

Identification of *HLA-A*0201*/*-A*2402*-restricted CTL epitope-peptides derived from a novel cancer/testis antigen, *MCAK*, and induction of a specific antitumor immune response

MEGUMI KAWAMOTO^{1,3*}, FUMIAKI TANAKA^{1,3*}, KOSHI MIMORI¹, HIROSHI INOUE¹,
YUKIO KAMOHARA¹ and MASAKI MORI^{1,2}

¹Department of Surgery, Medical Institute of Bioregulation, Kyushu University, 4546 Tsurumihara, Beppu 874-0838;

²Department of Gastroenterological Surgery, Osaka University Graduate School of Medicine, Osaka 565-0871, Japan

Received July 21, 2010; Accepted September 23, 2010

DOI: 10.3892/or.2010.1101

Abstract. Cancer immunotherapy is a potential therapeutic strategy, in addition to surgical treatment, radiotherapy, and chemotherapy. Cancer-specific immunotherapy, such as the MAGE peptide vaccine, has been utilized clinically. However, there are inherent limits to the effectiveness of vaccination using a single antigen because of the expression frequency of cancer-specific antigens on tumor cells. Thus, identification of a new cancer-specific antigen is needed. In this study, we examined the possibility of using cancer-specific immunotherapy based upon mitotic centromere-associated kinesin (MCAK) which was previously identified as a novel cancer/testis antigen. To evaluate the feasibility of developing cancer immunotherapy using MCAK peptides, we studied *HLA-A*0201* and **2402* as targets for CTLs in the context of HLA class I molecules. By using a peptide with a sequence of AINPELLQL (amino acid positions 63-71 in MCAK, *HLA-A*0201*) and FFEIYNGKL (amino acid positions 401-409 in MCAK, *HLA-A*2402*), CTL responses could be induced from unseparated PBMCs by stimulation of freshly isolated, peptide-pulsed PBMCs as antigen-presenting cells (APCs) and also by using interleukin-7 and keyhole limpet hemocyanin in primary culture. The induced CTLs could lyse *HLA-A*0201*2402* colon and gastric cancer cells expressing MCAK, as well as the peptide-pulsed target cells,

in an HLA class I, and CD8 restricted manner. The identification of the *MCAK/HLA-A*0201* and **2402* peptides suggests the possibility of designing peptide-based immunotherapeutic approaches that might prove effective in treating patients with MCAK-positive cancer.

Introduction

In cancer treatment, remarkable progress has been made recently through genetic and molecular approaches. In cancer immunotherapy, a target antigen must be highly expressed only in cancer cells. The conventional immunopotentialization therapy was expected to induce an antitumor effect by immunoreaction which was activated non-specifically. On the other hand, the recent study is focused on that how we let it elicit specific immune response effectively. In particular: i) identification of cancer specific antigen and its peptide shown by human histocompatibility leukocyte antigen; HLA, ii) the development of the activation method of a T cell recognition. The target antigen of the cancer immunotherapy is needed to be highly expressed only in cancer cells and not expressed in normal cells. At present, cancer/testis (CT) antigens are considered as one of the suitable targets. The antigen groups were expressed in various kinds of cancer tissue and only in a testis and thymus, in some antigens, in the normal tissue. The *MAGE* gene characteristic of human melanoma is representative (1). Testis cells have little or no expression of MHC molecules and little ability to present an antigen to T cells. Therefore, CT antigens are considered good targets for cancer immunotherapy. As for CTLs recognizing the CT antigen, they are thought to attack cancer cells selectively without attacking the testis cells which have no MHC molecule (2).

We recently reported that mitotic centromere-associated kinesin (MCAK) was overexpressed in gastric cancer cells, mediating poor prognosis (3), only in cancer cells and the in testis of the adult man in the normal tissue (4). Considering this result, we hypothesized that MCAK could be a CT antigen. MCAK is a member of the kinesin family. It is found throughout the cell and is especially concentrated at the centromeres, kinetochores and spindle poles (5). Kinesin family (KIF) proteins with the kinesin motor catalytic domain

Correspondence to: Professor Masaki Mori, Department of Gastro-enterological Surgery, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
E-mail: mmori@gesurg.med.osaka-u.ac.jp

*Contributed equally

Abbreviations: MCAK, mitotic centromere-associated kinesin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RT-PCR, reverse transcription-polymerase chain reaction; PBMCs, peripheral blood mononuclear cells

Key words: cancer/testis antigen, cytotoxic T lymphocyte, immunotherapy

and the coiled-coil domain are microtubule-dependent molecular motors (6-9). Members of the kinesin superfamily play an important role in intracellular transport and cell division (10). Unlike other kinesins that transport cargo, MCAK and the other members of the Kin-I subfamily of kinesins catalyze microtubule disassembly, a key aspect of normal chromosome movement (5). Regarding the association of MCAK with cancer, MCAK was reported to be highly expressed in colon cancer tissue (11), breast cancer (12,13) and gastric cancer (3). Therefore, MCAK is a suitable target antigen for cancer-specific immunotherapy.

In this study, we identified MCAK peptides, which could induce CTL, to clarify the possibility of cancer specific immunotherapy using novel cancer/testis antigen MCAK as target antigen.

Materials and methods

Cell lines. The human chronic myeloid leukemic cell line K562, colon cancer cell line RCM1, colo201, colo205, colo320DM, DLD1, WiDr, and the gastric cancer cell line NUGC3 were supplied by the Japanese Collection of Research Bioresources Cell Bank (Tokyo, Japan). The gastric cancer cell lines MKN45, KATOIII, AZ521 were obtained from the Cell Resource Center for Biomedical Research, Tohoku University. RCM1 was maintained in 45% RPMI-1640 with 45% Ham's F12, 10% fetal bovine serum. Other cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μ g/ml streptomycin.

