ELSEVIER

European Journal of Cancer 41 (2005) 1323–1330

European Journal of Cancer

www.ejconline.com

A unique gene having homology with the kinesin family member 18A encodes a tumour-associated antigen recognised by cytotoxic T lymphocytes from HLA-A2+ colon cancer patients

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Received 29 September 2004; received in revised form 15 February 2005; accepted 17 February 2005
Available online 27 April 2005

Abstract

Colon cancer is one of the major malignant tumours for which the development of a new treatment modality is needed. To provide the scientific basis for a specific immunotherapy for colon cancer, we looked for tumour-associated antigens recognised by cytotoxic T lymphocytes (CTLs) from human leukocyte antigen (HLA)-A2+ colon cancer patients. We report here a unique gene, 3362 base-pairs (bp) long, which has homology with the kinesin family member 18A. This gene was expressed at the mRNA level in the majority of tumour cells, but not in any normal tissues tested except for testis and lung. Two of 16 peptides with HLA-A2-binding motifs were recognised by tumour-reactive CTLs. In addition, these two peptides had the ability to induce HLA-A2-restricted and cancer-reactive CTLs from peripheral blood mononuclear cells (PBMCs) of colon cancer patients with several HLA-A2 subtypes. Overall, this study provides new information about a colon cancer-related antigen that might be an appropriate target for specific immunotherapy in HLA-A2+ colon cancer patients.

Keywords: Colon cancer; Tumour antigens; Kinesin family, HLA-A2; CTL; Peptide

1. Introduction

Colon cancer is one of the most common malignancies worldwide, and the prognosis of advanced colon cancer with distant metastases is extremely poor despite recent clinical trials with chemotherapeutic agents [1]. There is therefore a need for a new treatment modality. One such therapy might be specific immunotherapy, as recent advances in tumour immunology have enabled the identification of many genes encoding tumour antigens and their peptides that are recognised by cytotoxic T lymphocytes (CTLs) [2]. Several colon cancer-related antigens have been described previously [3–8]. Although specific immunotherapies for colon cancer patients using peptides, RNA, or lysates have been conducted [9–11], the clinical responses observed so far have been unsatisfactory. Further studies are therefore needed, and one of these might be the identification of additional colon cancer-related antigens recognised by CTLs.

We previously identified relatively large numbers of tumour-associated antigens and their CTL-directed

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doi:10.1016/j.ejca.2005.02.025

peptides by means of cDNA expression cloning methods with cDNAs of oesophageal, lung and pancreatic cancer cells [12–18]. Some of these peptides were used in the clinical study of cancer vaccines for colon cancer patients, but major tumour regression was rarely obtained in those clinical trials [19,20]. In this study, in order to increase peptide candidates suitable for an anti-cancer vaccine, we attempted to identify additional colon cancer-associated antigens and CTL-directed peptides. We describe here one antigen and two peptides applicable to specific immunotherapy of human leukocyte antigen (HLA)-A2+ colon cancer.

2. Materials and methods

2.1. CTL line

The parental HLA-A2-restricted and tumour-reactive OK-CTL line was established from tumour-infiltrating lymphocytes (TILs) of a colon cancer patient (HLA-A*0207/*3101, -B46/51, -Cw1), as reported previously [18]. This patient has survived more than 5 years since the surgical removal of the main colon tumours without any sign of recurrence. The OK-CTL line with an 80% CD3+CD4-CD8+ phenotype showed both HLA-A2-restricted and tumour-specific CTL activity as measured by both ^{51}Cr-release and interferon (IFN)-γ production assays [18]. One sub-line of this OK-CTL line, OK-CTLf, was used as an indicator in the following experiments.

