SPANDIDOS PUBLICATIONS

Kinesin superfamily protein-derived peptides with the ability to induce glioma-reactive cytotoxic T lymphocytes in human leukocyte antigen-A24+ glioma patients

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Abstract. One promising modality in the treatment of malignant glioma is specific immunotherapy. However, this modality requires information about target antigens and their epitope peptides that are recognized by T cells. In this study, we searched for new target candidates in specific immunotherapy for malignant glioma by utilizing cDNA microarray technology to compare gene expressions in malignant glioma tissues to those in benign glioma and a panel of normal tissues. The selected genes included three members of the kinesin superfamily proteins (KIFs): KIF1C, KIF3C, and KIF21B. RT-PCR showed that these three genes were expressed in the majority of glioma cell lines. These antigen-derived 25 peptides, which had the ability to bind to human leukocyte antigen (HLA)-A24 molecules, were first screened for their ability to be recognized by the immunoglobulin G of glioma patients, and then tested for their potential to induce peptide-specific and glioma-reactive cytotoxic T lymphocytes (CTLs) from the peripheral blood mononuclear cells of HLA-A24+ glioma patients. The results showed that the $KIF1C_{149-158}$ and $KIF3C_{512-520}$ peptides efficiently induced HLA-A24-restricted and glioma-reactive $CD8^+$ T cells. These results suggest the existence of KIF-reactive CTL precursors in glioma patients, and should facilitate the development of specific immunotherapies for malignant glioma.

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Introduction

There has been little improvement in the prognosis and survival of malignant glioma patients over the past decade (1,2), despite the fact that aggressively combined treatment modalities have been developed. Therefore, the development of a new treatment modality is needed, and one option is specific immunotherapy. Advances in tumor immunology have led to the discovery of several cancer-associated antigens and their epitope peptides that can be recognized by cancer-reactive cytotoxic T lymphocytes (CTLs) (3). Glioma-associated antigens and their peptides have also been reported (4-6), but their numbers are very limited. Thus, there is a need for the identification of therapeutically useful glioma-associated antigens and their epitope peptides.

The development of cDNA microarray technology has enabled comprehensive profiling of the gene expressions of malignant cells (7,8). This approach leads to the identification of genes whose expression patterns are pre-dominant in malignant cells. We have also identified new target antigens of malignant glioma by using cDNA microassay technology to compare the gene expressions in malignant glioma tissues with those in benign glioma and normal brain tissues. As a result, 17 candidates were selected, including three kinesin superfamily proteins (KIFs): KIF1C, KIFC3, and KIF21B. In this study, we clarify the potential of these three KIF antigens as vaccine candidates.

Materials and methods

Samples and cDNA microarray. Samples were obtained from 52 glioma patients who underwent surgery. The study was approved by the Ethics Committee of Niigata University, and complete written informed consent was obtained from all of the patients. They consisted of 16 grade II, 14 grade III, and 22 grade IV cases based on the WHO grade classification (9,10). Genetic analysis was performed using agilent cDNA microarrays (Agilent Technologies, Palo Alto, CA, USA). Total RNA (20 μg) was reverse transcribed using an Agilent direct-label cDNA synthesis kit (Agilent Technologies) according

to the manufacturer's directions. Labeled cDNA was purified using QIAquick PCR Purification columns (Qiagen, Valencia, CA, USA) and then concentrated by vacuum centrifugation. cDNA was suspended in a hybridization buffer, and hybridized to Agilent human 1 cDNA microarrays (Agilent Technologies) for 17 h at 65˚C according to the Agilent protocol. To avoid the generation of false between-group differences by randomly pairing the glioma samples on the two channel cDNA arrays, each sample was individually labeled, and co-hybridized with a normal brain sample labeled with a complementary dye. Normal brain samples were generated by pooling equal amounts of RNA from each control sample, and labeling them individually as well. In addition, cyanine dye switch hybridizations were performed for each sample. Normal brain samples were purchased from Clontech (Tokyo, Japan). After the chips were washed using 5% SSC/0.1% SDS solution, the fluorescence intensity was measured using a laser scanner, and analyzed using Feature Extraction Software (ver. A.4.0.45; Agilent Technologies) according to the manufacturer's instructions. A total of 12,729 genes were analyzed.

