Identification of EphB6 variant-derived epitope peptides recognized by cytotoxic T-lymphocytes from HLA-A24+ malignant glioma patients

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Abstract. We found previously that EphB6, a member of the erythropoietin-producing hepatocyte (Eph) receptor tyrosine kinase family, was preferentially expressed in malignant gliomas. In the present study, RT-PCR revealed a putative secretory variant form of human EphB6 that was expressed in the majority of glioma cell lines, though not in normal tissues. The variant has a unique 54 amino acid sequence that is not found in the normal EphB6. Therefore, we attempted to determine the antigenic peptides unique to the variant for immunotherapy. The two variant-derived peptides had the ability to bind to HLA-A2402 molecules and each of them could induce cytotoxic T-lymphocytes (CTLs) *in vitro* in peripheral blood mononuclear cells of HLA-A24+ glioma patients. Furthermore, the cytotoxicity was mediated by peptide-specific CD8+ T cells in an HLA-A24 restricted manner. Taken together, the two peptides derived from the variant of EphB6 might be appropriate targets for peptide-based specific immunotherapy to HLA-A24+ patients with malignant glioma.

Introduction

Despite advances in neurosurgery, radiation and chemotherapy, the prognosis for patients with malignant glioma is poor (1,2). Therefore, the development of new treatment modalities is needed. Recent advances in tumor immunology have led to the discovery of several cancer antigen peptides that are recognized by cancer-specific cytotoxic T-lymphocytes (CTLs) and that have been applied in clinical trials of cancer vaccines (3-6). However, the clinical effects of the vaccines are very limited (3,4). Therefore, the identification of new antigen peptides related to the malignant glioma is important for the development of specific immunotherapy for malignant glioma patients.

We attempted to identify new target antigens of malignant glioma using a cDNA microarray that enabled us to perform a comprehensive profiling of the gene expression levels and to compare those in malignant glioma tissues with those in benign glioma and normal brain tissues. As a result, we determined that the erythropoietin-producing hepatocyte (Eph) gene was predominantly expressed in malignant gliomas (Yamanaka *et al* unpublished data). EphB6 is a member of the Eph receptor tyrosine kinase family expressed in a variety of human tissues and has been shown to lack protein kinase activity (7-9). In the present study, we found a 54 amino acid (aa) sequence unique to the alternative splicing variant form of EphB6 expressed in malignant glioma cells and identified two epitope peptides recognized by CTLs.

Materials and methods

Patients. Peripheral blood mononuclear cells (PBMCs) were obtained from HLA-A24+ malignant glioma patients and healthy donors after written informed consent was obtained. The Institutional Ethics Review Board of Kurume University approved this study protocol. None of the participants were infected with HIV. The PBMCs were prepared by Ficoll-Conray density gradient centrifugation. Each of the samples were cryopreserved until use in the experiments. The HLA type (HLA-A24+ or –A24-) was determined by flow cytometry using an anti-HLA-A24 monoclonal antibody (mAb) (One Lambda, Canoga, CA).

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Peptides. Each of the peptides used in this study had >90% purity and were obtained from Hokkaido System Science (Sapporo, Japan) or GeneNet (Fukuoka, Japan). The names and sequences of the peptides are as follows: EphB6v-1, SYPHNNFPF; EphB6v-2, SFEPPSLLL; EBV-A24 (an Epstein-Barr virus-derived HLA-A2402-restricted peptide), TYGPVFMCL; Flu-A24 (an influenza virus-derived HLA-A2402-restricted peptide), RFYIQMCYEL; HIV-A24 (a human immunodeficiency virus-derived HLA-A2402-restricted peptide), RYLRQQLLGI; EBV-A2 (an Epstein-Barr virus-derived HLA-A0201-restricted peptide), GLCTLVAML. Peptides were dissolved in dimethylsulfoxide (DMSO) at 10 mg/ml and stored at -20˚C.

