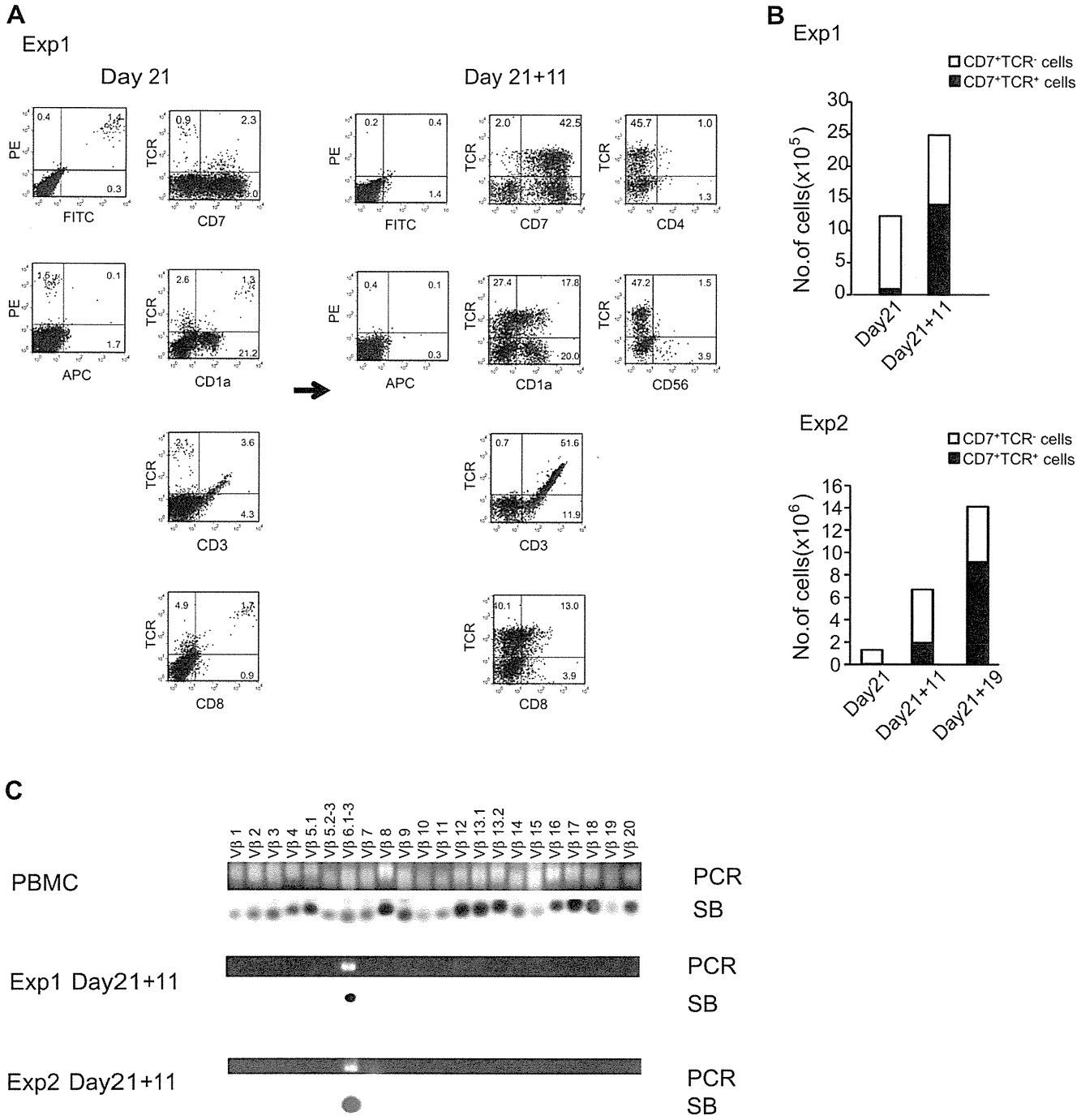

Figure 6. Generation of T cell precursors from TCR-transduced hematopoietic progenitors. $CD34^+$ cells (4×10^4 cells/well) were transduced with the TCR during coculture on telomerized stromal cells for 7 days, and then recultured on D1D4 stromal cells for 14 days in the presence of 3GF. Cultured cells were then recultured on Delta-1–transduced OP-9 stromal cells in the presence of Flt3L (5 ng/mL) and IL-7 (5 ng/mL) for an additional 11 or 19 days. The phenotype **(A)** and number **(B)** of $CD7^+TCR^-$ and $CD7^+TCR^+$ cells were assessed. Data are the mean of duplicate cultures. **(C)** PCR analysis of Vβ in peripheral blood mononuclear cells (PBMCs) or cells cocultured for 11 days on Delta-1–expressing OP9 stromal cells after coculture on telomerized stromal cells and then D1D4 stromal cells for 21 days. PCR products were evaluated using Southern blot (SB) analysis.