Cell lines. The glioma cell lines used in this study were as follows: KNS-81, KALS-1, KINGS-1, Becher, and no. 11, C1R-A24, a human leukocyte antigen (HLA)-A*2402-expressing subline of C1R lymphoma (Dr M. Takiguchi, Kumamoto University, Japan).

Reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated from the cancer cell lines using RNAzol™ B (Tel-Test Inc., Friendswood, TX, USA). cDNA was prepared using the SuperScript™ Preamplification System for First Strand cDNA Synthesis (Invitrogen, Carlsland, CA, USA), and amplified using the following primers: 5'-CAAG TGTGTGGTCAGCATGC-3' (sense) and 5'-CTCTGGTTCT CACTAAGCG-3' (anti-sense) for KIF1C, 5'-TATGATGCC AGCTCCAAGC-3' (sense) and 5'-ATTCTTGGTGACGAA GGAGG-3' (anti-sense) for KIF3C, 5'-AATGTGATCAGCG CCTTAGG-3' (sense) and 5'-TGTAGCATGGCATTCT CTCG-3' (anti-sense) for KIF21B, and 5'-CTTCGCGGGCG ACGATGC-3' (sense) and 5'-CGTACATGGCTGGG GTGTTG-3' (anti-sense) for *ß-actin*.

PCR was performed using TaqDNA polymerase in a DNA thermal cycler (iCycler; Bio-Rad Laboratories, Hercules, CA, USA) for 30 cycles of 95˚C for 30 sec, 60˚C for 30 sec, and 72˚C for 30 sec. The PCR products were separated by electrophoresis on 2% agarose gels.

Measurement of anti-peptide antibody. The levels of anti-peptide immunoglobulin G (IgG) were measured by the Luminex™ system as previously reported (11). Briefly, the plasma was incubated with 25 μl peptide-coupled color-coded beads for 2 h at room temperature on a plate shaker. After incubation, the mixture was washed with a vacuum manifold apparatus and incubated with 100 μl biotinylated goat anti-human IgG (γ chain-specific) for 1 h at room temperature. The plate was then washed, and 100 μl streptavidin-PE was added to each well and incubated for 30 min at room temperature on a plate shaker. The bound beads were washed three times followed by the addition of 100 μl Tween-PBS to each well. Fifty microliters of each sample were then examined using the Luminex™ system.

Induction of peptide-specific CTLs from PBMCs. Peptides with >90% purity were purchased from Hokkaido System Science (Sapporo, Japan). The influenza (Flu) virus, EB virus, and HIV-derived peptides were used as the controls binding to the HLA-A24 alleles. Assays for the detection of peptide-specific CTLs were performed according to a previously reported method with several modifications (12). Briefly, PBMCs were incubated with 10 μg/ml of each peptide in quadruplicate in a 96-well microplate (Nunc, Roskilde, Denmark). The culture medium consisted of 45% RPMI-1640, 45% AIM-V medium (Life Technologies, Gaithersburg, MD, USA), 10% FBS, 100 units/ml interleukin (IL)-2, and 0.1 mM MEM nonessential amino acid solution (Life Technologies). On the 15th day of culturing, the cells were separated into four wells. The cells in two wells were cultured with the corresponding peptide-pulsed C1R-A24 cells, and those in the other two wells were cultured with HIV peptide-pulsed C1R-A24 cells. After an 18-h incubation, the supernatant was collected, and the interferon (IFN)-γ production was determined by ELISA. The successful induction of peptide-specific CTLs was judged to be positive when the P-value was <0.05 by the two-tailed Student's t-test and the difference in IFN-γ production compared with the HIV peptide was >100 pg/ml.

Cytotoxicity assay. Peptide-stimulated PBMCs were tested for their cytotoxicity against no. 11 (HLA-A24-negative) and KNS-81 (HLA-A24-positive) by a standard 6-h ^{51}Cr-release assay. PHA-activated T cells from HLA-A24-positive healthy donors were used as the negative control. After the CD8$^+$ T cells were positively isolated using a CD8-positive isolation kit (Dynal, Oslo, Norway), 2,000 ^{51}Cr-labeled cells per well were cultured with effector cells in 96-round-well plates. The specific ^{51}Cr-release was calculated according to the following formula: % specific lysis = (test sample release - spontaneous release) x100/(maximum release - spontaneous release). Spontaneous release was determined using the supernatant of the sample incubated with no effector cells, and the maximum release was then determined by the supernatant of the sample incubated with 1% Triton X (Wako Pure Chemical Industries, Osaka, Japan). In some of the experiments, anti-HLA class I (W6/32, mouse IgG2a), anti-HLA-DR (L243, mouse IgG2a), and anti-CD14 (JML-H14, mouse IgG2a) mAb were added to the wells at the initiation of the culture. The specificity of the peptide-stimulated PBMCs was also confirmed by a cold inhibition assay. Twenty thousand unlabeled C1R-A24 cells, which were pre-pulsed with either the HIV peptide or a corresponding peptide, were used as cold target cells.