Cell lines. The glioma cell lines used in our study were T98G, KALS-1, KNS-81, Becher, No. 10 and No. 11. C1R-A2402 is an HLA-A2402-expressing transfectant line of C1R lymphoma (kindly provided by Dr M. Takiguchi, Kumamoto University, Japan) and was cultured in RPMI-1640 medium supplemented with 10% FBS and 500 μg/ml hygromycin B (Invitrogen, Carlsbad, CA). RMA-S-A2402/K^b is a transfectant cell line of murine RMA-S cells expressing the chimeric gene of *HLA-A2402/K^b* as reported previously (10). T98G-A2402, a transfectant of the *HLA-A2402* gene, was established as follows: T98G cells were transfected with the *HLA-A2402* gene, which had been cloned into the expression vector *pCR3.1* (Invitrogen) (10), using FuGENE6 transfection reagent (Roche, Mannheim, Germany) and stable cell lines were obtained by geneticin (1 mg/ml) selection.

Reverse transcriptase-polymerase chain reaction (RT-PCR) and cloning of the PCR products. Total RNA was purified from cell lines using RNAzol™ B (Tel-Test, Friendswood, TX). Template cDNAs were prepared using the SuperScript™ Preamplification System for first strand cDNA synthesis (Invitrogen). The primers were as follows: 5'-TCAGCACTC ATGCTACACTGG-3' (sense) and 5'-TCTGCCTGGTCAT AGTAGCG-3' (anti-sense) for EphB6, and 5'-ACAGTCAG CCGCATCTTCTT-3' (sense) and 5'-AGGGGTCTACATG GCAACTG-3' (anti-sense) for GAPDH. RT-PCR was performed using Taq DNA polymerase (ExTaq, Takara, Otsu, Japan) in a DNA thermal cycler (iCycler; Bio-Rad Laboratories, Hercules, CA) for 35 cycles at 98˚C for 10 sec, 55˚C for 30 sec and 72˚C for 60 sec and the PCR products were separated by electrophoresis on 2% agarose gel. For sequencing of the PCR products, the products were cloned into an expression vector, *pcDNA™ 3.1D/V5-His TOPO* (Invitrogen), according to the manufacturer's instructions. After the transformation of bacteria (TOP10F; Invitrogen), isolated clones were sequenced.

HLA stabilization assay. The ability of peptides to bind to HLA-A2402 molecules was examined by an HLA stabilization assay using RMA-S-A2402/K^b cells as previously reported (10,11) with minor modifications. Briefly, the cells were cultured at 26˚C for 20 h in RPMI-1640 medium supplemented with 20% FBS. Then the cells were incubated in Opti-MEM (Invitrogen) supplemented with human ß$_2$ microglobulin (2 μg/ml) and various concentrations (1-100 μmol/l) of peptides at 26˚C for 2 h followed by incubation at 37˚C for 3 h. After washing with PBS, the cells were incubated with anti-human MHC class I mAb (clone: PT85A, VMRD, Pullman, WA) for 30 min on ice, followed by staining with Alexa Fluor 488 goat anti-mouse IgG (Invitrogen) for 30 min on ice. After washing, the fluorescence intensity of the cells was analyzed by a EPICS-XL (Beckman Coulter, Fullerton, CA). Binding activity was evaluated by the mean fluorescence intensity of HLA-A2402 molecules.

Measurement of anti-peptide antibody. The peptide-coupled beads were prepared as reported previously (12). In brief, 100 μl of xMAP color-coded beads (Luminex Corp, Austin, TX) were washed with 0.1 mol/l 2-morpholinoethanesulfonic acid (MES) buffer (pH 7.0) and then added to 100 μl of peptide (1.25 mg/ml in MES buffer). The reaction mixture was then incubated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 1 mg/ml; Pierce, Rockford, IL) at 37˚C for 20 min in darkness and the reaction was repeated twice. After the incubation, the beads were washed with 0.05% Tween-20-PBS and further treated with 100 μl of 1 mol/l Tris (hydroxymethyl) aminomethane•HCl for 15 min at 37˚C. The beads were re-suspended in 500 μl of 0.05% NaN_3-Block-Ace (Yukijirushi, Sapporo, Japan) and adjusted to 10^6 beads/ml.

