.

Results: High cell-surface expression of HER2 was observed in all the cell lines. HER2-targeted/4-1BB-costimulated CAR T cells specifically recognized the synovial sarcoma cells, secreted interferon gamma and tumor necrosis factor alpha, and exerted cytotoxic effects in these cells.

Conclusion: To the best of our knowledge, this is the first study to indicate that HER2-targeted CAR T cells are directly effective against molecularly defined synovial sarcoma cells. Furthermore, our findings might set the basis for developing improved CAR T cell-based therapies for chemo-refractory or relapsed synovial sarcoma.

Introduction

Synovial sarcoma, which is characterized by the specific SS18 (also known as SYT)-SSX1/2/4 fusion gene, is a mesenchymal tumor that constitutes approximately 5–10% of soft-tissue sarcomas. Synovial sarcoma does not strictly originate from the synovial tissue and occurs anywhere in the human body. It is most prevalent in individuals aging between 15 and 40 years, with a slight male dominance [1]. Definitive therapy for synovial sarcoma involves radical resection. Patients with an unresectable primary lesion or metastatic lesions are administered chemotherapy and radiotherapy. The 5-year overall survival reportedly ranges from 56% to 76%, but patients who present with metastatic lesions have poorer outcomes than other patients, with a 5-year overall survival lesser than 10% [1,2]. Therefore, the development of new therapies for synovial sarcoma is warranted.

Cancer immunotherapy is one of the promising candidate novel therapies for metastatic, refractory, and/or relapsed synovial sarcoma. In particular, adoptive cell therapies utilizing genetically modified autologous T cells to express chimeric antigen receptor (CAR) have shown remarkable clinical success in hematological malignancies, and

Abbreviations: CAR, chimeric antigen receptor; scFv, single-chain variable fragment; HER2, human epidermal growth factor receptor 2; FBS, fetal bovine serum; E:T, effector: target; RTCA, real-time cell analysis; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon gamma.

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have been further propelled to the forefront of next-generation experimental cell therapies for solid tumors as well [3,4]. CAR is composed of an extracellular single-chain variable fragment (scFv) derived from a monoclonal antibody that recognizes surface antigens, a transmembrane spacer, and an intracellular signaling/activation domain(s). Many tumor-associated antigens have been identified as targets for CAR T-cell therapy for solid tumors [5]. However, no studies have investigated the activity of CAR against synovial sarcoma.

Human epidermal growth factor receptor 2 (HER2) is a 185-kDa transmembrane tyrosine kinase receptor, which promotes cell proliferation, differentiation, and survival through the RAS-RAF-MAPK and PI3K-Akt signaling pathways [6]. HER2-targeted humanized monoclonal antibody trastuzumab has long been used to treat patients with HER2-overexpressing breast cancer and metastatic gastric or gastroesophageal junction adenocarcinoma [7]. HER2 has attracted significant attention in recent times as a novel target for CAR-T cell therapy for bone and soft tissue sarcomas, including osteosarcoma and rhabdomyosarcoma [5,8]. The current clinical trial uses CD28-costimulated HER2-targeted CAR T cells [8,9]. In this study, we developed a novel CAR gene construct to enforce human immune cells to express HER2-targeted CAR containing 4-BB and CD3ζ signaling domains and analyzed the effect of CAR T cells on synovial sarcoma cells. Our findings might lay the foundation for future studies developing improved CAR T cell-based therapies for chemo-refractory or relapsed synovial sarcoma.

Materials and methods

Experimental cell lines and detection of HER2 expression

The cell line SYO-1 was a generous gift from Dr. Akira Kawai (Department of Orthopedic Surgery, Okayama University, Okayama, Japan) [10]. The cell line HS-SY-II was a generous gift from Dr. Hiroshi Sonobe (Department of Pathology, Kochi Medical School, Kochi, Japan) [11]. The cell line Yamato-SS was obtained from RIKEN BRC Cell Bank (Tsukuba, Japan) [12]. The cell line SW982 was obtained from the American Type Culture Collection (Rockville, MD, USA). The cell lines were screened for fusion mRNA using reverse transcriptase PCR [13]. HS-SY-II and Yamato-SS cells expressed the SS18-SSX1 fusion transcript; SYO-1 cells expressed the SS18-SSX2 fusion transcript. As SW982 cells expressed no fusion transcripts, they were excluded from this study. All three cell lines (SYO-1, HS-SY-II, and Yamato-SS) were maintained in Dulbecco's Modified Eagle Medium (Sigma-Aldrich Japan, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS). For detection of surface HER2 expression, cultured cells were harvested using trypsin-EDTA (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and washed once with phosphate-buffered saline. Expression of HER2 was detected by PE anti-human HER-2 antibody (BioLegend, San Diego, CA, USA) and was analyzed by a FACSCaliber flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). PE Mouse IgG1, κ Isotype Ctrl Antibody (BioLegend) was used as the control.