Total RNA extraction. Frozen tissue specimens were homogenized in guanidium thiocyanate, and total RNA was obtained by ultracentrifugation through a cesium chloride cushion, as described previously (11).

Quantitative real-time PCR. Total RNA was extracted from each bulk sample and cDNA was synthesized from 8.0 μ g total RNA as described previously (14). The following primers were used to amplify the *MCAK*: sense primer, 5'-GATGGAAGCCTGCTCTAACG-3', and antisense primer 5'-GAGCAGATTCCGCTTTGTTC-3'. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; sense primer 5'-TTGG TATCGTGGAAGGACTCA-3' and antisense primer 5'-TG TCATCATATTTGGCAGGTTT-3') was used as an internal control. Reactions were performed in a Light Cycler system (Roche Applied Science, Indianapolis, IN) using the Light Cycler Fast Start DNA Master SYBR-Green I kit (Roche Diagnostics, Mannheim, Germany). Details of each reaction are described elsewhere (15). Briefly, thermal cycling for all genes was initiated with a denaturation step of 95°C for 10 min, followed by 40 cycles at 95°C for 10 sec, 66°C (60°C for *GAPDH*) for 10 sec, and 72°C for each optimal length (1 sec/25 bp). All calculated concentrations of target genes were divided by the amount of the endogenous reference (*GAPDH*) to obtain normalized *MCAK* expression values. Each assay was performed in triplicate.

Synthetic peptide. To screen for *HLA-A*0201* and *-A*2402*-restricted peptide sequences of MCAK, we used two epitope

prediction algorithms (BIMAS, http://bimas.dcrf.nih.gov/molbio/hla_bind; SYFPEITHI, <http://syfpeithi.bmi-heidelberg.com>). Five peptides of nine residues were found to contain the binding motif for *HLA-A*0201* and *-A*2402* with a highest predicted binding in both algorithms. Peptides used for CTL induction were purchased from Sigma-Aldrich (St. Louis, MO) and were purified by repeated ether precipitations. Purity was determined by analytical reverse phase high-performance liquid chromatography and proven to be $\geq 95\%$ pure. Peptides were dissolved in DMSO and stored at -20°C before use.

CTL induction. CTL induction *in vitro* was performed according to the procedure described previously (16,17). In brief, PBMCs of healthy donors (*HLA-A*0201* or *-A*2402*) were collected by centrifugation on a Ficoll-Paque density gradient. Written informed consent was obtained from all healthy donors. PBMCs were pre-pulsed by purified peptides at a final concentration of 20 μ g/ml for 2 h at 37°C. Cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 50 μ M 2-mercaptoethanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan), with the addition of keyhole limpet hemocyanin (5 μ g/ml, Calbiochem-Novabiochem Co., San Diego, CA) and IL-7 (25 ng/ml; Peptotech EC Ltd., London, UK). On day three, recombinant IL-2 (Peptotech) was then added to the culture at 50 IU/ml. Responder cells were restimulated every seven days with freshly isolated autologous PBMCs that had been prepulsed with peptide and treated with mitomycin C (Kyowa Hakko Co., Ltd., Osaka, Japan). Cultures were fed with fresh medium containing IL-2 one day after stimulation. CTL activity was assessed on day 21.

Phenotypic analysis. Flow cytometric analysis of the CTLs was performed using a FACScan. The cells were stained with murine anti-human monoclonal antibodies against CD3, CD4 and CD8 (Becton-Dickinson, San Jose, CA). Isotype-matched murine antibodies (Becton-Dickinson) served as negative controls.

Cytotoxicity assay. Target cells were labeled with 100 μ Ci of ^{51}Cr for 1 h at 37°C and labeled cells were then washed and resuspended. Peptide-pulsed targets, colo205, colo320DM cells, were prepared by incubating the cells with peptides for 2 h at 37°C and then labeling them with ^{51}Cr . Effector cells were placed in each well of round-bottomed microtiter plates in duplicate. Labeled target cells (1×10^4 cells/well) were incubated with various numbers of effector cells for 4 h at 37°C. Radioactivity of the culture supernatant was measured with an automated γ counter and the percentage of specific lysis was calculated according to the following formula: (experimental ^{51}Cr release - spontaneous ^{51}Cr release)/(maximum ^{51}Cr release - spontaneous ^{51}Cr release) $\times 100$.

Inhibition of cytotoxicity with monoclonal antibody. Appropriate target cells were incubated with monoclonal antibodies at a final concentration of 20 μ g/ml for 1 h at 4°C prior to the assay for cytotoxicity. The monoclonal antibodies

