対象として血清抗NY-ESO-1抗体価を測定し陽性率を算出した。既存の腫瘍マーカーについて検討する目的で胃癌腫瘍マーカーに関するシステマチックレビューを行った。

（倫理面への配慮）

食道癌臨床生体試料を用いた研究に関しては、東邦大学医学部倫理委員会ならびに東邦大学医療センター大森病院倫理委員会において、研究計画が承認されており、承認された研究計画に従って研究対象であるすべての食道癌患者から文書による臨床研究の承諾を得ている。血液サンプルは、個人情報保護法に従って、連結可能匿名化して保管している。「多施設共同研究による新規腫瘍マーカーの探索と有用性の検討（研究代表者　島田英昭）」審査番号22-047ならびに審査番号21-074

C. 研究結果

消化管癌症例全体での陽性例は 813 例中 113 例(14%)であり、健常者陽性率は 0%であった。臓器別陽性率は、食道扁平上皮癌:31%、胃癌:10%、大腸癌:8%であった。扁平上皮癌と腺癌との比較では、扁平上皮癌で有意に陽性率が高かった(31% vs 9%, P<0.001)。Stage I 症例では、食道癌:16%、胃癌:8%、大腸癌:10%であった。胃癌における既存の腫瘍マーカーとしてはCEA, CA19-9, CA72-4が有用であるが stage I における陽性率は低く早期診断には有用ではない。

D. 考察

胃・大腸腺癌に比較して、食道扁平上皮癌において有意に高い陽性率を示した。stage Iにおいても高い陽性率であった。

E. 結論

NY-ESO-1 抗原タンパクを標的とする血清自己抗体検出系を確立した。食道扁平上皮癌における陽性率は既存の腫瘍マーカーと同等の陽性率であり、特に stage I における陽性率が高かった。

F. 研究発表

1. 論文発表

1) Tokita K, Shimada H et al. Clathrin heavy chain is a useful immunohistochemical markerfor esophageal squamous intraepithelial neoplasia. Esophagus 2013 DOI 10.1007/s10388-013-0381-9

2) Shimada H et al. Clinical significance of serum tumor markers for gastric cancer:a systematic review of literature by the Task Force of the JapaneseGastric Cancer Association. Gastric Cancer 17:26-33, 2013 DOI:10.1007/s10120-013-0259-5

2. 学会発表

1) Shimada H et al. Clinical Significance of Serum SEREX Antibody in Esophageal Carcinomas. 72th JCA, Yokohama, 2013

G. 知的所有権の出願・取得状況

1. 特許取得

なし.

2. 実用新案登録

なし.

3. その他

なし.

III. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kageyama S, Wada H, Muro K, Niwa Y, Ueda S, Miyata H, Takiguchi S, Sugino SH, Miyahara Y, Ikeda H, Imai N, Sato E, Yamada T, Osako M, Ohnishi M, Harada N, Hishida T, Doki Y, Shiku H.	Dose-dependent effects of NY-ESO-1 protein vaccine complexed with cholesteryl pullulan (CHP-NY-ESO-1) on immune responses and survival benefits of esophageal cancer patients.	J Transl Med	11(1)	246	2013
Miyazaki Y, Fujiwara H, Asai H, Ochi F, Ochi T, Azuma T, Ishida T, Okamoto S, Mineno J, Kuzushima K, Shiku H, Yasukawa M.	Development of a novel redirected T-cell-based adoptive immunotherapy targeting human telomerase reverse transcriptase for adult T-cell leukemia.	Blood	13;121(24)	4894-901	2013
Muraoka D., Nishikawa H., Noguchi T., Wang L., Harada N., Sato E., Luescher I., Nakayama E., Kato T., Shiku H.	Establishment of animal models to analyze the kinetics and distribution of human tumor antigen-specific CD8+ T cells.	Vaccine	19;31(17)	2110-8	2013
Iwami K., Natsume A., Ohno M., Ikeda H., Mineno J., Nukaya I., Okamoto S., Fujiwara H., Yasukawa M., Shiku H., Wakabayashi T.	Adoptive transfer of genetically modified Wilms' tumor 1-specific T cells in a novel malignant skull base meningioma model.	Neuro-Oncology	15(6)	747-58	2013
Asai H., Fujiwara H., An J., Ochi T., Miyazaki Y., Nagai K., Okamoto S., Mineno J., Kuzushima K., Shiku H., Inoue H., Yasukawa M.	Co-Introduced Functional CCR2 Potentiates In Vivo Anti-Lung Cancer Functionality Mediated by T Cells Double Gene-Modified to Express WT1-Specific T-Cell Receptor.	PLoS One	8	e56820	2013

Hirayama M., Nishikawa H., Nagata Y., Tsuji T., Kato T., Kageyama S., Ueda S., Sugiyama D., Hori S., Sakaguchi S., Ritter G., Old LJ., Gnjatic S., Shiku H.	Overcoming regulatory T-cell suppression by a lyophilized preparation of Streptococcus pyogenes.	Eur. J. Immunol.	43(4)	989-1000	2013
Gupta A., Nuber N., Esslinger C., Wittenbrink M., Treder M., Landshammer A., Noguchi T., Kelly M., Gnjatic S., Ritter E., von Boehmer L., Nishikawa H., Shiku H., Old L., Ritter G., Knuth A., van den Broek M.	A novel human-derived antibody against NY-ESO-1 improves the efficacy of chemotherapy.	Cancer Immunology	13	3. Epub	2013
Takahashi T., Shiku H.	Cell surface antigens: invaluable landmarks reflecting the nature of cells.	Cancer Immunology	12	2	2012
Fujiwara S, Wada H, Kawada J, Kawabata R, Takahashi T, Fujita J, Hirao T, Shibata K, Makari Y, Iijima S, Nishikawa H, Jungbluth AA, Nakamura Y, Kurokawa Y, Yamasaki M, Miyata H, Nakajima K, Takiguchi S, Nakayama E, Mori M, Doki Y	NY-ESO-1 antibody as a novel tumour marker of gastric cancer.	Br J Cancer	108(5)	1119-25	2013
Tsujinaka T, Yamamoto K, Fujita J, Endo S, Kawada J, Nakahira S, Shimokawa T, Kobayashi S, Yamasaki M, Akamaru Y, Miyamoto A, Mizushima T, Shimizu J, Umeshita K, Ito T, Doki Y, Mori M	Subcuticular sutures versus staples for skin closure after open gastrointestinal surgery: a phase 3, multicentre, open-label, randomised controlled trial	Lancet	382(9898)	1105-12	2013

Ogino T, Nishimura J, Barman S, Kayama H, Uematsu S, Okuzaki D, Osawa H, Haraguchi N, Uemura M, Hata T, Takemasa I, Mizushima T, Yamamoto H, Takeda K, Doki Y, Mori M.	Increased Th17-Inducing Activity of CD14(+) CD163(low) Myeloid Cells in Intestinal Lamina Propria of Patients With Crohn's Disease.	Gastroenterology	145(6)	1380-91	2013
和田尚，西塔拓郎，長瀬博次，土岐祐一郎	"特集　治療効果の判定基準と臨床試験のendpoint　RECISTとirResponse Criteria 4) Mogamulizumabの効果判定"	腫瘍内科	12(4)	394-400	2013
平野聡	膵・胆道癌治療の新たな展開　−非手術療法後のAdjuvant Surgery−	北海道医学雑誌	88（2-3）	55-57	2013
平野聡，田中栄一，土川貴裕，松本譲，加藤健太郎，海老原裕磨，中村透，七戸俊明	消化器外科手術アトラス　肝門部胆管癌に対する門脈分岐部切除を伴う肝右葉・尾状葉・胆管切除術	消化器外科	36(4)	395-404	2013
平野聡，田中栄一，土川貴裕，松本譲，中村透，七戸俊明	Ⅲ膵臓　6. 腹腔動脈合併尾側膵切除（DP-CAR）	手術　臨時増刊号　最新肝胆膵脾手術アトラス	67(6)	899-905	2013
Gunji S et al.	Effects of rikkunshito, a kampo medicine, on quality of life after proximal gastrectomy.	J Surg Res	185(2)	575-580	2013
Yamamoto T, Nakayama K, Hirano H, Tomonaga T, Ishihama Y, Yamada T, Kondo T, Kodera Y, Sato Y, Araki N, Mamitsuka H, Goshima N.	Integrated View of the Human Chromosome X - centric Proteome Project	J. Proteome Res.	12	58−61	2013
Ishida A, Tsumura K, Oue M, Takenaka Y, Shigeri Y, Goshima N, Ishihara Y, Hirano T, Baba H, Sueyoshi N, Kameshita I, Yamazaki T.	An active C-terminally truncated form of Ca2+/calmodulin-dependent protein kinase phosphatase-N (CaMKP-N/PPM1E)	BioMed Research International	2013	doi:10.1155/2013/134813	2013