Among the cytokines, SCF, Flt3L, and TPO coordinately promoted the generation of proT and proB cells from human hematopoietic progenitors on stromal cells. Conversely, these effects were inhibited by IL-3 and GM-CSF by directly acting on hematopoietic progenitors. Similar effects by cytokines were observed in the generation of plasmacytoid dendritic cells belonging to the lymphoid lineage (data not shown) [44]. An inhibitory effect of IL-3 on B cell development has been suggested by other studies using murine stromal cells [19,45],

but our data are the first to demonstrate that IL-3 suppresses the generation of various types of lymphoid precursors on human bone marrow stromal cells. Because no effect was observed with other cytokines, such as G-CSF, IL-6, and IL-15, a different approach, such as engineered production of Hox B4 protein from stromal cells, would be required to obtain higher numbers of T cell precursors [46].

Delta-1 or -4 expression on stromal cells similarly promoted pre–T cell differentiation, and their coexpression additively promoted preT cell differentiation. It remains uncertain whether Delta-1 and -4 ligands bind to distinct Notch receptors or identical Notch receptors with different affinities [7,11–13,42,47]. The mechanism of the additive effect of Delta-1 and -4 cannot be explained by HES-1 gene expression. However, our study suggests that coexpression of Delta-1 and -4 on stromal cells induces a higher percentage of hematopoietic progenitors to differentiate into pre–T cells. Notably, although even bone marrow stromal cells transduced with Delta-1 and -4 did not support T cell differentiation into the $CD1a^+$ cell stage, Delta ligand expression on human thymic stromal cells promotes differentiation into $CD7^+CD1a^+$ cells that are detectable in the thymus [48]. These data imply that not only Delta ligand-mediated Notch signaling, but also unknown signals from thymic stromal cells are required for T cell differentiation into the $CD7^+CD1a^+$ stage.

Transduction of the TCR into hematopoietic progenitors followed by coculture on Delta-transduced human bone marrow stromal cells led to the generation of pre-T cells expressing the TCR, although the transduction efficiency of the TCR into hematopoietic progenitors appeared remarkably lower than that in previous studies targeting mature T cells [35]. Nonetheless, these TCR-transduced T cell precursors, upon coculture with Delta-1–expressing OP-9 murine stromal cells, promptly and remarkably proliferated and differentiated toward $CD8^+$ cells, relative to that of nontransduced T cell precursors. Although it has been speculated that TCR-transduced T lymphoid precursors differentiated toward $CD8^+$ cells rather than $CD4^+$ cells, presumably because of a lack of human leukocyte antigen class II expression on OP-9 stromal cells [49], it is interesting to note that similar rapid growth has been observed in other studies by coculture of TCR-transduced hematopoietic progenitors on Delta-1–expressing OP-9 murine stromal cells from the beginning of cultures [27,28,49]. Further investigation is required to elucidate whether such rapid proliferation of TCR-transduced T cell precursors occurs at or beyond the $CD1a^+$ stage, or by a specific interaction with OP-9 murine stromal cells. Nevertheless, these studies will contribute to our understanding of the regulation of human early T lymphopoiesis on bone marrow stromal cells and to the development of novel therapies with T cell precursors.

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Conflict of interest disclosure

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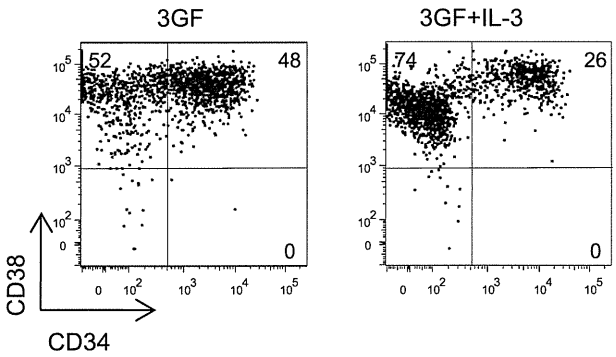
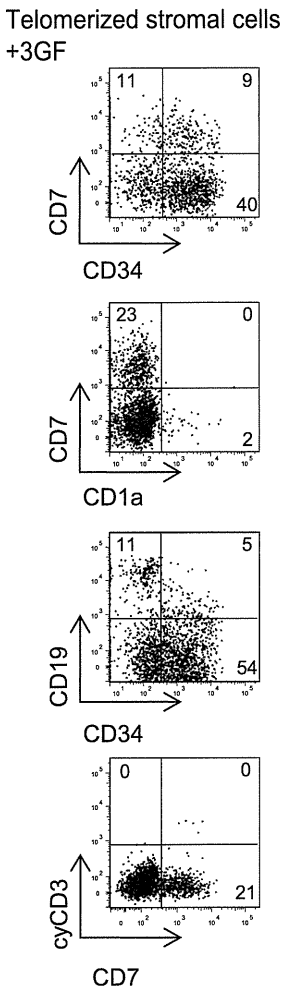
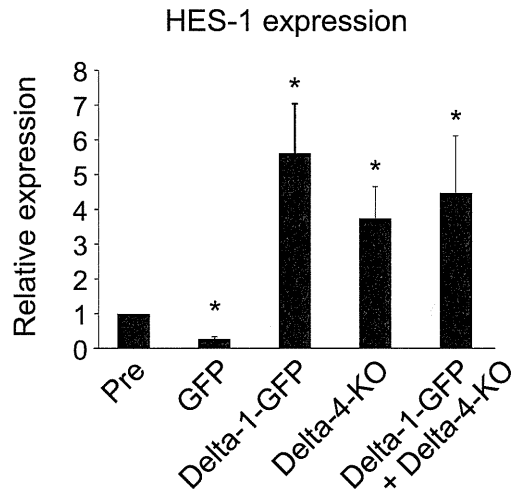


Supplementary Figure 1. Effect of IL-3 on the differentiation of hematopoietic progenitors. $CD34^{+}CD38^{lo/-}CD7^{-}CD19^{-}CD10^{-}$ cells were cultured with telomerized stromal cells and 3GF in the presence or absence of IL-3. The expression of CD34 and CD38 after exclusion of the $CD14^{+}$ population is shown.