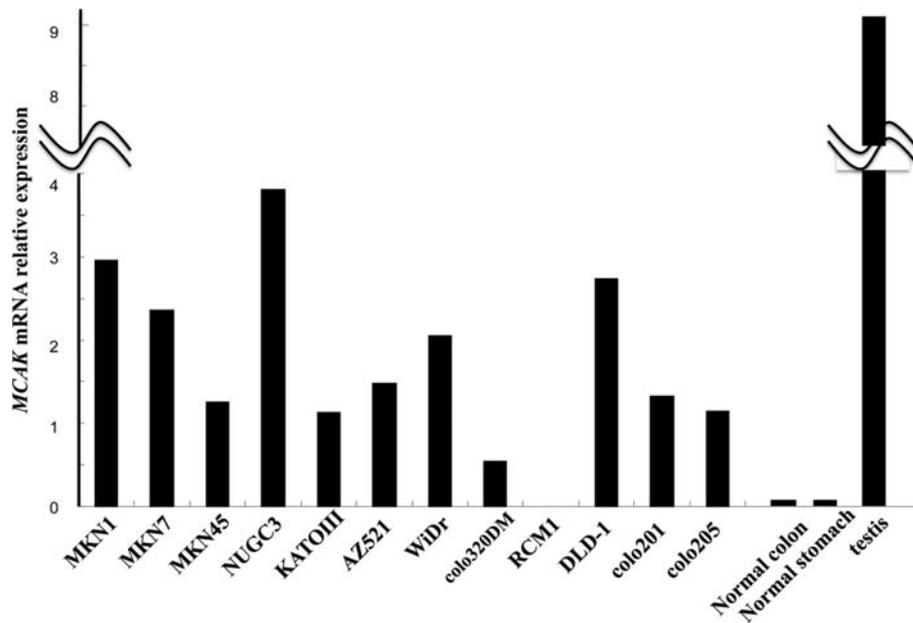


Figure 1. Expression of the *MCAK* gene in colorectal and gastric cancer cell lines detected by quantitative RT-PCR. The *MCAK* gene was highly expressed in NUGC3 (gastric cancer cell line, *HLA-A*2402*⁺) and DLD-1 (colorectal cancer cell line, *HLA-A*0201*⁺), marginally expressed in colo320DM (colorectal cancer cell line, *HLA-A*2402*⁺) and colo205 (colorectal cancer cell line, *HLA-A*0201*⁺), and not expressed in RCM-1 (colorectal cancer cell line, *HLA-A*0201*⁻). Expression of the *MCAK* gene analyzed by RT-PCR in normal colon, stomach, and testis tissues is shown. Each cDNA template was synthesized from 8.0 μ g commercially available mRNA (Human RNA Master Panel II, Clontech) encompassing 20 normal tissues, obtained from multiple, disease-free individuals. Experiments were repeated three times. Representative data are shown.

Table I. *HLA-A*0201* binding motif containing 9-mer peptides derived from the sequence of *MCAK*.

Start position	Amino acid sequence	Binding score (BIMAS)	Binding score (SYFPEITHI)
288	LLLVHEPKL	134	28
375	AMASRDVFL	85	27
421	VLEDGKQQV	31	24
519	SLLALKECI	19	23
63	AINPELLQL	7	23

used were anti-*HLA* class I, CD4, CD8 and control IgG antibody (Abcam, Cambridge, UK).

Statistical evaluation. Statistical analysis was performed using a repeated measure ANOVA method when comparing the cytotoxic activity in an individual group. Differences were considered significant at $p < 0.05$.

Results

Expression of *MCAK* mRNA. We analyzed the expression of *MCAK* mRNA in all cell lines used in this study (Fig. 1). The *MCAK* gene was expressed in all cell lines except RCM-1 (colon cancer cell line, *HLA-A0201*⁻, *2402*⁺). The *MCAK* gene was overexpressed in DLD-1 (colon cancer cell line, *HLA-A0201*⁺, *2402*⁺), and NUGC3 (gastric cancer cell line, *HLA-A0201*⁻, *2402*⁺). The *MCAK* gene was expressed at low levels in colo205 (colon cancer cell line, *HLA-A0201*⁺, *2402*⁻) and colo320DM (colon cancer cell line, *HLA-A0201*⁻, *2402*⁺) (Fig. 1).

Screening of peptides from *MCAK*. We pulsed PBMCs obtained from healthy donors using five peptides (Tables I and II). After 21 days of culture, we performed a cytotoxicity assay against cancer cell lines DLD-1 (which expressed both *HLA-A*0201* and *-A*2402* and the *MCAK* gene) and K562 which has high cytotoxic activity for the natural killer cells. High specific cytotoxic activity and low NK activity were generated using 63: AINPELLQ (*HLA-A*0201*) and 401: FFEIYNGKL (*HLA-A*2402*) peptide in five peptide in the DLD-1 cell line (Fig. 2).

Characterization of the cytotoxic effectors. A flow cytometric analysis was performed before and after cultivation using peptide AINPELLQL (*HLA-A*0201*) and FFEIYNGKL (*HLA-A*2402*). Following stimulation by AINPELLQL (*HLA-A*0201*), the percentage of CD4⁺ cells gradually decreased after the seventh day, whereas the CD8⁺ cells gradually increased. After exposure to FFEIYNGKL (*HLA-A*2402*), the percentage of CD4⁺ cells continued to increase until day 14 and thereafter decreased, whereas CD8⁺ cells

Table II. *HLA-A*2402* binding motif containing 9-mer peptides derived from the sequence of MCAK.

Start position	Amino acid sequence	Binding score (BIMAS)	Binding score (SYFPEITHI)
580	RYADRVKEL	528	23
404	IYNGKLFDL	300	22
324	VYRFTARPL	200	21
401	FFEIYNGKL	33	18
231	EFARMIKEF	11	17

Start position indicates the number of amino acids from N-terminal of MCAK. Binding score is derived from 'BIMAS' and 'SYFPEITHI' as described in Materials and methods.