Okita K, Yamakawa T, Matsumura Y, Sato Y, Amano N, Watanabe A, Goshima N, Yamanaka S.	An Efficient Non-viral Method to Generate Integration-Free Human iPS Cells from Cord Blood and Peripheral Blood Cells	Stem Cells	31(3)	458-466	2013
Yugawa T, Nishino K, Ohno S, Nakahara T, Fujita M, Goshima N, Umezawa A, Kiyono T.	Noncanonical NOTCH signaling limits self-renewal of human epithelial and induced pluripotent stem cells through ROCK activation	Mol Cell Biol.	33 (22)	4434 – 4447	2013
Tokita K, Seimiya M, Matsushita K, Tomonaga T, Onodera K, Ohki S, Tanizawa T, Uesato M, Shimada H, Matusbara H, Nakatani Y, Nomura F	Clathrin heavy chain is a useful immunohistochemical marker for esophageal squamous intraepithelial neoplasia.	Esophagus	DOI: 10.1007/s10388-013-0381-9		Published online: 2013
Shimada H, Noie T, Ohashi M, Oba K, Takahashi Y.	Clinical significance of serum tumor markers for gastric cancer: a systematic review of literature by the Task Force of the Japanese Gastric Cancer Association.	Gastric Cancer	17(1)	26-33	2014

Ⅳ．研究成果の刊行物･別刷

Kageyama et al. Journal of Translational Medicine 2013, 11:246
http://www.translational-medicine.com/content/11/1/246

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RESEARCH Open Access

Dose-dependent effects of NY-ESO-1 protein vaccine complexed with cholesteryl pullulan (CHP-NY-ESO-1) on immune responses and survival benefits of esophageal cancer patients

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Abstract

Background: Cholesteryl pullulan (CHP) is a novel antigen delivery system for cancer vaccines. This study evaluated the safety, immune responses and clinical outcomes of patients who received the CHP-NY-ESO-1 complex vaccine, Drug code: IMF-001.

Methods: Patients with advanced/metastatic esophageal cancer were enrolled and subcutaneously vaccinated with either 100 μg or 200 μg of NY-ESO-1 protein complexed with CHP. The primary endpoints were safety and humoral immune responses, and the secondary endpoint was clinical efficacy.

Results: A total of 25 patients were enrolled. Thirteen and twelve patients were repeatedly vaccinated with 100 μg or 200 μg of CHP-NY-ESO-1 with a median of 8 or 9.5 doses, respectively. No serious adverse events related to the vaccine were observed. Three out of 13 patients in the 100-μg cohort and 7 out of 12 patients in the 200-μg cohort were positive for anti-NY-ESO-1 antibodies at baseline. In the 100-μg cohort, an antibody response was observed in 5 out of 10 pre-antibody-negatives patients, and the antibody levels were augmented in 2 pre-antibody-positive patients after vaccination. In the 200-μg cohort, all 5 pre-antibody-negative patients became seropositive, and the antibody level was amplified in all 7 pre-antibody-positive patients. No tumor shrinkage was observed. The patients who received 200 μg of CHP-NY-ESO-1 survived longer than patients receiving 100 μg of CHP-NY-ESO-1, even those who exhibited unresponsiveness to previous therapies or had higher tumor burdens.

Conclusions: The safety and immunogenicity of CHP-NY-ESO-1 vaccine were confirmed. The 200 μg dose more efficiently induced immune responses and suggested better survival benefits. (Clinical trial registration number NCT01003808).

Keywords: Esophageal cancer, Cancer vaccine, NY-ESO-1, Cholesteryl pullulan (CHP)

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Background

Complexes of cholesteryl pullulan (CHP) nano-particles that contain a tumor antigen are a new type of cancer vaccine with a novel antigen delivery system that presents multiple epitope peptides to both the MHC class I and class II pathways [1-4]. We have been developing CHP-protein human cancer vaccines that efficiently induce immune responses against multiple T cell epitopes for various HLA types. Previous clinical studies using CHP-HER2 and CHP-NY-ESO-1 vaccines showed that these vaccines could be administered repeatedly without serious adverse effects, and both vaccines induced antigen-specific $CD4^+$ and $CD8^+$ T cell immunity as well as humoral immunity [5-7].

Because the NY-ESO-1 antigen is a cancer-testis antigen that is exclusively expressed in the tumor tissue, aside from expression in the normal testis and placenta, this antigen is considered an ideal target for cancer immunotherapy [8,9].

The appropriate dose for NY-ESO-1 protein vaccine has not been determined, although doses up to 100 μg have been examined, in which a higher dose was more immunogenic compared to lower doses of 10 μg and 30 μg [10].

We conducted a dose-escalating trial with CHP-NY-ESO-1 vaccine doses of 100 μg and 200 μg for esophageal cancer patients who were resistant to standard therapies. We evaluated the safety and immune responses to the NY-ESO-1 antigen over the vaccination period, and explored the clinical impact on esophageal cancer patients with a poor prognosis.

In this study, we analyzed IgG antibody responses as antigen-specific immune responses. Although T cells that are induced by a cancer vaccine should be evaluated as an immune-monitoring marker, T cells can be difficult to detect directly and quantitatively assess, whereas IgG titers measured by ELISA could act as a suitable immune-monitoring marker. Analyzing antibody responses induced by CHP-NY-ESO-1 vaccine, the 200 μg-dose more efficiently induced immune responses and suggested better survival benefits.

Materials and methods

Preparation of CHP-NY-ESO-1 complex vaccine

CHP-NY-ESO-1 complex vaccine (Drug code: IMF-001) was provided by ImmunoFrontier, Inc. (Tokyo, Japan). All processes were performed following current Good Manufacturing Practices (cGMP) conditions. The toxicity of the drug products was assessed using animal models, and stability was monitored during the clinical trial using representative samples of the investigational drug product.

Study design

This study was a phase 1, open-label, multi-institutional, dose-escalating clinical trial of the CHP-NY-ESO-1 complex vaccine administered subcutaneously to patients with unresectable, advanced, or refractory esophageal tumors that expressed the NY-ESO-1 antigen. The primary objective was to determine the maximum tolerated dose (MTD) and the biological recommended dose, and the secondary objective was to assess clinical efficacy.

Patients were eligible for entry, if they had a performance status of 0, 1, or 2, were at least 20 years old, had a life expectancy of 4 months or more, and did not have impaired organ function. Patients were ineligible if they were positive for HIV antibody, had multiple cancers, autoimmune disease, serious allergy history, or active brain metastasis, or received previous chemotherapy, systemic steroid or immunosuppressive therapy within less than 4 weeks.

The patients were divided into the following two cohorts of 10 patients each: Cohort 1, 100 μg of the NY-ESO-1 protein every two weeks, and Cohort 2, 200 μg of the NY-ESO-1 protein every two weeks. When a patient withdrew from the trial within three vaccinations, they were replaced with an additional patient.

Clinical responses were assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST ver1.1) [11] and its modified version. The modified version is based on immune-related Response Criteria (ir-RC) [12] and includes the following: Tumor responses were assessed every 6 weeks. Even if disease progression was observed within the first 12 weeks, PD (progressive disease) was not judged. When disease progression was observed after 18 weeks, PD was determined.

Each patient received 6 administrations. However, the treatment could be continued beyond this period if the patient wished to maintain treatment and met the following criteria: 1) no evidence of tumor progression or worsening of performance status (PS), and 2) an anti-NY-ESO-1 antibody response was confirmed. Safety was evaluated according to the National Cancer Institute Common Terminology Criteria for Adverse Events ver.3.0 (NCI-CTCAE ver.3.0) [13]. All the safety information was collected and evaluated, and dose escalation was judged by the Independent Data and Safety Committee.

The study was performed in accordance with the current version of the Declaration of Helsinki and Good Clinical Practice. Written informed consent was obtained from all patients participating in this study. The protocol was approved by the institutional review board at each site. The clinical trial was sponsored by ImmunoFrontier, Inc. (Tokyo, Japan), and registered as ID: NCT01003808 of ClinicalTrials.Gov.

Expression of NY-ESO-1 antigen

NY-ESO-1 expression was assessed by immunohistochemistry with the monoclonal antibody, E978 (Sigma-Aldrich, Saint Louis, MO), [9] or quantitative RealTime-PCR (qRT-PCR) using specific primers [14].

