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Discovery of predictive biomarker candidates for intrinsic resistance to FOLFOX chemotherapy in colon cancer using a top down LC-MALDI approach

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ABSTRACT

Objective: Chemoresistance of cancer cells to chemotherapeutics is a main obstacle in chemotherapy to a successful outcome especially in first line, but also in later stages of chemotherapy. In the therapy of colorectal cancer, combinational chemotherapies such as FOLFOX have been shown to overcome resistances to single agents, but the response rates are still low. Although, chemotherapeutic agents are still the backbone of colorectal cancer therapy, molecular determinants of chemoresistance are still lacking. Therefore, patient tailored therapy of colorectal cancer is still a huge challenge. Since the proteome of each cell is responsible for fundamental biological processes and also makes up the bulk of pharmaceutical targets and potential biomarkers, the search for predictive protein biomarker candidates is promising. **Materials and Methods:** In this study, we used a viability assay to classify cell lines chemosensitive or chemoresistant to the FOLFOX chemotherapy *in vitro*. Subsequently, a newly developed top down LC-MALDI-MS-MS workflow was used to analyze the low molecular weight proteome of the cell lines in order to discover protein biomarker for intrinsic chemoresistance to FOLFOX chemotherapy. Initially, a technical validation of a selected biomarker was conducted. **Results:** The used cell lines were classified in chemosensitive and chemoresistant based on their chemosensitivity to FOLFOX at different time points. The top down LC-MALDI-MS-MS workflow subsequently resulted in the identification of several protein biomarker candidates, differentially regulated between the chemosensitive and chemoresistant groups. **Conclusion:** Findings from discovery studies need to be validated in a stringent manner in order to find robust and meaningful biomarker candidates. The discovery of predictive biomarkers for chemoresistance and the identification of molecular mechanisms underlying intrinsic chemoresistance could tremendously promote individualized chemotherapy.

KEY WORDS: Colorectal cancer, chemosensitivity, FOLFOX therapy, proteomics, predictive protein biomarker

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INTRODUCTION

Colorectal cancer is the third most commonly diagnosed cancer in males and the second in females, with over 1.2 million new cancer cases and 608,700 cancer related deaths in 2008 [1]. Despite the advances being made in early detection of colorectal cancer, approximately half of all patients develop metastatic disease [2]. The prognosis for these patients is poor, although palliative chemotherapy has been shown to be able to prolong survival and to improve the quality of life compared with the best supportive care [3]. Chemoresistance of cancer cells to chemotherapeutics is a main obstacle in chemotherapy to a successful outcome in first line therapy. It has been hypothesized that selection pressure resulting from tumor internal evolution can lead to subpopulations of cell clones, carrying certain cellular mechanism that can be summarized under the term "intrinsic chemoresistance." Cellular mechanisms of intrinsic chemo resistance are mainly characterized by the fact that they lead to increased tolerance of cancer cells to chemotherapeutics. These cells are most likely to survive first line chemotherapy and arise as recurrence disease.

Chemotherapeutic agents are still the backbone of colorectal cancer therapy, but molecular determinants of chemoresistance are still lacking [4]. In order to overcome therapeutic failures, colorectal cancer has been studied in detail on the genetic level resulting in an initial understanding of the sequence of mutational changes and expression patterns during development, progression and their impact on individual drug response [5-7]. Furthermore gene expression studies focusing on individual drug response have been conducted to add to the complex picture of molecular networks underlying

chemoresistance [8]. However, this has not yet answered the clinical demands for improved diagnosis and prediction of response to chemotherapy.

Patient tailored therapy for colorectal cancer is still a huge challenge. In the therapy of colorectal cancer, combinational chemotherapies like FOLFOX have been shown to overcome resistances to single agents but the response rates are still low [9]. The discovery of predictive biomarkers for chemoresistance and the identification of molecular mechanisms underlying intrinsic chemoresistance could tremendously promote individualized chemotherapy.