2.2. Cell lines

Tumour cell lines used as target cells were HLA-A*0207+ Ca9-22 oral squamous cell carcinoma (SCC) cells, HLA-A*0201+ SW620 colon adenocarcinoma cells, HLA-A*0206+ KE3 oesophageal SCC cells, HLA-A2- COLO320 colon adenocarcinoma, HLA-A2- RERF-LC–MS lung adenocarcinoma, autologous Epstein-Barr virus (EBV) transformed B (EBV-B) cells and phytohaemagglutinin (PHA)-activated T cells. SW620 tumour cells were used for preparation of the cDNA library. COS-7 cells or T2 cells were used as the target of a gene-expression cloning method or for pulsing peptides, as reported previously [18]. SW620 and HLA-A*0201+ Panc-1 pancreatic adenocarcinoma was used in the Northern blot analysis. Tumour cell lines used for quantitative real-time polymerase chain reaction (RT-PCR) were colon cancer (SW620, COLO201, COLO205, SW480, KM12LM), lung cancer (QG56, Sq-1, 11–18, LK87, 1087, PC9), liver cancer (Hep-G2, KIM-1, KMCH-1), oesophageal cancer (KE3, KE4, KE8, TE9, TE11), gastric cancer (MKN45, SSTW9), ovarian cancer (TOC-2, KOC-2S, RMUG-C), glioma (KNS42, KALS-1, KNGS-1) and leukaemia (HPBMTL, K562, THP-1, RAJI).

2.3. Identification of a cDNA clone

A previously reported cDNA-expression cloning method [12] was used to identify genes coding for tumour antigens recognised by the OK-CTLf sub-line. A cDNA library was prepared from poly (A)+ RNA of the SW620 colon tumour cells and inserted into the expression vector pCMV-SPORT-2 (Invitrogen, Carlsbad, CA, United States of America (USA)). The cDNA clone of HLA-A*0207, -A*2601, or -A*2402 was obtained by reverse RT-PCR and inserted into the eukaryotic expression vector pCR3 (Invitrogen). COS-7 cells (5×10^3) were co-transfected with cDNA and plasmids encoding HLA class I gene by Lipofectamine (Invitrogen) and cultured for 2 d, followed by addition of the OK-CTLf (5×10^4 cell/well). After an 18-h incubation, the level of IFN-γ in supernatants was determined by enzyme-linked immunosorbent assay (ELISA). The homology of sequences of cloned genes was analysed using databases in the European Molecular Biology Laboratory GenBank/DDBJ homepage and SEREX database (www-ludwig.unil.ch/SEREX.html). A BLASTP search was performed using the non-redundant Swiss-Prot database, and a motif scan in a predicted protein sequence using the Prosite and ISREC (hits.isb-sib.ch/cgi-bin/PFSCAN) databases. In Northern blot analysis, total RNAs (10 μg/lane), extracted from the peripheral blood mononuclear cells (PBMCs) and PHA-blast cells of healthy volunteers and from SW620 and Panc-1 tumour cells, were employed with ^{32}P-labelled probe cDNA of a 1109-base-pair (bp) cDNA fragment at positions 2146–3254 of SW#108 digested with *AvaII*.

2.4. Quantitative PCR

Gene expression was quantified by RT-PCR using ABI PRISM 7000 (Applied Biosystems, Foster City, CA, USA) as reported previously [21]. RT-PCR of cDNA specimens was conducted in a total volume of 12.5 μl with 1 × TaqMan Master mix (Applied Biosystems), and 1.25 μl of mixture of primers and probe. Primers and TaqMan probes used in this study were purchased from Applied Biosystems (Assay ID#: Hs00229692). Thermal cycler parameters included 2 min at 50 °C, 10 min at 95 °C, and 40 cycles involving degeneration at 95 °C for 15 s and annealing extension at 60 °C for 1 min. RNAs were extracted using the RNA-Bee RNA isolation reagent (Tel-Test, Inc., Friendswood, TX, USA) method according to the manufacturer's instructions. Total RNAs from normal tissues (colon, liver, lung testis, spleen, placenta, heart, muscle, stomach, kidney and brain) were purchased

from Sawaday Technology (Tokyo, Japan). Complimentary DNA (cDNA) of mRNA was prepared from 5 μg of total RNA by SuperScript Preamplification System (Invitrogen) according to the manufacturer's instructions.

