have demonstrated that cancer vaccines might sometimes show worse clinical outcomes.[6,7] Therefore, it would be critical to identify biomarkers that accurately portray anti-tumor immune responses and predict prognosis in treated patients.[3,6] With regard to post-vaccination biomarkers, several factors, including CTL responses, Th1 responses, delayed-type hypersensitivity (DTH) and autoimmunity, have been reported to be associated with clinical responses in some clinical trials.[20-23] However, as they have not been always reproducible in other studies, there are currently no validated prognostic or predictive biomarkers in widespread use.

We also investigated immunological biomarkers in 500 advanced cancer patients who received PPV from October 2000 to October 2008.[16] By the statistical analysis in this patient population, both lymphocyte counts prior to the vaccination ($p = 0.0095$) and increased IgG responses ($p = 0.0116$) to the vaccine peptides, along with performance status ($p < 0.0001$), were well correlated with overall survival.

To identify biomarkers useful for selecting appropriate patients before vaccination, we further addressed pre-vaccination prognostic markers in patients with several different types of advanced cancers who underwent PPV. In CRPC treated with PPV ($n = 40$), a comprehensive study of soluble factors and gene expression profiles by microarray analysis demonstrated that higher IL-6 level and granulocytic myeloid-derived suppressor cells (MDSC) in the peripheral blood before vaccination were closely associated with poorer prognosis.[24] In patients with refractory non-small cell lung cancer ($n = 41$), multivariate Cox regression analyses showed that higher C-reactive protein (CRP) level before vaccination was a significant predictor of unfavorable overall survival (HR = 10.115, 95% CI = 2.447–41.806, $p = 0.001$).[25] In addition, in refractory biliary tract cancer patients ($n = 28$), higher IL-6 and lower albumin levels before vaccination were significantly unfavorable factors for overall survival [HR = 1.123, 95% CI = 1.008–1.252, $p = 0.035$; HR = 0.158, 95% CI = 0.029–0.860, $p = 0.033$; respectively].[26] Collectively, these findings have demonstrated that less inflammation may contribute to better responses to PPV, suggesting that evaluation of the inflammatory factors before vaccination could be useful for selecting appropriate cancer patients for PPV. Based on these findings, an early phase clinical trial is currently underway to show whether the blockage of IL-6-mediated inflammatory signaling with a humanized anti-IL-6 receptor monoclonal antibody, tocilizumab, would be beneficial for enhancing the immune and/or clinical responses of PPV.[27]

Conclusions

The field of cancer immunotherapy has drastically moved forward during the past 20 years, but there have been several issues to be addressed for success of cancer vaccine development. In view of complexity and diversity of immunological characters of tumors and immune cell repertoires, we have developed a new concept of PPV. In the clinical trials conducted during the past several years, we have shown promising results of PPV as a new treatment modality for patients with various types of advanced cancers. Further randomized phase III clinical trials would be essential to prove clinical benefits of PPV. In addition, novel biomarkers for selecting patients who would most benefit from PPV remain to be identified.

Disclosure of Potential Conflicts of Interest

The authors have no conflict of interest and financial relationships to disclose.

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Review Article

Next-generation peptide vaccines for advanced cancer

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Many clinical trials of peptide vaccines have been carried out since the first clinical trial of a melanoma antigen gene-1-derived peptide-based vaccine was reported in 1995. The earlier generations of peptide vaccines were composed of one to several human leukocyte antigen class I-restricted CTL-epitope peptides of a single human leukocyte antigen type. Currently, various types of next-generation peptide vaccines are under development. In this review, we focus on the clinical trials of the following categories of peptide vaccines mainly published from 2008 to 2012: (i) multivalent long peptide vaccines; (ii) multi-peptide vaccines consisting of CTL- and helper-epitopes; (iii) peptide cocktail vaccines; (iv) hybrid peptide vaccines; (v) personalized peptide vaccines; and (vi) peptide-pulsed dendritic cell vaccines. (*Cancer Sci* 2013; 104: 15–21)

A cDNA-expression cloning technique to identify genes and peptides of tumor-associated antigens was first reported by van der Bruggen *et al.* in 1991.[1] Subsequently, a technique using autologous antibodies was introduced for identification of genes and peptides recognized by the host immune system.[2] These advanced techniques have provided a large number of antigens and peptides applicable as cancer vaccines. Many clinical trials of peptide vaccines have been carried out since the first clinical trial of a melanoma antigen gene-1 (MAGE-1)-derived peptide-based vaccine was reported in 1996 by Hu *et al.*[3] The earlier generations of peptide vaccines were composed of one to several human leukocyte antigen (HLA)-class I-restricted peptides of a single HLA-type. The peptides were emulsified with Montanide ISA51, a clinical grade of Freund's incomplete adjuvant, or pulsed on antigen-presenting cells and used for vaccination. Various types of new generation peptide vaccines have since been developed (Figs 1,2). In this review, we discuss the recent clinical trials of the latest generation of peptide-based cancer vaccines mainly published from 2008 to 2012.

Multivalent long peptide vaccines

The classical types of peptide vaccines only contain one to several epitope peptides, which are recognized by CTLs or helper T cells. In contrast, the mother proteins of the peptide vaccines usually contain several HLA-type restricted epitopes recognized by both CTLs and helper T cells. Although the importance of helper T cells in the induction of CTLs has been established and protein vaccines are able to induce both CTLs and helper T cells, the protein vaccines have several demerits in terms of manufacturing and safety controls. To avoid these drawbacks, synthetic long peptide vaccines have been developed. Synthetic long peptide vaccines are predominantly taken up by antigen presenting cells (APCs), where they are processed for presentation by both MHC class I and II molecules.

Several clinical studies using mixes of synthetic long peptides have been reported, as mixes of synthetic long peptide are likely to contain multiple HLA class I and II T-cell epitopes, which allows the use of this type of peptide vaccine in all patients irrespective of the type of HLA of each patient. Kenter *et al.*[4] carried out a phase I study of high-risk type human papilloma virus (HPV) 16 E6 and E7 overlapping long peptides in end-stage cervical cancer patients. Cocktails of nine E6 peptides and/or four E7 peptides, each 25–35-mer, covering the entire sequences of E6 and E7 proteins, were given s.c. with Montanide ISA51 four times at 3-week intervals. Co-injection of E6 and E7 long peptides induced a strong and broad T-cell response dominated by immunity against E6. Subsequently, they carried out a phase II study of this vaccine in patients with HPV-positive grade 3 vulvar intraepithelial neoplasia.[5] Vulvar intraepithelial neoplasia is a chronic disorder caused by HPV 16. At 3 months after the last vaccination, 12 of 20 patients (60%) had clinical responses and reported relief of symptoms. Five women had complete regression of the lesions. At 12 months of follow-up, 15 of 19 patients (79%) had clinical responses with a complete response in 9 of 19 patients (47%).

A synthetic long peptide vaccine targeted for p53 was reported by Speetjens *et al.*[6] The p53 synthetic long peptide vaccine consisted of 10 synthetic 25–30-mer long overlapping peptides, spanning amino acids 70–248 of the wild type p53 protein. Ten patients with metastatic colorectal cancer were vaccinated with this vaccine. The p53-specific T cell responses were induced in 9 of 10 patients as measured by γ-interferon (IFN-γ). Subsequently, a phase II study of a p53 synthetic long overlapping peptide vaccine in patients with ovarian cancer was carried out by the same group.[7] Twenty patients with recurrent elevation of CA-125 were immunized with the vaccine. Stable disease, as determined by CA-125 levels and computed tomography scans, was observed in 2/20 (10%) patients as the best clinical response, but no relationship was found with vaccine-induced immunity. Interferon-γ-producing p53-specific T-cell responses were induced in all patients who received all four immunizations. Interestingly, the IFN-γ secreted cells were CD4 T-cells and no CD8 T-cell/CTL responses were detected. The absence of CD8 T-cell/CTL responses may be attributable to the dominant production of

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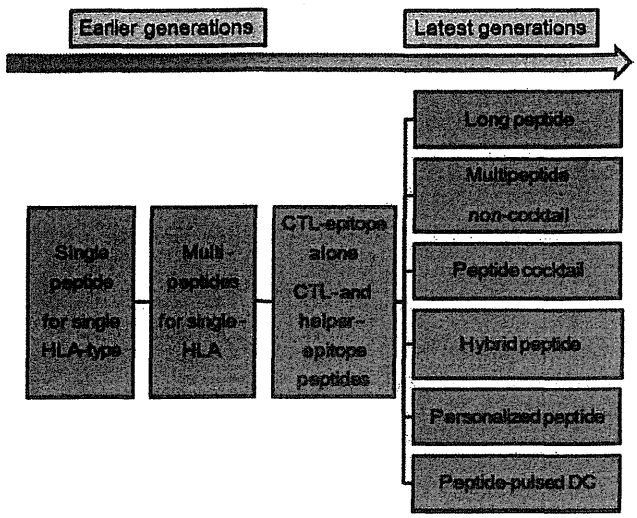

Fig. 1. Transition of peptide vaccine development for advanced cancer. DC, dendritic cells.

