Generation of novel complete HLA class I monoallelic cell lines used in an MHC stabilization assay for neoantigen evaluation

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Received January 17, 2023; Accepted May 16, 2023

DOI: 10.3892/ol.2023.13910

Abstract. Immunogenic neoantigens derived from somatic mutations in cancer have been identified through clinical studies with the cloning of tumor-infiltrating T cells, and cancer driver gene mutation-derived epitopes have been reported; however, these are rare. At present, the validation of epitopes predicted *in silico* is difficult as human T-cell clonal diversity cannot be reproduced *in vitro* or in experimental animal models. To confirm the epitope peptides presented by human leukocyte antigen (HLA) class I molecules predicted *in silico*, biochemical methods such as major histocompatibility complex (MHC) stabilization assays and mass spectrometry-mediated identification have been developed based on HLA-A*02:01 monoallelic T2 cells and HLA-C*01:02 monoallelic LCL721.221 cells. Therefore, in the present study, to prevent confusion due to peptide cross-presentation among HLA molecules, HLA class I monoallelic B-cell clones were generated from the TISI cell line by knocking out HLA-ABC and *TAP2*, and knocking in HLA alleles. To explore cancer driver mutations as potential targets for immunotherapy, exome sequencing data from 5,143 patients with cancer enrolled in a comprehensive genome analysis project at the Shizuoka Cancer Center were used to identify somatic amino acid substituted mutations and the 50 most frequent mutations in five genes, *TP53*, *EGFR*, *PIK3CA*, *KRAS* and *BRAF*, were identified. Using NetMHC4.1, the present study predicted whether epitopes derived from these mutations are presented on major HLA-ABC alleles in Japanese individuals and synthesized 138 peptides for MHC stabilization assays. The authors also attempted to examine the candidate epitopes at physiological temperatures by using antibody clone G46-2.6, which can detect HLA-ABC, independent of β2-microglobulin association. In the assays, although the peptide-induced HLA expression levels were associated with the predicted affinities, the respective HLA alleles exhibited varying degrees of responsiveness, and unexpectedly, p53-mutant epitopes with predicted weak affinities exhibited strong responses. These results suggested that MHC stabilization assays using completely monoallelic HLA-expressing B-cell lines are useful for evaluating the presentation of neoantigen epitopes.

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Abbreviations: MHC, major histocompatibility complex; HLA, human leukocyte antigen; TCR, T-cell receptor; AAS, amino acid substitution; TCGA, The Cancer Genome Atlas; ICI, immune checkpoint inhibitor; ICB, immune checkpoint blockade

Key words: HLA, MHC, antigen peptide transporter 2, neoantigen, driver, immunotherapy, CRISPR

Introduction

Recently, with improvements in sequencing technology, information on human cancer genomes has increased. Tumor mutation burden (TMB) and mutation-derived neoantigens are novel findings obtained from cancer genome sequencing (1-3). TMB-high, microsatellite instability-high (MSI-high) or mismatch repair-deficient (dMMR) tumors, which leads to a number of neoantigens, are known predictive markers for the efficacy of immune checkpoint inhibitor (ICI) therapies (4-6); pembrolizumab became the first drug approved for the treatment of cancer selectively according to these biomarkers rather than the primary tumor site. The advent of cancer

immunotherapy has been a revolution for cancer patients, and indications for ICIs and combination therapies have expanded to various cancer types, ranging from melanoma, colorectal cancer, gastric cancer, hepatocellular carcinoma and others over the past decade (7). However, the use of ICIs, which allows the immune system to comprehensively target antigens, is known to cause immune-related adverse effects (8), hyperprogressive disease (9) and an increased risk of early mortality (10); it has also not benefited for the patients with low TMB tumors. On the other hand, tebentafusp, which targets specific cancer antigen epitopes on human leucocyte antigen (HLA), has also entered clinical use (11). Therefore, in the development and selection of immune-oncology therapeutics, the detection of whether a tumor has a mutation that can be targeted by the patient's immune system has become a crucial factor.

The majority of neoantigens are derived from passenger mutations that are not involved in carcinogenesis, and they are more likely to exert immunogenic effects than to be cancer driver mutations (12). In general, driver mutations are considered to be difficult antigens to target, as they are associated with cancer development and exert immunomodulatory activity in the tumor microenvironment (13-15). Nevertheless, researchers have successfully cloned and used driver mutation-specific tumor-infiltrating T-cell (TIL) or T-cell receptor (TCR) repertoires for adoptive immunotherapy (16,17). Small clinical trials of immunotherapy using driver mutation-derived neoantigens have been performed (18,19), and moderate anti-tumor effects have been verified. In particular, Chen *et al* (20) demonstrated that vaccines targeting common driver mutations are applicable to metastatic cancers, and the combination of vaccines and immunomodulatory therapies may constitute a promising regimen. However, the combined diversity of HLAs, antigen-presenting molecules and mutation-derived epitopes is a major obstacle to the clinical development of cancer vaccines. Although the accurate prediction of epitopes is an essential component of personalized immunotherapy, it is difficult to validate *in silico* predicted epitopes as human T-cell clonal diversity cannot be reproduced *in vitro* or *in vivo*.

To confirm predicted antigen epitopes, immunological and biochemical approaches such as major histocompatibility complex (MHC) stabilization assays or mass spectrometry (MS)-mediated identification have been developed based on HLA-B-, HLA-C-null T2 cells (CVCL_2211) (21) or HLA-class-I-null LCL721.221 cells (CVCL_6263, ATCC CRL-1855), to which HLA-alleles have been transferred (22-24); however, HLA-C expression in LCL721.221 cells was not found to be completely abrogated (25); therefore, this cell line was discontinued by the American Type Culture Collection (ATCC). The present study generated antigen peptide transporter 2 (*TAP2*)-knockout (KO) and monoallelic HLA-class-I-expressing B-cell lines derived from the TISI cell line (CVCL_E851) (26) for MHC stabilization assays, screened frequent hot spot mutations in 5,143 cancer patients treated at the Shizuoka Cancer Center Hospital and identified driver mutation-based neoantigen epitope candidates.

Materials and methods

Comprehensive cancer research project HOPE. A total of 5,143 cancer cases were analyzed in the High-tech Omics-based Patient Evaluation (HOPE) project, which has been conducted in Shizuoka Cancer Center since 2014 using multiomics analyses, such as whole-exome sequencing (WES) and gene expression profiling (27). For the WES analysis, somatic mutations were identified by comparing data on tumors and corresponding blood samples. Total exonic mutations for each sequenced tumor included single-nucleotide variants and indel/frameshift mutations. All nonsynonymous mutations detected in the cases were collected and screened.

