A SRM/MRM based targeted proteomics strategy for quantification of potential biomarkers of TKI sensitivity in EGFR mutated lung adenocarcinoma

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Introduction

Lung cancer remains the leading cause of cancer-related deaths worldwide. Epidermal growth factor receptor (*EGFR*) is the primary driver oncogene in 10-30% lung adenocarcinoma. Kinase domain mutations in EGFR (L858R and E746-A750 deletion) provide sensitivity to EGFR-specific tyrosine kinase inhibitors (TKIs). Despite good tumor inhibitory effects, after approximately a year, patients acquire resistance to EGFR TKIs. About 60% of the acquired resistance can be attributed to a mutation in the gatekeeper residue of the kinase domain of the receptor (T970M).

There are limited options for circumventing acquired resistance to the 1st generation EGFR-TKIs, gefitinib or erlotinib. Recently, the FDA approved the 3rd generation EGFR TKI, osimertinib for treatment of patients harboring EGFR T790M mutation. However, acquired resistance to osimertinib is inevitable, underscoring the unmet need of identifying potential markers of EGFR TKI response and resistance. We have previously identified several kinases and adaptor proteins from SILAC-based quantitative MS methods that were significantly differentially expressed, suggesting that these are a subset of potential biomarkers of TKI response. We are currently carrying out verification studies utilizing the MS method most often used to complement shotgun approach known as selected/multiple reaction monitoring (SRM/MRM) because of its high sensitivity, speed and dynamic range.

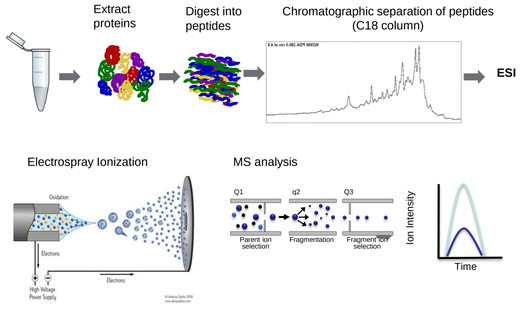

Figure 1: Workflow of a SRM analysis

Although, the MS-based shotgun proteomics approaches have revolutionized the pace of protein identification and relative quantification in complex samples, several technical limitations exist that make it unsuitable for absolute quantification, i.e. only the high abundant part of the proteome is reproducibly identified, protein targets present in lower stoichiometric amounts are stochastically identified leading to missing data and unreliable quantification. In order to overcome some of the limitations of these data dependent acquisition methods, we have utilized a more robust method of targeted proteomics via multiple reaction monitoring to carry out quantification of these potential biomarkers in the lung adenocarcinoma cells.

Methods

We performed SRM/MRM-MS using a nano-flow liquid chromatography instrument (Agilent Infinity 1200 series) coupled to a nano-electrospray ionization source attached to a Triple Quadrupole MS (Agilent 6495). In this method, digested proteins are chromatographically separated and ionized in an Agilent Chipcube LC, followed by mass analysis in a triple quadrupole MS.

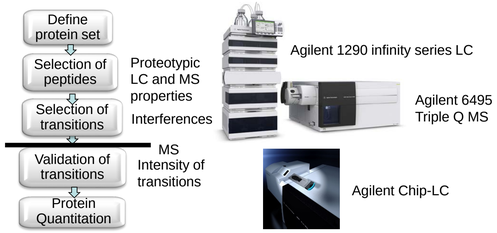

Figure 2: Approach for developing the targeted assays using the heavy isotope labelled versions of the target peptides.

Protein names	Gene names	Targets	Sequence
Neuroblast differentiation-associated protein	AHNAK	Y-160	RVTAY(P)TVDVTGR(13C6,15N4)
		Y-715	VKGE Y(P) DMTVP K(13C6,15N2)
Caveolin-1	CAV1	Y-6	Y(P) VD S(P) EGHLYTVPI R(13C6,15N4)
		Y-14	YVDSEGHL Y(P) TVPI R(13C6,15N4)
Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 2	INPPL1	Y-1135	TLSEVD Y(P) APAGPA R(13C6,15N4)
Signal transducer and activator of transcription 5A	STAT5A	Y-694	AVDG Y(P) VKPQI K(13C6,15N2)
E3 ubiquitin-protein ligase CBL	CBL	Y-141	M Y(P) EENSQP R(13C6,15N4)
Dual adapter for phosphotyrosine and 3-phosphotyrosine and 3-phosphoinositide	DAPP1	Y-139	KVEEPSI Y(P) ESV R(13C6,15N4)
ERBB receptor feedback inhibitor 1	ERRFI1	Y-394	VSSTHY(P)YLLPERPPYLDK(13C6,15N2)
Enhancer of filamentation 1	NEDD9	Y-164	TGHGYV Y(P) EYPS R(13C6,15N4)
Neurofibromin	NF1	Y-2579	RVAETD Y(P) EMETQ R(13C6,15N4)
Epidermal growth factor receptor	EGFR	Y-998	MHLPSPTDSNFY(P)R(13C6,15N4)
		Y-1172	GSHQISLDNPDY(P)QQDFFPK(13C6,15N2)
		Y-1197	GSTAENAEY(P)LR(13C6,15N4)

Table1: List of target phospho-tyrosine peptides and their heavy isotope labelled standards used for assay design and validation.

