# Multilayered omics-based analysis of a head and neck cancer model of cisplatin resistance reveals intratumoral heterogeneity and treatment-induced clonal selection

Supplementary Information

*DNA extraction and NGS library preparation (cell lines)*

Genomic DNA (gDNA) was extracted with the Invisorb Spin Tissue Mini Kit (Stratec, Birkenfeld, Germany). HaloPlex library preparation was performed as recommended by the manufacturer (Agilent, protocol version F1, July 2015). Briefly, the gDNA was digested and the HaloPlex probe library was hybridized in the presence of the indexing primer cassette. The biotinylated DNA-probe hybrids were captured by magnetic purification of the samples with streptavidin-coated magnetic beads. Fragmented targets were amplified via PCR at cycling conditions recommended by the manufacturer. Subsequent to the PCR reaction, the final library was purified using AMPure XP magnetic beads (Beckman Coulter, Krefeld, Germany) and quantified using the library quantification kit Illumina TruSeq (Bio-Rad Laboratories, Munich, Germany) and the QX200 ddPCR System (Bio-Rad Laboratories).

*Targeted NGS of patient tumor samples*

Tissue slices were stained with hematoxylin and eosin and reviewed by a pathologist (K.J.) to delineate tumor areas and to quantify tumor cell contents. Only tumor samples with a minimum tumor cell content of 30% were further processed. After deparaffinization using xylol and ethanol (two times), tumor areas were macrodissected and tissue pellets were dried at 55 °C for 10 min. Genomic DNA was extracted using the High Pure FFPET DNA Isolation Kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer’s instructions and stored at -20°C until further processing. HaloPlexHS library preparation was performed according to the manufacturer’s instructions (Agilent, protocol version C0, December 2015). Libraries were pooled and subjected to PE sequencing on the Illumina NextSeq500 platform with the NextSeq Midoutput Sequencing Kit, producing 150 bp PE reads. After duplicate removal, a mean sequencing depth of 170-fold (range: 60-330) was achieved in the whole exonic *TP53* target region.

*Microarray-based transcriptome analysis*

Total RNA extraction from the FaDu cell line and subclones was performed with the High Pure RNA Isolation Kit (Roche). Eluted samples were shipped to the genomic core facility of the German Cancer Research Center (Heidelberg, Germany) for microarray mRNA analysis. RNA quality was checked by gel analysis using the total RNA Nano chip assay on an Agilent 2100 Bioanalyzer (Agilent Technologies GmbH, Berlin, Germany). Only samples with RNA index values >8.5 were selected for expression profiling. RNA concentrations were determined using the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). Biotin-labeled cRNA samples for hybridization on the HumanHT-12 v4 Expression BeadChip array (Illumina) were prepared according to Illumina's recommended sample labeling procedure. Briefly, 500ng total RNA was used for complementary DNA (cDNA) synthesis, followed by an amplification/labeling step (in vitro transcription) to synthesize biotin-labeled cRNA according to the Illumina® Total Prep™ RNA Amplification Kit (Life Technologies). Biotin-16-UTP was purchased from Roche Applied Science (Penzberg, Germany). The cRNA was column-purified and eluted in 60 µl of water. Quality of cRNA was controlled using the RNA Nano Chip Assay on an Agilent 2100 Bioanalyzer and spectrophotometrically quantified (NanoDrop).

Hybridization was performed at 58°C, in GEX-HCB buffer (Illumina) at a concentration of 100ng cRNA/µl, unsealed in a wet chamber for 20hs. Spike-in controls for low, medium and highly abundant RNAs were added, as well as mismatch control and biotinylation control oligonucleotides. Microarrays were washed once in High Temp Wash buffer (Illumina) at 55°C and then twice in E1BC buffer (Illumina) at room temperature for 5 min. After blocking for 5 min in 4ml of 1% (wt/vol) Blocker Casein in phosphate buffered saline Hammarsten grade (Pierce Biotechnology, Rockford, IL), array signals are developed by a 10-min incubation in 2ml of 1µg/ml Cy3-streptavidin (Amersham Biosciences, Buckinghamshire, UK) solution and 1% blocking solution. After a final wash in E1BC, the arrays are dried and scanned using an iScan array scanner.

Data extraction was done for all beads individually, and outliers were removed when the absolute difference to the median was greater than 2.5 times the median absolute deviation. All remaining bead-level data points were then quantile normalized (1).