Prediction of epitope peptides in silico. We used the NetMHCpan 4.1 (Immune Epitope Database, https://www.iedb.org/) to assess HLA presentation of 8 to 11-mer peptide epitopes from cancer driver gene mutation to predict affinity (IC_{50}) on the basis of the binding affinity (BA) model or to predict rank scores (%Rank) on the basis of the eluted ligand (EL) model.

Cell lines. TISI human B-lymphoblastoid cell line (B-LCL) (CVCL_E851) with homozygous HLA-class-I loci (26) were supplied by Takara Shuzo Co., Ltd. (Otsu, Shiga, Japan), and we verified the HLA types by Sanger sequencing using SeCore SBT kit (#5300025, One Lambda, Thermo Fisher Scientific, Waltham, Massachusetts, USA). T2 cells were purchased from American Type Culture Collection (#CRL-1992, ATCC, Manassas, VA, USA). These cell lines were maintained in RPMI-1640 medium (#R8758, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) supplemented with 10% (v/v) FBS (#10437, Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and 1x penicillin-streptomycin (#151401222, Gibco). Newly constructed TAP2-KO HLA-ABC monoallelic cell clones were maintained in RPMI-1640 medium supplemented with 10% (v/v) FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate (#11360070, Gibco), 1x MEM nonessential amino acids (#11140050, Gibco), and 55 μg/ml 2-mercaptoethanol (#21985-023, Gibco).

Flow cytometry and antibodies. Monoallelic cell cloning was performed using a PE-labeled anti-HLA-ABC monoclonal antibody (clone: G46-2.6, BD Biosciences, Franklin Lakes, New Jersey, USA) to stain HLA-ABC KO or knock-in (KI) cells and a FACSAria cell sorter (BD Biosciences) for sorting single cells to 96-well plates. For the MHC stabilization assay, we used PE-labeled anti-HLA-ABC antibody clone G46-2.6 diluted 1:10 and clone W6/32 (Dako Denmark A/S, Dako North America, Inc., Carpinteria, California, USA) diluted 10 μg/ml with FACSCanto flow cytometer (BD Biosciences) for analysis. PE-labeled anti-mouse Ig polyclonal antibody (#550589, BD Biosciences) was used as the secondary antibody. FlowJo software ver.8.8.7 (Tomy digital biology Co., Ltd. Tokyo, Japan) was used for analysis.

Construction of TAP2-deficient and HLA class I monoallelic cell lines. Using the CRISPR/Cas9 system (Invitrogen, Thermo Fisher Scientific), *TAP2* and *HLA-A*, *HLA-B*, and *HLA-C* genes were knocked out in the TISI cell line using synthetic gRNAs (#A35510, Invitrogen) (Table SI). Each synthetic HLA-class-I allele cDNA (Genewiz, Tokyo, Japan) corresponding to a TISI-HLA-A*24:02:01:01-deleted site was transcribed to single-stranded DNA and isolated from double strand using agarose gel (#50070, Lonza) electrophoresis

with ethidium bromide (#315-90051, Nippongene), and Purified single-stranded DNA was knocked in the TAP2- and HLA-ABC-KO TISI subclone using a paired sgRNA. Each KO and KI site was confirmed by PCR and Sanger sequencing. All sgRNAs and primer pairs are shown in Tables SI and SII.

Reagents and solvents for peptide synthesis. Fmoc-amino acids were obtained from CEM Corp. (Matthews, NC, USA). Fmoc-amino acid-Wang-resins were obtained from Gyros Protein Technologies Inc. (Tucson, AZ, USA). H-Pro-Barlos resin and reagents for peptide synthesis, 1-[bis(dimethylamino) methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU), N,N'-diisopropylcarbodiimide (DIC), Oxyma Pure [Ethyl-2-cyano-2-(hydroxyimino)acetate], N,N-diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), triisopropylsilane (TIPS), and methylene chloride (DCM), were purchased from Watanabe Chem. IND., LTD. (Hiroshima, Japan). Piperidine, pyrrolidine, phenol and ethanedithiol (EDT) were obtained from Fujifilm Wako Pure Chem. Corp. (Tokyo, Japan). N,N'-Dimethylformamide (DMF), N-methyl-2-pyrrolidone (NMP), HPLC grade acetonitrile (ACN) and water were obtained from Kanto Chem. Co. Inc. (Tokyo, Japan).

Peptide synthesis. Neoantigen peptides were synthesized via solid-phase chemistry using an Fmoc protection strategy. Starting from 0.05 mmol of Fmoc-amino acid-Wang-resin, protected peptide resins were constructed using 0.5 mmol of Fmoc-amino acids with tert-butyl-based side-chain-protecting groups on peptide synthesizers with the peptide synthesizer Tribute (Gyros Protein Technologies Inc.) at room temperature or with the microwave peptide synthesizer Liberty PRIME (CEM Corp.) at high temperature (105˚C). As Fmoc removal (deprotection) and peptide bond formation (coupling) reagents, 20% piperidine/NMP and HATU/DIPEA for Tribute and 20% pyrrolidine/DMF and DIC/Oxyma for Liberty PRIME, respectively, were used. The synthesis of peptides bearing Pro at the C-terminus was carried out starting with H-Pro-Barlos-resin using a Tribute synthesizer. After final deprotection of the Nα-Fmoc group of the N-terminal amino acid, the partially protected peptide resin obtained was washed with DCM and MeOH and dried *in vacuo*. Removal of side-chain-protecting groups and cleavage of the resin were performed by treatment with a TFA-containing scavenger cocktail (TFA-phenol-EDT-thioanisole-H2O-TIPS; 90-2-2-2-2-2) at room temperature for 1.5-3 h. The product was precipitated with ether, collected by centrifugation, and dried *in vacuo*. The resulting crude peptides were purified using a 1525 Binary HPLC Pump equipped with a 2489UV/VIS detector (Waters Corp., Milford, MA, USA) with a YMC-Actus Triart C18 column, 5 μm, 20x150 mm (YMC Corp. Kyoto, Japan). The purity and mass spectrum of the purified peptides were analyzed using an Alliance e2695 HPLC System equipped with a 2489UV/VIS detector and an ACQITY QDa mass detector (Waters Corp.) with an XSelect CSH C18 column, 2.5 μm, 3x75 mm column (Waters Corp.). All synthetic peptides are shown in Table SIII.