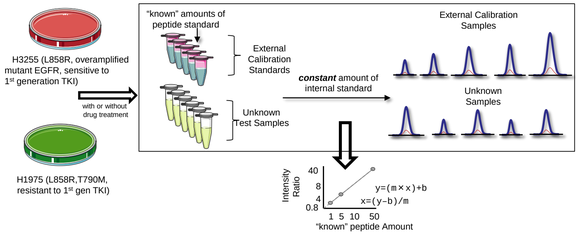

Figure 3: Experiment design for the absolute quantification of the target phospho-peptides

Results and discussion

Round 1 nanoLC MS/MS: SRM Signal Intensity Rank and Fragmentation Fingerprint using Skyline 3.5

- *Spectral library* from individual peptide injections on the triple Q
- Export methods with transition list parameters (*m/z* Precursors, *m/z* Products, & Collision Energies)

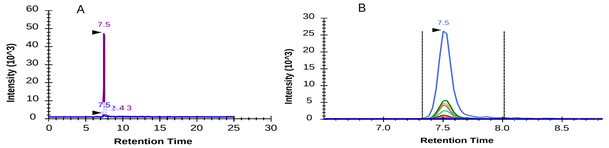

Figure 4A: The 2+ and 3+ precursors of CAV1 Y-14 and the co-eluting product ions. **B.** from all the monitored transitions. Similarly chromatogram libraries were built for all the target peptides.

Round 2 nanoLC MS/MS: iRT Calibration and retention time normalization

- Edit and annotate all peptides with non-existent or ambiguous chromatogram
- Export methods for remaining peptides for retention time calibration

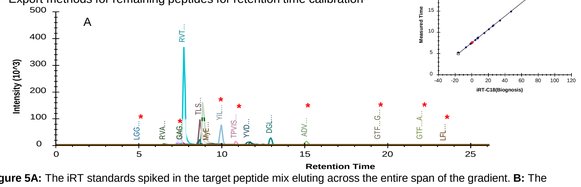

Figure 5A: The iRT standards spiked in the target peptide mix eluting across the entire span of the gradient. **B:** The linear regression obtained for calculation of the iRT values and retention time prediction

Round 3 nanoLC MS/MS: Scheduling MRM assays for optimum duty cycle and sensitivity

- Update chromatogram libraries with the iRT values
- Export scheduled methods for target peptides

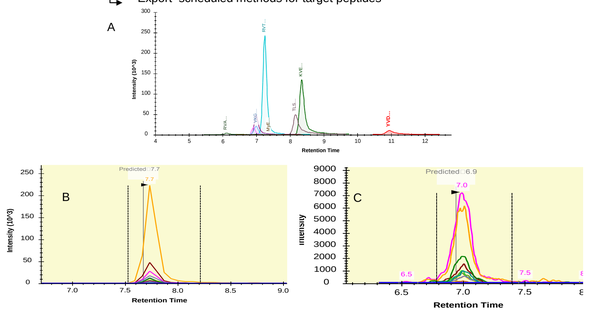

Figure 6A: The scheduled MRM with 2 minute windows across the predicted retention times. **B:** Chromatogram from unscheduled method. **C:** chromatogram from scheduled method.

Round 4 nanoLC MS/MS: Running the optimized scheduled MRM assays for screening the endogenous peptides

- Phospho-tyrosine enriched samples spiked with constant amount of heavy labelled standards
- Peak area ratios used for quantitation

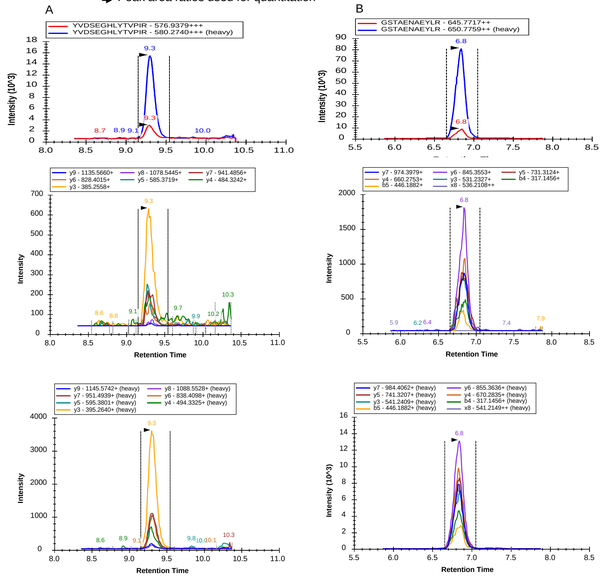

Figure 7: The MS1 and MS2 chromatograms for the target **(A)** CAV1 Y-14 and **(B)** EGFR-Y-1197 in the H1975 cell line.

We are currently validating these assays in human lung adenocarcinoma cell lines with known EGFR mutations with or without treatment with 1st and 3rd generation EGFR TKIs.

Conclusions

- We have shown the development of quantification assays using MS-based targeted proteomics methods for testing potential biomarkers of drug response.
- An external multi-point calibration curve will be used in conjunction with these optimized assays for accurate and precise quantification of these phospho-tyrosine markers.
- A systematic approach was taken to optimize and validate robust assays that will be used *in vitro* and can be translated to clinical samples.