Immunoaffinity-Based Multiplexed Targeted Mass Spectrometric Assay for Ovarian Cancer Biomarker Verification.

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INTRODUCTION

Ovarian cancer is the fifth leading cause of death in women in the Unites States. The disease is frequently asymptomatic, until it has advanced to an untreatable stage and spread well beyond the ovaries. Although serum cancer antigen -125 (CA-125) levels and transvaginal ultrasound study have contributed to earlier detection of ovarian cancer, these screening methods are still not used routinely to screen for ovarian cancer. Furthermore, CA-125 does not provide the accuracy needed for early diagnosis or differentiate it from malignant non-ovarian disease. Hence a panel of biomarkers is necessary to improve the specificity and sensitivity for early detection.

The advances in genomics and proteomics technologies have led to discovery of a large number of putative biomarkers, but these candidates must be rigorously verified in plasma or serum for blood-based biomarker development. This requires analytical techniques that can robustly measure proteins in complex biological matrices such as plasma with large dynamic concentration range of upto 12 orders of magnitude. Targeted mass spectrometry (MS)-based proteomic approaches such as selected reaction monitoring (plural MRM) in conjunction with isotopically labeled standards is emerging as a viable alternative and/or complimentary technique to ELISA for protein quantification. Unlike shotgun MS used in biomarker discovery, the MRM-MS approach measures one or more selected peptides that are unique to the target protein (i.e.“proteotypic”) and serves as quantitative stoichiometric surrogate for protein concentrations in the sample **(Figure 1)**. The assays are specific, precise, higher throughput and multiplex-able. Thus, MRM-based assays have the potential to enable large-scale verification of hundreds of candidate biomarkers. Furthermore, the sensitivity of MRM assays can be substantially increased at the sample level by multidimensional fractionation, depletion or by enriching for specific target peptides as shown in Figure 1.

In the present study, we use an immunoaffinity peptide enrichment based liquid chromatography-targeted MS approach to verify two ovarian cancer biomarkers CA-125 and Mesothelin using a cohort of clinically well-characterized serum samples and quantitatively characterize the behavior of these biomarkers.

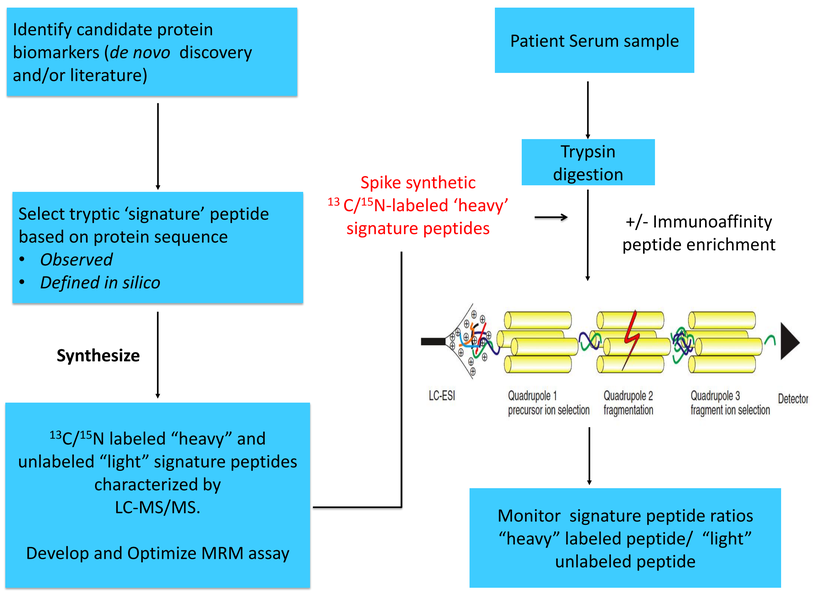

Figure 1: Workflow for candidate biomarker verification by multiple reaction monitoring using stable isotope-labeled internal standards and liquid chromatography-tandem mass spectrometry.

METHODS

The preliminary biomarker verification study was done on a set of 20 blinded and randomized samples including serous ovarian cancer samples and benign disease cases. The workflow is illustrated in **Figure 2** below. Both the anti-peptide antibodies and the proteotypic peptides for CA-125 (ELGPYTLDR) and Mesothelin (LLGPHVEGLK) were purchased from SISCAPA Assay Technologies..

Selection and optimization of transitions

The unlabeled ‘light’ and labeled ‘heavy’ proteotypic peptides for CA-125 and Mesothelin were characterized by LC-MS/MS. The three most abundant transitions were chosen for each peptide with preference given to y-ions to ensure that the heavy peptide MRM transitions contained the isotope label.
A total of 12 transitions (3 for each heavy and light peptide) chosen for the assay.

Forward and Reverse response curves

Forward curve: Varying amounts of unlabeled peptide and a constant amount of labeled peptide are spiked into undepleted plasma digest followed by immunoaffinity enrichment.
Used to determine endogenous analyte level.

Reverse curve: Varying amounts of labeled peptide and a constant amount of unlabeled peptide are spiked into undepleted plasma digest followed by immunoaffinity enrichment.
Used to determine LOD of the assay.