Results

mRNA expression of three KIF genes in glioma and normal tissues. Samples obtained from 52 glioma patients were analyzed using an agilent cDNA microarray. The fluorescence intensity of 36 malignant glioma tissues was compared to those of 16 benign glioma and normal brain tissues. A total of 12,729 genes were analyzed in this manner, and 17 genes

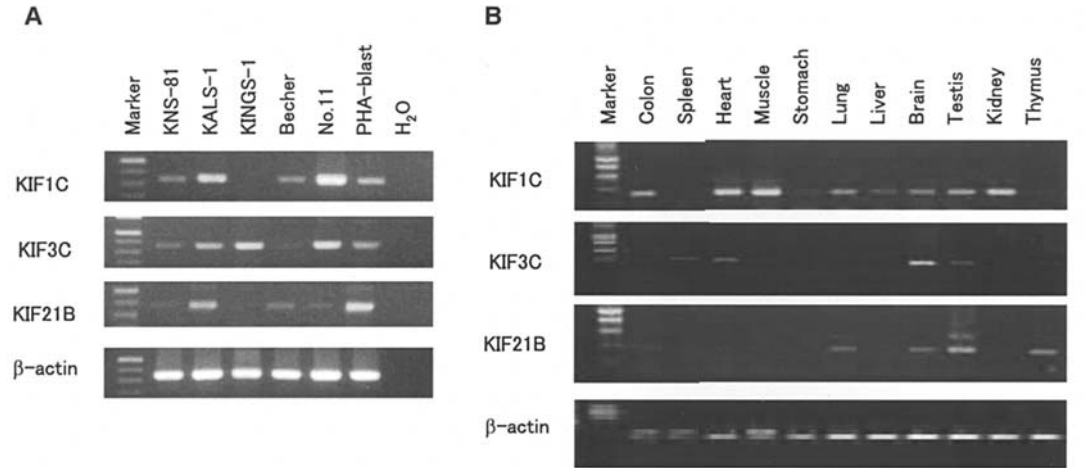

Figure 1. The mRNA expression of the three kinesin superfamily protein (KIF) genes in the glioma cell lines and normal tissues. Five glioma cell lines (A) and a panel of normal tissues (B) were examined for their mRNA expression of KIFs. The glioma cell lines tested were KNS-81 (malignant glioma), KALS-1 (glioblastoma), KINGS-1 (anaplastic astrocytoma), Becher (astrocytoma), and no. 11 (anaplastic astrocytoma). The expression of ß-actin was assayed as the control.

Table I. Kinesin-derived peptide candidates binding to the HLA-A24 molecules.

Peptides	Amino acid sequence	Length	Binding score
KIF1C			
73-82	VYRDIGEEML	10	240
149-157	IYCERVRDL	9	240
149-158	IYCERVRDLL	10	336
331-339	NYEETLSTL	9	360
340-348	RYADRTKQI	9	120
725-734	VYQIPQRRRL	10	300
756-764	CYEVALADF	9	150
968-977	RFVPPHDCKL	10	79.2
KIF3C			
137-145	QYLVRASYL	9	300
147-155	IYQEEIRDL	9	360
147-156	IYQEEIRDLL	10	504
174-182	VYIKDLSSF	9	150
347-355	SYDESLSTL	9	240
458-467	NYLQEQKERL	10	300
512-520	KYKAMESKL	9	440
607-615	EYIRVRQDL	9	504
629-637	GYLIIENFI	9	126
702-710	RYRAENIMF	9	200
702-711	RYRAENIMFL	10	400
KIF21B			
220-228	GYASTDEEI	9	55
473-482	KYCSHSGLVF	10	200
621-630	HYDGIECLAI	10	50
670-678	AFIPGRPML	9	36
670-679	AFIPGRPMLL	10	36
731-740	NYVPGLTPCL	10	432