Serum samples

To analyze antigen-specific antibody responses, sera were collected at baseline and two weeks after each vaccination. All sera were stored at −80°C until analysis.

Antibody responses to NY-ESO-1 antigen

NY-ESO-1-specific antibodies in the sera were measured by ELISA as described previously [15]. Briefly, recombinant NY-ESO-1 proteins (His-tag and GST-tag) and NY-ESO-1 peptides were absorbed onto immunoplates (442404; Nunc, Roskilde, Denmark) at a concentration of 10 ng/50 μL/well at 4°C. The collected serum samples were diluted from 1:400 to 1:102,400. After washing and blocking the plate, the sera were added and incubated for 10 h. After washing, goat anti-human IgG (H + L chain) (MBL, Nagoya, Japan) conjugated with peroxidase (The Binding Site, San Diego, CA) was added. After adding the TMB substrate (Pierce, Rockford, IL), the plate was read using a Microplate Reader (model 550; Bio-Rad, Hercules, CA).

Serum samples for 80 healthy volunteers were evaluated to determine a cut-off level for the anti-NY-ESO-1 antibody based on the optical density $(OD)_{450-550}$ absorption value. The cut-off level of anti-NY-ESO-1 IgG was 0.182. A sample was considered to be positive for anti-NY-ESO-1 antibodies if the optical density $(OD)_{450-550}$ absorption value in the ELISA was at the cut-off level or higher at a serum dilution of 1:400. The immune responses of patients with pre-existing anti-NY-ESO-1 antibodies were judged as augmentation if the serum diluted 4-fold or more remained positive.

Statistical analysis

Rates of the immune responses between the patients in Cohort 1 and Cohort 2 were compared by Fisher's exact test, and the survival curve was estimated using the Kaplan–Meier method and compared by the log-rank test. In order to adjust the confounding factors, Cox proportional hazards model was applied. All analyses were done using SAS 9.2 (SAS Institute Inc., Cary, NC).

Results and discussion

Patient characteristics and clinical safety

A total of 25 patients were enrolled in the clinical trial. All patients had unresectable, advanced, or refractory esophageal cancers. The tumor cells in all of these patients were NY-ESO-1-positive, in which the positivity was determined by immunohistochemistry and qRT-PCR for 24 patients and one patient, respectively. All patients received standard chemotherapy and/or other cancer therapies including radiotherapy and surgery, which were ultimately ineffective (Table 1).

Cohort 1 consisted of 13 patients who were given 100 μg of the vaccine; Cohort 2 consisted of 12 patients who were given 200 μg of the vaccine. The patients in Cohort 1 and Cohort 2 received 2 to 27 vaccinations with a median of 8 doses and 3 to 21 vaccinations with a median of 9.5 doses, respectively (Table 1). No dose-limiting toxicity (DLT) was observed. All the patients except one developed transient, grade 1 skin reactions at the injection sites. Other adverse events included swallowing disturbance (n = 8), diarrhea (n = 3), and fever (n = 2), in which events of grade 3 or 4 were included. These events were considered unrelated to the CHP-NY-ESO-1 vaccination. Based on the laboratory data, decreased lymphocyte counts were observed (n = 10), which were all grade 3. These patients had lymphopenia at baseline, probably due to the previous chemotherapies. During the course of the vaccinations, they developed grade 3 lymphopenia, which were shifted from the other grade of the pre-vaccine lymphopenia. Other changes included decreased Na levels (n = 4), decreased hemoglobin levels (n = 3), elevated transaminase levels (n = 2) and elevated uric acid (n = 2) (Table 2). These adverse events were changed from the decreased or elevated levels at baseline. They did not affect the vaccine continuation. Therefore, the changes were considered not related or unlikely related to the vaccination.

Table 1 Patients demographics

	100 μg	200 μg
No. patients enrolled	13	12
Sex		
Male	13	11
Female	0	1
Age		
Median	69	64.5
Range	49-72	53-79
Prior therapy		
Surgery	6	5
Radiotherapy	11	7
Chemotherapy	13	12
Pre-existing antibody to NY-ESO-1 antigen	3	7
No. vaccinations		
Median	8	9.5
Range	2-27	3-21

Immune responses to NY-ESO-1 protein

As shown Table 3, 3 out of the 13 patients, and 7 out of 12 patients had pre-existing antibodies to NY-ESO-1, while the remaining 10 and 5 patients did not have this reactivity in Cohort 1 and Cohort 2, respectively.

To evaluate the antibody responses after vaccination, serum samples collected at the serial vaccinations were analyzed using an antigen-specific IgG ELISA. In three patients of 100–02, 100–3 and 200–7 who were vaccinated three times, the serum samples from 1st and 2nd

Table 2 Adverse events during CHP-NY-ESO-1 vaccinations

Adverse event	100 μg(n = 13)						200 μg(n = 12)						Total
	Grade						Grade						
	1	2	3	4	5	Subtotal	1	2	3	4	5	Subtotal	
Skin reaction	12	0	0	0	0	12	12	0	0	0	0	12	24
Swallowing disturbance	0	0	3	0	0	3	0	0	4	1	0	5	8
Diarrhea	0	0	2	0	0	2	1	0	0	0	0	1	3
Fever	2	0	0	0	0	2	0	0	0	0	0	0	2
Decreased lymphocytes count	0	0	7	0	0	7	0	0	3	0	0	3	10
Decreased Na level	0	0	2	0	0	2	0	0	2	0	0	2	4
Decreased Hb level	0	0	3	0	0	3	0	0	0	0	0	0	3
Elevated ALT/AST level	0	0	2	0	0	2	0	0	0	0	0	0	2
Elevated uric acid level	0	0	1	1	0	2	0	0	0	0	0	0	2

NOTE: Events occurring more than once are listed. Events of disease progression are not listed.

vaccination were assayed. In Cohort 1, out of 10 pre-antibody-negative patients, 5 became seropositive. Two out of 3 pre-antibody-positive patients had augmented antibody responses. In total, 7 of 13 (53.8%) patients exhibited immune responses. Five pre-antibody-negative and 7 pre-antibody-positive patients in Cohort 2 became positive or were augmented, yielding 12 out of 12 or 100% responsiveness. The 200-μg dose was more immunogenic than the 100-μg dose ($p = 0.015$, Fisher's exact test). In Cohort 1, immune reactions were observed after a median of 2 cycles, with a range of 1 to 4 vaccine cycles. In Cohort 2, the immune responses were also evident after a median of 2 cycles with a range of 1 to 5 cycles (Table 3). The chronological appearance of the immune responses and antibody titers are shown in Figure 1. The antibody intensities appeared more quickly and at a higher titer in patients in Cohort 2 (200 μg) than those in Cohort 1(100 μg). In addition to His-tag NY-ESO-1 protein, we tested serum reactivities to GST-tag NY-ESO-1 protein and NY-ESO-1 peptides. We confirmed specific reactions to NY-ESO-1 antigen in these sera.

Clinical responses and long-term follow-up

There were no cases of tumor shrinkage with partial response (PR) or complete response (CR) in any of the 25 patients. At the assessment that occurred every 6 weeks after vaccination, stable disease (SD) was observed in 3 patients in Cohort 1 and 6 patients in Cohort 2 (Table 4). There was no discordance in the evaluations between RECIST ver1.1 [11] and its modified version [12].

Table 3 Antibody responses in patients vaccinated with 100 μg or 200 μg of CHP-NY-ESO-1

100 μg				200 μg			
pt No.	Vaccination cycle	Baseline (dilution titer)	Antibody response (cycle*)	pt No.	Vaccination cycle	Baseline (dilution titer)	Antibody response (cycle*)
100-01	9	negative	responded(4)	200-01	15	negative	responded(2)
100-02	3	negative	no response**	200-02	9	negative	responded(2)
100-03	3	negative	no response**	200-03	8	positive (x1,600)	responded(5)
100-04	7	negative	no response	200-04	21	negative	responded(2)
100-05	2	negative	no response	200-05	3	negative	responded(2)
100-06	16	positive (x6,400)	responded(1)	200-06	10	positive (x400)	responded(1)
100-07	9	positive (x25,600)	no response	200-07	3	positive (x25,600)	responded(2)**
100-08	10	negative	responded(1)	200-08	11	positive (x400)	responded(1)
100-09	5	negative	no response	200-09	18	positive (x400)	responded(3)
100-10	27	positive (x400)	responded(3)	200-10	11	positive (x400)	responded(2)
100-11	8	negative	responded(2)	200-11	3	positive (x400)	responded(2)
100-12	8	negative	responded(2)	200-12	9	negative	responded(1)
100-13	26	negative	responded(2)				
antibody response rate			53.8%***				100%***

*vaccine cycles with which antibody responses appeared. **antibody responses assayed after two vaccinations.***p = 0.015(Fisher's exact test).

