To evaluate the response of NFκB *in vitro*, B16F10 NFκB transfectants or B16F10 CMV control cells (1×10^5/well) were cultured in a 96-well plate and treated with TNF-α (0.1–100 ng/mL). After incubation for 6 h, luciferase activity was measured with a multiplate reader (2030 ARVO X; Perkin Elmer Life Sciences, Boston, MA, USA).

Analysis of NFκB activation with TRAIL. The B16F10 NFκB cells (1.25×10^5/well) were cocultured with either TRAIL-expressing 2PK3 (TRAIL-2PK3) cells or control 2PK3 cells (1.25×10^5, 6.25×10^4, 1.25×10^4/well) in a 96-well plate. After 6 h incubation, luciferase activity was measured with a multiplate reader. To evaluate the specificity of TRAIL on NFκB activation in B16F10 cells, TRAIL-2PK3 (1×10^5/well) cells were pretreated with anti-TRAIL mAb (clone N2B2, 10 μg/mL) at 37°C for 1 h then cocultured with B16F10 NFκB cells at 37°C for 6 h. After the incubation, luciferase activity was measured with a multiplate reader.

Cell proliferation assay. The B16F10 CMV cells (5×10^3/well) were cocultured with TRAIL-2PK3 or control 2PK3 cells (1×10^4, 5×10^3, 2.5×10^3/well) in a 96-well plate for 48 h at 37°C. After the incubation, luciferase activity was measured with a multiplate reader.

Gelatin zymography. The B16F10 NFκB cells (5×10^5/well) were cultured with serum-free medium in a 24-well plate then treated with TNF-α (50 ng/mL) or cocultured with TRAIL-2PK3 (5×10^5/well) for another 48 h. After the incubation, cell-free supernatants were collected and mixed with sample buffer containing 2% SDS (without 2-mercaptoethanol) and incubated at 37°C for 20 min. Comparative gelatin zymography was carried out on 10% SDS-PAGE with 0.1% gelatin. Samples were electrophoresed at 10 mA for 4–5 h at 4°C. Gels were washed with buffer containing 2.5% Triton X-100 and 0.01 M Tris-HCl for 2 h at 4°C and washed with 0.01 M Tris-HCl for 40 min at room temperature. Gels were incubated in the buffer containing 0.05 M Tris-HCl, 0.5 mM $CaCl_2$, and 1 μM $ZnCl_2$ for 48 h at 37°C. After the incubation, gels were stained with Coomassie Brilliant Blue for 6 h and destained with 5% acetic acid and 10% methanol. The bands were quantified using ImageQuant LAS 4010 (GE Healthcare Japan, Tokyo, Japan).

Experimental lung metastasis model. Inbred wild-type C57BL/6 mice were purchased from Japan SLC (Tokyo, Japan). All experiments were carried out according to the guidelines of the Care and Use of Laboratory Animals of the University of Toyama (Toyama, Japan). The B16F10 CMV cells were inoculated i.v. (3×10^5) with or without pretreatment with anti-DR5 mAb (30 min, 4°C). Mice were injected with D-luciferin (150 mg/kg i.p.; Promega) 4 days after the tumor inoculation, then the lungs were removed 20 min after the D-luciferin injection to measure luminescence using an *in vivo* imaging system (IVIS Lumina II; Caliper Life Sciences, Hopkinton, MA, USA). The data was presented as the mean luminescence ± SEM.

Statistical analysis. Data were analyzed for statistical significance using Student's *t*-test. *P*-values <0.05 were considered significant.

Results

Establishment of NFκB-mediated luciferase gene stably expressing B16F10 cells. In order to determine whether TRAIL–DR5 interaction may have biological roles in B16F10 metastatic melanoma cells through NFκB-mediated inflammatory signals, we established luciferase gene-expressing B16F10 cells under an NFκB reporter (B16F10 NFκB cells). We first characterized the association between cell numbers and luciferase activity of B16F10 NFκB cells or control B16F10 CMV cells under stable cell culture conditions. There was a strong correlation between luciferase activity and cell number not only in B16F10 CMV cells (Fig. S1A,B) but also in B16F10 NFκB cells (Fig. S1C,D). These results clearly indicated that the luminescence represents cell number or viability without any stimulation in those reporter cells.

We further examined the response of B16F10 NFκB cells to TNF-α, known to be a typical inflammatory cytokine to activate the NFκB pathway. As shown in Figure 1, TNF-α induced luciferase activity in a dose-dependent manner (Fig. 1a) and

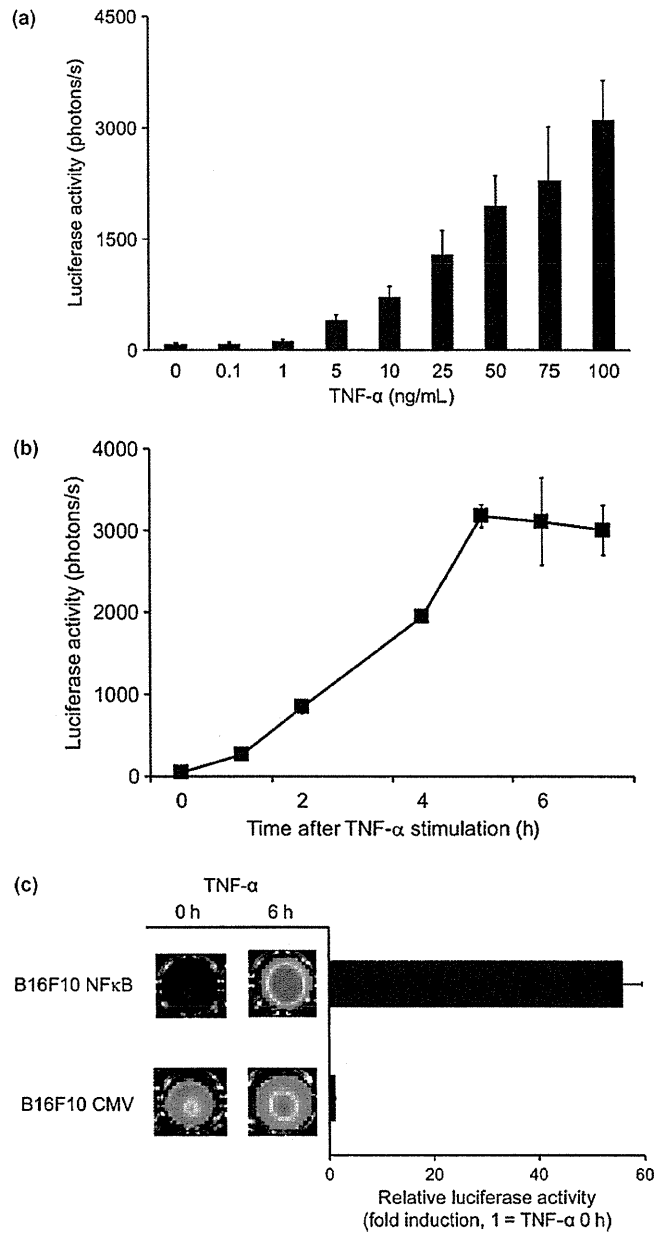

Fig. 1. Nuclear factor-κB (NFκB) activation after tumor necrosis factor-α (TNF-α) treatment in B16F10 mouse melanoma cells. (a) B16F10 NFκB cells were incubated with indicated concentrations of TNF-α for 6 h and the luminescence was measured. (b) B16F10 NFκB cells were treated with TNF-α (100 ng/mL) and the luminescence was measured at the indicated time after the TNF-α stimulation. (c) B16F10 NFκB and B16F10 CMV cells were stimulated TNF-α (100 ng/mL) for 6 h. The luminescence was measured at 0 h and 6 h after TNF-α stimulation. Error bars represent SEM.

appeared to have a peak response at 6–8 h after the TNF-α stimulation (Fig. 1b). Such induction of luciferase activity in response to TNF-α was specific for B16F10 cells expressing NFκB reporter, because B16F10 CMV cells did not show any response in its luciferase activity after TNF-α stimulation (Fig. 1c). These results indicate that B16F10 NFκB cells but not B16F10 CMV cells induce their luminescence in response to inflammatory stimulation through the NFκB pathway.

Interaction between TRAIL and DR5 activates NFκB in B16F10 cells. It is known that highly metastatic B16F10 melanoma cells are resistant to TRAIL-induced apoptosis despite their expression of DR5 receptor (Fig. S1E,F). To investigate whether TRAIL–DR5 interaction activates the inflammatory signaling pathway in B16F10 cells, we tested B16F10 NFκB cells to monitor NFκB activation in response to TRAIL stimulation. After coculture with TRAIL-2PK3 transfectants, B16F10 NFκB cells showed increased luminescence, but not with control 2PK3 cells (Fig. 2a). The reporter activity was associated with the amount of TRAIL availability within the coculture (Fig. 2a). Importantly, such induction of reporter activity was diminished in the presence of anti-TRAIL mAb (Fig. 2b). Furthermore, the activation of TRAIL receptor by agonistic anti-DR5 mAb also activated NFκB reporter in a dose-dependent manner (Fig. S2). Collectively, these results indicate that TRAIL–DR5 interaction activates the NFκB pathway in B16F10 cells.

Interaction between TRAIL and DR5 functionally activates B16F10 cells. We next examined whether TRAIL shows any functional roles in B16F10 cells in association with NFκB activation. In concert with NFκB activation, the proliferation rate of B16F10 cells was increased after 48 h of coculture with TRAIL-2PK3 cells but not with control 2PK3 cells (Fig. 3a). In addition to its activity in promoting proliferation, TRAIL–DR5 interaction also increased the production of MMP-9 from B16F10 cells, which is known to be a typical target molecule for NFκB activation. As shown in Figure 3(B), the activity of MMP-9 in the cell culture supernatant of TRAIL-stimulated B16F10 cells was higher than that of the control, and the induction of MMP-9 by TRAIL stimulation was comparable to TNF-α. These results clearly indicate that TRAIL–DR5 functionally activates B16F10 cells to facilitate their proliferation and MMP-9 production.

Finally, we examined the physiological significance of TRAIL–DR5 interactions in cancer metastasis. In an experimental lung metastasis model of B16F10 melanoma cells, we found that the activation of TRAIL receptor by pretreatment with agonistic anti-DR5 mAb enhanced the metastatic colonization of B16F10 cells (Fig. 4). Together with the functional role of TRAIL engagement in B16F10 cells *in vitro*, these results strongly imply that TRAIL–DR5 interactions have a physiological potential to enhance metastasis of B16F10 melanoma cells rather than to induce apoptosis of the cells expressing these receptors.

Discussion

Tumor necrosis factor-related apoptosis-inducing ligand is associated with one of the important effector pathways in the tumor immune surveillance, and the TRAIL signal has been known to induce the suppression of tumor metastasis by inducing apoptosis of malignantly transformed cells.[4,10,12,30] In contrast, it is also suggested that TRAIL may be involved in

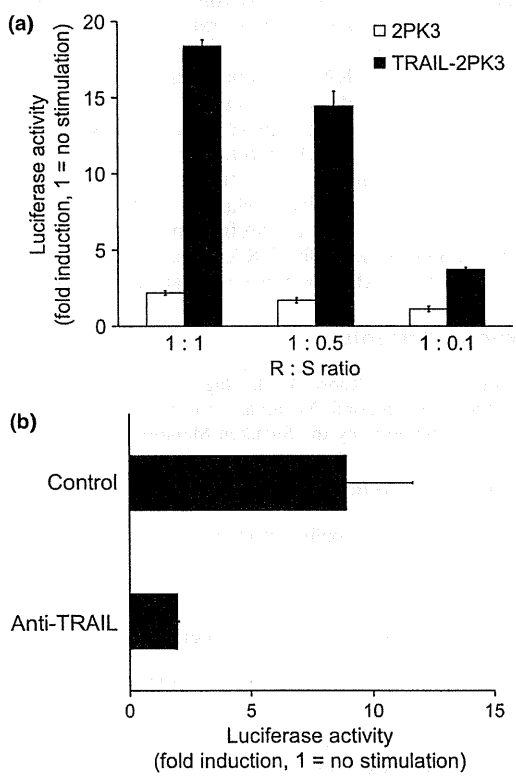

Fig. 2. Nuclear factor-κB (NFκB) activation through tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)–DR5 interaction in B16F10 mouse melanoma cells. (a) B16F10 NFκB cells were cocultured with TRAIL-2PK3 or 2PK3 at indicated responder (R):stimulator (S) ratios (B16F10 NFκB : TRAIL-2PK3 or 2PK3). After 6 h incubation, the luminescence was measured. (b) B16F10 NFκB cells were cocultured with TRAIL-2PK3 (at R:S 1:1) and N2B2 (10 μg/mL). After 6 h incubation, the luminescence was measured. Error bars represent SEM.