HLA, human leukocyte antigen; KIF, kinesin superfamily protein. The peptide binding score was calculated based on the predicted half-time of dissociation from the HLA class I molecules as obtained from the website (Bioinformatics and Molecular Analysis Section, Computed Bioscience and Engineering Laboratory, Division of Computer Research and Technology, NIH).

were found to be more highly expressed in malignant glioma tissues than in benign glioma and a panel of normal tissues, including the brain, adrenal gland, bone marrow, colon, liver, fetal liver, heart, kidneys, lungs, mammary gland, prostate, salivary glands, muscles, intestines, spinal cord, spleen, stomach, testes, thymus, bronchia, thyroid, and uterus (R.Y., unpublished data). These 17 genes included 3 KIF genes, KIF1C, KIF3C, and KIF21B, which were the focus of this study.

We first examined the mRNA expression of these genes by RT-PCR (Fig. 1A). The mRNA expression of all three genes was detected in four of the five glioma cell lines. The mRNA expression of all three genes was also detected in the PHA-activated T cells and some normal tissues (Fig. 1B). The mRNA of the KIF1C gene was more widely expressed than that of the other two KIF genes in the normal tissues. mRNA of the KIF3C gene was detected in the spleen, heart, brain, and testes. The mRNA expression of the KIF21B gene was detected in the lungs, brain, testes, and thymus.

Detection of IgG reactive to the KIF-derived peptides. Next, we investigated the immunogenicity of these three KIFs. We prepared 25 peptides derived from 3 KIFs: Eight for KIF1C, 11 for KIF3C, and 6 for KIF21B, based on the binding motifs to the HLA-A24 molecules (13) (Table I). We next examined their ability to be recognized by the IgGs of glioma patients, as IgGs reactive to CTL-directed peptides are detectable in the plasma of patients with different types of cancer (14), as well as the levels of IgG well correlated with the clinical responses of patients who received a peptide vaccination as reported previously (15,16). IgGs reactive to a corresponding peptide were judged to be significant when the immunofluorescence intensity in 1:100-diluted plasma exceeded 1.25x that of the control samples with no peptides. The results of the KIF1C peptides are shown in Table II. Among the 8 peptides, 3 KIF1C peptides, $KIF1C_{149-157}$, $KIF1C_{149-158}$, and $KIF1C_{340-348}$, were recognized by the IgGs in the plasma of glioma patients more frequently than those of the other 3 peptides. A similar result was observed in the samples from the healthy donors. The same assay was performed on the 11

Table II. KIF peptide-specific IgG in the plasma of glioma patients and healthy donors.

Subject	KIF1C peptide								
	73-82	149-157	149-158	331-339	340-348	725-734	756-764	968-977	No peptide
	Immunofluoresence intensity								
Patient									
1	57.8	**98.5**	**114.5**	33.5	**103**	65.5	36.5	77	74
2	80.5	**117.5**	**98.5**	39.5	**102**	85.3	45	**86**	68.5
3	46.8	**124.3**	**100**	22	**85.5**	61.3	28.8	**83.5**	48.5
4	17.5	29.5	**55.8**	10.5	26	19.5	11.5	27	36
5	**119**	**132**	**163**	53.5	**173.5**	98.5	58	97	93.5
6	**388.5**	**1066**	**706**	86.5	**595.5**	**385**	185.8	**533.8**	155.5
7	68.5	**108**	**105**	35.5	**103.8**	**134.3**	40.3	74.8	66.5
8	11.5	12.3	16	8.5	14.8	10	6.5	12.5	30.5
9	11	27.3	19.5	5.5	17.5	9.3	7.5	19.5	28
10	**1585**	**1688.3**	**1264**	485.4	**2174.5**	**1187.8**	658	**1132.5**	640
Total	3/10	7/10	8/10	0/10	7/10	4/10	0/10	4/10	
Healthy donor									
1	**122.5**	**122.3**	**213**	70	**141.8**	99,5	87.3	117	97
2	19.5	31.8	41	13.5	21.5	19.5	14	23.5	36
3	64.5	**113.3**	**112**	29	**74.5**	66.8	46.8	**76.5**	55.3
4	29.5	47.8	55.8	16.5	45.3	30.5	20	33	49.5
5	50	**87.8**	**245.8**	35	59	52.5	40.3	58.5	61
6	**97**	**134.8**	**189.5**	40	**156**	**87**	47.5	**94**	69.3
7	**648.5**	**468.8**	442	233	**1599**	**660**	196	**498.5**	369.5
8	**6937.5**	**4962.8**	**4708**	4133	**9638**	**4984**	3893.5	**5813**	3604.5
9	**36.5**	**39.5**	**97.5**	22.5	**34**	**35.3**	27	32.3	26.5
10	**96.5**	**110.3**	**147.5**	62.5	**121.5**	**93.8**	**82**	**94**	56.3
Total	6/10	8/10	7/10	0/10	7/10	5/10	1/10	5/10	