2.5. Peptides and CTL assay

The only difference in the peptide sequence between HLA-A*0201 and HLA-A*0207 was the 123rd amino acid, which was Y or C, respectively. This amino acid was located in the coil region in the 2nd structure but not in the α-helix or β-sheet, which was involved in peptide binding, and thus this difference might not influence the binding of the peptide. Consequently, peptides capable of binding to the HLA-A*0207 molecules were searched at the literature level with regard to peptides for HLA-A*0201-binding motifs [22], and 16 different peptides (>70% purity) were synthesised for screening. To identify CTL-directed epitopes, we incubated the OK-CTLf for 18 h with T2 cells pre-pulsed with each peptide at different doses for 2 h, followed by harvesting of supernatant to measure IFN-γ by ELISA. Two peptides at positions 101–109 and 576–585 with >90% purity were provided for the *in vitro* CTL induction. An HIV-derived peptide (SLYNTYATL) with an HLA-A2-binding motif was used as a negative control. All peptides were dissolved with dimethyl sulphoxide at a dose of 10 mg/ml.

2.6. CTL induction

PBMCs (1×10^5/well) obtained from HLA-A2+ cancer patients were incubated with 10 μM of the peptide in 96-well round plate in the presence of 100 U/ml IL-2, as previously reported [18]. At culture days 3, 6 and 9, the culture supernatant of each well was replaced in fresh medium containing 10 μM of the corresponding peptide for re-stimulation of the cells. After 15 d, the cultured cells were additionally cultured with IL-2 100 U/ml without the peptide stimulation for approximately 15 d. Approximately 1 month after starting the *in vitro* culture, the cultured cells were used as effector cells in a CTL assay. In some experiments, 20 μg/ml of anti-HLA class I (W6/32), anti-HLA class II (H-DR-1), anti-CD4 (Nu-Th/I), anti-CD8 (Nu-Ts/c) and anti-HLA-A2 (BB7.2) were used. Anti-CD14 (JML-H14, IgG2a) served as an isotype-matched control mAb. A two-tailed Student's *t*-test was employed for the statistical analysis.

3. Results

The HLA-A2-restricted and tumour-specific CTL line was established from TILs of a patient with colon adenocarcinoma [18]. One of the sub-lines (OK-CTLf) with an 80% CD3+CD4-CD8+ phenotype showed HLA-A2-restricted and tumour-specific CTL activity as measured by IFN-γ production assay (data not shown) and ^{51}Cr-release assay (Fig. 1). Namely, the OK-CTLf cells showed cytotoxicity against tumour cells with different HLA-A2 subtypes, but not to any of HLA-A2- tumour cells, autologous PHA-blasts or autologous EBV-B cells. With this OK-CTLf line, 1000 pools of 100–150 cDNA clones (a total of 1–1.5×10^5 cDNA clones) from the cDNA library of SW620 tumour cells were screened by the gene-expression cloning method, followed by selection to identify specific cDNAs within positive pools. After repeated experiments, we identified one cDNA clone of 3362 bp length (SW#108: Accession No. AB062483). Representative results of the CTL assays are shown in Fig. 2. This OK-CTLf line showed the reactivity against COS-7 cells only when co-transfected with both the SW#108 gene and the HLA-A*0207 cDNA. However, they did not react to COS-7 cells when transfected with the SW#108 gene alone or co-transfected with the HLA-A*0207 cDNA and a cDNA clone #001 taken as a negative control. In addition, this CTLf line failed to react to COS-7 cells transfected with the SW#108 gene and HLA-A*2402 or HLA-A*2601 cDNA.

Nucleotide sequencing of the SW#108 gene revealed that it was highly similar to the H. sapiens kinesin family member 18A gene (accession number BC048347, NP_112494.2) and they were completely identical at the amino acid level. Although the kinesin family consists of a variety of molecules with different expression patterns in various tissues, the family's fundamental