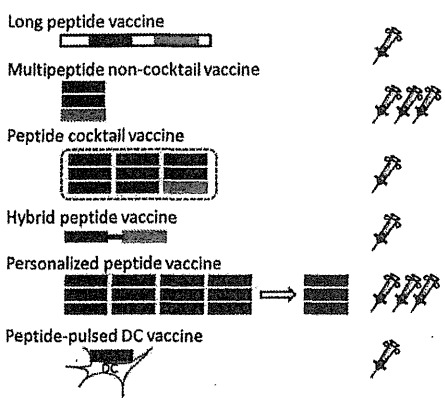

Fig. 2. Various types of latest generation peptide vaccines. The number of syringes indicates that of the final preparation for injection. Green, CTL-epitopes; orange, helper-epitopes. DC, dendritic cells.

Th2 cytokines, whose inhibitory effects on CTL induction are well known, although the vaccine immunization resulted in the expansion of p53-specific Th1 and Th2 CD4 T-cell responses.

Kakimi *et al.*[8] carried out a phase I trial of an NY-ESO-1 synthetic long peptide vaccine. A 20-mer NY-ESO-1f peptide, which includes multiple epitopes recognized by antibodies, and CD4 and CD8 cells, was given along with OK-432 and Montanide ISA51 to patients with advanced cancers. Both CD4 and CD8 T cell responses, as well as NY-ESO-1 antibody, were increased or induced in 9 of 10 patients.

Multipeptide vaccines consisting of CTL- and helper-epitopes

As mentioned above, helper T cells play crucial roles in the induction of CTLs. Some of the latest generation of peptide vaccines consist of HLA class-II restricted helper epitope peptides recognized by CD4 T cells in addition to class-I restricted CTL-epitope peptides to induce both CTLs and helper T cells. Numerous helper epitopes had been identified from the same target molecules of CTL-epitope vaccines and co-used as cancer vaccines.[9–17] A helper epitope peptide

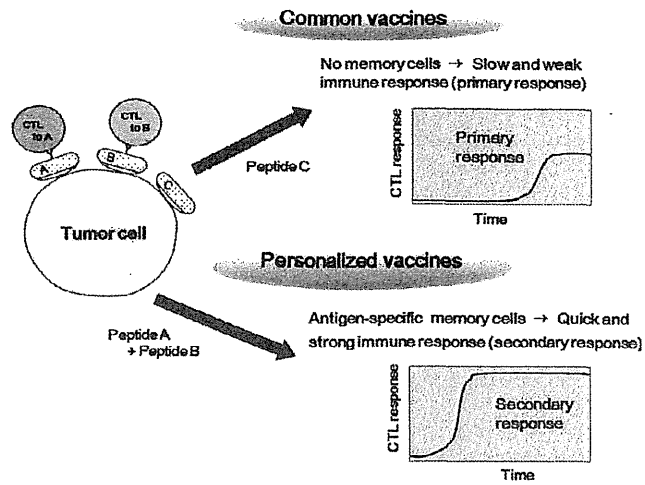

Fig. 3. Personalized peptide vaccine. In the classical type of vaccine, peptides derived from tumor-specific or overexpressed antigens are used as vaccine peptides and often mismatched to the pre-existing immunity of patients. In personalized peptide vaccines, appropriate peptides for vaccination are screened and selected from a panel of vaccine candidates in each patient, based on pre-existing host immunity and HLA types.

capable of binding pan HLA-DR (pan-DR epitope [PADRE]) has been reported,[18] and a clinical trial of a peptide vaccine using this helper epitope was reported. Kuball *et al.*[15] carried out a phase I study of CTL-epitope peptides of Wilms' tumor gene, proteinase 3, and mucin 1, and PADRE or mucin 1-helper epitope peptide with Montanide ISA51 and CpG oligonucleotide. Each peptide was formulated independently of the others and injected at a separate site. An increase in PADRE-specific CD4 T cells was observed after vaccination but these appeared unable to produce interleukin 2 (IL2), and the regulatory T cells were increased. This study indicates that helper epitope peptides have the potential to induce both helper T cells and regulatory T cells.

Peptide cocktail vaccines

Different peptides have different binding affinities to the corresponding HLA molecules. Therefore, if different CTL-epitope peptides with different binding affinities are loaded to APCs, there may be competition among the individual peptides to bind HLA molecules on the APCs. To prevent this, individual peptides of multipeptide vaccines were formulated independently of each other and injected at separate sites in most of the former clinical trials. In our case, a maximum of four peptides were individually mixed with Montanide ISA51 and injected s.c. at different sites on the same day. The maximum number of four peptides was similar to the maximum acceptable number of doses for patients on the same day, and no more than five peptides were used for vaccination. One of the strategies for overcoming the limitation of peptide number is the use of multipeptide cocktail vaccines. The multipeptide cocktail vaccines have no limitation of peptide number, as one preparation can contain more than 10 peptides. However, the issue of competition between the individual peptides of a cocktail vaccine for the binding of HLA molecules on the APCs still remains.

Different types of multipeptide cocktail vaccines have been developed, that is, vaccines consisting of CTL-epitope peptides alone,[19–21] or CTL-epitope and helper-epitope peptides.[9–13,16,17] The number of component peptides in the cocktail vaccines varies from around four to more than 10. Barve

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Table 1. Immunological and clinical responses to personalized peptide vaccines for advanced cancer

Disease status	Phase	HLA restriction	Total no. of patients	Humoral response (%)	Cellular response (%)	Clinical response (%)	MST (months)	Grade 3/4 toxicities	Ref. no.
Advanced CRPC	PI	A24	10	60	40	SD 50	Not ref.	0	31
Advanced CRPC	PI	A24	13	91	55	PR 63	24	G3, 5%	32
Advanced CRPC	PI	A2	10	70	40	SD 30	22	0	33
Advanced CRPC	PI/II	A24	16	50	71	PR 43	17	0	37
Advanced CRPC	PI/II	A2/A24	58	88	78	PR 24	17	G3, 7%	38
Localized PC	PII	A24	10	80	80	PR 20	Not ref.	0	39
Advanced CRPC	PI, extension	A24	15	47	67	PR 13	24	0	46
Advanced CRPC	PII, randomized	A2/A24	57	64	50	PFS 8.5 (vaccine) vs 2.8M (control)	22.4 (vaccine) vs 16.1M (control)	0	44
Advanced CRPC	PII	A2/A24/ A3sup/A26	42	44	34	PR 12	17.8	0	49
Advanced malignant glioma	PI	A2/A24	21	40–64	50–82	PR 24, SD 38	Not reached	0	36
Advanced glioblastoma multiforme	PI, extension	A24	12	17	75	PR 17, SD 42	10.6	0	47
Advanced corolectal cancer	PI	A24	10	70	50	PR 10	Not ref.	0	34
Advanced corolectal cancer	PI/II	A2/A24	7	71	57	SD 14	Not ref.	G3, 20%	40
Advanced pancreatic cancer	PI	A2/A24	13	69	69	PR 15, SD 54	7.6	0	41
Non-resectable pancreatic cancer	PII	A2/A24	21	72	78	PR 33, SD 43	9	0	45
Advanced gastric cancer	PI	A2/A24	13	80	50	SD 45	Not ref.	0	30
Advanced lung cancer	PI	A24	10	40	40	SD 80	15.2	0	29
Refractory SCLC	PII	A2/A24/ A3sup/A26	10	83	83	SD 20	6.2	G3, 4%	50
Refractory NSCLC	PII	A2/A24/ A3sup/A26	41	49	34	SD 56	10.1	G3, 7%	42
Metastatic RCC	PI	A2/A24	10	80	5	SD 60	23	0	43
Malignant melanoma	PI	A2/A24	7	57	86	SD 43	Not ref.	0	28
Recurrent gynecologic cancer	PI	A2/A24	14	86	85	SD 36	Not ref.	G3, 8%	35
Advanced urotherial cancer	PI	A2/A24	10	80	80	CR 10, PR 10	24	0	48

A3sup, A3 super type; CR, complete response; CRPC, castration-resistant prostate cancer; G3, grade 3; HLA, human leukocyte antigen; M, months; MST, median survival time; Not ref., not referred; NSCLC, non-small-cell lung cancer; PI, phase I clinical trial; PII, phase II clinical trial; PC, prostate cancer; PD, progressive disease; PFS, progression-free survival; PR, partial response; RCC, renal cell carcinoma; Ref., reference; SCLC, small-cell lung cancer; SD, stable disease.

