IPD Project Details

Project ID: IPD6238

Project Title: XPO1 is a critical player for Bortezomib resistance in Multiple Myeloma: A quantitative proteomic approach

Description: Among the blood cancers, 13% mortality is caused by Multiple myeloma (MM) type of haematological malignancy. In spite of therapeutic advances in chemotherapy treatment, still MM remains an incurable disease is mainly due to emergence of chemoresistance. At present time, FDA approved bortezomib is the first line drug for MM treatment. However, like other chemotherapy, MM patients are acquiring resistance against bortezomib. The present study aims to identify and validate bortezomib resistant protein targets in MM using iTRAQ and label free quantitative proteomic approaches. 125 differentially expressed proteins were commonly found in both approaches with similar differential expression pattern. XPO1 protein was selected for further validation as its significant high expression was observed in both iTRAQ and label free analysis. Bioinformatic analysis of these common differentially expressed proteins showed a clear cluster of proteins such as SMC1A, RCC2, CSE1, NUP88, NUP50, TPR, HSPA14, DYNLL1, RAD21 and RANBP2 being associated with XPO1. Functional studies like cell count assay, flow cytometry assay and soft agar assay proved that XPO1 knock down in RPMI 8226R cell line results in re-sensitization to bortezomib drug.

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Sample Preparation: I) Sample preparation and iTRAQ proteomic analysis RPMI 8226 parental and RPMI 8226R resistant cells were washed twice with ice cold PBS. The cells were lysed in 0.5 M triethyl ammonium bicarbonate (TEAB) buffer which contains 7 M urea. The cell lysate mixtures were centrifuged at 15,000 × g and 4 °C for 15 min. The supernatant was collected and mixed with four volumes of ?20 °C pre-chilled acetone and was kept overnight at ?20 °C. Protein pellet was collected and supernatant was discarded after centrifugation at 8,000 × g and 4 °C for 15 min. Pellet containing proteins was re-dissolved in 0.5 M TEAB buffer with 7 M urea. Protein concentration was estimated using 2D quant kit (GE healthcare) according to the manufacturer’s protocol.

Peptide Separation: For each sample, 100 µg protein was taken and all protein samples were reduced, alkylated, and digested with trypsin for overnight. Digested peptides from all the samples were iTRAQ labelled following the manufacturer's instructions as described in the iTRAQ protocol (Sciex). The labelled samples were pooled and concentrated by vacuum centrifugation using SpeedVac (Savant- SPD 1010, Thermo Electron Corporation). Labelled peptide samples were fractionated by strong cation exchange (SCX) chromatography using Poly-Sulfoethyl A column; 100 x 4.6 mm, 5 µm, 300°A, (PolyLC, Columbia, MD) using Shimadzu HPLC. The fractionated peptide samples were again concentrated through SpeedVac and desalted using C18 ZipTips (Millipore) before subjecting them to the LC-MS/MS analysis. Tryptic digested and desalted labelled peptides were analyzed using Orbitrap fusion mass spectrometer (Thermo scientific™) coupled to EASY-nLC 1200 (Thermo scientific™) equipped with EASY- Spray nano flow column (50 cm x 75 µm ID, PepMap C18). Peptides were separated using a 140 min gradient of 5 to 95% phase B (80% Acetonitrile and 0.1% formic acid) at a flow rate of 300 nL/min. The scan sequence began with MS1 spectrum from mass range 375-1700 m/z using Orbitrap analyser at resolution 60,000; having automatic gain control (AGC) target of 4 × 105 and maximum injection time of 50 ms. MS2 precursors were fragmented by high energy collision-induced dissociation (HCD) and analyzed using the Orbitrap analyser of Thermo Scientific Orbitrap fusion mass spectrometer (NCE11 35; AGC 5 × 104; maximum injection time 22 ms, resolution 15,000 at 200 m/z). II) Sample preparation and LFQ proteomic analysis RPMI 8226 parental and RPMI 8226R resistant cells were washed twice with PBS. The cells were lysed in a Urea lysis buffer (7 M urea, 2M thiourea and 2% CHAPS). The cell lysate mixtures were centrifuged at 15,000 g and 4 °C for 15 min. The supernatant was collected and mixed with four volumes of chilled acetone in a separate clean microcentrifuge tube and was kept overnight at ?20°C. The resultant protein precipitate was centrifuged at 8,000 × g, 4 °C for 15 min and the protein pellet was redissolved using the dissolution buffer (7 M urea in 50 mM ABC buffer). Final protein concentration was estimated using 2D quant kit (GE healthcare). Label free analysis was performed using equal amount of protein (100 ?g) per sample. Proteins were reduced with DTT, alkylated with IAA, and digested by trypsin at a ratio of 1:50. Further, the peptides from trypsin digested proteome were desalted and reconstituted in LC-MS grade water with 0.1% formic acid. 1 µg peptides mix of sample were analyzed for each sample in triplicates by Orbitrap Fusion mass-spectrometer (Thermo Scientific™, USA) coupled to EASY-nLC 1200 nano-flow liquid chromatography system (Thermo Scientific™, USA) equipped with EASY- Spray column (50 cm x 75 µm ID, PepMap C18). Peptides were separated by using a 140 min gradient of 5 to 95% phase B (0.1% formic acid in 80% acetonitrile) at a flow rate of 300 nL/min. The Orbitrap MS acquisition parameters were the same as mentioned in the iTRAQ analysis section above.