The levels of anti-peptide IgG or IgM in plasma were measured by using a Luminex™ system as previously reported (12). In brief, diluted sample plasma was incubated with 65 μl of peptide-coupled xMAP beads for 2 h at 37˚C on a plate shaker. After incubation, the mixture was washed with a vacuum manifold apparatus and incubated with 100 μl of 1:200-diluted biotinylated goat anti-human IgG (γ chain-specific) (2.5 μg/ml; Vector Laboratories Inc, Burlingame, CA, USA) or IgM (2.5 μg/ml; Vector) for 1 h at 37˚C. The plate was then washed, 100 μl of 1:200-diluted streptavidin-PE (Molecular Probes, Eugene, OR) was added to each well and the plate was incubated for 30 min at 37˚C on a plate shaker. The beads were washed three times followed by an addition of 100 μl of 0.05% Tween-20-PBS to each well and then analyzed by a Luminex system. The level of the antibody was administered in fluorescence intensity (FI) units.

Induction of peptide-specific CTLs from PBMCs. Peptide-specific CTLs were detected as described in our previous report (13) with minor modifications. Flu-A24 and EBV-A24 peptides were used as positive controls and HIV-A24 peptides were used as a negative control. PBMCs ($1x10^5$ cells/well) were incubated with 10 μg/ml of each peptide in quadruplicate in a U-bottom type 96-well microculture plate (Nunc, Roskilde, Denmark) in 200 μl of a culture medium consisting of 45% RPMI-1640, 45% AIM-V medium (Life Technologies, Gaithersburg, MD), 10% FBS, 100 U/ml interleukin-2 (IL-2), and 0.1 mmol/l MEM nonessential amino acid solution (Life Technologies). On day 13-15 of culture, cells in each well were washed and aliquoted into four wells. The cells in two wells were cultured with the corresponding peptide-pulsed C1R-A2402 cells and those in the remaining two wells were cultured with HIV-A24 peptide-pulsed C1R-A2402 cells. After 24-30 h of incubation at 37˚C, the supernatants were collected and the interferon-γ (IFN-γ) concentration was measured by enzyme-linked immunosorbent assay (ELISA).

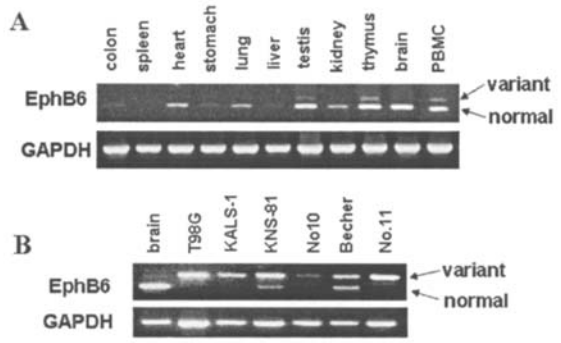

Figure 1. Expression of *EphB6* in normal tissues (A) and malignant glioma cell lines (B) at mRNA levels were examined by RT-PCR. *GAPDH* was used as an internal control.

The assay was carried out in 8 wells and the results were considered positive if the values of the test peptide were significantly higher than that of the HIV-A24 peptide at $p<0.05$.

Cytotoxicity assay. Peptide-stimulated PBMCs were tested for their cytotoxicity against the glioma cell lines T98G (HLA-A24$^-$) and T98G-A2402 (HLA-A24$^+$) by a standard 6-h ^{51}Cr-release assay. PBMCs from HLA-A24$^+$ healthy donors were cultured with 20 μg/ml of phytohemagglutinin (PHA-P, DIFCO, Detroit, MI) for 3 days and used as PHA blasts. CD8$^+$ T cells purified from the peptide-stimulated PBMCs using a CD8-positive isolation kit (Dynal, Oslo, Norway) were incubated with $2x10^3$ cells/well of ^{51}Cr-labeled target cells at various effector/target (E/T) ratios in a U-bottom type 96-well microculture plate. The specific ^{51}Cr-release was calculated according to the following formula: % specific lysis = (test sample release - spontaneous release) / (maximum release – spontaneous release) x 100 (%). Spontaneous release was determined using the supernatant of the target cells incubated with no effector cells and the maximum release was determined by a supernatant of the target cells incubated with 1% Triton X-100 (Wako Pure Chemical, Osaka, Japan). In certain experiments, anti-HLA class I (W6/32, mouse IgG2a) or isotype-matched control (anti-CD14) mAb was added to the wells at the initiation of the assay. The specificity of peptide-stimulated PBMCs was also confirmed by a cold target inhibition assay. Cells ($2x10^3$)/well of unlabeled C1R-A2402 cells pre-pulsed with either the HIV-A24 or corresponding peptide were used as cold target cells.