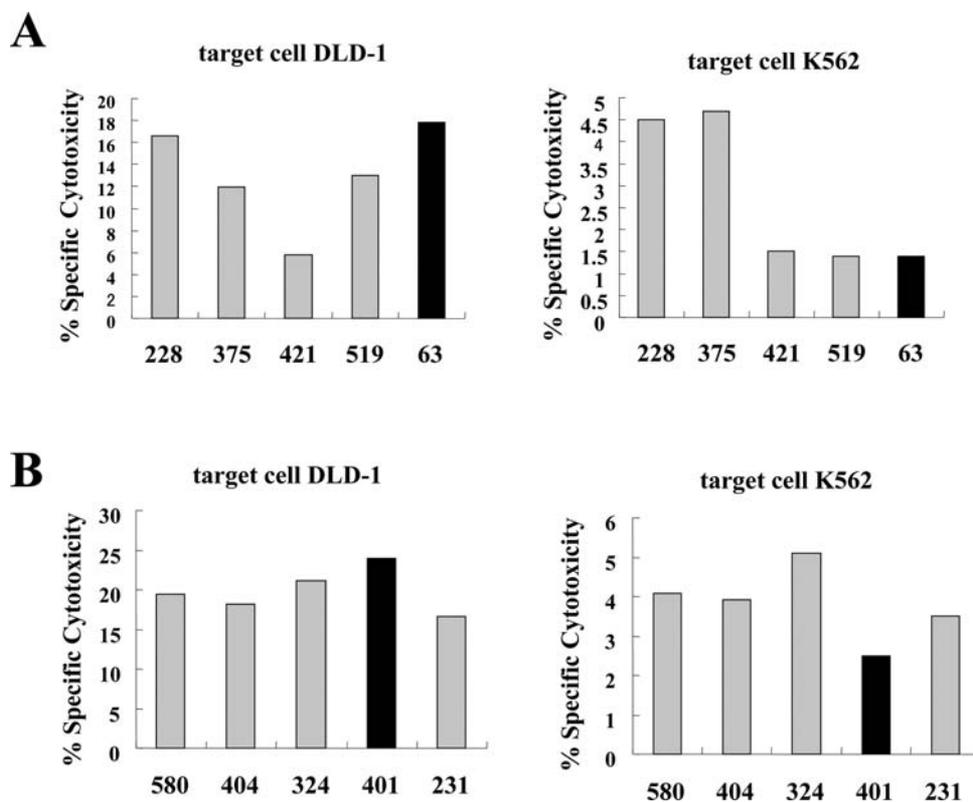


Figure 2. Lysis of DLD-1 and K562 cells by effector cells induced by five candidate peptides of *HLA-A*0201* (A), *HLA-A*2402* (B). The cytotoxic activity of effector cells was assessed against DLD-1 (colorectal cancer cell line, *HLA-A*2402*⁺/*HLA-A*0201*⁺, MCAK⁺) and K562 (chronic myeloid leukemic cell line, *HLA-A*⁻) at an E/T ratio of 10:1. Each group of effector cells was generated from PBMCs by pulsing with each HLA allele of five peptides (Tables I and II), and re-stimulated with peptide-pulsed autologous PBMC two times. After 21 days, the effector cells were co-cultured with ⁵¹Cr-labeled DLD-1 cells and K562 for 4 h and radioactivity of the culture supernatant was determined with a γ counter. The effector cells pulsed with peptides 63: AINPELLQL (*HLA-A*0201*) and 401: FFEIYNGKL (*HLA-A*2402*) exhibited high specific cytotoxicity and low NK activity. Experiments were repeated at least three times. Representative data are shown.

increased after day 14. As for both, on day 21, 70% of the cells were positive for CD8 (Fig. 3).

*HLA-A*0201* and **2402*-restriction of 63: AINPELLQL and 401: FFEIYNGKL peptides from MCAK. We performed cytotoxicity assays against the colon cancer cell lines colo205 and colo320DM which express MCAK marginally and do not express *HLA-A*0201*, or *HLA-A*2402*. After 21 days of culture, the effector cells induced by using the two peptides exhibited significantly higher cytotoxicity against

the peptide-pulsed colo205 and colo320DM cells than non-pulsed colo205 and colo320DM cells (Fig. 4).

Specific cytotoxic activity of effector cells induced by MCAK-derived peptide. We assessed the anti-tumor activity of induced effector cells against various cancer cell lines. Effector cells induced by AINPELLQL (*HLA-A*0201*) and FFEIYNGKL (*HLA-A*2402*) showed high toxicity against the DLD-1 cell line (MCAK⁺, *HLA-A*0201*⁺) and NUGC3 (MCAK⁺, *HLA-A*2402*⁺). However, cytolysis was signi-

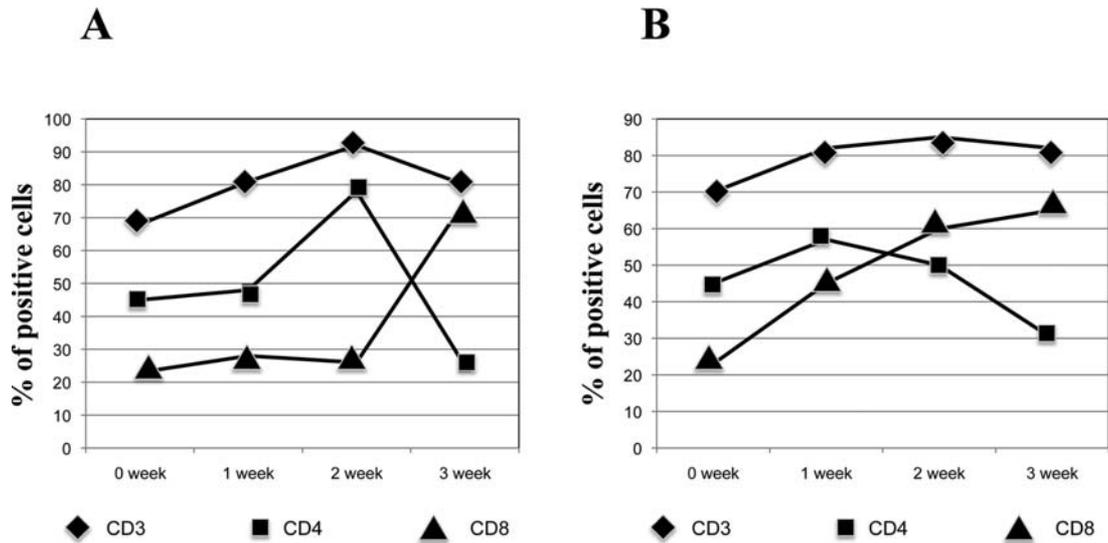


Figure 3. Phenotype of the effector cells stimulated with MCAK peptide 63: AINPELLQL (*HLA-A*0201*) and 401: FFEIYNGKL (*HLA-A*2402*). Effector cells generated from healthy donors were stimulated with the MCAK/*HLA-A*0201* peptide 63: AINPELLQL (*HLA-A*0201*) (A) and /*HLA-A*2402* peptide (B) 401: FFEIYNGKL, and these cells were stained with anti-CD3, anti-CD4, or anti-CD8 monoclonal antibody. Experiments were repeated at least four times. Representative data are shown.