Since all cellular and tissue development and regulation events are dominated by expression differences on the protein and posttranslational level, proteomics techniques are a key element for any biomarker discovery and validation strategy. Previous work on cancer cell resistance to apoptosis has already pointed out that anti-apoptotic proteins are involved in mechanism of resistance to anticancer treatment [10], and therefore resistance to FOLFOX therapy may also involve anti-apoptotic proteins. In general, the proteome of each cell is responsible for fundamental biological processes and also makes up the bulk of pharmaceutical targets. Consequently, the determination of significant associations between protein expression patterns and in *vitro* chemosensitivity may enable the discovery of new predictive biomarkers for the improvement of treatment. Ultimately, these biomarkers need to be validated in the clinical setting and a validated molecular technique needs to be established to provide a biomarker based companion diagnostic. Tailored chemotherapy for individual patients could increase the response rate to therapy and spare patients from ineffective therapy and associated side effects. In the present study, we report on a newly established LC-MALDI top down workflow for the analysis of low molecular weight, basal protein expression patterns in a diverse panel of colorectal cell cultures in order to identify biomarker candidates for intrinsic resistance to FOLFOX chemotherapy.

MATERIALS AND METHODS

Materials

All standard laboratory chemicals for proteomic analysis including acetonitrile (AcN), trifluoroactetic acid (TFA), formic acid (FA), iodoacetamide, ammonium bicarbonate and ammonium phosphate were high performance liquid chromatography (HPLC)-grade or better from Sigma (Steinheim, Germany). *Rapi*Gest™ SF cleavable detergent was purchased from Waters. Methanol was from Roth (Karlsruhe, Germany). Sinapinic acid and the MALDI mass calibration standard, consisting of protein and peptide calibration standards were purchased from Bruker (Bremen, Germany). Dithiothreitol (DTT), sodium dodecylsulfate (SDS), and trypsin proteomic grade were purchased from Sigma (Steinheim, Germany). Cell culture media (DMEM/F12), D-PBS and MEM vitamins were obtained from PAA (Cölbe, Germany). Foetal calf serum and supplements (penicillin/streptomycin, L-glutamine, gentamicin) were purchased from Invitrogen (Karlsruhe, Germany). Transferrin and collagen I was obtained from Roche (Mannheim, Germany), fetuin and insulin from Sigma (Steinheim, Germany). Cell culture plastic ware was purchased from Sarstedt (Nümbrecht, Germany), Biozym (Oldendorf, Germany) and Greiner Bio One (Frickenhausen, Germany). Dimethyl sulfoxide, 5-Fluorouracil (5-FU), leucovorin (LV) and Oxaliplatin (Oxa) were from Sigma (Steinheim, Germany). Drugs were diluted in culture medium shortly before use. All other chemicals were obtained from standard commercial sources.

Cell Lines

Fourteen cell cultures, consisting of two primary mixed culture, three primary clonal cell lines and nine secondary cell lines, were used for this study [Table 1]. Secondary cell lines were purchased from Cell Line Service, Eppelheim, Germany. The primary mixed cultures originated from tumor specimen collected and prepared under standardized conditions at clinics and laboratories belonging to the network of Indivumed GmbH. The patients gave written consent and IRB approval was obtained by the responsible IRB board of the physicians association in Hamburg, Germany. Briefly, tumor tissues were mechanically minced under sterile conditions and enzymatically digested with collagenase solution (2 mg/ml) (Serva, Heidelberg, Germany) at 37°C for 60 min. Cell suspensions were filtered through a 420 μm pore steel mesh as well as 100 μm and 70 μm filters and centrifuged at 500 × g for 3 min. Erythrocytes were lysed by resuspending the cell pellet in 1-2 mL Easy Lysis Solution (Dako, Hamburg, Germany) for 10 min. Primary cell yields and viability were determined by trypan blue exclusion test (Sigma, Steinheim, Germany) using a Neubauer chamber. Clonal cell lines from the mixed culture were established by single cell preparations. Primary cell lines were cultured in a DMEM/F12 based medium supplemented with 10% fetal bovine serum, in collagen coated flasks at 37°C and 5% CO_2.