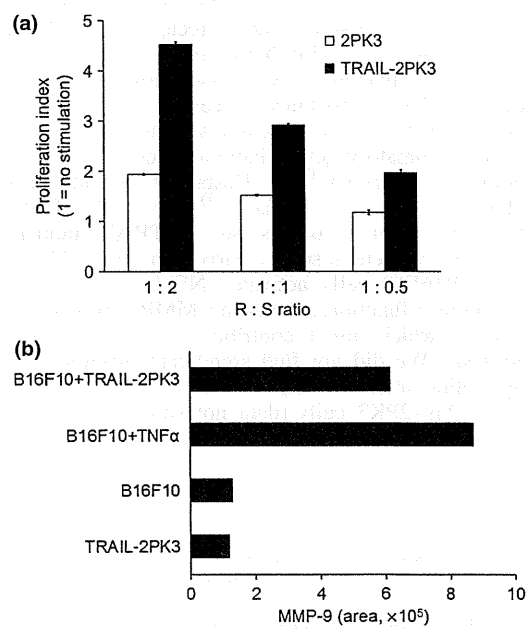

Fig. 3. Functional roles of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)–DR5 pathway in B16F10 mouse melanoma cells. (a) B16F10 CMV cells were cocultured with TRAIL-2PK3 or control 2PK3 cells at responder (R):stimulator (S) ratio of 1:1 for 48 h and luminescence was measured. Error bars represent SEM. (b) B16F10 cells were stimulated with tumor necrosis factor-α (TNF-α; 50 ng/mL) or cocultured with TRAIL-2PK3 (at R:S 1:1) for 48 h, and the cell-free supernatant was collected. Gelatin zymography was used to determine MMP-9 production and the band intensity was measured.

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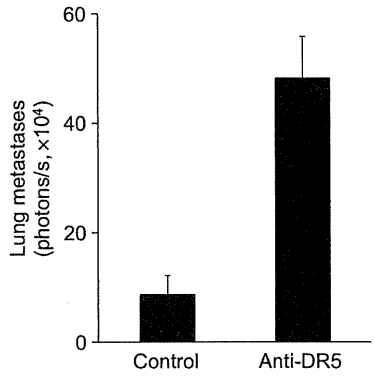

Fig. 4. Stimulation of tumor necrosis factor-related apoptosis-inducing ligand receptor enhances experimental metastasis of B16F10 mouse melanoma cells. B16F10 CMV cells were inoculated i.v. with or without pretreatment with anti-DR5 mAb. Lungs were removed 4 days after tumor inoculation to measure luminescence for determining lung metastasis. Data presented as the mean luminescence ± SEM.

cancer cell activation by providing inflammatory signals similar to other TNF superfamily members.[3,13,15–18] Previous studies have indicated that NFκB activation can be critical for acquiring resistance to TRAIL-induced apoptosis in some tumor cells.[19,27,28] In the present study, we showed the contribution of TRAIL–DR5 interaction to the activation of the NFκB pathway in B16F10 mouse melanoma cells, which is resistant to the TRAIL-induced apoptosis pathway. The TRAIL–DR5 interaction also plays a functional role in B16F10 cells by inducing their proliferation, MMP-9 production, and acquisition of metastatic potential *in vivo*.

It has been shown that cancer cells can evade TRAIL-induced apoptosis or acquire TRAIL resistance through several different mechanisms.[6,7,12,15] One particular mechanism can be the lower expression of death receptors for TRAIL, such as DR4 and DR5.[19–22] Furthermore, the intrinsic activation of anti-apoptotic machinery was also shown to be involved in acquiring TRAIL resistance in cancer cells.[12,31–33] It is also suggested that NFκB can be a key regulator for the expression of pro-inflammatory genes, including those for cancer cell proliferation and survival.[25,26] Despite the significant expression of DR5 on their cell surface,[24] murine B16F10 melanoma cells were known to be resistant to TRAIL inducing apoptosis.[23] Our present results clearly show that TRAIL stimulation in B16F10 cells activates NFκB and further promotes their cellular functions, including MMP-9 production and proliferation, which might contribute to cancer progression and metastasis. We did not find significant differences in B16F10 proliferation after a relatively short time (24 h) of coculture with TRAIL-2PK3 cells (data not shown), therefore, TRAIL–DR5 interaction may require more persistent interaction with its receptor in promoting B16F10 cell proliferation compared to its induction of apoptosis, which is generally seen 8–16 h after TRAIL ligation. Considering the TRAIL receptor ligation activates the NFκB pathway through interaction with the TNF receptor 1-associated death domain adaptor protein to recruit receptor-interacting protein kinase and TRAF2,[5,13,15,17] similar mechanisms might underlie the TRAIL-induced activation of NFκB in B16F10 cells to induce such cellular functions. Further study will be required to determine which signaling pathway is involved in the functional activation of B16F10 cells in response to TRAIL. Known to specifically express on host immune cells such as natural killer cells, dendritic cells, and activated T cells, TRAIL plays an important role in anti-tumor immune responses.[23,34–37] In contrast, we have shown that B16F10 melanoma cells may use TRAIL–DR5 interaction to promote their metastatic potential. Consistent with our current finding, it has been reported that TRAIL enhanced survival and/or proliferation in TRAIL-resistant primary leukemia cells in an NFκB-dependent manner.[38] Interestingly, it has also been reported that the NFκB pathway plays a role in the induction and maintenance of epithelial–mesenchymal transition,[26,39,40] considered to be an important process of tumor invasion and metastasis spread. Furthermore, the metastasis of TRAIL-resistant human pancreatic ductal carcinoma was promoted by TRAIL in a xenograft model.[41] Additional study is clearly required to determine whether endogenous TRAIL could be involved in the malignant progression of B16 melanoma cells; however, our current findings support a contrary role of the TRAIL–DR5 pathway in the inflammatory tumor microenvironment, in inducing the metastatic potential of cancer cells rather than inducing apoptosis in B16 melanoma cells. Considering several clinical trials of agonistic human TRAIL receptor antibodies have been undertaken to test their efficacy in cancer patients,[42–46] it would be very important to characterize patients' cancer cell types in their response to TRAIL-induced apoptosis. Collectively, our present findings propose a pro-tumor role of TRAIL–DR5 interaction in murine B16F10 melanoma cells by enhancing metastatic potential. Thus, a careful approach is required in the clinical application of the TRAIL pathway in cancer treatment, especially in TRAIL apoptosis-resistant cancer cell types.

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Disclosure Statement

The authors have no conflict of interest.

References

1 Wiley SR, Schooley K, Smolak PJ *et al.* Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity* 1995; **3**: 673–82.
2 Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A, Ashkenazi A. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J Biol Chem* 1996; **271**: 12687–90.
3 Ashkenazi A. Targeting death and decoy receptors of the tumour-necrosis factor superfamily. *Nat Rev Cancer* 2002; **2**: 420–30.
4 Yagita H, Takeda K, Hayakawa Y, Smyth MJ, Okumura K. TRAIL and its receptors as targets for cancer therapy. *Cancer Sci* 2004; **95**: 777–83.
5 Gonzalvez F, Ashkenazi A. New insights into apoptosis signaling by Apo2L /TRAIL. *Oncogene* 2010; **29**: 4752–65.
6 Wang S, El-Deiry WS. TRAIL and apoptosis induction by TNF-family death receptors. *Oncogene* 2003; **22**: 8628–33.
7 Holoch PA, Griffith TS. TNF-related apoptosis-inducing ligand (TRAIL): a new path to anti-cancer therapies. *Eur J Pharmacol* 2009; **625**: 63–72.
8 Ashkenazi A, Pai RC, Fong S *et al.* Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest.* 1999; **104**: 155–62.
9 Ichikawa K, Liu W, Zhao L *et al.* Tumoricidal activity of a novel anti-human DR5 monoclonal antibody without hepatocyte cytotoxicity. *Nat Med* 2001; **7**: 954–60.
10 Seki N, Hayakawa Y, Brooks AD *et al.* Tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis is an important endogenous mechanism for resistance to liver metastases in murine renal cancer. *Cancer Res* 2003; **63**: 207–13.