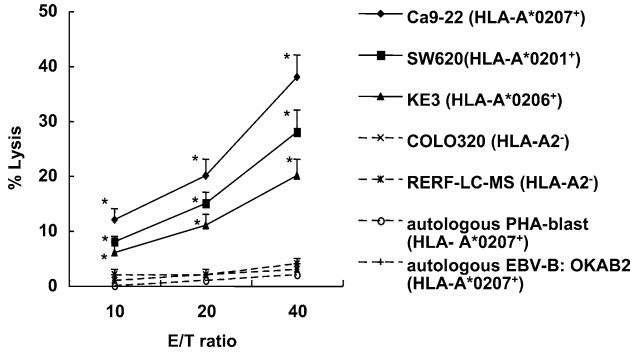

Fig. 1. HLA-A2-restricted cytotoxicity of the cytotoxic T lymphocytes, OK-CTLf cells. Cytotoxicity of OK-CTLf against seven target cells was tested by a 6-h ^{51}Cr-release assay at different E/T ratios in triplicate. Target cells were HLA-A*0207+ Ca9-22 head and neck SCC, HLA-A*0201+ SW620 colon adenocarcinoma, HLA-A*0206+ KE3 oesophageal squamous cell carcinoma (SCC), HLA-A*2402+ COLO320 colon adenocarcinoma and HLA-A*1101+ RERF-LC–MS lung adenocarcinoma, autologous phytohaemagglutinin (PHA)-blastoid cells and autologous Epstein-Barr virus (EBV)-B cells (OKAB2). A two-tailed Student's *t*-test was used for statistical analysis (*$P < 0.05$) against a negative control (HLA-A2- COLO320 cell line). E/T, effector/target.

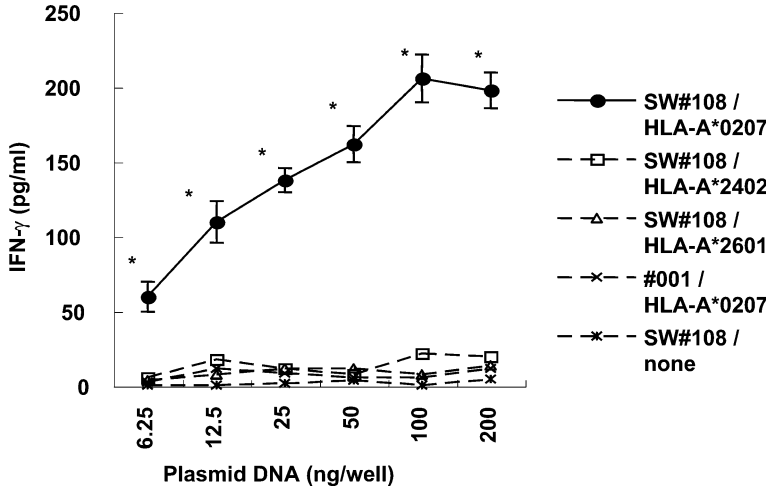

Fig. 2. HLA-A*0207-restricted recognition of the SW#108 gene product by the cytotoxic T lymphocytes, OK-CTLf cells. Different amounts of the SW#108 gene and 100 ng of HLA-A*0207, -A*2402, or -A*2601 cDNA were co-transfected into COS-7 cells, incubated for 48 h, and then tested for their ability to stimulate interferon (IFN)-γ release by the OK-CTLf line. The background of IFN-γ release by the CTLs in response to COS-7 cells (under 50 pg/ml) was subtracted from the values in the figure. The cDNA #001 was used as an irrelevant cDNA clone that was not recognised by the OK-CTLf cells. Values represent the means of triplicate assays. A two-tailed Student's *t*-test was used (*$P < 0.05$).

function is believed to be the action of a microtubule-based motor protein involved in intracellular organella transport [23]. A motif scan of this protein sequence by ISREC suggested that it possesses two possible transmembrane helices with scores above 500. Except for these findings, there is no information available on the SW#108 gene.

We next analysed the mRNA expression of the SW#108 gene by Northern blot analysis (Fig. 3(a)). The length of the SW#108 mRNA was approximately 3800 bases, and its expression level was high in Panc-1 and SW620 tumour cells, very low in PHA-blast cells, and undetectable in PBMCs. We further investigated the mRNA expression of this gene by quantitative RT-PCR (Fig. 3(b)). SW#108 mRNA expression was standardised by β-actin mRNA expression. SW#108 mRNA expression in SW620 was calculated as expression level 1, and the relative expression of SW#108 mRNA in the other tumour cells and normal tissues or cells are demonstrated in Fig. 3(b). As a result, SW#108 mRNA was expressed in the majority of tumour cells tested, but was not detectable in normal tissues except for the testis and the lung. These results indicate that this gene was preferentially expressed in the majority of tumour cells.