MHC stabilization assay. The TAP2-KO monoallelic HLA class I-expressing TISI cell lines were precultured with 10,000 U interferon (IFN)-α (Smiferon300, Sumitomo Pharma, Osaka, Japan) to restore baseline HLA expression. $1x10^5$ HLA monoallelic cells were incubated with 25 μM peptide in RPMI 1640 medium with 3 μg/ml beta-2 microglobulin (#21985023, Sigma-Aldrich), 55 μM 2-melcaptoethanol and 0.1% BSA (#A7906, Sigma-Aldrich) at 37˚C with 5% CO_2 for 18 h. Cells cultured with 0.5% DMSO without peptide were used as negative controls. Following incubation, the cells were stained with PE-labeled anti-HLA-ABC antibody (clone: G46-2.6) diluted 1:10 at RT (23˚C) for 60 min, washed 3 times with PBS supplemented with 0.5% BSA and 2 mM EDTA at 4˚C, and fixed with 0.4% paraformaldehyde in PBS with 0.1% BSA and 2 mM EDTA. HLA class I expression increase (ΔHLA) was measured using a FACSCanto flow cytometer (BD Biosciences). The ΔHLA was obtained by calculating the geometric mean of the fluorescence intensity (MFI) using the following formula:

ΔHLA (% control)={(Sample MFI)-(DMSO cont. MFI)}/{(DMSO cont. MFI)-(Unstained background MFI)} x100

Statistical analysis. To determine the significance of differences between HLA expression levels, Kruskal-Wallis test followed by Steel's multiple comparison test was performed and $P<0.05$ was considered to indicate a statistically significant difference. The Kruskal-Wallis test, the Steel's multiple comparison test, and Pearson or Spearman correlation coefficients were calculated using EZR version 1.55 (Saitama Medical Center, Jichi Medical University, Saitama, Japan) (28). All means and standard deviations in the tables are based on three or more independent experiments.

Results

Generation of TAP2-KO HLA-class-I monoallelic B-lymphoblastoid cell lines. The MHC stabilization assay was improved with the use of HLA-partially-null B-lymphoblastoid cell lines to confirm epitope peptide presentation by HLA molecules (21,22,24). To perform a more reliable MHC stabilization assay, *HLA-A*-, *HLA-B*-, *HLA-C*- and *TAP2*-KO clones derived from the TISI cell line were generated (Figs. S1-S3) and the respective single HLA alleles were knocked in using the CRISPR/Cas9 system (Fig. S4). TISI cells were selected as they constitute a B-lymphocyte line with the ability to cross-present exogenous antigens on HLA molecules and carry homologous HLA-class-I loci. The HLA allele cDNA was knocked in the loci of HLA-A*24:02:01 (Fig. 1A and B). HLA class I expression was not detected in the TAP2, HLA-A, HLA-B and HLA-C-KO TISI clone (Fig. 1C), and the cells subjected to HLA class I cDNA knock-in exhibited HLA class I on their cell surface (Fig. 1D). Each clone was confirmed using genomic DNA sequencing.

Validation of the MHC stabilization assay using T2 cells and the newly generated monoallelic HLA cells. The principle of the MHC stabilization assay is that peptides binding to HLA molecules stabilize and increase HLA expression levels on the cell surface. The first study was based on TAP2-deficient and HLA-A*02:01 monoallelic T2 cells and antibody clone W6/32, which can bind HLA-ABC associated with β-2 microglobulin (β2m) and requires 26˚C culture conditions to prevent β2m dissociation (21,29). On the other hand, antibody clone G46-2.6

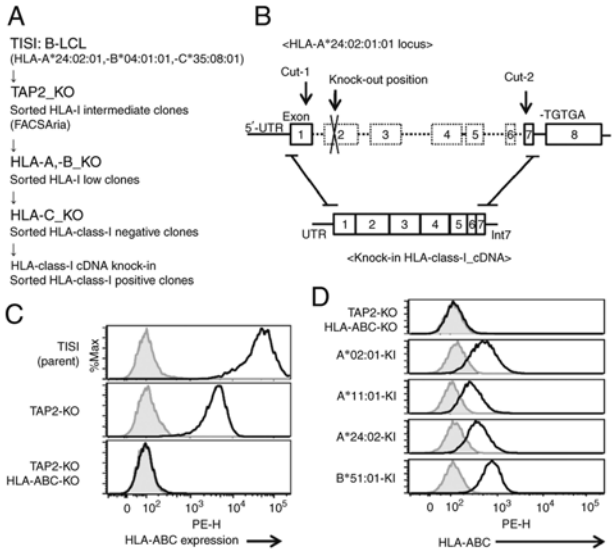


Figure 1. Generation of *TAP2*-KO HLA-class-I monoallelic B-cell lines. (A) Procedure for generating TAP2-deficient and single-HLA-class-I allele-expressing cell lines. (B) HLA allele cDNA knock-in sites using CRISPR/Cas9. (C) HLA class I expression on the cell surface of the TAP2-KO and HLA-ABC-KO cell line. (D) HLA-class-I expression on the respective HLA allele knock-in cell lines. B-LCL, B-lymphoblastoid cell line; TAP2, antigen peptide transporter 2; HLA, human leukocyte antigen; KO, knockout; KI, knock-in.

binds to HLA-ABC with or without β2m, which permits evaluation under physiological 37˚C culture conditions, preventing low-affinity peptide binding at a low temperature (30) or the effect of bovine β2m in FBS (31). The present study therefore used clone G46-2.6, which enabled clearer observations. The addition of β2m to the assay culture supernatant was necessary for the detection of HLA by antibody clone W6/32, although it exerted a minimal effect on HLA expression (Figs. 2A and S5). In addition, interferons in the culture supernatant can easily alter HLA expression levels. Since HLA expression was extremely low immediately following knock-in cell cloning, the assays were performed after culturing the cells with IFN-α for 1 week to restore the HLA expression levels.

The results revealed increased HLA expression levels (ΔHLA) in MHC stabilization assays using the in-house-generated monoallelic HLA knock-in cell lines, although the predicted peptide/HLA IC_{50} did not necessarily associate with ΔHLA (Fig. 2B). To assess the usability of the novel monoallelic HLA cells, the MHC stabilization assays were compared between the T2 cells and the monoallelic HLA-A*02:01 knock-in cell line using synthetic peptides (Table SIII). Although the knock-in cell line exhibited low HLA-A expression levels, it exhibited a good correlation with the T2 cells in the original method (Fig. 2C). Furthermore, there was a slight difference between the HLA-A*11:01 homo knock-in and hemi knock-in cell lines (Fig. 2D).