11 Takeda K, Hayakawa Y, Smyth MJ *et al.* Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. *Nat Med* 2001; **7**: 94–100.
12 Dimberg LY, Anderson CK, Camidge R, Behbakht K, Thorburn A, Ford HL. On the TRAIL to successful cancer therapy? Predicting and counteracting resistance against TRAIL-based therapeutics. *Oncogene* 2013; **32**: 1341–50.
13 Falschlehner C, Emmerich CH, Gerlach B, Walczak H. TRAIL signalling: decisions between life and death. *Int J Biochem Cell Biol* 2007; **39**: 1462–75.
14 Schneider P, Olson D, Tardivel A *et al.* Identification of a new murine tumor necrosis factor receptor locus that contains two novel murine receptors for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). *J Biol Chem* 2003; **278**: 5444–54.
15 Hersey P, Zhang XD. How melanoma cells evade trail-induced apoptosis. *Nat Rev Cancer* 2001; **1**: 142–50.
16 Chaudhary PM, Eby M, Jasmin A, Bookwalter A, Murray J, Hood L. Death receptor 5, a new member of the TNFR family, and DR4 induce FADD-dependent apoptosis and activate the NF-kappaB pathway. *Immunity* 1997; **7**: 821–30.
17 Hu WH, Johnson H, Shu HB. Tumor necrosis factor-related apoptosis-inducing ligand receptors signal NF-kappaB and JNK activation and apoptosis through distinct pathways. *J Biol Chem* 1999; **274**: 30603–10.
18 Schneider P, Thome M, Burns K *et al.* TRAIL receptors 1 (DR4) and 2 (DR5) signal FADD-dependent apoptosis and activate NF-kappaB. *Immunity* 1997; **7**: 831–6.
19 Khanbolooki S, Nawrocki ST, Arumugam T *et al.* Nuclear factor-kappaB maintains TRAIL resistance in human pancreatic cancer cells. *Mol Cancer Ther* 2006; **5**: 2251–60.
20 Büneker C, Mohr A, Zwacka RM. The TRAIL-receptor-1: TRAIL-receptor-3 and -4 ratio is a predictor for TRAIL sensitivity of cancer cells. *Oncol Rep* 2009; **21**: 1289–95.
21 Nguyen T, Zhang XD, Hersey P. Relative resistance of fresh isolates of melanoma to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. *Clin Cancer Res* 2001; **7**: 966s–73s.
22 Zhang XD, Franco A, Myers K, Gray C, Nguyen T, Hersey P. Relation of TNF-related apoptosis-inducing ligand (TRAIL) receptor and FLICE-inhibitory protein expression to TRAIL-induced apoptosis of melanoma. *Cancer Res* 1999; **59**: 2747–53.
23 Kayagaki N, Yamaguchi N, Nakayama M *et al.* Expression and function of TNF-related apoptosis-inducing ligand on murine activated NK cells. *J Immunol.* 1999; **163**: 1906–13.
24 Drobits B, Holcmann M, Amberg N *et al.* Imiquimod clears tumors in mice independent of adaptive immunity by converting pDCs into tumor-killing effector cells. *J Clin Invest.* 2012; **122**: 575–85.
25 Karin M, Greten FR. NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol* 2005; **5**: 749–59.
26 Aggarwal BB, Gehlot P. Inflammation and cancer: how friendly is the relationship for cancer patients? *Curr Opin Pharmacol* 2009; **9**: 351–69.
27 Franco AV, Zhang XD, Van Berkel E *et al.* The role of NF-kappa B in TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis of melanoma cells. *J Immunol.* 2001; **166**: 5337–45.
28 Deeb D, Jiang H, Gao X *et al.* Curcumin sensitizes prostate cancer cells to tumor necrosis factor-related apoptosis-inducing ligand/Apo2L by inhibiting nuclear factor-kappaB through suppression of IkappaBalpha phosphorylation. *Mol Cancer Ther* 2004; **3**: 803–12.
29 Wang CY, Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS. NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. *Science* 1998; **281**: 1680–3.
30 Wajant H, Pfizenmaier K, Scheurich P. TNF-related apoptosis inducing ligand (TRAIL) and its receptors in tumor surveillance and cancer therapy. *Apoptosis* 2002; **7**: 449–59.
31 Hinz S, Trauzold A, Boenicke L *et al.* Bcl-XL protects pancreatic adenocarcinoma cells against CD95- and TRAIL-receptor-mediated apoptosis. *Oncogene* 2000; **19**: 5477–86.
32 Schimmer AD, Welsh K, Pinilla C *et al.* Small-molecule antagonists of apoptosis suppressor XIAP exhibit broad antitumor activity. *Cancer Cell* 2004; **5**: 25–35.
33 Sinicrope FA, Penington RC, Tang XM. Tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis is inhibited by Bcl-2 but restored by the small molecule Bcl-2 inhibitor, HA 14–1, in human colon cancer cells. *Clin Cancer Res* 2004; **10**: 8284–92.
34 Takeda K, Smyth MJ, Cretney E *et al.* Involvement of tumor necrosis factor-related apoptosis-inducing ligand in NK cell-mediated and IFN-gamma-dependent suppression of subcutaneous tumor growth. *Cell Immunol* 2001; **214**: 194–200.
35 Smyth MJ, Takeda K, Hayakawa Y, Peschon JJ, van den Brink MR, Yagita H. Nature's TRAIL–on a path to cancer immunotherapy. *Immunity* 2003; **18**: 1–6.
36 Griffith TS, Wiley SR, Kubin MZ, Sedger LM, Maliszewski CR, Fanger NA. Monocyte-mediated tumoricidal activity via the tumor necrosis factor-related cytokine. *TRAIL. J Exp Med.* 1999; **189**: 1343–54.
37 Fanger NA, Maliszewski CR, Schooley K, Griffith TS. Human dendritic cells mediate cellular apoptosis via tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). *J Exp Med* 1999; **190**: 1155–64.
38 Ehrhardt H, Fulda S, Schmid I, Hiscott J, Debatin KM, Jeremias I. TRAIL induced survival and proliferation in cancer cells resistant towards TRAIL-induced apoptosis mediated by NF-kappaB. *Oncogene* 2003; **22**: 3842–52.
39 Wu Y, Zhou BP. TNF-alpha/NF-kappaB/Snail pathway in cancer cell migration and invasion. *Br J Cancer* 2010; **102**: 639–44.
40 Min C, Eddy SF, Sherr DH, Sonenshein GE. NF-kappaB and epithelial to mesenchymal transition of cancer. *J Cell Biochem* 2008; **104**: 733–44.
41 Trauzold A, Siegmund D, Schniewind B *et al.* TRAIL promotes metastasis of human pancreatic ductal adenocarcinoma. *Oncogene* 2006; **25**: 7434–9.
42 Herbst RS, Kurzrock R, Hong DS *et al.* A first-in-human study of conatumumab in adult patients with advanced solid tumors. *Clin Cancer Res* 2010; **16**: 5883–91.
43 Wakelee HA, Patnaik A, Sikic BI *et al.* Phase I and pharmacokinetic study of lexatumumab (HGS-ETR2) given every 2 weeks in patients with advanced solid tumors. *Ann Oncol* 2010; **21**: 376–81.
44 Camidge DR. Apomab: an agonist monoclonal antibody directed against Death Receptor 5/TRAIL-Receptor 2 for use in the treatment of solid tumors. *Expert Opin Biol Ther.* 2008; **8**: 1167–76.
45 Tolcher AW, Mita M, Meropol NJ *et al.* Phase I pharmacokinetic and biologic correlative study of mapatumumab, a fully human monoclonal antibody with agonist activity to tumor necrosis factor-related apoptosis-inducing ligand receptor-1. *J Clin Oncol* 2007; **25**: 1390–5.
46 Hotte SJ, Hirte HW, Chen EX *et al.* A phase 1 study of mapatumumab (fully human monoclonal antibody to TRAIL-R1) in patients with advanced solid malignancies. *Clin Cancer Res* 2008; **14**: 3450–5.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Establishment of B16F10 CMV and B16F10 nuclear factor-κB (NFκB) mouse melanoma cells.

Fig. S2. Nuclear factor-κB (NFκB) activation by DR5 stimulation in B16F10 mouse melanoma cells.

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Clinical Cancer Research

Long-term Vaccination with Multiple Peptides Derived from Cancer-Testis Antigens Can Maintain a Specific T-cell Response and Achieve Disease Stability in Advanced Biliary Tract Cancer

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Cancer Therapy: Clinical

Clinical Cancer Research

Long-term Vaccination with Multiple Peptides Derived from Cancer-Testis Antigens Can Maintain a Specific T-cell Response and Achieve Disease Stability in Advanced Biliary Tract Cancer

Atsushi Aruga[1,2], Nobuhiro Takeshita[1], Yoshihito Kotera[1], Ryuji Okuyama[1], Norimasa Matsushita[1], Takehiro Ohta[1], Kazuyoshi Takeda[3], and Masakazu Yamamoto[1]

Abstract

Purpose: The prognosis of patients with advanced biliary tract cancer (BTC) is extremely poor and there are only a few standard treatments. We conducted a phase I trial to investigate the safety, immune response, and antitumor effect of vaccination with four peptides derived from cancer-testis antigens, with a focus on their fluctuations during long-term vaccination until the disease had progressed.

Experimental Design: Nine patients with advanced BTC who had unresectable tumors and were refractory to standard chemotherapy were enrolled. HLA-A*2402–restricted epitope peptides, lymphocyte antigen 6 complex locus K, TTK protein kinase, insulin-like growth factor-II mRNA-binding protein 3, and DEP domain containing 1 were vaccinated subcutaneously once a week at doses of 0.5, 1, or 2 mg and continued until disease progression. The adverse events were assessed by Common Terminology Criteria for Adverse Events and the immune response was monitored by an enzyme-linked immunospot assay or by flow cytometry. The clinical effects observed were tumor response, progression-free survival (PFS), and overall survival (OS).

Results: Four-peptide vaccination was well tolerated. No grade 3 or 4 adverse events were observed. Peptide-specific T-cell immune responses were observed in seven of nine patients and clinical responses were observed in six of nine patients. The median PFS and OS were 156 and 380 days. The injection site reaction and CTL induction seemed to be prognostic factors of both PFS and OS.

Conclusions: Four-peptide vaccination was well tolerated and seemed to provide some clinical benefit to some patients. These immunologic and clinical responses were maintained over the long term through continuous vaccinations. *Clin Cancer Res; 19(8); 2224–31. ©2013 AACR.*

Introduction

Biliary tract cancer (BTC) is not a common disease worldwide, but is prevalent in East Asia and Latin America. The occurrence rate is gradually increasing and there is a high mortality rate because most cases of BTC are not diagnosed until advanced and inoperable. At this time, very few standard treatments have been established for BTC (1, 2), and thus development of new treatment modalities is urgently needed. Recently, cancer vaccines using synthetic peptides have been undergoing development throughout the world, and their safety and clinical efficacy have been reported (3, 4). Cancer peptide vaccines are capable of inducing antigen-specific CTLs *in vivo* (5). In this study, we selected 4 cancer-testis antigens that were overexpressed in nearly 100% of BTC cancer cells, as revealed by cDNA microarray technology coupled with laser microdissection in a previous study. Patients were enrolled on the basis of unresectable BTC refractory to standard chemotherapy, and no additional diagnostic procedures were needed, except for genotyping for HLA-A*2402. This study was conducted as a phase I study to assess the safety and antigen-specific immune response of a 4-peptide vaccination in patients with advanced BTC. Patients were vaccinated on a continuous basis over the long term until their disease had progressed, a time when we assessed the safety of the vaccination by CTCAE v3.0 as a primary endpoint and the antigen-specific immune response and clinical benefit as secondary endpoints.

Materials and Methods

Patient eligibility

Patients with unresectable BTC (intrahepatic bile duct cancer, extrahepatic bile duct cancer, or gallbladder cancer)

Authors' Affiliations: [1]Department of Gastroenterological Surgery; [2]Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University; and [3]Department of Immunology, Juntendo School of Medicine, Tokyo, Japan

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Atsushi Aruga, Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo, Japan. Phone: 81-3-5367-9945; Fax: 81-3-3359-6046; E-mail: aruga@abmes.twmu.ac.jp

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Translational Relevance

Numerous clinical reports have shown that peptide vaccines can induce peptide-specific CTLs to mediate tumor-specific responses *in vivo*. However, there is currently no suitable peptide vaccine for biliary tract cancer (BTC). In addition, the immunologic and clinical responses of peptide vaccines injected over the long term have not been sufficiently investigated. In this phase I clinical study, we investigated the safety, antitumor effect, and immunologic response of a multiple-peptide vaccination administered until the signs of disease progression. Our results showed that a four-peptide vaccine induced each of the respective peptide-specific CTLs, and these responses lasted throughout a long-term vaccination without any serious adverse events. These observations suggest that multiple-peptide vaccination could be a novel and promising therapy for patients with BTC.

who were refractory to standard chemotherapy were eligible for this study. All patients were required to have an HLA-A type of A*2402. Additional inclusion criteria consisted of age between 20 and 80 years, absence of severe organ function impairment, white blood cell count between 2,000 and 10,000/mm^3, hemoglobin >8 mg/dL, platelet count >100,000/mm^3, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) <100 IU/L, and total bilirubin <2 mg/dL. Performance status measured by the Eastern Cooperative Oncology Group (ECOG) scale was 0 to 2. It was required that there should be at least 4-week interval since the last chemotherapy. The exclusion criteria consisted of pregnancy, serious infections, severe underlying disease, severe allergic disease, and a judgment of unsuitability by the principal investigator.

Study design and endpoints

This was a phase I study. Patients who received standard chemotherapy under a diagnosis of inoperable BTC between April 2008 and March 2009 were invited to participate after providing their informed consent. The HLA-A genotypes of these patients were examined, and the 9 patients with an HLA-A type of A*2402 were enrolled. Four peptides were used for the vaccine, lymphocyte antigen 6 complex locus K (LY6K)–177 (RYCNLEGPPI; ref. 6), TTK protein kinase (TTK)–567 (SYRNEIAYL; ref. 7), insulin-like growth factor-II mRNA-binding protein 3 (IMP3)–508 (KTVNELQNL; ref. 8), and DEP domain containing 1 (DEPDC1; EYYELFVNI; ref. 9). These peptides were chosen from a large number of antigens identified by using cDNA microarray technology coupled with laser microdissection because they were the most highly overexpressed in BTC samples in a previous study. The purity (>97%) of the peptides was determined by analytic high-performance liquid chromatography (HPLC) and mass spectrometry analysis. The endotoxin levels and bioburden of these peptides were tested and determined to be acceptable based on the GMP grade for the vaccines (NeoMPS Inc.). These peptides were mixed with incomplete Freund's adjuvant (IFA; Montanide ISA51, SEPPIC), which has been proven safe and used in many clinical studies, and injected subcutaneously into the inguinal or the axicilla site. Each of the 4 peptides at doses of 0.5, 1, or 2 mg was injected subcutaneously into 3 patients once a week until the eighth vaccination and once or twice a week after the ninth vaccination as a monotherapy until the patient was judged to exhibit disease progression. This dose escalation design was chosen on the basis of limitations in the production of the emulsion component. The primary endpoint in this study was the assessment of toxicities caused by the vaccination based on the Common Terminology Criteria for Adverse Events version 3 (CTCAE v.3). The secondary endpoint was the assessment of the immunologic response, tumor response, progression-free survival (PFS), and overall survival (OS) from the first dose given. For the image analysis, computed tomography (CT) scan or ultrasound was conducted during the prevaccination period and every fourth vaccination until the disease had progressed. This study was approved by the Institutional Review Board at Tokyo Women's Medical University (Tokyo, Japan) and was registered with the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR number, 000003207). Informed consent was obtained from all the patients and the procedures followed were in accordance with the Declaration of Helsinki.