Transduction of T cells with CAR gene

MSCV-IRES-GFP vector plasmid, pEQ-PAM3(-E), and pRDF plasmids were obtained from St. Jude Vector Development and Production Shared Resource (Memphis, TN, USA). ScFv that recognizes human HER2 is derived from the monoclonal antibody FRP5 [14]. We used the splicing by overlap-extension PCR method to ligate gene fragments to create HER2-targeted CAR gene containing scFv-FRP5, CD8α hinge and transmembrane domains, and 4–1BB costimulatory and CD3ζ signaling domains. The cell line HEK293T was obtained from American Type Culture Collection and the cells (3×10^6) were seeded in culture dishes and transfected with the CAR gene using Fugene HD (Promega, Madison, WI, USA) according to the manufacturer's instructions. HeLa cells (1×10^5), obtained from American Type Culture Collection, were used for measuring retroviral titers. Primary human mononuclear cells were obtained from three healthy volunteers. CD3-positive T cells were isolated using RosetteSep™ human T cell enrichment cocktail (STEMCELL Technology, Vancouver, Canada). T cells were incubated with Dynabeads human T-activator CD3/CD28 (Thermo Fisher Scientific, Waltham, MA, USA) in the presence of 200 IU/mL of recombinant human interleukin-2 (rhIL-2; Peprotech, Rocky Hill, NJ, USA) in RPMI-1640 supplemented with 10% FBS. After 48–72 h, T cells were transduced with the CAR gene using Retronection (TakaraBio, Otsu, Japan) according to the manufacturer's instructions with slight modifications. The transduced cells were maintained in RPMI-1640 supplemented with 10% FBS, and 200 IU/mL rhIL-2 was added to the cells every 2–3 days. Surface expression of HER2-targeted CAR on T cells was detected using recombinant human ErbB2/Her2 Fc Chimera Avi-tag Protein (R&D systems, Minneapolis, MN, USA). The immunophenotype of the HER2-targeted CAR T cells was assessed by staining with PerCP anti-CD3 antibody, PE anti-CD56 antibody, PE anti-CD4 antibody, and PerCP anti-CD8 antibody (BioLegend, San Diego, CA, USA) and was analyzed by a FACSCaliber flow cytometer.

Cytokine secretion assay

Synovial sarcoma cells (1×10^5) were seeded in a 96-well flat-bottom plate and allowed to adhere for 12–24 h. HER2-targeted CAR T cells were added to the plate at an effector (E): target (T) ratio of 1:1. In 200 μL of culture medium supplemented with 10% FBS and 10 IU/mL of rhIL-2, synovial sarcoma and HER2-targeted CAR T cells were co-cultured for 24 h. IFN-γ and TNF-α levels in the supernatant were measured using Cytometric Bead Array (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Cytotoxicity assay

Synovial sarcoma cells (2×10^5) were seeded in a 96-well flat-bottom plate and allowed to adhere for 12–24 h. HER2-targeted CAR T cells were added to the plate at an E:T ratio of 4:1 in the presence of low-dose rhIL-2 (10 IU/mL). After 7 days, the growth of tumor cells was observed using an optical microscope (CKX41SF, Olympus Corporation, Tokyo, Japan; magnification x100). The same procedure was used for the WST-8 assay except for the E:T ratios (4:1, 2:1, and 1:1). After 7 days, residual tumor cells were analyzed by the WST-8 assay using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions.

Real-time cell analysis (RTCA)

Synovial sarcoma cells (1×10^4) were seeded in an iCELLigence™ E-plate (ACEA Biosciences, San Diego, CA, USA) and allowed to adhere for 12–24 h. On the following day, HER2-targeted CAR T cells were added at E:T ratios of 2:1 and 1:1 in the presence of low-dose rhIL-2 (10 IU/mL). Cellular growth impedance of cells attached to the plate was measured continuously every 1 h for up to 7 days using real-time cell analyzer iCELLigenceTM (ACEA Biosciences) according to the manufacturer's instructions.

Statistical analysis

Statistical analyses were conducted using EZR software (version 4.0.0) as previously described [15]. Differences in the antitumor effect and cytokine production between cells were analyzed by the Student's *t*-test. All experiments were performed using three technical replicates, and the data are shown as mean ± standard deviation (SD). *P*-value <0.05 was considered statistically significant.

Results

Expression of HER2 on synovial sarcoma cell surface

To examine whether synovial sarcoma cells could be targeted by HER2-targeted CAR T cells, we first examined the expression of HER2 on the surface of three types of synovial sarcoma cells (SYO-1, Yamato-SS, and HS-SY-II). All three cell lines showed high expression of HER2 (Fig. 1).

Establishment of the gene construct and expression of HER2-targeted CARs on T-cell surface

Fig. 2A illustrates the newly created second-generation 4-1BB-costimulated HER2-targeted CAR gene construct. CAR is composed of an extracellular antigen binding domain (scFv), CD8α hinge and transmembrane domains as well as 4-1BB and CD3ζ intracellular signaling domains [16]. The scFv construct is derived from the monoclonal antibody FRP5 [14]. T cells transduced with HER2-targeted CAR gene showed surface expression of HER2-targeted CAR protein, as demonstrated by the recombinant human HER2 Fc chimeric protein (Fig. 2B). The HER2-targeted BB-ζ CAR T cells proliferated exponentially, by an average of 20.3 ± 7.6-fold on day 7, 230.6 ± 76.1-fold on day 14, and 1960.5 ± 129.1-fold on day 21 for three independent donors. The average CD3-positive cell percentage of the final products used for the subsequent functional assays was 92.6 ± 5.3%. The CD8-positive cell percentage was averaged to 51.3 ± 12.7%, whereas the CD4-positive cell percentage was averaged to 45.0 ± 12.6% (Fig. 2C).