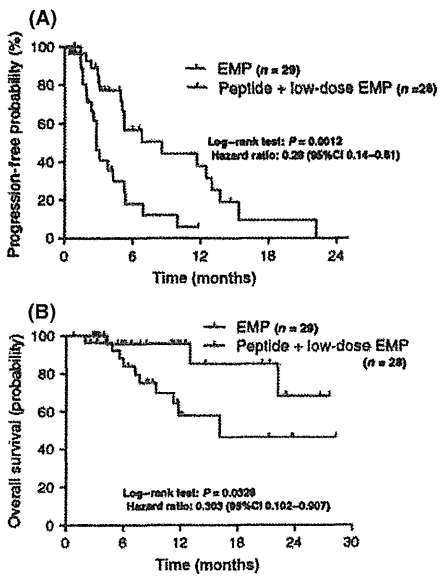

Fig. 4. Randomized phase II trial of personalized peptide vaccine (PPV) plus low-dose estramustine phosphate (EMP) versus standard-dose EMP in patients with castration-resistant prostate cancer. Patients were randomized into groups receiving either PPV plus low-dose EMP (280 mg/day) or standard-dose EMP (560 mg/day). (A) Duration of progression-free survival in the first treatment. (B) Overall survival of patients treated with PPV plus low-dose EMP and standard-dose EMP. CI, confidence interval.

et al.[9] carried out a phase I/II study of a cocktail vaccine IDM-2101 consisting of nine CTL-epitope peptides and the PADRE helper-epitope peptide with Montanide ISA51 in patients with metastatic non-small-cell lung cancer. No significant adverse events were noted except for low-grade erythema and pain at the injection site. One-year survival in the treated patients was 60%, and median overall survival was 17.3 months. One complete response case was observed in the total of 63 patients. Feyerabend and colleagues reported cocktail vaccines for patients with prostate cancer.[12] The cocktail vaccine consisted of 13 synthetic peptides, 11 HLA-A*0201 restricted CTL epitopes and two helper epitopes derived from prostate tumor antigens. A phase I/II trial of the vaccine was carried out in HLA-A2-positive patients with hormone-sensitive prostate cancer with biochemical recurrence after primary surgical treatment. The same group also developed another cocktail vaccine for renal cell cancer.[17] The vaccine, IMA901, consisted of nine HLA-A*0201 restricted CTL-epitopes and one helper epitope from renal cell cancer antigens with hepatitis B virus epitope as a marker peptide. A randomized phase II trial with a single dose of cyclophosphamide reduced the number of regulatory T cells and confirmed that immune responses to the vaccine component peptides were associated with longer overall survival.

Hybrid peptide vaccines

Peptide sequences of most of the single epitope vaccines as well as multi-epitope long peptide vaccines are native sequences with or without modification of anchor amino acids. Some of the latest generation of peptide vaccines are of hybrid-type, that is, a peptide fused with two epitopes. The Ii-Key/HER-2/neu hybrid peptide vaccine is a fusion peptide made up of the Ii-Key 4-mer peptide and human epidermal growth factor receptor-2 (HER-2)/neu (776–790) helper epitope peptide.[22,23] The Ii protein catalyzes direct charging

Table 2. Pros and cons of the latest generation of peptide vaccines

	Pros						Cons				
Vaccine type	Induction of CTL	Induction of Th	Applicable for multi-HLA type	Activation of memory T-cells	High efficiency of antigen presentation	Synthetic chemicals	No induction of Th	Possible induction of Treg	Not applicable for multi-HLA type	Multi formula	Induction of primary response	Biologics
Long peptide vaccine	Yes	Yes	Yes	No	No	Yes	No	Yes	No	No	Yes	No
Multipeptide non-cocktail vaccine	Yes	Yes	Yes	No	No	Yes	No	Yes	No	Yes	Yes	No
Peptide cocktail vaccine	Yes	Yes	Yes	No	No	Yes	No	Yes	No	No	Yes	No
Hybrid peptide vaccine	Yes	Yes		No	No	Yes	No	Yes	Yes	No	Yes	No
Personalized peptide vaccine	Yes	No	Yes	Yes	No	Yes	Yes	No	No	Yes	No	No
Peptide-pulsed DC vaccine	Yes	No	No	No	Yes	No	Yes/No	No	Yes	No	Yes	Yes

DC, dendritic cell; HLA, human leukocyte antigen; Th, helper T-cells; Treg, regulatory T-cells.

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of MHC class II epitopes to the peptide-binding groove, circumventing the need for intracellular epitope processing, and the shortest active sequence of the Ii protein is the Ii/Key peptide.[24] Holmes *et al.*[22] and Perez *et al.* [23] reported the results of phase I studies of the Ii-Key/HER -2/neu hybrid peptide vaccine in patients with prostate cancer. Significant decreases in circulating regulatory T cell frequencies, plasma HER-2/neu, and serum transforming growth factor-β levels were observed when compared with the native HER-2/neu (776–790) peptide vaccination.

Takahashi and colleagues developed a hybrid peptide of a helper-epitope and CTL-epitope of MAGE-A4.[25] The phase I study of the vaccine was carried out in patients with advanced cancers who were vaccinated with MAGE-A4-H/K-HELP combined with OK432 and Montanide ISA51. In a case report, there were no severe side-effects except for a skin reaction at the injection site. The vaccine induced MAGE-A4-specific Th1 and Tc1 immune responses and the production of MAGE-A4-specific complement-fixing IgG antibodies. Tumor growth and the carcinoembryonic antigen tumor marker were significantly decreased in the final diagnosis.

Personalized peptide vaccines

Virtually all prevaccination patients already have a weak immunity to cancer cells. However, the characteristics of cancer cells and of the immunological status against cancers differ widely among patients, even among those with the same histological types of cancer and identical HLA types. One of the reasons for the low clinical efficacies of the earlier generations of peptide vaccines might be a mismatch between the vaccine peptides and pre-existing immunity to the cancer cells. We therefore attempted to optimize the vaccine peptides so that they were appropriately matched to the pre-existing immunity of each patient (Fig. 3). There are two ways to detect pre-existing immunity, detection of CTL-precursors and detection of IgG in the peripheral blood. The PBMCs were cultured with vaccine peptide panels and the CTL responses to each peptide were measured. The second method is to detect IgG antibodies to the vaccine peptide panels. It is well known that the production of the IgG class of antibodies requires T-cell help. Therefore, the presence of a specific IgG indicates the presence of helper T cells. We carried out a series of clinical trials using personalized peptide vaccines (PPVs) for advanced cancer patients.[26–50] In this PPV formulation, appropriate peptide antigens for vaccination are screened and selected from a panel of vaccine candidates in each patient, based on pre-existing host immunity as mentioned above. Currently, we use 31 HLA class I-restricted peptide candidates, which were identified from a variety of tumor-associated antigens mainly through the cDNA expression cloning method with tumor-infiltrating T-lymphocyte lines, 12 peptides for HLA-A2, 14 peptides for HLA-A24, 9 peptides for HLA-A3 supertype (A3, A11, A31, or A33), and 4 peptides for HLA-A26. The safety and potential immunological effects of these vaccine candidates have been shown in previous clinical studies.[26,27] A maximum of four peptides, which were selected based on the results of HLA typing and the pre-existing immune responses specific to each of the 31 different vaccine candidates, were injected s.c. with Montanide ISA51 weekly or bi-weekly.

Currently, we evaluate the pre-existing immune responses to vaccine candidates by B cell responses, but not by T cell responses, as the performance characteristics, such as the sensitivity and reproducibility, of the current T cell assays are far from satisfactory. In contrast to these drawbacks inherent to T cell assays, B cell assays have more potential for screening and/or monitoring antigen-specific immune responses even to HLA class I-restricted peptides. For example, we have recently published several papers describing the clear correlations between clinical benefits and antigen-specific B cell responses measured by IgG antibody production in patient plasma after vaccination. Notably, the multiplex bead-based Luminex technology that we have developed for monitoring B cell responses allow simple, quick, and highly reproducible high-throughput screening of IgG responses specific to large numbers of peptide antigens with a tiny amount of plasma.

In the clinical trials of PPV carried out during the past decade, we have shown promising results in various types of cancers.[26–50] Table 1 shows the summary of the immunological and clinical responses in 460 advanced cancer patients who received PPV. The best clinical responses assessed in the 436 evaluable patients were a partial response in 43 patients (10%), stable disease in 144 patients (33%), and progressive disease in 249 patients (57%), with a median overall survival of 9.9 months. Of note, a recent phase II randomized clinical trial of PPV for 57 castration-resistant prostate cancer patients showed that patients receiving PPV in combination with low-dose estramustine phosphate (EMP) showed a significantly longer progression-free (median survival time, 8.5 months *vs* 2.8 months; hazard ratio, 0.28 [95% confidence interval, 0.14–0.61]; $P = 0.0012$) and overall survival (median survival time, undefined *vs* 16.1 months; hazard ratio, 0.30 [95% confidence interval, 0.1–0.91]; $P = 0.0328$) than those receiving standard-dose EMP alone, suggesting the feasibility of this combination therapy (Fig. 4).[44] In addition, PPV was also used in an early phase clinical trial of patients with recurrent or progressive glioblastoma multiforme, one of the most aggressive brain tumors, with a median overall survival of 10.6 months.[47] Based on these promising results, randomized phase III trials are currently underway in glioblastoma. To prove the clinical benefits of PPV for accelerating cancer vaccine development, further randomized phase III trials would also be recommended in other types of cancers.

Peptide-pulsed dendritic cell vaccines

Many clinical trials of dendritic cell (DC)-based vaccinations using autologous DC and tumor-associated antigen peptides have been carried out to assess the ability of these vaccines to induce clinical responses in cancer patients.[51–54] Rahma *et al.*[54] carried out a comparative study of DC-based vaccine versus non-DC-based authentic peptide vaccine. Twenty-one advanced ovarian cancer patients were divided two groups: arm A received a p53 CTL-epitope peptide with Montanide with IL2; arm B received the same peptide-pulsed DCs with IL2. The median progression-free survival and overall survival were 4.2 (arm A) *i* 8.7 (arm B) months and 40.8 (arm A) versus 29.6 (arm B) months, respectively. This study suggests that the simple peptide vaccination and labor-consuming DC-based vaccination therapy are similarly effective.

