which enhanced the recruitment of CTLs in the tumor (25). We hypothesize that the αPD-1 Ab affected chemokine expression, which resulted in recruitment of vaccine-induced CTLs to the tumor. In the present study, the experimental model did not show a change in the expression of CXCL10. However, the expression of CCL3 was elevated by the combination treatment with vaccine and αPD-1 Ab. Furthermore, blocking PD-1 decreased the expression of inhibitory receptors in peptide-specific CTLs at the tumor site. Recently, mouse models revealed that peptide/IFA vaccination increased the antigen-driven expression of the inhibitory receptors PD-1, LAG-3, CTLA-4, and Tim-3 in CTLs, suggesting partial exhaustion (37). PD-1 blockade might be a rational strategy that could be used to rescue CTLs in a state of exhaustion. Interestingly, αPD-1 Ab therapy did not decrease LAG-3 expression in TILs; however, CTLA-4 expression was decreased, suggesting the partial rescue of CTL from exhaustion. A previous study reported that dual treatment with αLAG-3 and αPD-1 Ab was effective in mice with established tumors (38) as well as during the *in vitro* expansion of human NY-ESO-1-specific CTLs (39). Furthermore, Sierro *et al* reported that blocking both PD-1 and PD-L1 might further enhance the antitumor effects of tumor vaccines in mouse models (40).

Based on the results of this clinical trial, the GPC3 peptide vaccine has fewer side effects due to its antigen specificity (8). Enhancing GPC3 peptide vaccine therapy is considered to be promising in terms of sustained tumor control in HCC patients. These data suggest that use of αPD-1 Ab could enhance the antitumor effects of a peptide vaccine, and provide the foundation for the clinical development of a combination therapy.

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Identification of HLA-A2 or HLA-A24-restricted CTL epitopes for potential HSP105-targeted immunotherapy in colorectal cancer

YU SAWADA[1,2*], HIROYUKI KOMORI[3,4*], YOSHIYUKI TSUNODA[1,5,6*], MANAMI SHIMOMURA[1], MARI TAKAHASHI[1], HIDEO BABA[4], MASAAKI ITO[5], NORIO SAITO[5], HIROYUKI KUWANO[6], ITARU ENDO[2], YASUHARU NISHIMURA[3] and TETSUYA NAKATSURA[1]

[1]Division of Cancer Immunotherapy, Exploratory Oncology Research and Clinical Trial Center, National Cancer Center, Kashiwa 277-8577; [2]Department of Gastroenterological Surgery, Yokohama City University Graduate School of Medicine, Yokohama 236-0004; Departments of [3]Immunogenetics and [4]Gastroenterological Surgery, Graduate School of Medical Sciences, Kumamoto University, Kumamoto 860-8556; [5]Colorectal and Pelvic Surgery Division, National Cancer Center Hospital East, Kashiwa 277-8577; [6]Department of General Surgical Science (Surgery I), Gunma University Graduate School of Medicine, Maebashi 371-8511, Japan

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Abstract. We previously reported that heat shock protein 105 (HSP105) is overexpressed in a variety of human cancers, including colorectal, pancreatic and esophageal cancer and has proven to be a novel biomarker for the immunohistochemical detection of these cancers. In the present study, we used HLA-transgenic mice (Tgm) and the peripheral blood mononuclear cells (PBMCs) of colorectal cancer patients to identify HLA-A2 and HLA-A24-restricted HSP105 epitopes, as a means of expanding the application of HSP105-based immunotherapy to HLA-A2- or HLA-A24-positive cancer patients. In addition, we investigated by *ex vivo* IFN-γ ELISPOT assay whether the HSP105-derived peptide of cytotoxic T cells (CTLs) exists in PBMCs of pre-surgical colorectal cancer patients. We found that four peptides, HSP105 A2-7 (RLMNDMTAV), HSP105 A2-12 (KLMSSNSTDL), HSP105 A24-1 (NYGIYKQDL) and HSP105 A24-7 (EYVYEFRDKL), are potential HLA-A2 or HLA-A24-restricted CTL HSP105-derived epitopes. HSP105-specific IFN-γ-secreting T cells were detected in 14 of 21 pre-surgical patients with colorectal cancer in response to stimulation with these four peptides. Our study raises the possibility that these HSP105 peptides are applicable to cancer immunotherapy in patients with HSP105-expressing cancer, particularly colorectal cancer.

Correspondence to: Dr Tetsuya Nakatsura, Division of Cancer Immunotherapy, Exploratory Oncology Research and Clinical Trial Center, National Cancer Center, 6-5-1 Kashiwanoha, Kashiwa 277-8577, Japan
E-mail: tnakatsu@east.ncc.go.jp

*Contributed equally

Key words: cancer immunotherapy, cytotoxic T cell, colorectal cancer, heat shock protein 105, HLA-transgenic mice

Introduction

Colorectal cancer is one of the most prevalent cancers and a major cause of mortality worldwide (1). Although adjuvant systemic chemotherapy or chemoradiation can confer a limited but significant survival advantage, novel and more effective therapies are needed. To improve survival rates, new therapeutic agents have been investigated. Immunotherapy for colorectal cancer is a promising candidate treatment, and there is evidence that host immune responses can influence survival (2). Ideal targets for immunotherapy are gene products overexpressed in cancer cells but silenced in normal tissues, with the exception of immune-privileged tissues, such as that of the testis.

We previously reported that heat shock protein 105 (HSP105), identified by SEREX, is overexpressed in a variety of human cancers, including colorectal, pancreatic and esophageal cancer, but with little to no expression in normal tissues aside from the testis (3,4). HSP105 is a stress protein induced by various stressors and belongs to the HSP105/110 family and plays an important role as a chaperone under physiological conditions (5). Using immunohistochemical analysis, we previously found that HSP105 was specifically overexpressed in 44 of 53 (83.0%) colorectal cancer patients (4). It has also been reported that DNA vaccination with both HSP105 and bone marrow-derived dendritic cells (BM-DCs) pulsed with HSP105 led to tumor rejection of colorectal cancer but did not induce an autoimmune reaction in mice (6-8).

This suggests that HSP105 presents a useful tumor-specific antigen target for immunotherapy. However, HSP105-derived epitope peptides of $CD8^+$ T cells have not been identified. The gene frequency of HLA-A24 (A*24:02) is relatively high in Asian populations, especially the Japanese, but low in Caucasians. On the other hand, the gene frequency of HLA-A2 (A*02:01) is high among several ethnic groups, including Asians and Caucasians (9). Therefore, HLA-A2 or HLA-A24-restricted cytotoxic T cell (CTL) HSP105 epitopes could be extremely

useful for immunotherapy in a large portion of patients worldwide. In the present study, we identified human HSP105-derived CTL epitopes restricted by HLA-A2 or HLA-A24 using HLA-transgenic mice (Tgm) and examined whether these epitope-based peptides could activate HSP105-reactive CTLs in peripheral blood mononuclear cells (PBMCs) of patients with colorectal cancer.

Materials and methods

Mice. HLA-A2.1 (HHD) Tgm, H-2D$^{b-/-}$β2m$^{-/-}$ double-knockout mice introduced with the human β2m-HLA-A2.1(α1 α2)-H-2D^{b} (α3 transmembrane cytoplasmic) (HHD) mono-chain gene construct were generated in the Departmente SIDA-Retrovirus, Unite d' Immunite Cellulaire Antivirale, Institut Pasteur, Paris, France (10,11) and were kindly provided by Dr F.A. Lemonier. HLA-A24.2 (HHD) Tgm were purchased from Japan SLC, Inc. (Shizuoka, Japan). Female 6- to 8-week-old BALB/c mice (H-2K^{d}) and BALB/c nude mice, purchased from Charles River Japan (Yokohama, Japan), were maintained and handled in accordance with animal care policy.

Cell lines. The human colorectal cancer cell line SW620 (endogenously expressing HSP105 and HLA-A*02:01, 24:02) and human liver cancer cell line HepG2 (HSP105-low expressing and HLA-A*02:01, 24:02), were kindly provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan). Murine colorectal cancer cells, Colon26 (C26) (endogenously expressing HSP105 and H-2K^{d}) were kindly provided by Dr Kyoichi Shimomura (Fujisawa Pharmaceutical Co., Osaka, Japan). T2 cells (a TAP-deficient and HLA-A*02:01-positive cell line) were provided by Kyogo Ito of Kurume University. Cells were maintained *in vitro* in RPMI-1640 or DMEM supplemented with 10% FCS.

RNA interference. Small interfering RNAs targeting human HSP105 were chemically synthesized by Dharmacon Research (HSP105-siRNA and luciferase; Lafayette, CO, USA) as previously described (12), with the following siRNA sequences: HSP105-siRNA, UUGGCUGCAACUCCGAUU GTT and luciferase, CGUACGCGGAAUACUUCGATT. The transfection of siRNA oligonucleotides was carried out using Oligofectamine (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's guidelines.

Peptides. Human HSP105-derived peptides, identical in amino acid sequence with mouse HSP105 and expressing the binding motifs for HLA-A*02:01- and HLA-A*24:02-encoded molecules, were designed with BIMAS software (BioInformatics and Molecular Analysis Section; Center for Information Technology, NIH, MD, USA). We purchased a total of 16 versions of peptides carrying the HLA-A2 (A*0201)-binding motifs and 9 versions of peptides carrying the HLA-A24 (A*2402)-binding motifs from Biologica (Tokyo, Japan) (Table I).

