Efficacy of immunotherapy targeting the neoantigen derived from epidermal growth factor receptor T790M/C797S mutation in non–small cell lung cancer

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Abstract
Lung cancer is the leading cause of cancer-related deaths worldwide. Epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKI) often have good clinical activity against non–small cell lung cancer (NSCLC) with activating EGFR mutations. Osimertinib, which is a third-generation EGFR-TKI, has a clinical effect even on NSCLC harboring the threonine to methionine change at codon 790 of EGFR (EGFR T790M) mutation that causes TKI resistance. However, most NSCLC patients develop acquired resistance to osimertinib within approximately 1 year, and 40% of these patients have the EGFR T790M and cysteine to serine change at codon 797 (C797S) mutations. Therefore, there is an urgent need for the development of novel treatment strategies for NSCLC patients with the EGFR T790M/C797S mutation. In this study, we identified the EGFR T790M/C797S mutation-derived peptide (790-799) (MQLMPFGSLL) that binds the human leukocyte antigen (HLA)-A*02:01, and successfully established EGFR T790M/C797S-peptide-specific CTL clones from human PBMC of HLA-A2 healthy donors. One established CTL clone demonstrated adequate cytotoxicity against T2 cells pulsed with the EGFR T790M/C797S peptide. This CTL clone also had high reactivity against cancer cells that expressed an endogenous EGFR T790M/C797S peptide using an interferon-γ (IFN-γ) enzyme-linked immunospot (ELISPOT) assay. In addition, we demonstrated using a mouse model that EGFR T790M/C797S peptide-specific CTL were induced by EGFR T790M/C797S peptide vaccine in vivo. These findings suggest that an immunotherapy targeting a neoantigen derived from EGFR T790M/C797S mutation could be a useful novel therapeutic strategy for NSCLC patients with EGFR-TKI resistance, especially those resistant to osimertinib.

KEYWORDS
C797S mutation, EGFR T790M, immunotherapy, neoantigen, non–small cell lung cancer
INTRODUCTION

Lung cancer is a highly aggressive malignant disease with a poor prognosis. In the United States, there are 220,000 new cases and 150,000 deaths per year from lung cancer, while in Japan, lung cancer is the leading cause of cancer-related deaths. In addition, the prevalence of lung cancer continues to increase worldwide, despite the slight decline of cases in Western countries and Japan. Lung cancer can be classified into two main types: small cell lung cancer and non–small cell lung cancer (NSCLC), the latter of which accounts for approximately 85% of all lung cancer cases.

Mutations in the epidermal growth factor receptor (EGFR) are observed in various types of cancer, and NSCLC is no exception. In fact, approximately 30%-40% and 15% of patients with NSCLC have EGFR mutations in Japan and Western countries, respectively. Activating EGFR mutations cause tumor cell differentiation and proliferation by inducing intracellular signaling pathways, including the signal transducer and activator of transcription (STAT), mitogen-activated protein kinase (MARK) and the phosphatidylinositol 3-kinase (PI3K) pathway. Therefore, EGFR mutations are good targets for cancer therapy, and several antibodies that inhibit the activating EGFR pathway have been developed. In particular, first-generation or second-generation EGFR-tyrosine kinase inhibitors (EGFR-TKI), such as gefitinib, erlotinib and afatinib, which are molecular target drugs, have been approved for NSCLC patients with activating EGFR mutations. A meta-analysis of randomized trials of treatment-naïve patients with advanced NSCLC showed that patients treated with EGFR-TKI had better progression-free survival (PFS) compared with the patients treated with standard chemotherapy; the median PFS in the NSCLC patients with EGFR-TKI and chemotherapy were 11.0 and 5.6 months, respectively. Based on these results, EGFR-TKI are currently a standard first-line therapy for advanced NSCLC patients with EGFR-TKI-sensitizing mutations (EGFRm).