Results

Expression of the EphB6 gene in normal tissues and glioma cell lines. We examined the mRNA expression of the *EphB6* gene by RT-PCR. As shown in Fig. 1, the *EphB6* gene was ubiquitously though differentially expressed in the various normal tissues, as well as in glioma cell lines. An extra band of PCR product was also seen in certain tissues, i.e., the testis, thymus, brain and PBMCs. The extra products were more abundantly and more clearly detected in the glioma cell lines than the products of normal size. Therefore, the two different sizes of PCR products were further subjected to sequencing.

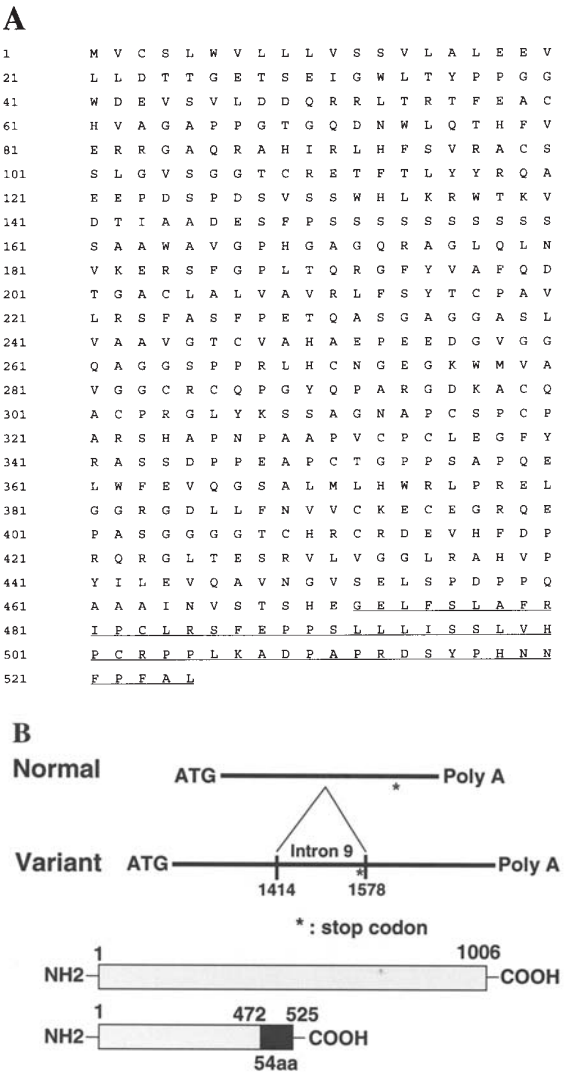

Figure 2. Deduced amino acid sequence of EphB6v (A) and a schematic diagram of *EphB6* genes (B) are shown. (A) A unique 54 amino acid sequence in the C-terminal of EphB6v is underlined. (B) Insertion of 165 bp derived from intron 9 was observed at nucleotide position 1414-1578 in *EphB6v* cDNA and there is a stop codon at position 1576. The EphB6v protein is a truncated form of normal EphB6 and a unique 54 amino acid sequence encoded by intron 9 is located at aa position 472-525.

The nucleotide sequence of the PCR product of regular size agreed with the previously reported sequence of the *EphB6* transcript (data not shown). In contrast, the nucleotide sequence of the extra product contained intron 9. The deduced aa sequence and structure are shown in Fig. 2 (GenBank accession number, EU054308). The intron 9 included a stop codon at nucleotide position 1576. Therefore, the extra PCR product is an alternative splicing variant form of *EphB6* and the deduced amino acid sequence of the variant form possesses a unique 54 aa sequence that is not found in normal EphB6.