Induction of HSP105-reactive CTLs in Tgm. Peptide immunizations in mice were performed as previously described (13). In brief, bone marrow (BM) cells (2x10^{6}) from HLA-A2 or HLA-A24 Tgm were cultured in RPMI-1640 medium supplemented with 10% FCS, GM-CSF (5 ng/ml) and 2-mercaptoethanol (0.8 ng/ml) for 7 days in 10-cm plastic dishes. These BM-DCs were pulsed with the two HSP105 peptide mixtures (1 μmol/l each peptide) for 2 h at 37˚C. We primed the HLA-A2 or HLA-A24 Tgm with the syngeneic BM-DC vaccine (5x10^{5}/mice) into the peritoneal cavity twice, once per week. Seven days following the last immunization, the spleens were collected and CD4^{-} spleen cells were isolated by negative selection with anti-CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) to exclude any nonspecific IFN-γ production from the CD4^{+} spleen cells co-cultured with the BM-DCs. The CD4^{-} spleen cells (2x10^{6}/well) were stimulated with syngeneic BM-DCs (2x10^{5}/well) that had been pulsed with each peptide *in vitro*. After 6 days, the frequency of cells producing IFN-γ/2x10^{4} CD4^{-} spleen cells upon stimulation with syngeneic BM-DCs (1x10^{4}/well), pulsed with or without each peptide, was assayed using an enzyme-linked immunospot (ELISPOT) assay as previously described (13).

Identification of a CTL epitope in BALB/c mice. The peptide immunizations in mice were performed as previously described (14). Splenocytes removed from mice 7 days following the last immunization were harvested and cultured in 24-well culture plates (2.5x10^{6}/well) in 45% RPMI, 45% AIMV, 10% FCS and supplemented with recombinant human interleukin 2 (100 U/ml), 2-mercaptoethanol (50 μmol/l) and each peptide (10 μmol/l). After 5 days, the cytotoxicity of these cells against target cells was assayed using standard 6-h ^{51}Cr release assays (15).

Blood samples. Blood samples from cancer patients were collected during routine diagnostic procedures after obtaining formal consent from patients at the Kumamoto University Hospital, from April to September 2006 and from patients at the National Cancer Center Hospital East, from December 2006 to March 2007. The study was approved by the local ethics committee, and informed consent was obtained from all patients.

Induction of HSP105-reactive human CTLs. We isolated PBMCs from heparinized blood of HLA-A24^{+} and/or HLA-A2^{+} Japanese patients with colorectal cancer using Ficoll-Conray density gradient centrifugation; peripheral monocyte-derived dendritic cells (DCs) were generated as previously described (16,17). CD8^{+} T cells were isolated with CD8 microbeads (Miltenyl Biotec, Bergisch Gladbach, Germany) from PBMCs of the same donor and peptide-reactive CD8^{+} CTLs were generated. Five days following the last stimulation, the cytotoxic activities of the CTLs against cancer cell lines were measured by ^{51}Cr-release assay as previously described (15). For these assays, CTLs were co-cultured with each cancer cell line, as the target cells (5x10^{3}/well), at the indicated effector/target ratio.

In vivo tumor challenge. Subcutaneous tumors were induced in mice by injecting 1x10^{4} SW620 cells suspended in 100 μl PBS or Hanks' balanced salt solution (Gibco, Grand Island, NY, USA) into the backs of BALB/c nude mice. Tumor incidence and volumes were assessed weekly using calipers and tumor areas were measured. Results are presented as mean tumor areas ± SD.

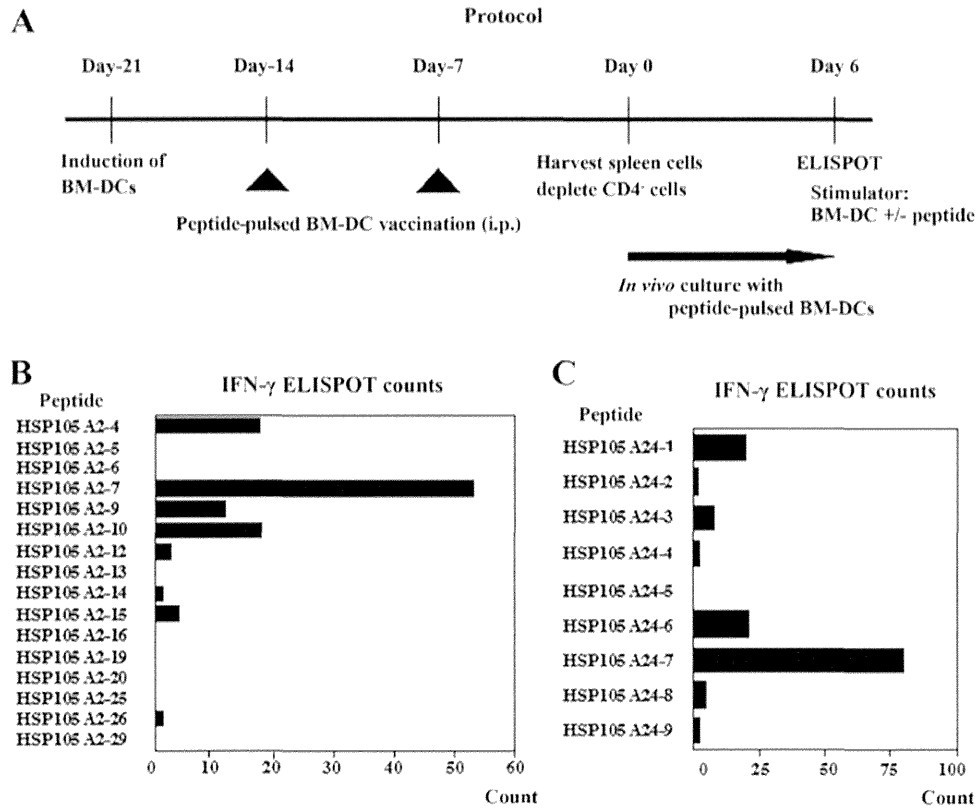

Figure 1. Identification of HLA-A2 or HLA-A24-restricted CTL epitopes of HSP105 using HLA-A2.1 Tgm and HLA-A24 Tgm. (A) The protocol used for identification of HLA-A2 or HLA-A24-restricted CTL epitopes of HSP105 is shown. We primed the HLA Tgm with BM-DCs ($5x10^5$) pulsed with the mixture of HSP105-derived peptides carrying the HLA-A2 or HLA-A24 binding motif into the peritoneal cavity once a week for 2 weeks. Seven days after the last DC vaccination, spleens were collected and $CD4^-$ spleen cells ($2x10^6$/well) were stimulated with syngeneic BM-DCs ($2x10^5$/well) pulsed with each peptide *in vitro* for 6 days. We used these cultured $CD4^-$ spleen cells as responder cells in the IFN-γ ELISPOT assay. (B) The bar graphs show the IFN-γ ELISPOT counts per $2x10^4$ $CD4^-$ spleen cells co-cultured with HLA-A2-restricted peptide-pulsed BM-DCs after normalization to counts from cells co-cultured with BM-DCs without peptide loading. (C) The bar graphs show the IFN-γ ELISPOT counts in the HLA-A24-restricted peptides. The columns represent the means from duplicate assays.

Ex vivo IFN-γ ELISPOT assay in peripheral blood in pre-surgical colorectal cancer patients. Ex vivo IFN-γ ELISPOT assays were performed to determine tumor-specific interferon-γ (IFN-γ)-secreting T cells. The 96-well plates were coated with anti-human IFN-γ (BD Biosciences Co., Ltd., USA). After an overnight incubation at 4˚C, the wells were washed and blocked with complete medium for 2 h at room temperature. A total of $1x10^6$ unfractionated PBMCs were added in duplicate wells and incubated at 37˚C for 18-20 h with or without peptides at 0.2 μl/well (1-10 μM). The plate was washed and then incubated with 5 μg/ml biotinylated anti-human IFN-γ antibody for 2 h at room temperature. After washing away the antibodies, streptavidin-HRP was added for 1 h. Finally, the plate was washed and replaced with fresh substrate solution and the reaction was terminated by washing with distilled water. The HLA-A2-restricted CMV peptide (NLVPMVATV) and HLA-A24 restricted CMV peptide (QYDPVAALF), which includes an epitope derived from the CMV pp65 protein, were used as positive controls.

Histological and immunohistochemical analysis. To investigate whether $CD8^+$ T cells infiltrated normal tissues triggered by the HSP105-derived peptide vaccine, we performed immunohistochemical staining with a monoclonal antibody against CD8 (1:100; LifeSpan BioSciences, Inc., Seattle, WA, USA) in tissue specimens from HLA-A2 Tgm immunized with the HSP105 peptides, as previously described (7). Immunohistochemical staining with rabbit polyclonal antibodies against HSP105 (1:200; Santa Cruz Biotechnology, Inc.; Santa Cruz, CA, USA) was performed according to the manufacturer's instructions.

Results

Identification of HLA-A2-or HLA-A24-restricted CTL epitopes in HLA Tgm. We designed pools of HSP105 peptides possessing amino acid sequences conserved between humans and mice that have a highly predicted binding score to HLA-A2 (pool of 16 different peptides) or HLA-A24 (A*24:02) (pool of 9 different peptides) (Table I). $CD4^-$ spleen cells were obtained from Tgm immunized twice i.p. with BM-DCs that had been pulsed with each peptide mixture; the spleen cells were then stimulated *in vitro*, again with the BM-DCs pulsed with each peptide mixture (Fig. 1A).

The IFN-γ ELISPOT counts, normalized to those of spleen cells co-cultured with BM-DCs without peptide loading, clearly indicated a HSP105 A2-7 peptide-specific response in the $CD4^-$ spleen cells (Fig. 1B). These $CD4^-$ spleen cells ($2x10^4$/well) showed 55±29.7 spot counts/well in response to the BM-DCs pulsed with the HSP105 A2-7 peptide, whereas they showed 23±31.1 spot counts/well in the presence of

Table I. HSP105-derived peptides conserved between human and mouse HSP105 predicted to bind to HLA-A2 or HLA-A24.