Supplementary Figure 2. T and B cell differentiation on nontransduced telomerized stromal cells. The phenotypes of $CD34^{+}$ cells (4×10^{4} cells/well) after coculture on nontransduced telomerized stromal cells in the presence of 3GF for 3 weeks are shown.



Supplementary Figure 3. Comparison of HES-1 expression. Relative expression of HES-1 mRNA in uncultured $CD34^{+}$ cells and cells generated by coculture of $CD34^{+}$ cells on stromal cells transduced with GFP, Delta-1–GFP, Delta-4–KO or Delta-1–GFP plus Delta-4–KO vectors in the presence of 3GF for 24 hours are shown. Relative gene expression was calculated as the fold induction compared with untreated $CD34^{+}$ cells. Data are the means ± SD of triplicate cultures. Quantitative reverse transcriptase PCR was performed by modification of a previously published method [33]. Hairy and enhancer of split homolog-1 (HES-1) primers were obtained from Assays on-Demand (Assay ID: Hs00172878_m1; Applied Biosystems, Foster, CA, USA). PCR conditions were as follows: initial denaturation at 95°C for 15 min, and then 50 cycles of denaturation at 94°C for 1 min, and annealing and extension at 60°C for 1 min. Transcript quantification was performed in triplicate for each sample. Gene expression was normalized to that of endogenous glyceraldehyde-3-phosphate dehydrogenase as an internal standard (Pre-Developed TaqMan Assay Reagents, 4326317E; Applied Biosystems). Relative gene expression was calculated as a fold induction compared with that in untreated $CD34^{+}$ cells.

INTERNAL MEDICINE

□ CASE REPORT □

Amelanotic Malignant Melanoma of Unknown Primary Origin Metastasizing to the Bone Marrow: A Case Report and Review of the Literature

Tomotaka Suzuki[1], Shigeru Kusumoto[2], Shinsuke Iida[2], Toyohiro Tada[3] and Fumiko Mori[1]

Abstract

We herein describe the case of a 77-year-old Japanese man who presented with progressive thrombocytopenia. No lymphadenopathies, bone lesions, hepatosplenomegaly or masses within any internal organs were detectable. Bone marrow smears revealed diffuse infiltration of large atypical cells morphologically resembling mature lymphoid neoplasms. A flow cytometric analysis showed that the tumor cells strongly expressed CD56 without myeloid or lymphoid antigens, suggesting that they were non-hematologic in origin. Ultimately, amelanotic malignant melanoma of unknown primary origin was diagnosed based on positive immunostaining for S100 proteins, HMB-45 and Melan-A. This case illustrates the usefulness of flow cytometric analyses for making such diagnoses. We also review the available literature on similar cases.

Key words: malignant melanoma, unknown primary origin, bone marrow, amelanotic, CD56

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Introduction

Although more than 90% of malignant melanomas have a cutaneous origin, melanomas may present metastatically in the absence of an identified primary lesion (1); such tumors are designated "melanomas of unknown primary origin" (MUP). Furthermore, some malignant melanomas are "amelanotic," meaning that they do not contain histopathologically detectable melanin pigment, which often makes diagnosing MUP difficult (2, 3). We herein report a case of bone marrow metastasis of amelanotic MUP and describe the usefulness of flow cytometric analyses for diagnosing this disease, with a review of the literature.

Case Report

A 77-year-old Japanese man was referred to our hospital due to progressive thrombocytopenia that had developed over the previous two months. His medical history included myocardial infarction, diabetes mellitus and early gastric cancer that was completely resected via endoscopy. On a physical examination, the patient exhibited general weakness and significant emaciation (body mass index: 17 kg/m^2) without other significant findings, including superficial lymphadenopathies or skin lesions. He also had no fever or night sweats.

Peripheral blood tests showed thrombocytopenia (platelet count: 2.6×10^4/μL), normocytic anemia (a hemoglobin level of 10.3 g/dL with an absolute reticulocyte count of 4.5×10^4/μL) and mild leukocytosis (1.1×10^4/μL with 10% myelocytes, 4% metamyelocytes, 74% neutrophils, 9% lymphocytes, 2% monocytes, 1% eosinocytes and 4% erythroblasts). Additional laboratory tests showed an elevated FDP level of 153 μg/dL, suggesting the presence of coexisting disseminated intravascular coagulation (DIC). No lymphadenopathies, bone sclerotic or lytic lesions, hepatomegaly, splenomegaly or masses within any internal organs were detectable on contrast-enhanced computed tomography.

Bone marrow aspiration indicated hypercellular marrow with diffuse involvement of tumor cells and strongly suppressed hematopoiesis, as revealed on May-Giemsa staining.