Determination of T cell epitope peptides. Putative HLA-A24-binding peptides derived from the EphB6 variant (EphB6v) were analyzed using BIMAS software (Bioinformatics and Molecular Analysis Section, NIH; http://wye.cit.nih.gov/molbio/hla_bind/). Two 9-mer peptides (EphB6v-1 at position

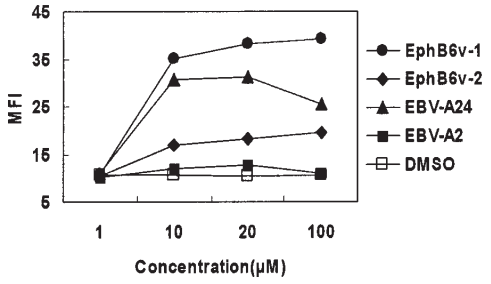

Figure 3. Binding capacity of EphB6v-1 and -2 peptides to HLA-A2402 molecules was examined by an HLA stabilizing assay using RMA-S-A2402/K^b cells. Each point indicates the mean fluorescence intensity (MFI) of HLA expression of the duplicate assay.

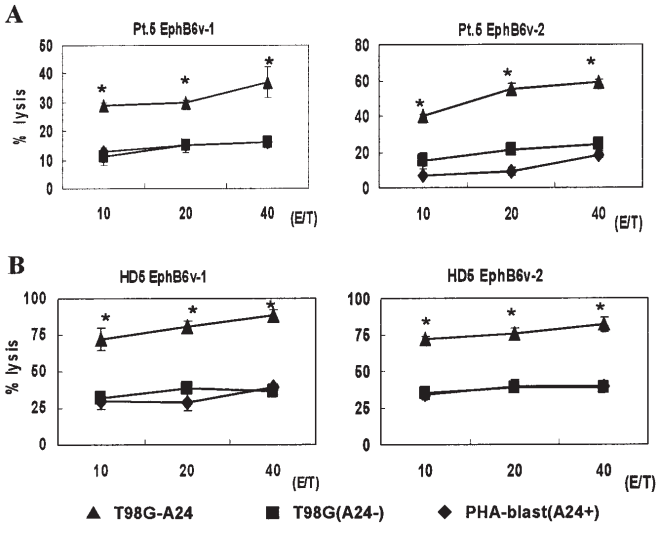

Figure 4. The cytotoxicity of peptide-induced CTLs from HLA-A24$^+$ patients (A) and healthy donors (B) to T98G, T98G-A2402 and PHA blasts was examined by a 6-h ^{51}Cr-release assay. Representative results are shown.

515 and EphB6v-2 at position 486) with the highest binding scores were selected for further analyses. The BIMAS-binding scores of the half-life of peptide-MHC dissociation of EphB6v-1 and -2 are 150 and 36, respectively. To confirm the binding of these two peptides to HLA-A2402 molecules, we performed an HLA stabilization assay. RMA-S-A2402/K^b cells incubated at 26˚C showed a much higher HLA surface expression level and this level rapidly decreased after the incubation at 37˚C because of the instability of HLA expression without the binding peptide (11). Therefore, we used this cell line to evaluate the binding capacities of EphB6v peptides to HLA-A2402 molecules. As shown in Fig. 3, the EphB6v-1 and EphB6v-2 peptides, as well as the positive control EBV-A24, stabilized HLA-A2402 expression on RMA-S-A2402/K^b cells, when compared with the negative control peptide (EBV-A2) or solvent (DMSO) alone. The results also suggest that the binding affinity of EphB6v-1 is higher than that of EphB6v-2 with the agreement of BIMAS scores.

Induction of EphB6v peptide-specific CTLs from the PBMCs of HLA-A24$^+$ donors. We then attempted to test the ability of the EphB6v-1 and -2 peptides to induce peptide-specific CTLs from the PBMCs of HLA-A24$^+$ glioma patients. The PBMCs were stimulated *in vitro* with each of the peptides or control peptides and then examined for their IFN-γ production in response to corresponding peptide-pulsed C1R-A2402 cells. The results showed that EphB6v-1 and -2 successfully induced peptide-specific CTLs in 7 and 9 of the 13 cancer patients, respectively (Table I). EBV-A24 and Flu-A24 peptides were used as positive controls. We also examined whether the EphB6v-1 and -2 peptides had the potential to generate peptide-specific CTLs from HLA-A24$^+$ healthy donors and detected IFN-γ production in 5 and 6 of the 11 healthy donors, respectively (Table I). These results indicate that the EphB6v-1 and -2 peptides possessed the ability to induce peptide-specific CTLs from the glioma patients and healthy donors.