KIF, kinesin superfamily protein; IgG, immunoglobulin G. The IgG reactive to a corresponding peptide was judged to be significant when the absorbance in a l:100-diluted plasma was >1.25x the absorbance of the no peptide control samples. Significance was evaluated for each of the plasma samples, and the positive results are shown in bold.

KIF3C-derived and 6 KIF21B-derived peptides, and the results are summarized in Table III. IgGs reactive to 3 KIF3C peptides, $KIF3C_{458-467}$, $KIF3C_{512-520}$, $KIF3C_{702-710}$, and 2 KIF21B peptides, $KIF21B_{220-228}$ and $KIF21B_{621-630}$, were recognized by the IgGs in the plasma of glioma patients and healthy donors more frequently than those of the other peptides. Based on these findings, these 8 KIF peptides (3 for KIF1C, 3 for KIF3C, and 2 for KIF21B) were the focus of the following experiments.

Induction of KIF peptide-specific CTLs from the PBMCs of glioma patients. We next examined whether or not 8 KIF peptide candidates that were frequently recognized by the IgGs in glioma patients possess the potential 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 examined for their IFN-γ production in response to the corresponding peptide-pulsed C1R-A24 cells (Table IV). The successful induction of the peptide-specific CTLs was judged to be positive when the P-value was <0.05 and when the difference in IFN-γ production compared to the control HIV peptide exceeded 100 pg/ml. The positive results are shown in bold. All 8 peptides induced corresponding peptide-reactive CTLs from the PBMCs of 3 or 4 of the 10 HLA-A24$^+$ glioma patients. In contrast, only the $KIF1C_{149-158}$ peptide induced peptide-specific CTLs in 1 of the 5 HLA-A24$^+$ healthy donors, and the other 7 KIF peptides failed to generate peptide-specific CTLs in any of the 5 healthy donors (data not shown).

Induction of glioma-reactive CTLs from HLA-A24$^+$ glioma patients. It was crucial to determine whether or not these KIF

Summary of the IgGs reactive to the KIF-derived n the plasma of patients and healthy donors.

Peptides	Positive cases/total cases		
	Patients	Healthy donors	Total
KIF1C			
73-82	3/10	6/10	9/20
149-157	7/10	8/10	15/20
149-158	8/10	7/10	15/20
331-339	0/10	0/10	0/20
340-348	7/10	7/10	14/20
725-734	3/10	5/10	8/20
756-764	0/10	1/10	1/20
968-977	4/10	5/10	9/20
KIF3C			
137-145	2/10	1/10	3/20
147-155	0/10	1/10	1/20
147-156	0/10	1/10	1/20
174-182	1/10	2/10	3/20
347-355	0/10	0/10	0/20
458-467	4/10	4/10	8/20
512-520	5/10	4/10	9/20
607-615	2/10	3/10	5/20
629-637	0/10	1/10	1/20
702-710	7/10	9/10	16/20
702-711	2/10	5/10	7/20
KIF21B			
220-228	2/10	3/10	5/20
473-482	2/10	2/10	4/20
621-630	2/10	5/10	7/20
670-678	2/10	2/10	4/20
670-679	2/10	2/10	4/20
731-740	1/10	1/10	2/20

IgG, immunoglobulin G; KIF, kinesin superfamily protein.

peptides could induce HLA-A24-restricted and glioma-reactive CTLs. To this end, the PBMCs from HLA-A24$^+$ glioma patients were stimulated with each of the 8 KIF peptides, and it was determined whether or not the peptide-reactive CTLs can show cytotoxicity against the glioma cells. Among the 8 KIF peptides, only the KIF1C$_{149\text{-}158}$ and KIF3C$_{512\text{-}520}$ peptides efficiently induced glioma-reactive CTLs from the PBMCs of HLA-A24$^+$ glioma patients (Fig. 2). The PBMCs from patients 3 and 5, which were stimulated *in vitro* with either the KIF1C$_{149\text{-}158}$, or KIF3C$_{512\text{-}520}$ peptide, exhibited a higher level of cytotoxicity against the HLA-A24$^+$ KNS-81 cells than against the HLA-A24$^-$ no. 11 cells and the HLA-A24$^+$ T cell blasts.