Peptides	Position	Subsequent residue listing	HLA-A2 binding score
HSP105 A2-4	120-128	MLLTKLKET	107
HSP105 A2-5	141-149	VISVPSFFT	55
HSP105 A2-6	155-163	SVLDAAQIV	37
HSP105 A2-7	169-177	RLMNDMTAV	591
HSP105 A2-9	202-210	DMGHSAFQV	21
HSP105 A2-10	222-230	VLGTAFDPFL	759
HSP105 A2-12	275-284	KLMSSNSTDL	276
HSP105 A2-13	276-284	LMSSNSTDL	26
HSP105 A2-14	300-309	KMNRSQFEEL	50
HSP105 A2-15	304-313	SQFEELCAEL	32
HSP105 A2-16	313-321	LLQKIEVPL	36
HSP105 A2-19	434-442	FLRRGPFEL	43
HSP105 A2-20	458-467	KIGRFVVQNT	76
HSP105 A2-25	668-676	LLTETEDWL	401
HSP105 A2-26	675-684	WLYEEGEDQA	146
HSP105 A2-29	757-765	EVMEWMNNV	15

Peptides	Position	Subsequent residue listing	HLA-A24 binding score
HSP105 A24-1	180-188	NYGIYKQDL	240
HSP105 A24-2	214-223	AFNKGKLKVL	30
HSP105 A24-3	251-260	KYKLDAKSKI	110
HSP105 A24-4	305-313	QFEELCAEL	47
HSP105 A24-5	433-442	TFLRRGPFEL	33
HSP105 A24-6	613-622	MYIETEGKMI	90
HSP105 A24-7	640-649	EYVYEFRDKL	330
HSP105 A24-8	725-733	HYAKIAADF	140
HSP105 A24-9	739-748	KYNHIDESEM	82

The binding scores were estimated by using BIMAS software: http//bimas.dcrt.nih.gov/cgi-bin/molbio/ken_parker_comboform.

BM-DCs pulsed with the HSP105 A2-4 peptide. A similarly strong response was observed for the HSP105 A24-7 peptide (Fig. 1C). $CD4^-$ spleen cells ($2x10^4$/well) showed 79.5±27.6 spot counts/well in response to the BM-DCs pulsed with the HSP105 A24-7 peptide, whereas they showed 20.5±14.8 spot counts/well in the presence of BM-DCs with the HSP A24-6 peptide. These assays were performed twice with similar results and they suggest that the HSP105 A2-7 and A24-7 peptides are potential CTL epitope peptides in both HLA Tgm and humans.

Identification of a CTL epitope in BALB/c mice and CTLs that are cytotoxic against C26 tumors in mice. There were similar structural motifs within the peptides that bound to human HLA-A24 and mice K^d. We selected those peptides with binding motifs for both HLA-A24 and K^d molecules and prepared 9 different synthetic peptides (HSP105-1-9). When we tested these peptides for their potential to induce *in vitro* tumor reactive CTLs in spleen cells derived from BALB/c mice immunized with the HSP105 peptides, only the HSP105 24-1 peptide-induced CTLs showed specific cytotoxicity against C26 tumors ($HSP105^+$, H-$2K^d$) (Fig. 2). The cytotoxicity against C26 was attenuated by HSP105 siRNA. These findings indicate that the HSP105 A24-1 peptide has the capacity to induce tumor reactive CTLs and that peptide vaccination-primed CTLs are reactive to this peptide *in vivo*. We would expect this HSP105 A24-1 (NYGIYKQDL) peptide to also be an epitope for human CTLs.

HSP105-reactive CTLs from PBMCs of HLA-A2-positive colorectal cancer patients and CTLs induce cytotoxicity against HSP105-expressing cancer cells. We generated a CTL line from PBMCs of colorectal patients by stimulation with the HSP105 A2-12 peptide. As shown by ^{51}Cr release assays, the resulting CTLs showed HSP105-specific cytotoxicity against SW620 cells ($HSP105^{+++}$, HLA-A2) and against T2 cells pulsed with the HSP105 A2-12 peptide ($HSP105^-$, HLA-A2), but not against HepG2 cells ($HSP105^{\pm}$, HLA-A2) or T2 cells pulsed with an irrelevant peptide (Fig. 3A). HSP105 siRNA decreased the cytotoxicity against SW620 cells. We investigated the effects of the HSP105 A2-12 peptide-reactive CTL lines on the mice implanted with the SW620 cells. Fourteen days after inoculation of HSP105 A2-12 peptide-reactive CTLs, there was an apparent reduction in tumor size in the SW620 compared to that in untreated mice (Fig. 3B). These results clearly indicate the efficacy of HSP105 A2-12 (KLMSSNSTDL) peptide-reactive CTL injection therapy for $HSP105^+$ tumors in mice.

Detection of HSP105-specific CTLs in peripheral blood of pre-surgical patients with colorectal cancer. Our results suggest that the four peptides, HSP105 A2-7 (RLMNDMTAV), HSP105 A2-12 (KLMSSNSTDL), HSP105 A24-1 (NYGIYK QDL) and HSP105 A24-7 (EYVYEFRDKL), are HSP105-derived, HLA-A2, or HLA-A24-restricted CTL epitopes. To determine the frequencies of the HSP105-derived T cells specific for these peptide in pre-surgical colorectal cancer patients, we analyzed the PBMC responses for each peptide using the ELISPOT assay. HSP105 expression was detected in 20 of 21 (95%) patients, consistent with previous studies (4). HSP105-specific T cells secreting IFN-γ were detected in patients stimulated with the HSP105 A2-7 (4 patients), HSP105 A2-12 (6 patients), HSP105 A24-1 (2 patients) and HSP105 A24-7 (6 patients) peptides (Table II). ELISPOT assay detected positive IFN-γ responses to at least one of the HSP105-derived peptides in PBMCs in 14 of the 21 patients. In contrast to the results for colorectal cancer patients, the 4 peptides were not recognized by PBMCs from healthy donors. Both the ratio of normal donors who showed positive T-cell responses to CMV-derived peptides and the frequencies of the specific T cells were identical to those of the colorectal cancer patients (data not shown).

HSP105-derived peptide immunization does not induce autoimmunity in HLA-A2 Tgm. HSP105 in normal adult mice is expressed in only certain tissues, and expression in these tissues is less than that in C26 tumor cells, suggesting a low

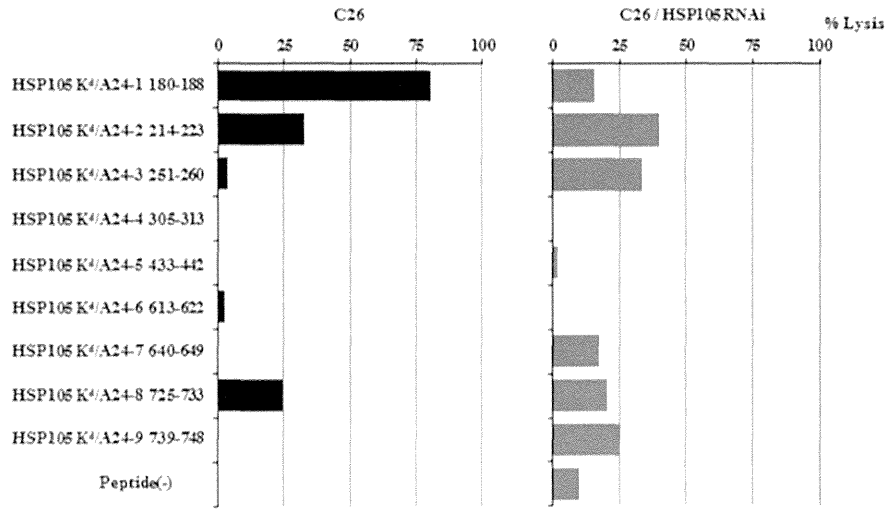

Figure 2. Identification of an HSP105-derived HLA-A24 and K^d-restricted CTL epitope. BALB/c mice were immunized with 9 HSP105 peptides. Using the ^{51}Cr release assay, sensitized spleen cells that had been stimulated *in vitro* with each HSP105 peptide (10 μmol/l) and cultured for 5 days with 100 U/ml interleukin-2 were examined for CTL activity against C26 cells and C26 cells transfected with HSP105 siRNA (C26/HSP105 RNAi). Values represent the percentage of specific cell lysis, based on the mean values from triplicate assays.

Table II. Expression of HSP105 in colorectal cancer tissue and quantification of HSP-specific CTLs in colorectal cancer patients.

						[c]Spot number of peptide-specific CTLs				
HLA-A2-positive patients	Age (yrs.)	Gender	HLA	Stage[a] of tumor	HSP105 expression[b]	HSP105 A2-7		HSP105 A2-12		CMV
1	62	M	0201/2601	IIIB	++	27	+	126	+	160
5	79	M	0207/1101	IIIB	++	0	-	2	-	10
6	51	M	0201/0206	I	+	0	-	49	+	136
8	55	M	0206/2402	I	±	0	-	0	-	66
11	69	M	0206/2402	IIIC	+	143	+	0	-	0
12	61	M	0201/3303	I	±	2	-	45	+	367
13	64	F	0201/2601	IIIC	±	0	-	2	-	254
14	66	M	0206/2402	IIIC	-	13	+	0	-	58
15	78	M	0201/1101	IIA	+	0	-	5	+	57
16	51	F	0206/2601	IV	±	31	+	7	+	15
17	63	F	0206/1101	IIA	++	0	-	25	+	96
HLA-A2402-positive patients						HSP105 A24-1		HSP105 A24-7		CMV
2	64	F	2402	IV	++	2	-	44	+	6
3	60	M	2402/3101	IIIC	++	0	-	0	-	11
4	71	F	2402/3101	IIA	++	25	+	51	+	12
7	47	M	2402/3101	IIIA	++	4	-	6	+	3
9	66	M	2402	IV	++	8	+	6	+	7
10	60	M	2402/3101	I	++	1	-	19	+	26
18	64	M	1101/2402	IV	+	0	-	2	-	40
20	46	F	1101/2402	IIIB	++	4	-	7	+	5
21	66	F	2402	I	++	3	-	0	-	38

F, female; M, male. [a]Stage, staging was performed according to the TNM classification (Union for International Cancer Control; UICC). [b]HSP105 expression, staining intensity of tumor cells was scored on a scale according to the following four grades: -, absent; ±, weak; +, moderate; ++, strong. [c]Spot number indicates the number of peptide-specific CTLs calculated by subtracting the spot number in a well of no peptide. -, Spot number <5; +, Spot number ≥5.