However, despite the initial high tumor response to EGFR-TKI, most patients experience cancer progression after 9-13 months of treatment, with approximately 60%-70% of those patients harboring the EGFR T790M resistance mutation. The slight decline of cases in Western countries and Japan. Consequently, it is reported that osimertinib provides adequate anti–tumor effect against various carcinomas. Cancer immunotherapy has recently attracted attention as a fourth treatment method that produces promising anti–tumor effects through a significantly different approach than existing therapies. In fact, several cancer immunotherapies have been reported to result in adequate anti–tumor effects against various types of cancer.

Cancer immunotherapy was initially limited to melanoma, but recently, it has been reported that immunotherapy targeting the neoantigen, which is a cancer-specific antigen derived from mutated amino acid sequences, has a remarkable anti–tumor effect against various carcinomas. Neoantigens often have the advantage of being highly immunogenic and having a high affinity for T-cell receptors (TCR) on cancer-specific CTL. Indeed, we previously demonstrated that in NSCLC patients with the T790M mutations, immunotherapy targeting the EGFR T790M mutation-derived antigen could be a treatment option resulting in better anti–tumor effect.

We hypothesized that cancer cells harboring the EGFR T790M/C797S mutation could be targeted by activated immune cells. In the present study, we identified a human leukocyte antigen (HLA)-A2-restricted EGFR T790M/C797S mutation-derived epitope. Our results suggested that the immunotherapy targeting the EGFR T790M/C797S mutation-derived antigen with a significantly different approach from EGFR-TKI could be a novel treatment strategy for advanced NSCLC patients with EGFR T790M/C797S mutations who are resistant to osimertinib.

MATERIALS AND METHODS

2.1 Cell lines

The human lymphoblastoid T2 cell line (HLA-A*02:01, TAP), the mouse lymphoma cell line RMA-S-HHD (transfected HLA-A*02:01, TAP), and the SK-HEP-1 (HLA-A*02:01/A*24:02) human ductal cell line were maintained in our laboratory and used as target cells. The T2 and RMA-S-HHD cells were cultured in RPMI1640 (Sigma Chemical) medium supplemented with 10% FBS (Gibco-BRL) and 1% penicillin-streptomycin-glutamine (Gibco-BRL). The SK-HEP-1 cells were cultured in DMEM (Sigma Chemical) supplemented with 10% FBS (Gibco-BRL) and 1% penicillin-streptomycin-glutamine (Gibco-BRL). The EGFR expression vectors in which wild-type EGFR, EGFR T790/C797S, EGFR C797S or EGFR T790M expression plasmid inserted into the pEF1α-IRES-AcGFP (Takara Bio) were kindly provided by Dr Tetsuro Sasada (Kanagawa Cancer Center Research Institute). These EGFR expression vectors were transfected in SK-HEP-1...
with Lipofectamine 2000 Transfection Reagent (Invitrogen Life Technologies) according to the manufacturer’s instructions. SK-HEP-1 cells transfected EGFR mutations were cultured in DMEM with G418 Sulfate (Merck) at 800 μg/mL every 3-4 days.

2.2 | PBMC collection

Peripheral blood samples were collected from four HLA-A*02:01-positive healthy donors who provided informed consent. PBMC were isolated by density centrifugation using Ficoll-Hypaque (Pharmacia) and frozen in liquid nitrogen until use.

2.3 | Epitope prediction and synthesis

The epitope prediction software NetMHC3.4 Server and BIMAS were used to predict peptides that bind to HLA-A2. EGFR T790/C797S mutation-derived peptides (purity >95%) were purchased from Scrum. The peptides were dissolved in dimethylformamide (Wako Pure Chemical Industries) to a final concentration of 10 mg/mL and stored in liquid nitrogen until use. An HLA-A*02:01-restricted glypican-3 (GPC3)144-152 peptide (FVGEFFTDV) (American Peptide) was used as a positive control in the peptide-binding assay. An HLA-A2-restricted glypican-3 (GPC3)144-152 peptide (FVGEFFTDV) (American Peptide) was used as a negative control peptide in the CTL assay.

