Development of a Multiplexed Targeted SRM Assay for NCI's Top Tumor Associated Antigens

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Recently, the NCI prioritized the top tumor associated cancer antigens (TAA) based on predefined criteria, including clinical efficacy (Cheever et al 2009). To facilitate these measurements in clinically relevant samples, a single 252 analyte multiplexed SRM assay was developed using JPT's SpikeMix™ TAA Sets. The assay was deployed against 12 different cancer cell lines including two sets of isogenic-paired set (treatment-naive and acquired resistance to a tyrosine kinase inhibitor) of HER2-positive breast cancer cell lines to characterize expression changes of these TAA in acquired drug resistance.

Introduction

The NCI conducted a pilot project to prioritize tumor associated cancer antigens to develop a priority-ranked list of cancer targets (Cheever et al 2009), resulting in the selection of 75 "well-vetted" protein targets based on predefined criteria, including clinical efficacy. JPT (Germany) has recently created a commercially available panel of 252 pre-mixed non-labeled tryptic peptides or C13/N15 C-terminal residue stable isotopically labeled (SIL) tryptic peptides corresponding to 65 of these Tumor Associated Antigen targets (**SpikeMix™ TAA**) for use in targeted SRM assays.

Here we describe the development of a single 252 analyte multiplexed SRM assay on a Waters ACQUITY UPLC M-Class System operating in a three-fraction two-dimensional LC/LC (high pH / low pH) mode coupled to a Waters TQ-S mass spectrometer through an ionKey/MS interface. To define linear range, limit of detection and limit of quantitation, calibration curves were generate for each SIL labeled TAA analyte over the range 50 amol to 100 fmol on-column in the presence of 8 ug of digested HepG2 cell lysate background which had been spiked with 40 fmol of each non-labeled peptide.

The finalized targeted SRM assay was deployed against 12 different cancer cell line whole cell lysates (i.e. no sub-fractionation), including breast cancer, lung cancer, brain cancer, etc. in triplicate. The assay was deployed on two sets of isogenic-paired set (treatment-naive and acquired resistance to a tyrosine kinase inhibitor) of HER2-positive breast cancer cell lines (triplicate biological preparations) to characterize expression changes of these TAA in acquired drug resistance.

Materials & Methods

Pelleted cells were lysed with probe sonication in the presence of 0.2% Rapigest/50 mM ammonium bicarbonate, pH 10.0. Solubilized protein was subjected to miniBradford assay in duplicate. Lysates were normalized to 2 ug/uL and were reduced/alkylated/trypsin digested (18hr @ 37C). Digested samples were sub-alliquoted, lyophilized to dryness, and stored at -80C until use. Lyophilized pre-mixed SpikeMix™ TAA-heavy (JPT, ~100 pmol/peptide) or SpikeMix™ TAA-light (JPT, ~100 pmol/peptide) were resuspended in 200 mM ammonium formate, pH 10, vortexted, sub-alliquoted, and stored in -80C at 200 fmol/uL.

Samples were subjected to a three-fraction LC/LC separation on a Waters ACQUITY 2D M-Class UPLC with Dual Trapping by loading peptides onto a 300um x 50mm 5 um BEH C18 (A: 20 mM ammonium formate, pH 10.0 / B: CAN) column and eluting at 13.7%B, 18.4%B, and 50%B for 3 min @ 2 ul/min. 1st Dimension