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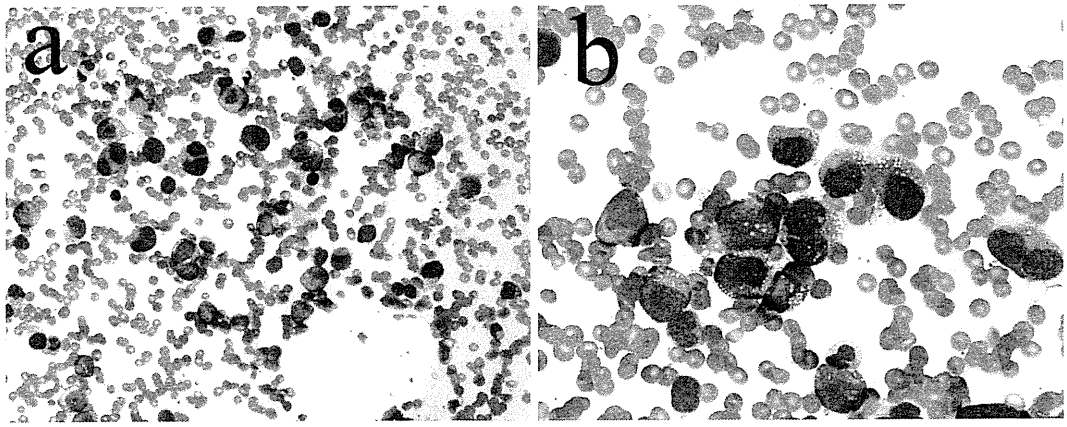

Figure 1. **Bone marrow aspiration smear showing infiltration of large atypical cells. The cells had vacuoles in the cytoplasm (a: original magnification ×200, b: ×400 with May-Giemsa staining).**

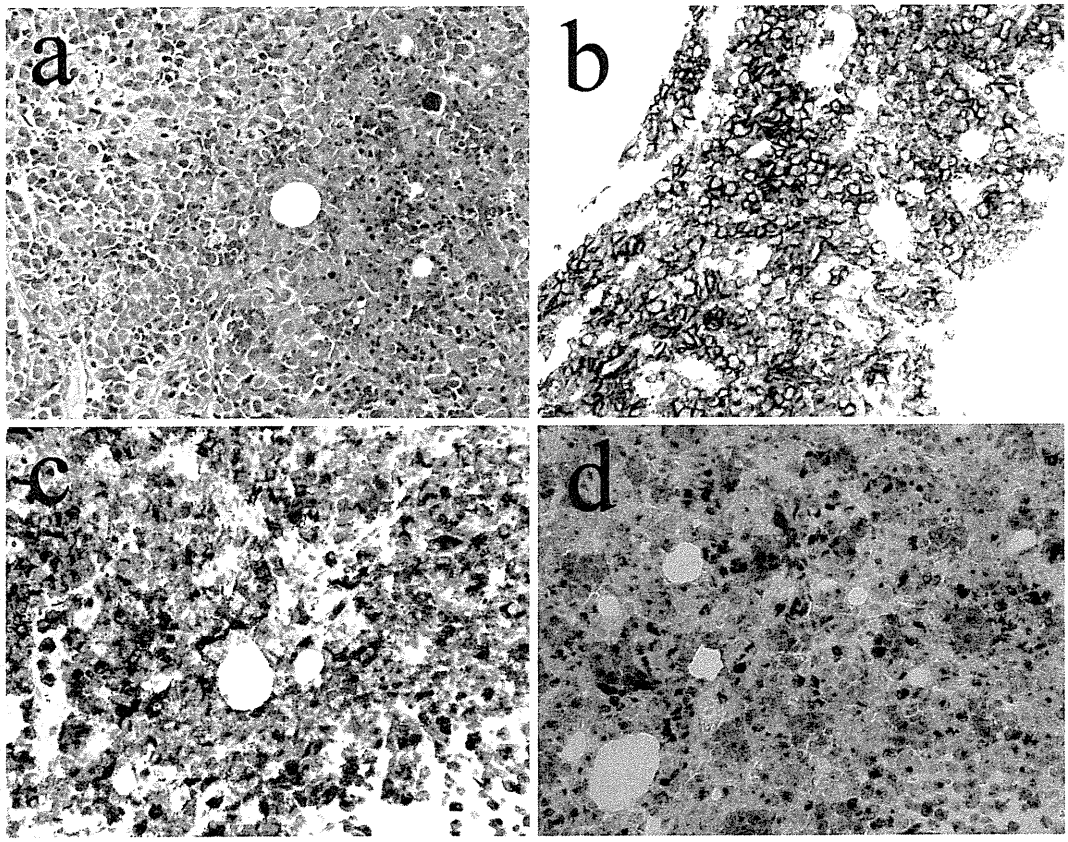

Figure 2. **Bone marrow biopsy specimens showing diffuse infiltration of tumor cells with some clear nucleoli (a: ×400 with Hematoxylin and Eosin staining). The cells were positive for CD56 (b: ×400), HMB-45 (c: ×400) and Melan-A (d: ×400).**

The tumor cells were non-cohesive and large with abundant vacuoles in basophilic cytoplasm. More than two nuclei were evident in 5% to 10% of infiltrating cells. The tumor cells were negative for myeloperoxidase staining and, based on their morphology, were suspected of being derived from mature lymphoid neoplasms (Fig. 1a, b).