2.4 | Peptide-binding assay

To determine the binding ability of the predicted peptides to HLA-A*02:01 molecules, an in vitro cellular binding assay was performed as reported previously. Briefly, after incubation of the T2 cells in culture medium at 26°C overnight, T2 cells were washed in PBS and suspended in 1 mL Opti-MEM (Gibco-BRL) with a peptide at 100 μg/mL and incubated at 26°C for 3 hours and then 37°C for 2.5 hours. After washing with PBS, HLA-A2 expression was measured by flow cytometry using FITC-conjugated HLA-A2 monoclonal antibodies (clone BB7.2; MBL). Mean fluorescence intensity (MFI) was analyzed using FlowJo software (Tomy Digital Biology). An HLA-A*02:01-restricted GPC3 peptide (FVGEFFTDV) (American Peptide) was used as a positive control in the peptide-binding assay.

2.5 | Induction of peptide-specific CTL

The PBMC obtained from healthy donors with HLA-A*02:01 were co-cultured at a density of 2 × 10^6 cells/well with 10 μg/mL EGFR T790M/C797S mutation-derived peptides in 45% RPMI 1640 and 45% AIM-V (Gibco-BRL) medium supplemented with 10% human AB serum and recombinant human interleukin (IL)-2 at 37°C for 14 days. Then, CD8+ cells were isolated from the peptide-stimulated PBMC using human CD8 microbeads (Miltenyi Biotec GmbH).

2.6 | Interferon-γ enzyme-linked immunospot analysis

Specific secretion of interferon-γ (IFN-γ) from human CTL in response to stimulator cells was assayed using the IFN-γ enzyme-linked immunospot (ELISPOT) kit (BD Biosciences) according to the manufacturer’s instructions. Stimulator cells were pulsed with peptide for 2 h at room temperature and then washed three times. Responder cells were incubated with stimulator cells for 20 hours. The resulting spots were counted using an ELIPHOTO counter (Minerva Tech). HIV-gag (77-85) (SLYNTYATL) (American Peptide) was used as a negative peptide control in the CTL assay.

2.7 | CD107a assay and establishment of CTL clones

The CD8+ cells that were isolated from cultured cells with human CD8 microbeads were incubated with target cells at a 2:1 ratio for 3.5 hours at 37°C. A CD107a-specific monoclonal antibody (BD Biosciences) was included during the incubation period. CD8+CD107a+ cells were sorted using a FACSAria II cell sorter (BD Biosciences). The target cells used were peptide (10 μg/mL)-pulsed T2 cells and RMA-S-HHD. Sorted CTL were stimulated and the CTL clones were established as described previously.21

2.8 | Cytotoxicity assay

The cytotoxic activity was analyzed using the Terascan VPC system (Minerva Tech). The CTL clones were used as the effector cells. T2 target cells were labeled with calcein-AM (Dojindo Molecular Technologies) solution for 30 minutes at 37°C. The labeled cells were then co-cultured with the effector cells for 4-6 hours. Fluorescence intensity was measured before and after the culture period and specific cytotoxic activity was calculated using the following formula:

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\% \text{ cytotoxicity} = \left(1 - \frac{\text{average fluorescence of the sample wells} - \text{average fluorescence of the maximal release control wells}}{\text{average fluorescence of the minimal release control wells} - \text{average fluorescence of the maximal release control wells}}\right) \times 100\%.
\]

2.9 | Determination of recognition efficiency

Calcein-AM-labeled target T2 cells were pulsed with a range of peptide concentrations starting at 10^{-5} mol/L and decreasing by log steps to 10^{-12} mol/L. The CTL clones were incubated with target T2 cells at a 10:1 effector/target ratio for 4-6 hours. For each CTL clone, percentage cytotoxicity was plotted against peptide concentration. The peptide concentration at which the curve crossed 50% cytotoxicity was defined as the recognition efficiency of that clone.
2.10 | HLA-A*02:01 transgenic mice and peptide vaccine administration

HLA-A*02:01 transgenic mice (A2-Tg mice) are H-2D\(^b\) β2m double knockout mice transformed with a human β2m-HLA-A2.1 (α1α2)-H-2D\(^b\) (α3 transmembrane cytoplasmic) monochain construct that was generated in the Department SIDA-Retrovirus, Unité d’Immunologie Cellulaire Antivirale, Institut Pasteur, France and kindly provided by Dr F. A. Lemonnier.\(^{33,34}\) The A2-Tg mice were maintained under the institutional guidelines set by the Animal Research Committee of the National Cancer Center Hospital East, Japan. The mice were housed under specific-pathogen-free (SPF) conditions with a 12-hour light cycle and access to food and water ad libitum. Six-to-ten-week-old mice were used in all experiments.