Conclusion

Many investigators have attempted to develop more effective cancer vaccines, and in this review we discussed the resulting progress in the latest generation of peptide vaccines. The pros and cons of each type of vaccine are shown in Table 2. Each study used different adjuvants, cytokines, and/or other combination therapies with different doses. Moreover, the individual peptides themselves had different immunological and clinical potency as well as different amino acid sequences. Therefore, it is very hard to conclude that one type of vaccine was more efficient than another. The role of immune checkpoint molecules, such as CTLA-4 and programmed cell death-1, on antitumor immunity was clarified, and promising results have been reported in the clinical trials using combination therapies

with peptide vaccines and immune checkpoint blockades.[55–57] Further randomized phase III trials would be essential to prove the clinical benefits of these vaccine therapies, including immune checkpoint blockade combination therapies.

Disclosure Statement

The author Akira Yamada is an Executive Officer for Green Peptide Company, Ltd.

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Advantages of high *b*-value diffusion-weighted imaging to diagnose pseudo-responses in patients with recurrent glioma after bevacizumab treatment

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ABSTRACT

Background: The diagnosis of pseudo-responses after bevacizumab treatment is difficult. Because diffusion-weighted imaging (DWI) is associated with cell density, it may facilitate the differentiation between true- and pseudo-responses. Furthermore, as high *b*-value DWI is even more sensitive to diffusion, it has been reported to be diagnostically useful in various clinical settings.

Materials and methods: Between September 2008 and May 2011, 10 patients (5 males, 5 females; age range 6–65 years) with recurrent glioma were treated with bevacizumab. All underwent pre- and post-treatment MRI including T2- or FLAIR imaging, post-gadolinium contrast T1-weighted imaging, and DWI with *b*-1000 and *b*-4000. Response rates were evaluated by MacDonald- and by response assessment in neuro-oncology working group (RANO) criteria. We also assessed the response rate by calculating the size of high intensity areas using high *b*-value diffusion-weighted criteria. Prognostic factors were evaluated using Kaplan–Meier survival curves (log-rank test).

Results: It was easier to identify pseudo-responses with RANO- than MacDonald criteria, however the reduction of edema by bevacizumab rendered the early diagnosis of tumor progression difficult by RANO criteria. In some patients with recurrent glioma treated with bevacizumab, high *b*-value diffusion-weighted criteria did, while MacDonald- and RANO criteria did not identify pseudo-responses at an early point after the start of therapy.

Discussion and conclusion: High *b*-value DWI reflects cell density more accurately than regular *b*-value DWI. Our findings suggest that in patients with recurrent glioma, high *b*-value diffusion-weighted criteria are useful for the differentiation between pseudo- and true responses to treatment with bevacizumab.

1. Introduction

Glioblastoma is the most common malignant primary neoplasm of the central nervous system. Despite aggressive treatment, it almost always recurs with fatal consequences. As vascular endothelial growth factor (VEGF) and its receptors are highly expressed in glioblastoma, VEGF may constitute an important molecular target in its treatment. VEGF increases vascular permeability and contributes to contrast enhancement and the peritumoral edema associated with these tumors. Anti-angiogenic agents, especially those targeting VEGF such as bevacizumab, can significantly reduce vascular permeability. This results in diminution of the enhanced lesion irrespective of changes in the tumor size. Therefore, it is very difficult to determine the responder status of glioma patients treated with bevacizumab on conventional MR images and some tumors thought to have responded to bevacizumab therapy exhibit progression without manifesting an increase in the size of the gadolinium-enhanced tumor. This phenomenon, defined as a "pseudo-response", has been observed immediately after the start of treatment and renders the accurate assessment of a true tumor response difficult [1–3]. Emerging evidence of survival prolongation in patients who responded to bevacizumab [4] suggests that it exerts antitumor effects. Reliable means to assess the treatment response and the progression of these tumors addressed with anti-angiogenic agents must be developed.

The response assessment based on neuro-oncology working group (RANO) criteria takes into account increases in the enhanced tumor size, the T2/FLAIR high size, the dose of corticosteroids, and clinical symptoms. Using RANO criteria, it may be possible to identify tumor progression after treatment with bevacizumab because post-treatment the non-enhanced tumor area tends to increase without an increase in the size of the enhanced tumor. This may

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also be reflected by an increase in the size of the T2/FLAIR high-intense lesion. On the other hand, as treatment with bevacizumab may reduce the size of brain edema, it may be difficult to distinguish between true- and pseudo-response at an early point after treatment with bevacizumab.

As the apparent diffusion coefficient (ADC) calculated from diffusion-weighted (DW) images is associated with tumor cellularity [5], it is considered an important biomarker of cancer [6,7]. The ADC has also been used to assess the response of brain tumors to therapy [7] and to predict survival in patients with newly diagnosed glioblastoma [8]. DWI studies at higher diffusion gradient strength (*b*-values) have been used for the diagnosis of acute stroke [9], the assessment of lesion-to-normal contrast in neurodegenerative diseases [10], the prediction of the glioma grade [11], and for the differentiation between glioblastoma and malignant lymphoma [12]. The aim of this study was to evaluate whether RANO criteria and DW imaging including high *b*-value DW (HBDW) imaging could assess the pseudo-response after treatment with bevacizumab. Here we show that HBDW imaging may represent a useful tool for the diagnosis of pseudo-responses in glioblastoma patients treated with bevacizumab.

2. Materials and methods

2.1. Patients and MR imaging

Between September 2008 and May 2011, 10 patients (5 males, 5 females; age range 6–65 years) with recurrent glioma were treated with bevacizumab in our institutions. Recurrence before the administration of bevacizumab was defined by MacDonald criteria [13].

All MRI studies were performed on a 3 T superconducting system (Signa Excite HD 3.0T; GE Medical Systems, Milwaukee, WI, USA). All patients underwent pre- and post-treatment magnetic resonance (MR) imaging including T2- (TR 4800 ms, TE 100 ms, echo train length 18, field-of-view (FOV) 22 cm × 22 cm, matrix size 512 × 320/2NEX, section thickness 6 mm, intersection gap 1.0 mm, 1 acquisition) or FLAIR imaging (TR 10,000 ms, TE 140.0 ms, inversion recovery time 2400.0 ms, FOV 22 cm × 22 cm, matrix size 288 × 160/1NEX, section thickness 6 mm, intersection gap 1.0 mm, 2 acquisitions), gadolinium-enhanced T1-weighted imaging (TR 450 ms, TE 18 ms, FOV 22 cm × 22 cm, matrix size 256 × 192/1NEX, section thickness 6 mm, intersection gap 1.0 mm, 2 acquisitions), and DW imaging at $b = 1000$ and $b = 4000$ s/mm. The parameters at *b*-1000 and *b*-4000 DWI were: 8-channel phased array head coil, TR 5000 ms, TE 66.2 ms (*b*-1000) and TR 5000 ms and TE 96.4 ms (*b*-4000), NEX 1, FOV 220, slice thickness 6 mm, gap 1.0 mm, number of slices 20, data acquisition matrix 128 × 128, scan time 20 s (*b*-1000) and 40 s (*b*-4000).

2.2. Response after treatment with bevacizumab

The response rate was determined using 3 different methods. Under MacDonald criteria [13], the enhanced tumor size was calculated and defined as complete response (CR = disappearance of all enhanced target lesions), partial response (PR = a 50% decrease from the baseline), stable disease (SD = neither PR- nor progressive disease (PD) criteria are met), PD (a 25% increase over the smallest sum recorded or the appearance of new lesions), the clinical assessment and corticosteroid dose were also recorded. Under the criteria of the response assessment in neuro-oncology (RANO) working group [2], factors such as enhanced tumor size, T2/FLAIR high size, dose of corticosteroids, and clinical symptoms were taken into account. At visual inspection, HBDW (*b*-4000) imaging was superior to regular *b*-value based (*b*-1000) DW imaging for the assessment of size changes of high-intense lesions. Therefore, under the third method we calculated the size of the high-intense lesion on HBDW images using its two dimensional measurements and established HBDW criteria where CR = disappearance of all high intensity lesions on HBDW images, PR = a 50% decrease from the baseline of high intensity lesions observed on HBDW images, SD = neither PR nor PD criteria are met, PD = a 25% increase over the smallest sum recorded or the appearance of new DW high lesions on HBDW images.

2.3. Statistical analysis

Statistical analyses were with PRISM version 5.0 (GraphPad Software Inc., La Jolla, CA, USA). The survival time of patients with recurrent glioma was measured from the time of initial treatment with bevacizumab to the time of death or last follow-up. To evaluate prognostic values we performed Kaplan–Meier survival analysis (log-rank test) that incorporated the response to bevacizumab based on MacDonald-, RANO-, or HBDW criteria.

3. Results

Table 1 presents a summary of our patients. Their age ranged from 6 to 65 years (mean 42.5 years, median 40 years). Based on MacDonald criteria, the initial response rate was CR, $n = 4$; PR, $n = 4$; SD, $n = 1$; PD, $n = 1$; under RANO criteria it was CR, $n = 2$; PR, $n = 3$; SD, $n = 3$; PD, $n = 2$, and under HBDW criteria, the initial response rate was PR, $n = 3$; SD, $n = 3$; PD, $n = 4$ patients.