Cytotoxicity of EphB6v peptide-specific CTLs from the PBMCs of HLA-A24$^+$ donors. The cytotoxicity of EphB6v peptide-induced CTLs from the PBMCs of HLA-A24$^+$ glioma patients was further examined by a ^{51}Cr-release assay. PBMCs from the patients were repeatedly stimulated *in vitro* with each of the two peptides and their cytotoxicity against T98G (HLA-A24$^-$) glioma cells, T98G-A2402 (HLA-A24$^+$) cells and PHA blasts (HLA-A24$^+$) was measured. The PHA blasts from HLA-A24$^+$ healthy donors were used as a non-tumorous control of HLA-A24$^+$ cells. As shown in Fig. 4, CTLs induced from PBMCs from the glioma patients and healthy donors exhibited higher levels of cytotoxicity against T98G-A2402 cells than against T98G cells and PHA blasts. The cytotoxicity of EphB6v-1 or -2 peptide-induced CTLs from the glioma patients and healthy donors was significantly inhibited by the addition of cold targets; namely, cold C1R-A2402 cells pulsed with the corresponding peptide, though not those pulsed with the HIV peptide, inhibited cytotoxicity (Fig. 5A and B). The cytotoxicity of the peptide-induced CTLs against the T98G-A2402 cells was also significantly inhibited by an addition of anti-HLA class I mAb (W6/32), though not by an addition of isotype-matched anti-CD14 control mAb (Fig. 5C and D). These results indicated that EphB6v-1 or -2 peptide-induced CTLs could exhibit cytotoxic activity against glioma cells in an HLA-A24-restricted and antigen-specific manner.

Detection of IgG or IgM reactive to the EphB6v-1 and -2 peptides. We previously reported that IgG antibodies reactive to the CTL epitope peptides were frequently detected in the plasma of cancer patients and the presence of the antibodies correlated well to the clinical effects of the peptide vaccination (4,14). Plasma levels of IgG and IgM reactive to EphB6v-1 and -2 peptides of the 16 glioma patients and 15 healthy donors were measured. As shown in Fig. 6, IgG and IgM reactive to the EphB6v-1 and -2 peptides were detected in the plasma of the glioma patients and healthy donors and there was no significant difference in the level of the antibody between the patients and healthy donors.

Discussion

Based on the sequence homology, structure and binding affinity, the Eph receptors were divided into two groups, EphA