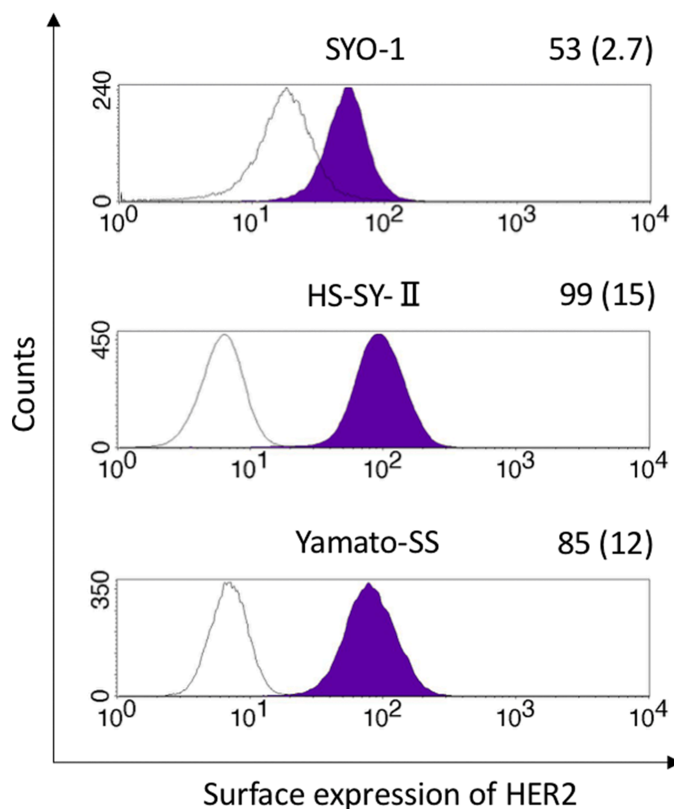

Fig. 1. Surface expression of human epidermal growth factor receptor 2 (HER2) on synovial sarcoma cells.
Each histogram shows the signal intensity of phycoerythrin (PE) channels when the cells were stained with PE anti-human HER2 antibody (filled area) or PE Mouse IgG1, κ Isotype Ctrl Antibody (open area). Mean fluorescence intensity of the filled histogram and the ratio of intensities of the filled and open histograms in parentheses are presented at the top right corner of each histogram. The experiments were performed at least three times, and the representative data are shown.

Specific recognition and cytotoxicity of synovial sarcoma cells by HER2-targeted CAR T cells

A cytokine secretion assay was performed to analyze whether HER2-targeted CAR T cells had a specific response to synovial sarcoma cells. The HER2-targeted CAR T cells released significantly higher levels of tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) into the culture medium than T cells transduced with the empty vector (mock), upon 24 h exposure to synovial sarcoma cells (Fig. 3).

To investigate HER2-specific cytotoxic activity of CAR T cells, SYO-1 synovial sarcoma cells were co-cultured with or without effector cells for 1 week at an E:T ratio of 4:1. Viable SYO-1 cells were rarely observed in the co-culture with HER2-targeted CAR T cells, whereas SYO-1 cells proliferated in the co-culture with mock-transduced T cells or no effector cells (Fig. 4A). The antitumor effect of HER2-targeted CAR T cells was measured quantitatively using a cell proliferation assay (WST-8 assay) and by conducting RTCA. In the WST-8 assay, HER2-targeted CAR T cells from three healthy T-cell donors showed significantly higher antitumor effects against SYO-1, HS-SY-II, and Yamato-SS cells in a dose-dependent manner than mock-transduced control T cells at almost all E:T ratios (Fig. 4B). RTCA showed that HER2-targeted CAR T cells exerted significant growth-suppressive effects on all three cell lines at E:T ratios of 2:1 and 1:1, compared to mock-transduced T cells (Fig. 5).

Discussion

In this study, we constructed a new 4-1BB-costimulated HER2-targeted CAR gene. We sought to show the potential of CAR T-cell therapy in treating refractory or relapsed synovial sarcoma, which have a dismal prognosis with existing treatment modalities. For this purpose, we used three cell lines that have been shown to express the chimeric gene SS18-SSX1/2/4, which results in genetic lesions with disease-causing property and diagnostic value. First, we observed that all three cells expressed high levels of HER2 on the cell surface. Hence, we considered that HER2 might be a vital target for cell therapy for patients with synovial sarcoma who are refractory to conventional chemotherapy and radiotherapy. Next, our in vitro analysis showed that T cells transduced with the HER2-targeted CAR gene designed herein specifically recognized the synovial sarcoma cells, secreted IFN-γ and TNF-α, and exerted strong cytotoxicity in these cells. To the best of our knowledge, our study is the first to indicate that molecularly defined synovial sarcoma cells are sensitive to HER2-targeted CAR T cells.