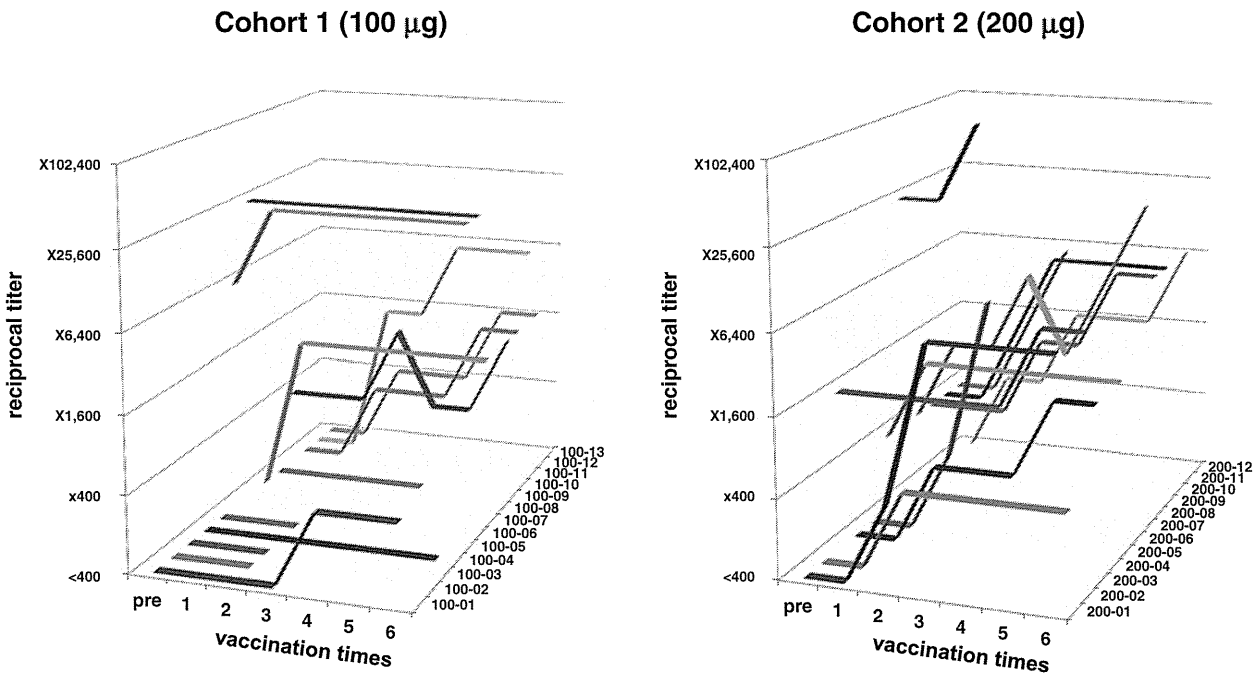

Figure 1 Antibody responses to recombinant NY-ESO-1 protein as determined by ELISA. Sera from 13 patients in Cohort 1 (100-μg dose) and 12 patients in Cohort 2 (200-μg dose) were collected at each vaccination, and serially diluted by 400, 1,600, 6,400, 25,600 and 102,400. Reciprocal titers were determined based on the maximally diluted sera, which showed a higher OD (optical density of 450–500) value than the cut-off level for NY-ESO-1 antibody positivity.

The disease progression-free survival time was 11 weeks on average, with a median of 6 weeks and range of 4 to 52 weeks. In Cohort 1 (n = 13), patients who were vaccinated with 100 μg of CHP-NY-ESO-1 survived without disease progression for 11 weeks on average, with a median of 6 weeks and range of 4 to 52 weeks. In Cohort 2 (n = 12) in which patients received the 200-μg dose, the patients were progression-free for 10 weeks on average, with a median of 8.5 weeks and range of 6 to 18 weeks (Table 4). There was no difference between the two cohorts (p = 0.748, Figure 2-A).

The overall survival time was 33 weeks on average, with a median of 31 weeks and range of 4 to 72 weeks. In Cohort 1 (n = 13), the patients survived for 25 weeks on average, with a median of 23 weeks and range of 4 to 60 weeks. In Cohort 2 (n = 12), they survived for 41 weeks on average, with a median of 41 weeks and range of 8 to 72 weeks (Table 4). The patients vaccinated with 200 μg of CHP-NY-ESO-1 had statistically longer survival than those who received the 100-μg dose (p = 0.050, Figure 2-B). Each cohort included three patients who were vaccinated three times or less because of early disease progression, and were withdrawn from this study, respectively. Having excluded those 6 patients, the patients vaccinated with 200 μg-vaccine still had longer survival than those with 100 μg-vaccinations (data not shown).

When the survival of patients who had responded to previous therapies (n = 12) was compared to non-responders (n = 13), the responders lived longer than the non-responders after vaccination (p = 0.005, Figure 2-C). The patients who never responded to previous therapies and received the 200-μg dose (n = 6) significantly lived longer than those who received the 100-μg dose (n = 7) (p = 0.029, Figure 2-D).

When the survival of patients who had tumors with a maximal diameter of 30 mm or less, including non-measurable lesions (n = 13) was compared with those with diameters more than 30 mm (n = 12), the patients with higher tumor burdens had shorter life spans (p = 0.021, Figure 2-E). Among patients with higher tumor burdens, patients who were vaccinated with the 200-μg dose (n = 4) lived longer than those who received the 100-μg dose (n = 8), (p = 0.037, Figure 2-F).

Using Cox proportional hazards models, the vaccine dose and the responsiveness to previous therapy were independent factors that influenced the overall survival, which showed p = 0.011 with HR 3.595 (95%CI 1.335-9.678) and p = 0.002 with HR 0.194 (95%CI 0.068-0.553), respectively. Also, the vaccine dose and the tumors sizes including non-measurable disease independently affected the overall survival, showing p = 0.040 with HR 2.630 (95%CI 1.045-6.614) and p = 0.020 with HR 0.322 (95%CI 0.124-0.833), respectively.

Table 4 Baseline clinical profiles and responses after CHP-NY-ESO-1 vaccinations

100 μg						200 μg					
pt No.	Response to previous therapies (duration time, weeks)	*sum of tumor diameters (mm)	Tumor response (BOR)	Time-to-progression (weeks)	Survival (weeks)	pt No.	Response to previous therapies (duration time, weeks)	*sum of tumor diameters (mm)	Tumor response (BOR)	Time-to-progression (weeks)	Survival (weeks)
100-01	PR (4)	NA	PD	6	31	200-01	PR (29)	24	SD	17	70
100-02	SD	53	NE	4	6	200-02	NE	25	SD	18	33
100-03	NE	144	NE	4	6	200-03	PR (32)	55	PD	6	37
100-04	PD	182	PD	5	17	200-04	PR (30)	NA	PD	6	50
100-05	CR (38)	101	NE	4	4	200-05	PR (32)	NA	PD	6	72
100-06	SD	69	PD	6	31	200-06	NE	32	SD	18	54
100-07	CR (15)	78	PD	6	29	200-07	NE	205	NE	6	8
100-08	NE	39	SD	18	23	200-08	PR (12)	16	SD	11	33
100-09	SD	18	PD	6	8	200-09	CR (96)	88	PD	6	45
100-10	CR (24)	NA	SD	11	60	200-10	SD	NA	SD	12	48
100-11	SD	31	SD	12	20	200-11	SD	NA	NE	6	12
100-12	PR (9)	NA	NE	16	28	200-12	SD	NA	SD	12	33
100-13	PR (16)	NA	NE	52	59						

*target lesions determined based on RECIST criteria.