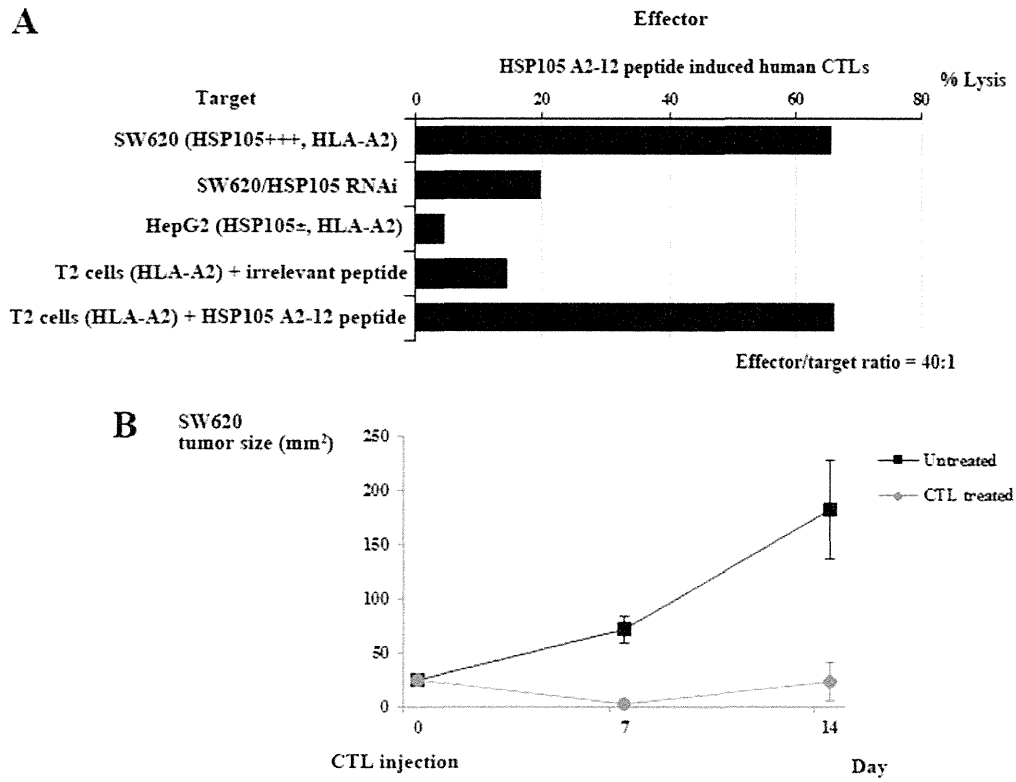

Figure 3. CTL induction from PBMCs of HLA-A2-positive cancer patients. (A) HSP105 peptide-reactive CTLs were generated from $CD8^+$ T cells of HLA-$A2^+$ colorectal cancer patients. After three or four stimulations with autologous monocyte-derived DCs pulsed with the HSP105 A2-12 peptides, the CTLs were subjected to a standard ^{51}Cr release assay at the indicated effector/target ratio (40/1). Their cytotoxicity against SW620 cells ($HSP105^{+++}$, HLA-A2), SW620 cells transfected with HSP105 siRNA ($HSP105^-$), HepG2 cells ($HSP105^{\pm}$, HLA-A2), T2 cells pulsed with an irrelevant peptide ($HSP105^-$, HLA-A2) and T2 cells pulsed with the HSP105 A2-12 epitope peptide were all examined by ^{51}Cr release assay. Values represent the percentage of specific cell lysis, based on the mean values from triplicate assays. (B) There was marked growth inhibition of SW620 cells ($HSP105^+$) engrafted into nude mice after intratumoral injection of human CTLs induced by the HSP105 peptides. When tumor size reached 25 mm^2 on day 9 after s.c. tumor implantation, human CTLs ($3x10^6$) reactive to the HLA-A2-restricted HSP105 peptide, generated from an HLA-$A2^+$ donor, were i.t. inoculated. Tumor sizes in nude mice administered the HSP105 epitope peptide-induced CTL lines (n=3), or no treatment (n=3), are shown. The mean tumor size (mm^2) for each group of mice was expressed, and bars represent SD.

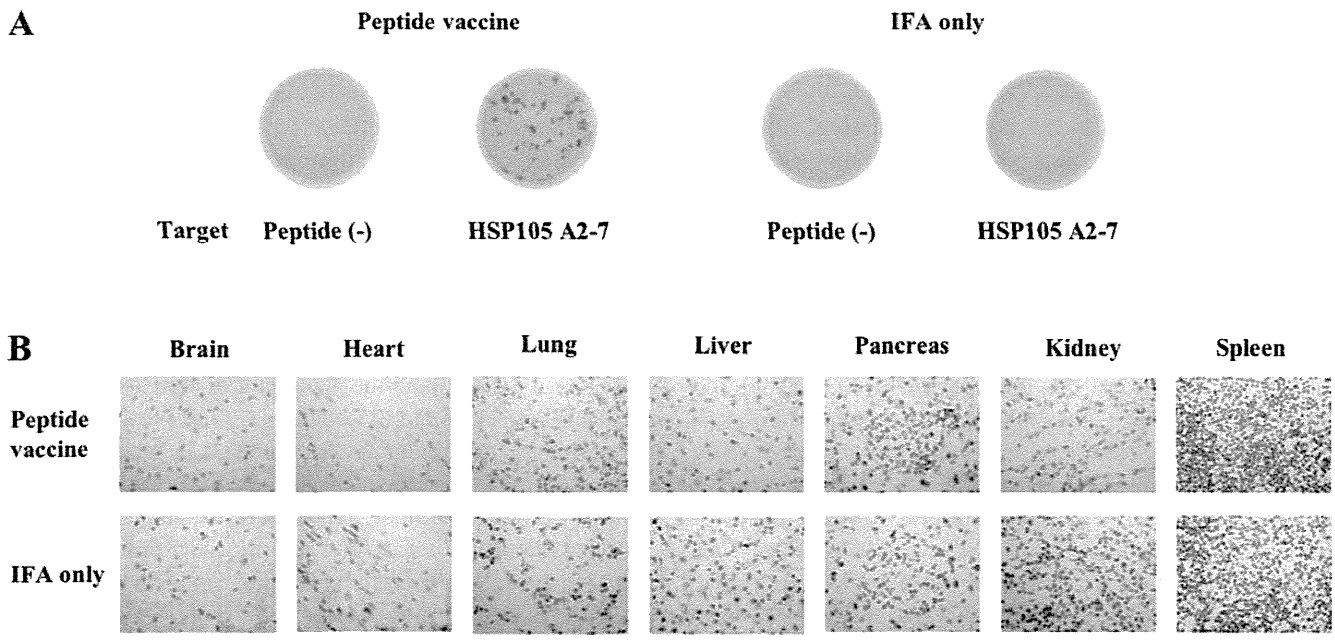

Figure 4. The HSP105 peptide vaccine induces HSP105 peptide-specific CTLs, while CD8 T cells do not infiltrate into normal tissues. (A) HSP105 peptide-specific CTLs were induced in the spleen cells of immunized mice with the HSP105 peptide vaccine. IFN-γ ELISPOT assays were performed using BM-DCs pulsed with HSP105 A2-7 and non-pulsed BM-DCs as target cells. The representative data are shown (n=6). (B) Immunohistochemical staining with anti-CD8 mAb was performed in tissue specimens of HLA-A2 Tgm immunized with the HSP105 A2-7 and A2-12 peptides. The tissue specimens were removed and analyzed 7 days after the second vaccination (original magnification, x400). The representative data are shown (n=3).

risk of damage to normal tissues posed by HSP105 antigen-induced immune responses (6). To investigate whether immunization of the mice with HSP105-derived peptides causes autoimmunity, HLA-A2 Tgm were immunized with the HSP105 A2-7 and A2-12 peptides emulsified in incomplete Freund's adjuvant at 7-day intervals and then sacrificed 7 days after the second vaccination. Using the IFN-γ ELISPOT assay, we confirmed the induction of HSP105 peptide-specific CTLs in the spleen cells of immunized mice (Fig. 4A). We did not detect any pathological changes, such as $CD8^+$ lymphocyte infiltration or tissue destruction/repair, in the brain, heart, lung, liver, pancreas, or kidney of HLA-A2 Tgm (Fig. 4B). These results indicate that the HSP105 peptide-reactive $CD8^+$ CTLs did not attack the healthy tissue specimens that we evaluated.

Discussion

Heat shock proteins (HSPs) have essential functions in the regulation of protein folding, conformation, assembly and sorting. They function as molecular chaperones to maintain the native conformational states of proteins, preventing protein aggregation (18). HSPs are classified into several families based on their molecular weight, including HSP105/110, HSP90, HSP70, HSP60, HSP40 and HSP27 (19). HSP105 is a stress protein within the HSP105/110 family that we previously reported to be overexpressed in a variety of human cancers but with little to no expression in normal tissues, aside from the testis. Thus, HSP105 presents a promising candidate for a target antigen in cancer immunotherapy (3-7). In particular, HSP105 is specifically overexpressed in colorectal cancer (83%) (4). Furthermore, HSP105 is expressed in highly metastatic colon cancer cell lines and its expression is correlated with advanced clinical cancer stages and positive lymph node involvement (20). When considering immunogenic target molecules for cancer immunotherapy, it is important to select a tumor antigen that does not run the risk of becoming lost during immunoediting (21). We reported previously that siRNA-mediated suppression of HSP105 protein expression induced apoptosis in various types of cancer cells, but not in fibroblasts (12). Therefore, it is possible that tumor cells do not lose HSP105 expression, allowing for continued growth.

