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Original Article

Discovery and Validation of Predictive Biomarkers of Survival for Non-small Cell Lung Cancer Patients Undergoing Radical Radiotherapy: Two Proteins With Predictive Value

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ABSTRACT

Lung cancer is the most frequent cause of cancer-related death world-wide. Radiotherapy alone or in conjunction with chemotherapy is the standard treatment for locally advanced non-small cell lung cancer (NSCLC). Currently there is no predictive marker with clinical utility to guide treatment decisions in NSCLC patients undergoing radiotherapy. Identification of such markers would allow treatment options to be considered for more effective therapy. To enable the identification of appropriate protein biomarkers, plasma samples were collected from patients with non-small cell lung cancer before and during radiotherapy for longitudinal comparison following a protocol that carries sufficient power for effective discovery proteomics. Plasma samples from patients pre- and during radiotherapy who had survived >18 mo were compared to the same time points from patients who survived <14 mo using an 8 channel isobaric tagging tandem mass spectrometry discovery proteomics platform. Over 650 proteins were detected and relatively quantified. Proteins which showed a change during radiotherapy were selected for validation using an orthogonal antibody-based approach. Two of these proteins were verified in a separate patient cohort: values of CRP and LRG1 combined gave a highly significant indication of extended survival post one week of radiotherapy treatment.

1. Introduction

In the era of personalised medicine, biomarkers are required for the stratification of patients allowing therapy to be tailored. This could include molecular histology of disease to allow driver mutation targeted therapy, for example EGFR tyrosine kinase inhibitors for lung cancer patients (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004). Biomarkers which can be used as early markers of response to treatment would be particularly useful in the clinic as well as in drug development, allowing patients therapy to be tailored as early as possible (Beretta, 2007). To be used routinely in the clinic, a biomarker would have to be measurable in a non-invasive readily accessible tissue or biofluid. Plasma as well as urine is routinely used in clinics for the diagnosis of a variety of diseases. For example, monitoring prostate specific antigen levels in blood has been used for screening and monitoring progression of prostate cancer (reviewed in Lilja et al., 2008).

A major issue for identification of protein biomarkers is the high dynamic range of protein content in plasma (of the order of 10^{10} Polanski and Anderson, 2007) that can make mask lower abundance proteins reducing the opportunity for detection with current instrumentation. However advances in mass spectrometry and liquid chromatography coupled to the depletion of highly abundant proteins have allowed the plasma proteome to be investigated with approximately 6 orders of magnitude penetration allowing identification of so called tissue leakage proteins which are predicted to be rich in biomarkers (Rodriguez-Suarez and Whetton, 2013; Zhou et al., 2012). Another

Abbreviations: AC, adenocarcinoma; CEA, carcinoembryonic antigen; CRP, C-reactive protein; EGFR, epidermal growth factor receptor; FDR, false discovery rate; IL-6, Interleukin 6; iTRAQ, isobaric tagging for relative and absolute quantification; LBP, lipopolysaccharide binding protein; LRG1, leucine-rich alpha-2-glycoprotein; mo, months; MS/MS, tandem mass spectrometry; NSCLC, non-small cell lung cancer; PCA, principal component analysis; SCLC, small cell lung cancer; SqCC, squamous cell carcinoma; TEAB, triethyl ammonium bicarbonate; VEGF, vascular endothelial growth factor; v/v, volume/volume.

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challenge of biomarker discovery is the large variation present both between individuals in a population and in an individual over time. We have previously published an analysis showing that with the appropriate use of longitudinal samples our isobaric tagging plasma proteomics workflow can be used to identify biomarkers from clinical studies with as few as three patients per group with a power of 0.8 for the 70% least variant proteins (Zhou et al., 2012). We have coupled this approach to our newly published bioinformatics technique which more accurately estimates specific protein technical variation, this additional modelling allows more proteins to be identified as differentially expressed with sufficient power (Zhou et al., 2013). To show the utility of these methodologies we have investigated if plasma markers with clinical utility can be identified in non-small cell lung cancer (NSCLC) patients undergoing radical radiotherapy in a deliberately small cohort (3 vs 3) using a longitudinal sampling approach. Two baseline samples prior to the start of radiotherapy were analysed from each patient, allowing the baseline variation of each protein to be assessed, and thus significant changes during radiotherapy identified. These changes were then validated in a second independent cohort of twenty three patients using a second methodology.

Patients diagnosed with lung cancer have a 5 year survival rate of <10% in Britain (Parkin et al., 2005). Globally there are over 1.2 million deaths related to lung cancer per annum (Crino et al., 2010). Surgery remains the mainstay curative treatment for this disease. However the majority of patients present with disease that is too advanced to be resected or have multiple comorbidities precluding surgery. As a consequence radical radiotherapy, either alone or combined with chemotherapy, plays a major role in the treatment of patients with locally advanced lung cancer (Crino et al., 2010). Radiotherapy is known to cause acute and late toxicity in patients due to damage to surrounding normal tissue. An example of thoracic radiation toxicity is pneumonitis and pulmonary fibrosis which can be life threatening and can affect patients' quality of life and treatment outcome (reviewed in Abratt and Morgan, 2002). Therefore assessment of response to treatment such as radical radiotherapy is a valid and useful contribution to determining treatment options in those undergoing radical radiotherapy. We therefore considered if we can find predictive factors for survival after radical radiotherapy by use of our proteomics pipeline.