However, an immunophenotypic analysis of bone marrow aspiration using flow cytometry (FCM) showed a strong CD56 expression in the tumor cells; however, myeloid and lymphoid antigens, including CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD11c, CD16, CD19, CD20, CD23, CD25, CD30, CD33, CD34, CD43 and CD45, were absent.

The bone marrow biopsy specimen showed diffuse infiltration of large, rounded neoplastic cells with clear nucleoli without evidence of melanin pigmentation (Fig. 2a). An immunohistochemical examination demonstrated tumor cells positive for CD56 (Fig. 2b), S100 proteins, HMB-45 (Fig. 2c) and Melan-A (Fig. 2d) and negative for CD3, granzyme, keratin, synaptophysin and chromogranin A. These results confirmed that the patient had metastasis of

Table. **Three Case Reports of Malignant Melanoma of Unknown Primary Origin with Metastasis to the Bone Marrow**

	Reference No.9	Reference No.2	current case
Age/sex	34/male	22/male	77/male
Chief complaint	axillary mass	weakness, weight loss	thrombocytopenia
Blood examination			
WBC (/μL)	2,900	7,800	11,400
Hb (g/dL)	7.1	5.5	10.2
Plt (/μL)	20,000	65,000	20,000
Leukoerythroblastosis	Yes	Yes	Yes
Other metastasis sites	lymph node	lymph node	none
Amelanotic/melanotic	melanotic	melanotic	amelanotic
Flow cytometric analysis	performed CD56 was not described	not performed	performed CD56 was positive
Positive immunostains	S 100 proteins, HMB-45	S 100 proteins, HMB-45	S 100 proteins, HMB-45, Melan-A
Prognosis	died 3 weeks after diagnosis	not available	died 1 week after diagnosis

malignant melanoma to the bone marrow.

We carefully reexamined the patient after making the diagnosis, although no evidence of concomitant cutaneous or mucosal primary lesions was identified and no previous history of skin lesions or surgery was reported. Although the patient had been periodically examined by an ophthalmologist due to his diabetic retinopathy, uveal melanoma had not been detected. He experienced continuous nasal and gastrointestinal bleeding as a result of rapidly aggravating DIC and died approximately one week after the diagnosis on palliative treatment.

Discussion

The incidence of MUP varies from 4% to 8% of patients with malignant melanoma (4-7). Standard criteria for diagnosing MUP have been described by Das Gupta (8); the present patient met these diagnostic criteria. Baab et al. reported the metastatic site at diagnosis in 98 cases of MUP to be as follows: lymph node metastases only (55%), subcutaneous metastases with or without lymph node involvement (13%) and visceral metastases with or without lymph node involvement (32%). Among the visceral cases, metastasis to the bone was observed in only two patients (2% of the MUP cases) although little information was available regarding bone marrow metastasis of MUP (4). Therefore, MUP with metastasis to the bone marrow is rare. To the best of our knowledge, there are only two case reports of bone marrow metastasis of MUP (2, 9) (Table).

The histopathological findings of MUP suggest that the tumor cells are comprised of polygonal or spindle cells with prominent nucleoli (2). On the other hand, they may also exhibit varying cellular morphology with high-grade anaplastic cellular features (10). Because making the morphological diagnosis of MUP is often difficult, approximately 10% of MUP lymph node biopsies may be misdiagnosed as anaplastic carcinoma instead of MUP (5). Importantly, most melanoma cells have melanin pigment in their cytoplasm, which is a major clue to suspect MUP; however, the diagnosis of MUP is established based on immunostaining for markers such as HMB-45, Melan-A and S-100 proteins (11, 12).

However, if the MUP cells are amelanotic, the tumor cells will lack melanin pigment, as observed in the present case, and it becomes difficult to identify malignant melanoma based on histopathology (10). Because there are multiple differential diagnoses to be considered in such cases, the diagnostic process is complicated. Giuliano et al. reported that 50 of 2,881 melanoma patients (1.7%) had amelanotic malignant melanoma, including 29 amelanotic primary sites and 21 amelanotic metastases from a melanotic primary site. Only three patients presented with an absence of pigment in both the primary and metastatic sites (3). Interestingly, amelanotic melanoma has been reported to be associated with an even more aggressive clinical course and a high incidence of metastasis (13-15), similar to the fulminant course observed in the present case.

FCM is an essential tool for diagnosing hematological malignancies in order to confirm the immunophenotype of leukemia and lymphoma (16). However, there is little evidence regarding the clinical significance of FCM for diagnosing non-hematological malignancies. In the presented case, the tumor cells were strongly positive for CD56 but expressed no T-cell, B-cell or myeloid antigens, suggesting that they were more likely non-hematological in origin. CD56 is a cell adhesion protein called NCAM1 (neural cell adhesion molecule 1) that is known to be expressed in hematological malignancies, such as NK/T cell neoplasms, malignant myeloma and some cases of acute leukemia. However, CD56 is also expressed in some non-hematological malignancies, such as melanoma (17), small cell carcinoma (18), rhabdomyosarcoma (19), Merkel cell carcinoma (20), neuroblastoma (21) and neuroendocrine tumors/carcinoma (22).