KIF peptide-specific and CD8$^+$ T cell-dependent cytotoxicity against glioma cells. We also identified the cells responsible for the cytotoxicity of the peptide-stimulated PBMCs. Purified CD8$^+$ T cells were used in the following experiments. As shown in Fig. 3A, the cytotoxicity of the KIF1C$_{149\text{-}158}$ and KIF3C$_{512\text{-}520}$ peptide-induced CD8$^+$ T cells from the HLA-A24$^+$ glioma patients against the KNS-81 cells was significantly inhibited by the addition of anti-HLA class I mAb, but not by the addition of anti-HLA class II (HLA-DR) or anti-CD14 mAb as an isotype-matched control. Furthermore, the cytotoxicity was significantly inhibited by the addition of unlabeled C1R-A24 cells that were pre-pulsed with corresponding KIF peptides, but not by that of HIV peptide-pulsed unlabeled C1R-A24 cells (Fig. 3B). Taken together, the cytotoxicity of the KIF1C$_{149\text{-}158}$ and KIF3C$_{512\text{-}520}$ peptide-stimulated PBMCs against the KNS-81 cells could be ascribed to the corresponding peptide-specific CD8$^+$ T cells.

Discussion

In this study, we applied cDNA microarray technology to identify new vaccine targets in specific immunotherapy for malignant glioma, and examined the potential of three KIFs as vaccine candidates. Although several glioma-associated antigens have been identified to date (4-6), the three KIFs examined here could be used as additional target antigens.

The KIF proteins are a large superfamily of molecular motors (17). KIFs use the energy of ATP hydrolysis to translocate cargo along microtubules, whereas their biological function is acting as a microtubule-based motor protein involved in intracellular organella transport (18). Because of their ATP activity, kinesins are considered as targets for anti-mitotic drug development (19). Certain studies have shown evidence that the kinesin family forms complexes with the proteins involved in tumorigenesis, such as the Aurora kinases (20) and BRCA2 (21). The anti-tumor activity of kinesin inhibitors has also been reported (22,23). Kinesins could be interesting targets in the field of anti-cancer molecular therapeutics.

KIF1C contains an N-terminal motor domain followed by a U104 domain. It has been reported to be expressed in all human tissues examined, and is required for the retrograde transport of Golgi vesicles to the endoplasmic reticulum (24). KIF3C is a subunit of the KIF3 family that forms the kinesin-II motor complex (25). In mice, KIF3C has been reported to be expressed in the nervous system during embryonic development and is up-regulated during neuroblastoma differentiation (26). As for KIF21B, mouse Kif21b was isolated from a retina cDNA library, and has been shown to be expressed in the brain and spleen, and to a lesser degree in the testes (27), but there is no information about human KIF21B. We have recently reported that a unique gene having homology with the KIF18A encodes a tumor-associated antigen recognized by the CTLs from colon cancer patients (28). However, this is the first report suggesting that KIFs could be target antigens in the treatment of malignant glioma.

It is unknown whether or not these antigens are superior to previously reported antigens as vaccine candidates. Due to their preferential expression in malignant glioma tissues compared to benign glioma and a panel of normal tissues in the results of the cDNA microarray, we tested the possibility of whether these gene products and their peptides could be

Table IV. Induction of peptide-specific CTLs from the PBMCs of HLA-A24+ glioma patients or healthy donors.