Screening of amino acid substituted (AAS) driver mutations in 5,143 cancer patients. The HOPE cohort comprised 5,521 tumor specimens derived from 5,143 patients treated at the Shizuoka Cancer Center Hospital between January, 2014 and March, 2019. The major cancer types were colon (18.4%), lung (16.5%), rectal (13.3%) and stomach (10.8%) cancers (27). As cancer-driving gene mutations are high-frequency mutations at specific sites in the genome due to evolutionary convergence (32), mutation frequency data were used to select the driver mutations assessed in the present study. This approach is consistent with the objective of screening for mutations that are common in the cancer patient population.

The results revealed 21 AAS mutations with >1% frequency in the HOPE cohort, and these mutations were found in only five cancer driver genes (Fig. 3A). The highly frequent mutations mostly overlapped with those in The Cancer Genome Atlas (TCGA) Cancer HotSpots (33). In the Japanese population, the KRAS G12S and G13D mutants were the most common, whereas KRAS G12R was less common. EGFR mutations were also more common in the Japanese population; however, as the resected primary tumors were the main samples, the EGFR T790M mutation associated with drug resistance was found in only 1 patient in this cohort (Table SIV). The eight most frequent TP53 AAS mutations were prominent at six positions. *TP53* is a tumor suppressor gene; however, these dominant-negative mutations appear to be high-frequency targets as the mutants function as oncogenes (Fig. 3B).

The present study first evaluated the frequent mutation-derived AAS epitope presentation on HLA molecules through the NetMHCpan4.1 binding-affinity (BA) model and the eluted-ligand (EL) model to predict binding affinities (IC_{50}) and rank scores (%Rank). In the Japanese dominant HLA alleles (34), a significant number of peptides were predicted to have a high affinity for HLA-A and a smaller number for HLA-B and HLA-C (Fig. 3C).

Correlation of the in silico prediction of AAS epitopes with MHC stabilization assays using the newly generated cell lines. The present study compared *in silico* predictions and *in vitro* assays of the epitopes derived from driver mutations with >0.2% frequency. A total of 138 epitope peptides were synthesized with a predicted affinity (IC_{50}) <500 nM or IC_{50} <1,000 nM and %Rank <1% for the 10 most frequently appearing HLA-A, -B and -C alleles in the Japanese population, and two peptides derived from cytomegalovirus (Table SIII). For each HLA allele, synthesized peptides with predicted IC_{50} <10,000 nM were used for the assays, and six to eight peptides with predicted IC_{50} >10,000 nM were used as negative controls. All assay results are presented in Table SV.

As was expected, ΔHLA correlated with the predicted scores and affinities, particularly for HLA-A*33:03 with the peptide predicted IC_{50} <1,000 nM (Figs. 4 and 5). The BA model IC_{50} <1,000 nM or the EL model %Rank <1% are often used as cut-off criteria in the epitope predictions; however, for HLA-A*24:02 and HLA-A*11:01, peptides with predicted IC_{50} >1,000 nM or with %Rank ranging from 2 to 4% were found to increase HLA expression. Of note, two 9-mer epitopes derived from *TP53* driver mutations exhibited extremely high responses to HLA-A*11:01 despite predicted IC_{50} >5,000 nM, which was more notable than the two 10-mer epitopes with higher predicted affinities (Fig. 5); thus, these mutations are potential new target candidates. Furthermore, the majority of the peptides, including the KRAS G12 or G13 mutation and rich in valine were presented on HLA-A*11:01 regardless of the variant of the mutation.