The A2-Tg mice were intradermally injected twice at a week interval at the base of the tail with 100 \(μL\) EGFR T790M/C797S mutation-derived peptide vaccine. The peptide vaccine consisted of a combination of peptide : 7% NaHCO\(_3\) : incomplete Freund’s adjuvant (IFA) (1:9:10). Two weeks after the last administration of the peptide vaccine, the mice were killed and the spleens were removed.

2.11 | Generation of bone marrow-derived dendritic cells

Bone marrow-derived dendritic cells (BM-DC) were generated as described previously.\(^{35}\) In brief, bone marrow cells (\(2 \times 10^6\) cells) derived from A2-Tg mice were cultured in RPMI 1640 (Sigma Chemical) containing 10% FBS (Gibco-BRL), 50 \(μM\) 2-mercaptoethanol (2-ME) and 10 ng/mL murine granulocyte-macrophage colony-stimulating factor (mGM-CSF). After a week of culture, floating cells were collected and used as BM-DC.

2.12 | In vitro stimulation of splenocytes derived from peptide-administered mice

A week after the last administration of the peptide vaccine, splenocytes were collected and CD8\(^+\) cells were isolated by positive magnetic cell sorting with anti-CD8 microbeads (Miltenyi Biotec GmbH) according to the manufacturer’s protocol. The CD8\(^+\) cells were co-cultured with peptide-pulsed BM-DC for a week and the antigen-specific T-cell frequency was determined.

3 | RESULTS

3.1 | Identification of HLA-A*02:01-restricted epidermal growth factor receptor T790M/C797S-derived peptides

We selected four 9-mer or 10-mer peptides that were predicted to have high binding affinity for HLA-A*02:01 molecules using NetMHC3.4 Server and BIMAS software as the candidate HLA-A*02:01-restricted EGFR T790M/C797S-derived CTL epitopes (Table 1). T790M/C797S-A is a nine-mer peptide, while T790M/C797S-B, -C and -D are 10-mer peptides. Of these four candidate peptides, T790M/C797S-B and -D had higher binding ability to HLA-A*02:01 molecules.

Next, by in vitro cellular peptide-binding assay with HLA-A2 TAP-deficient T2 cell line, we evaluated the binding affinity of the four candidate peptides for the HLA-A*02:01 molecules (Figure 1). The peptide-binding assay showed that all four peptides were able to bind HLA-A*02:01 molecules; MFI of T790M/C797S-A, -B -C and -D were 262.5, 3298.0, 188.0 and 1569.0, respectively. In particular, the binding capability of the T790M/C797S-B peptide was higher than those of the other candidate peptides. In addition, the T790M/C797S-B peptide showed high binding ability for the HLA-A*02:01 molecules even at low peptide concentrations (Figure S1). Therefore, based on the above results, we judged that T790M/C797S-B peptide was the most suitable as HLA-A*02:01-restricted EGFR T790M/C797S-derived CTL epitope, and performed further analysis of T790M/C797S-B peptide as follows.

3.2 | Induction of epidermal growth factor receptor T790M/C797S-derived peptide-specific CTL from human PBMC

To evaluate the immunogenic potential of the T790M/C797S-B peptide, we attempted to induce T790M/C797S-B-specific CTL from PBMC obtained from four healthy donors. In detail,

| Peptide name    | Position | Length (mer) | Sequence     | NetMHC 3.4 ANN IC50\(^a\) (nM) | BIMAS score\(^b\) |
|-----------------|----------|--------------|--------------|--------------------------------|-------------------|
| T790M/C797S-A   | 789-797  | 9            | IMQLMPFGS    | 3317                           | 0.531             |
| T790M/C797S-B   | 790-799  | 10           | MQLMPFGSLL   | 581                            | 51.770            |
| T790M/C797S-C   | 788-797  | 10           | LIMQLMPFGS   | 1176                           | 0.374             |
| T790M/C797S-D   | 789-798  | 10           | IMQLMPFGSL   | 511                            | 6.478             |

\(^a\)NetMHC 3.4 ANN IC50 is an evaluation of the ability of binding to HLA calculated with NetMHC 3.4 software.