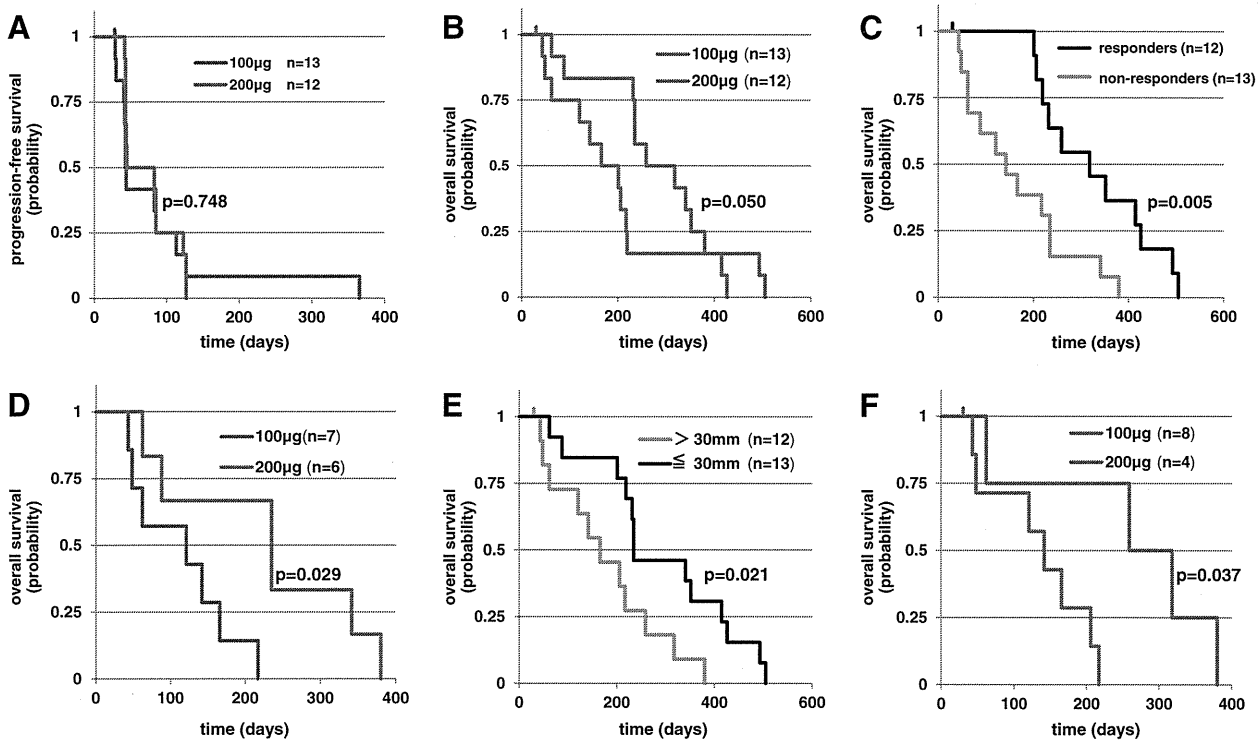

Figure 2 Kaplan-Meier survival curves of patients who were alive after the CHP-NY-ESO-1 vaccinations. A: Progression-free survival (PFS) of patients assigned to Cohort 1 (100-μg dose, n = 13) or Cohort 2 (200-μg dose, n = 12). The probability of PFS of the patients who received 200 μg of the vaccine and those who received the 100-μg dose were similar (log-rank test, p = 0.748). **B**: Overall survival (OS) of patients assigned to Cohort 1 or Cohort 2. The patients who received 200 μg of the vaccine survived longer than those who received the 100-μg dose. (log-rank test, p = 0.050). **C**: OS of patients vaccinated with either 100 μg or 200 μg of CHP-NY-ESO-1 compared between patients who responded to previous therapies and those who never responded. The patients who responded to previous therapies and were vaccinated with CHP-NY-ESO-1 survived longer than non-responders. (log-rank test, p = 0.0050). **D**: OS of patients vaccinated with 100 μg or 200 μg of CHP-NY-ESO-1 who never responded to previous therapies. The patients who received 200 μg of the vaccine survived longer than those who received the 100-μg dose (log-rank test, p = 0.029). **E**: OS of patients vaccinated with 100 μg or 200 μg of CHP-NY-ESO-1 compared with either baseline tumor burdens of >30 mm (sum of the tumor diameters) or 30 mm or less including non-measurable lesions. The patients with a tumor burden higher than 30 mm had reduced survival compared to those with smaller burdens (log-rank test, p = 0.021). **F**: OS of patients who received 100 μg or 200 μg of CHP-NY-ESO-1 who had baseline tumor burdens greater than 30 mm. The patients who received 200 μg of the vaccine survived longer than those who received the 100-μg dose (log-rank test, p = 0.037).

This study was a phase 1 dose-escalating clinical trial that examined two doses of the CHP-NY-ESO-1 vaccine in esophageal cancer patients. The primary goals were to evaluate the vaccine safety and immune responses to the NY-ESO-1 antigen, and we further explored the clinical effects on esophageal cancer patients with a poor prognosis.

CHP consists of a hydrophobic polysaccharide pullulan containing chemically introduced cholesterol groups, which spontaneously aggregate to form nano-sized particles that can contain antigen proteins. Using this system as a vaccine, tumor antigen proteins delivered to antigen-presenting cells can stimulate both antigen-specific CD4+ T cells and CD8+ T cells. In a pre-clinical study, dendritic cells pulsed with the CHP-NY-ESO-1 complex could induce both NY-ESO-1-specific CD4+ and CD8+ T cells [4]. Previous clinical studies using CHP-HER2 and CHP-NY-ESO-1 vaccines have shown that these vaccines can induce antigen-specific $CD4^+$ and $CD8^+$ T cell immunity in cancer patients [5-7].

In the current study, we found that CHP-NY-ESO-1 was clinically safe and that the immune responses to the NY-ESO-1 antigen, which were evaluated based on IgG antibody titers, showed a dose-dependent effect between the 100-μg dose and 200-μg. Furthermore, the survival rates of patients who were vaccinated with the 200-μg dose were superior to those who received the 100-μg dose. The patients had recurrent or metastatic esophageal tumors that exhibited clinical resistance to chemotherapy or radiotherapy. The first 13 patients were enrolled to Cohort 1, and the next 12 patients were included in Cohort 2. As the clinical backgrounds of the two cohorts were similar, it was reasonable to make a comparative consideration.

As the previous NY-ESO-1 protein vaccine trials have demonstrated, the toxicity of the CHP-vaccine was very

mild. Grade 3 swallowing disturbances were seen, which were likely related to the progression of esophageal cancer. The other grade 3 events included diarrhea, which was not related to the vaccine. The only related events were grade 1 skin reactions at the injection sites.

Previous vaccine trials have used recombinant full-length NY-ESO-1 protein with various adjuvants. Melanoma patients were divided into three cohorts that were vaccinated with 10 μg, 30 μg or 100 μg of the NY-ESO-1 protein in combination with the saponin adjuvant ISCOMATRIX [10]. The 100-μg dose of NY-ESO-1 induced more immune responses than the other two doses. The responses were evaluated based on IgG antibody titers and delayed-type hypersensitivity (DTH) of skin reactions. In the CHP system, a single 100-μg dose of CHP-NY-ESO-1 was examined with or without the adjuvant OK-432 [6,7,16]. These reports suggested that the 100-μg dose of CHP-NY-ESO-1 is sufficient to induce immune responses. The current trial was designed to determine whether the NY-ESO-1 protein vaccine has potential dose-dependent effects on immunogenicity in patients with homogeneous backgrounds. By assessing humoral immune responses in the cohorts that received 100 μg and 200 μg of the vaccine, the responses appeared in the early phases. We initially intended to analyze antibodies using samples from patients who were vaccinated for at least 4 cycles, as we thought it could take at least 4 cycles to detect immune responses. In the overall data acquisition, samples from all 25 patients were analyzed, which included sera from at least two vaccinations. In conclusion, we found that the 200-μg dose was more efficient than the 100-μg dose.

The other reports included vaccine studies using recombinant NY-ESO-1 protein in combination with Imiquimod and CpG [17,18]. In these studies, the NY-ESO-1 protein was given at doses of 100 μg, and 100 μg or 400 μg, respectively. Based on the patients' sera, the 400-μg dose might have induced more antibody responses than the 100-μg dose, but this was not statistically analyzed. Combined with these reports, the NY-ESO-1 protein might be immunogenic at increasing doses of 10 μg, 30 μg, 100 μg and 200 μg. Since dose-limited toxicity (DLT) was not observed at the higher dose of 200 μg in this study, additional dose increments might be acceptable to determine whether higher doses can induce stronger immune responses.

In this study, we explored a long-term clinical outcome of the NY-ESO-1 protein vaccine. This study was not initially designed to detect a statistical significance of the clinical effect between the 2 cohorts. Instead, we made a comparison to find out if there might include a positive signal for further clinical trials of this vaccine. The NY-ESO-1 protein vaccine with the adjuvant ISCOMATRIX suggested that melanoma patients who were vaccinated after standard therapy tended to have fewer relapses [10], which were not statistically analyzed. The other studies reported that vaccinations with NY-ESO-1-expressing poxvirus vectors and NY-ESO-1 overlapping peptides both prolonged progression-free survivals in ovarian cancer patients who did not have measurable disease after standard therapy [19,20]. In this study, most of the patients developed disease-progression in 6 months, and there was no difference between the patients vaccinated with 100 μg and 200 μg of the CHP-NY-ESO-1, as the previous studies demonstrated that disease-progression occurs in the early phase of vaccinations [12,21].