Here we report on the proteomic analysis of samples from the prospective study, RADAR, in which patients with small cell lung cancer or NSCLC who are treated with radical radiotherapy are asked to donate blood for research into toxicity and predicting outcome to treatment. The materials collected for this study were longitudinal in nature with samples taken prior to the radiotherapy and during treatment. This allowed us to look at proteins which can act as predictive markers of survival early in the radiotherapy treatment using global discovery proteomics. The results of this proteomic analysis are detailed below with potential markers identified and validated in an independent cohort.

2. Materials and Methods

2.1. Sample Collection

Blood was collected from donors in lithium heparin coated tubes and centrifuged within 30 min of collection at 2500 ×*g* for 15 min at 4 °C before aliquots of the plasma layer were stored at −80 °C. Samples were collected at the following time points for each patient; before RT, during RT (days 2, 3, 8, then weekly) and on completion after RT (months 1, 3, 6) (Fig. 1). Blood samples were taken from 29 randomised patients with lung cancer enrolled in the RADAR study at the Christie Hospital, Manchester, UK following written informed consent with ethical approval from the Central Manchester Local Research Ethics Committee. This proteomic analysis was undertaken on two samples per patient collected prior to the start of radiotherapy and a third sample on day 8 of the treatment regimen.

2.2. Proteomic Workflow and Experimental Setup

The experiment and workflow was carried out as in Fig. 1. A 50 μl aliquot of each sample in the study was pooled and used as a pooled internal control sample, analysed in duplicate in each isobaric tagging for relative and absolute quantification (iTRAQ) experiment to test technical variation. Each iTRAQ experiment consisted of two internal control samples (channels 119 and 121) and six samples from two patients randomised into the remaining channels. The study used three iTRAQ experiments to analyse samples from six patients.

2.3. Protein Depletion, Digestion and Labelling

Abundant proteins were removed from plasma using an Agilent Mars14 chromatography column following the manufacturers' protocol (Agilent, Palo Alto, CA, USA). Depleted samples were concentrated and exchanged into 1 M triethyl ammonium bicarbonate (TEAB) using 4 ml spin concentrators with a 5 KDa molecular weight cutoff filter (Agilent Palo Alto, CA, USA) as per manufacturer's instructions. The protein concentration in buffer-exchanged samples was measured using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA). 50 μg of each sample was reduced with the addition of 1/10th of the sample volume of 50 mM tris(2-carboxyethyl)phosphine for 1 h at 60 °C. Cysteine residues were then alkylated by the addition of 1/20th of the total sample volume of 200 mM methyl thiomethanesulfonate (in isopropanol) before incubation for 10 min at room temperature. Protein was digested by the addition of 5 μg of porcine trypsin (Promega, Madison, WI, USA), followed by overnight incubation at 37 °C. The digested protein samples were isobarically tagged with 8plex iTRAQ reagents according to the manufacturers' instructions (ABSCIEX, Framingham, MA, USA). After labelling the samples were dried at 60 °C in a DNA concentrator (GeneVac, Ipswich, UK) and then stored at −20 °C.

2.4. High pH Reverse Phase Chromatography

Isobarically tagged samples were reconstituted in 100 μl of buffer A (99.5% water adjusted to pH 10.5 with ammonium hydroxide) and appropriate samples pooled prior to being loaded onto a 100 mm × 4.6 mm 3 μm C18 HPLC columns (Agilent Palo Alto, CA, USA). Peptides were eluted by the application of a linear 30 min gradient up to 50% buffer B (Acetonitrile, 0.1% (v/v) ammonium hydroxide) with 70 × 15 s fractions collected from 4 min. Fractions were dried in a DNA concentrator (GeneVac Ipswich, UK) at 60 °C and stored at −20 °C.

2.5. Mass Spectrometry (MS/MS)

Samples were reconstituted in 30 μl of samples loading buffer (20 mM citric acid, 2% (v/v) acetonitrile and 0.1% (v/v) formic acid). 3 μl of each fraction was then loaded onto a nanoACQUITY UPLC Symmetry C18 Trap, 5 μm, 180 μm × 20 mm (Waters, Milford, MA, USA) at 15 μl/min of 3% (v/v) acetonitrile, 0.1% (v/v) formic acid for 5 min. Analytical separation of the peptides was performed using nanoACQUITY UPLC BEH C18 Column, 1.7 μm, 75 μm × 250 mm (Waters, Milford, MA, USA). Briefly, peptides were separated over a 91 min solvent gradient from 3% (v/v) acetonitrile, 0.1% (v/v) formic acid to 40% (v/v) acetonitrile, 0.1% (v/v) formic acid. MS was carried out by a TripleTOF 5600 (ABSciex, Framingham, MA, USA) set up to analyse the top 20 ions by MS/MS per MS scan. The MS scanned between 350 and 1250 m/z with an accumulation time of 250 ms. Ions were only selected for MS/MS if they were over 150 counts per a second and had a charge state of between 2 and 5, ions previously selected were excluded for 30 s. The MS/MS was carried out in high sensitivity mode with 100 ms accumulation time and a rolling collision energy based upon mass and charge with a spread of 20. The MS/MS scanned between 100 and 1600 m/z.