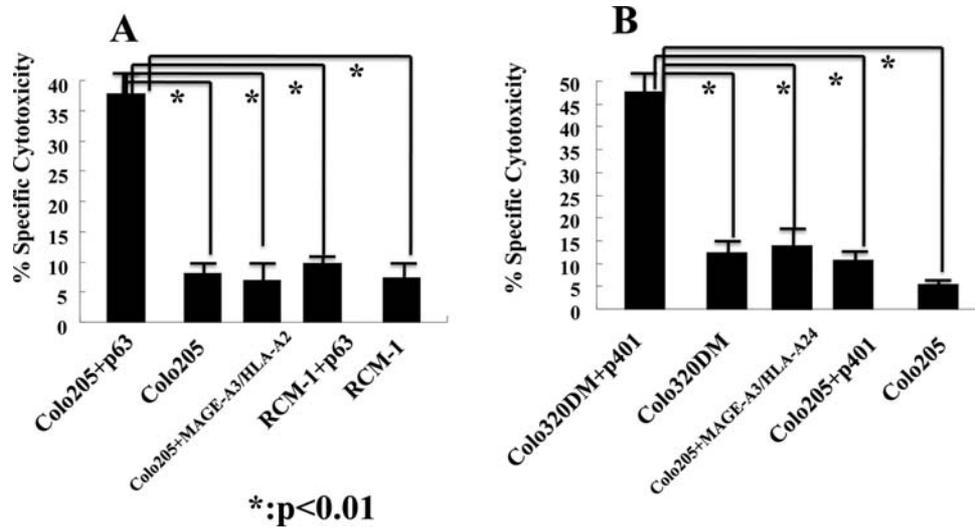


Figure 4. The cytotoxic activity of effector cells against the colon cancer cell lines with or without peptide at an E/T ratio of 5:1. PBMCs from a healthy donor were peptide-pulsed and stimulated with mitomycin-treated autologous PBMCs every seven days. After 21 days of incubation, the effector cells were co-cultured with ⁵¹Cr-labeled target cells in a 4-h cytotoxicity assay at various E/T ratios. Specific cytotoxicity of the effector cells stimulated with MCAK/*HLA-A*0201* peptide (A) 63: AINPELLQL (*HLA-A*0201*) and (B) 401: FFEIYNGKL (*HLA-A*2402*) against colo205 (colon cancer cell line, *HLA-A0201*⁺, *HLA-A*2402*⁻, MCAK⁻), RCM-1 (colorectal cancer cell line, *HLA-A0201*⁻, MCAK⁻), colo320DM (colorectal cancer cell line, *HLA-A*2402*⁺, MCAK⁻). As irrelevant peptides restricted by HLA-A2, MAGE-A3/HLA-A2 peptide (FLWGPRALV), and peptide with *HLA-A2402*, MAGE-A3/*HLA-A2402* (IMPKAGLLI) were used. Data represent the mean ± SD of cytotoxicity. There was a significant difference between cytolytic activity against colo205 pulsed with p63, colo205 pulsed with p401 cells and those against other targets (p<0.01 for all). Experiments against cells pulsed with irrelevant peptides were repeated twice, others were repeated at least four times.

ificantly diminished against colo205 (*HLA-A*0201*⁺) and MKN45 (*HLA-A*2402*⁺), which expressed MCAK marginally (Fig. 5).

To determine whether the AINPELLQL (*HLA-A*0201*) and FFEIYNGKL (*HLA-A*2402*)-induced effector cells recognized MCAK expressing targets in an HLA-restricted manner, HLA class I, anti-CD4, CD8, and control IgG monoclonal antibodies were used to confirm the recognition by effectors. Cytotoxic activity of the effector cells against DLD-1 (*HLA-A*0201*⁺) and NUGC3 (*HLA-A*2402*⁺) was

significantly eliminated by the anti-HLA-class-I and -CD8 antibody. Marginal inhibition was observed when we used an anti-CD4 antibody (Fig. 6). These results suggested that induced effectors mainly lysed target cells expressing MCAK in an HLA-class I, CD8-restricted manner.

Discussion

MCAK is controlled by aurora kinase which has been implicated in tumorigenesis (18). Aurora kinase encompasses