\(^b\)BIMAS score is an evaluation of the ability of binding to HLA calculated with BIMAS software.
PBMC from four healthy donors with HLA-A*02:01 were cultured for 14 days with stimulation by T790M/C797S-B peptide every 3-4 days (Figure 2A). Then, we isolated CD8$^+$ cells from the peptide-stimulated human PBMC using human CD8 microbeads and evaluated the specificity of these isolated CD8$^+$ cells for the T790M/C797S-B peptide using an IFN-γ ELISPOT assay (Figure 2B). In donor 2, the spot number of CD8$^+$ cells targeting the T790M/C797S-B-pulsed T2 cell line was adequately higher than that of CD8$^+$ cells targeting no peptide- or HIV-gag peptide-pulsed T2 cell line; the spot number of no peptide, HIV-gag peptide and T790M/C797S-B peptide, were 20, 18 and 197, respectively. These results suggested that the T790M/C797S-B peptide had immunogenic potential and that T790M/C797S-B-specific CTL could be induced from human PBMC of donor 2.

In contrast, IFN-γ ELISPOT assay for T790M/C797S-D, which had the second highest binding ability to the HLA-A*02:01 molecules, showed that T790M/C797S-D-specific CTL was not induced from PBMC of a healthy donor having HLA-A*02:01 (Figure S2).

### 3.3 Establishment of T790M/C797S-B-specific CTL clones from human PBMC using a CD107a assay

We next attempted to establish T790M/C797S-B-specific CTL clones from human PBMC from healthy donor 2. We performed a CD107a assay against CD8$^+$ cells from donor 2 that were co-cultured with T790M/C797S-B-pulsed T2 cells, using cell sorting with a CD107a antibody. The data for the flow cytometry analysis is shown in Figure 2C. The population of CD8$^+$CD107a$^+$ cells represented 0.4% of all stimulated cells. These cells were sorted as T790M/C797S-B-specific CTL clones in each well of a 96-well plate, using a FACSAria II cell sorter. In addition, we demonstrated with an IFN-γ ELISPOT assay that the established CTL clones had adequate T790M/C797S-B peptide specificity. In particular, one CTL clone (clone 1) had a more sufficiently sensitive reactivity to T790M/C797S-B-pulsed T2 cells compared to HIV-gag peptide-pulsed T2 cells; the spot numbers of T790M/C797S-B peptide and HIV-gag peptide were 563 and 0, respectively (Figure 2D). These results indicated that T790M/C797S-B-specific CTL clones were successfully established from human PBMC from a healthy donor.

### 3.4 Evaluation of reactivity of T790M/C797S-B-specific CTL clones

We analyzed the reactivity of T790M/C797S-B-specific CTL clones with T2 cells pulsed with HIV-gag, wild-type EGFR, EGFR T790M/C797S, EGFR C797S and EGFR T790M peptides using IFN-γ ELISPOT assay (Figure 3A). The T790M/C797S-B-specific CTL clones specifically recognized T2 cells pulsed with EGFR T790M/C797S peptide but not HIV-gag peptide-pulsed T2 cells. In addition, the T790M/C797S-B-specific CTL clones showed sensitive reactivity to T2 cells pulsed with the EGFR C797S peptide. Conversely, T2 cells pulsed with wild-type EGFR and EGFR T790M peptides were not recognized by the T790M/C797S-B-specific CTL clones.