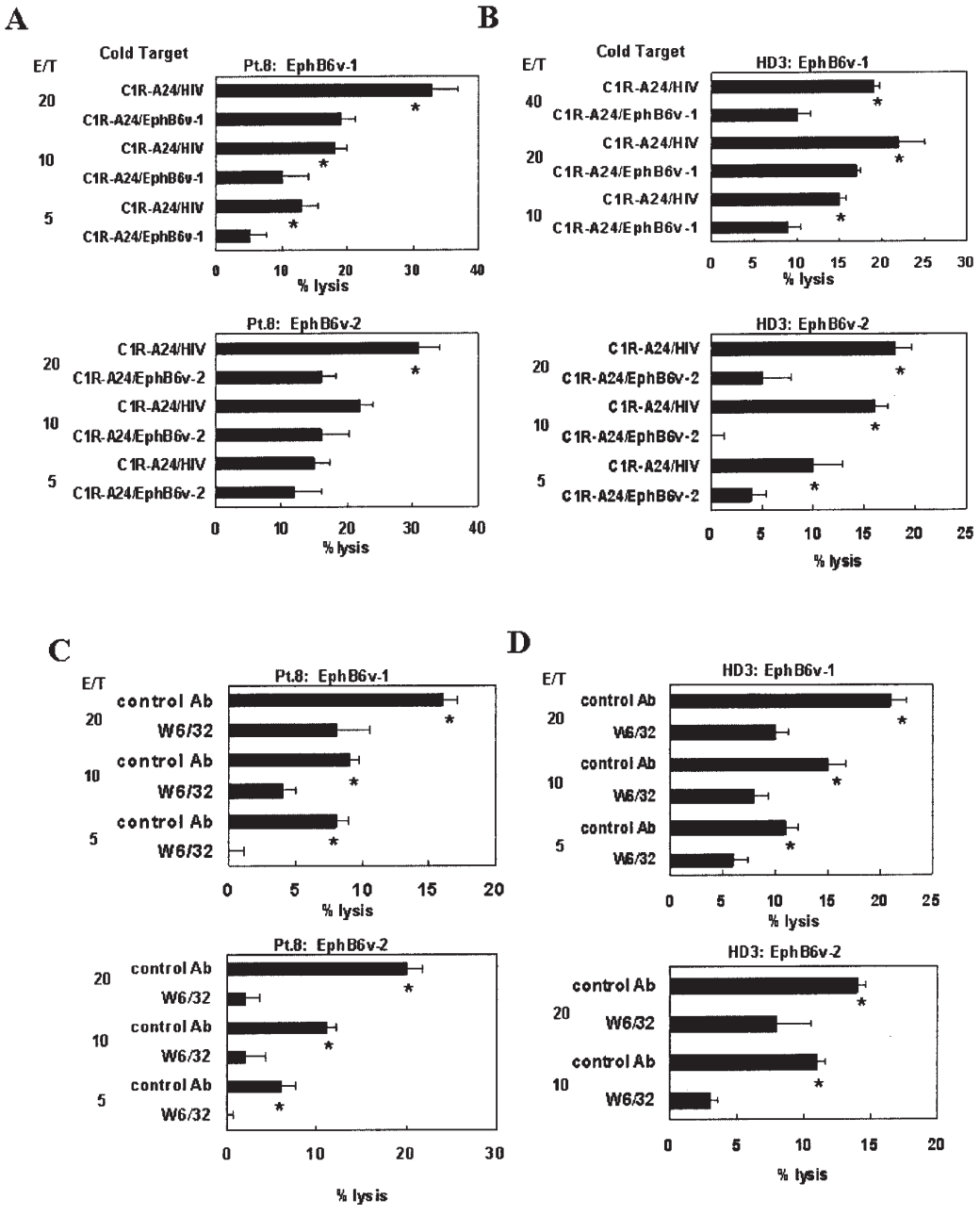

Figure 5. Cold target inhibition and antibody blocking of the cytotoxicity of EphB6v peptide-induced CTLs from malignant glioma patients and healthy donors. Purified $CD8^+$ T cells fraction of peptide-induced CTLs from HLA-$A24^+$ malignant glioma patients (A,C) or healthy donor (B,D) were tested for cytotoxicity against T98G-A2402 cells in the presence of cold C1R-A2402 cells preloaded with either the corresponding peptide or the HIV peptide (A,B) or anti-class I HLA (W6/32) mAb (C,D). Anti-CD14 mAb was also used as an isotype-matched control mAb. *Values are statistically significant from that of corresponding controls by the Student's t-test, $p<0.05$.

and EphB (15). Among the 14 members of the Eph receptor tyrosine kinase family, EphB6 is the only kinase-defective Eph receptor; this defect arises due to an alteration in the intracellular kinase domain (7,8). Most kinase-defective growth factor receptor proteins are associated with pathogenic conditions causing human or other mammalian disorders (7,9). Nevertheless, EphB6 has been reported to be expressed in a variety of normal human tissues, such as the brain, heart, retina and thymus, as well as in blood cells (7,9). The expression of EphB6 has also been reported in human small cell lung carcinomas (16) and neuroblastomas (17,18), whereas no or low expression of EphB6 is observed in malignant melanomas and metastatic cancer (17,19). Furthermore, a high expression of EphB6 is predictive of a significantly favorable prognosis in human neuroblastomas (18). Therefore, EphB6 is considered to be an inhibitory factor on the development of metastatic cancers.

In this study, we found a variant form of EphB6 that was preferentially expressed in malignant glioma cell lines. Furthermore, we found that the variant form has a unique 54 aa sequence encoded by intron 9 in the C-terminal which is not found in normal EphB6. The variant form lacks a transmembrane domain. Therefore, the variant form might be secreted from the cells. Several variant forms of EphB6 have been reported in mice and some of these also lack a transmembrane domain. Although the physiological function

Table I. CTL induction from glioma patients and healthy donors.