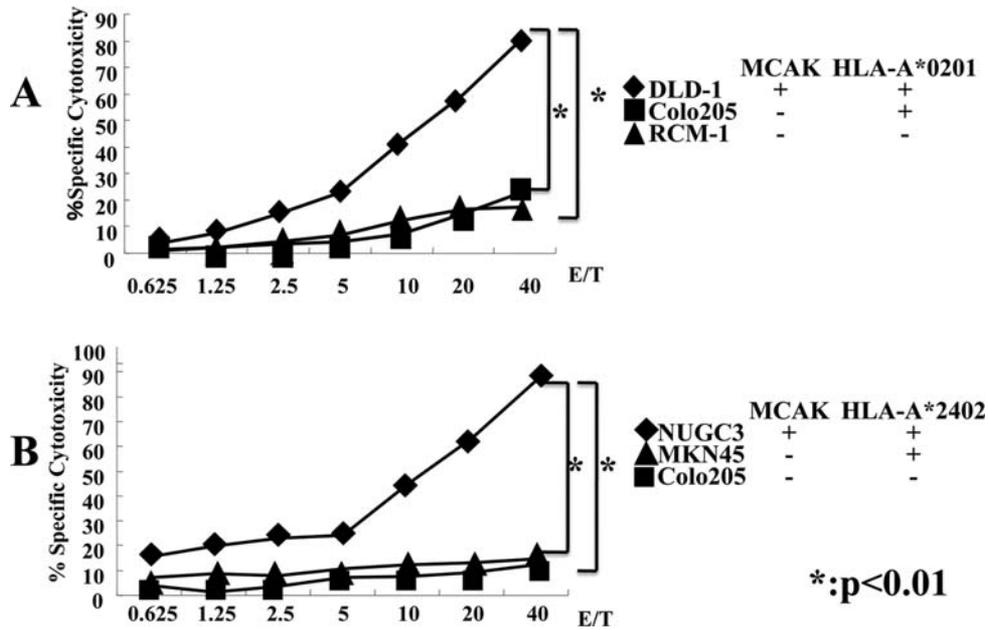


Figure 5. Specific lysis of CTLs induced with the peptide 63: AINPELLQL (*HLA-A*0201*) and 401: FFEIYNGKL (*HLA-A*2402*): the CTLs showed high cytotoxic activity against DLD-1 (*MCAK*⁺, *HLA-A*0201*⁺) and NUGC3 (*MCAK*⁺, *HLA-A*2402*⁺). On the other hand, CTLs did not show significant cytotoxicity activity against colo205 (*MCAK*⁻, *HLA-A*0201*⁺, *HLA-A*2402*⁻), or RCM-1 (*MCAK*⁻, *HLA-A*0201*⁻) or MKN45 (*MCAK*⁻, *HLA-A*2402*⁺). Data represent the means ± SD of cytotoxicity. There was a significant difference between cytolytic activity against DLD-1 (both *MCAK* and *HLA-A0201*), NUGC3 (both *MCAK* and *HLA-A2402*), and those against other targets (either/both *HLA* and/or *MCAK* expression was inadequate) ($p < 0.01$ for all).

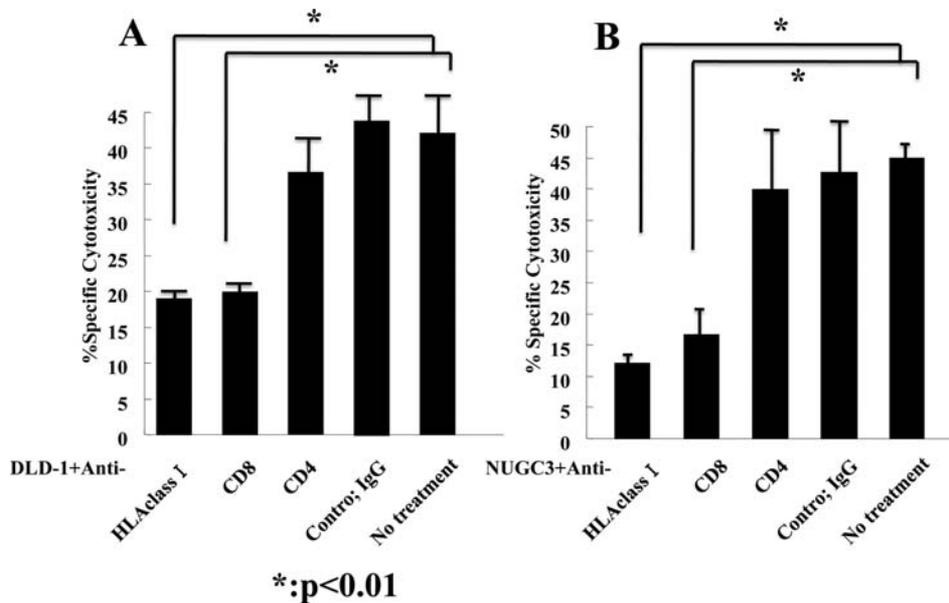


Figure 6. Inhibition of recognition of CTLs by anti-*HLA*-class I monoclonal antibody. Target cells, DLD-1 (*MCAK*⁺, *HLA-A*0201*⁺) and NUGC3 (*MCAK*⁺, *HLA-A*2402*⁺), were incubated with anti-*HLA*-class I, CD4, CD8, and control IgG monoclonal antibody (mAb) at a final concentration of 20 μg/ml for 1 h at 4°C before co-culture with effector cells stimulated with the *MCAK/HLA-A*0201* peptide (A) 63: AINPELLQL (*HLA-A*0201*) and (B) 401: FFEIYNGKL. The cytotoxic activity of the CTLs was estimated after co-culture with monoclonal antibody-treated cells or non-treated cells at an E/T ratio of 10:1. Data represent the means ± SD of cytotoxicity. There was a significant difference between cytolytic activities when treated with anti-class I and anti-CD8 antibody, but not with anti-CD4 or control IgG ($p < 0.01$ for both DLD-1 and NUGC3). Experiments using anti-class I mAb and parent cells were repeated three times, then experiments using anti-CD4, CD8 and control IgG experiments were repeated twice.

three known family members, aurora-A, -B and -C. The function of aurora C is unknown, but aurora A and aurora B played an important roles in cell division. In addition, aurora-A and -B are overexpressed in a variety of tumor cell lines, suggesting their potential role in gene instability and

inducing tumor progression (5). Aurora-B kinase inhibitor was considered a cancer drug target due to its induction of cytotaxis and apoptosis. A phase I trial has been carried out with chronic myelogenous leukemia patients using aurora-B kinase inhibitor (19). Aurora B is enriched at merotelic

attachment sites, where it regulates MCAK (20). MCAK is regulated in its localization and microtubule depolymerization activity by Aurora B phosphorylates (23). Overexpression of aurora B in cancer cells promotes the microtubule depolymerization activity of MCAK, and this may promote cancer cell immortality.