Advances in molecular biology and tumor immunology have paved the way for identification of a large number of tumor-associated antigens (TAAs) and antigenic peptides recognized by tumor reactive CTLs; hence, peptide-based cancer immunotherapy has become an intensely studied field (22,23). Several HSPs, including HSP70, HSP90 and gp96, bind and deliver (through receptor-mediated endocytosis of HSP) antigenic peptides to the antigen-processing pathway of antigen-presenting cells (APCs) and these peptides are then presented on major histocompatibility complex (MHC) class I molecules. This HSP-mediated pathway has been demonstrated to evoke potent antiviral and antitumor immune responses (24). On the other hand, many researchers have identified MHC class I-presenting peptide epitopes derived from HSP (25). Furthermore, HSP105 itself may induce $CD8^+$ T cells to become reactive towards tumor cells that express HSP105, using HSP105-DNA and HSP105-pulsed DC vaccines in mice (6-8).

We found 4 peptides [HSP105 A2-7 (RLMNDMTAV), HSP105 A2-12 (KLMSSNSTDL), HSP105 A24-1 (NYGI YKQDL) and HSP105 A24-7 (EYVYEFRDKL)] to be potential HSP105-derived, HLA-A2 or A24-restricted CTL epitopes. There was a discrepancy between the expected HSP105 CTL epitopes in Tgm and in PBMCs of colorectal cancer patient. To identify the HSP105-derived CTL epitope peptides, we analyzed the PBMC responses to each of the 4 peptides in colorectal cancer patients using the *ex vivo* IFN-γ ELISPOT assay.

In this study, we used an *ex vivo* assay to detect HSP105-specific IFN-γ-secreting T cells in PBMCs from 14 of 21 pre-surgical patients with colorectal cancer. Generally, CTLs specific for tumor antigens cannot be detected directly *ex vivo*; rather only after expansion by repeated *in vitro* stimulation with the antigenic peptide in the appropriate antigen-presenting cells. This is attributed to assay sensitivity and the low frequency of tumor antigen-specific CTLs (26). HSP105-specific CTLs in PBMCs, which can be detected directly *ex vivo* without *in vitro* stimulation, provide strong immunological evidence of HSP105-derived CTL epitopes, which we were able to identify in this study. However, because the prognosis of the pre-surgical patients was affected by various factors, it was difficult to evaluate the correlation between a positive CTL response before surgery and clinical improvement at the present stage; an increase in the number of patients at each stage and further analyses of this relationship are necessary.

Although the SEREX method facilitated the identification of tumor antigens that could be recognized by antibodies and $CD4^+$ T cells, few of their T-cell epitopes have been determined (27). We previously reported in mice that HSP105-DNA and HSP105-pulsed DC vaccines induced a reaction in $CD4^+$ T cells and $CD8^+$ T cells towards tumor cells expressing HSP105 (6-8). HSP105 was identified by SEREX (3) and thus, HSP105-specific $CD4^+$ T cell reactions may be induced by HSP105 immunization. It was shown that antigen-specific $CD4^+$ T cells are required to activate memory $CD8^+$ T cells into fully functional effector killer cells (28). We are now preparing a clinical trial to investigate HSP105-based immunotherapy for HSP105-expressing tumors, including those from colorectal cancer. We plan to use the HSP105 epitope peptides identified in this study as an initial attempt. We expect that HSP105-based immunotherapy will be a novel treatment strategy for colorectal cancer patients.

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Review Article

Clinical Development of Immune Checkpoint Inhibitors

Ayumu Ito,[1] Shunsuke Kondo,[2,3] Kohei Tada,[4] and Shigehisa Kitano[2,4]

[1]*Department of Hematopoietic Stem Cell Transplantation, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan*
[2]*Department of Experimental Therapeutics, Exploratory Oncology Research and Clinical Trial Center (EPOC), National Cancer Center, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan*
[3]*Department of Hepatobiliary and Pancreatic Oncology, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan*
[4]*Division of Cancer Immunotherapy, Exploratory Oncology Research and Clinical Trial Center (EPOC), National Cancer Center, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan*

Correspondence should be addressed to Shigehisa Kitano; skitano@ncc.go.jp

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Recent progress in cancer immunotherapy has been remarkable. Most striking are the clinical development and approval of immunomodulators, also known as immune checkpoint inhibitors. These monoclonal antibodies (mAb) are directed to immune checkpoint molecules, which are expressed on immune cells and mediate signals to attenuate excessive immune reactions. Although mAbs targeting tumor associated antigens, such as anti-CD20 mAb and anti-Her2 mAb, directly recognize tumor cells and induce cell death, immune checkpoint inhibitors restore and augment the antitumor immune activities of cytotoxic T cells by blocking immune checkpoint molecules on T cells or their ligands on antigen presenting and tumor cells. Based on preclinical data, many clinical trials have demonstrated the acceptable safety profiles and efficacies of immune checkpoint inhibitors in a variety of cancers. The first in class approved immune checkpoint inhibitor is ipilimumab, an anti-CTLA-4 (cytotoxic T lymphocyte antigen-4) mAb. Two pivotal phase III randomized controlled trials demonstrated a survival benefit in patients with metastatic melanoma. In 2011, the US Food and Drug Administration (FDA) approved ipilimumab for metastatic melanoma. Several clinical trials have since investigated new agents, alone and in combination, for various cancers. In this review, we discuss the current development status of and future challenges in utilizing immune checkpoint inhibitors.

1. Introduction

In this decade, remarkable progress has been made in the clinical application of cancer immunotherapies. Most notable is the emergence of immune checkpoint inhibitors. Large-scale clinical trials have shown their feasibility and efficacy for patients with advanced malignancies. The therapeutic targets, or "immune checkpoints," are also known as coinhibitory molecules or costimulatory molecules expressed on T cells.

As the name implies, costimulatory/inhibitory molecules mediate positive/negative signals that modify MHC-TCR (major histocompatibility complex-T-cell receptor) signaling pathways. These signals each regulate T-cell survival, proliferation, differentiation, or responsiveness to cognate antigens. The net effect depends on the balance among signals [1]. T-cell activation requires costimulatory signals. If they contact antigens without costimulatory ligands on antigen presenting cells (APCs), T cells remain inactivated in a state of anergy.

Coinhibitory molecules induce T-cell dysfunction (so called "T-cell exhaustion") or apoptosis. Employing this inhibitory pathway, the immune system can attenuate excessive immune reactions and ensure self-tolerance, which is important for maintaining immune homeostasis. These functions involve programmed cell death protein-1 (PD-1), programmed cell death-1 ligand-1/2 (PD-L1/2), cytotoxic T lymphocyte antigen-4 (CTLA-4), lymphocyte-activation gene 3 (LAG-3), T-cell immunoglobulin mucin-3 (TIM-3), and B and T lymphocyte attenuator (BTLA). Tumor cells harness

these suppressive effects as one of their "immunoediting" mechanisms [2]. As shown in recent clinical trials, immune checkpoint blockade with monoclonal antibody promotes endogenous antitumor activities of immune cells and achieves clinically significant benefits for cancer patients [3, 4].

In this review, we focus on the current development status of and future challenges in utilizing immune checkpoint inhibitors, especially CTLA-4, PD-1, and PD-L1.

2. Anti-CTLA-4 Antibody

CTLA-4 (also known as CD152) is a member of the CD28 family of receptors [21, 22]. CTLA-4 is inducibly expressed on the surfaces of activated conventional $CD4^+$ and $CD8^+$ T cells. CTLA-4 binds to ligands B7.1 (CD80) and B7.2 (CD86) on APCs, where it competes with costimulatory receptor CD28 to bind with shared ligands. As CTLA-4 binds with higher affinity than CD28, it reduces CD28-dependent costimulation. CTLA-4 also mediates direct inhibitory effects on the MHC-TCR pathway [23]. CTLA-4 recruits 2 phosphatases, SHP-2 and PP2A, to its intracellular YVKM domain. SHP-2 dephosphorylates the CD3ζ chain, attenuating the TCR signal. PP2A inhibits downstream Akt phosphorylation, further impairing TCR signaling. Furthermore, CTLA-4 is constitutively and highly expressed on $CD4^+CD25^+FOXP3^+$ regulatory T cells (T regs) and plays a role in their suppressive functions [24–26]. CTLA-4 knockout mice have a lethal autoimmune-like syndrome. Prominent infiltration of $CD4^+$ T cells is detected in multiple organs. Thus, CTLA-4 is considered to be indispensable for maintaining immune homeostasis.

In the tumor microenvironment, CTLA-4 suppresses antitumor immune activities. In animal models, it has been shown that CTLA-4 blockade leads to reactivation of the antitumor immune response and tumor shrinkage [27–29]. The mechanism of action has not yet been fully elucidated. Observations made to date suggest that anti-CTLA-4 antibodies function not only by blocking inhibitory signals from reaching effector T cells but also by depleting regulatory T cells in the tumor microenvironment [30, 31]. For use in humans, based on preclinical studies, two anti-CTLA-4 antibodies have been developed: ipilimumab (Bristol-Myers Squibb) and tremelimumab (Pfizer).

2.1. Ipilimumab. Ipilimumab is a fully humanized IgG1 monoclonal antibody that inhibits CTLA-4 [32, 33].

Early clinical trials evaluated ipilimumab in patients with a variety of malignancies, including melanoma, prostate cancer, renal cell carcinoma, and non-Hodgkin lymphoma [34–45]. Some of these studies combined ipilimumab with a peptide vaccine, chemotherapy, or IL-2. Based on preclinical data, ipilimumab was administered at a dose range of 0.1–20 mg/kg, employing single or multiple dosing schedules (every 3-4 weeks).

A phase I study evaluated a single 3 mg/kg dose of ipilimumab for patients with metastatic hormone-refractory prostate cancer. Two (14%) of 14 patients showed ≧50% decline in prostate specific antigen. One (7%) patient developed grade 3 rash/pruritus requiring systemic corticosteroid administration [36]. Another phase I trial combined ipilimumab (administered at 3 mg/kg every 3 weeks) with a glycoprotein (gp) 100 peptide vaccine for patients with metastatic melanoma. Three (21%) of 14 patients responded to this treatment, including 2 showing complete responses (CRs). Grade 3 to 4 immune-related adverse events (irAEs) occurred in 6 (43%) patients. These irAEs included dermatitis, enterocolitis, hepatitis, and hypophysitis [34]. On the whole, irAEs were mild and manageable with therapy discontinuation and/or appropriate treatments, including corticosteroids.