Cell Viability Assay (Perkin Elmer)

Cell viability was assessed by intracellular adenosine triphosphate (ATP)-level analysis according to the manufacturer's protocol using the ATPlite Luminescence ATP Detection Assay System (PerkinElmer, Boston, MA). Cells were seeded 384-well microtiter plates at densities adjusted to the individual growth rate of the cell lines, ranging from 500 to 1500 cells per well and pre incubated for 24 h. The cells were then treated with various concentrations of FOLFOX combination treatment and then further incubated for additional 48 h, 72 h and 96 h following which ATP content was measured. The concentrations of the drugs used were summarized in fold FOLFOX dilutions, wherein 1 fold FOLFOX corresponds to 500 μM 5-FU, 100 μM LV and 20 μM Oxa. Samples were tested in quadruplicate. The resulting luminescence was read using a FLUOstar OPTIMA® system (BMG Laboratories, Offenburg, Germany). The results are displayed as the percentage of viability at a given concentration in reference to the untreated control. Data were analyzed as means ± standard deviation and IC50 values were calculated from the dose response curves using GraphPad Prism® Version 5.0 (GraphPad Software, San Diego, CA, USA).

Table 1: Basic data of primary cell cultures and secondary cell lines used in this study

T-test bucket	Identity of biomarker candidates	UniProt identifier	*P* value	Fold change SIR
2470.4s: 15858.49 m/z	SOD [Cu-Zn]-homo sapiens (Human)	SODC HUMAN	0.0001	−1.86
1491.5s: 12332.08 m/z	ATPase inhibitor, mitochondrial-homo sapiens (Human)	ATIF1JHUMAN	0.0027	4.94
1475.4s: 6170.38 m/z	Ubiquitin-60S ribosomal protein L40-homo sapiens (Human)	RL40 HUMAN	0.0001	3.61
2337.5s: 11204.95 m/z	Dermcidin (Preproteolysin)- homo sapiens (Human)	DCD HUMAN	0.0063	3.31
3000.2s: 13926.29 m/z	Thioredoxin domain-containing protein 17-homo sapiens (Human)	TXD17 HUMAN	0.0054	2.68
2406.9s: 6921.07 m/z	DNA-directed RNA polymerases I, II, and III subunit RPABC4 OS=homo sapiens	RPAB4 HUMAN	0.0015	−1.79
2660.9s: 13727.06 m/z	Histone H2B Type 1 (H2B.1 A)-homo sapiens (Human)	H2B1C HUMAN	0.0002	−3.39
1665.6s: 8373.76 m/z	Cysteine-rich protein 1 (CRIP) - homo sapiens (Human)	CRIP1JHUMAN	0.0011	1.81
2817.1s: 8867.83 m/z	Cytochrome c oxidase polypeptide VIc precursor (EC 1.9.3.1) - Homo sapiens (Human)	COX6C HUMAN	0.0017	−2.08
2673.0s: 8144.58 m/z	Uncharacterized protein C20orf52 - Homo sapiens (Human) R0M01	CT052 HUMAN	0.0030	−2.14
2738.9s: 18435.83 m/z	Thioredoxin, mitochondrial precursor (Mt-Trx) (MTRX) (Thioredoxin-2) - Homo sapiens (Human)	THIOM HUMAN	0.0049	−2.1
2626.6s: 11040.89 m/z	Loss of heterozygosity 3 chromosomal region 2 gene A protein - Homo sapiens (Human)	L3R2AJHUMAN	0.0121	−2.11
2571.6s: 13985.36 m/z	Small nuclear ribonucleoprotein Sm D3 (snRNP core protein D3) (Sm-D3) - Homo sapiens (Human)	SMD3 HUMAN	0.0130	−1.73
2632.0s: 15209.69 m/z	E-FABP - homo sapiens (Human)	FABPE HUMAN	0.0130	−2.18
2591.4s: 10292.36 m/z	Dynein light chain 1, cytoplasmic (dynein light chain LC8-type 1) - homo sapiens (Human)	DYL1 HUMAN	0.0134	−1.64
2369.0s: 21805.30 m/z	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 9 OS- homo sapiens	NDUB9_HUMAN	0.0139	−2.27
2646.1s: 7939.90 m/z	40S ribosomal protein S28 - homo sapiens (Human)	RS28 HUMAN	0.0147	−1.66
2636.3s: 6929.97 m/z	IADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 1 (EC 1.6.5.3) - Homo sapiens (Human)	NDUB I_HUMAN	0.0200	−1.52
2789.8s: 12777.80 m/z	SH3 domain-binding glutamic acid-rich-like protein - homo sapiens (Human)	SH3L1 HUMAN	0.0272	−2.17
2843.5s: 17899.84 m/z	Ubiquitin-conjugating enzyme E2 L3 (EC 6.3.2.19) - homo sapiens (Human)	UB2L3 HUMAN	0.0302	−1.96
2376.1s: 21973.62 m/z	Peptidyl-prolyl cis-trans isomerase F, mitochondrial OS=homo sapiens (Human)	PPIF HUMAN	0.0361	−3.26