In our previous study, MCAK was considered to be a CT antigen and was overexpressed in gastric cancer, suggesting the possibility of being a target gene for cancer specific immunotherapy (3). Therefore, in this study, we asked whether *HLA-A*0201* and *-A*2402*-restricted MCAK-specific CTL induction was possible. *HLA-A*0201* and *-A*2402* are commonly expressed alleles in Caucasian and Asian individuals. In Japanese population, the *HLA-A*0201* expression rate is 20-30%, and *HLA-A*2402* is expressed in 60% (4,22,23). We stimulated PBMCs of *HLA-A*0201* and *HLA-A*2402* positive healthy donors with five peptides selected from computer-based epitope predictions. We investigated cytotoxic activity against the colon cancer cell line DLD-1 and the chronic myelocytic leukemia-derived cell line K562. 63: AINPELLQL (*HLA-A*0201*) and 401: FFEIYNGKL (*HLA-A*2402*) peptides revealed the highest specific cytotoxic activity. In addition, effector cells generated with MCAK-derived peptide AINPELLQL (*HLA-A*0201*) and FFEIYNGKL (*HLA-A*2402*) demonstrated high cytotoxicity limited to DLD1 and NUGC3 which overexpressed MCAK and were HLA allele-positive (Fig. 5).

To determine the mechanism of immunological response in the recognition of antigen by the MCAK-reactive CTLs, anti-HLA class I monoclonal antibodies were tested for their capacity to inhibit the ⁵¹Cr-releasing assay by MCAK-specific CTLs when the cells were cultured with DLD1 or NUGC3 cells. Cytolysis induced by MCAK-specific CTLs was significantly inhibited by anti-HLA class I and anti-CD8 monoclonal antibodies, indicating that the CTLs recognize the MCAK-derived epitopes in an HLA class I, and CD8-restricted manner (Fig. 6). Because effector cells obtained in this study included CD4⁺ cells, we determined the contribution of the cytotoxic activity of CD4⁺ cells. Consistent with our previous studies using MAGE-A3 derived CTLs, only a marginal effect was observed (16,17). In this study, effector cells were generated in conditioned medium containing KLH, modified from the method of Plebanski *et al* (24). Using this method, we previously identified MAGE-A3/ HLA-A24 (17), NY-ESO-1/HLA-A24 (25), and STK31/HLA-A2 peptides (26) after generation of effector cells. KLH generates immunoreactive B cells, and CD4⁺ and CD8⁺ T cells. In this study, we were unable to obtain PBMC from cancer patients. In the next study, we shall examine the generation of CTLs from patients with cancer.

MAGE-3, NY-ESO-1, and SSX-2 are known as CT antigens that are recognized in CTL and are over-expressed in colon and gastric cancers (27,28). Generally, the incidence of CT antigen expression in various tumor types has a low frequency in colon and gastric cancers (29). However, MCAK is overexpressed in gastric cancer (3), colon cancer (11), and breast cancer (12). It is known that the expression of the CT antigens, MAGE and NY-ESO-1, cross-over (29,30). This narrows the adaptation range of the cancer immunotherapy. However, in case of patients with

gastric cancer, MCAK expression was observed in 28% of the cases without the expression of MAGE and NY-ESO-1 (3). Furthermore, there is a report that MCAK participates in the growth and carcinogenesis of breast cancer, and MCAK expression is high in a colon cancer cell line which is a 5-FU resistant strain (31). MCAK immunotherapy might be effective after treatment of 5FU therapy in cancer patients.

In this study, we identified novel cancer/testis antigen MCAK peptides which could induce CTLs to lyse cancer cells in an HLA-A2- or HLA-A24-restricted manner. Our results provide evidence that this methodology to identify CT antigens is useful and MCAK is a promising target for cancer immunotherapy for colorectal, and gastric cancers.

Acknowledgements

This study was supported by Japan Society for the Promotion of Science (JSPS) Grant-in-Aid for Scientific Research, grant numbers 21679006, 20390360, 20591547, 21591644, 21592014 and 21229015.

References

1. van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, Knuth A and Boon T: A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 254: 1643-1647, 1991.
2. Boon T, Cerottini JC, Van den Eynde B, van der Bruggen P and Van Pel A: Tumor antigens recognized by T lymphocytes. *Annu Rev Immunol* 12: 337-365, 1994.
3. Nakamura Y, Tanaka F, Haraguchi N, Mimori K, Matsumoto T, Inoue H, Yanaga K and Mori M: Clinicopathological and biological significance of mitotic centromere-associated kinesin overexpression in human gastric cancer. *Br J Cancer* 97: 543-549, 2007.
4. Scanlan MJ, Welt S, Gordon CM, Chen YT, Gure AO, Stockert E, Jungbluth AA, Ritter G, Jäger D, Jäger E, Knuth A and Old LJ: Cancer-related serological recognition of human colon cancer: identification of potential diagnostic and immunotherapeutic targets. *Cancer Res* 62: 4041-4047, 2002.
5. Gary J: Gorbsky Mitosis: MCAK under the Aura of Aurora B. *Curr Biol* 14: 346-348, 2004.
6. Brady ST: A novel brain ATPase with properties expected for the fast axonal transport motor. *Nature* 317: 73-75, 1985.
7. Vale RD, Reese TS and Sheetz MP: Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. *Cell* 42: 39-50, 1985.
8. Hirokawa N, Pfister KK, Yorifuji H, Wagner MC, Brady ST and Bloom GS: Submolecular domains of bovine brain kinesin identified by electron microscopy and monoclonal antibody decoration. *Cell* 56: 867-878, 1989.
9. Endow SA: Kinesin motors as molecular machines. *Bioessays* 25: 1212-1219, 2003.
10. Wittmann T, Hyman A and Desai A: The spindle: a dynamic assembly of microtubules and motors. *Nat Cell Biol* 3: 28-34, 2001.
11. Mori M, Barnard GF, Staniunas RJ, Jessup JM, Steele GD Jr and Chen LB: Prothymosin- α mRNA expression correlates with that of c-myc in human colon cancer. *Oncogene* 8: 2821-2826, 1993.
12. Shimo A, Tanikawa C, Nishidate T, Meng-Lay L, Matsuda K, Park JH, Ueki T, Ohta T, Hirata K, Fukuda M, Nakamura Y and Katagiri T: Involvement of kinesin family member 2C/ mitotic centromere-associated kinesin overexpression in mammary carcinogenesis. *Cancer Sci* 99: 62-70, 2008.
13. Shimo A, Nishidate T, Ohta T, Fukuda M, Nakamura Y and Katagiri T: Elevated expression of protein regulator of cyto-kinesis 1, involved in the growth of breast cancer cells. *Cancer Sci* 98: 174-181, 2007.
14. Mori M, Mimori K, Yoshikawa Y, Tanaka F and Sugimachi K: Analysis of the gene-expression profile regarding the progression of human gastric carcinoma. *Surgery* 131: S39-S47, 2002.