Cell-based cancer immunotherapy has been one of the promising strategies for treating metastatic, refractory, and/or relapsed synovial sarcoma. Recently, T cell receptor-T cell therapy targeting cancer-testis antigen NY-ESO-1 via genetically-engineered autologous T cells expressing an affinity-enhanced T cell receptor, which recognizes an HLA-A2-restricted NY-ESO-1/LAGE1a-derived peptide, has been investigated in a pilot phase I/II trial [17]. In this trial, 6 of 12 patients infused with the cell product show an antitumor response that lasts over several months [17]. However, in the same trial, 120 patients with synovial sarcoma were screened for HLA-A*02 and NY-ESO-1 expression, which resulted in the enrollment of only 15 patients with relapsed and/or metastatic synovial sarcoma. In addition, we previously found defective or downregulated expression of HLA class I in about 30% of the tumors from patients with synovial sarcoma, which could be one of evasion mechanisms from cytotoxic T cell attack [18]. Despite the promising clinical responses, the applicability of this cell therapy may not be sufficiently high to cover most patients with metastatic, chemo-refractory, and/or relapsed synovial sarcoma. In contrast, CAR T cells could directly recognize the target antigen expressed on the surface of cancer cells, without the need for HLA selection in patients (i.e., antigen processing and HLA presentation were not required).