Measurement of immunologic response

Lymphocyte preparation for immunologic monitoring. The performance of the immunologic assay at the central laboratory was periodically standardized and validated by Clinical Laboratory Improvements Amendments (CLIA) and the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines. Peripheral blood lymphocytes (PBL) were obtained from the patients at the prevaccination period and after every fourth vaccination. Peripheral blood was taken by venipuncture, collected in an EDTA tube, and transferred to the center laboratory within 24 hours at room temperature. Within 24 hours of blood collection, PBLs were isolated using Ficoll-Paque Plus (GE Healthcare Bio-Sciences) density gradient solution and were stored at −80°C in cell stock media (Juji Field) without serum at 5×10^6 cells/mL. After thawing, the cell viability was confirmed to be more than 90% by Trypan-blue dye staining.

Enzyme-linked immunospot assay. The peptide-specific CTL response was estimated by enzyme-linked immunospot (ELISPOT) assay following *in vitro* sensitization. Frozen peripheral blood mononuclear cells (PBMC) derived from the same patient were thawed at the same time, and the viability was confirmed to be more than 90%. PBMCs (5×10^5/mL) were cultured with 10 μg/mL of the respective peptide and 100 IU/mL of interleukin (IL)-2 (Novartis) at 37°C for 2 weeks. The peptide was added to the culture at day 0 and 7. Following CD4$^+$ cell depletion by a Dynal CD4

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Table 1. Patient characteristics

No.	Age/sex	Tumor site: Primary[a]	Tumor site: Metastases	Previous therapy[b]	Peptide dose, mg	Number of vaccines	Clinical response[c]	PFS, d	OS, d	TM[d]	ISR[e] (Grade)	Lymphocyte number (%)	Peptide-specific CTL[f]: LY6K	TTK	IMP3	DEPDC1
1	64/M	IBD	Liver/peritoneum	GEM, CDDP, TS-1	0.5	4	PD	47	87	NT	1	955 (8.3)	1+	1+	0	1+
2	75/M	GB	Liver/peritoneum	GEM, TS-1	0.5	4	PD	31	66	NT	0	1,390 (17.3)	NA	NA	NA	NA
3	67/M	GB	Lymph nodes	Ope, GEM, AFTV	0.5	54	CA	491	639	Decrease	2	1,801 (39.4)	3+	3+	2+	3+
4	65/F	IBD	Lung/lymph nodes/bone	Ope, GEM	1.0	29	Stable disease	372	1,044	ND	2	1,521 (34.1)	3+	1+	1+	3+
5	59/F	GB	Liver/lymph nodes	Ope, GEM, TS-1	1.0	19	Stable disease	176	380	Decrease	2	2,124 (22.1)	3+	1+	1+	3+
6	76/F	EBD	Liver/lymph nodes	Ope, GEM, CBDCA, VP-16	1.0	9	PD	64	109	Increase	1	1,450 (30.2)	3+	1+	1+	3+
7	69/F	IBD	Liver	Ope, GEM, TS-1	2.0	35	Stable disease	428	764	NC	2	1,216 (23.2)	3+	2+	1+	3+
8	74/M	IBD	Lung	Ope, GEM, TS-1	2.0	16	Stable disease	156	466	ND	2	2,399 (38.2)	3+	3+	1+	3+
9	78/F	EBD	Liver/peritoneum	GEM, TS-1	2.0	16	CA	137	179	Decrease	1	1,249 (36.1)	3+	1+	1+	3+

[a]Primary tumor site: EBD, extrahepatic bile duct; GB, gallbladder; IDB, intrahepatic bile duct.
[b]Previous therapy: CBDCA, carboplatin; GEM, gemcitabine; CDDP, cisplatin.
[c]Clinical response: CA, clinical activity. CA means that CR or PR was not achieved and tumor regression occurred.
[d]NC, no change; ND, not detected; NT, not tested; TM, tumor marker.
[e]ISR: injection site reaction evaluated according to CTCAE v3.0.
[f]CTL: CTLs were assessed by the algorithm shown in Supplementary Fig. S2. NA, not analyzed.

Positive Isolation Kit (Invitrogen), an IFN-γ ELISPOT assay was conducted using a Human IFN-γ ELISpot PLUS kit (MabTech) according to the manufacturer's instructions. Briefly, HLA-A*2402–positive B lymphoblast TISI cells (IHWG Cell and Gene Bank) were incubated with 20 μg/mL of vaccinated peptides overnight, and then the residual peptide in the media was washed out to prepare peptide-pulsed TISI cells as the stimulation cells. Prepared $CD4^-$ cells were cultured with peptide-pulsed TISI cells (2×10^4 cells/well) at a 1:1, 1:2, 1:4, or 1:8 mixture ratio of responder cells to stimulator cells (R:S ratio) on a 96-well plate (Millipore) at 37°C overnight. Nonpeptide-pulsed TISI cells were used as negative control stimulator cells. To confirm IFN-γ productivity, responder cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin (3 μg/mL) overnight, then applied to an IFN-γ ELISPOT assay (2.5×10^3 cells/well) without stimulator cells. All ELISPOT assays were conducted in triplicate wells. The plates were analyzed by an automated ELISPOT reader, ImmunoSPOT S4 (Cellular Technology, Ltd.) and ImmunoSpot Professional Software Version 5.0 (Cellular Technology, Ltd.). The number of peptide-specific spots was calculated by subtracting the number of spots in the control well from the number of spots in the well with peptide-pulsed TISI cells. The sensitivity of our ELISPOT assay was estimated as an approximately average level by an ELISPOT panel of the Cancer Immunotherapy Consortium [CIC (http://www.cancerresearch.org/consortium/assay-panels/)].

Flow cytometry assay. The expression of peptide-specific T-cell receptors was analyzed on a FACS-Canto II flow cytometer (Becton Dickinson) using LY6K-derived epitope peptide-MHC pentamer–phycoerythrin (PE; ProImmune, Ltd.), TTK, or DEPDC1-derived epitope peptide-MHC dextramer–PE (Immudex) according to the manufacturer's instructions. HIV-derived epitope peptide (RYLRDQQLL)-MHC pentamer or dextramer–PE was used as a negative control. Briefly, the *in vitro* cultured T cells were incubated with peptide-MHC pentamer or dextramer–PE for 10 minutes at room temperature, then treated with fluorescein isothiocyanate (FITC)–conjugated anti-human CD8 monoclonal antibody (mAb), allophycocyanin (APC)-conjugated anti-human CD3 mAb, PE-Cy7–conjugated anti-human CD4 mAb, and 7-aminoactinomycin D (BD Pharmingen) at 4°C for 20 minutes. Conventional 2-color phenotypical analysis was also conducted with FITC-conjugated anti-human CD3, CD4, and CD8 mAb plus PE-conjugated anti-human CD28, CD57, and CD62L mAb (BD Pharmingen) and CXCR3 plus CCR4 mAb (R&D Systems) to assess the change of T-helper cell (T_H)1/T_H2 subsets, cytotoxic cell subset, and central memory/effector memory subsets.

Statistical analysis

Statistical analyses of prognostic factors of PFS or OS were done using the Kaplan–Meier method and evaluated by log-rank test. A P value less than 0.05 was considered to indicate a statistically significant difference. All statistical analyses were conducted using SPSS statistics software.

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Table 2. Adverse events assessed by CTCAE v3.0

Adverse events	Total (%)	Grade 1 (%)	Grade 2 (%)	Grade 3 (%)	Grade 4 (%)
Hemoglobin	6 (66.7)	5 (55.6)	1 (11.1)	0	0
Lymphopenia	2 (22.2)	2 (22.2)	0	0	0
Injection site reaction	8 (88.9)	3 (33.3)	5 (55.6)	0	0

NOTE: Hemoglobin and lymphopenia were observed before the first vaccination. No other adverse events were seen throughout the period of peptide vaccination.

Results

Patient characteristics

Nine patients (4 males and 5 females; median age, 70 years; range, 59–78) whose HLA type was A*2402 were enrolled in this study (Table 1). Their primary tumor site was the intrahepatic bile duct in 4 cases, the extrahepatic bile duct in 2 cases, and the gallbladder in 3 cases. They had several metastases to the liver, lungs, lymph nodes, peritoneum, and bone. Previous therapies consisted of operation, gemcitabine, cisplatin, tegafur–gimeracil–oteracil potassium (TS-1), carboplatin, or etoposide (VP-16). Two patients dropped out after the first follow-up study and 1 patient dropped out after second study. Six patients were vaccinated more than 16 times, with the maximum number being 54 times.

Assessment of toxicity

Toxicity was assessed by CTCAE v3.0. Eight of 9 patients developed grade 1 or 2 injection site reactions. Low hemoglobin and lymphopenia were observed before the first vaccination and were not worsened throughout the vaccination term. No other adverse events were seen through peptide vaccination. Therefore, the multiple-peptide vaccine therapy was well tolerated without any adverse events of grade 3 or higher (Table 2) up to a dose of 2 mg for each peptide, or a total of 8 mg for all 4 peptides.

Antigen-specific immune response

In the ELISPOT assay, one or more wells showed 25 spots or more observed in 7 of 9 patients (Supplementary Fig. S1). Table 1 summarizes the responses to each antigen in each patient based on the algorithm given in Supplementary Fig. S2. The number of peptide-specific IFN-γ spots per section increased with the number of vaccinations (Fig. 1A and B), and the number of LY6K-specific CTLs also increased (Fig. 1C) gradually. These immune responses were not found for all antigens and were not found in all patients. In particular, the anti-LY6K and DEPDC1 responses were greater than the responses to TTK or IMP3. In the patient receiving vaccination for the longest period of time, patient 3, these immune responses were observed over the long term with vaccination (Fig. 2A). However, patient 3 might not be a representative case, as the immune responses to antigens were already elevated before vaccination in this patient. The reason for the early elevation of antigens in this patient might be that he had received the standard chemotherapy plus the autologous formalin-fixed tumor vaccine (AFTV; ref. 10) at approximately 1 year before enrolling in this study. The phenotypical analysis was shown in Figs. 1D and 2B.

Clinical response

Two patients exhibited a clinical activity indicating tumor regression in some targets (Fig. 2C and D) but did not achieve a complete remission (CR) or partial response (PR), 4 had stable disease, and 3 had progressive disease (PD) as judged after the eighth vaccination. The 6 patients who were judged to have clinical activity or stable disease continued to be administered the vaccination until their disease was judged to be PD. Although stable disease was achieved through long-term vaccination, all of the patients eventually showed disease progression, and all had died within 3 years of the first vaccination. The median PFS of all patients after the first vaccination was 156 days (Fig. 3A) and the median OS was 380 days (Fig. 3B). In the univariate analysis of the prognostic factors, the patients who developed grade 2 local skin reaction at the vaccination site, peptide-specific CTLs (i.e., CTLs with over 25 IFN-γ spots), or a type I immune condition (i.e., a $CXCR3^+CCR4^-$ T-cell ratio of over 8%) showed a longer survival time than those with either PFS or OS (Table 3). These parameters were therefore considered prognostic factors.