SISCAPA assay for patient samples

50 fmoles of the heavy labeled peptide was spiked into 10 µL of undepleted patient serum digest followed by immunoaffinity enrichment.

Quantitation of endogenous peptides

The captured target peptides were eluted and analyzed by LC-MRM/MS using an Agilent 1260 Infinity HPLC/Chip cube system interfaced with an Agilent 6490 QQQ-MS.

The peak area ratio of light to heavy peptides (L/H) were estimated and used to determine the endogenous peptide concentration in patient samples.

RESULTS

The forward and reverse peptide addition response curves were done in triplicate and the LOD for both CA-125 and Mesothelin was determined to be 55 fmoles/ml.

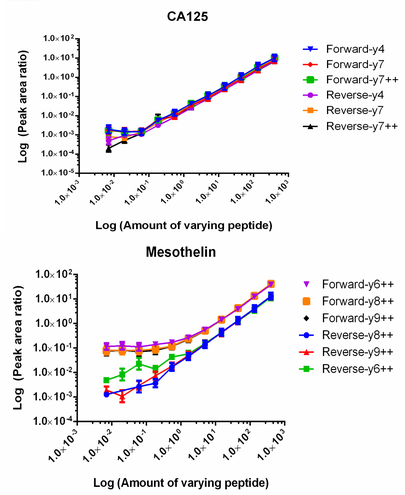

Figure 3: Forward and Reverse peptide addition curves for CA-125 and Mesothelin in 10 µl of undepleted serum digest

The immunoaffinity-based MRM assay was performed in triplicate on all the 20 patient samples. Peak area ratios of light to heavy peptide was determined for each transition monitored. The average peak area ratio was used to determine the endogenous concentration of CA-125 and Mesothelin in the patient samples (**Figure 4**). The MRM assay was further validated by comparing the MRM quantification results with the corresponding ELISA assay. A high degree of correlation was observed between the two immunoassays (R^2= 0.85 and 0.94 for CA-125 and Mesothelin, respectively) (**Figure 5**) despite the different methods of quantification.

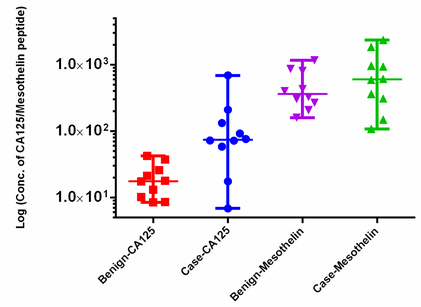

Figure 4: Quantification of CA-125 and Mesothelin by immunoaffinity-based MRM assay. The peak area ratios were calculated for three different transitions for both biomarkers and the average ratio was used to calculate endogenous protein concentration. The CA-125 and Mesothelin concentration profiles are categorized into benign (ovarian disease) and case (ovarian cancer) groups.

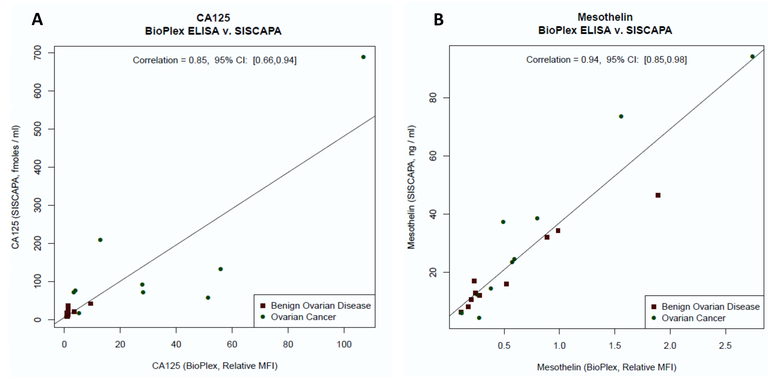

Figure 5: Comparison of quantification of the MRM assay versus the corresponding ELISA assay in serum samples for CA-125 (A) and Mesothelin (B).

DISCUSSION

The triplicate analysis of the two protein candidates across 20 serum samples demonstrated that the immunoaffinity-based multiplexed quantitative assay is robust and replicable. Unlike ELISA that measures the target protein only in its free form, the targeted mass spectrometry approach measures the total protein concentration in the serum in a denatured setting. Further more, because the anti-peptide antibodies bind both labeled and unlabeled monitor peptides equally, the quantitative information is preserved throughout the capture and elution and reflects the total protein concentration in a complex biological matrix. The specificity conferred by the fragmentation pattern of the target peptides, allows the immunoaffinity-based assay to act much like a sandwich ELISA with the MS/MS fragmentation acting as the secondary antibody.

The pilot study described above has now been expanded to include a larger cohort of 130 well characterized clinical samples that also includes healthy age-matched controls. Further validation and evaluation of the differences between the two different quantitative approaches may have a significant impact on the future application of MS-based assays for protein biomarker quantification and transferring this technology for a low cost, routine clinical diagnostics platform.

Acknowledgements: This research is funded by the Canary Foundation.