To determine epitope peptides recognised by the OK-CTLf, we prepared 16 different SW#108-derived peptides based on the HLA-A2 molecule-binding motif [22]. These peptides were individually pulsed on T2 cells, followed by testing their ability to stimulate IFN-γ release by the OK-CTLf cells. An HIV-derived peptide with an HLA-A2-binding motif was used as a negative control. The peptide sequence and position of the 1st amino acid of the peptides are shown on the right side of the column. Among them, two peptides at positions 101–109 and 576–585 were recognised by the OK-CTLf cells (Fig. 4(a)). The increase in IFN-γ production was dependent on the amounts of peptides added to the culture in both cases (Fig. 4(b)). Although a sudden drop was observed in the SW#108 101–109 peptide curve at a dose of 10^4 ng/ml, this seemed to be due not to toxicity of the peptide but to dimethyl sulphoxide in which the peptides was dissolved.

It is crucial to determine whether these peptides could induce HLA-restricted and peptide-specific CTLs cytotoxic to colon tumour cells. Therefore, we tested the two peptides for their ability to induce CTLs from PBMCs of six HLA-A2+ colon cancer patients (subtypes; two HLA-A*0201, two A*0206, and two A*0207) and five HLA-A2+ healthy donors (one HLA-A*0201 and four HLA-A*0206). Their PBMCs were stimulated with each of the two peptides (10 μg/ml) in the presence of IL-2 (100 U/ml) every 3 d for 15 d followed by incubation with IL-2 alone for an additional 15 d. The peptide-stimulated PBMCs were used for CTL assay after approximately 1 month's culture. The cultured cells were tested their cytotoxicity by a 6-h ^{51}Cr-release assay against HLA-A2+SW620 tumour cells (Fig. 5(a)). HLA-A2- QG56 tumour cells and HLA-A2+ PHA-blast cells were also used as controls. As a result, the SW#108 101-109 and SW#108 576–585 peptides induced significant levels of HLA-A2-restricted cytotoxicity against HLA-A2+ SW620 tumour cells from the PBMCs from colon cancer patients with several HLA-A2 subtypes. In addition, cytotoxicity levels were significantly inhibited by anti-class I (W6/32) or anti-CD8 mAb, but not by the other mAbs tested. Anti-peptide specificity of CTLs was further confirmed by cold inhibition assay (Fig. 5(b)). The cytotoxicity of CTLs was

