Absolute quantification of tumor antigens using embedded MHC-I isotopologue calibrants

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Abstract

Targeted immunotherapies have varying thresholds of antigen density required for an optimal antitumor immune response, and thus absolute quantification of peptide major histocompatibility complex (pMHC) antigen expression is necessary to inform appropriate therapeutic strategies (1–3). Previously, T cell receptor (TCR)-mimetic antibodies have been used to estimate copy numbers but require a specific high-affinity antibody for each target of interest, limiting broad applicability (4). Mass spectrometry-based approaches historically rely on exogenous heavy isotope-labeled peptide standards for single-point estimation (5, 6), failing to account for sample processing losses (7) and ion suppression (8).

We previously reported a technique to perform absolute quantification with an internal calibration curve, combining heavy isotope-labeled MHCs (hipMHCs) with tandem mass tags (8). While this method was successful in capturing endogenous expression of target pMHCs, substantial ion suppression limited standard curves to a 10-fold range and required replicate samples, limiting the method’s ease of use and suitability for low-quantity material.

To circumvent these limitations, we developed SureQuant-IsoMHC, a method for high-sensitivity absolute quantification of MHC-I peptides from in vitro and in vivo samples. SureQuant-IsoMHC uses a series of heavy isotope-coded peptide standards (isotopologues) and SureQuant internal standard-triggered parallel reaction monitoring (IS-PRM) (9) to generate an embedded standard curve to estimate endogenous expression levels of 18 melanoma antigens. Here, we apply SureQuant-IsoMHC to profile changes in pMHC expression in a melanoma cell line with binimetinib (MEKi) treatment and exemplify the approach by profiling antigen levels using limited input material from human melanoma tumor punch biopsies.

Results and Discussion

Four isotopologues were synthesized per target with an increasing number of heavy (1 to 4H) amino acids (Fig. 14). HipMHCs were generated using the 1H, 2H, and 3H standards, quantified by an enzyme-linked immunosorbent assay (ELISA), and added to the cell lysate at a ratio of 1:10:100 to generate a multipoint calibration curve with a 100-fold dynamic range. Endogenous and isotopically labeled pMHCs were enriched (8), and prior to analysis a high concentration of the 4H standard was added exogenously to serve as the IS trigger for SureQuant quantitation. Integrated product ion areas were summed, and a linear fit of the 1 to 3H isotopologues was used to determine the endogenous concentration.

We selected a panel of 18 pMHC targets (Iso18 panel) for SureQuant-IsoMHC quantification from a multiplexed, discovery immunopeptidomics analysis of BRAF mutant melanoma SKMEL5 cells treated with binimetinib for 72 h. MEKi treatment increased surface HLA expression and resulted in dynamic changes in pMHC abundances relative to the dimethyl sulfoxide (DMSO)-treated control (Fig. 1B and C and Dataset S24), in agreement with previous literature (10). Iso18 target peptides were predicted to bind HLA-A*02:01, increased in presentation following MEK inhibition, and spanned a range of abundances within the immunopeptidome (Fig. 1D and Dataset S2B). This panel includes peptides derived from several well-studied tumor-associated antigens (TAAs), e.g., PMEL (gp100) and DCT (TRYP2).

To evaluate the linear intensity response of the Iso18 isotopologues against a relevant background, peptides were added exogenously at four concentrations (0.1 to 100 fmol) to a prepurified mixture of MHC peptides (Dataset S34). For further evaluation, hipMHCs of half the panel were spiked in across five concentrations (0.1 to 1,000 fmol) to 7.5 × 106 SKMEL5 cells (Dataset S3B). The magnitude of ratio compression within the 1- to 100-fmol titration varied from 1.75 to 9.35×, emphasizing the need for multipoint calibrants for accurate quantitation. A sensitivity analysis showed five or more detectable transitions at 10 attomole across all peptides with one exception (KLDVGNAEV), suggesting most endogenous targets present at approximately one copy/cell should be detectable by SureQuant-based targeting (Dataset S3C).

We applied SureQuant-IsoMHC to quantify changes in expression of the Iso18 panel in SKMEL5 cells pre MEKi, titrating 1H/2H/3H hipMHCs into 7.5 × 106 cells (5 mg lysate) in triplicate. As expected, the 1H, 2H, 3H and endogenous (L [light]) peptides were triggered by the identification of 4H for all Iso18 peptides. For example, in the first DMSO replicate of “SLDDYNHLV,” integrated intensities approximated expected ratios, and a linear fit determined the endogenous concentration at 15.5 fmol or ~1,200 molecules/cell (Fig. 2A and B).