Overexpression of HER2 has been observed in 5–30% of invasive

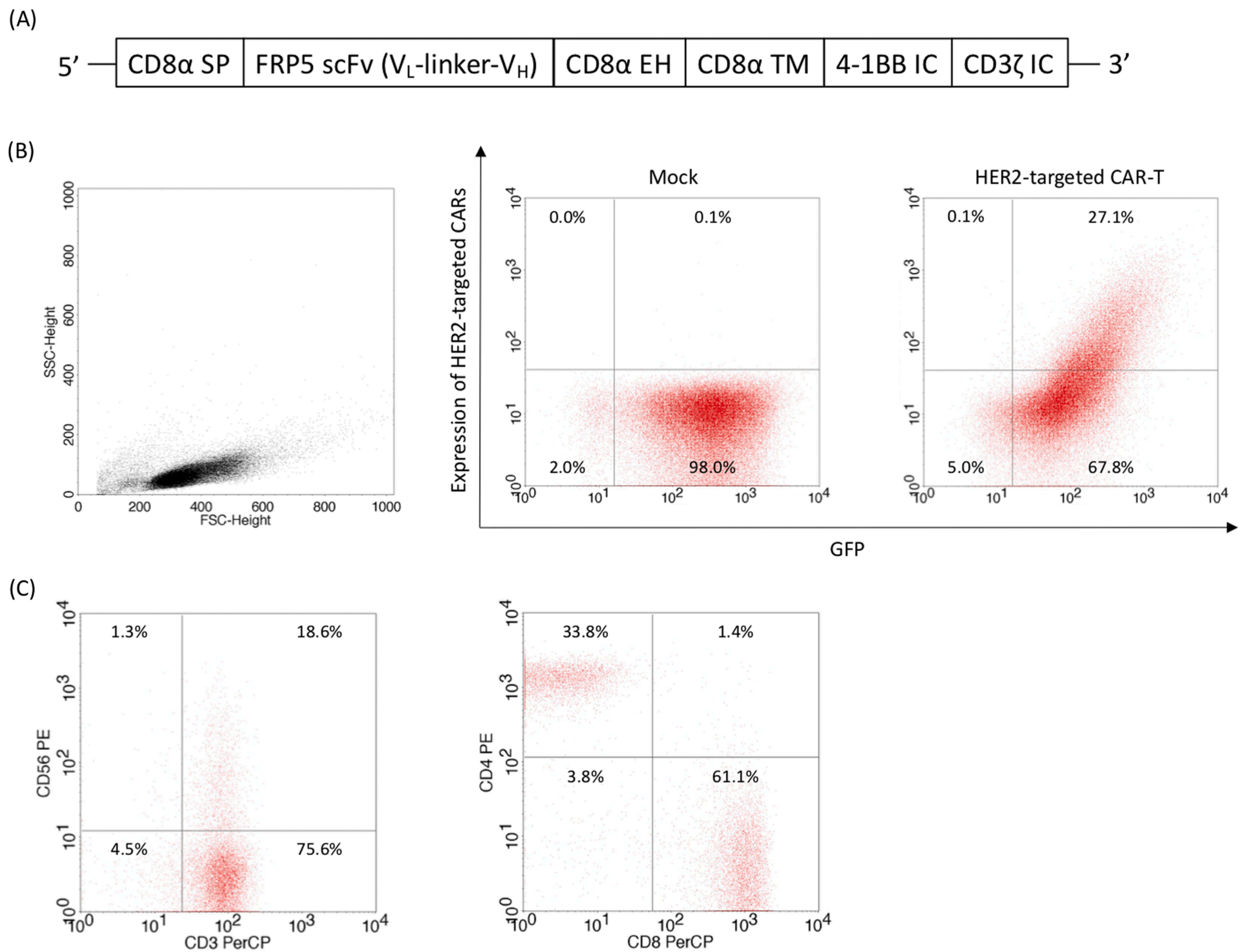

Fig. 2. Establishment of the gene construct and T-cell surface expression of HER2-targeted chimeric antigen receptor (CAR).
(A) Construction of the second-generation anti-HER2 CAR gene. SP: signal peptide domain, scFv: single-chain variable fragment domain, EH: extracellular hinge domain, TM: transmembrane domain, IC: intracellular domain.
(B) Expression of HER2-targeted CARs on the surface of transduced T cells. The horizontal axis represents the level of green fluorescent protein (GFP). The vertical axis represents the surface expression levels of HER2-targeted CAR, as demonstrated by the recombinant human HER2 Fc chimeric protein, followed by staining with PE-conjugated F(ab')2 fragment goat anti-human IgG secondary antibody.
(C) Purity and phenotype of HER2-targeted CAR T cells analyzed through functional assays. The cells were stained with CD3 PerCP/CD56 PE or CD8 PerCP/CD4 PE.

breast, 10–30% of gastric, 0–83% of esophageal, 20–30% of ovarian, 14–80% of endometrial, and approximately 20% of lung cancers [7]. Immunohistochemical analysis has shown that 78% of osteosarcomas and 33% of rhabdomyosarcomas exhibit HER2 expression [19,20]. Furthermore, 20–50% clinical specimens of synovial sarcoma are HER2-positive [21–24]. Ahmed et al. [25] reported that HER2-specific CAR T cells exhibit powerful cytotoxicity in osteosarcoma cells that express HER2 at low levels and cannot be recognized by monoclonal antibodies with the same specificity. Preclinical studies and clinical trials of HER2-targeted CAR T-cell therapy have been performed on solid tumors, including colorectal, breast, gastric, and lung cancers, glioblastoma, and some sarcomas [26]. Subsequently, a clinical trial of HER2-targeted CAR T-cell therapy against HER2-positive sarcomas has been conducted [8]. In this trial, HER2-targeted CAR T cells were infused into 19 patients, including 16 osteosarcoma, 1 Ewing sarcoma, 1 desmoplastic small round cell tumor, and 1 primitive neuroectodermal tumor. None of the patients had serious adverse events, and 4 of 19 patients had stable disease after cell infusion. Recently, the same trial reported long-term tumor control with multiple CAR T-cell infusions in a 7-year-old male with rhabdomyosarcoma with bone marrow metastases [27]. Additionally, several clinical trials of HER2-targeted CAR T-cell therapy for pediatric brain tumors are currently ongoing [28].

Three monoclonal antibody clones, 4D5, CHA21, and FRP5, have been used to form anti-HER2 scFv in HER2-targeted CAR T cells [25,29, 30]. The clone 4D5 is used for trastuzumab, and has also been widely applied in designing HER2-targeted CAR gene constructs. Notably, HER2-targeted CAR T cells administered to a fatal patient reported by the National Cancer Institute of the United States utilized the clone 4D5 [29]. In this patient, it was presumed that scFv-4D5-bearing CAR T cells are sensitive to a low level of HER2 protein expressed on normal lung epithelial cells. Nonetheless, the underlying causes of the fatal reaction may be attributed to the high affinity of scFv-4D5 [29], costimulatory domains (two costimulatory domains, CD28 and 4-1BB), and high cell dose (10^{10} cells). In order to avoid the adverse features of scFv-4D5, scFv-FRP5 has been employed in recent clinical trials. The HER2-targeted CAR T cells used in the clinical trials performed by Baylor College of Medicine, Houston, USA, for sarcomas and brain tumors, such as glioblastoma, utilize CD28-costimulated CAR gene [8,9,