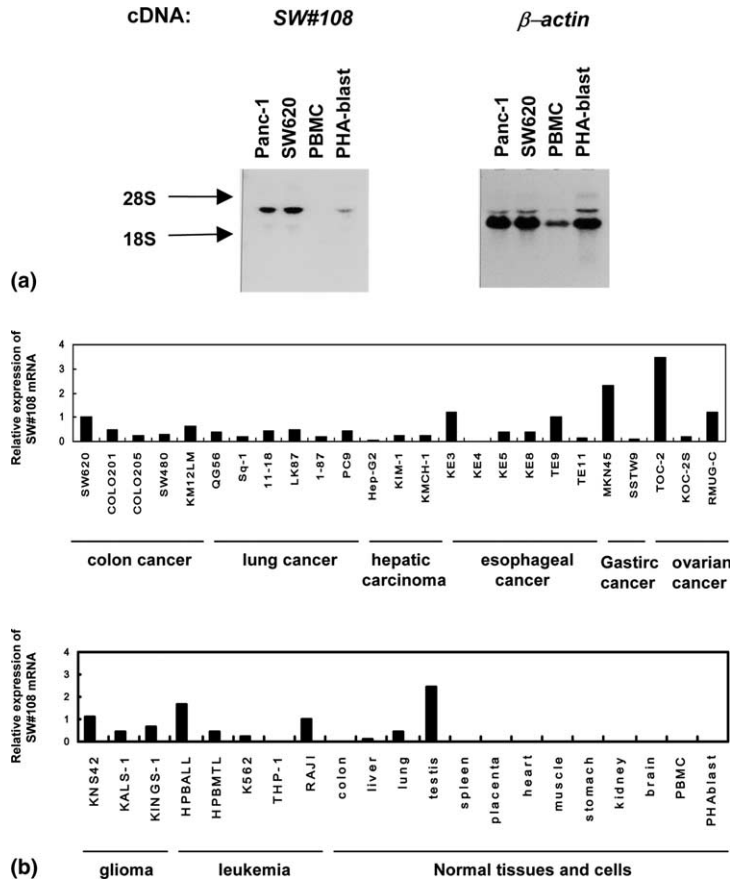

Fig. 3. SW#108 mRNA expression. (a) Northern blot analysis was performed. Ten μg total RNA were provided for Northern blot analysis. Expression of β-actin was assayed as a control. (b) Real-time polymerase chain reaction (RT-PCR) was performed using RNA from various tumour cell lines, normal tissues and cells. The SW#108 mRNA expression was standardised by the β-actin mRNA expression. The SW#108 mRNA expression in SW620 was calculated as expression level 1, and the relative expression of the SW#108 mRNA in other samples are demonstrated. PBMC, peripheral blood mononuclear cells; PHA, phytohaemagglutinin.

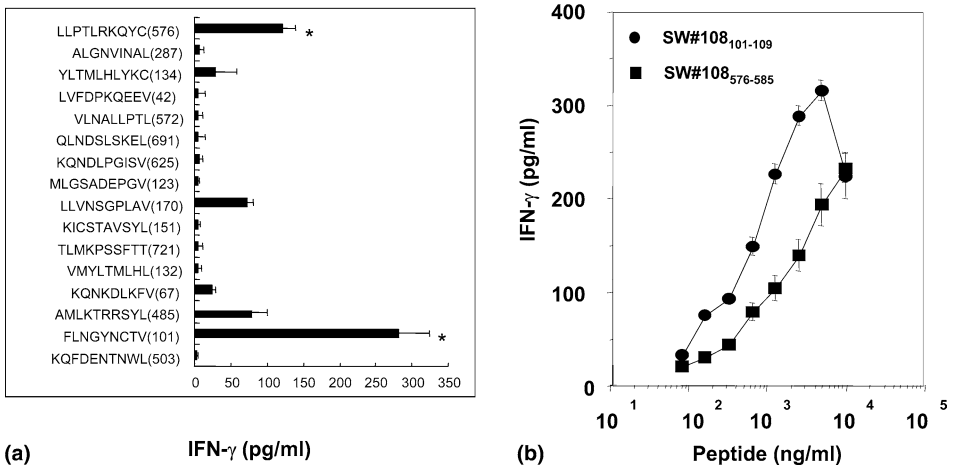

Fig. 4. Recognition of two epitope peptides by the cytotoxic T lymphocytes, OK-CTLf cells. (a) Each of the peptides (9–10 mer) with an HLA-A2 binding motif was loaded onto T2 cells. The OK-CTLf cells were then added and incubated for 18 h, followed by collection of cell-free supernatant for measurement of interferon (IFN)-γ. Values indicate the means of triplicate assays. The background of IFN-γ release by the CTLs (under 50 pg/ml) in response to the T2 cells alone was subtracted from the values in the figure. Peptides on T2 cells that could stimulate a significant amount of IFN-γ production are indicated (*$P < 0.05$ by two-tailed Student's t-test and 100 pg/ml > [IFN-γ]). (b) The level of IFN-γ production was dependent on the doses of peptides in both cases. Values are the mean of the triplicate assays.