We also evaluated the cytotoxic activity of the T790M/C797S-B-specific CTL clones against T2 cells pulsed with each peptide. The T790M/C797S-B-specific CTL clones specifically lysed T2 cells pulsed with EGFR T790M/C797S and EGFR C797S peptides but not T2 cells pulsed with HIV-gag, wild-type EGFR and EGFR T790M peptides (Figure 3B). These results demonstrated that the T790M/C797S-B-specific CTL clones had sensitivity to EGFR T790M/C797S peptide but not wild-type EGFR peptide, and also had adequately cross-reactivity against the EGFR C797S peptide.
3.5 | Analysis of functional avidity of T790M/C797S-B-specific CTL clones

We evaluated the cytotoxicity profiles of the T790M/C797S-B-specific CTL clones against T2 cells pulsed with a decreasing concentration series from $10^{-5}$ to $10^{-13}$ M of the EGFR T790M/C797S peptide (Figure 3C). The peptide concentration at 50% cytotoxicity was defined as the recognition efficiency of the clone, which was $10^{-8}$ M of EGFR T790M/C797S peptide. This result indicated that the T790M/C797S-B-specific CTL clone had a high affinity for the EGFR T790M/C797S peptide.

3.6 | Evaluation of reactivity of T790M/C797S-B-specific CTL clones against cancer cell lines that express endogenous epidermal growth factor receptor T790M/C797S peptide

We assessed the ability of the T790M/C797S-B-specific CTL clone to recognize cancer cell lines that expressed endogenous EGFR T790M/C797S peptide. We used SK-HEP-1/T790M/C797S transfectants in which the EGFR T790M/C797S mutation was expressed as the target cells. The CTL clone was incubated with SK-HEP-1 or SK-HEP-1/T790M/C797S, and the reactivity of T790M/C797S-B-specific CTL...
clone to endogenous peptides was evaluated using IFN-γ ELISPOT assay (Figure 3D). Production of IFN-γ in T790M/C797S-B-specific CTL clone was detected against SK-HEP-1/T790M/C797S (316 spots) but not SK-HEP-1 (0 spots). Thus, we confirmed that high-affinity CTL had a highly sensitive response to cancer cells that express endogenous EGFR T790M/C797S peptide.

3.7 | Induction of T790M/C797S-B-specific CTL by administration of T790M/C797S-B peptide vaccine in vivo

Finally, we investigated whether antigen-specific CTL could be induced by T790M/C797S-B peptide vaccination in vivo by using a mouse model (Figure 4A). Two HLA-A2 transgenic mice were injected with the peptide vaccine twice at a week interval. A week after the second peptide vaccine administration, the spleens of these mice were harvested and CD8+ cells derived from the spleen were isolated using CD8 microbeads. These CD8+ cells were stimulated for 1 week with BM-DC pulsed with the T790M/C797S-B peptide, and using IFN-γ ELISPOT assay, we evaluated the specificity of CD8+ cells for the T790M/C797S-B peptide (Figure 4B). We found that the IFN-γ ELISPOT reactivity of the CD8+ cells targeted to T790M/C797S-B-pulsed RMA-S-HHD cells was much higher compared to that of CD8+ cells targeted to RMA-S-HHD cells pulsed with an HIV peptide. In addition, we performed the CD107a assay on CD8+ cells that were co-cultured with RMA-S-HHD cells pulsed with T790M/C797S-B or HIV peptides. The proportion of CD8+CD107a+ cells in