Discussion

BTC is well known as a disease with an extremely poor prognosis. Operation in the early stage is the only curative treatment of BTC, but unfortunately most of these lesions are not found until the late stage. There are only a few standard chemotherapies for this disease, that is, gemcitabine, gemcitabine plus cisplatin, and/or TS-1. Both PFS and OS of the patients treated with the standard chemotherapies were almost the same as the data of the patients in this study although they were enrolled after the failure of the standard chemotherapies. This result indicated the potential of the peptide vaccine for improving PFS and OS in patients with BTC. In this study, no CR or PR was seen, but long-term stable disease was seen in some patients, and thus the OS seemed to improve. This

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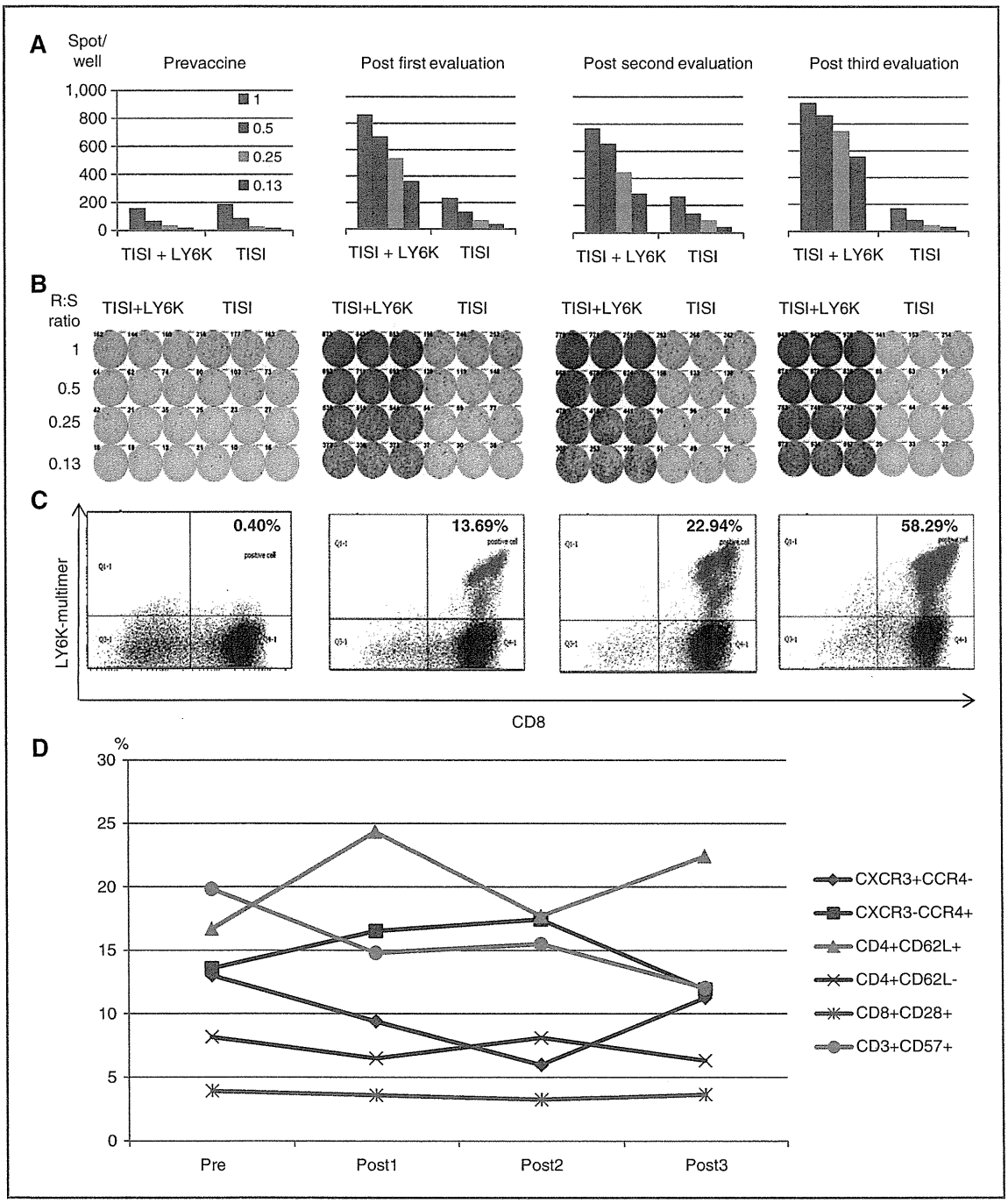

Figure 1. Immunologic monitoring in case 8. A, LY6K peptide-specific IFN-γ-positive spots at several R:S ratio. B, IFN-γ ELISPOT assay for LY6K. C, the value of LY6K-multimer positive/CD8 positive cells. D, phenotype analysis of lymphocytes by flow cytometry.

is a special characteristic of cancer vaccine therapy; therefore, we should plan a phase II study to assess the PFS and/or OS in a randomized study.

There have been numerous clinical trials on cancer vaccine therapy, and the safety, immune response, and clinical effects have already been reported. Dendritic cell

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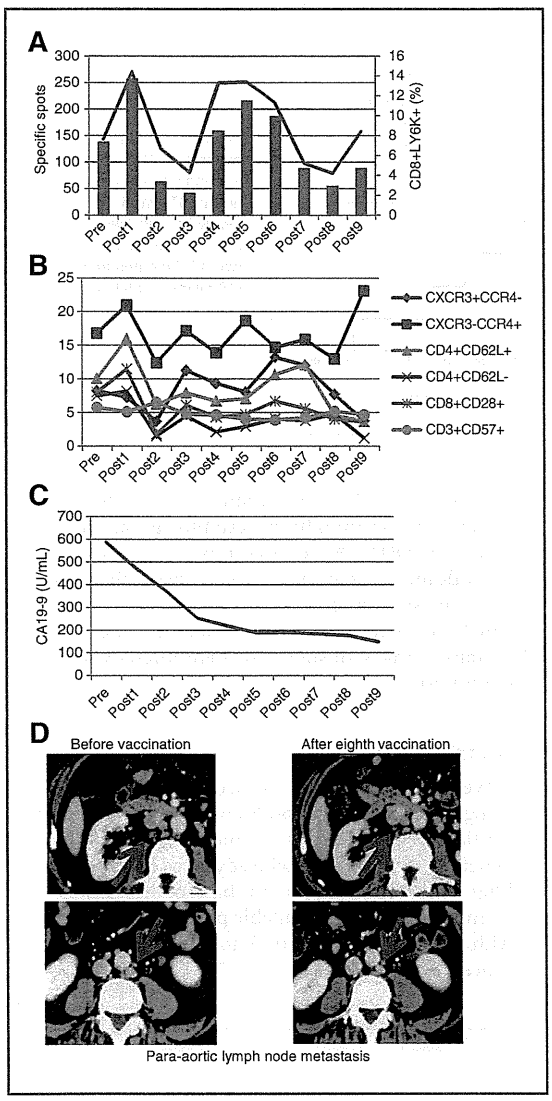

Figure 2. Immunologic and clinical response assessment in case 3. A, LY6K peptide-specific IFN-γ ELISPOT assay (bar) and pentamer analysis (line). The R:S ratio is 0.25. B, phenotype analysis of lymphocytes by flow cytometry. C, serum dosage of CA19-9. D, CT imaging of para-aortic lymph node metastases before and after vaccination. The tumor size was diminished from 40 to 21 mm. Several lymph node metastases regressed markedly, but not all achieved a CR or PR.

vaccine therapies in particular have been investigated for a long time. We previously reported the clinical use of a dendritic cell vaccine in an adjuvant setting for intrahepatic bile duct cancer (11), and a similar trial was also reported by another group (12). The dendritic cell vaccine seems to be a useful tool for adjuvant therapy, but it is difficult to harvest and induce the dendritic cells, and the high cost and severe regulations of the cell processing are also major problems. Therefore, the peptide vaccine is expected to be developed as an attractive alternative for cancer vaccine therapy. The peptides used in this study have already been used in different combinations in other clinical trials for esophageal cancer (13, 14) or bladder cancer (15). These reports have shown the safety of these peptides and their ability to induce peptide-specific CTLs *in vivo* when injected individually. Our study is the first trial to use injection of a mixture of 4 peptides into one site, and our results showed that each of the peptide-specific CTLs was induced *in vivo*. The immune responses to the 4 peptides were not equal. Each of the 4 peptides was synthesized using the most immunogenic sequence measured in a previous *in vitro* study. There might be some differences in the immunogenic reaction among these 4 peptides. This result is meaningful in part because a single vaccination of mixed peptides would be less painful for a patient than 4 separate vaccinations of the individual peptides. In our previous study, these 4 antigens were expressed on almost all BTCs (data not shown). Therefore, it is not necessary to test the expression of antigens on each tumor. At present, there are very few trials to develop new therapeutics for BTC, and thus this peptide vaccine must be developed immediately.

There are many candidates for peptides that have already undergone clinical trials (16–18). The results of these previous studies suggest that peptide-specific CTL induction is needed to achieve a clinical effect by peptide vaccine therapy. The ability to induce peptide-specific CTLs is not equal among all peptides, and the 4 peptides that we used here were very effective. In particular, LY6K and DEPDC1 are very hopeful candidates for inducing a strong CTL response, and thereby improving the PFS and OS. In the blood examination, patients with a lymphocyte count more than 1,500 tended to show a better prognosis.

Although peptide vaccines are a hopeful candidate for cancer therapy, their clinical efficacy is currently limited. To obtain a good result in the clinical trials with immunotherapy, an important problem to be solved is the immune suppression in cancer patients. Regulatory T cells are one of the most critical factors in the suppression of immune response. Nonmyeloablative chemotherapy to deplete the regulatory T cells is a promising technique to overcome these problems (19). A CCR4 antagonist or anti-CCR4 mAb that has already been approved in Japan might be a useful tool, because the regulatory T cells express CCR4 (20, 21). Another method using denileukin diftitox has also been examined in animal models and human models (22, 23). The regulation of the host immune condition is crucial for obtaining a good immune response in a clinical study. An anti-CTLA-4 mAb (ipilimimab) has also been approved for melanoma (24), and anti-PD-1 (25) or anti-PD-L1 (26) showed promising results in some clinical studies. A combination therapy could be a more successful anticancer strategy for cancer immunotherapy in the future.

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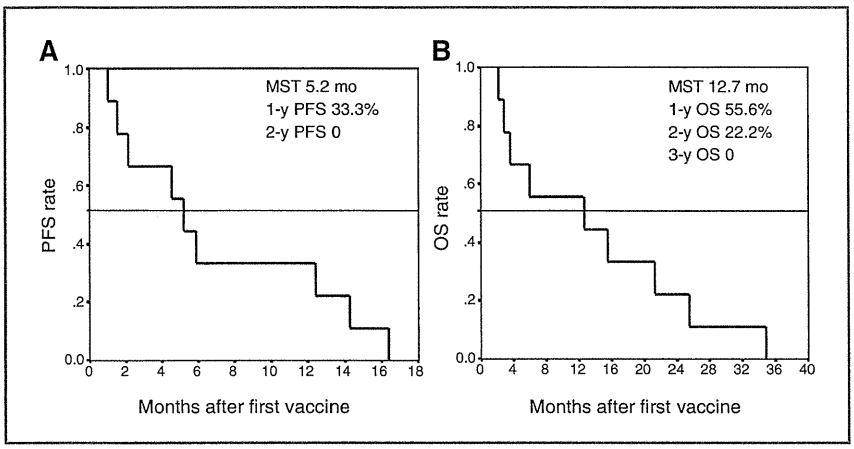

Figure 3. PFS and OS in all enrolled patients. A, PFS after first vaccination. The mean survival time (MST) was 5.2 months and the 1-year PFS ratio was 33.3%. B, OS after first vaccination. The MST was 12.7 months and the 1-year OS ratio was 55.6%.

At this stage, there is only one cancer vaccine, Sipuleucel-T, which was approved by the U.S. Food and Drug Administration (FDA) in 2011 (27). However, several phase III randomized trials of cancer peptide vaccines are ongoing throughout the world, and new candidates are coming soon. In this study, we showed that long-term vaccination with a multiple cancer peptide vaccine was feasible and resulted in the prolongation of PFS and OS in patients with advanced BTC. To obtain success in a clinical study, the next goal in the progress of cancer vaccines might be an adjuvant therapy after curative operation. Another possibility would be a combination with first-line chemotherapy, but we have not yet evaluated the ability of chemotherapy to induce antigen-specific CTLs *in vivo*. We should be careful when combining an immunotherapy and chemotherapy in order that these modalities do not counteract each other.