A phase II trial compared 3 doses (0.3, 3, or 10 mg/kg) administered every 3 weeks for a total of 4 doses. Eligible patients were permitted to receive reinduction therapy (at a dose of 10 mg/kg) or maintenance therapy (administered at the previously assigned dose level every 12 weeks). The overall response rate (ORR) in the 10 mg/kg arm was superior to those in the other arms (11.1% versus 4.2% versus 0.0%), but irAEs were also higher in the 10 mg/kg arm [43]. The optimal dosing and scheduling are as yet unknown. A phase III randomized trial (NCT01515189) is currently comparing 2 doses (3 mg/kg versus 10 mg/kg). No consensus has yet been reached on the relative significance of reinduction versus maintenance therapy [46, 47]. A prospective study comparing reinduction therapy versus the physician's choice of chemotherapy (NCT00495066) is currently underway.

Based on pivotal phase III randomized controlled trials (RCTs) showing survival benefit, ipilimumab was approved by the US Food and Drug Administration (FDA) for metastatic melanoma [5, 6]. In the landmark phase III trial for patients with previously treated metastatic melanoma, ipilimumab (administered at 3 mg/kg every 3 weeks for a total of 4 doses) with or without the gp 100 peptide vaccination was compared with the gp 100 peptide vaccine alone. Eligible patients were permitted to receive reinduction therapy. The median OSs in the ipilimumab-containing arms were significantly superior to that in the gp 100 alone arm (10.1 months in ipilimumab/gp 100, 10.0 months in ipilimumab alone, and 6.4 months in gp 100 alone, hazard ratio (HR) 0.68; $P < 0.001$). Grade 3 to 4 irAEs were seen in 10–15% of patients in the ipilimumab-containing arms, while 3% in the gp 100 alone arm experienced irAEs. There were 14 treatment-related deaths (2.1%), including 7 patients with irAEs [5]. Long-term follow-up analysis confirmed an approximately 20% survival rate for patients in the ipilimumab-containing arms. Safety profiles in long-term survivors were comparable among the 3 groups, and new onset irAEs after the last dose of ipilimumab were infrequent (8%; all grades) [48]. The other phase III trial compared ipilimumab (at 10 mg/kg every 3 weeks for 4 doses)/dacarbazine with dacarbazine/placebo, followed by maintenance therapy with ipilimumab or placebo administered every 12 weeks for eligible patients. Overall survival (OS) was significantly longer in the ipilimumab/dacarbazine arm (11.2 versus 9.1 months), and the higher survival rates were durable (47.3% versus 36.3% at 1 year, 28.5% versus 17.9% at 2 years, 20.8% versus 12.2% at 3 years, HR for death 0.72; $P < 0.001$). Grade 3 to 4 AEs were seen in more patients in the ipilimumab/dacarbazine arm (56.3% versus 27.5%;

$P < 0.001$). No drug-related deaths occurred among those in the ipilimumab/dacarbazine arm [6].

The analysis of the collected data from 12 previous clinical trials, which include 1861 ipilimumab-treated patients with advanced melanoma, demonstrated a median OS of 11.4 months and 3-year OS rate of 22%. The OS curve started to show plateau around year 3, which was independent of the dose of ipilimumab (3 or 10 mg/kg), therapy line (treatment-naïve or not), or use of maintenance therapy [49].

2.2. Tremelimumab. Tremelimumab is a human IgG2 monoclonal antibody that blocks CTLA-4 [50].

Early clinical trials on tremelimumab monotherapy showed response rates of 2–17%, and these responses were durable (>150 days) [51–57]. Based on preclinical and clinical data, the standard regimen is 15 mg/kg every 90 days. Most adverse events were mild and manageable. These adverse events included skin rash, diarrhea, and endocrine abnormalities.

A phase III study compared tremelimumab (15 mg/kg every 3 months) with chemotherapy (physician's choice) in patients with untreated advanced melanoma [7]. This study demonstrated no benefits in either ORR (10.7% versus 9.8%) or OS (12.6 mo versus 10.7 mo), but a superior response duration was seen (35.8 versus 13.7 months). This observation might be explained by patient selection bias (exclusion of patients with lactate dehydrogenase (LDH) >2x upper limit of normal), drug crossover (to ipilimumab) in the control arm, and even a potentially suboptimal dosing regimen. Tremelimumab is still being investigated for other tumors, both alone and as combination therapy (Table 1).

3. Anti-PD-1 Antibodies

Programmed cell death protein-1 (PD-1; also known as CD279), like CTLA-4, is a coinhibitory CD28-family molecule [22]. While CTLA-4 works in the early phase of naïve-T-cell activation, PD-1 functions mainly in the late phase, in which PD-1 induces exhaustion or anergy in effector T cells. Thus, PD-1 is considered to play an important role in chronic inflammation such as that associated with viral infection or tumor exposure [58]. PD-1 is expressed on activated T cells, T regs [59], activated B cells, NK cells, and monocytes. It binds to the B7-family ligands PD-L1 (programmed death ligand-1, B7-H1) and PD-L2 (programmed death ligand-2, B7-DC) on APCs. PD-1 has cytoplasmic domain motifs known as ITIM (immunoreceptor tyrosine-based inhibitory motif) and ITSM (immunoreceptor tyrosine-based switch motif) [23]. When these motifs are phosphorylated, they recruit two inhibitory phosphatases, SHP-1 and SHP-2 (SHP: SH2-containing-phosphatase). These phosphatases dephosphorylate the CD3ζ chain, decreasing TCR signaling. Although the inhibitory mechanisms of CTLA-4 and PD-1 have some similarity in terms of inhibiting Akt activation, CTLA-4 can also interfere with Akt independently via PP2A [23]. PD-1 knockout mice show a milder lupus-like syndrome than CTLA-4 knockout mice [60].

Tumor cells utilize the PD-1-PD-L1/2 pathway to evade immune-cell attack [61]. Blockade of this pathway was shown to restore and augment antitumor immune activities [62].

3.1. Nivolumab (BMS-936558/ONO-4538). Nivolumab is a fully humanized IgG4 monoclonal antibody that blocks PD-1 [62].

Phase I studies tested nivolumab in such cancers as melanoma, non-small cell carcinoma of the lung (NSCLC), ovarian cancer, and renal cell carcinoma. These studies showed response rates of approximately 20–30%, durable tumor regression (>1 year), and an acceptable safety profile, with Grade 3 to 4 irAEs developing in about 20% of patients [8, 9, 63–65]. In long-term follow-up of the phase I trial for advanced melanoma, median OS was 16.8 months and survival rates were 62% at 1 year and 43% at 2 years. The patients requiring discontinuation of treatment maintained their tumor responses for at least 16 months (16–56 months). Long-term safety profiles were acceptable and similar to those described in a previous report [8]. The preliminary results of a phase I study evaluating nivolumab (at 3 mg/kg q2w) for untreated advanced NSCLC were recently reported. The ORR was 30% with 2 complete remissions (CRs), as measured by RECIST. ORR and progression-free survival (PFS) correlated with PD-L1 positivity (67% versus 0% for ORR, 45.6 mo versus 36.1 mo for median PFS). AEs were generally manageable and grade 3 to 4 AEs occurred in 3 patients, including rash, increased transaminase, and hyperglycemia [66].

Recently the interim analysis report of a phase III study (NCT01721746), comparing nivolumab monotherapy (at 3 mg/kg q2w) with investigator's choice chemotherapy in ipilimumab-refractory advanced melanoma, was shown. The ORRs were 32% in the nivolumab arm and 11% in the control arm, with the median duration of response in the nivolumab arm not reached. Grade 3 to 4 drug-related AEs were less frequent in the nivolumab arm (9% versus 31%) [10]. Another phase III study (NCT01721772) compared nivolumab monotherapy (at 3 mg/kg q2w) with dacarbazine in 418 patients with previously untreated stage III or IV melanoma. This study was stopped ahead of schedule and unblinded after independent data monitoring committee found significant survival superiority in nivolumab over dacarbazine. The results from the double-blind part of the study before the stoppage showed that the OS rate at 1 year was significantly higher in the nivolumab arm (72.9% versus 42.1%, HR for death 0.42; $P < 0.001$), and the median PFS was also significantly longer in the nivolumab arm (5.1 versus 2.2 months, HR for death or progression 0.43; $P < 0.001$). Grade 3 to 4 drug-related AEs occurred in more patients in the dacarbazine arm (11.7% versus 17.6%). No drug-related deaths occurred in both arms [11]. A phase II study (NCT01927419) of nivolumab in combination with ipilimumab compared with ipilimumab alone for advanced melanoma is currently ongoing (recruitment has been completed).

In 2013, nivolumab received Fast Track designation for the treatment of NSCLC, melanoma, and renal cell carcinoma (RCC) from the FDA. In April 2014, a rolling submission to the FDA for nivolumab in third-line pretreated NSCLC was started. In May 2014, nivolumab received a Breakthrough

Table 1

Target molecule	Drug name	Phase	Status/NCT number	Disease	Number of patients	Study design	Response	Survival	Treatment-related adverse events (≧Gr3)	Reference
CTLA-4	Ipilimumab	III	Completed (NCT00094653)	Melanoma	676	Endpoint: safety/efficacy Ipi + gp100 versus Ipi versus gp100	Ipi + gp100: ORR 5.7%; SD 14.4%	Ipi + gp100 versus gp100: 10.1 versus 6.4 mos	Ipi + gp100: drug-related 17.4%; irAEs 10.2%; diarrhea 4.5%; fatigue 5.0%; dyspnea 3.7%; anemia 2.9%; endocrine abnl. 11%; AST↑ 0.5%; ALT↑ 0.3%	[5]
		III	Completed (NCT00324155)	Melanoma	502	Endpoint: efficacy Ipi + DTIC versus PBO + DITC	Ipi + DTIC: ORR 15.2%; SD 18.0%	Ipi + DTIC versus PBO + DTIC: 11.2 versus 9.1 mos	Ipi + DTIC: immune-related 41.7%; pruritus 2.0%; rash 1.2%; diarrhea 4.0%; colitis 6.1%; AST↑ 17.4%; ALT↑ 20.7%	[6]
	Tremelimumab	III	Completed (NCT00257205)	Melanoma	655	Endpoint: efficacy treme. versus chemo.	ORR 10.7%	Treme. versus chemo.: 12.6 versus 10.7 mos (NS)	52%; diarrhea/colitis 18%; fatigue 6%; rash 2%; pruritus 1%; dyspnea 3%; hypothalamus and pituitary disorders 1%; hepatitis 1%	[7]

Table 1: Continued.