	KIF1C			KIF3C			KIF21B			
Subiects	149-157	149-158	340-348	458-467	512-520	702-710	220-228	621-630	EBV	Flu
	IFN-γ (pg/ml)									
Patient										
1	92	0	66	34	24	11	0	0	0	29
2	0	11	16	0	0	0	29	10	0	0
3	**224**	30	**222**	30	48	73	56	0	58	**167**
4	50	0	80	**110**	27	0	**149**	0	0	0
5	0	**123**	**593**	66	**345**	**110**	**346**	**194**	0	**255**
6	**121**	**145**	**221**	55	91	**357**	**170**	**154**	71	**137**
7	**142**	38	65	**138**	**211**	**243**	51	93	**142**	95
8	0	**203**	96	38	285	96	**215**	**198**	0	0
9	**162**	0	52	**162**	0	22	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0
Total	4/10	3/10	3/10	3/10	3/10	3/10	4/10	3/10	1/10	3/10

CTLs, cytotoxic T lymphocytes; HLA, human leukocyte antigen; KIF, kinesin superfamily protein; IFN-γ, interferon γ; EBV, EB virus; Flu, influenza virus. The PBMCs from HLA-A24+ glioma patients and healthy donors were stimulated *in vitro* with the indicated peptides as described in Materials and methods. On day 15, the cultured PBMCs were tested for their reactivity to C1R-A24 cells, which were pre-pulsed with a corresponding peptide. The HIV peptide was used as the control. We show the significant values of P<0 05 by the two-tailed Student's t-test and the difference of 100 pg/ml in the IFN-γ production compared with the response to the HIV peptide. The positive results are shown in bold.

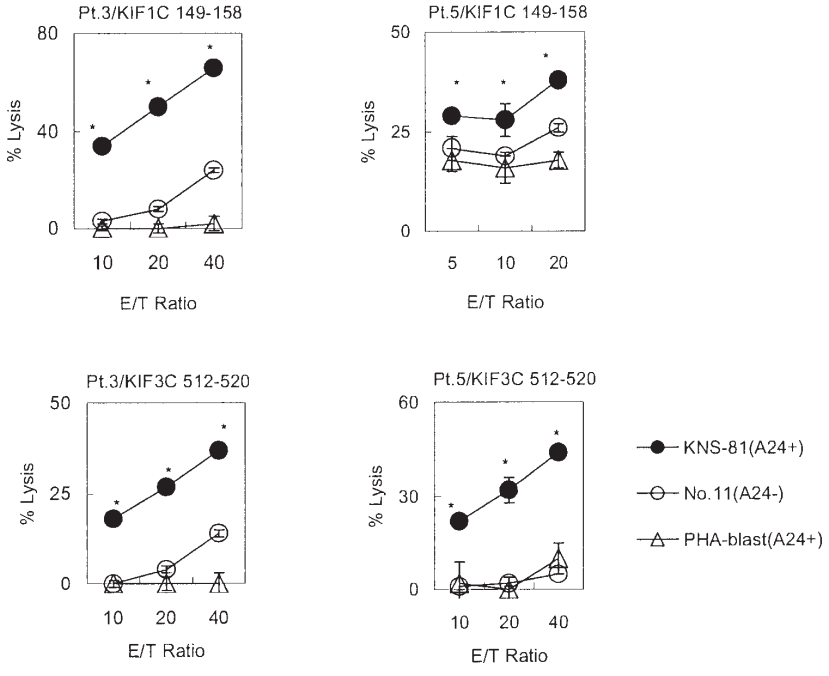

Figure 2. The induction of glioma-reactive cytotoxic T lymphocytes (CTLs) from human leukocyte antigen (HLA)-A24+ glioma patients with two kinesin superfamily protein (KIF) peptides. The KIF1C$_{149-158}$ and KIF3C$_{512-520}$ peptides were tested for their ability to induce CTLs from the PBMCs of two HLA-A24+ glioma patients. The PBMCs were stimulated with each of the KIF peptides (10 μg/ml) in the presence of interleukin (IL)-2 (100 units/ml) every 3 days for 15 days, followed by incubation with IL-2 alone for an additional 15 days. Their cytotoxicity against the HLA-A24+ KNS-81 cells was tested, and the HLA-A24- no. 11 cells and the HLA-A24+ PHA-blast cells were used as the negative controls. The CTL assay was performed by a standard 6-h ^{51}Cr-release assay in triplicate determinations. The two-tailed Student's t-test was employed for the statistical analysis (*P<0.05) against the negative controls.

target molecules in specific immunotherapy for glioma patients. To find the CTL epitopes, we first screened the KIF peptides by their ability to be recognized by the IgGs in glioma patients. This screening was performed based on our previous observations in the clinical trials of a peptide-based vaccine against a variety of cancer types. In those trials, we