CRIP: Cysteine-rich intestinal protein, ATP: Adenosine triphosphate, SOD: Superoxide dismutase, E-FABP: Fatty acid-binding protein, epidermal

Cell Lysis and Sample Preparation for Proteomic Analysis

Cells were lysed in an organic solution consisting of 50% AcN and 0.5% TFA. Lysis was performed for 10 min on ice followed by a 10 min ultrasonic treatment. Lysates were then centrifuged for 5 min at 16873 × g (4°C) and the supernatant was immediately transferred to LoBind reaction tubes (Eppendorf, Hamburg, Germany). Lysates were then dried in a vacuum centrifuge 5301 (Eppendorf, Hamburg, Germany) and resuspended in 0.1% FA. The protein concentration was determined using the Bicinchoninic Acid Protein assay kit (Sigma, Steinheim, Germany).

Reverse Phase - HPLC (RP-HPLC)

Proteins of whole cell lysates were separated by RP chromatography using a HPLC system 1200 (Agilent, Böblingen, Germany) with a 4.6 mm × 50 mm mRP (macroporous reversed phase) column (Agilent, Böblingen, Germany). Solvent A was 0.1% TFA in ultrapure water, solvent B was 0.1% TFA in 99.9% AcN. 75 μg of protein amount was injected at a LC flow rate of 300 μl/min. The column was heated constantly to 60°C and proteins were eluted by a 67 min gradient from 2% to 40% solvent B during the first 51 min followed by a 15 min increase to 60% B. UV absorption at 214 nm was used to monitor the separation and quality of the protein/peptide separation. After each sample separation at least four successive cycles of blank injections (50 μl of 0.1 % TFA in ultrapure water), followed by short gradient separations (25 min) were run through the LC system in order to equilibrate the column and remove remaining proteins. LC fractions were collected every 36.6 s (=183 μl) into a LoBind deepwell plate (Eppendorf, Hamburg, Germany) resulting in a total number of 94 fractions per sample. A volume of 50 μl of each LC fraction was transferred to a 96-well plate dried in a vacuum concentrator 5301 (Eppendorf, Hamburg, Germany). Each dried fraction was re-suspended in 3.5 μl Sinapinic acid and 3 μl per fraction were spotted onto a polished steel target, shortly before MALDI-MS acquisition.

MALDI-MS Measurement and Data Analysis

An ultraflex III MALDI-MS instrument equipped with a 200 Hz smartbeam laser was used for the acquisition of linear as well as reflector and LIFT spectra. All software packages including MALDI-MS data acquisition, post processing of spectra and statistical analysis were obtained from Bruker Daltonik (Bremen, Germany). Polish steel targets and calibrants (monoisotopic masses: Angiotension II, 1046.54; Angiotensin I, 1296.68; Neurotensin, 1672.91; Renin Substrate, 1758.93; ACTH clip 1-17, 2093.08; ACTH clip 18-39, 2465.19; ACTH clip 1-24, 2932.58; ACTH clip 7-38, 3657.92; Insulin, 5734.51; Ubiquitin, 8565.76; Cytochrom C, 12360.97; Myoglobin, 16952.30) were used for calibration. In addition, an external standard, consisting of a cell lysate with a spiked insulin concentration of XX fmol/μl was used to adjust the laser energy throughout the complete study. We aimed to find the right laser energy to reach reproducible intensity values for insulin between 3000 and 6000 arbitrary units and a resolution of