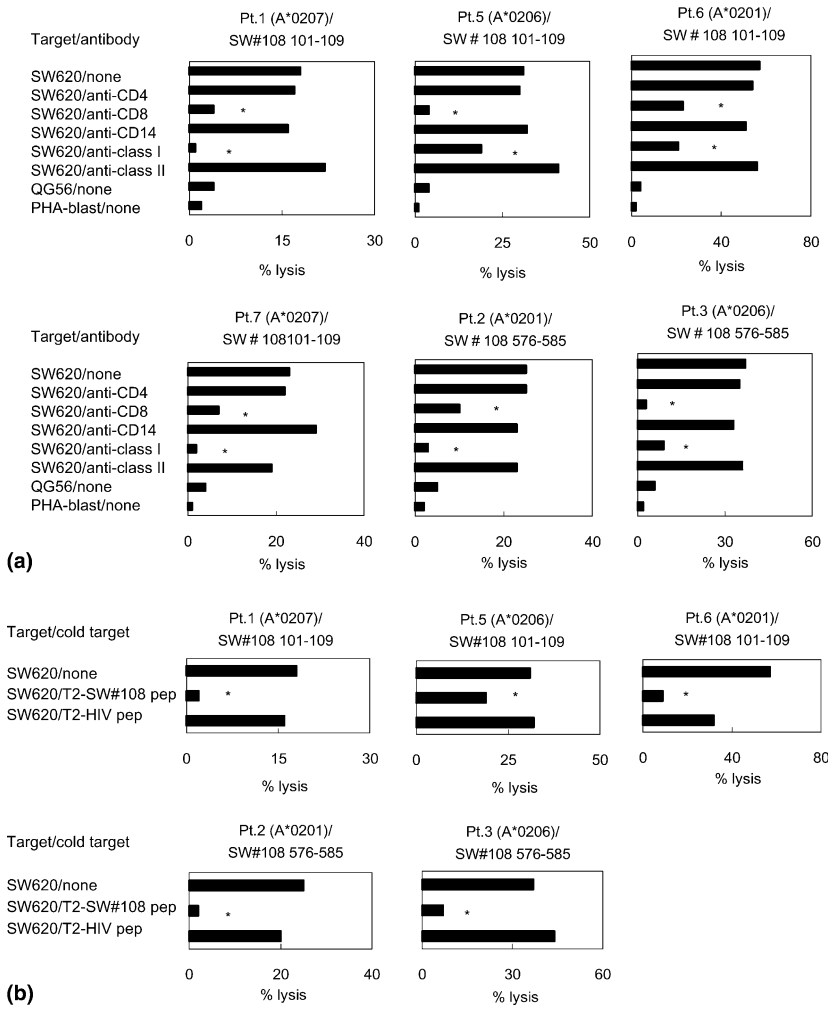

Fig. 5. Peptide-specific and HLA class I-restricted cytotoxicity of peptide-stimulated peripheral blood mononuclear cells (PBMCs) from colon cancer patients. The SW#108 101–109 and SW#108 576–585 peptides were tested for their ability to induce cytotoxic T lymphocytes, CTLs from peripheral blood mononuclear cells (PBMCs) of 6 colon cancer patients with several HLA-A2 subtypes. Patients 2 and 6 had A*0201 genotype, patients 3 and 5 had A*0206 genotype, and patients 1 and 7 had A*0207 genotype. PBMCs were stimulated with each of the peptides (10 μg/ml) in the presence of IL-2 (100 U/ml) every 3 d for 15 d, followed by incubation with interleukin (IL)-2 alone for an additional 15 d. (a) Their cytotoxicity against HLA-A*0201+ SW620 tumour cells was tested in the presence or absence of the indicated monoclonal antibody (mAb). The CTL assay was performed by a standard 6-h ^{51}Cr-release assay at an effector to target cell ratio of 10:1 in the triplicate determinants. HLA-A2-QG56 tumour cells and HLA-A*0201+ PHA-blast cells were used as negative controls. (b) The peptide specificity of the CTLs was also confirmed by cold inhibition assay. Unlabelled T2 cells pulsed with a corresponding SW#108 peptide or an irrelevant HIV peptide were added at a hot to cold target ratio of 20:1. This assay was not performed in the case of patient 7 because of the limitation of peptide-stimulated PBMCs. The result of patient 7 was not shown because the peptide-stimulated PBMCs were not enough to perform the assay. A two-tailed Student's *t*-test was used for statistical analysis in this study; a P value < 0.05 was considered significant.

inhibited by the addition of corresponding peptide-pulsed unlabelled T2 cells, but not by that of the HIV peptide-pulsed unlabelled T2 cells. The cold inhibition assay was not performed in the case of patient 7 because of the limitation of peptide-stimulated PBMCs. In contrast to cancer patients, the peptide-stimulated PBMCs from any of five healthy donors did not exhibit HLA-A2-restricted cytotoxicity against tumour cells (data not shown). These results suggest that these two peptides had the ability to induce HLA-A2-restricted CTLs and their CTL activity was largely mediated by peptide-reactive and HLA-class I-restricted CD8+ T cells.