**FIGURE 3** Functional analysis of the T790M/C797S-B-specific CTL clone. A, Reactivity of the T790M/C797S-B-specific CTL clone with each peptide. Interferon-γ (IFN-γ) enzyme-linked immunospot (ELISPOT) assay against T2 cells pulsed with each peptide was performed in duplicate, for which representative data are shown. The T2 cells pulsed with HIV-gag, wild-type epidermal growth factor receptor (EGFR), EGFR T790M, EGFR C797S or EGFR T790M/C797S peptides were used as target cells (effector, 1 × 10^5 cells/well; target, 1 × 10^4 cells/well). B, Cytotoxic activity of the T790M/C797S-B-specific CTL clone against T2 cells pulsed with HIV-gag (blue), wild-type EGFR (purple), EGFR T790M (green), EGFR C797S (orange), and EGFR T790M/C797S (red) analyzed with a cytotoxicity assay. C, The T790M/C797S-B peptide-specific avidity of the T790M/C797S-B-specific CTL clone. The CTL clones were tested for their avidities using various concentrations of EGFR T790M/C797S (red) or HIV-gag (blue) peptide-pulsed T2 targets. The peptide concentration at which the curve crossed 50% cytotoxicity was defined as the recognition efficiency of the clone. The effector/target ratio was 10. D, The ability of the T790M/C797S-B-specific CTL clone to recognize the cancer cell line that expressed endogenous EGFR T790M/C797S peptide. IFN-γ ELISPOT assay was carried out against the cancer cell lines. SK-HEP-1 cells and SK-HEP-1/T790M/C797S cells expressing the EGFR T790M/C797S mutation were used as target cells (effector, 1 × 10^5 cells/well; target, 1 × 10^4 cells/well). The bars indicate IFN-γ ELISPOT counts.
CD8+ cells co-cultured with T790M/C797S-B-pulsed RMA-S-HHD cells was much higher than that in CD8+ cells co-cultured with HIV peptide-pulsed RMA-S-HHD cells (7.9% vs 0.5%, respectively). These results suggest that administration of T790M/C797S-B peptide vaccine could induce T790M/C797S-B-specific CTL in vivo.

4 | DISCUSSION

Epidermal growth factor receptor-tyrosine kinase inhibitors show significant clinical effects against NSCLC expressing activating EGFR mutations.8–11 However, even with osimertinib, which has an anti-tumor effect on NSCLC patients with the EGFR T790M mutation, the NSCLC patients develop acquired resistance to EGFR-TKI without exception, in approximately 1 year.14,19,21 Therefore, to overcome these resistance issues, as a different approach from conventional therapies, we found the possibility of novel immunotherapy targeting an EGFR T790M/C797S mutation-derived antigen against NSCLC patients that have developed EGFR-TKI resistance, especially to osimertinib.

Within the last 10 years, there has been rapid development of cancer immunotherapy, including immune checkpoint inhibitor therapy, which has greatly revolutionized standard cancer treatment. Indeed, clinical trials for immunotherapies targeting tumor-associated antigens in cancer patients have been conducted worldwide.36–38 Previously, we identified GPC3, which is a cancer-specific antigen, and performed clinical trials for a GPC3 peptide vaccination in patients with several cancer types, including advanced hepatocellular carcinoma (HCC).32,39–44 As a result of these trials, we confirmed an adequate anti-tumor effect in advanced HCC with the GPC3 peptide vaccination, which led to better overall survival in these HCC patients.39,41 In addition, mutation-derived antigens in cancer cells are unique because they are not expressed in normal tissues. Thus, these antigens would not be expected to induce immunotolerance and may have higher immunogenicity compared to other antigens. Indeed, in melanoma patients who experienced dramatic therapeutic effects after adoptive cell therapy with tumor-infiltrating lymphocytes, the mutated antigen-derived epitope was immunodominant and was recognized by tumor-reactive T cells.45,46 In addition, for patients with several cancer types, personalized vaccine therapy using a tumor-specific mutated antigen had good clinical effects.29,47 As described above, mutation-derived cancer antigens involved in proliferation of tumor cell and drug resistance can be ideal targets for cancer vaccine therapies. Here, we have identified
the EGFR-T790M/C797S-B peptide, which is a new tumor-associated CTL epitope derived from the EGFR T790M/C797S mutation, as a novel therapeutic target against EGFR-TKI-resistant NSCLC patients. Furthermore, we confirmed that T790M/C797S-B-specific CTL can be induced from PBMC of healthy donors.

In this study, we used a CD107a mobilization assay to generate T790M/C797S-B-specific CTL clones from healthy donor PBMC. Rubio et al showed that the cell sorting using a CD107a mobilization assay was useful for identifying and isolating functional tumor-reactive T cells with high affinity for PBMC of cancer patients after vaccination.48 We also previously established several GPC3-specific CTL clones from PBMC of advanced HCC patients vaccinated with GPC3 peptide vaccine using single-cell sorting with a CD107a antibody.32 In addition, we demonstrated that these CTL clones were capable of killing HCC cells expressing GPC3.32 In this study, we successfully established EGFR T790M/C797S-B-specific CTL clones with high affinity and antigen-specific killing activity against tumor cells, using a CD107a mobilization assay.