In this report, we showed the safety, immune response, and clinical use of a peptide vaccine in patients with advanced BTC. We anticipate that this immunotherapy will eventually be established as the standard therapy for BTC. We are planning to advance to a phase II randomized study in an advanced cancer setting, an adjuvant setting after curative operation or a study in which the peptide vaccine would be the first choice therapy along with standard chemotherapy to verify our hypothesis.

Conclusions

We have shown that a cancer peptide vaccine therapy using a mixture of 4 peptides was well tolerated, induced peptide-specific CTLs, and seemed to provide some clinical benefit in some patients with advanced BTC throughout the long-term vaccination. On the basis of these results, a phase II clinical study with a suitable protocol is warranted along with subsequent clinical trials to verify the usefulness of the cancer peptide vaccine.

Table 3. Prognostic factors of PFS or OS

Factors	PFS	OS
Sex (male/female)	0.954	0.297
Age (≥65/<65)	0.728	0.544
Primary tumor site (I/G, I/E, G/E)[a]	0.679, 0.207, 0.364	0.235, 0.207, 0.364
LY6K CTL spots (≥25/<25)	0.002	0.002
TTK CTL spots (≥25/<25)	0.017	0.005
DEPDC1 CTL spots (≥25/<25)	0.002	0.002
LY6K multimer + CTLs (≥10%/<10%)	0.113	0.840
$CXCR3^{+}CCR4^{-}$ (≥8%/<8%)	0.017	0.005
Skin reaction of vaccine site (≥G2/<G2)	0.003	0.003
Vaccine dose (0.5 mg/1 mg, 0.5 mg/2 mg, 1 mg/2 mg)	0.988, 0.988, 0.694	0.343, 0.343, 0.832
Lymphocyte (%; ≥30%/<30%)	0.545	0.423
Lymphocyte (number; ≥1,500/<1,500)	0.155	0.155

[a]Primary tumor site: E, extrahepatic bile duct; G, gallbladder; I, intrahepatic bile duct.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A. Aruga, T. Ohta
Development of methodology: A. Aruga, T. Ohta
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Aruga, N. Takeshita, N. Matsushita
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Takeda
Writing, review, and/or revision of the manuscript: A. Aruga
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Kotera, R. Okuyama
Study supervision: M. Yamamoto

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References

1. Okusaka T, Nakachi K, Fukutomi A, Mizuno N, Ohkawa S, Funakoshi A, et al. Gemcitabine alone or in combination with cisplatin in patients with biliary tract cancer: a comparative multicenter study in Japan. Br J Cancer 2010;103:469–74.
2. Valle J, Wasan H, Palmer DH, Cunningham D, Anthoney A, Maraveyas A, et al. Cisplatin plus gemcitabine versus gemcitabine for biliary tract cancer. N Engl J Med 2010;362:1273–81.
3. Sangha R, Butts C. L-BLP25: a peptide vaccine strategy in non–small cell lung cancer. Clin Cancer Res 2007;13(15Suppl):4652s–4s.
4. Schwartzentruber DJ, Lawson DH, Richards JM, Conry RM, Miller DM, Treisman J, et al. gp100 peptide vaccine and interleukin-2 in patients with advanced melanoma. N Engl J Med 2011;364:2119–27.
5. Sawada Y, Yoshikawa T, Nobuoka D, Shirakawa H, Kuronuma T, Motomura Y, et al. Phase I trial of a glypican-3-derived peptide vaccine for advanced hepatocellular carcinoma: immunologic evidence and potential for improving overall survival. Clin Cancer Res 2012;18:3686–96.
6. Ishikawa N, Takano A, Yasui W, Inai K, Nishimura H, Ito H, et al. Cancer-testis antigen lymphocyte anteigen 6 complex lolus K is a serologic biomarker and a therapeutic target for lung and esophageal carcinomas. Cancer Res 2007;67:11601–11.
7. Suda T, Tsunoda T, Daigo Y, Nakamura Y, Tahara H. Identification of human leukocyte antigen-A24-restricted epitope peptides derived from gene products upregulated in lung and esophageal cancers as novel targets for immunotherapy. Cancer Sci 2007;98:1803–8.
8. Mizukami Y, Kono K, Daigo Y, Rakano A, Tsunoda T, Kawaguchi Y, et al. Detection of novel cancer-testis antigen-specific T-cell responses in TIL, regional lymph nodes, and PBL in patients with esophageal squamous cell carcinoma. Cancer Sci 2008;99:1448–54.
9. Kanehira M, Harada Y, Takata R, Shuin T, Miki T, Fujioka T, et al. Involvement of upregulation of DEPDC1 (DEP domain containing 1) in bladder carcinogenesis. Oncogene 2007;26:6448–55.
10. Muragaki Y, Maruyama T, Iseki H, Tanaka M, Shinohara C, Takakura K, et al. Phase I/II trial of autologous formalin-fixed tumor vaccine concomitant with fractionated radiotherapy for initially-diagnosed Glioblastoma. J Neuro Surg 2011;115:248–55.
11. Shimizu K, Kotera Y, Aruga A, Takeshita N, Takasaki K, Yamamoto M. Clinical utilization of postoperative dendritic cell vaccine plus activated T-cell transfer in patients with intrahepatic cholangiocarcinoma. J Hepatobiliary Pancreat Sci 2012;19:171–8.
12. De Vleeschouwer S, Fieuws S, Rutkowski S, Van Calenbergh F, Van Loon J, Goffin J, et al. Postoperative adjuvant dendritic cell-based immunotherapy in patients with relapsed glioblastoma multiforme. Clin Cancer Res 2008;14:3098–104.
13. Kono K, Mizukami Y, Daigo Y, Takano A, Masuda K, Yoshida K, et al. Vaccination with multiple peptides derived from novel cancer-testis antigens can induce specific T-cell responses and clinical responses in advanced esophageal cancer. Cancer Sci 2009;100:1502–9.
14. Kono K, Iinuma H, Akutsu Y, Tanaka H, Hayashi N, Uchikado Y, et al. Multicenter, phase II clinical trial of cancer vaccination for advanced esophageal cancer with three peptides derived from novel cancer-testis antigens. J Transl Med 2012;10:141.
15. Obara W, Ohsawa R, Kanehira M, Takata R, Tsunoda T, Yoshida K, et al. Cancer peptide vaccine therapy developed from oncoantigens identified through genome-wide expression profile analysis for bladder cancer. Jpn J Clin Oncol 2012;42:591–600.
16. Miyazawa M, Ohsawa R, Tsunoda T, Hirono S, Kawai M, Tani M, et al. Phase I clinical trial using peptide vaccine for human vascular endothelial growth factor receptor 2 in combination with gemcitabine for patients with advanced pancreatic cancer. Cancer Sci 2010;101:433–9.
17. Ohno S, Okuyama R, Aruga A, Sugiyama H, Yamamoto M. Phase I trial of Wilm's tumor 1 (WT1) peptide vaccine with GM-CSF or CpG in patients with solid malignancy. Anticancer Res 2012;32:2263–70.
18. Noguchi M, Kakuma T, Uemura H, Nasu Y, Kumon H, Hirao Y, et al. A randomized phase II trial of personalized peptide vaccine plus low dose estramustine phosphate (EMP) versus standard dose EMP in patients with castration resistant prostate cancer. Cancer Immunol Immunother 2010;59:1001–9.
19. Koike N, Pilon-Thomas S, Mule JJ. Nonmyeloablative chemotherapy followed by T-cell adoptive transfer and dendritic cell-based vaccination results in rejection of established melanoma. J Immunother 2008;31:402–12.
20. Pere H, Montier Y, Bayry J, Quintin-Colonna F, Merillon N, Dransart E, et al. A CCR4 antagonist combined with vaccines induces antigen-specific CD8+ T cells and tumor immunity against self antigens. Blood 2012;118:4853–62.
21. Ishida T, Joh T, Uike N, Yamamoto K, Utsunomiya A, Yoshida S, et al. Defucosylated anti-CCR4 monoclonal antibody (KW-0761) for relapsed adult T-cell leukemia-lymphoma: a multicenter phase II study. J Clin Oncol 2012;30:837–42.
22. Matsushita N, Pilon-Thomas SA, Martin LM, Riker AI. Comparative methodologies of regulatory T cell depletion in a murine melanoma model. J Immunol Methods 2008;333:167–79.
23. Morse MA, Hobeika AC, Osada T, Serra D, Niedzwiechi D, Lyerly DH, et al. Depletion of human regulatory T cells specifically enhances antigen-specific immune responses to cancer vaccines. Blood 2010;112:610–8.
24. Robert C, Thomas L, Bondarenko I, O'Day S, Weber J, Garbe C, et al. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. N Engl J Med 2011;364:2517–26.
25. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med 2012;366:2443–54.
26. Brahmer JR, Tykodi SS, Chow LQM, Hwu W-J, Topalian SL, Hwu P, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N Engl J Med 2012;366:2455–65.
27. Cheever MA, Higano CS. PROVENGE (Sipuleucel-T) in prostate cancer: the first FDA-approved therapeutic cancer vaccine. Clin Cancer Res 2011;17:3520–6.

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Phase I/II clinical trial using HLA-A24-restricted peptide vaccine derived from KIF20A for patients with advanced pancreatic cancer

Shingo Asahara[1*], Kazuyoshi Takeda[2], Kenji Yamao[3], Hiroyuki Maguchi[4] and Hiroki Yamaue[5]

Abstract

Background: We previously developed an immunotherapy treatment utilizing a cancer vaccine reagent KIF20A-66 in order to treat pancreatic cancer. KIF20A-66 is HLA-A24-restricted epitope peptide derived from KIF20A, a member of kinesin super family protein 20A that is significantly transactivated in pancreatic cancer. In this report, we further demonstrated non-randomized, open-label, single centered phase I/II clinical trial of immunotherapy using the KIF20A-66 peptide for the patients with advanced pancreatic cancer.

Methods: Vaccination was performed to the patients with metastatic pancreatic cancer, in whom gemcitabine-based therapy had failed. In phase I study, KIF20A-66 peptide was subcutaneously injected weekly in a dose-escalation manner (doses of 1.0 and 3.0 mg/body, 6 patients/1 cohort). After safety was assessed, phase II study was conducted using 3.0 mg of KIF20A-66 peptide.

Results: KIF20A-66 peptide vaccination was well tolerated in the doses we examined and tumor responses after 1 month of the treatment were evaluated. Among 29 patients who completed one course of the treatment at least, stable disease (SD) was found in 21 cases, while progressive disease (PD) was found in 8 cases, indicating that the disease control rate was 72%. Objective tumor shrinkage was observed in 8 cases, including 1 case of complete response (CR). The median survival time (MST) and progression free survival time (PFS) were 142 days and 56 days, respectively. These results clearly demonstrate that overall survival of the patients was significantly prolonged, compared to the historical controls of 9 cases with unmatched HLA in the same hospital (MST: 83 days), as well as 81 cases in our and other hospitals (MST: 63 days).

Conclusion: The patients vaccinated with KIF20A-66 peptide had better prognosis than the control group with best supportive care (BSC). Thus, we concluded that KIF20A-66 vaccination is significantly effective as an immunotherapy against advanced pancreatic cancer. KIF20A-66 peptide was well tolerable in the dose of either 1.0 mg or 3.0 mg/body, and effectively induced peptide-specific response of cytotoxic T lymphocyte (CTL). Further clinical study using this peptide is a promising approach for advanced pancreatic cancer to achieve high potential benefit for better prognosis.

Clinical trial registration: UMIN-CTR, number UMIN000004919

Keywords: KIF20A, Peptide vaccine, Pancreatic cancer

Introduction

Pancreatic cancer remains one of the most challenging conditions to treat, due to extremely poor prognosis with the overall five-year survival of less than 10% [1-3]. During the last decades, gemcitabine has been the standard single-agent chemotherapy for unresectable pancreatic cancer [4,5]. Regarding combination chemotherapy, several phase III trials of gemcitabine-based multi-drug regimens have been attempted, whereas significant improvement in survival has not been observed [6-14]. Although TS-1, a prodrug of 5-FU, has been employed as a major alternative approach in a variety of solid tumors, the single-agent treatment of TS-1 yielded non-inferiority result against the gemcitabine treatment [15]. After all, once pancreatic cancer became

* Correspondence: s.asahara@chibatoku.or.jp
[1]Department of Internal Medicine, Chiba Tokushukai Hospital, Chiba, Japan
Full list of author information is available at the end of the article

refractory to gemcitabine, there is virtually no effective treatment for the patients. Hence, novel strategy providing better survival benefit is urgently required, in particular, for the patients with advanced pancreatic cancer.