over 600 with 3000 applied laser shots. Subsequently, sample measurements were performed under these adjusted laser conditions by accumulating 3000 laser shots on random positions of each target spot in the linear mode. Three replicates of every sample were processed independently to reveal the technical reproducibility of the workflow. Initially, after data acquisition all peaks with a minimum signal to noise ratio of 3 were labeled during post processing and were subsequently used for statistical analysis. Measurements were controlled by the software WARP-LC 1.2 and peak detection, spectra smoothing and baseline correction were done in FlexAnalyis software 3.0. WARP-LC was used to generate non-redundant compound lists which served as input files for the statistical data analysis by ProfileAnalysis (Version 2.0; Bruker Daltonik, Bremen, Germany).

Statistical Analysis of the LC-MALDI Data

Profile analysis was used to align the compound lists to generate buckets with a defined retention time and *m/z* value window, which allowed the comparison over multiple sample data sets. Normalization of peak intensities was done on the total intensity of all selected buckets. Differential peptide analysis was then performed with the goal to discover robust and significant expression differences between groups. Only *m/z* ions displaying a minimum averaged peak intensity difference of 1.5 between groups and a $P < 0.01$ were considered for further analysis such as protein identification.

In Gel Digestion

RP-HPLC fractions containing the respective target proteins were dried in a vacuum centrifuge (Eppendorf Vacuum concentrator, 5301) and separated by SDS-PAGE on a 4-12 % SDS Bis Tris gel (Invitrogen). Gel bands at the molecular size of interest were excised and washed. DTT (100 mM) was added to a final concentration of 10 mM and reduction was maintained at 56°C for 45 min followed by alkylation with iodoacetamide (15 mM final concentration) for 30 min in the dark (room temperature [RT]). Trypsin at a concentration of 0.2 $\mu g/\mu l$ was added and enzymatic digestion was performed overnight at 37°C. TFA was added until a final concentration of 1% to stop further trypsin digestion. Peptides were subsequently extracted from the gel bands by sonification and extraction buffer (50% AcN/1% TFA). After 45 min the samples were centrifuged at 16000 × g for 10 min at 4°C to separate the supernatant from the remaining gel pieces and the supernatants were stored in aliquots at −80°C.

Identification of peptides by MS/MS

Tryptic peptides resulting from the in gel digestion were subsequently subjected to capillary HPLC (cap-LC, 1200 Agilent system) using a 0.3 mm × 15 mm C18 mRP column (Agilent). Solvent A was 0.1% TFA in ultrapure water, solvent B was 0.1% TFA in 99.9% AcN. Four μl of tryptic digest were injected at a LC flow rate of 4 μl/min and the resulting LC fractions were spotted every 15 s (=1 μl) by a fraction collector (FC Proteineer, Bruker Daltonics) to spots of a-cyano-4-hydroxycinnamic acid matrix Prespotted AnchorChip (PAC) target. After each sample separation at least two successive cycles of blank injections (3 μl of 0.1% TFA in ultrapure water), followed by short gradient separations (30 min) were run through the LC system in order to equilibrate the column and remove remaining tryptic peptides. The PAC target spots were dried at RT, sealed in plastic bags and stored in the dark for a maximum time of 24 h before MALDI-MS data were acquired. Directly before a MALDI measurement, the PAC target was dipped two times carefully for 5 s in 500 ml of 10 mM ammonium phosphate buffer containing 0.1% TFA (4-8°C) shortly. The peptide identification strategy started with a MALDI-MS detection step in the reflector mode with the goal to identify as many peptides as possible by the second acquisition round, which comprises of MS/MS data generation by the LIFT mode. Target Peptides were identified by MALDI MS/MS in the LIFT mode using a semi-automated spectra accumulation procedure. Target precursor masses were first selected based on minimal S/N of 10 by WARP-LC. The respective molecular parent ions were selected in a timed ion gate at 8 kV and detected by accumulating 400-800 laser shots. In the LIFT mode, post source decay fragments were further accelerated by 19 keV. Depending on the peak abundance between 1500 and 5000 laser shots were accumulated in the LIFT mode to reach high quality MS/MS spectra. Biotools version 3.2. (Bruker Daltonics) was used to submit MS/MS peak lists to database searches using MASCOT (Matrix Science). For the database search the following criteria were used: Enzyme: None or trypsin; variable modifications: Oxidation M; carbamidomethyl; mass tolerance: 50 ppm; MS/MS tolerance: 0.7 Da; peptide charge: +1; two missed cleavages were allowed. Ion scores of a minimal probability of 95% ($P < 0.05$) were accepted and regarded as significant hits.