15. Ogawa K, Utsunomiya T, Mimori K, Tanaka F and Mori M: Clinical significance of human kallikrein gene 6 messenger RNA expression in colorectal cancer. *Clin Cancer Res* 11: 2889-2893, 2005.
16. Tanaka F, Fujie T, Go H, Mori M, and Akiyoshi T. Efficient induction of antitumor cytotoxic T lymphocytes from a healthy donor using HLA-A2-restricted MAGE-3 peptide in vitro. *Cancer Immunol Immunother* 44: 21-26, 1997.
17. Tanaka F, Fujie T, Tahara K, Mori M and Akiyoshi T: Induction of antitumor cytotoxic T lymphocytes with a MAGE-3-encoded synthetic peptide presented by human leukocytes antigen-A24. *Cancer Res* 57: 4465-4468, 1997.
18. Lan W, Zhang X, Kline-Smith SL, *et al*: Aurora B phosphorylates centromeric MCAK and regulates its localization and microtubule depolymerization activity. *Curr Biol* 14: 273-286, 2004.
19. Giles FJ, Cortes J, Jones D, Bergstrom D, Kantarjian H and Freedman SJ: MK-0457, a novel kinase inhibitor, is active in patients with chronic myeloid leukemia or acute lymphocytic leukemia with the T315I BCR-ABL mutation. *Blood* 109: 500-502, 2007.
20. Mashino K, Sadanaga N, Tanaka F, Yamaguchi H, Nagashima H, Inoue H, Sugimachi K and Mori M: Expression of multiple cancer-testis antigen genes in gastrointestinal and breast carcinomas. *Br J Cancer* 85: 713-720, 2001.
21. Knowlton AL, Lan W and Stukenberg PT: Aurora B is enriched at merotelic attachment sites, where it regulates MCAK. *Curr Biol* 16: 1705-1710, 2006.
22. Van Elsas A, Nijman HW, Van der Minne CE, Mourer JS, Kast WM, Melief CJ and Schrier PI: Induction and characterization of cytotoxic T-lymphocytes recognizing a mutated p21ras peptide presented by *HLA-A*0201*. *Int J Cancer* 4: 389-396, 1995.
23. Okano F, Storkus WJ, Chambers WH, Pollack IF and Okada H: Identification of a novel *HLA-A*0201*-restricted, cytotoxic T lymphocyte epitope in a human glioma-associated antigen, interleukin 13 receptor alpha2 chain. *Clin Cancer Res* 8: 2851-2855, 2002.
24. Plebanski M, Allsopp CE, Aido M, Reyburn H and Hill AV: Induction of peptide-specific primary cytotoxic T lymphocytes response from human peripheral blood. *Eur J Immunol* 25: 1783-1787, 1995.
25. Yamaguchi H, Tanaka F, Ohta M, Inoue H and Mori M: Identification of HLA-A24-restricted CTL epitope from cancer-testis antigen, NY-ESO-1, and induction of a specific antitumor immune response. *Clin Cancer Res* 10: 890-896, 2004.
26. Yokoe T, Tanaka F, Mimori K, Inoue H, Ohmachi T, Kusunoki M and Mori M: Efficient identification of a novel cancer/testis antigen for immunotherapy using three-step microarray analysis. *Cancer Res* 68: 1074-1082, 2008.
27. Scanlan MJ, Welt S, Gordon CM, Chen YT, Gure AO, Stockert E, Jungbluth AA, Ritter G, Jäger D, Jäger E, Knuth A and Old LJ: Cancer-related serological recognition of human colon cancer: identification of potential diagnostic and immunotherapeutic targets. *Cancer Res* 62: 4041-4047, 2002.
28. Inoue H, Mori M, Honda M, Li J, Shibuta K, Mimori K, Ueo H and Akiyoshi T: The expression of tumor-rejection antigen 'MAGE' genes in human gastric carcinoma. *Gastroenterology* 109: 1522-1525, 1995.
29. Sahin U, Tureci O and Schmitt H: Human neoplasms elicit multiple specific immune responses in the autologous host. *Proc Natl Acad Sci USA* 92: 11810-11813, 1995.
30. Imanishi T, Akazawa T, Kimura A, Tokunaga K and Gojobori T: Allele and haplotype frequencies for HLA and complement loci in various ethnic groups. *Oxford Sci Publ* 1: 1065-1220, 1992.
31. Wang W, Cassidy J, O'Brien V, Ryan KM and Collie-Duguid E: Mechanistic and predictive profiling of 5-Fluorouracil resistance in human cancer cells. *Cancer Res* 64: 8167-8176, 2004.