Human leukocyte antigen class I molecules play an important role in the anti-tumor immune system because these molecules present tumor-specific antigens to CTL that have cancer cell recognition and killing functions.49 Therefore, even if immunotherapies, including peptide vaccines or TCR gene transfer T-cell therapy, induced peptide-specific CTL into the tumor microenvironment, it cannot be expected that these immunotherapies result in adequate anti-tumor effects unless the peptides are presented on the HLA class I molecule at the surface of the cancer cell. In fact, several studies have reported that HLA class I expression could be a prognostic factor in a variety of cancers.50-52 In this study, we clarified that by using cancer cell lines that transfected the EGFR T790M/C797S mutation gene, EGFR T790M/C797S-B peptides are endogenously presented on HLA class I molecules, and are recognized by the peptide-specific CTL clones with high sensitivity. In addition, our results using the A2-Tg mouse model show that EGFR T790M/C797S-B peptide administration could induce peptide-specific CTL in vivo. Therefore, in the future, the EGFR T790M/C797S-B peptide could be remarkably useful as a cancer peptide vaccine against the EGFR T790M/C797S mutation and HLA-A2.

In our study, the EGFR T790M/C797S-B-specific CTL clones did not show activity against wild-type EGFR or EGFR T790M peptides. This result suggested that immunotherapy using EGFR T790M/C797S-B peptide may have no anti-tumor effect in NSCLC patients without the EGFR C797S mutation, at least theoretically. Recently, immunotherapy in combination with conventional cancer therapy including chemotherapy, radiation therapy and molecular targeted therapy, was reported to have a synergistic effect on patients with several types of cancer.53 As one of the mechanisms, several chemotherapeutic agents can lead the upregulation of tumor-association antigen expression or the improvement of tumor cell resistance to specific CTL.54 In addition, Pollack et al showed that use of EGFR-TKI augments the IFN-γ-induced expression of MHC classes I and II by A431 malignant human keratinocytes.55 Therefore, we propose that a combination of immunotherapy using the EGFR T790M/C797S-B peptide and conventional therapy, including EGFR-TKI treatment and chemotherapy, could be the best option for the initial treatment of NSCLC patients.

In conclusion, we identified the EGFR T790M/C797S-B peptide as a novel HLA-A2-restricted EGFR T790M/C797S-derived CTL epitope. We also successfully established EGFR T790M/C797S-B-specific CTL clones from human PBMC and demonstrated that these clones had the ability to recognize and lyse T2 cells pulsed with the EGFR T790M/C797S peptide. These CTL clones had a highly sensitive response to cancer cells that expressed endogenous EGFR T790M/C797S peptide. In addition, the proof of induction of EGFR T790M/C797S-B-specific CTL in the A2-Tg mouse model in vivo indicated that vaccine therapy using the EGFR T790M/C797S-B peptide could have an adequate anti-tumor effect. These results suggest that immunotherapy targeting the EGFR T790M/C797S mutation could be useful as a novel therapeutic strategy against NSCLC patients with EGFR-TKI resistance.

ACKNOWLEDGEMENTS
This study was supported in part by the National Cancer Center Research and Development Fund (25-7 and 28-A-B). We would like to thank Dr Tetsuro Sasada (Kanagawa Cancer Center Research Institute, Yokohama, Japan) for providing the EGFR expression vectors. In addition, we wish to thank Editage (www.editage.jp) for English language editing.

DISCLOSURE STATEMENT
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
YA, YS, TY and TN designed the study. YA, YS and TY analyzed the data. YA wrote the manuscript. All authors reviewed and accepted the manuscript.

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**SUPPORTING INFORMATION**
Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Akazawa Y, Saito Y, Yoshikawa T, et al. Efficacy of immunotherapy targeting the neoantigen derived from epidermal growth factor receptor T790M/C797S mutation in non–small cell lung cancer. *Cancer Sci*. 2020;111:2736–2746. [https://doi.org/10.1111/cas.14451](https://doi.org/10.1111/cas.14451)