*Phosphoproteome analysis*

Cells were seeded in 150x25mm petri dishes. When the cultures reached a confluency of 70-80%, cells were washed twice with cold PBS and lysed with 7M Urea/2M Thiourea/50mM Tris-HCl at pH8 containing Complete EDTA-free protease and PhosSTOP inhibitor cocktails (Roche Diagnostics). We used 2mg of total protein for subsequent analysis.

Briefly, lysates were centrifuged for 30min at 20,800x g and protein concentrations quantified using Bradford assays (Bio-Rad Laboratories). Following protein reduction with 10mM DTT and alkylation using 30mM iodacetamide lysates were diluted with 50mM Tris-HCl to 1M Urea. Overnight trypsin (Promega, Fitchburg, WI) digestion was carried out at a ratio of 1:20 at 37°C. The next day, samples were acidified to a pH of 4.0 and particulates were removed by centrifugation for 30min at 2,500x g.

Peptides were desalted and dimethyl labeled on column as previously described with slight modifications (2). OASIS HLB (Waters, Milford, MA) cartridges were washed with acetonitrile (ACN) and conditioned with 0.1% formic acid (FA). After sample loading peptides were washed with 0.1% FA and labelled with “light” (CH2O + NaBH3CN), “medium” (CD2O + NaBH3CN) or “heavy” (13CD2O + NaBD3CN) labeling reagents (all from Sigma-Aldrich, St. Louis, MO). We used the light label for an internal standard in each labeling triplet, comprising of a mixed lysate of the FaDu clones, to allow for inter-triplett analysis and comparisons. Subsequent washing with 0.1% FA labeled peptides were eluted with 0.1% FA/80% ACN, concentrated in a SpeedVac and stored at -80°C.

Enrichment of phosphorylated peptides was performed based on the method of Thingholm et al. (3). First, for each labeling triplet the internal standard (light) and one medium and one heavy labeled FaDu clone were pooled. 40µL of 500mg/mL Titansphere TiO 10µm (GL Sciences Inc., Torrance, CA) in 80% ACN/0.2% TFA were washed with 0.6% NH4OH (Merck, Darmstadt, Germany) and sample buffer (80mg/mL glycolic acid/80% ACN/2% TFA solution). Sample buffer (2x) was added 1:1 to the pooled peptide solutions and particulates were removed by centrifugation for 10min at 16,000x g. Peptides were then sequentially incubated with 500mg/ml TiO2 (2hs, 1h) and 100mg/ml TiO2 (overnight, 1h, 1h) for a total of 5 incubation steps. Following loading of the TiO2-peptide solution onto a self-prepared microcolumn, beads were washed with 80mg/mL GA/80% ACN/2% TFA, 80% ACN/0.2%TFA and 20% ACN. Consecutive elution of phosphopeptides was executed by three times addition of 30µL of 0.6% NH4OH. All eluates from the same sample were pooled, concentrated in a SpeedVac and stored at -80°C. Samples were desalted using C18 Tips (Thermo Scientific, Waltham, MA) according to the manufacturer’s instructions.

Samples were analyzed in technical replicates using a QExactive mass spectrometer (Thermo Scientific) and a U3000 RSLC nano HPLC system (Dionex, Sunnyvale, CA) coupled to a 50cm long by 75-micron internal diameter EASYspray column. Samples were resolved using a 4-hour gradient with the mass spectrometer run in Data Dependent Analysis mode in which the 20 most intense multiply charged precursors were selected for fragmentation. The mass spectrometer settings were as follows: Precursor resolution, 70,000; AGC target, 3,000,000; maximum fill time, 250 ms; MSMS resolution, 17,500; AGC target ,100,000; maximum fill time, 120 ms; isolation window, 3 Da; NCE, 30; underfill ratio 10%. Data were processed and quantified using Proteome Discoverer 1.4 (Thermo Scientific) and Mascot (MatrixScience, London, United Kingdom). Site localization probabilities were calculated by PhosphoRS 3.154 which is implemented in Proteome Discoverer. The statistical programming language R (https://www.r-project.org) was used for subsequent data analysis. Peptide data were extracted from Proteome Discoverer and analyzed using an in-house R script. Kinase enrichment analysis was performed using the web-based tool (<http://www.maayanlab.net/KEA2/index.html>) developed by Avi Ma’ayan and colleagues (4).

References:

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