In contrast, we found that dose-dependent effects of the CHP-NY-ESO-1 vaccine on overall survival of patients with advanced/metastatic esophageal cancer. Analyzing other clinical categories, both the baseline tumor sizes and the tumor responsiveness to previous therapies were significant factors influencing the overall survival. Using Cox proportional hazards models, it was indicated that the tumor sizes and the vaccine doses independently influenced the survival. In the same way, the responsiveness to previous therapies and the vaccine doses independently affected the survival. Therefore, it is suggested that the higher dose of CHP-NY-ESO-1 vaccine played a role in prolongation of the overall survival in the esophageal cancer patients.

In addition, the higher-dose of the vaccine provided significant survival benefit in patients who never responded to the previous therapies or had larger tumor burdens than the lower dose vaccinations. It is difficult to discuss why the patients with a poorer prognosis were more benefited from the 200-μg dose of the vaccine than 100-μg. It might be speculated that the dose-dependency clinical benefits were more often observable in patients with a poorer prognosis, because they might have needed more immune responses in order to survive longer by preventing disease deterioration.

In the previous CHP-NY-ESO-1 vaccine study, which was a phase 1 study that enrolled various types of NY-ESO-1-expressing cancer patients, tumor regression was observed in two out of four esophageal cancer patients [6]. However, tumor shrinkage is rarely observed in cancer vaccine therapies, although some disease stabilization is seen. This study shows that clinical benefits, such as long-term survival, can be detected if a clinical trial is designed in a comparative way. The results were not compared to unvaccinated controls, and it is not possible to directly determine the effects of the vaccine, but is possible to reasonably interpret the effects of immune response on the clinical outcomes.

Conclusions

The safety and immunogenicity of the CHP-NY-ESO-1 vaccine were confirmed in the patients with antigen-expressing esophageal cancer. The 200-μg dose efficiently induced antigen-specific immune responses and suggested better survival benefits, even for patients with a poorer prognosis. In future clinical trials, 200 μg will be the recommended dose.

Abbreviations
BOR: Best overall response; NA: Not available; NE: Not evaluable.

Competing interests
This study is supported by ImmunoFrontier, Inc. and Naozumi Harada is an employee, and Mami Ohnishi and Tadashi Hishida are former employees of ImmunoFrontier, Inc. Hiroshi Shiku is a stockholder of ImmunoFrontier, Inc.

Authors' contributions
SK, HW, KM, YN, SU, HM, ST and YD treated patients and provided the clinical data. SHS and YM worked on immune responses. HI, NI and ES evaluated tumor antigen expression. TY, MOs and MOh worked on the study statistics. NH and TH were responsible for manufacturing the study drug. SK and HS wrote the manuscript. All authors read and approved the final manuscript.

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References

1. Gu XG, Schmitt M, Hiasa A, Nagata Y, Ikeda H, Sasaki Y, Akiyoshi K, Sunamoto J, Nakamura H, Kuribayashi K, Shiku H: **A novel hydrophobized polysaccharide/oncoprotein complex vaccine induces in vitro and in vivo cellular and humoral immune responses against HER2-expressing murine sarcomas.** *Cancer Res* 1998, **58**:3385–3390.
2. Ikuta Y, Katayama N, Wang L, Okugawa T, Takahashi Y, Schmitt M, Gu X, Watanabe M, Akiyoshi K, Nakamura H, Kuribayashi K, Sunamoto J, Shiku H: **Presentation of a major histocompatibility complex class 1-binding peptide by monocyte-derived dendritic cells incorporating hydrophobized polysaccharide-truncated HER2 protein complex: implications for a polyvalent immuno-cell therapy.** *Blood* 2002, **99**:3717–3724.
3. Wang L, Ikeda H, Ikuta Y, Schmitt M, Miyahara Y, Takahashi Y, Gu X, Nagata Y, Sasaki Y, Akiyoshi K, Sunamoto J, Nakamura H, Kuribayashi K, Shiku H: **Bone marrow-derived dendritic cells incorporate and process hydrophobized polysaccharide/oncoprotein complex as antigen presenting cells.** *Int J Oncol* 1999, **14**:695–701.
4. Hasegawa K, Noguchi Y, Koizumi F, Uenaka A, Tanaka M, Shimono M, Nakamura H, Shiku H, Gnjatic S, Murphy R, Hiramatsu Y, Old LJ, Nakayama E: **In vitro stimulation of CD8 and CD4 T cells by dendritic cells loaded with a complex of cholesterol-bearing hydrophobized pullulan and NY-ESO-1 protein: Identification of a new HLA-DR15-binding CD4 T-cell epitope.** *Clin Cancer Res* 2006, **12**:1921–1927.
5. Kitano S, Kageyama S, Nagata Y, Miyahara Y, Hiasa A, Naota H, Okumura S, Imai H, Shiraishi T, Masuya M, Nishikawa M, Sunamoto J, Akiyoshi K, Kanematsu T, Scott AM, Murphy R, Hoffman EW, Old LJ, Shiku H: **HER2-specific T-cell immune responses in patients vaccinated with truncated HER2 protein complexed with nanogels of cholesteryl pullulan.** *Clin Cancer Res* 2006, **12**:7397–7405.
6. Uenaka A, Wada H, Isobe M, Saika T, Tsuji K, Sato E, Sato S, Noguchi Y, Kawabata R, Yasuda T, Doki Y, Kumon H, Iwatsuki K, Shiku H, Monden M, Jungbluth AA, Ritter G, Murphy R, Hoffman E, Old LJ, Nakayama E: **T cell immunomonitoring and tumor responses in patients immunized with a complex of cholesterol-bearing hydrophobized pullulan (CHP) and NY-ESO-1 protein.** *Cancer Immun* 2007, **7**:9–19.
7. Kawabata R, Wada H, Isobe M, Saika T, Sato S, Uenaka A, Miyata H, Yasuda T, Doki Y, Noguchi Y, Kumon H, Tsuji K, Iwatsuki K, Shiku H, Ritter G, Murphy R, Hoffman E, Old LJ, Monden M, Nakayama E: **Antibody response against NY-ESO-1 in CHP-NY-ESO-1 vaccinated patients.** *Int J Cancer* 2007, **120**:2178–2184.
8. Chen YT, Scanlan MJ, Sahin U, Türeci O, Gure AO, Tsang S, Williamson B, Stockert E, Pfreundschuh M, Old LJ: **A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening.** *Proc Natl Acad Sci U S A* 1997, **94**:1914–1918.
9. Jungbluth AA, Chen YT, Stockert E, Busam KJ, Kolb D, Iversen K, Coplan K, Williamson B, Altorki N, Old LJ: **Immunohistochemical analysis of NY-ESO-1 antigen expression in normal and malignant human tissues.** *Int J Cancer* 2001, **92**:856–860.
10. Davis ID, Chen W, Jackson H, Parente P, Shackleton M, Hopkins W, Chen Q, Dimopoulos N, Luke T, Murphy R, Scott AM, Maraskovsky E, McArthur G, MacGregor D, Sturrock S, Tai TY, Green S, Cuthbertson A, Maher D, Miloradovic L, Mitchell SV, Ritter G, Jungbluth AA, Chen YT, Gnjatic S, Hoffman EW, Old LJ, Cebon JS: **Recombinant NY-ESO-1 protein with ISCOMATRIX adjuvant induces broad integrated antibody and CD4(+) and CD8(+) T cell responses in humans.** *Proc Natl Acad Sci U S A* 2004, **101**:10697–10702.
11. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, Dancey J, Arbuck S, Gwyther S, Mooney M, Rubinstein L, Shankar L, Dodd L, Kaplan R, Lacombe D, Verweij J: **New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1).** *Eur J Cancer* 2009, **45**:228–247.
12. Wolchok JD, Hoos A, O'Day S, Weber JS, Hamid O, Lebbé C, Maio M, Binder M, Bohnsack O, Nichol G, Humphrey R, Hodi FS: **Guidelines for the evaluation of immune therapy activity in solid tumors: immune-related response criteria.** *Clin Cancer Res* 2009, **15**:7412–7420.
13. Trotti A, Colevas AD, Setser A, Rusch V, Jaques D, Budach V, Langer C, Murphy B, Cumberlin R, Coleman CN, Rubin P: **CTCAE v3.0: development of a comprehensive grading system for the adverse effects of cancer treatment.** *Semin Radiat Oncol* 2003, **13**:176–181.
14. Fujita S, Wada H, Jungbluth AA, Sato S, Nakata T, Noguchi Y, Doki Y, Yasui M, Sugita Y, Yasuda T, Yano M, Ono T, Chen YT, Higashiyama M, Gnjatic S, Old LJ, Nakayama E, Monden M: **NY-ESO-1 expression and immunogenicity in esophageal cancer.** *Clin Cancer Res* 2004, **10**:6551–6558.
15. Stockert E, Jäger E, Chen YT, Scanlan MJ, Gout I, Karbach J, Arand M, Knuth A, Old LJ: **A survey of the humoral immune response of cancer patients to a panel of human tumor antigens.** *J Exp Med* 1998, **187**:1349–1354.
16. Aoki M, Ueda S, Nishikawa H, Kitano S, Hirayama M, Ikeda H, Toyoda H, Tanaka K, Kanai M, Takabayashi A, Imai H, Shiraishi T, Sato E, Wada H, Nakayama E, Takei Y, Katayama N, Shiku H, Kageyama S: **Antibody responses against NY-ESO-1 and HER2 antigens in patients vaccinated with combinations of cholesteryl pullulan (CHP)-NY-ESO-1 and CHP-HER2 with OK-432.** *Vaccine* 2009, **27**:6854–6861.
17. Adams S, O'Neill DW, Nonaka D, Hardin E, Chiriboga L, Siu K, Cruz CM, Angiulli A, Angiulli F, Ritter E, Holman RM, Shapiro RL, Berman RS, Berner N, Shao Y, Manches O, Pan L, Venhaus RR, Hoffman EW, Jungbluth A, Gnjatic S, Old L, Pavlick AC, Bhardwaj N: **Immunization of malignant melanoma patients with full-length NY-ESO-1 protein using TLR7 agonist imiquimod as vaccine adjuvant.** *J Immunol* 2008, **181**:776–784.
18. Valmori D, Souleimanian NE, Tosello V, Bhardwaj N, Adams S, O'Neill D, Pavlick A, Escalon JB, Cruz CM, Angiulli A, Angiulli F, Mears G, *et al*: **Vaccination with NY-ESO-1 protein and CpG in Montanide induces integrated antibody/Th1 responses and CD8 T cells through cross-priming.** *Proc Natl Acad Sci U S A* 2007, **104**:8947–8952.
19. Odunsi K, Matsuzaki J, Karbach J, Neumann A, Mhawech-Fauceglia P, Miller A, Beck A, Morrison CD, Ritter G, Godoy H, Lele S, Dupont N, *et al*: **Efficacy**