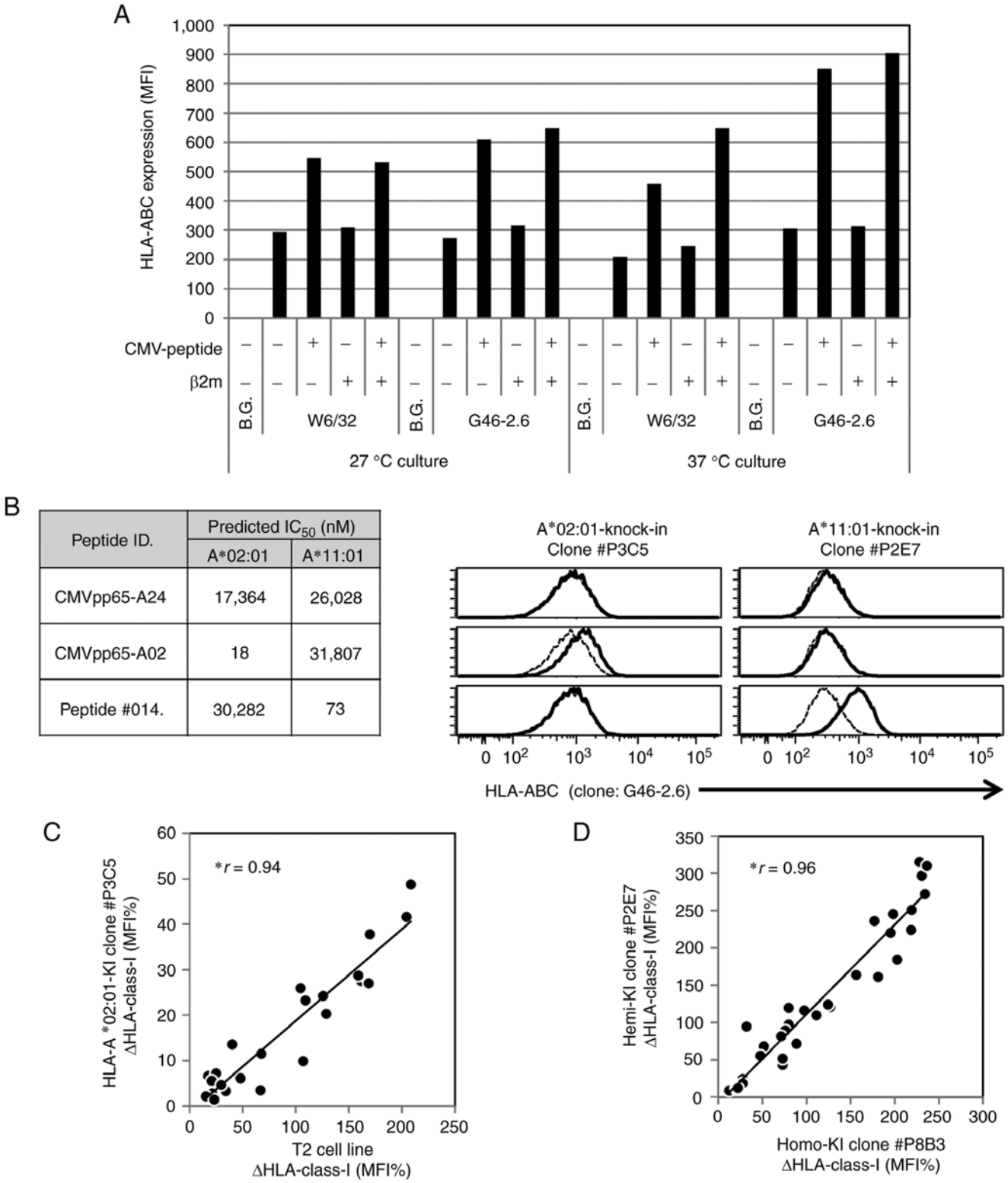


Figure 2. Validation of the MHC stabilization assay using the newly generated monoallelic cell lines. (A) Differences between the anti-HLA-ABC antibody clone W6/32 and the clone G46-2.6 in T2 cells under several culture conditions. (B) Representative increased HLA expression (ΔHLA) on the monoallelic HLA-class-I cell lines induced by immunogenic peptides. (C) Consistency of the MHC stabilization assays for the A*02:01 KI clone P3C5 and the T2 cell line. (D) Consistency of the MHC stabilization assays for the HLA-A*11:01 homo-KI and hemi-KI clones. The figures present representative data from the two to four experiments. *r values denote Pearson's correlation coefficient. HLA, human leukocyte antigen; KI, knock-in.

Candidate epitopes based on driver mutations identified through the MHC stabilization assay. The epitopes candidates were screened on the basis of the following criteria (Fig. 6): BA_IC_{50} <1,000 nM, EL_%Rank <1%, no proline in the three forward positions to interfere with peptide processing (35), cysteine content <2 molecules (36,37) and ΔHLA >20% in the MHC stabilization assay with the synthetic peptides. Finally, 27 candidate epitopes were identified in the present study (Table I).

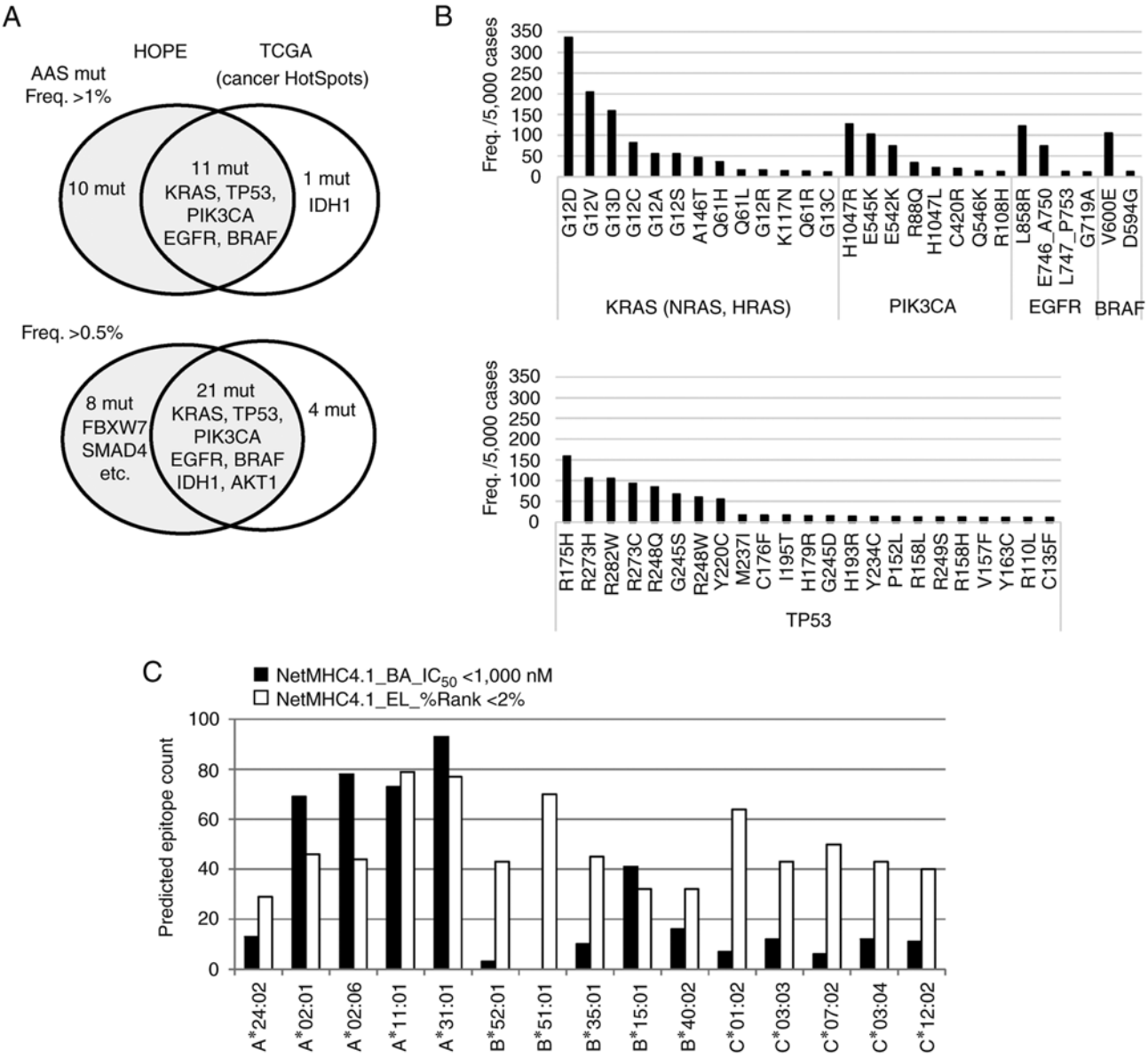


Figure 3. AAS cancer driver gene mutations in 5,143 cancer patients. (A) Number of frequently occurring AAS mutations in the HOPE and TCGA cohorts. (B) Total number of the five most frequent cancer driver gene mutations found with >0.2% (>10 patients) frequency in the HOPE cohort. (C) Respective number of 8- to 11-mer AAS epitopes with a frequency of >0.2% predicted to be presented on the major HLA-A, HLA-B, or HLA-C types common in the Japanese population using NetMHC4.1. AAS, amino acid substituted; TCGA, The Cancer Genome Atlas; HLA, human leukocyte antigen.