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The authors declare no competing interest.

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Endogenous pMHC levels spanned a wide range, and in all cases MEKi treatment increased expression (Fig. 2C). The PRUNE2-derived peptide, “GOVEIVTKV,” had an estimated 20 molecules/cell with DMSO and 250 with MEKi treatment, whereas the SLC45A2 sequence, “RLLGTEFQV,” had ~40,000 molecules/cell with DMSO and ~144,000 with MEKi. SureQuant-IsoMHC provided accurate quantification across nearly four orders of magnitude, highlighting the wide diversity in expression levels of tumor antigens in the immunopeptidome. The success of targeted immunotherapies will depend in part on the ability to confidently identify and quantify an antigen target(s) for each patient. To evaluate the levels of selected TAAbs in patient tumors, we applied SureQuant-IsoMHC to 10 HLA-A2*01+ human melanoma punch biopsies to identify/quantify the expression of antigens used in targeted immunotherapies, verifying the presence and concentration of target antigens in small quantities of patient tumor specimens.

As there are many antigen-specific targeted immunotherapies in clinical development, verifying the presence and concentration of target antigens in small quantities of patient tumor specimens is of increasing importance. SureQuant-IsoMHC provides a high sensitivity, highly reproducible solution for the accurate quantification of even low-abundance target antigens. Here we targeted well-characterized tumor antigens; however, this method may be similarly leveraged for predicted neoantigens or viral epitopes using minimal tumor material. These lowly abundant targets have historically been challenging to identify using DDA, even with large amounts of sample (11), rendering SureQuant-IsoMHC an attractive solution.

Future studies may utilize SureQuant-IsoMHC to characterize the expression levels of known or predicted antigens across a larger tumor cohort and expand beyond HLA-A*02:01. These data may be sensitivity in detecting and quantifying low-abundance epitopes.

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**Materials and Methods**

Detailed descriptions are provided in SI Appendix. Patients with metastatic melanoma at Massachusetts General Hospital (Boston, MA) provided written informed consent for the collection of tissue and blood samples for research and genomic profiling. This study was approved by the Dana-Farber/Harvard Cancer Center Institutional Review Board (DF/HCC Protocol 11-181). Tumor samples were collected, snap frozen, and stored at −80 °C prior to analysis.

hiMHCs of isotopologues were generated using ultraviolet (UV)-mediated peptide, quantified by ELISA, and added into SKMELS cell and tumor lysates. Heavy and endogenous pMHCs were purified by immunoprecipitation using a pan-specific HLA class I antibody (w6/32), and peptides were isolated by size exclusion filtration as previously described (8). The 4H trigger peptide was added exogenously, and peptides were subsequently analyzed using the

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**Fig. 1.** Experimental workflow and peptide panel selection. (A) Schematic of SureQuant-IsoMHC sample preparation and analysis. (B) HLA-A/B/C surface expression of tumor specimens (n = 5). (C) Mean change in surface expression for pMHCs, mean centered (n = 3). Iso18 panel peptides are in blue. (D) Abundance of Iso18 panel peptides (blue) relative to background MHC-I peptides.
and interpolation of endogenous concentration for peptides (concentration per 5 mg lysate). (Stopfer et al. PNAS) Fig. 2. SureQuant-IsoMHC quantification in vitro and in vivo. (Data are included in the article and/or dataset identifier PXD024917. Dataset S7 contains a file map. All other study mass spectrometry data files have been deposited in the Data Availability.)

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Fig. 2. SureQuant-IsoMHC quantification in vitro and in vivo. (A) Product ion traces for “SLDDYNHLV.” (B) Linear fit of summed intensities ($r^2 = 0.997$) and interpolation of endogenous concentration for “SLDDYNHLV” with DMSO. (C) Copies-per-cell estimates of pMHCs with DMSO/10 nM MEKi, $n = 3$ except where “X” = L below limit of detection and “Y” = H below intensity threshold. Error bars ± SD; *extrapolated. (D) Tumor analysis workflow. (E) Peptide concentrations per 5 mg lysate. (F) T1 Iso18 concentrations. Dotted line is lowest calibration point, *pMHCs identified with DDA. (G) Number of unique peptides (Left, black) and Iso18 panel peptides (Right, red) identified with DDA.