of vaccination with recombinant vaccinia and fowlpox vectors expressing NY-ESO-1 antigen in ovarian cancer and melanoma patients. *Proc Natl Acad Sci U S A* 2012, **109**:5797–5802.

20. Sabbatini P, Tsuji T, Ferran L, Ritter E, Sedrak C, Tuballes K, Jungbluth AA, Ritter G, Aghajanian C, Bell-McGuinn K, Hensley ML, Konner J, *et al*: **Phase I trial of overlapping long peptides from a tumor self-antigen and poly-ICLC shows rapid induction of integrated immune response in ovarian cancer patients.** *Clin Cancer Res* 2012, **18**:6497–6508.
21. Small EJ, Schellhammer PF, Higano CS, Redfern CH, Nemunaitis JJ, Valone FH, Verjee SS, Jones LA, Hershberg RM: **Placebo-controlled phase III trial of immunologic therapy with Sipuleucel-T (APC8015) in patients with metastatic, asymptomatic hormone refractory prostate cancer.** *J Clin Oncol* 2006, **24**:3089–3094.

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Development of a novel redirected T-cell–based adoptive immunotherapy targeting human telomerase reverse transcriptase for adult T-cell leukemia

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Regular Article

LYMPHOID NEOPLASIA

Development of a novel redirected T-cell–based adoptive immunotherapy targeting human telomerase reverse transcriptase for adult T-cell leukemia

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Key Points

- The efficacy and safety of a novel redirected T-cell–based adoptive immunotherapy targeting hTERT for patients with adult T-cell leukemia.
- hTERT-specific T-cell receptor gene-transduced $CD8^+$ T cells lyse ATL cells, but not normal cells, both in vitro and in vivo.

Although adult T-cell leukemia (ATL) has a poor prognosis, successful allogeneic hematopoietic stem cell transplantation (allo-HSCT) in some cases suggests that a cellular immune-mediated strategy can be effective. So far, however, no effective target for anti-ATL immunotherapy has been defined. Here we demonstrated for the first time that human telomerase reverse transcriptase (hTERT) is a promising therapeutic target for ATL, and we developed a novel redirected T-cell–based immunotherapy targeting hTERT. *hTERT* messenger RNA was produced abundantly in ATL tumor cells but not in steady-state normal cells. Rearranged human leukocyte antigen-A*24:02 (HLA-A*24:02) –restricted and $hTERT_{461-469}$ nonameric peptide-specific T-cell receptor (TCR) α/β genes were cloned from our previously established cytotoxic T lymphocyte clone (K3-1) and inserted into a novel retroviral TCR expression vector encoding small interfering RNAs for endogenous *TCR* genes in redirected T cells (hTERT-*siTCR* vector). Consequently, allogeneic or autologous gene-modified $CD8^+$ T cells prepared using the hTERT-*siTCR* vector successfully killed ATL tumor cells, but not normal cells including steady-state hematopoietic progenitors, in an HLA-A*24:02-restricted manner both in vitro and in vivo. Our experimental observations support the development of a novel hTERT-targeting redirected T-cell–based adoptive immunotherapy for ATL patients, especially those for whom suitable allo-HSCT donors are lacking. (*Blood.* 2013;121(24):4894-4901)

Introduction

Adult T-cell leukemia (ATL) is an aggressive peripheral T-cell neoplasm caused by human T-cell lymphotropic virus I (HTLV-I).[1] It is estimated that there are more than 1 million HTLV-I carriers in Japan, about 5% of whom develop ATL at around 60 years of age or older.[2] Because ATL tumor cells soon acquire chemotherapy resistance and compromise host immunity against infectious pathogens, ATL has a poor prognosis.[3] Although most ATL patients are ineligible for allogeneic hematopoietic stem cell transplantation (allo-HSCT) because of advanced age, age-related comorbidity, or lack of suitable donors,[4] the number of ATL patients who are treated successfully with allo-HSCT and achieve prolonged survival has been increasing.[5] The graft-versus-ATL effect observed in ATL patients treated successfully with allo-HSCT[5] strongly suggests that a cellular immune-mediated approach for ATL can be clinically effective. With regard to cellular immunotherapy for ATL (unlike Epstein-Barr virus [EBV]-associated malignancy[6]), targeting of antigens associated with HTLV-I (the causative virus of ATL) such as Tax[7] and HBZ[8] still remains controversial, and the recently proposed NY-ESO-1[9] (a cancer-testis antigen) still awaits clinical validation. Thus, at this time, no effective therapeutic target antigen for anti-ATL immunotherapy has been clinically defined.

Human telomerase reverse transcriptase (hTERT), which is a component of human telomerase and a catalytic subunit for telomere elongation, is activated in almost all cancer cells, including hematologic malignancies, but not in normal cells.[10] In HTLV-I–infected cells and ATL tumor cells, Tax or interleukin-2 (IL-2) signaling strongly activates the *hTERT* promoter through the nuclear factor-κB or PI3K pathway,[11-13] suggesting that expression of hTERT protein would be upregulated in ATL tumor cells. Clinical trials of anticancer immunotherapy targeting hTERT have already been conducted, and both the safety and induction of immune responses to hTERT have been reproducibly confirmed.[10,14-17] In our previous studies, we defined a [human leukocyte antigen] HLA-A*24:02-restricted $hTERT_{461-469}$ nonameric peptide (VYGFVRACL) that was capable of inducing antileukemia cytotoxic T lymphocytes (CTLs),[18] and we subsequently established a CTL clone, K3-1, specific for this

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epitope.[19] We previously conducted a phase I/II clinical trial of hTERT peptide vaccine for treatment of HLA-A*24:02$^+$ patients with lung cancer and metastatic renal cell cancer.[20] These achievements strongly encouraged us to further explore cellular immune-mediated treatment of ATL targeting hTERT. Because of concern over the potential regulatory T-cell function of ATL tumor cells,[21] in this study we focused on developing a redirected T-cell–based immunotherapy targeting hTERT rather than using an hTERT$_{461\text{-}469}$ peptide vaccine. Recently developed forms of anticancer immunotherapy using gene-modified T cells that redirect defined tumor-associated antigens have been shown to have clinical promise.[22-25] To this end, therefore, we first cloned the rearranged HLA-A*24:02-restricted and hTERT$_{461\text{-}469}$-specific T-cell receptor α/β (*TCR-α/β*) genes from K3-1 and inserted them into a novel *TCR* gene expression vector carrying silencers for endogenous TCRs (*siTCR* vector)[26] in redirected T cells (hTERT-*siTCR* vector). Notably, we used a souped-up second-generation 2A peptide-based *siTCR* vector that achieved an increased level of expression of the introduced TCR.[27]

In this study, we used the newly established hTERT-*siTCR* vector to examine the feasibility of a novel redirected T-cell–based adoptive immunotherapy targeting hTERT for treatment of ATL.