Farionla et al. evaluated the CD56 expression in neuroendocrine tumors obtained from fine-needle aspiration biopsy specimens and reported the utility of the CD56 expression in the absence of lymphoid antigens for specifically diag-

nosing neuroendocrine tumors (22). On the other hand, few reports regarding the usefulness of FCM in diagnosing malignant melanoma have been published. Bhagwati et al. performed FCM on samples of bone marrow metastasis obtained from a patient with MUP. However, the authors did not test for CD56 positivity and obtained little information for diagnosing malignant melanoma using FCM (9).

We herein reported a case of amelanotic MUP with metastasis exclusively to the bone marrow and demonstrated the usefulness of FCM for diagnosing malignant melanoma, especially when the tumor cells at the biopsy site are amelanotic.

The authors state that they have no Conflict of Interest (COI).

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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).

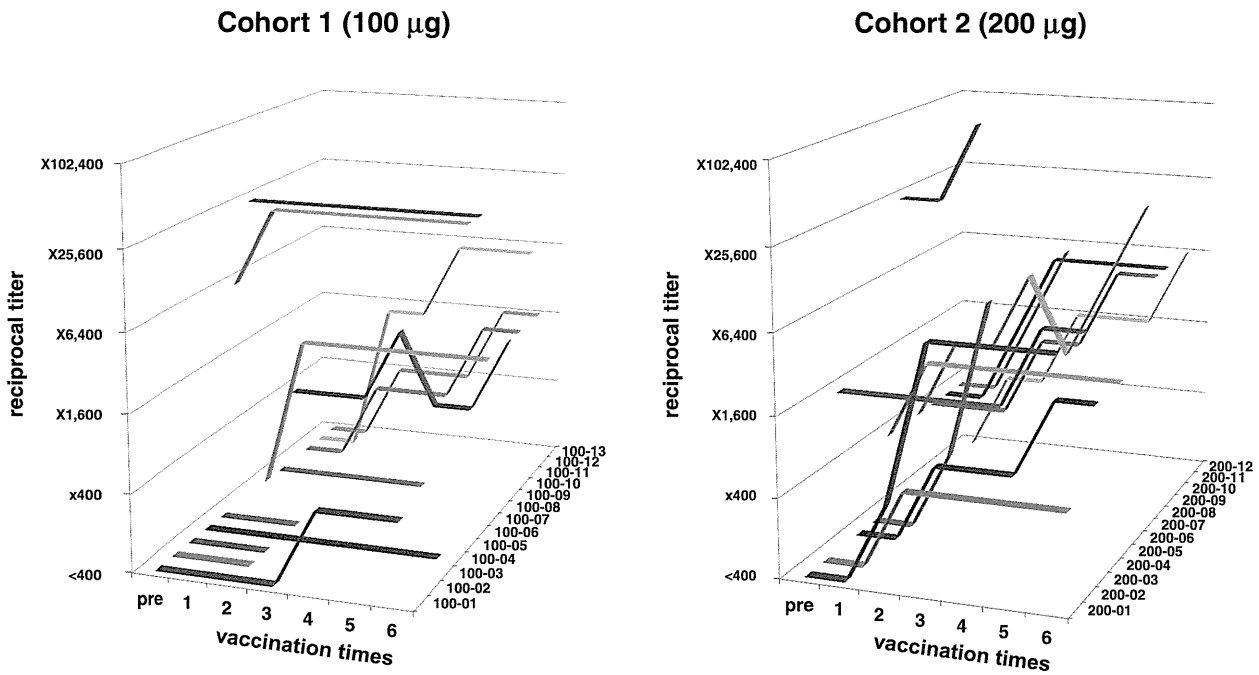

Figure 1 Antibody responses to recombinant NY-ESO-1 protein as determined by ELISA. Sera from 13 patients in Cohort 1 (100-μg dose) and 12 patients in Cohort 2 (200-μg dose) were collected at each vaccination, and serially diluted by 400, 1,600, 6,400, 25,600 and 102,400. Reciprocal titers were determined 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.