After bevacizumab administration, the enhanced lesion disappeared in 5 tumors and based on MacDonald criteria CR was recorded. In 3 patients there was a decrease in the size of both the T2/FLAIR- and the HBDW high intense lesion; based on RANO and HBDW criteria, PR was recorded (Fig. 1, case 5). These patients are currently alive without recurrence and their treatment with bevacizumab continues.

In some patients the high intensity lesion on T2/FLAIR- and HBDW images increased (Fig. 2, case 1). They were categorized as PD under RANO or HBDW criteria. After continued treatment with bevacizumab, they were recorded as PD. In some patients there was a decrease in the size of the T2/FLAIR high intense lesion after one cycle of bevacizumab. However, in 2 patients the high intense lesion became larger on HBDW images (Fig. 3, case 2) and based on RANO criteria PD was recorded after the continuation of bevacizumab treatment.

We performed Kaplan–Meier survival analysis based on the response rate determined by MacDonald-, RANO-, and HBDW criteria. Under MacDonald criteria we observed no statistical difference between CR/PR- and SD/PD patients (Fig. 4A). Under RANO criteria there was a statistical difference between CR/PR- and SD/PD patients ($p = 0.0153$, Fig. 4B) and under HBDW criteria the difference was more obvious and CR/PR patients survived longer ($p = 0.0152$, Fig. 4C).

4. Discussion

Our study documents that in patients with recurrent glioblastoma, DWI is the superior imaging technique for the diagnosis of pseudo-responses and that HBDW imaging is particularly advantageous. We also show that RANO- is superior to MacDonald criteria because the size of non-enhanced tumors increases after bevacizumab treatment. On the other hand, as the strong effect of bevacizumab against brain edema may produce a decrease in the T2/FLAIR high intense area, this may hide the extension of the tumor area shown as an increase in the T2/FLAIR high intense area. HBDW imaging clearly demonstrated the extent of the tumor area at an early time point after the start of treatment with bevacizumab.

Table 1
Summary of patients treated with bevacizumab.

Case	Age	Gender	Disease	After 1 cycle			After 2–5 cycles			Overall survival	
				MacDonald criteria	RANO criteria	HBDW criteria	MacDonald criteria	RANO criteria	HBDW criteria	Months	Current status
1	47	F	Glioblastoma rec.	CR	PD	PD	PD	PD	PD	5.0	Dead
2	42	F	Glioblastoma rec.	PR	PR	PD	PD	PD	PD	3.3	Dead
3	65	M	Glioblastoma rec.	SD	SD	SD	PD	PD	PD	6.1	Dead
4	45	M	Glioblastoma rec.	CR	CR	PR	CR	CR	PR	19.1	Alive
5	37	M	Glioblastoma rec.	PR	PR	PR	CR	CR	PR	5.8	Alive
6	43	M	Anaplastic astrocytoma rec.	CR	CR	PR	CR	CR	CR	30.2	Alive
7	65	F	Anaplastic astrocytoma rec.	PR	PR	PD	SD	SD	PD	6.2	Dead
8	13	F	PNET rec.	PD	PD	PD	PD	PD	PD	3.1	Dead
9	37	M	Pontine glioma rec.	CR	SD	SD	PD	PD	PD	2.3	Dead
10	6	F	Pontine glioma rec.	PR	SD	SD	PD	PD	PD	2.0	Dead

RANO, response assessment in neuro-oncology working group; HBDW, high *b*-value diffusion-weighted; rec., recurrence; PNET, primitive neuroectodermal tumor; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

There is an apparent increase in the tendency for infiltrating tumor progression after anti-angiogenic treatment; this is discernible on T2-weighted- and FLAIR images [14]. This may be attributable to the recruitment of existing blood vessels. Under RANO criteria, the most recent response criteria for glioma, T2/FLAIR images are taken into account [2]. However, other factors, e.g. post-irradiation- and/or postoperative changes, chemotherapy, tumor infiltration, and tumor-induced edema, may produce changes on T2-weighted- or FLAIR images, pointing to the need for clear response criteria.

Newer imaging techniques such as PET, MR spectroscopy, and perfusion- and diffusion-weighted imaging that provide functional information may be more reliable in the assessment of tumor activity during anti-angiogenic treatment [2]. Tumor-cell density decreases if treatment is effective. On the other hand, ineffective treatment or tumor recurrence results in increased tumor-cell density and the size of the high cell density area increases due to an increase in the tumor size [7].

Ours is the first documentation that HBDW (*b*-4000) imaging at MRI more effectively distinguishes between pseudo- and true responses than other MRI techniques including standard *b*-1000 DW imaging. HBDW imaging has been found to be useful for the diagnosis of acute infarction [9], degenerative diseases [10], for glioma grading [11], and for the differentiation between glioblastoma and malignant lymphoma [12]. Preclinical studies using HBDW support our findings that HBDW may be useful for the early detection of responses to chemotherapy [15]. At HBDW there is more contrast at the tissues of interest than at regular *b* value imaging [16] and it has been reported that there are slow- and fast diffusion components that correspond with intra- and extracellular

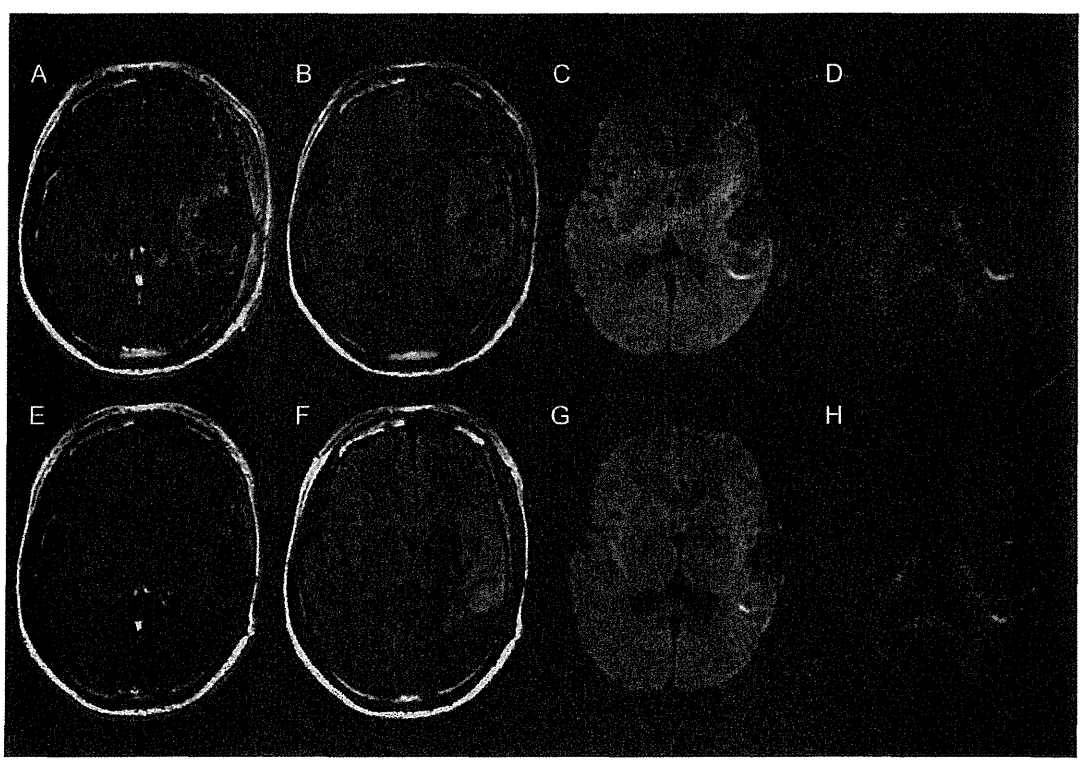

Fig. 1. This 37-year-old man with glioblastoma underwent radical surgery and radiotherapy with concomitant and adjuvant chemotherapy with temozolomide. Gadolinium (Gd)-enhanced T1-weighted- (A) and FLAIR images (B), DW images at *b*-1000 s/mm^2 (DWI_{1000}) (C) and at *b*-4000 DWI (DWI_{4000}) (D) were acquired before treatment with bevacizumab. Gd-enhanced T1-weighted images showed a marked decrease in the enhanced lesion (E). On FLAIR images (F), DWI_{1000} (G), and DWI_{4000} (H) the high intensity area was decreased. At present, 6 months post-treatment, there has been no tumor recurrence and he continues to receive bevacizumab treatment.

