PROTEOGENOMIC APPROACHES FOR EXPLORING THE DARK IMMUNOPEPTIDOME

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Background Comprehensive coverage of the class I human leukocyte antigen (HLA-I) immunopeptidome can help inform the development of cancer immunotherapies. Currently, cancer-specific antigen targets are predicted from somatic mutations identified by whole exome sequencing of canonical protein-coding genomic regions. Novel or unannotated open reading frames (nuORFs) may provide an additional source of cancer-specific antigens. However, detection of cancer-specific antigens by mass spectrometry is limited by large sample input requirements and computational challenges in spectral matching to large protein databases. Ribosome profiling (Ribo-Seq) can narrow the scope of the ‘dark proteome’ by identifying active translation of nuORFs. Here, we present an optimized immunopeptidomics workflow and integrate Ribo-Seq data with peptidomics to characterize the dark immunopeptidome.

Methods HLA-I peptide complexes in A375 melanoma cells were immunoprecipitated with W6/32 antibody using an automated workflow on an AssayMap Bravo (Agilent). HLA peptides were acid eluted, then reduced, alkylated and desalted, or fractionated with SDB-XC stage tips before analysis using optimized trapped ion mobility separation and parallel accumulation-serial fragmentation (PASEF) methods on a timsTOF SCP mass spectrometer (Bruker). Ribo-Seq was performed on the same cell line to generate a cell type specific protein database containing both actively translated nuORFs and canonical protein sequences. Immunopeptidomics data were then searched against a human reference proteome in combination with a cell type specific or a pan sample database.

Results Our optimized immunopeptidomics workflow shows improved sensitivity, demonstrated by the identification of over 1,000 unique HLA-I peptides from as little as one million A375 melanoma cells in a single mass spectrometry run. Five to ten million melanoma cells routinely identified 3,000 – 4,000 peptides in a single run. For larger sample inputs, fractionation further increased peptide identification to enable routine identification of over 10,000 peptides. This increased depth allowed for the discovery and characterization of peptides from novel antigen sources. In A375 melanoma cells, approximately 1–4% of HLA-I peptides identified map to nuORFs identified by Ribo-Seq. These nuORFs include retained introns, as well as up- and down-stream ORFs. nuORF-derived peptides were enriched in the immunopeptidome relative to the proteome suggesting differential processing and presentation of dark matter antigens.

Conclusions We have developed an immunopeptidomics workflow that combines sensitive mass-spectrometry with Ribo-Seq to study dark matter antigens. Our results demonstrate that novel unannotated open reading frames substantially contribute to the immunopeptidome. Dark matter antigens may serve as attractive targets for cancer-specific immunotherapies.

http://dx.doi.org/10.1136/jitc-2023-SITC2023.0145