Target molecule	Drug name	Phase	Status/NCT number	Disease	Number of patients	Study design	Response	Survival	Treatment-related adverse events (≧Gr3)	Reference
PD-1	Nivolumab (BMS-936558/ONO-4538)	I	Ongoing (not recruiting) (NCT00730639)	Melanoma	107	Endpoint: safety/efficacy 5 dosing regimens	ORR 30.8%; median duration of response 104 wks; SD (≧24 wks) 6.5%	OS 16.8 mos; PFS 3.7 mos	22.4%; fatigue 1.9%; diarrhea 1.9%; abdominal pain 1.9%; lymphopenia 2.8%	[8]
		I	Ongoing (not recruiting) (NCT01176461)	Melanoma	90	Endpoint: safety/efficacy 3 dosing regimens	ORR 25%; SD (≧24 wks) 21%	PFS (at 24 wks) 46%	5.6%; rash 2.2%; interstitial pneumonitis 2.2%	[9]
		III	Ongoing (not recruiting) (NCT 01721772)	Melanoma	370	Endpoint: efficacy Nivo. versus ICC	ORR 32% versus 11%	NA	9% versus 31%	[10]
		III	Completed (NCT01721772)	Melanoma	418	Endpoint: efficacy Nivo. versus dacarbazine	ORR 40.0% versus 13.9%	OS (at 1 yr) 72.9% versus 42.1%, median PFS 5.1 versus 2.2 mo	11.7% versus 17.6%; fatigue 0.5%; diarrhea 1.0%; rash 0.5%; vomiting 0.5%	[11]
	Pidilizumab (CT-011)	II	Completed (NCT01435369)	Melanoma	103	Endpoint: safety/efficacy 2 dosing regimens	ORR 5.9%	OS (at 1 yr): 64.5%	NA	[12]
	Pembrolizumab (MK-3475)	I	Ongoing (not recruiting) (NCT01295827)	Melanoma	135	Endpoint: safety/efficacy 3 dosing regimens	ORR 38% by RECIST and 37% by irRC	Median PFS >7 mos	13%; hypothyroidism 1%; diarrhea 1%; fatigue 1%; AST↑ 1%; renal failure 1%; rash 2%; pruritus 1%	[13]
		I	Ongoing (not recruiting) (NCT01295827)	Untreated NSCLC	57	Endpoint: safety/efficacy 3 dosing regimens	ORR 26% by RECIST and 47% by irRC	Median OS NR; OS at 1 yr 80%; median PFS 45.6%; PFS at 24 wks 70%	CK↑ 2%; pericardial effusion 2%; pneumonitis 2%; acute kidney injury 2%	[14]
		I	Ongoing (not recruiting) (NCT01848834)	Head and neck cancer	60	Endpoint: safety/efficacy single arm	ORR 19.6% in total, 20.0% in HPV+, and 19.4% in HPV−;	NA	Gr3–5 16.7%; Rash 3.3%	[15]
		I	Ongoing (not recruiting) (NCT01848834)	Gastric cancer	39	Endpoint: safety/efficacy single arm	ORR 30.2% by RECIST	NA	7.7%; hypoxia 2.6%; peripheral neuropathy 2.6%; pneumonia 2.6%	[16]

Table 1: Continued.

Target molecule	Drug name	Phase	Status/NCT number	Disease	Number of patients	Study design	Response	Survival	Treatment-related adverse events (≧Gr3)	Reference
	BMS-936559	I	Ongoing (not recruiting) (NCT00729664)	Melanoma	52	Endpoint: safety 4 dose levels	ORR 17%; SD (≧24 wks) 27%	PFS (at 24 wks) 42%	9%; fatigue 1%; infusion reaction 1%; lymphopenia 1%	[17]
				NSCLC	49		ORR 10%; SD (≧24 wks) 12%	PFS (at 24 wks) 31%		
				Ovarian cancer	17		ORR 6%; SD (≧24 wks) 18%	PFS (at 24 wks) 22%		
				Renal cell carcinoma	17		ORR 12%; SD (≧24 wks) 41%	PFS (at 24 wks) 53%		
PD-L1	MPDL3280A	I	Recruiting (NCT01375842)	Urothelial bladder cancer	68	Endpoint: safety/efficacy/ biomarker single arm	ORR: PD-L1 + 43% (at 6 wks) and 52% (at 12 wks); PD-L1 − 11% (at 6 wks);	NA	4%; no irAE	[18]
	MEDI4736	I	Recruiting (NCT01693562)	Advanced solid tumors	26 (as of Jan 2014)	Endpoint: safety/efficacy single arm	PR 15.4%; disease control rate (≧12 wks) 46%	NA	Any Gr 34%; Gr3/4 0%; no DLT; no MTD	[19]
	MSB0019718C	I	Recruiting (NCT01772004)	Refractory malignancies	27 (as of Jan 2014)	Endpoint: safety single arm	NA	NA	Treatment discontinuation 52.2% (8.7% for AEs); drug-related AEs 11.1%; DLT 3.7% (CPK↑, myositis, and myocarditis)	[20]

Abbreviations: NSCLC, non-small cell lung cancer; Ipi, Ipilimumab; gp100, glycoprotein 100 peptide vaccine; DITC, dacarbazine; PBO, placebo; ORR, objective response rate; PR, partial response; SD, stable disease; mo, month; wk, week; RECIST, response evaluation criteria in solid tumors; irRC, immune-related response criteria; HPV, human papillomavirus; NA, not available; NS, not significant; NR, not reached; OS, overall survival; PFS, progression-free survival; AE, adverse event; irAE, immune-related adverse event; Gr, Grade; abnl., abnormality; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CPK, creatine phosphokinase; DLT, dose limiting toxicity; MTD, maximum tolerance dose; ICC, investigator's choise chemotherapy.

Therapy designation for non-Hodgkin lymphoma from the FDA. In Japan, in July 2014, nivolumab received manufacturing and marketing approval for unresectable melanoma from the domestic regulator, the Ministry of Health Labor and Welfare, which made nivolumab the first in anti-PD-1 antibody to receive regulatory approval in the world.

3.2. Pidilizumab (CT-011). Pidilizumab (CT-011) is a humanized IgG-1κ monoclonal antibody that blocks PD-1. In animal models, an antitumor effect was achieved with BAT monoclonal antibody (a murine mAb developed against a membrane preparation of a Burkitt lymphoma cell line), from which pidilizumab is derived [67, 68].

In humans, the safety and tolerability of the single dose regimen were shown in a phase I study of patients with advanced hematologic malignancies [69]. No treatment-related toxicities occurred and the maximum tolerated dose was not identified in this trial (0.2–6 mg/kg).

Pidilizumab has been tested in phase II trials, as monotherapy for patients with diffuse large B-cell lymphoma after autologous hematopoietic stem-cell transplantation [70] and as combined therapy with rituximab for relapsed follicular lymphoma [71]. Both trials showed promising efficacies even in high-risk patients.

The results of a phase II trial in patients with pretreated advanced melanoma were recently reported. ORR was 5.9%, measured by immune-related response criteria (irRC), and the OS rate at 1 year was 64.5%. The patients who had been pretreated with ipilimumab (51% of patients) tended to experience a higher rate of immune-related stable disease (irSD) and longer PFS (2.8 mo versus 1.9 mo) [12].

3.3. Pembrolizumab (MK-3475, Formally Known as Lambrolizumab). Pembrolizumab (MK-3475) is a humanized monoclonal IgG-4κ antibody that blocks PD-1.

A phase I dose-escalation study evaluated three dose levels, 1 mg/kg, 3 mg/kg, and 10 mg/kg, administered every 2 weeks, in patients with multiple solid tumors [72]. All dose levels were found to be safe, and the maximum tolerated dose was not identified. Clinical responses were observed at all dose levels. Another phase I study tested 3 regimens (2 mg/kg every 3 weeks and 10 mg/kg every 2 or 3 weeks) in patients with advanced melanoma [13]. AEs were generally mild and grade 3 to 4 AEs were seen in 13% of patients. The ORRs ranged from 38% to 52%, in the biweekly 10 mg/kg cohort (measured by RECIST), showing no significant differences. These responses were durable, with the median PFS exceeding 7 months for all three regimens.

An ongoing phase II trial is now comparing 2 dose levels of pembrolizumab with investigator-choice chemotherapy in patients with previously treated advanced melanoma (NCT01704287). Another ongoing phase II trial is also evaluating 2 dose schedules of pembrolizumab (10 mg/kg q2w or q3w) compared with ipilimumab (3 mg/kg q3w) for advanced melanoma (NCT01866319).

In April 2013, pembrolizumab received the Breakthrough Therapy designation for advanced melanoma from the FDA. After being reviewed under the FDA's Accelerated Approval program, in September 2014, pembrolizumab received approval for treatment of patients with advanced melanoma by the FDA.