Cancer immunotherapy is a promising approach to fight against cancer, and thus we have conducted research and development of peptide vaccines targeting tumor-specific antigens [16-19]. Briefly, we identified dozens of cancer-testis or oncofetal proteins from more than 1,000 clinical cancer tissues using cDNA microarray including 32,000 genes or ESTs [20]. Utilizing the result of this genome-wide expression profile analysis, we tried to establish an epitope peptide derived from the tumor-associated antigen mentioned above, which is applicable for cancer peptide vaccination [21,22]. KIF20A, kinesin family member 20A, is one of the candidates of such target antigen, as it was up-regulated in the majority of pancreatic cancer [23]. Therefore, we developed an epitope peptide, namely KIF20A-66, restricted to HLA-A*2402 that is the most common HLA-A allele in a Japanese population [24]. We here report the results of a phase I/II clinical trial using KIF20A-66 mono peptide as cancer immunotherapy for the patients with advanced pancreatic cancer.

Methods

Patient eligibility

Patients with unresectable or metastatic pancreatic cancer, who were resistant to gemcitabine and TS-1 treatments or unable to continue the treatment of gemcitabine or TS-1 because of severe adverse events, were enrolled in this trial from March 2009 to February 2010 at Chiba Tokushukai Hospital. The eligibility criteria are as follows: unresectable pancreatic cancer with metastatic, recurrent and/or locally advanced disease based on diagnostic imaging using computed tomography (CT) and histological examinations. Other entry criteria included the HLA-A*2402-positive status, an Eastern Cooperative Oncology Group (ECOG) performance status of 0–2, age of 20–85 years, life expectancy of at least 2 months, adequate respiratory, and liver and kidney functions for vaccination treatment. The exclusion criteria are as follows: pregnancy or lactation, active infection, other active malignancy, non-recovered injury, and treatment with immunosuppressive agents or steroid. Written informed consent was obtained from each individual patient, and the study was approved by Tokushukai Group Ethical Committee. The study was registered at University Hospital Medical Information Network (UMIN) Center with the Clinical Trial Registration number UMIN000004919.

Control group

Clinical data used as the control group (BSC, multi-center, n = 81) in this study were obtained from our and other hospitals where written informed consent was obtained at each institution. Clinical information of each patient utilized in our statistical analysis includes age at diagnosis, sex, performance status at the endpoint of the Standard Chemotherapy, treatment status at primary lesion, median survival time, and mean survival time. This study was approved by the institutional review board at each institution.

Study design and end points

This study is a non-randomized, open-label phase I/II clinical trial with dose escalation of KIF20A-66 peptide mono-therapy. The primary end point of phase I part was safety of peptide vaccination and tolerance for phase II part. The primary end point of phase II part was antitumor effects assessed by CT scan in accordance with the Response Evaluation Criteria in Solid Tumors (RECIST) criteria version 1.1. The secondary end points were overall survival (OS), progression free survival (PFS), immunological responses assessed by CTL induction specific to the KIF20A-66 peptide and the injection site reactions (ISRs). In phase II part, the information of 9 patients with best supportive care in the Chiba Tokushukai Hospital from January 2007 to January 2009 was used as a historical control.

Treatment protocol

After emulsified with Incomplete Freund's adjuvant (Montanide ISA51VG, SEPPIC, France), KIF20A-66 peptide in the amount of 1.0 or 3.0 mg/body was subcutaneously administered on days 1, 8, 15 and 22 in a 28 days-treatment cycle. After two cycles of the vaccination, the peptide was administrated once in every two weeks until tumor progression was observed in the patient.

Toxicity assessment

The toxicity was assessed based on the Common Terminology Criteria for Adverse Events version 3.0 (CTCAE v3.0).

Peptides

The KIF20A-66 peptide (KVYLRVRPLL) was synthesized and its quality was analyzed by American Peptide Company Inc. (Sunnyvale, CA). The epitope peptide derived from HIV-Env peptide (RYLRDQQLL), restricted to HLA-A*2402, was used as a control to evaluate CTL response.

Enzyme-linked immunospot (ELISPOT) assay

To evaluate the peptide-specific CTL response, ELISPOT assay was performed after *in vitro* sensitization [16]. Briefly, frozen Peripheral Blood Mononuclear Cells (PBMC) derived from the same patient were thawed, cultured with respective peptide and IL-2 (Novartis, Emeryville, CA) (IVS), and harvested after two weeks. Followed by

$CD4^+$ cell depletion, IFN-γ ELISPOT assay was performed utilizing HLA-A*2402-positive TISI cells (IHWG Cell and Gene Bank, Seattle, WA) stimulated by either vaccinated peptide or HIV-Env peptide (as control). Reaction in a MultiScreen-IP 96-plate (Millipore, Bedford, MA) was measured by an automated ELISPOT reader, Immuno-SPOT S4 (Cellular Technology Ltd, Cleveland, OH) with ImmunoSpot Professional Software Version 5.0 (Cellular Technology Ltd). All ELISPOT assays were performed in triplicate. The number of peptide-specific spots was calculated by subtracting the number of the spots of control cells from that of the cells stimulated by vaccinated peptide. The peptide-specific T cell response was classified into four grades (–, +, ++, and +++), according to the algorithm flow chart described in our previous report (+++ : the content rate of CTL is more than 0.2% , ++ : 0.02 - 0.2%, + : 0.01 - 0.02%, –: less than 0.01%) [25]. Sensitivity of ELISPOT assay was estimated as approximate average level utilizing proficiency panels conducted by Cancer Immunotherapy Consortium (CIC) in 2009 and 2011 [26].

Flow cytometry

Expression of peptide specific T cell receptor (TCR) was examined by FACS-CantoII (Becton Dickinson, San Jose, CA) using KIF20A-66/HLA-A*2402 dextramer-PE (KI F20A-dextramer) according to the manufacturer's instruction (Immudex, Copenhagen, Denmark). HIV-A24 epitope peptide (RYLRDQQLL)/MHC-dextramer (HIV-dextramer) was used as negative control. Briefly, cells were incubated with peptide-HLA-A*2402 dextramer-PE for 10 minutes at room temperature, then treated with FITC-conjugated anti-human CD8 monoclonal antibody (mAb), APC-conjugated anti-human CD3 mAb, PE-Cy7-conjugated anti-human CD4 mAb, and 7-AAD (BD Biosciences, San Jose, CA) at 4°C for 20 minutes. Analysis gate was set on the staining profiles using HIV-dextramer, and positive cell percentage ($dextramer^+$ cells/$CD3^+$ $CD4^-$ $CD8^+$ cells) was calculated by subtracting the percentage of HIV-$dextramer^+$ from that of KIF20A-$dextramer^+$.

Statistical analysis

StatView version 5.0 (SAS Institute Japan Ltd., Japan) was used for statistical analysis. TTP and OS curves were estimated using the Kaplan-Meier methodology and analyzed with a log-rank test. Mann–Whitney U test and Chi-square test were used to compare patient characteristics.

Results

The peptide vaccine treatment

A total of 31 patients with chemotherapy-refractory pancreatic cancer were enrolled in this trial. 16 patients had unresectable tumor and 15 had recurrent one after surgery. Tables 1 and 2 indicate clinicopathological information of the 31 patients, as well as the patients in control group, who received best supportive care in our and other hospitals (Table 1). The peptide in the amount of either 1.0 mg or 3.0 mg per body was examined in this phase I/II study. These dosages were well tolerated in the 31 patients with advanced pancreatic cancer. There is no severe adverse event (SAE) related to the peptide vaccine in the 1.0 mg/body-injected group, except the immunological response at injection sites. As well, no SAE was observed in the first 6 patients in the 3.0 mg/body-injected group during the first cycle in the treatment. Hence, we determined that 3.0 mg per body is an appropriate dose for phase II part in this study.

Immunological injection site reactions (ISRs) of all the 31 patients were evaluated. Clinical responses of 29 patients out of 31, who received at least one treatment cycle (4 injections), were evaluated by immuno-monitoring. ISRs, including adverse reactions on the skin in grades 1–3, was observed in 23 patients out of 29. It should be noted that there were two patients who were incompatible with further vaccination treatment due to the exclusion criteria, such as autoimmune hepatitis and interstitial pneumonia. The patient, who experienced grade 3 autoimmune hepatitis after 11 months of vaccination, was recovered after drug withdrawal. Another patient with the interstitial pneumonia was well recovered by hospital treatment without any steroid therapy. In these cases, we could not rule out the possibility whether these adverse events were related to vaccine treatments or not.

Clinical outcomes of eligible patients

Among the 29 patients examined in this trial, 21 patients yielded the status of "stable disease" (SD), while 8 resulted in "progressive disease" (PD) after one cycle of the treatment (injections of the peptide vaccine for 4 times) (Table 2). The rate of disease control at the time of one cycle was calculated to be 72%. 8 patients showed objective tumor response at target lesions (Figure 1). On the other hand, according to RECIST criteria, the other patients were not classified as partial response (PR), since the ratio of tumor shrinkage was insufficient. One patient (case 9) achieved "complete response" (CR) after SD over the long term (Table 2, Figures 1a, 2, and 3). The rate of objective response to the total was calculated to be 25.8%.

Case 9 describes a 33-year-old female ended up with CR after 25 months including a long period of SD (Figure 1a). This patient underwent pancreatoduodenectomy in November 2008 and was diagnosed with giant cell pancreatic cancer. Adjuvant chemotherapy utilizing gemcitabine was discontinued at the one course

Table 1 Clinical status and profile of the patients

	KIF20A peptide vaccine treatment	Best supportive care	
	Chiba (n = 31) *	Chiba (n = 9) *	Multi-center (n = 81) **
Age (average, (range))	61.3 (33–80)	64 (53–82)	64.5 (41–85)
Sex (Male: Female)	17:14	5:4	49:32
Performance status (0:1:2:3)	11:8:12:0	1:3:3:2	13:28:36:0 ***
Status of primary lesion (Resected: Unresected)	15:16	1:8	23:58
Median survival time (days)	142.0 ± 23.7	83.0 ± 33.5	62.0 ± 6.5
Mean survival time (days)	171.8 ± 23.8	93.3 ± 14.8	91.1 ± 11.6

*, Clinical data obtained at our institution, Chiba Tokushukai Hospital.
**, Clinical data of Multi-center (n = 81) include those obtained from Chiba and other three hospitals.
***, 4 cases were excluded, since Performance Status was not determined.

of drug administration, due to severe adverse reactions including hematopoietic toxicity. In February 2009, a progressive solitary liver metastasis was diagnosed (Figure 1a). There was no clinical sign of inflammation at the time of April 13th, 2009. White blood cell count ($2.8 \times 10^3/\mu$-l) and CRP level (0.02 mg/dl) were within normal limits. Vaccination started on April 23rd, 2009, and the tumor kept stable condition during the administration. After 8 months, shrinkage of the tumor size was observed. Vaccination was discontinued after 11 months, because the level of liver enzyme was increased and thus autoimmune hepatitis was suspected. Nonetheless, the tumor continued to shrink and became undetectable by CT 25 months after the start of administration. At the time of the submission of this manuscript, there is no sign of relapse or metastasis, and the general condition of the patient has been kept well with the performance status (PS) of zero.

Case 14 reports a 60-year-old male who showed objective response (Figure 1b). After pancreatoduodenectomy, gemcitabine treatment started in October 2008 and liver metastasis was found 3 months later. Followed by TS-1 chemotherapy, we found that metastatic lesions in the liver progressed after the condition of SD during 3 cycles of TS-1 treatment. After 1 cycle of the peptide vaccine, one target lesion of liver metastases located at S8 was shrunken. This lesion kept shrinking until September 2009, and became hardly detectable by CT scan. Similarly, a metastatic lesion in the lymph node was significantly shrunken until September 2009. However, the other target lesion (S4) in the liver showed no response to the vaccine treatment and the tumor progression was promoted after 2 cycles. Finally, the patient died at 220 days after the start of the vaccination.