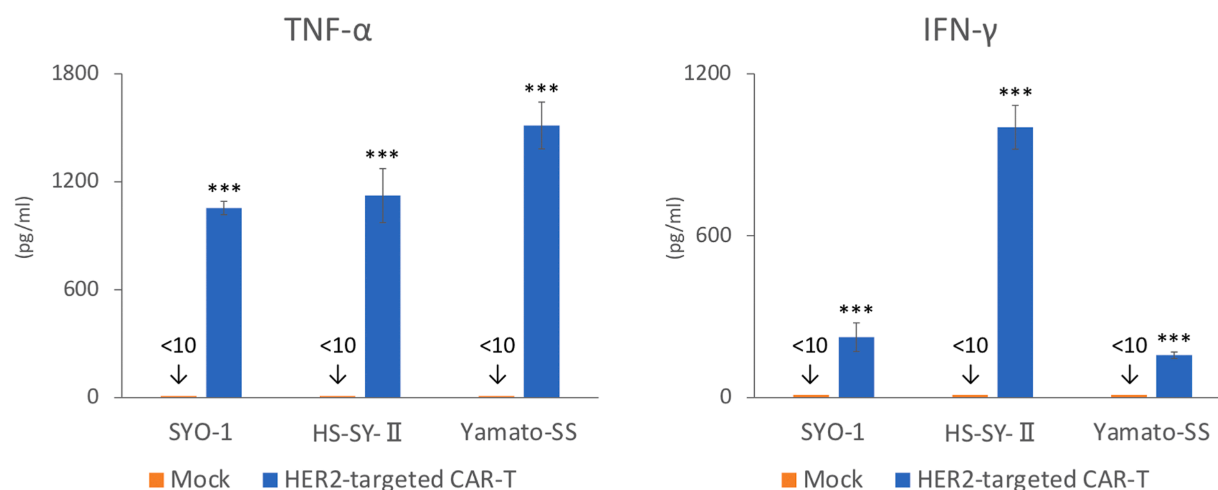

Fig. 3. Secretion of tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) by CAR T cells assessed by cytometric bead array after 24 h exposure to synovial sarcoma cells.
HER2-targeted CAR T cells (blue bars) produced significantly higher levels of TNF-α and IFN-γ than T cells transduced with the empty vector (mock; orange bars). Data are presented as the mean ± standard deviation (SD) of triplicate experiments. The data are representative results from experiments using at least 2 healthy donors. $^{***}P < 0.001$.

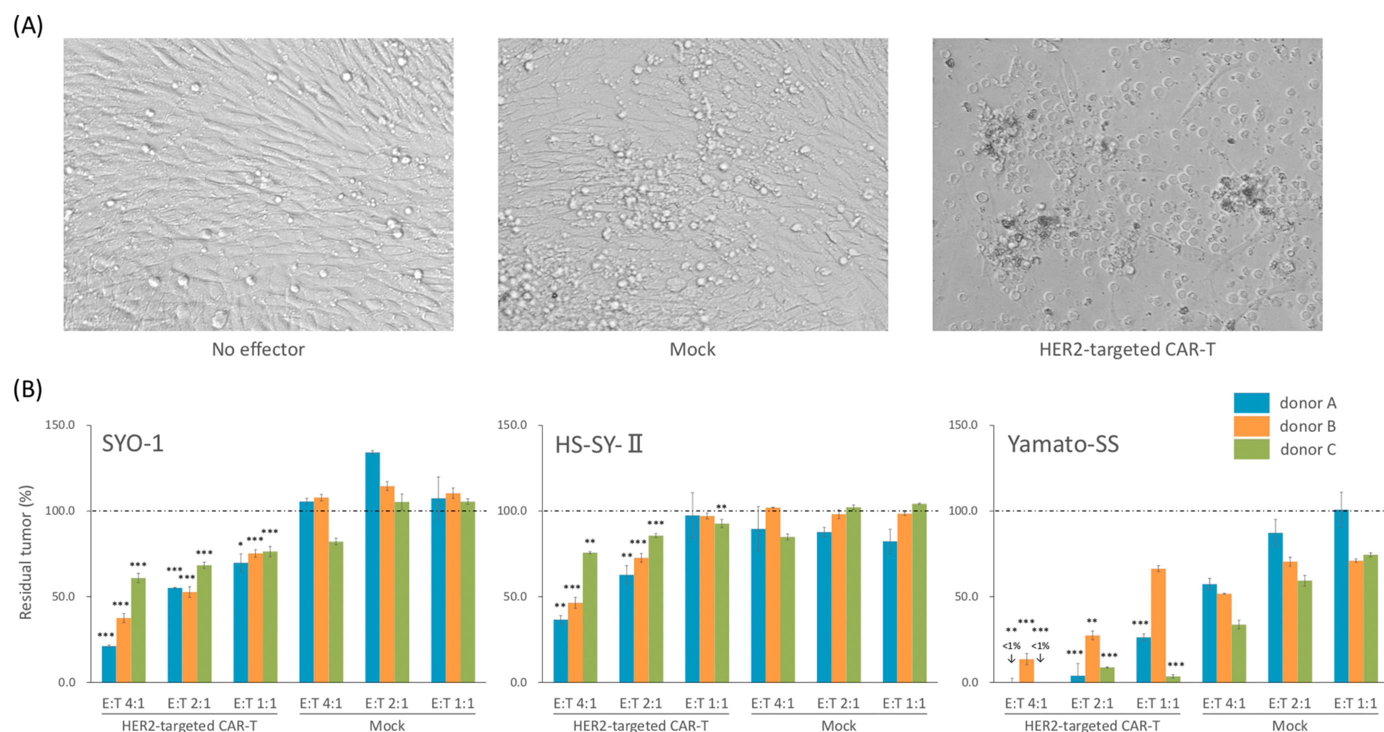

Fig. 4. Antitumor effect of HER2-targeted CAR T cells on synovial sarcoma cells.
(A) Synovial sarcoma cell line SYO-1 was co-cultured with HER2-targeted CAR T cells or mock-transduced T cells at an effector (E): target (T) ratio of 4:1, or cultured without any effector cells for 7 days. Visualization under an inverted microscope shows that in the culture with HER2-targeted CAR T cells, spindle-shaped tumor cells are rarely observed, and round shaped cohered CAR T cells are observed (magnification x100). Similar results were obtained with all three cell lines, and representative pictures are presented.
(B) HER2-targeted CAR T cells from three healthy T-cell donors were co-cultured for 7 days with synovial sarcoma cells at E:T ratios ranging from 1:1 to 4:1. HER2-targeted CAR T cells show significantly higher antitumor effect than mock-transduced T cells. Some inter-individual variation is noticeable. The y-axis represents the percentage of residual tumors (compared with tumor cells without effector cells). Data are presented as the mean ± standard deviation (SD) of triplicate experiments. The data are representative of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