4. Discussion

This study identified a unique gene that encodes a tumour-associated antigen recognised by HLA-A2-restricted and colon cancer-reactive CTLs. The SW#108 gene was highly homologous with the kinesin family member 18A gene. Regarding the kinesin gene, 41 genes from human origin and 268 genes from all species have been registered as a kinesin family, and those genes are divided into 10 subfamilies, according to the kinesin home-page (www.proweb.org/kinesin/Kinesin.html). However, the SW#108 gene homologue, HsPKFZp434

(AL136819), has not yet been grouped into any subfamily. Although the kinesin family consists of relatively large numbers of different molecules, its biological function is believed to be the action of a microtubule-based motor protein involved in intracellular organella transport [23]. Because of their adenosine triphosphatase activity, kinesins are considered as targets for anti-mitotic drug development [24]. In addition, recent studies showed evidence that the kinesin family form complexes with proteins involved in tumourgenesis, such as the Aurora kinases [25] and BRCA2 [26]. However, no information about the function of kinesin family member 18A gene is available. If this gene plays crucial roles in cancer survival and growth, it could be a promising target in specific immunotherapy. This will be the subject of a future study.

Regarding the expression profile, some members of the kinesin family, such as neuronal kinesin, seem to be expressed preferentially in tumour cells, whereas others, such as ubiquitous kinesin, are ubiquitously expressed [27]. In this study, we showed that SW#108 mRNA was preferentially detected in various types of cancer cells, as well as in normal testis and lung. In spite of the expression in the testis, the SW#108 antigen seems not to be the cancer-testis antigen [28] because of its expression in the lung.

Several colon cancer-related antigens, including CEA, HER2/neu, MUC-1 and ras-oncogene, have been identified so far [3–8]. We suppose that the SW#108 antigen is an additional antigen. In addition, it is unknown whether or not this antigen is superior to previously reported antigens as a candidate in specific immunotherapy. However, because of the preferential expression in cancer cells, we tested the possibility that this gene product and its peptides could be a target molecule in specific immunotherapy for colon cancer patients. We looked at whether SW#108 peptide-specific CTLs could be induced from the PBMCs of HLA-A2+ colon cancer patients, and whether such CTLs could show cytotoxicity against colon cancer cells. It was found that both the SW#108 101–109 and SW#108 576–585 peptides had the potential to induce colon cancer-reactive CTLs from colon cancer patients. Such CTLs showed no cytotoxicity against PHA-activated normal PBMCs. Because this gene was expressed in the lung at a moderate level, it is critical to determine whether SW#108 peptide-specific CTLs could show cytotoxicity against normal lung tissues. However, this was not tested as we could not obtain normal lung tissues. At present, the SW#108 antigen and its peptides are not candidates for clinical trials.

More than 1500 types of tumour antigens were identified by the serological analysis of recombinant cDNA expression cloning (SEREX) method [29]. There was partial homology at the protein level between SW#108 and ID680, one of the tumour antigens identified by the SEREX method with sera of colon cancer patients. Because the SEREX method utilises immunoglobulin G to identify candidates of cancer antigens, the SW#108 gene product could also be recognised by the humoral immune system.

The HLA-A2 allele is found in 23% of Black Africans, 53% of Chinese, 40% of Japanese and 50% of Caucasians [30]. HLA-A*0201 is found in the majority of HLA-A2+ Caucasians and also in 45% of HLA-A2+ Japanese, while HLA-A*0206 is found in 36% of HLA-A2+ Japanese. HLA-A*0207 molecules share similar peptide-binding motifs with HLA-A*0201 molecules [22]. Regarding HLA-A2 subtypes, the SW#108 101–109 and SW#108 576–585 peptides had the ability to generate HLA-A2-restricted and colon cancer-reactive CTLs from colon cancer patients with several HLA-A2 subtypes. Further studies with more samples with different HLA-A2 subtypes are needed to confirm this observation.

Conflict of interest statement

None declared.

Acknowledgements

This study was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan (No. 12213134 to K.I. and Research Center of Innovative Cancer Therapy of 21th Centry COE Program for Medical Science to K.I. and S.S.), from the Japan Society for the Promotion of Science (No.14570526 to S.S.) and from the Ministry of Health and Welfare, Japan (No. H14-trans-002, 11–16, and H12-cancer-004 to K.I., and No. H14-cancer-033 to S.S.).

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