In case 24, a 74-year-old male also showed objective response (Figure 1c). After distal pancreatectomy in August 2007, adjuvant chemotherapy utilizing gemcitabine was performed for 6 months and then switched to TS-1 because of the side effect. Bone metastasis was found in the xiphoid process by CT scan in April 2009. Radiation therapy was performed to the xiphoid process in May 2009, but the tumor did not respond well. The patient was enrolled into the peptide vaccine trial in July 2009 after one month of cooling off period. Bone metastasis started to shrink after one cycle of the peptide vaccine treatment. The precordial pain was rapidly diminished and well controlled without opioid treatment. After the 5th shot of the peptide, Grade 3 interstitial pneumonia was observed and the treatment was discontinued. The patient was hospitalized in one week of treatment without any steroid therapy and then well recovered. Even without the vaccination, pain was well controlled and tumor markers kept decreasing for the next two months. After the re-progression of the disease, gemcitabine was administered and no clinical effect was observed. Since the patient desired to receive the peptide vaccine again, we obtained an approval of the re-entry of this case from the Ethical committee. The vaccine treatment was restarted with careful monitoring, while neither adverse events nor clinical effect was observed in this second round of drug administration. His overall survival period from the first day of administration was 495 days.

The median overall survival time of 31 patients was 142 days, and the progression free survival period was 56 days (Figures 4a and 4b). In comparison with the control group without the vaccine treatment, who are the patients visited Chiba Tokushukai Hospital in the period between January 2007 and January 2009 (MST: 83 days), overall survival of the patients with the KIF20A-peptide vaccination was statistically significant ($p = 0.0468$, MST: 142 vs. 83 days) (Figure 4c). Moreover, MST of the patients who received BSC was 63 days. Compared to the control group in multi-center, Overall Survival of the vaccinated patients was significantly improved ($p = 0.0020$, MST: 142 vs. 63 days) (Figure 4c). Taken together, we concluded that the cancer vaccination utilizing KIF20A-derived peptide was significantly effective as immunotherapy against advanced pancreatic cancer.

Table 2 Patient characteristics and clinical responses

No.	Age	Sex	Target lesion	Dose of peptide (mg)	Number of injection	Clinical response*	Objective Response	Response lesion	Injection site reaction(Grade)	CTL response	
										Pre-vaccination	Post-vaccination**
1	75	M	Local LNs	1	4	PD			0	N.A.	+
2	57	F	Local	1	11	PD			1	++	++
3	72	M	Liver	1	3	-			0	N.T.	N.T.
4	60	M	Lung, local LNs	1	19	SD	Yes	Lung metastasis	2	+	-
5	72	F	Primary , liver	1	12	PD			1	+	+++
6	65	F	Liver	1	4	PD			0	+	+
7	61	F	Local , liver	3	14	SD			2	+	+++
8	57	F	Primary, liver	3	10	SD			2	++	+++
9	33	F	Paraaortic LNs	3	29	SD	Yes(CR)	Liver metastasis	3	N.A.	+++
10	76	M	Liver	3	12	PD			2	-	++
11	55	F	Primary, lung	3	17	SD			1	+	-
12	58	M	Primary	3	5	PD			0	-	-
13	58	F	Live, lung, LNs	3	10	SD			1	-	++
14	60	M	Liver, LNs	3	17	SD	Yes	Liver metastasis, LNs	2	+++	+++
15	80	F	Liver, LNs, lung	3	5	PD			0	-	+
16	58	M	Primary, liver, lung	3	13	PD			1	-	++
17	49	M	Parimary	3	17	SD			2	+	+++
18	62	M	Primary, liver, LNs	3	7	SD			1	-	+++
19	61	M	Primarym, liver, lung, LNs	3	11	SD			2	-	+
20	58	M	LNs, lung	3	25	SD			2	+	+++
21	47	M	Primary, liver	3	13	SD			1	-	+
22	71	F	Liver, local LNs	3	7	SD	Yes	Liver metastasis	2	N.A.	++
23	50	M	Local, LNs	3	6	SD			0	N.A.	-
24	74	M	Bone	3	21	SD	Yes	Bone metastasis	2	N.A.	+++
25	69	F	Primary	3	2	-			0	N.T.	N.T.
26	80	M	Liver, lung	3	18	SD			1	+	+++
27	44	M	Liver, lung, local LNs	3	24	SD	Yes	Lung and liver metastasis	1	+	-
28	61	F	Peritoneal, local LNs	3	9	SD	Yes	Peritoneal metastasis	0	-	-
29	46	M	Liver	3	10	SD			2	-	+++
30	64	F	Liver	3	9	SD			2	-	+++
31	68	F	Liver	3	9	SD	Yes	Liver metastasis	2	+	+++

No.	PFS(day)	OS (day)	Pre-vaccination			Post-vaccination		
			WBC(/mm^3)	Lymphocyte (%)	Lymphocyte (/mm^3)	WBC(/mm^3)	Lymphocyte (%)	Lymphocyte (/mm^3)
1	36	36	7300	7	511	5300	10.5	557
2	26	108	7400	13	962	7900	8	632
3	31	31	7800	11	858	16200	10.5	1701
4	223	283	5100	21	1071	5200	10.5	546
5	24	128	2400	25.5	612	4200	10.8	454
6	26	40	4500	16.5	743	8000	4.1	328
7	55	155	4000	25	1000	6400	18.3	1171
8	56	145	4500	33	1485	14100	16	2256
9	>1219	>1219	2500	44.5	1113	3600	33	1188
10	27	142	2300	29.5	679	5800	11.5	667
11	112	225	2600	9	234	2200	11.5	253
12	32	32	4500	30	1350	2400	10.7	257
13	57	97	7100	15.5	1101	9100	10.5	956
14	169	220	2300	27	621	4100	19.5	800
15	24	44	8500	9.5	808	13300	4.8	638
16	28	182	4800	27	1296	6400	19.3	1235
17	169	309	6200	26.5	1643	7900	17.5	1383
18	93	93	4200	28	1176	6600	18.6	1228
19	57	105	10200	20.5	2091	28700	9	2583
20	169	332	10100	34	3434	7600	19.5	1482
21	56	249	6000	27.5	1650	9600	8	768
22	89	89	7000	11.5	805	5200	20	1040
23	148	148	7900	20	1580	11200	19	2128
24	415	495	3800	16	608	5600	17.8	997
25	11	11	7600	21.5	1634	7400	20.5	1517
26	112	207	6600	24	1584	7500	21.5	1613
27	115	317	2900	23.5	682	4000	25.5	1020
28	69	69	9000	26.5	2385	11200	7.5	840
29	52	388	4000	26	1040	5600	24.6	1378
30	56	69	4800	26.5	1272	8900	7.1	632
31	56	82	6800	33	2244	8300	19.5	1619

*Clinical response was evaluated one month after vaccination. PD, Progressive disease; SD, Stable disease; CR, Complete response; OR, Objective response.
**Best CTL response after vaccination. CTL responses were evaluated and classified based on the algorithm as described in Methods.
N.T. (Not Tested); CTL response was not tested in the samples in which PD was observed within one course of the treatment.
N.A. (Not Analyzed); CTL response was not analyzed because of the poor viability during the *in vitro* stimulation.

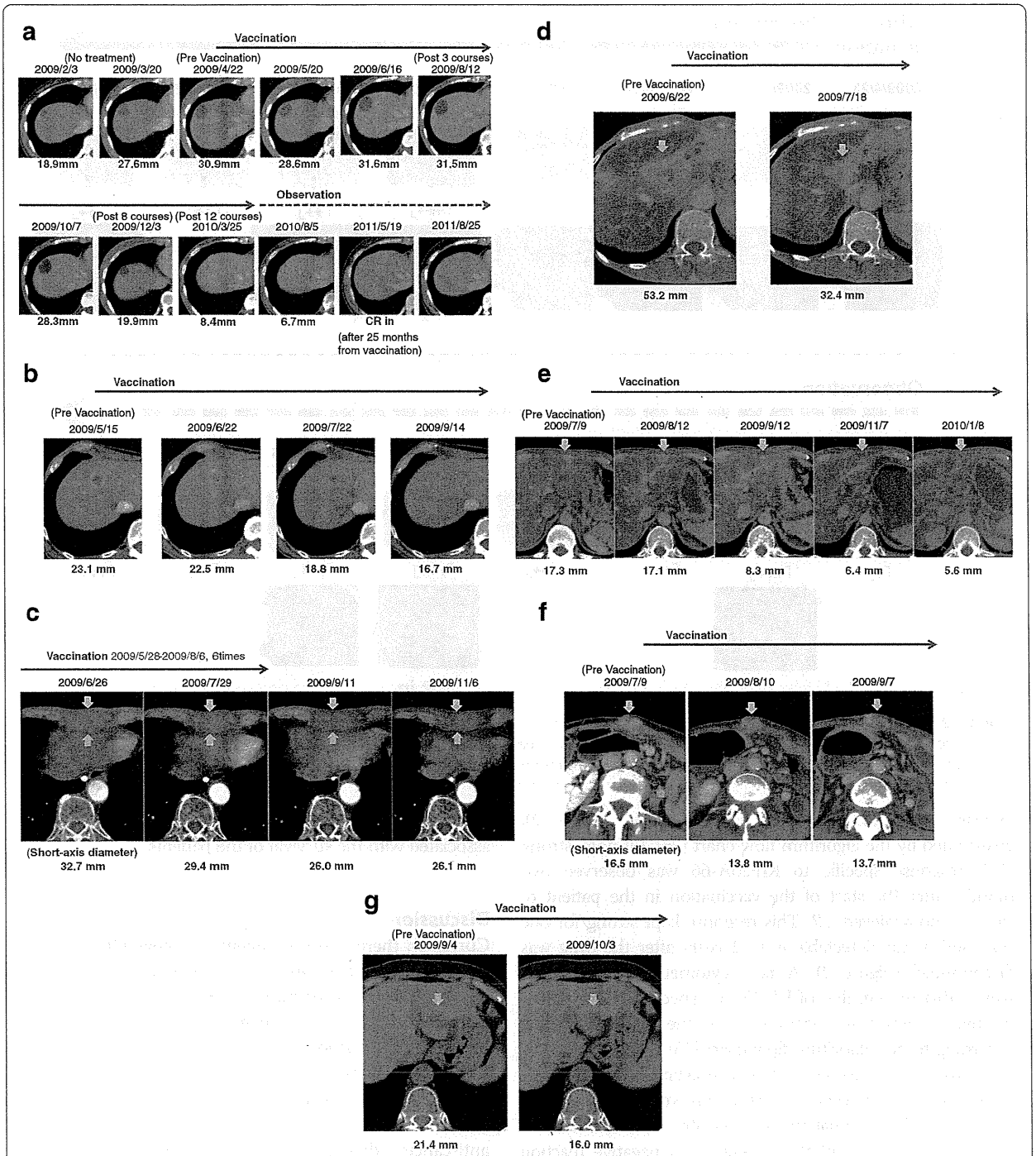

Figure 1 CT images of the target lesions showing objective tumor response. Representative CT images of the target lesions were shown, such as liver metastasis in case 9 **(a)**, liver metastasis in case 14 **(b)**, bone metastasis in xiphoid process in case 24 **(c)**, liver metastasis in case 22 **(d)**, liver metastasis in case 27 **(e)**, peritoneal metastasis in case 28 **(f)**, and liver metastasis in case 31 **(g)**. Arrow head indicates the target lesion.

CTL response and injection site reactions

We expected that the number of CTL responded to KIF20A peptide may be associated with the efficacy of the vaccine treatment. Therefore, CTL response was measured by ELISPOT assay in 29 patients who received the vaccination at least one cycle (Table 2). Among them, CTL responses in 24 patients were comparable in pre- and post-vaccination. In 16 patients out of 23 (70%), the