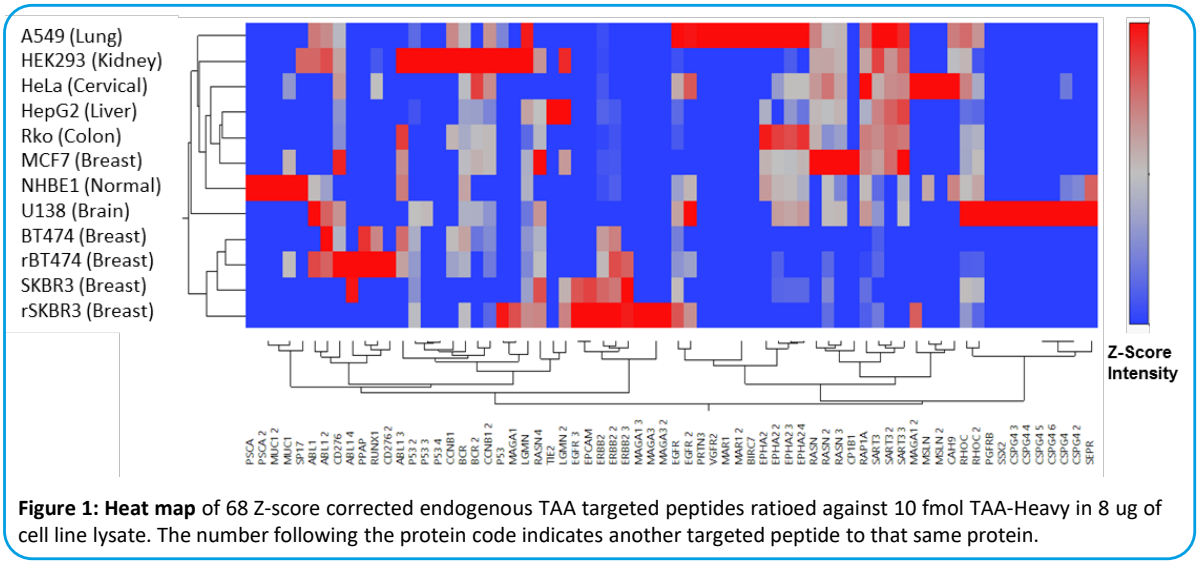

Figure 1: Heat map of 68 Z-score corrected endogenous TAA targeted peptides ratioed against 10 fmol TAA-Heavy in 8 ug of cell line lysate. The number following the protein code indicates another targeted peptide to that same protein.

elution solvent was diluted 10:1 with 0.1% formic acid in water for trapping prior to being switched in-line with the 2nd Dimension iKey. 2nd Dimension separation were accomplished on an 150 um x 100mm 1.7um BEH C18 ionKey from 7%B to 35%B over 18.5min @ 3 uL/min and 55°C with a total cycle time of 23 min/fraction.
Data were acquired on a Waters TQ-S operating in +nanoESI mode. Auto-dwell feature was enabled within MassLynx using 10 points across a 20s wide peak (typically 5 to 15 ms dwell). Up to three transitions per precursor monitored with charge state dependent CE settings. 3 min retention time scheduling windows. All data were analyzed within Skyline (MacCoss Laboratory, University of Washington).

Cell Line	A549	HEK293	HeLa	HepG2	MCF7	HBE1	Rko	U138	BT474	rBT474	SKBR3	rSKBR3
Cancer Source	Lung	Kidney	Cervical	Liver	Breast	Normal	Colon	Brain	Breast	Breast	Breast	Breast
Detectable TAA Peptides	31	31	26	24	26	30	24	31	20	26	19	24
Detectable TAA Proteins	18	17	16	13	14	17	12	16	13	15	11	13

Table 1: Overview of the measurable TAAs and corresponding cancer types.

Results

To begin development of the targeted assay, the pre-mixed SpikeMix™TAA-heavy product was analysed by open platform LC-MS/MS on a Synapt G2 QToF mass spectrometer (Waters) in the absence of background. Of the 252 peptides (65 proteins) in the solubilized sample, 234 peptides (62 proteins) were identified at a false discovery rate of 1.0%. For these analytes, preliminary SRM assays were developed within Skyline and transferred to the LC/LC-SRM platform targeting the top three transitions as determined from the open platform analysis. This resulted in the successful detection of 215 peptides (61 proteins). Calibration curves were acquired in duplicate in the presence of a HepG2 lysate background and an additional requirement of an R2 >0.9 was required over 50 amol – 100 fmol ranges to be considered in the final assay. The final assay contained 165 targeted peptides corresponding to 61 TAA proteins. The QuaSAR module within Skyline was used to assess the calibration curves of the analytes within the final assay. 48% of the 165 targeted TAA peptides had R2 values >0.99 and 79% had R2 values >0.97. LLOQ distributions indicated that 36% of the peptides were able to be quantitated as low as 100 amol 75% as low as 400 amol (**Figure 1**). The results of the QuaSAR analysis for each individual peptide, as well as the Skyline file containing the final targeted transitions have been made freely available at chorusproject.org.
To evaluate the method on various cancer types expected to have a range of expression levels of these particular antigens, the final assay was deployed on twelve different cancer cell types including lung, kidney, liver, and breast cancers as indicated in **Table 1**. The criteria for successful detection included at least one peptide above the LOQ with an S/N ratio >5 above background. Within each cancer type, the number of measurable TAA proteins ranged from 11 to 18. Interestingly, we found a very heterogeneous distribution of expression profiles in these particular antigens across the various cancer cell types.

Discussion & Conclusions

Here we describe and make available a targeted LC-SRM assay corresponding to the tumor associated antigens within JPT's SpikeMix™TAA-heavy product. Our results have been uploaded as QuaSAR files describing assay performance (r2, LLOQ, etc.) in a HepG2 lysate background. The spectra used to build the SRM assays as well as the Skyline document containing the final transitions have been made publically available at chorusproject.org. Initial deployment of the assay indicate the corresponding tumor associated antigens have diverse expression profiles in various cancer types, suggesting a potential impact of classifying unknown cancer types based on this panel of targeted analytes.

References

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The Company

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