Discussion

Although, the cancer antigen targeted DC vaccine was once approved by the FDA (38), the paradigm shift in immuno-oncology represented by T-cell-targeted immune checkpoint blockade (ICB) therapy has now made the cancer vaccines a niche. The ICB is highly effective for TMB-high or MSI-high tumors, and numerous clinical trials combining ICI and chemotherapy are underway (39). However, approaches targeting a specific cancer antigen have continued. A considerable number of cancer vaccine studies based on neoantigen and/or dendritic cells have demonstrated more than marginal antitumor effects; however, these peptides and vaccines exhibited more potent and more persistent antitumor effects when used in combination with immunomodulatory chemotherapy (20,40). Moreover, bi-specific antibody targeting the cancer-associated antigen epitope, restricted by a HLA allele, was approved by the FDA (11), and similar agents targeting driver mutations are also under development (41,42).

Cancer driver mutation-targeted immunotherapies are crucial in the field of clinical oncology, and validating the immunogenicity of neoantigen epitopes is critical, yet difficult in preclinical studies. Therefore, candidate cancer antigens identified via *in silico* prediction are being evaluated in immune cell-based assays. To confirm epitope peptide presentation on HLA molecules, methods such as isotope-labeled peptide binding to purified HLA molecules, MHC stabilization assays or MS-based assays have been performed. However, simple *in vitro* affinity measurements are not considered reliable, as peptide loading on HLA molecules is based on a peptide-loading complex in the endoplasmic

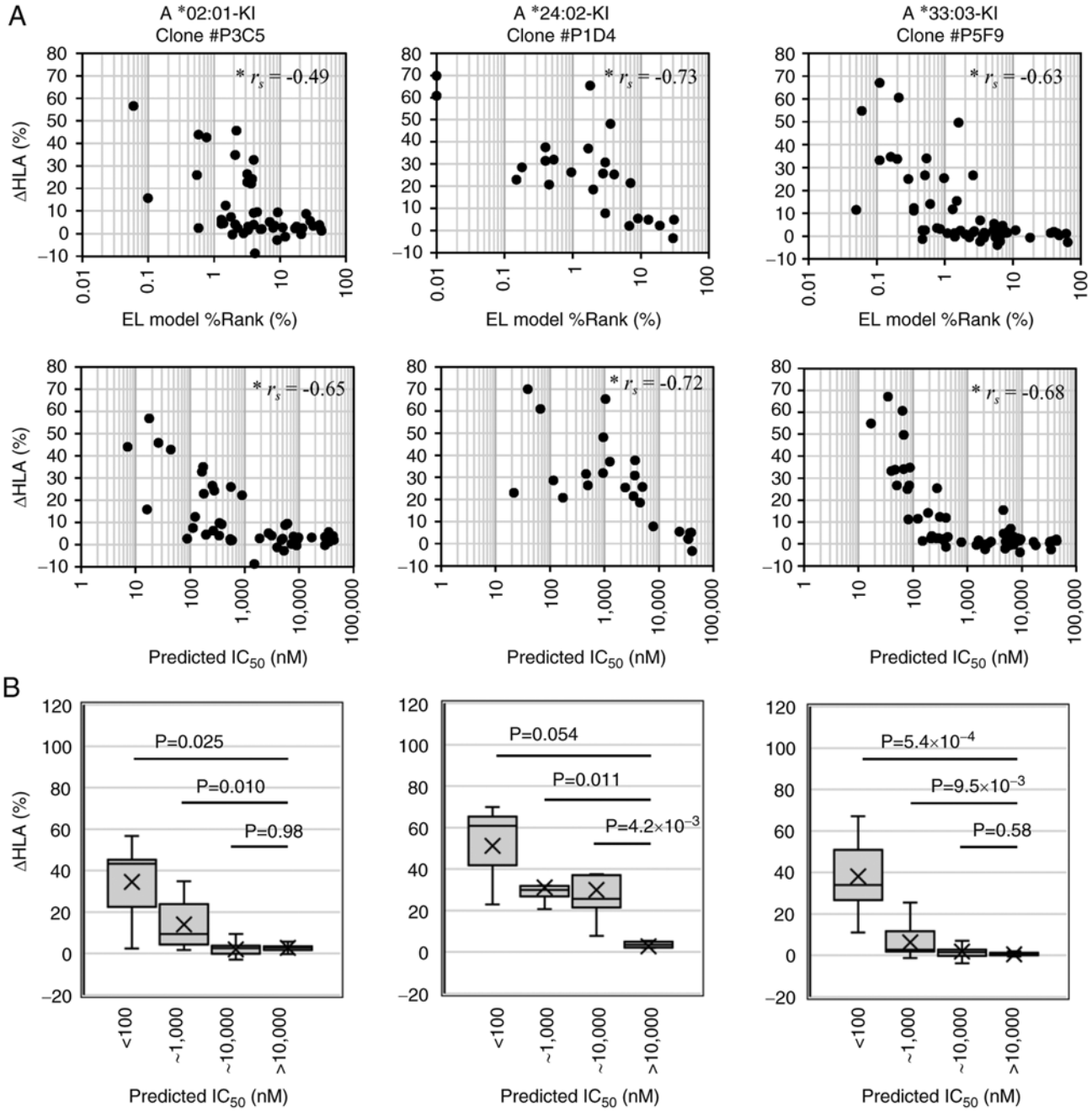
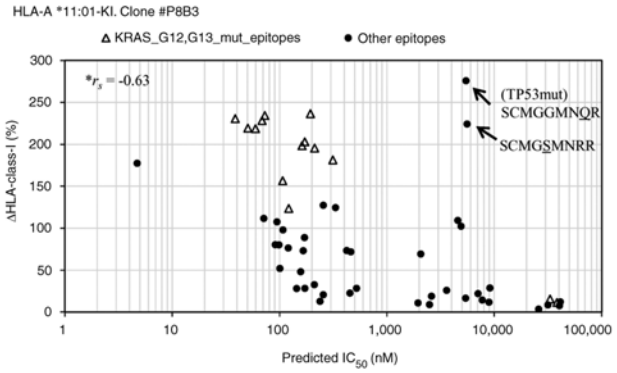