Patients and methods

Cell lines, freshly isolated leukemia cells, and normal cells

Approval for this study was obtained from the institutional review board of Ehime University Hospital. Written informed consent was obtained from all patients, healthy volunteers, and parents of cord blood donors in accordance with the Declaration of Helsinki.

B-lymphoblastoid cell lines (B-LCLs) were established by transformation of peripheral blood B lymphocytes with EBV. ATN-1,[28] TL-Om1,[29] HUT102[29], and TL-MAT[30] were human T-cell lines established from ATL patients, and TL-Su,[31] MT-1,[32] MT-2[32], and MT-4[33] were human T-cell lines transformed by HTLV-I infection. LCLs, T2-A24,[19] K562 (American Type Culture Collection [ATCC]), and human T-cell lines (except TL-Om1), maintenance of which requires 10 U/mL recombinant human IL-2 (rhIL-2) (R&D Systems), were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. The HLA-A*24:02 gene-transduced K562 (K562-A24) was maintained in culture medium supplemented with 1.0 μg/mL puromycin (Sigma-Aldrich). Peripheral blood mononuclear cells (PBMCs) from ATL patients and healthy donors and cord blood mononuclear cells (CBMCs) from healthy donors were isolated by density gradient centrifugation and stored in liquid nitrogen until use. All samples from ATL patients contained more than 90% ATL cells. CD4$^+$ T cells, CD14$^+$ cells from PBMCs, and CD34$^+$ cells from CBMCs were isolated by using CD4$^+$ cell-, CD14$^+$ cell-, or CD34$^+$ cell-isolating immunomagnetic beads (MACS beads; Miltenyi Biotec), respectively. IL-2–dependent CD4$^+$ cell lines induced by HTLV-I infection were generated as reported previously.[8]

Cloning of full-length *TCR* α and β chain genes and construction of hTERT-*siTCR* retroviral vector

HLA-A*24:02-restricted and hTERT$_{461\text{-}469}$ nonameric peptide (VYGFVRACL)-specific *TCR-α/β* genes were cloned from our previously established CTL clone, K3-1,[19] by using the 5′ rapid amplification of complementary DNA ends method (Clontech). The rearranged *TCR-α/β* genes of K3-1 expressed the germ line gene segments *TRAV29DV5/TRAJ34/TRAC* and *TRBV20-1/TRBJ2-1/TRBC2*, respectively. The retroviral vector expressing K3-1–derived *TCR* genes was constructed as reported previously.[26,27,34] Briefly, the constant regions of the hTERT-specific *TCR-α/β* genes were codon optimized and then integrated into a novel Splice-b2Aa-siTCR–based retroviral vector encoding small interfering RNAs that complementarily bind to the constant regions of the endogenous *TCR-α/β* genes (hTERT-*siTCR* vector).[27]

Establishment of hTERT-*siTCR*–transduced CD8$^+$ T-cell lines

Isolated CD8$^+$ T cells from PBMCs of healthy volunteers or ATL patients using CD8$^+$ cell-isolating MACS beads and stimulation with 1 μg/mL anti-CD3 monoclonal antibody (mAb; OKT-3; BioLegend) were cultured in GT-T503 (Takara Bio) supplemented with 5% human serum, 0.2% human albumin, 50 U/mL rhIL-2, 5 ng/mL rhIL-7 (R&D Systems), 10 ng/mL rhIL-15 (PeproTech), and 10 ng/mL rhIL-21 (Shenandoah Biotechnology). Then, CD8$^+$ T cells were transfected with the hTERT-*siTCR* retroviral vector using RetroNectin (Takara Bio) -coated plates as described previously.[34] In some experiments, because TRBV20-1 is specifically labeled with anti-Vβ2 mAb (IMGT Web resources: http://www.imgt.org/), Vβ2-positive cells among hTERT-*siTCR*–transduced CD8$^+$ T cells (hTERT-*siTCR*/CD8) were further isolated by using fluorescein isothiocyanate (FITC) –conjugated Vβ2 mAb (Beckman Coulter) and anti-FITC–conjugated MACS beads. To measure the expression levels of the introduced hTERT-specific TCR in gene-modified CD8$^+$ T cells, the cells were labeled with anti-CD8 (BD Biosciences) and anti-Vβ2 mAbs and phycoerythrin-conjugated HLA-A*24:02/hTERT$_{461\text{-}469}$ tetramer or HLA-A*24:02/HIV-1 Env$_{584\text{-}592}$ (RYLRDQQLL) tetramer, as a negative control.[19] Labeled cells were analyzed by using a Gallios flow cytometer (Beckman Coulter) and FlowJo Version 7.2.2 software (TreeStar). To expand the hTERT-*siTCR*/CD8 cells, they were stimulated weekly with mitomycin-C (Kyowa Hakko) –treated and hTERT$_{461\text{-}469}$ peptide–pulsed HLA-A*24:02$^+$ LCLs.

Cytotoxicity assays

Standard ^{51}Cr-release assays were performed as described previously.[35] Briefly, 5 × 10^3 unpulsed or peptide-pulsed target cells were labeled with ^{51}Cr ($Na_2{}^{51}CrO_4$; MP Bio Japan) and incubated at various ratios with effector cells in 200 μL of culture medium in 96-well round-bottomed plates. To assess HLA class I restriction, target cells were incubated with 10 μg/μL anti-HLA class I framework mAb (clone w6/32; ATCC) or a control anti-HLA -DR mAb (clone L243; ATCC) for 1 hour, then incubated with effector cells for 5 hours. The percentage of specific lysis was calculated as (experimental release cpm – spontaneous release cpm)/(maximal release cpm – spontaneous release cpm) × 100 (%). In some experiments, time-lapse imaging was used. Ten thousand ATL cells lentivirally gene-modified to express monomeric Azami-Green (Amalgaam) were cocultivated with 5 × 10^4 effector cells expressing hTERT-specific TCR (at an effector:target ratio of 5:1) for 12 hours in culture medium supplemented with 10 μg/mL propidium iodide (Sigma) to label dead cells red by using a glass dish for microscopic observation of live cells (iBIDI-dish1 Hi-Q4; Nikon). Images were acquired by using a systemic bio-imaging tool (BioStation IM; Nikon). To examine the cytotoxicity of these effector cells against early-differentiated and highly proliferating subsets of hematopoietic progenitor cells, CB-CD34$^+$ cells cultured by using a hematopoietic cell expansion medium (StemSpan CC100 and StemSpan SFEM; Stem Cell) for 7 days were subjected to flow-based cytotoxicity assay. 7-Aminoactinomycin D (7-AAD) –positive dead cells in each subset were examined by flow cytometry.

Quantitative analysis of hTERT mRNA expression

Quantitative real-time PCR (qRT-PCR) for *hTERT* messenger RNA (mRNA) was performed as described previously.[36] Briefly, after complementary DNA was synthesized, qRT-PCR for *hTERT* mRNA (NM_198253) was performed by using the QuantiTect SYBR green PCR Kit (QIAGEN) and primers as follows: forward, 5′-TTCTTGTTGGTGACACCTCACCTC-3′; reverse, 5′-CAGCCATACTCAGGGACACCTC-3′ (Takara Bio). Human hypoxanthine phosphoribosyltransferase 1 (*hHPRT1*) mRNA (NM_000194) was prepared and used as an internal control. Samples were analyzed by using an ABI Prism 7500 Sequence Detection System (Applied Biosystems). The expression level of *hTERT* mRNA was corrected by reference to that of *hHPRT1* mRNA, and the amount of *hTERT* mRNA relative to that in PBMCs was calculated by the comparative threshold cycle method. K562, which strongly expresses *hTERT* mRNA, was used as an internal control.