Donor	IFN-γ production (pg/ml)			
	EphB6v-1	EphB6v-2	EBV-A24	Flu-A24
Patient with malignant glioma				
Pt.1	38	45	-	172
Pt.2	-	-	-	-
Pt.3	-	-	-	-
Pt.4	-	-	-	-
Pt.5	188	30	71	-
Pt.6	-	-	91	-
Pt.7	206	134	-	228
Pt.8	382	323	-	467
Pt.9	221	96	-	-
Pt.10	-	28	-	-
Pt.11	-	41	-	-
Pt.12	16	30	134	56
Pt.13	50	47	407	68
Positive rate	7/13	9/13	4/13	5/13
Healthy donors				
HD1	-	317	68	68
HD2	27	-	-	-
HD3	-	93	-	-
HD4	235	36	220	-
HD5	-	-	-	-
HD6	-	118	175	48
HD7	-	-	1542	-
HD8	71	-	17	-
HD9	-	-	-	-
HD10	124	119	-	-
HD11	56	228	-	-
Positive rate	5/11	6/11	5/11	2/11

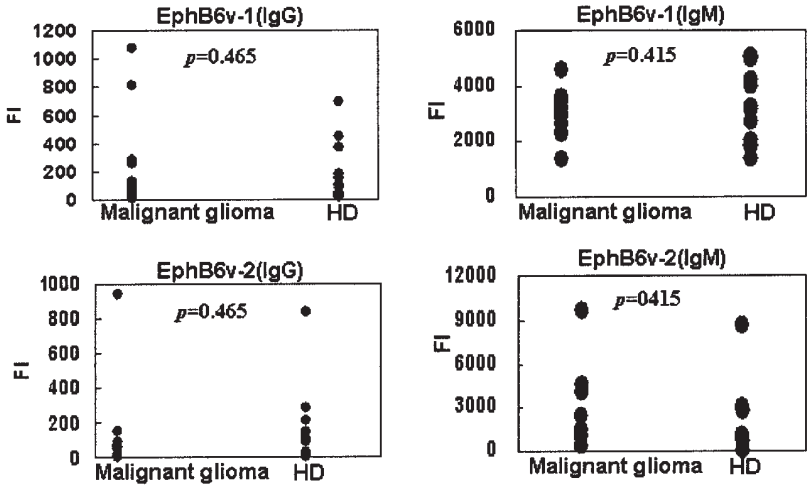

Figure 6. IgGs or IgMs reactive to EphB6v-1 or -2 in the plasma of malignant glioma patients or healthy donors. Peptide reactive IgG or IgM levels were measured by a Luminex system. Each point indicates the antibody level of 1:100 diluted plasma samples of malignant glioma patients (n=16) and healthy donors (n=15). P-values are calculated by Chi-square analysis.

of the variant form is unknown, this is the first report of a variant form of EphB6 in humans.

We focused on the unique 54 aa sequence in the variant form for further study and we identified two CTL epitope peptides that possessed the ability to induce CTLs in an HLA-A24-restricted manner. Significant levels of IgG and IgM antibodies reactive to the CTL epitope peptides were also detected in the majority of the patients and healthy donors. True antigens of the antibodies reactive to the peptide are unknown. These antibodies might cross-react with micro-organisms or intrinsic proteins. For example, the similarity of the peptide sequences of Cytomegalovirus, *Helicobacter pylori* and MAPKKK kinase to the EphB6v-1 peptide are 85, 77 and 75%, respectively and that of *Cryptosporidium parvum* to the EphB6v-2 peptide is 88%.

Our previous clinical studies of cancer vaccines indicated that the presence of IgG antibodies to the vaccine peptides accelerated the subsequent induction or enhancement of the two CTLs and IgG specific to the peptides by the peptide vaccination (4). These facts suggest that the two peptides derived from EphB6v may be good candidates for personalized peptide vaccines for patients with malignant glioma.

In conclusion, we identified two EphB6v-derived CTL epitope peptides suitable for use in personalized peptide vaccinations for malignant glioma patients. This study also provided new insights into the role of EphB6 in the brain and other cancers.

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