Figure 4. Correlation of the MHC stabilization assay ΔHLA and predicted peptide/HLA binding affinities or %Rank scores. (A) NetMHC4.1 predicted %Rank (%) or binding affinities (nM) are plotted on the x-axis, and the increased HLA expression (ΔHLA) on the monoallelic HLA cell lines following 18 h of culture with synthetic peptides are plotted on the y-axis. Each dot values represent the average of three independent experiments. *r_s values denote Spearman rank correlation coefficient. (B) ΔHLAs were plotted for each range of predicted affinity values. The data were analyzed for statistical significance using Kruskal-Wallis test followed by Steel's multiple comparison test. HLA, human leukocyte antigen.

reticulum (43). Moreover, the expression of multiple HLA-class-I alleles renders epitope prediction unreliable. The MHC stabilization assay with a single-HLA-expressing cell line is an efficient immunological tool with which to evaluate the CTL induction activity of antigen peptides (44). Research using HLA allele knock-in cells generated from the HLA-A- and HLA-B-null LCL721.221 cell line has reported large numbers of autoantigen epitopes with high affinity for HLA molecules based on analysis using high-throughput mass spectrometry systems (23). Furthermore, Kaseke *et al* (24) knocked out the *TAP1* gene to generate cells that can be used for MHC stabilization assays in evaluating viral antigens. The present study successfully generated the novel *TAP2*-KO and completely HLA-ABC null clones, as well as the HLA-A or HLA-B monoallelic clones with the B-lymphoblastoid TISI cell line using the CRISPR/Cas9 system to ensure the reliability of the MHC stabilization assay (Fig. 1). These monoallelic HLA-expressing cell lines were used to evaluate candidate epitopes for use as cancer vaccines.

Since the development of next-generation sequencing allowed for the identification of neoantigens derived from mutations, studies have been conducted to discover and



Mutation	Epitope peptide	HLA-A*11:01		
		EL_model %Rank (%)	Predicted IC_{50} (nM)	ΔHLA-I (%)
TP53_R248Q	SCMGGMNQR	2.00	5,475	275
	SSCMGGMNQR	0.49	171	89
TP53_G245S	SCMGSMNRR	2.70	5,603	224
	SSCMGSMNRR	0.78	145	28

Figure 5. Peptide groups with a marked HLA increase in the MHC stabilization assay with the HLA-A*11:01 allele KI clone. Predicted peptide/HLA affinities (nM) were plotted on the x-axis with ΔHLA (%) on the y-axis. KRAS_G12 or G13 mutation-derived epitopes are indicated by open triangles. Each dot values represent the average of three independent experiments. The predicted affinities and ΔHLA of the two distinct TP53 mutation-derived epitopes are indicated with EL_%Rank in the table. HLA, human leukocyte antigen.

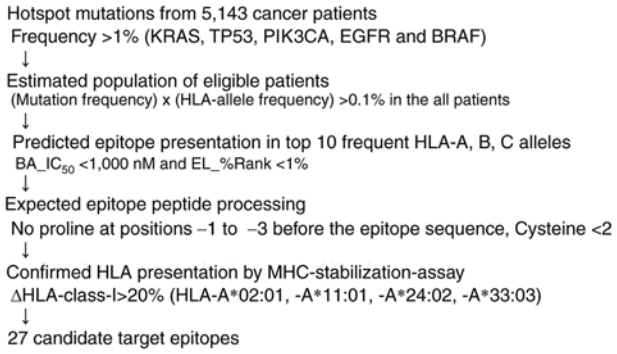


Figure 6. A procedure for screening immunogenic epitope peptides derived from cancer driver gene mutations in 5,143 cancer patients enrolled in the HOPE cohort. HLA, human leukocyte antigen.

characterize neoantigens (1,45,46). As a result, a number of neoantigens that may contribute to the prediction of a favorable response to ICB therapy have been identified thus far, and the majority of these neoantigens have been demonstrated to be products of passenger mutations (47,48). Passenger mutations are characterized as being i) diverse and abundant, but not conserved; and ii) likely to exhibit a decreased expression in recurrent cancers following ICB treatment and to not produce persistent cancer antigens (49,50). By contrast, driver mutations are exclusive and highly persistent, and maintained even at recurrent or metastatic tumor sites, and their functional inhibitions suppress tumor growth (51,52). Based on these advantages of driver mutations, small clinical trials of immunotherapy have been performed using driver mutation-derived neoantigen peptides or mRNA vaccines with or without ICB therapy (3), and the study (53) has reported that highly immunogenic mutations tend to be less likely to appear, even in oncogenes. However, KRAS- or p53-driver mutation-specific T-cells or TCR repertoires have been recently identified and utilized in patients with metastatic cancer as therapeutics, and a certain degree of efficacy has been observed (16,54,55). Therefore, frequent driver mutations are potential therapeutic targets. The present study screened frequent amino acid-substituted somatic mutations from the HOPE cohort. *KRAS* and *EGFR* mutations were more common in the HOPE cohort than in the TCGA cohort, apart from *KRAS*-G12R. (Table SIV). This finding is consistent with the fact that approximately half of the cases were colon, rectal and lung cancers, and EGFR mutations are common among Japanese non-smoking female patient with lung adenocarcinoma (56).

In the cancer patients who visited the Shizuoka Cancer Center Hospital, driver mutation-derived 27 candidate epitopes for the top four most frequent HLA-A alleles (Table I) were obtained by the procedure illustrated in Fig. 6 with some notable results. First, multiple targets were identified in HLA-A*11:01 and HLA-A*33:03, and the most frequent KRAS G12 and G13 mutation-derived hydrophobic epitopes were included among these targets. The phenotypic frequency of the A*11:01 allele was 17% in the Japanese population and ~12% in the European Caucasian population; the majority of tumors with KRAS mutations were targetable in these populations. Second, when comparing the *in silico*-predicted IC_{50}s with the HLA stabilization assay results, the epitope prediction for HLA-A*24:02 was underestimated compared to that for A*02:01, and that for A*33:03 was overestimated (Fig. 4 and Table SV). Third, strong antigen presentation was observed at two epitopes derived from TP53 mutations (Fig. 5) that were excluded from the candidate list (Table I), due to their predicted very low affinity, and this suggests that there may be some high-affinity epitopes among the peptides with low predicted affinities. HLA-A*24:02 is the most frequently expressed allele in East Asian populations, including Japanese. Although there is only one HLA-A*24:02-binding epitope included in the target list (Table I), experimental evaluation is likely to uncover additional mutation-derived potential epitopes. It may be possible to expand the number of driver mutation-derived targets. The accumulation of immunocytological data using such completely HLA monoallelic cells may be useful to improve the prediction of epitopes presented on HLA. More accurate prediction and validation of immune targetable driver mutations would complement the development of novel therapeutics through personalized procedure.