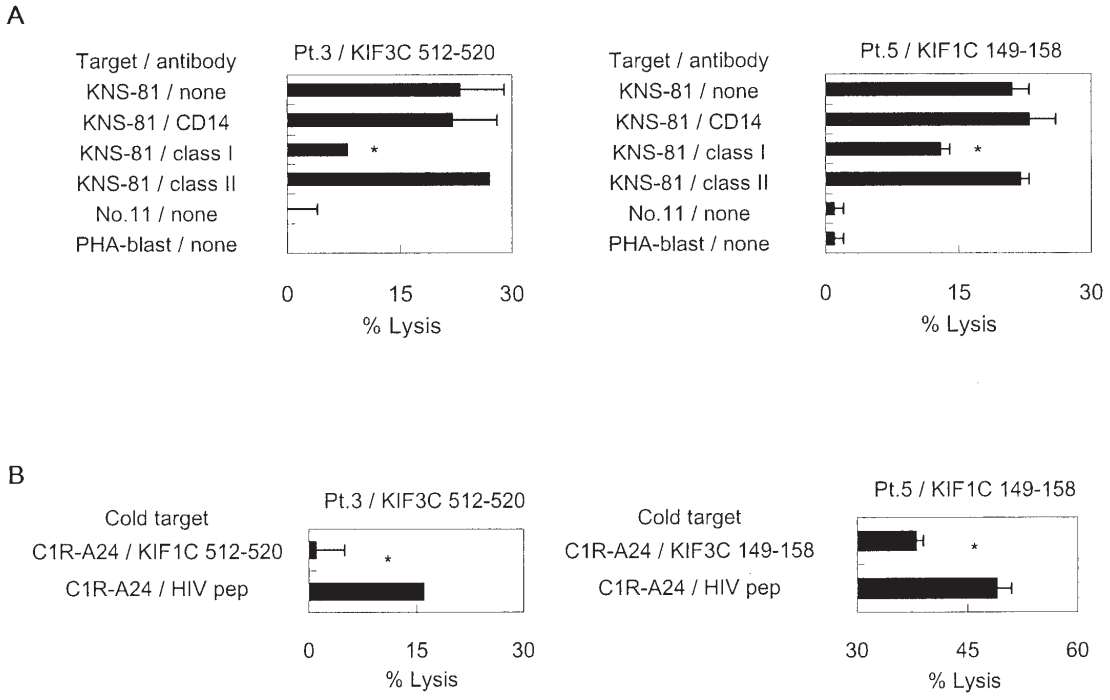

Figure 3. The kinesin superfamily protein (KIF) peptide-specific $CD8^+$ T cell-dependent cytotoxicity against the KNS-81 cells. (A) Purified $CD8^+$ T cells from the KIF peptide-stimulated PBMCs were tested for their cytotoxicity against the KNS-81 cells in the presence or absence of the indicated mAb. The cytotoxic T lymphocyte (CTL) assay was performed by a standard 6-h ^{51}Cr-release assay in triplicate determinations. The two-tailed Student's t-test was employed for the statistical analysis ($^*P<0.05$) against the negative controls. (B) The cytotoxicity of the purified $CD8^+$ T cells from the KIF peptide-stimulated PBMCs was examined by a cold inhibition assay. Unlabeled C1R-A24 cells that were pulsed with the corresponding KIF peptide or the irrelevant HIV peptide were added at a hot to cold target ratio of 10 to 1. The two-tailed Student's t-test was employed for the statistical analysis in this study; a P-value of <0.05 was considered significant.

observed that the peptide vaccination frequently resulted in the induction of IgGs reactive to administered CTL-directed peptides, and that the induction of IgGs reactive to vaccinated peptides was positively correlated with clinical responses and the survival of vaccinated patients (15,16). Indeed, with this approach, we have already identified several peptide candidates that are useful for a peptide-based vaccination (29,30). This approach should be practically useful when patient-derived PBMCs are limited and when there are too many peptide candidates for the *in vitro* stimulation assay. The point to be clarified is whether or not the identified CTL-directed peptides have the potential to induce glioma-reactive CTLs in patients.

In conclusion, we reported that the $KIF1C_{149-158}$ and $KIF3C_{512-520}$ peptides efficiently induced glioma-reactive CTLs from patients. The CTLs showed no cytotoxicity against the PHA-activated T cells, even though they were positive for the mRNA expression of the three KIFs. Therefore, these peptides could be used for peptide-based immunotherapy for glioma patients, although the protein expression of these antigens should be investigated in a panel of glioma and normal tissues before clinical use.

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