Westernblotting

Initial immunological validation of selected marker candidates was done by western blotting. The protein concentrations of the lysates were determined using the BCA protein assay kit from Pierce (Rockford, USA) according to the manufacturer's instructions. Briefly, a protein amount of 25 μg was separated by gel electrophoresis in a 4-12% SDS Bis Tris gel (Invitrogen). The separated proteins were electrotransferred to a polyvinylidene fluoride membrane (Immobilon P) using a tank blotting system (Invitrogen) according to the manufactures instructions. Blots were blocked by incubation for 2 h with 5% skim milk powder (w/v) in phosphate buffered saline containing 0.1% Tween-20. Immunoblot analysis was carried out with polyclonal antibodies raised against the full length Cu/Zn superoxide dismutase (SOD1) (Millipore) in a dilution of 1:2000 and a GAPDH (Ambion)antibody as loading control in a dilution of 1:4000 respectively, in the TRIS-buffered saline containing 0.1% Tween-20. Proteins were detected by enhanced chemiluminescence (Super Signal West Dura Chemiluminescence Substrate, Pierce/Thermo Scientific) using the Raytest detection system "Darkroom Evo III".

RESULTS

Characterization of Chemosensitivity to FOLFOX Combination Therapy

Individual chemosensitivity of cell lines was determined using the cell viability assay (Perkin Elmer). The IC50 of individual cell lines to a FOLFOX combination treatment was measured after 48 h, 72 h and 96 h of drug treatment. The analysis of single time points revealed time and dose dependent effects of the FOLFOX treatment. The used cell cultures showed different responses to FOLFOX treatment; therefore it was possible to define chemoresistant and chemosensitive groups based on the median IC50 of all cell lines over time. The groups differed significantly in chemosensitivity to FOLFOX treatment at every time point of the assay. The results are summarized in Figure 1. Based on these data one chemosensitive group of cell lines (n = 7) and one chemoresistant group (n = 7) was stratified for the subsequent proteomic studies.

Top down Proteomic Workflow

We developed a proteomic workflow for the detection of low molecular weight proteins and peptides. In contrast to the widely used bottom-up proteomic approach, which applies tryptic digestion of proteins prior to analysis, we analyzed cell lysates of cell lines by a top-down MALDI-MS without prior tryptic digestion. This approach should enable us to retain the intact molecular mass information of potential biomarker candidates, which is important e.g., for the elucidation of posttranslational modifications and truncations. Proteins from cell lysates were fractionated by RP-HPLC and subsequently analyzed by linear MALDI-MS in order to generate protein expression profiles in a mass range from 2.5 kDa up to 30 kDa, reflecting parts of the low molecular proteome. We were able to detect in average up to 4000 molecular features in these cell lysates in this mass range. Potential adducts as well as multiple charged ions contribute to this large number of features detected. This leads to the assumption that our workflow covers around 1000-1500 native peptides and proteins. Three typical LC-MALDI data of replicate measurements of the cell line HT-29 are displayed via a two-dimensional pseudo gel view (=survey view) in Figure 2. In this survey view the LC retention time scale is plotted on the y axis and the mass range on the x axis, whereas the relative peak intensity is displayed by the density.