Protein Characterization: I) iTRAQ Data analysis The protein identification and quantitation were performed by Proteome Discoverer (version 2.2, Thermo Scientific) through the Sequest HT database with 1% FDR and 1 missed cleavage permissible limits as input parameters. Database searching included search against all entries from

the Homo sapiens Uniprot database (download date 31 Oct. 2018). Total protein level precursor ion tolerance was set at 10 ppm. The product ion tolerance used for the data analysis was kept at 0.05 Da. iTRAQ tags on lysine residues and peptide N termini (+144.102 Da) and methylation of cysteine residues (+45.988 Da) were set as static modifications, while oxidation of methionine residues (+15.995 Da) was set as a variable modification. Peptide-spectra matches (PSMs) were adjusted to a false discovery rate (FDR) value of 0.01. For iTRAQ-based reporter ion quantitation, the summed signal-to-noise (S:N) ratio for each iTRAQ channel was extracted and the closest matching centroid to the expected mass of the iTRAQ reporter ion was found. For protein-level comparisons, PSMs were identified, quantified, and narrowed down to a 1% peptide FDR and then further to a final protein-level FDR of 1%. Proteins were quantified by summing reporter ion counts across all matching PSMs. The protein abundance ratios were exported for further analysis to Microsoft Excel. II) Data analysis for LFQ proteomic data The protein identification and quantitation were performed using the Proteome Discoverer software (version 2.2, Thermo Scientific) through the Sequest HT search engine database with 1% FDR and 1 missed cleavage as input parameters. Database searching included all entries from the Homo sapiens Uniprot reference proteome database (downloaded on 31/10/2018). Total protein level analysis was done by using a 10 ppm precursor ion tolerance. The product ion tolerance used for the data analysis was 0.05 Da. Oxidation of methionine residues (+15.995 Da) was set as a variable modification, whereas carbamidomethylation of cysteine residues (+57.021 Da) was set as static modification. Peptide-spectra matches (PSMs) were adjusted to a 0.01 FDR. For protein-level comparisons, PSMs were identified, quantified, and narrowed down to a 1% peptide FDR and then further to a final protein-level FDR of 1%. Proteins were quantified by summing the peptide ions area across all matching PSMs. All the triplicate samples' mean values were considered for the determination of fold change values. The protein abundance ratios were exported for further analysis to Microsoft Excel.

Experiment Type: Shotgun proteomics

Species: Homo sapiens - 9606

Tissue: Cell culture (bto:0000214), Plasma Cell (bto:0000392)

Cell Type: Cell culture, Plasma cell (cl:0000786)

Disease: Multiple myeloma (doid:9538)

Instrument Details: Orbitrap Fusion (MS:1002416)

Protein Modifications: monohydroxylated residue, iodoacetamide derivatized residue, iTRAQ4plex-116 reporter+balance reagent acylated residue

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