Besides melanoma, several early trials have showed the tolerability and antitumor effects of pembrolizumab in other tumors. The preliminary results of another phase I study evaluating pembrolizumab in untreated PD-L1-positive NSCLC were recently reported. The overall objective response rate was 25% (33% in the 2 mg/kg q3w, 20% in the 10 mg/kg q3w, and 31% in the 10 mg/kg q2w group), as measured by RECIST. AEs were generally mild and grade 3 to 4 AEs occurred in 3 patients, including pneumonitis requiring treatment discontinuation [14]. Another preliminary result was reported for the phase I trial of pembrolizumab as monotherapy, administered at 2 mg/kg every 2 weeks, to 60 patients with recurrent/metastatic head and neck cancers. Grade 3 to 4 drug-related AEs were reported in 16.7% of patients. The best ORR was 20% in all patients (assessed by RECIST 1.1). Efficacies were comparable between human papilloma virus- (HPV-) positive and HPV-negative patients (20.0% versus 19.4%) [15]. Another phase I study (NCT01848834) assessed pembrolizumab in the patients with previously treated advanced gastric cancer that expressed PD-L1. The enrolled 39 patients were treated with pembrolizumab at 10 mg/kg q2w. Median follow-up period was 6 months. Treatment-related AEs occurred in 24 patients (61.5%), and those of grade 3 to 5 occurred in 3 patients (pneumonitis, peripheral neuropathy, and hypoxia). ORR was 30.8% and disease control rate was 43.6%. Responses were mostly ongoing and the median response duration was not reached [16].

4. Anti-PD-L1 Antibodies

PD-L1 (also known as B7-H1 or CD274) and PD-L2 (also known as B7-DC or CD273) are inhibitory B7-family molecules that bind the PD-1 receptor. PD-L1 is inducibly expressed on a variety of hematopoietic and nonhematopoietic cells, including most human tumor cells and cells within the tumor microenvironment [61]. PD-L1 expression has been shown to correlate inversely with the clinical outcomes of some malignancies. PD-L2 is expressed on hematopoietic cells. PD-L1 knockout mice show infiltration of lymphocytes into nonlymphoid organs and exacerbation of preexisting autoimmune diseases [73, 74].

As mentioned above, the PD-1-PD-L1 axis is one of the main mechanisms by which cancer cells evade immune-cell attack [61]. Blockade of this pathway was shown to reinforce antitumor immune activities [62]. Because PD-L1 also interacts with CD80 [75, 76], anti-PD-L1 antibody might have optimal clinical potency against PD-1.

4.1. BMS-936559. BMS-936559 is a fully humanized IgG4 monoclonal anti-PD-L1 antibody. It inhibits the binding of PD-L1 to PD-1 and CD80. A phase I dose-escalation study evaluated BMS-936559 in 207 patients with selected cancers, including melanoma, NSCLC, ovarian cancer, and renal cell carcinoma. The study drug was administered at 4 dose levels (0.3–10 mg/kg) every 14 days, 3 times in each 6-week course for up to 16 cycles, when either CR or disease progression was confirmed. The ORRs were 6–17% and efficacy was durable

(>1 year in 8 of 16 patients who responded). Grade 3 to 4 irAEs, seen in 9% of the patients, were treatment-related in 5% [17].

4.2. MPDL3280A. MPDL3280A is a humanized IgG-1κ monoclonal anti-PD-L1 antibody. It is genetically engineered to modify the Fc domain, thereby impairing the antibody-dependent cellular cytotoxicity of PD-L1 expressing cells [77, 78].

A phase I trial of MPDL3280A as monotherapy for advanced melanoma achieved a response rate of 26% and PFS of 35% at 24 weeks. Grade 3 to 4 AEs were seen in 33% of patients [79]. The results of another phase I trial were recently reported. MPDL3280A was tested in patients with pretreated metastatic urothelial bladder cancer. ORR in PD-L1-positive patients was superior to that in PD-L1-negative patients (43% versus 11% at 6 weeks). ORR at 12 weeks was 52% in PD-L1-positive patients. Grade 3 to 4 AEs were seen in 4% of patients, with no irAEs [18]. The FDA has granted the Breakthrough Therapy designation to MPDL3280A.

4.3. MEDI4736. MEDI4736 is a humanized IgG-1κ monoclonal antibody that blocks PD-L1. MEDI4736 demonstrated tumor regression and improved survival in a mouse model.

A "first-time-in-human" phase I study evaluating the safety, tolerability, and pharmacokinetics of this agent in patients with advanced solid tumors is currently underway (NCT01693562). The interim report was recently presented. As of January 2014, 26 patients were receiving dose-escalation treatments and had been given a median of 5 (1–25) q2w and 4.5 (1–7) q3w doses of MEDI4736 across 6 cohorts (0.1–10 mg/kg q2w; 15 mg/kg q3w). No dose limiting toxicities (DLTs) or maximum tolerated dose was identified. Treatment-related AEs occurred in 34% of patients, but all were grade 1 to 2 and did not lead totreatment discontinuation. Four of the 26 patients showed partial responses (PRs). The rate (PR + stable disease $\geqq$ 12 weeks) was 46%. Clinical responses were durable, with 11 patients remaining in the study (2+ to 14.9+ months) [19]. Another phase I trial is now testing the combination of MEDI4736 plus tremelimumab (NCT01975831).

4.4. MSB0010718C. MSB0010718 is a fully humanized IgG1 monoclonal antibody directed to PD-L1. A phase I trial is currently testing MSB0010718 to assess its safety, tolerability, and pharmacokinetics in patients with refractory malignancies (NCT01772004). As of January 2014, 27 patients had been enrolled and were participating in a dose-escalation study (3 + 3 design; 1, 3, 10, and 20 mg/kg, q2w). Twenty-three patients had been followed for at least 4 weeks. Discontinuation of the treatment had been necessary in 12 patients (52.2%): 9 (39.1%) due to progression of disease, 2 (8.7%) for AEs, and 1 (4.3%) because the patient died. Grade 3 to 4 drug-related toxicities included laboratory abnormalities in 3 patients. One DLT was observed in 1 patient at dose level 4 (20 mg/kg): an irAE with creatine kinase elevation, myositis, and myocarditis [20].

5. Combination Therapy

Recent clinical trials have actively investigated the potential for synergistic effects by combining immune checkpoint inhibitors with other agents. The partner agents/therapies include other checkpoint agents, cytotoxic agents, anticancer vaccines, cytokines, and radiotherapy.

A phase I study evaluated combined therapy with ipilimumab plus nivolumab in patients with advanced melanoma [80]. The patients received ipilimumab once every 3 weeks for 4 doses and nivolumab once every 3 weeks for 8 doses concurrently. Then, eligible patients were permitted to receive both once every 12 weeks up to 8 doses. Grade 3 to 4 treatment-related AEs were seen in 53% of the concurrent-cohort patients but were mild and manageable. The maximum tolerated dose was 3 mg/kg of ipilimumab and 1 mg/kg of nivolumab, a dosing regimen at which 53% of patients showed responses. Recent follow-up surveys confirmed OS to be 94% at 1 year and 88% at 2 years in this cohort. An expansion cohort, with the patients receiving 3 mg/kg of ipilimumab and 1 mg/kg of nivolumab every 3 weeks for 4 doses and 1 mg/kg of nivolumab every 2 weeks until disease progression, is currently being evaluated in a phase II/III study [81]. A phase III trial (NCT01844505) evaluating this combination is currently ongoing (recruitment has been completed).

6. Biomarkers for Predicting Clinical Benefits and Adverse Reactions

Although immune checkpoint inhibitors have shown promising safety and efficacy, to date only a small proportion of patients have achieved long-term survival, with severe irAEs occurring on occasion. Biomarkers predicting clinical benefit may enable physicians to select individualized treatments for their patents and thereby maximize clinical benefits. Thus, there is an urgent need to identify "baseline (pretreatment)" biomarkers predicting responses or toxicities. Several biomarkers for examining T-cell proliferation or activation and other forms of antigen-specific immunity have been assessed in the context of immune checkpoint inhibitors.

Immunohistochemical PD-L1 expression in a tumor specimen is among the potential markers for PD-1-PD-L1-directed therapies. In a phase I study of nivolumab, though the data obtained are preliminary, an objective response was seen only in the patients who showed immunohistochemical PD-L1 expression in pretreatment tumor specimens [63]. These observations may support the strategy of selecting PD-L1-positive patients for therapy. However, PD-L1 expression on tumor cells is inducible and is susceptible to influences of the tumor microenvironment. Furthermore, technical advances in PD-L1 immunostaining are still needed. Also, the value of PD-L1 IHC staining as a predictive biomarker for combination therapy with nivolumab plus ipilimumab has yet to be validated [80]. As yet, the applicability and significance of PD-L1 expression as a baseline biomarker must be interpreted with caution and further prospective evaluations are needed, including the results of ongoing randomized

clinical trials that are prospectively evaluating PD-L1 IHC as a companion diagnostic platform (NCT01721746).

Another potential biomarker is pretreatment levels of monocytic myeloid-derived suppressor cells (m-MDSCs) [82, 83]. A recent retrospective study suggested higher pretreatment quantities of $Lin^{-}CD14^{+}HLA\text{-}DR^{low/-}$ m-MDSC to be associated with inferior OS in patients with metastatic melanoma treated with ipilimumab [83].

Recent genetic analysis using whole-exome sequencing showed the significance of somatic mutational load as predictive biomarker of clinical benefit in melanoma patients treated with CTLA-4 blockade. The neopeptide signature associated with clinical response was identified and predicted mutant peptides were verified to activate patient T cell *in vitro* [84].

Other potential predictive/prognostic biomarkers include the gene expression profiles obtained employing tumor biopsies [85, 86], CRP level [87], absolute lymphocyte and eosinophil counts [88], and LDH levels [89]. These possibilities await further research.

7. Conclusion

Immune checkpoint inhibitors have opened a new era of cancer immunotherapy. Since the FDA approval was obtained for the anti-CTLA-4 monoclonal antibody ipilimumab, several large-scale clinical trials have evaluated new agents both alone and in combinations with other conventional or new therapies. Future challenges include exploring new target molecules and immune cells, optimizing dosing regimens and combination therapies, validating the safety and efficacy of these novel treatment strategies in many other malignancies, establishing an immunomonitoring system to be applied during therapy, and identifying biomarkers predicting clinical responses and toxicities. Active, ongoing investigations are anticipated to provide further clinical benefits for patients with cancers that are currently refractory to treatment.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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