27]. In the present study, we developed a HER2-targeted CAR gene incorporating CD8α hinge and transmembrane domains along with 4-1BB costimulatory and CD3ζ signaling domains. It has been reported that T cells transduced with 4-1BB-costimulated CAR gene show longer persistence than those transduced with CD28-costimulated CAR gene in B-cell acute lymphoblastic leukemia [3,4,31]. In fact, a retrospective analysis of an exploratory clinical study comparing two types of CAR-T cell therapies with CD28 or 4-1BB costimulation in patients with B-cell acute lymphoblastic leukemia found that patients treated with the 4-1BB CAR-T cell therapy showed significantly higher overall survival rate

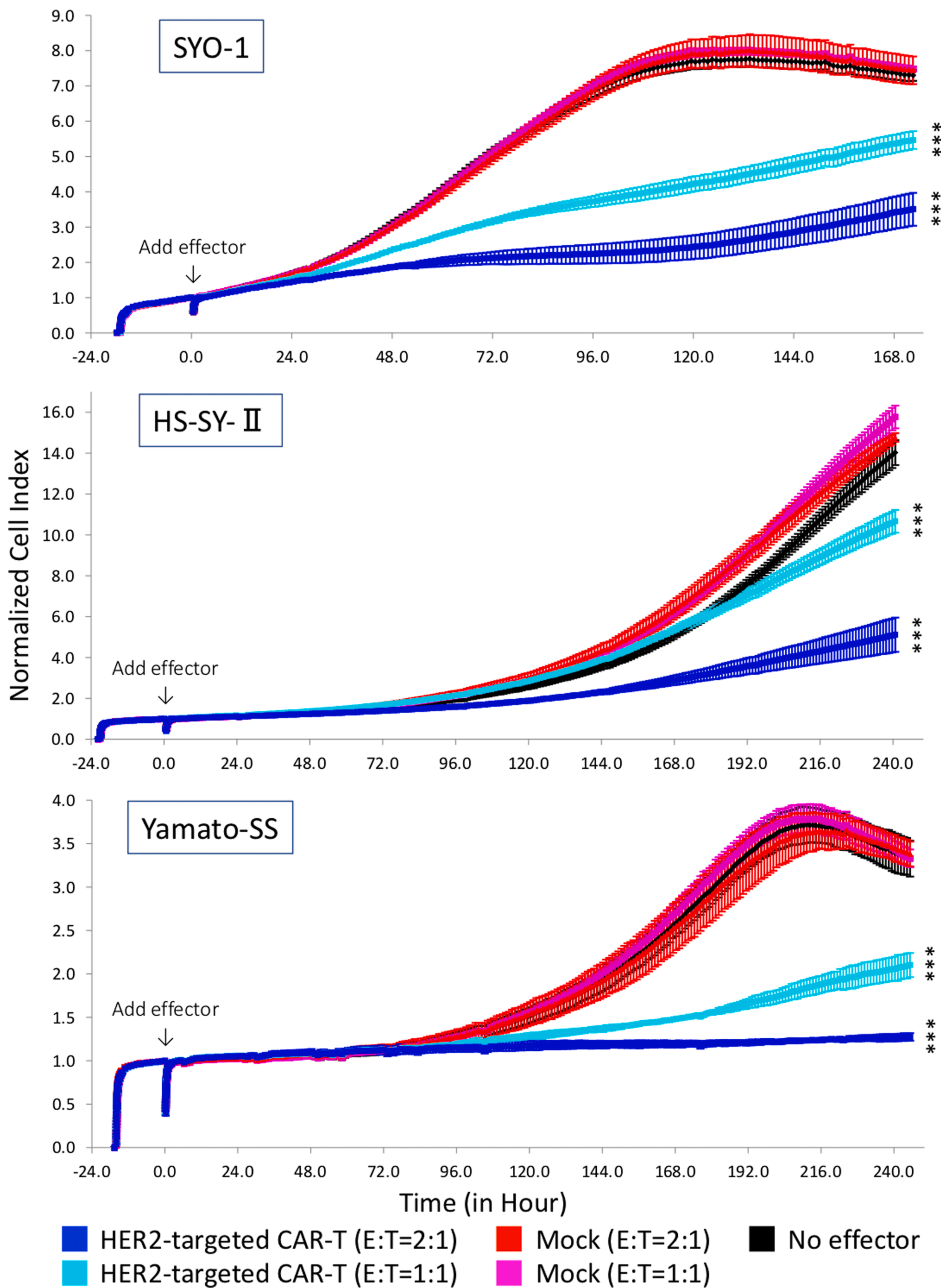

Fig. 5. Quantitative evaluation of the antitumor effect of HER2-targeted CAR T cells on synovial sarcoma cells using real-time cell analysis.
HER2-targeted CAR T cells and mock-transduced T cells were co-cultured with the synovial sarcoma cells at E:T ratios of 1:1 and 2:1. HER2-targeted CAR T cells significantly suppressed tumor cell growth in a dose-dependent manner compared with mock-transduced T cells. ***$P < 0.001$.

than those treated with the CD28 CAR-T cell therapy [32]. However, the suitability of using costimulatory domain for treating solid tumors remains controversial [4,33]. Therefore, future preclinical and clinical trials should compare the effectiveness of different costimulatory domains for improved CAR T cell persistence and tumor regulation.

