(1) Introduction

The hepatocyte growth factor (HGF) receptor, cMet, is a receptor tyrosine kinase which regulates cell proliferation, morphogenesis, and motility. Mutations in cMet, over-expression of cMet or aberrant cMet signalling is present in a variety of solid tumours including prostate cancer and is believed to participate in angiogenesis, tumour development, invasion, and metastasis. High cMet expression is observed in late stages and metastases of prostate cancer. Inhibition of cMet in prostate cancer cell lines had anti-angiogenic and anti-proliferative effects. Literature data suggests that cMet inhibition or knock-out is a therapeutic target of advanced prostate cancer. A number of drugs that reduce or inhibit the activity of c-Met are currently being developed including monoclonic antibodies (BMS-776707) and multikinase inhibitors (Crizotinib/Albiritinib, PF0341066).

(2) Material and Methods

Sample preparation: DU-145 were stimulated for 24 or 48 h with or without 50 ng/ml hepatocyte growth factor (HGF). Conditioned serum-free supernatant was concentrated using 3 KDa MWCO Amicon filter. Cells were lysed by ultrasonication.

Tandem Mass Tag TMTplex (TMT6) approach: Proteins were reduced, alkylated and digested with trypsin. Peptides were labelled with TMTplex reagents (ThermoFisher) at amino termini and lysine residues (labels releasing reporter ions during MS/MS at different m/z [2]). The combined samples were separated into 10 fractions by SD (size chromatography) and measured by LC-MS/MS (Orbitrap XL, ThermoFisher). Peptide and protein identification was performed using SEQUEST. Correction of reporter ion intensities was performed to adjust for isotope overlap and systematic bias by means of norm scaling. A linear mixed model (LME) algorithm was applied to analyze the data. The algorithm provides for each protein an estimate of the log2 signal intensity per group, p-values for differentially expressed proteins and corrects for random factors, i.e. effects of measuring different peptides per protein and different SC fractions.

Selected Reaction Monitoring by isotopic TMTplex approach: Proteins were reduced, alkylated and digested with trypsin. Each sample is labelled with a light TMT tag and then spiked with the heavy TMT-labelled reference. The lighter endogenous peptides co-elute with their equivalent heavier reference peptide and quantified by measuring the MS peak area for the light compared to heavy signals in a classical SRM workflow. Collision energy settings were experimentally optimized to achieve maximal signal intensity with lowest signal interference from co-eluting substances of targeted peptides.

(3) Discovery phase: Results

The effect of activating the cMet signalling pathway was studied by comparing the protein patterns of untreated control cells and cells exposed to HGF for 48 hours. To study the effect of inhibiting the HGF/cMet signalling pathway, cells were exposed to the first generation inhibitor SU11274 and to the second generation inhibitor PHA665752 in the presence of HGF. Conditioned supernatants and cell extracts were analysed applying the TMTplex-based workflow as shown in the left. Analysis of manipulated proteins were performed applying LC-MS/MS based approach. Biomarker candidates were identified by ANOVA and LME. Bidirectional hierarchical cluster analysis (HCA) was used to visualize abundance of biomarker candidates across the samples with activated and inhibited cMet pathway.

(4) Validation phase: Results

A subset of potential biomarkers were analysed by immunohasassay (ELISA) and by selected reaction monitoring methods in cell culture supernatant and cell culture lysate. For ELISA, nine antibodies were selected and regulations validated. For LC-SRM, we monitored 29 biomarker candidates comprising 42 peptides and 504 transitions.

(5) Conclusion

- The discovery phase resulted in the identification of ~2,500 proteins of which 170 were identified as potential biomarkers after statistical analysis (LME, ANOVA and PLS).
- For the majority of the tested analytes, Crizotinib treatment resulted in a significant change in abundance compared to untreated cells.
- During the validation phase, biomarker candidates were successfully monitored by ELISA and LC-SRM in cell culture, in xenograft mouse plasma samples and carcinoma biopsy.
- The final approach of the most important secretome biomarker candidates for prostate cancer monitors 9 proteins by ELISA and 29 proteins by LC-SRM.