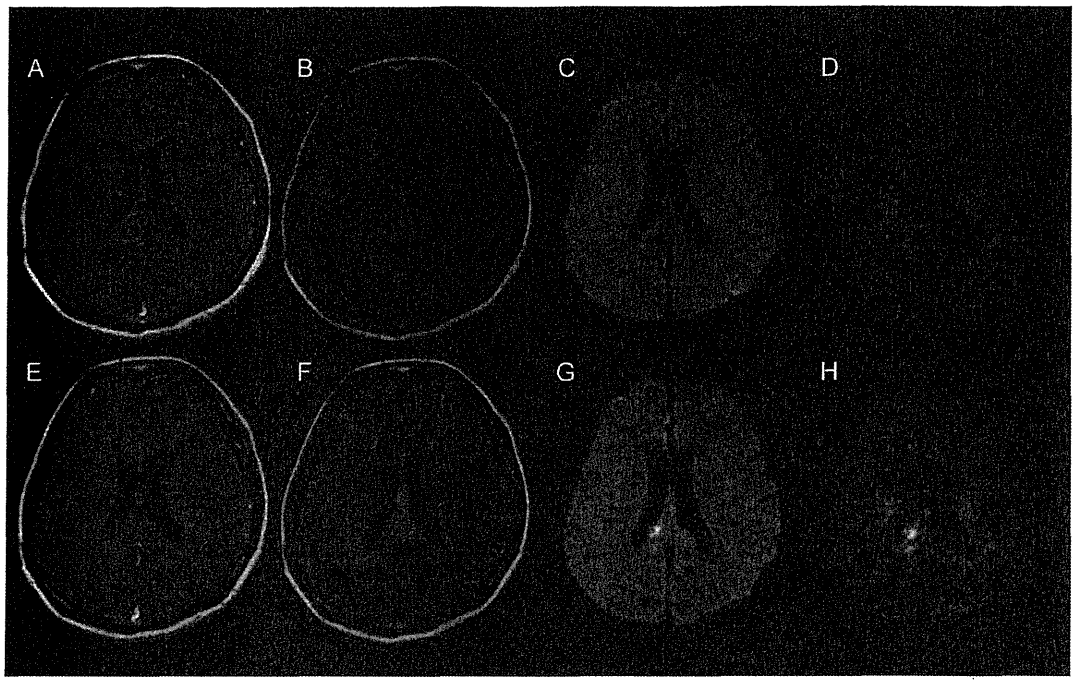

Fig. 2. This 47-year-old woman with glioblastoma underwent radical surgery and radiotherapy with concomitant and adjuvant chemotherapy with temozolomide. Gd-enhanced T1-weighted- (A) and FLAIR images (B) and DWI_{1000} (C) and DWI_{4000} (D) were acquired before treatment with bevacizumab. Gd-enhanced T1-weighted images showed a marked decrease in the enhanced lesion (E). On FLAIR images (F) and DWI_{1000} (G) and DWI_{4000} (H) the high-intensity area was increased. She died of tumor recurrence 5 months after the first course of bevacizumab.

diffusion, respectively [17,18]. Studies on multi-component diffusion in brain tissue demonstrated that the slow component is more sensitive at HBDW- than regular *b*-value DW imaging, suggesting that the ADC based on higher *b*-values reflects changes in tumor cellularity more accurately [12]. In fact, Doskaliyev et al. [12] found that the ADC is inversely associated with tumor cellularity and that this correlation is stronger with the ADC obtained at HBDW (*b*-4000) than regular b value DW (*b*-1000) imaging.

Calculation of the ADC should also be considered for the assessment of the tumor response. However, the ADC is associated with tumor cellularity but not with the tumor size. A decrease in cellularity after effective treatment would result in an increase in the ADC.

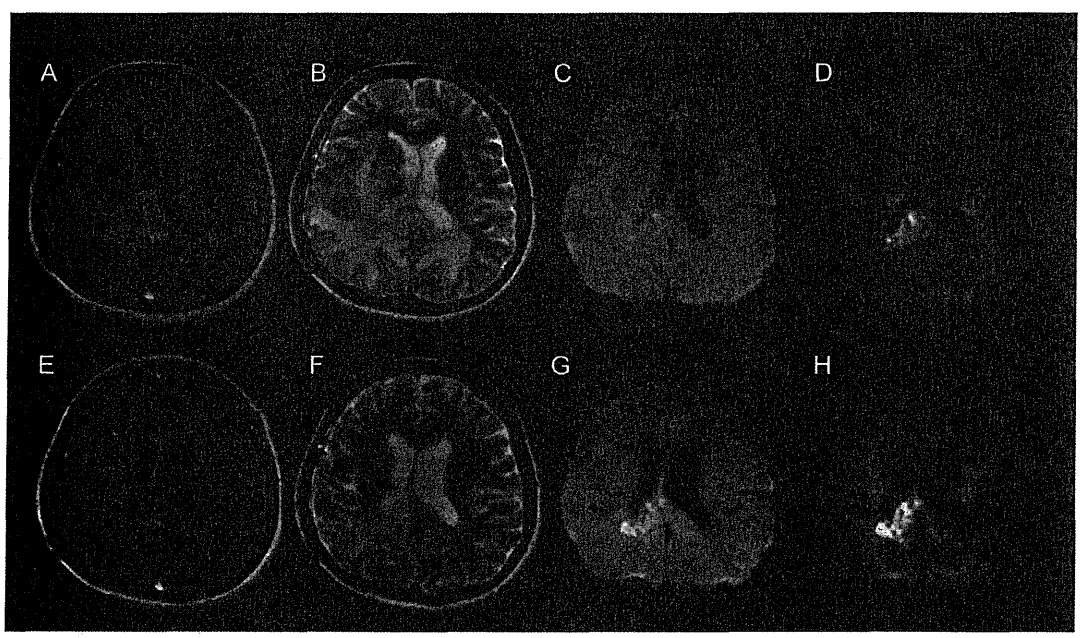

Fig. 3. This 42-year-old woman with glioblastoma underwent radical surgery and radiotherapy with concomitant and adjuvant chemotherapy with temozolomide. Gd-enhanced T1-weighted- (A) and T2-weighted images (B) and DWI_{1000} (C) and DWI_{4000} (D) were acquired before bevacizumab treatment. Gd-enhanced T1-weighted images showed a marked decrease in the enhanced lesion (E). On T2-weighted images we noted a marked decrease in the high intensity area, a decrease in the mass effect, and improvement in the midline shift (F). DWI_{1000} showed an increase in the high intensity area (G). On DWI_{4000} there was an obvious increase in the high-intensity area (H). She died of tumor recurrence 3 months after first course of bevacizumab.

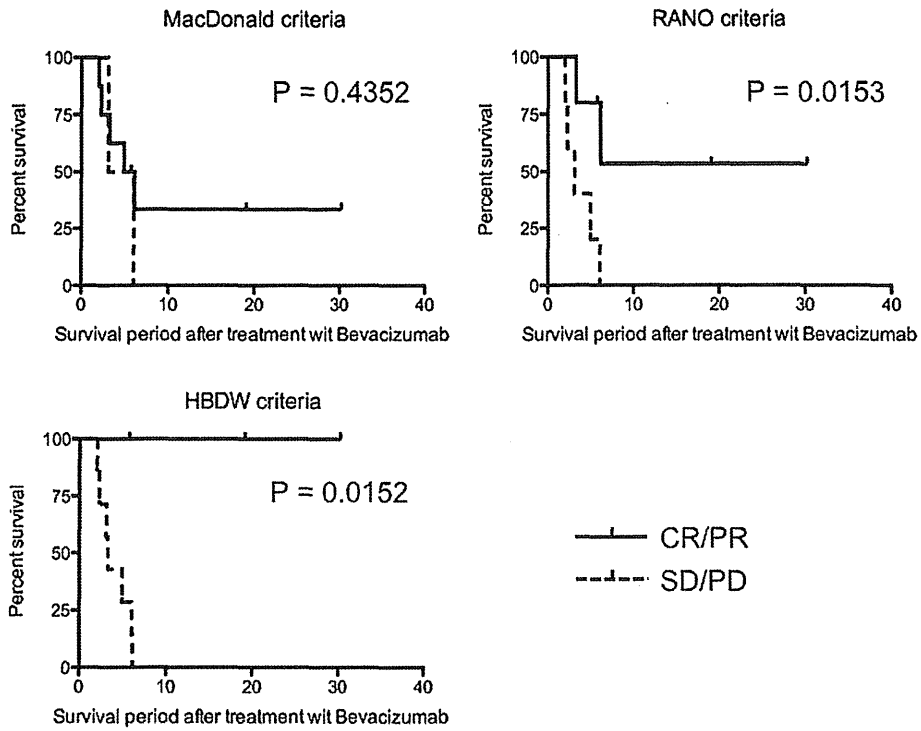

Fig. 4. Kaplan–Meier survival curves of all patients with recurrent glioma treated with bevacizumab (including 3 who remain alive) show the relationship between the evaluation criteria and the survival time measured from the date of surgery. Comparisons were between patients with complete response (CR)/partial response (PR) and stable disease (SD)/progressive disease (PD) using MacDonald criteria (A), between CR/PR and SD/PD using RANO criteria (B), and between CR/PR and SD/PD using DWI criteria.

However, calculation of the ADC at identical tumor sites before and after treatment is difficult. Moreover, it would be difficult to interpret changes in the ADC without evaluating the size of the tumor. In addition, use of the terms hyper- and hypocellularity instead of decreasing ADC and increasing ADC may be misleading since many pathologies and clinical scenarios affect ADC measurements.

The quantification of diffusion changes has evolved from the mean change in the ADC to a voxel-by-voxel approach termed the functional diffusion map (fDM) [7], a statistical method that prospectively compares heterogeneous ADC maps acquired after the start of therapy with pretreatment ADC maps. The two image data sets are co-registered and computationally analyzed to yield statistical maps of ADC changes as color overlays on anatomical images and to provide scatter plots of ADC changes to determine the tumor response in patients with brain tumors. Such information makes it possible to tailor treatments based on an early imaging biomarker readout in cases where an insufficient response is predicted. The fDM was proposed as an MRI biomarker for the quantification of the early brain tumor response to therapy [19].