The HLA multiplex and polymorphisms also cause graft-vs.-host disease and that is a major obstacle in cellular immunotherapy and regenerative medicine; HLA haplobanks, HLA monoallelic gene expression and HLA knock-in mice may further facilitate research and development (57-59). For clinical applications, the potential of antigenic epitopes needs to be carefully confirmed by CTL induction and/or humanized mouse models. In the future, the authors aim to perform a cancer immunotherapy study targeting these epitopes against solid tumors.

Table I. Candidate 27 epitopes of driver mutations

No.	AAS mutation	Freq.[a]	Sequence[b]	HLA					Estimated patient[d], %
				Allele	BA_ IC_{50}, nM	EL_Rank, %Rank	ΔHLA[c], %		
							Average	±SD	
1	KRAS_G12D	336	VVGA<u>D</u>GVGK	A*11:01	172	0.35	203	62	1.14
2	KRAS_G12D	336	VVVGA<u>D</u>GVGK	A*11:01	194	0.59	236	69	1.14
3	KRAS_G12V	204	VVGA<u>V</u>GVGK	A*11:01	39	0.10	230	77	0.69
4	KRAS_G12V	204	VVVGA<u>V</u>GVGK	A*11:01	69	0.35	228	69	0.69
5	KRAS_G13D	159	VVVGAG<u>D</u>VGK	A*11:01	213	0.59	195	59	0.54
6	KRAS_G13D	159	VVGAG<u>D</u>VGK	A*11:01	314	0.60	181	43	0.54
7	KRAS_G12C	82	VVGA<u>C</u>GVGK	A*11:01	73	0.52	234	73	0.28
8	KRAS_G12C	82	VVVGA<u>C</u>GVGK	A*11:01	122	0.59	123	30	0.28
9	KRAS_G12A	55	VVGA<u>A</u>GVGK	A*11:01	60	0.18	218	65	0.19
10	KRAS_G12S	55	VVGA<u>S</u>GVGK	A*11:01	51	0.15	219	67	0.19
11	TP53_R175H	159	VVR<u>H</u>CPHHER	A*33:03	79	0.29	25	8	0.45
12	TP53_R175H	159	EVVR<u>H</u>CPHHER	A*33:03	88	0.16	35	3	0.45
13	TP53_R248Q	85	SSCMGGMN<u>Q</u>R	A*11:01	171	0.49	89	10	0.29
14	TP53_G245S	67	SSCMG<u>S</u>MNRR	A*11:01	145	0.78	28	6	0.23
15	TP53_G245S	67	SSCMG<u>S</u>MNR	A*11:01	95	0.54	107	31	0.23
16	TP53_R248W	60	N<u>W</u>RPILTII	A*24:02	945	0.53	32	6	0.71
17	TP53_Y220C	55	VVP<u>C</u>EPPEV	A*02:01	564	0.55	26	7	0.23
18	PIK3CA_H1047R	127	FMKQMNDA<u>R</u>	A*33:03	68	0.54	34	13	0.36
19	PIK3CA_H1047R	127	YFMKQMNDA<u>R</u>	A*33:03	51	0.51	27	10	0.36
20	PIK3CA_H1047R	127	EYFMKQMNDA<u>R</u>	A*33:03	64	0.21	61	15	0.36
21	PIK3CA_E545K	102	STRDPLSEIT<u>K</u>	A*11:01	92	0.04	80	33	0.35
22	PIK3CA_E542K	74	AISTRDPLS<u>K</u>	A*11:01	99	0.25	80	9	0.25
23	PIK3CA_E542K	74	ISTRDPLS<u>K</u>	A*11:01	333	0.48	124	30	0.25
24	PIK3CA_E542K	74	KAISTRDPLS<u>K</u>	A*11:01	256	0.22	127	29	0.25
25	EGFR_L858R	122	KITDFG<u>R</u>AK	A*11:01	101	0.19	52	11	0.41
26	EGFR_E746_A750del	74	AI<u>K</u>TSPKANK	A*11:01	423	0.54	73	13	0.25
27	BRAF_V600E	105	KIGDFGLAT<u>E</u>K	A*11:01	108	0.28	98	4	0.36

[a]Mutation frequency in the HOPE cohort of 5,143 patients. [b]Underlined letters mean AAS positions. [c]ΔHLA means increasing HLA expression. [d]Estimated percentage of eligible patients among all patients. AAS, amino acid substitution; Freq., frequency.

Acknowledgements

Not applicable.

Funding

The present study was supported by a grant from JSPS KAKENHI (grant no. 20K07649), Japan.

Availability of data and materials

The somatic gene alterations in the HOPE cohort are available from the National Bioscience Database Center Human Database (Research ID, hum0127) as VCF or TSV format files (https://humandbs.biosciencedbc.jp/en/). The raw data supporting the conclusions of this article are available from the corresponding author upon reasonable request.

Authors' contributions

AI and YA were major contributors to the conception and design of the study, as well as in the drafting of the manuscript and were responsible for completing the study. TN, KUr, YS, KO, AS, YO, MTe, KUe, TM, YH, SY, HKa, TS, MTa, HKe and KY performed patient data acquisition and analysis, and TN, KO and TS contributed to these analyses and interpretations. NS, AK and YK contributed to the synthesis and supply of the peptides. AI performed the development of methodology, generated the genome editing cell lines and acquired the MHC stabilization assay data. AI and YA confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The experimental clinical research protocols of the present study were approved by the Institutional Review Board at the Shizuoka Cancer Center (Authorization no. 25-33). Written informed consent was obtained from all patients enrolled in the present HOPE project study. All experiments using clinical samples were performed in accordance with the Japanese ethical guidelines for human genome/gene analysis (Ministry of Health, Labour and Welfare 2017, https://www.mhlw.go.jp/stf/seisakunitsuite/bunya/hokabunya/kenkyujigyou/i-kenkyu/index.html).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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