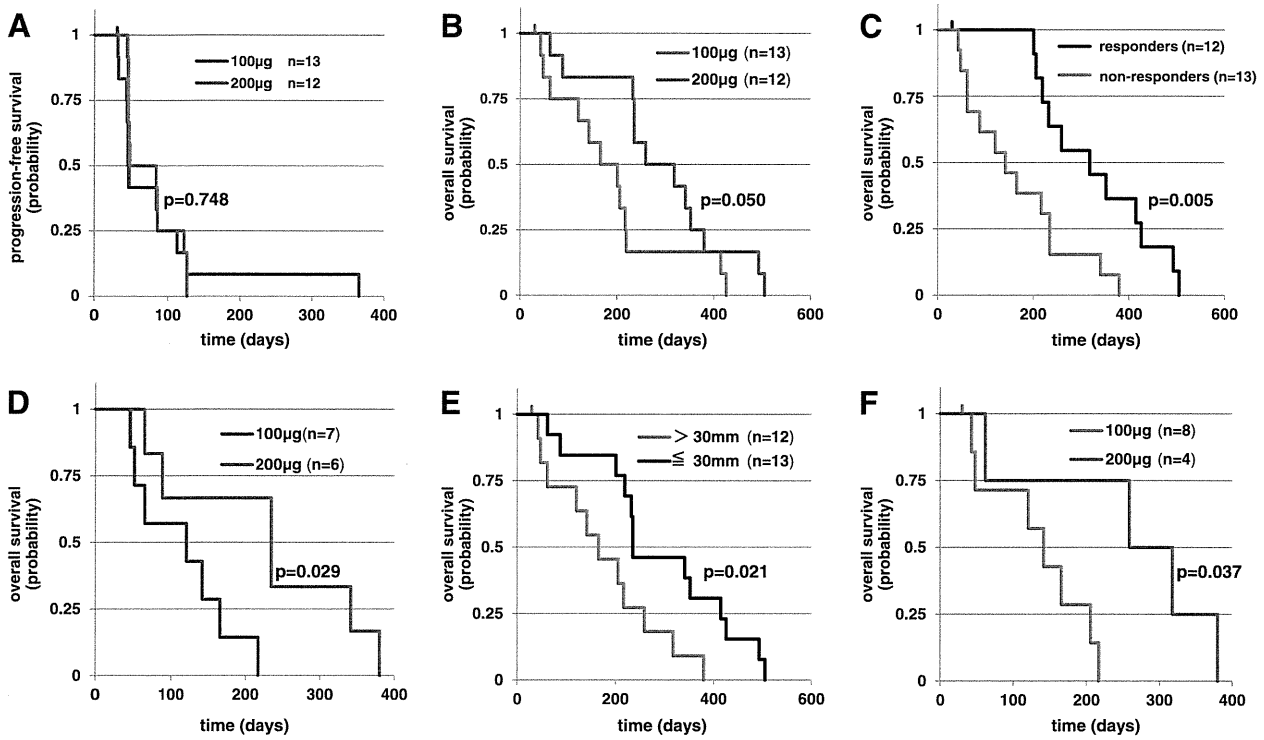

Figure 2 Kaplan-Meier survival curves of patients who were alive after the CHP-NY-ESO-1 vaccinations. A: Progression-free survival (PFS) of patients assigned to Cohort 1 (100-μg dose, n = 13) or Cohort 2 (200-μg dose, n = 12). The probability of PFS of the patients who received 200 μg of the vaccine 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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New findings of kinase switching in gastrointestinal stromal tumor under imatinib using phosphoproteomic analysis

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Despite the revolutionary effects of imatinib on advanced gastrointestinal stromal tumors (GISTs), most patients eventually develop disease progression following primary resistance or acquired resistance driven by secondary-resistant mutations. Even in radiographically vanishing lesions, pathology has revealed persistent viable cells during imatinib therapy, which could lead to the emergence of drug-resistant clones. To uncover the mechanisms underlying these clinical issues, here we examined imatinib-induced phosphoproteomic alterations in GIST-T1 cells, using our quantitative tyrosine phosphoproteomic analysis method, which combined immunoaffinity enrichment of phosphotyrosine-containing peptides with isobaric tags for relative and absolute quantitation (iTRAQ) technology. Using this approach, we identified 171 tyrosine phosphorylation sites spanning 134 proteins, with 11 proteins exhibiting greater than 1.5-fold increases in tyrosine phosphorylation. Among them, we evaluated FYN and focal adhesion kinase (FAK), both of which are reportedly involved in proliferation and malignant alteration of tumors. We confirmed increased tyrosine phosphorylation of both kinases by western blotting. Inhibition of FYN and FAK phosphorylation each increased tumor cell sensitivity to imatinib. Furthermore, a FAK-selective inhibitor (TAG372) induced apoptosis of imatinib-resistant GIST-T1 cells and decreased the imatinib IC_{50}. These results indicate that FYN or FAK might be potential therapeutic targets to overcome resistance to imatinib in GISTs. Additionally, we showed that the iTRAQ-based quantitative phosphotyrosine-focused phosphoproteomic approach is a powerful method for screening phosphoproteins associated with drug resistance.

The gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of the digestive tract, and is characterized by expression of the KIT (CD117) and/or DOG1 proteins. Most GISTs have oncogenic *KIT* or *PDFGRA* mutations, which is a key factor in sporadic GIST pathogenesis and proliferation.[1–3] This knowledge has facilitated the development of targeted therapies with tyrosine kinase inhibitors and led to the revolutionary treatment with imatinib mesylate (Glivec®; Novartis Pharmaceuticals). Recent clinical trials with advanced/unresectable GIST have shown that imatinib produces objective responses in ~50% of patients, and disease stabilization (stable disease) in another 30–40%. The corresponding 2-year overall survival rates range from 70 to 80%, indicating markedly improved patient outcomes compared with anecdotal data from cytotoxic chemotherapy in the preimatinib era.[4–6]

Despite imatinib's effectiveness, there remain several problems. First, GIST patients cannot stop taking the drug even if complete response is obtained, because discontinuation inevitably leads to reprogression and disease relapse.[7,8] Second, imatinib activity is limited by primary resistance to the drug in ~15% of patients, and secondary resistance eventually develops in more than 80% of patients.[5,9] Secondary resistance mainly occurs due to additional kinase domain mutations, which are thought to develop in viable tumor cells (persistent cells) during imatinib therapy. It is not yet known

Key words: GIST, imatinib, iTRAQ, proteomics

Abbreviations: FAK: focal adhesion kinase; GIST: gastrointestinal stromal tumor; iTRAQ: isobaric tags for relative and absolute quantitation; LC: liquid chromatography; MS/MS: tandem mass spectrometry; shRNA: small hairpin RNA; siRNA: small interfering RNA

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What's new?