Although the fDM approach is promising, technical and clinical challenges must be addressed [20]. First, the proper alignment of sequential images on baseline images is difficult but critical. A significant mass effect from tumor growth or intracranial pressure induced by edema may skew the registration between DW imaging datasets. Suspected tumor regions near gyri, sulci, or the ventricles may return false results due to misregistration effects. The proper choice of the *b*-value used for an accurate estimation of the ADC is an important aspect of fDM implementation that must be addressed. Also, the use of *b*-values greater than 1500 s/mm^2 results in a multi-exponential signal decay that may render a single estimate of the ADC inappropriate. Additional studies are necessary to confirm the usefulness of the fDM approach.

Our preliminary study has some limitations. First, we must consider that acute occlusion of the tumor vessels may produce high intensity on DW images. Time-course observations and monitoring of ADC changes may help to identify pseudo-responses. Second, although HBDW (*b*-4000) imaging was superior to regular *b* value-based (*b*-1000) DW imaging for the assessment of pseudo-response at visual inspection, HBDW imaging had the disadvantage of an inferior signal/noise ratio, and it may be possible to assess pseudo-responses by regular *b*-value DWI. Quantitative analysis that includes the tumor ADC value or the fDM approach is necessary to confirm the advantage of HBDW. In addition, the optimal *b*-value has not been determined. Third, tumors with lower cellularity and tumors with micro-necroses or micro-cysts may not show high intensity on HBDW images. Fourth, in our study the patients' age and the tumor histology were inhomogeneous. Fifth, the 6-mm slice thickness we used may be too high for an accurate assessment of the character of the lesion. Thinner slices may make it possible to characterize the lesions more accurately. Sixth, our study population was small and prospective studies on large patient populations, studies to identify the optimal *b*-value, and studies that include quantitative approach are necessary.

In conclusion, HBDW criteria could identify a pseudo-response earlier than RANO criteria. We presented evidence that HBDW imaging may represent a biomarker in glioma patients subjected to anti-angiogenic therapy.

Conflict of interest

None.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejrad.2011.10.018.

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Intracerebral Hemorrhage Despite Prophylactic Administration of Vitamin K in Infants

—Two Case Reports—

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Abstract

The incidence of vitamin K deficiency in infancy has decreased markedly, due to prophylactic administration of vitamin K during the neonatal period. However, vitamin K deficiency bleeding may occur during or after the neonatal period despite prophylactic administration in Japan. Two cases are reported of intracranial hemorrhage associated with coagulopathy in full-term infants who had received prophylactic administration of vitamin K. More reliable methods for prophylactic administration should be established.

Key words: infant, intracerebral hemorrhage, vitamin K, prophylactic administration, late type

Introduction

Vitamin K deficiency bleeding in infancy is a well-known risk factor for intracerebral hemorrhage in infants.[2] Prophylactic administration of vitamin K during the neonatal period is widespread and the prevalence of vitamin K deficiency has decreased markedly.[5,13] Vitamin K deficiency bleeding can be classified into three types according to the time of occurrence: Early type within the first 24 hours after birth, classic type between the 1st and 7th days, and late type between the 7th day and 6th month. Late type vitamin K deficiency bleeding is the most important, as intracranial hemorrhage (30–60%) can result, leading to high mortality (19.9–50%) and morbidity rates.[1,6,11,12] In contrast, the early and classic types tend to be less common causes of intracranial hemorrhage. Intracranial hemorrhage is observed as subarachnoid hemorrhage (50.0–85.7%), subdural hematoma (48.8–50.0%), intracerebral hemorrhage (42.9–58.3%), or intraventricular hemorrhage (10.7–41.6%). Multiple bleeding is observed in 66.6–69% of all hemorrhages.[1,7] We describe 2 cases of intracranial hemorrhage associated with vitamin K deficiency despite prophylactic administration of vitamin K, and discuss the causes of bleeding, and the treatment and prevention of vitamin K deficiency in infants.

Case Reports

Case 1: A 48-day-old boy was admitted to our hospital with a 4-day history of vomiting and poor feeding. The history given by the parents was consistent and no suspicion of abuse or non-accidental injury was raised. He was born at 39 weeks of gestation by spontaneous vaginal delivery, weighing 2518 g. The delivery was uncomplicated. He received oral prophylaxis with 2 mg of vitamin K on the day of birth, and again at ages 5 days and 1 month. He was breast-fed. Family history showed no obvious hemorrhagic diathesis and the mother was not taking any medication. On admission, neurological examination revealed the infant was somnolent, with tension at the anterior fontanel. Jaundice was evident. Computed tomography (CT) showed intraventricular, left parietal intracortical hemorrhage, and communicating hydrocephalus (Fig. 1). No mild bleeding or signs of cholestasis had occurred before intracranial hemorrhage. Laboratory studies revealed normal platelet count. However, blood coagulation studies indicated marked prolongation of pro-

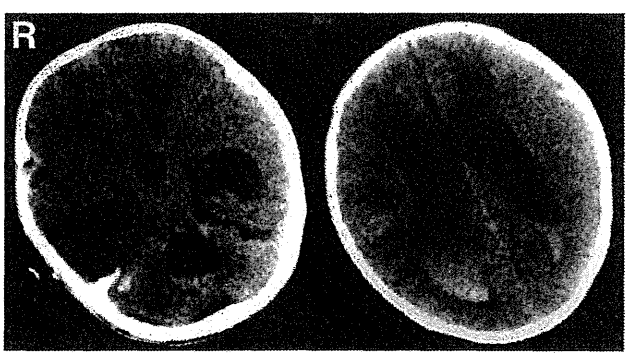

Fig. 1 Case 1. Preoperative computed tomography scans showing intraventricular, left parietal intracortical hemorrhage, and communicating hydrocephalus.

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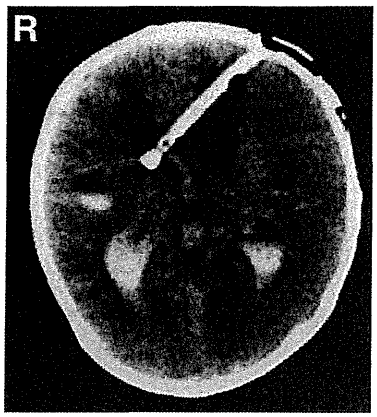

Fig. 2 Case 1. Postoperative computed tomography scan showing improvement in hydrocephalus, but an extensive ischemic lesion in the left hemisphere.

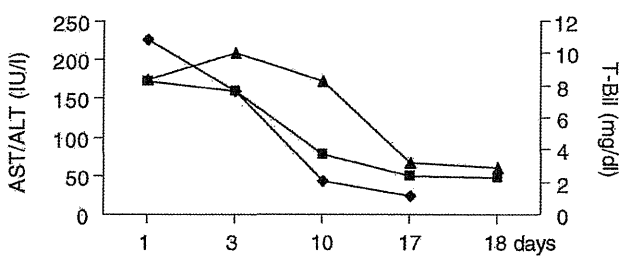

Fig. 3 Case 1. Liver function markers. ■: aspartate aminotransferase (AST), ▲: alanine aminotransferase (ALT), ◆: total bilirubin (T-Bil).

thrombin time (PT) and activated partial thromboplastin time (APTT). Coagulation activity was below the limits of detection, and protein-induced vitamin K absence or antagonist II (PIVKA-II) level was >75,000 mAU/ml (normal <40 mAU/ml). Liver function tests showed: total bilirubin, 10.9 mg/dl (normal 0.3–5.3 mg/dl); aspartate aminotransferase, 172 IU/l (normal 22–73 IU/l); alanine aminotransferase, 175 IU/l (normal 11–53 IU/l); γ-glutamyl transpeptidase (γ-GTP), 109 IU/l (normal 20–111 IU/ml); and alkaline phosphatase (ALP), 1493 IU/l (normal 469–1495 IU/ml). Vitamin K deficiency bleeding was suspected from the prolonged PT and APTT and elevated PIVKA-II, so vitamin K was administered. PT and APTT normalized and an extraventricular drainage catheter was placed. CT showed improvements in the hydrocephalus after placement of the catheter, but an extensive ischemic lesion was detected in the left hemisphere (Fig. 2). Eventually, the hydrocephalus required ventriculoperitoneal shunt placement. Neonatal hepatitis, biliary atresia, and inborn error of metabolism were considered as differential diagnoses for the causes of liver dysfunction. Cytomegalovirus and other virus tests yielded negative results. Abdominal echography showed no findings indicative of hepatitis or biliary atresia. Screening for inborn errors of metabolism also showed negative findings. Postoperative course was uneventful, and spontaneous improvements in liver dysfunction were seen without treatment (Fig. 3). The infant was discharged with slight right hemiparesis.

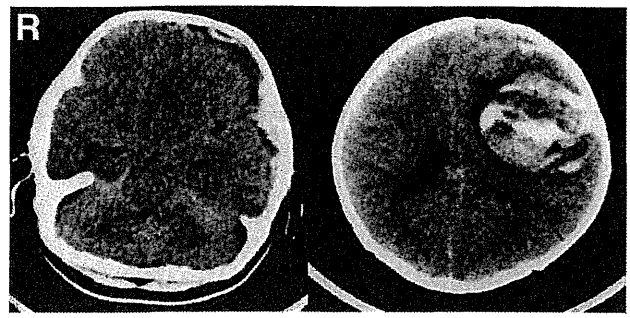

Fig. 4 Case 2. Preoperative computed tomography scans showing left frontal intracerebral hemorrhage with subdural hematoma.