Nonetheless, this study has a few limitations. First, we used only three cell lines for evaluation of the CAR T cells created in this study. This was because we avoided the use of cell lines that were said to be derived from synovial sarcoma but lacked the SS18-SSX1/2/4 fusion transcripts. Second, we did not evaluate the function of the CAR T cells in a preclinical xenograft model, although our in-vitro data demonstrated selective and highly potent antitumor effects on synovial sarcoma cells. Genetically modified T cells expressing scFv-FRP5-based CD28-costimulated CAR have been shown to exert powerful antitumor effects in preclinical mouse models and in a clinical trial [8,25,27]. Given that two different CD19-targeted CAR-T cell therapies, one with CD28 costimulation and the other with 4-1BB costimulation, showed similar results in complete response rate and event free survival in patients with refractory or relapsed B-cell lymphomas and that 4-1BB-costimulated CAR T cells seemed to have higher persistence after cell infusions than those with CD28 costimulation in patients with B-cell acute lymphoblastic leukemia [3,31], we expect that our CAR T cells harboring scFv-FRP5-based binding domain and 4-1BB costimulatory domain would also work in a preclinical model and in a clinical setting. Third, the CAR construct developed in this study may be further modified to have distinct structural diversities within the hinge and transmembrane domains for further improving CAR functions. Recently, to develop safer CAR-T cell therapy with a reduced probability of severe cytokine release syndrome, researchers made slight modifications such as addition of a few amino acids to the N- and C-terminal ends of CD8α hinge and transmembrane domains in the 4–1BB-costimulated CD19-targeted CAR; these modifications reduced cytokine secretion while preserving cytolytic and antiapoptotic properties [34]. Furthermore, Majzner et al. indicated that replacement of the hinge and transmembrane domains of CD8α with those of CD28 in 4-1BB-costimulated CAR construct resulted in enhanced capacity to recognize tumors with low antigen density and subsequent improvement in cytokine production and tumor eradication in in vitro experiments and preclinical models [35]. However, in our own experience, 4-1BB costimulated NKp44-based CAR incorporating CD28 hinge and transmembrane domains did not outperform the counterpart construct with CD8α hinge and transmembrane domains. Hence, we aim to test whether further modifications of the hinge and transmembrane domains in the current 4–1BB costimulated HER2-targeted CAR gene enhances (or diminishes) its function in future studies.

Conclusions

In conclusion, we developed a novel HER2-targeted 4-1BB-costimulated CAR gene. Some trials have reported an emergence of antigen-loss variants in solid tumors after treatment with CAR T cells [36]. Hence, to better target solid tumors with heterogeneous antigen expression patterns that may be prone to developing antigen-loss variants, some researchers have reported the usefulness of multiple targeting strategies [37]. Furthermore, we recently reported a novel CAR gene utilizing a ligand binding domain of natural cytotoxicity receptor NKp44 [38]. Thus, we speculated that dual targeting might reduce the potential risk of antigen loss in tumor cells, which causes tumor relapse. In the future, we will employ a combinatorial targeting strategy utilizing NKp44-based CAR and HER2-targeted CAR to target solid tumors including, synovial sarcoma.

Declaration of Competing Interest

Chihaya Imai reports patent royalties from Juno Therapeutics, and the other authors have no conflicts of interest to declare.

Ethics approval statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the ethical committee of Niigata University School of Medicine (approval #2015-2686).

Informed consent statement

Informed consent was obtained from all volunteers who participated in this study.

Consent for publication

Not applicable

Availability of data and materials

The data presented in this study are available upon request from the corresponding author.

CRediT authorship contribution statement

Yudai Murayama: Methodology, Investigation, Validation, Formal analysis, Writing - Original Draft; **Hiroyuki Kawashima**: Conceptualization, Resources, Supervision; **Nobuhiro Kubo**: Investigation; **Chansu Shina**: Methodology, Resources; **Yasushi Kasahara**: Methodology, Resources; **Masaru Imamura**: Funding acquisition; **Naoki Oike**: Resources; **Takashi Ariizumi**: Resources; **Akihiko Saitoh**: Resources; **Keichiro Mihara**: Resources; **Hajime Umezu**: Investigation; **Akira Ogose**: Conceptualization, Funding acquisition; **Chihaya Imai**: Project administration, Conceptualization, Resources, Supervision, Funding acquisition, Writing - Review & Editing.

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