While the targeted tyrosine kinase inhibitor imatinib can significantly improve two-year survival rates for patients with gastrointestinal stromal tumor (GIST), primary and secondary resistance mutations often limit its benefits. This study of the human GIST-T1 cell line suggests that imatinib-induced increases in tyrosine phosphorylation of FYN kinase and focal adhesion kinase (FAK) may be responsible for mediating some instances of imatinib resistance and therefore may be potential targets for killing persistent tumor cells and overcoming resistance. The findings also indicate that iTRAQ-based quantitative phosphotyrosine-focused proteomic analysis is a useful way of screening for phosphoproteins associated with drug resistance.

what mechanisms keep these persistent cells alive after shutdown of KIT signaling by imatinib.

Phosphorylation of protein kinases in signaling pathways is a key event in tumor cell survival and proliferation. Differential phosphoprotein analysis may provide clues to alternatively activated pathways and/or substituted kinases that may be activated after inhibition of main pathways, such as KIT.[10] Recent advances in mass spectrometry-based phosphoproteomics enable extensive profiling of serine-threonine kinases, while tyrosine kinase analysis has remained challenging in terms of quantity and quality. However, a recent method coupling peptide-level antiphosphotyrosine immunoaffinity purification with liquid chromatography (LC)-tandem mass spectrometry (MS/MS) has provided reasonable profiling for tyrosine phosphorylation.[11]

In the present study, we quantitatively measured the phosphoproteomic alterations induced by imatinib in a GIST-T1 cell line. We also examined the roles of some tyrosine kinases that were activated in persistent tumor cells after imatinib exposure.

Material and Methods

Cell lines

We previously established the human GIST cell line GIST-T1, which has a 57-nucleotide (V570-Y578) in-frame deletion in KIT exon 11.[12] The cell line identity was confirmed by DNA fingerprinting through short tandem repeat profiling, as previously described.[13] GIST-T1-R was established from GIST-T1 as an imatinib-resistant clone that arose from continuous culturing in 5 μM imatinib. The GIST-T1-R cells GIST-T1-R2 and GIST-T1-R8 each exhibit imatinib IC_{50} values of ~30 μM, which is ~1000 times that of the GIST-T1 parent.

Reagents and antibodies

Imatinib and TAG372—selective tyrosine kinase inhibitors for KIT and focal adhesion kinase (FAK), respectively—were synthesized and provided by Novartis Pharmaceuticals (Basel, Switzerland). The following primary antibodies were used: anti-phospho-Src Family (Tyr416) (1:1000), anti-phospho-ERK and anti-ERK from Cell Signaling Technology (Danvers, MA); anti-GAPDH from Santa Cruz Biotechnology (Santa Cruz, CA); anti-phospho-FAK (Tyr397) from Biosource (Camarillo, CA); anti-FAK from BD Transduction Laboratories (San Jose, CA) and anti-phosphotyrosine (clone 4G10) from Upstate Biotechnology (Lake Placid, NY). Detailed immunoprecipitation information is provided in the Supporting Information Materials and Methods section.

Peptide synthesis

A tyrosine-phosphorylated peptide (NVPLyK) derived from a trypsinized peptide sequence of yeast alpha-enolase was synthesized at Sigma Aldrich (Milwaukee, WI) using standard solid-phase peptide synthesis techniques and Fmoc chemistry.

Phosphopeptide immunoprecipitation

GIST-T1 cells were treated with 400 nM of imatinib for 0, 1, 6 and 24 hr. Tyrosine-phosphorylated peptides were purified using Cell Signaling PhosphoScan pTyr100 Kits (Beverly, MA) following the manufacturer's instructions with minor modification. Detailed information is provided in the Supporting Information Materials and Methods section.

iTRAQ labeling

After immunoprecipitation, peptides were dissolved in 9.8 M Urea (5 μL) and 1 M TEAB (20 μL). Following the manufacturer's protocol (Applied Biosystems, Foster City, CA), the samples were labeled with the isobaric tags for relative and absolute quantitation (iTRAQ) reagents as follows: GIST-T1 with reagent 114 (0 hr), GIST-T1 with reagent 115 (1 hr), GIST-T1 with reagent 116 (6 hr) and GIST-T1 with reagent 117 (24 hr). The labeled peptide samples were then pooled and desalted with Sep-Pak Light C18 Cartridges, and the peptides were dried in a SpeedVac. The labeled peptide mixtures were purified and fractionated into 14 fractions using strong cation exchange fractionation, as previously described.[13]

Quantitative mass spectrometric analysis

Nano LC-MS/MS analysis and iTRAQ data analysis were performed as described in the Supporting Information Materials and Methods section.

Small interfering RNA transfection

Commercial FAK small interfering RNA (siRNA) and nonspecific siRNA were obtained from QIAGEN. Cells were transfected with siRNA using Lipofectamine 2000 reagent