Case 2: A 60-day-old boy gradually became comatose over the course of 6 hours and was admitted to our hospital as an emergency. He had been born at 40 weeks of gestation by spontaneous vaginal delivery, weighing 3690 g. The delivery was uncomplicated. Vitamin K was administered transorally on the day of birth, and at ages 5 and 31 days. He was breast-fed and had a history of jaundice of the newborn. He also had a history of neonatal melena at age 1 month. However, he had not previously been brought to the outpatient service. Family history showed no obvious hemorrhagic diathesis and the mother was not taking any medication. On admission, neurological examination revealed comatose status with anisocoric pupils. The anterior fontanel showed tension. CT showed left frontal intracerebral hemorrhage with subdural hematoma (Fig. 4). The history given by parents was consistent and no suspicion of abuse or non-accidental injury was raised. Laboratory studies revealed normal platelet count. However, blood coagulation studies indicated marked prolongation of PT and APTT. Coagulation activity was below the limits of detection and PIVKA-II level was >49,322 mAU/ml. Liver function tests showed: total bilirubin, 5.6 mg/dl; aspartate aminotransferase, 236 IU/l; alanine aminotransferase, 221 IU/l; γ-GTP, 92 IU/l; and ALP, 1978 IU/l. Vitamin K deficiency bleeding was suspected from the prolonged PT and APTT and elevated PIVKA-II, so administration of vitamin K was started. Left fronto-temporo-parietal craniotomy was performed urgently and the hematoma was removed. During the operation, PT and APTT were checked and found to have normalized. No vascular abnormalities were observed in the hematoma cavity. Postoperative CT showed evacuation of the hematoma (Fig. 5). Differential diagnoses for the causes of liver dysfunction included neonatal hepatitis, biliary atresia, and inborn error of metabolism. The results of cytomegalovirus and other virus tests were negative. No findings suggesting hepatitis or biliary atresia were observed on abdominal echography. Furthermore, screening for inborn errors of metabolism also yielded negative results. Postoperative course was uneventful and liver dys-

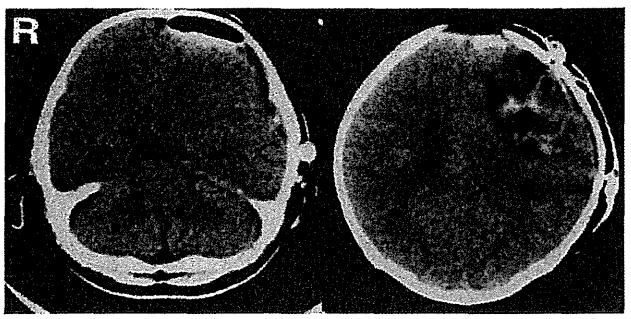

Fig. 5 Case 2. Postoperative computed tomography scans showing evacuation of the hematoma.

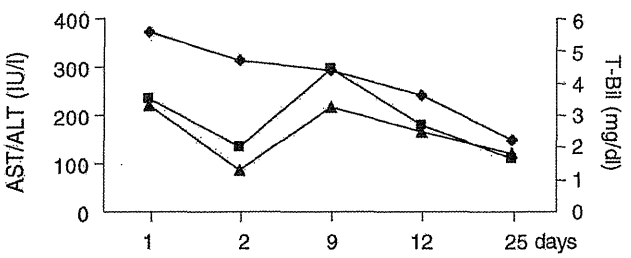

Fig. 6 Case 2. Liver function markers. ■: aspartate aminotransferase (AST), ▲: alanine aminotransferase (ALT), ◆: total bilirubin (T-Bil).

function spontaneously improved without treatment (Fig. 6). The patient was discharged without neurological deterioration.

Discussion

Late type vitamin K deficiency bleeding can be classified as idiopathic or secondary depending on the etiology. Causes of secondary bleeding are believed to originate in dysfunctional absorption of vitamin K.[1,17] This dysfunction is usually attributed to hepatic or intestinal disease, such as biliary atresia, cystic fibrosis, and α_1-antitrypsin deficiency. In a review of 131 reported cases of late type disease, 55 (42%) were associated with cholestatic disease,[5] including 22 cases of α_1-antitrypsin deficiency, 11 cases of biliary atresia, and 22 cases involving other forms of liver disorder.[18] Accordingly, secondary vitamin K deficiency contributing to liver disease must be suspected in patients with bleeding despite prophylactic administration of vitamin K, warranting detailed examinations to determine the primary disease. Our patients revealed liver dysfunction on admission, so various differential diagnoses for the causes of liver dysfunction were considered. Despite thorough investigations, the causes of liver dysfunction in these cases remained undetermined. However, we believe that an acquired disease, such as hepatitis, must have temporarily affected the absorption of vitamin K and resulted in late vitamin K deficiency bleeding, since liver dysfunction subsequently improved without treatment.

The American Stroke Association reviewed the literature on childhood stroke in 2008 and provided recommendations for treatment.[8] That report investigated non-traumatic hemorrhage as described, and recommended that children with severe coagulation factor deficiency should received appropriate coagulation factor replacement to prevent additional bleeding (Class I recommendations).[8] Therefore, vitamin K must be administered rapidly irrespective of the etiology in patients with vitamin K deficiency bleeding. Surgical treatment of supratentorial hematoma in infants is not recommended for most patients, based on evidence of surgical benefits.[8] However, surgery may help selected individuals with developing brain herniation or extremely elevated intracranial pressure (Class III recommendations).

Establishing prophylactic administration is another problem. In Japan, oral administration of 2 mg of vitamin K on the day of birth, and at 1 week and 1 month is recommended and widely applied.[6] As a result, the incidence of vitamin K deficiency in infancy has decreased markedly, particularly for the early and classic types in Japan, but late type vitamin K deficiency bleeding still occurs.[13] The 3 × 2-mg dose regime as used in Japan is associated with an incidence of late vitamin K deficiency bleeding of 0.44 per 100,000 births.[16] Therefore, this prophylactic regime cannot completely prevent the appearance of late vitamin K deficiency bleeding. A more reliable prophylactic regime is needed for patients with fat malabsorption/cholestasis, as these infants can be affected by late vitamin K deficiency bleeding. At present, no guidelines for methods of vitamin K prophylactic administration have been accepted worldwide. Administration method (oral/intramuscular), duration, and dose differ from country to country.[3,4,14,15,18] However, recent epidemiological studies have provided a more reliable prophylactic method. Comparison of the risks of vitamin K deficiency bleeding under different prophylactic regimens in infants with biliary atresia found that a daily dose of 25 μg of vitamin K failed to prevent bleeding, but weekly oral administration of 1 mg of vitamin K offered significantly higher protection to these infants and provided similar efficiency to 2 mg of intramuscular prophylaxis at birth. Neither of the prophylactic regimes of oral 1 mg provided weekly or 2 mg provided intramuscularly at birth were associated with intracranial hemorrhage.[14] No cases of vitamin K deficiency bleeding were encountered with weekly oral administration of 1 mg of vitamin K.[3,10,15] Late vitamin K deficiency bleeding with secondary etiology, as in our two patients, may be prevented using these prophylaxis regimens. Recommendations for prophylactic regimes should thus be revised based on these findings and a new plan is currently under consideration in Japan.[9]

We hope that more reliable methods of prophylactic administration will be established to resolve the problems associated with intracranial hemorrhage resulting from vitamin K deficiency.

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Phase II Study of Single-agent Bevacizumab in Japanese Patients with Recurrent Malignant Glioma†

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Objective: This single-arm, open-label, Phase II study evaluated the efficacy and safety of single-agent bevacizumab, a monoclonal antibody against vascular endothelial growth factor, in Japanese patients with recurrent malignant glioma.

Methods: Patients with histologically confirmed, measurable glioblastoma or World Health Organization Grade III glioma, previously treated with temozolomide plus radiotherapy, received 10 mg/kg bevacizumab intravenous infusion every 2 weeks. The primary endpoint was 6-month progression-free survival in the patients with recurrent glioblastoma.

Results: Of the 31 patients enrolled, 29 (93.5%) had glioblastoma and 2 (6.5%) had Grade III glioma. Eleven (35.5%) patients were receiving corticosteroids at baseline; 17 (54.8%) and 14 (45.2%) patients had experienced one or two relapses, respectively. The 6-month progression-free survival rate in the 29 patients with recurrent glioblastoma was 33.9% (90% confidence interval, 19.2–48.5) and the median progression-free survival was 3.3 months. The 1-year survival rate was 34.5% with a median overall survival of 10.5 months. There were eight responders (all partial responses) giving an objective response rate of 27.6%. The disease control rate was 79.3%. Eight of the 11 patients taking corticosteroids at baseline reduced their dose or discontinued corticosteroids during the study. Bevacizumab was well-tolerated and Grade $\geq$3 adverse events of special interest to bevacizumab were as follows: hypertension [3 (9.7%) patients], congestive heart failure [1 (3.2%) patient] and venous thromboembolism [1 (3.2%) patient]. One asymptomatic Grade 1 cerebral hemorrhage was observed, which resolved without treatment.

Conclusion: Single-agent bevacizumab provides clinical benefit for Japanese patients with recurrent glioblastoma.