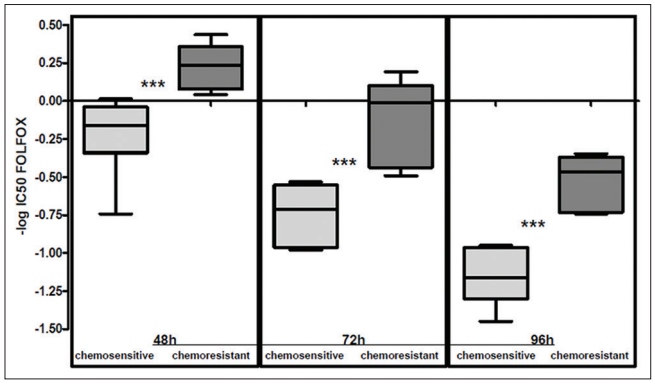

Figure 1: Chemosensitivity of sensitive and resistant cell culture groups to FOLFOX treatment, measured at 48, 72 and 96 h after drug treatment. The chemosensitivity differed significantly between these groups, P = 0.0007 for 48 h; P = 0.0003 for 72 h and P = 0.0001 for 96 h, respectively

Although, no exact quantitative information can be derived from those pictures the survey views give a first overview on retention time, signal intensity and general pattern reproducibility. For optimization of MALDI measurement reproducibility, the laser intensity for MALDI MS measurements was adjusted to an external standard consisting of a whole cell lysate with a defined amount of spiked insulin prior to every measurement. Assessment of reproducibility has been derived from the number of measured peaks (compounds) and the signal intensities of the same peaks within the technical replicates. The number of common, non-redundant compounds between individual samples ranged from 2500 to 3000, whereas the averaged CV for the number of compounds within the replicates was 11%. Statistical analysis of the LC-MALDI data sets by the Principal Components Analysis showed a weak clustering of chemoresistant against chemosensitive cell lines, as shown in Figure 3.

By comparing a panel of fourteen colorectal cancer cell cultures grouped into chemoresistant and chemosensitive, we found 95

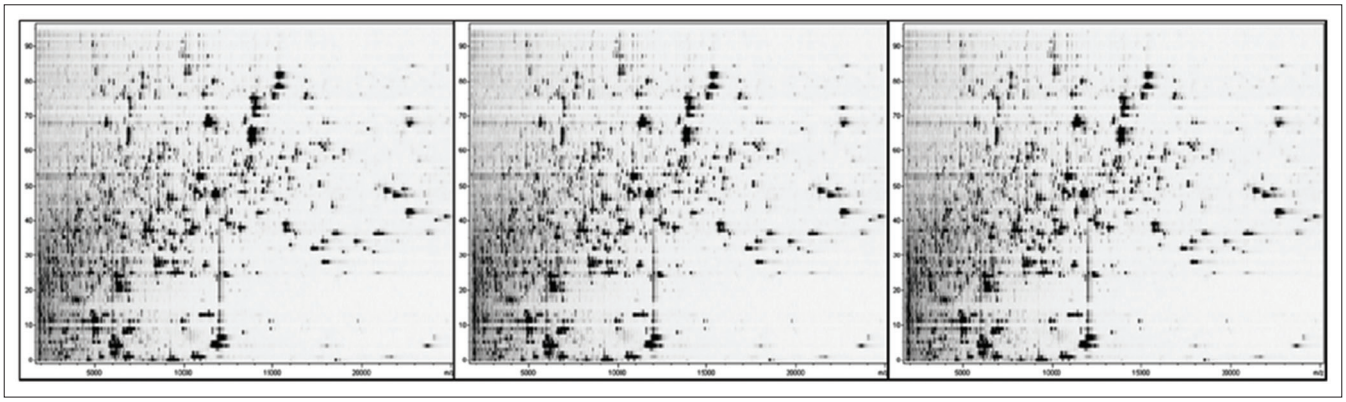

Figure 2: Replicate measurements of the cell line HT-29 displayed by a 2-D survey view visualizing the technical reproducibility of the top-down LC-MALDI workflow. Every LC fraction represents a single MALDI MS spectrum (linear mode)

compounds to be significantly (<0.01) regulated more than 1.5 fold between groups. These biomarker candidates could not be directly identified by MS/MS due to molecular weights above 4 kDa. Therefore, LC-fractions containing the biomarker candidate were fractionated using SDS-PAGE to further enrich the target protein, gel bands of respective molecular size were excised and in gel digestion was applied resulting peptides were again screened by LC-MALDI-MS-MS. The targeted identification approach using in gel digestion resulted in the identification of biomarker candidates from various cellular compartments, Table 2. Examples for identified biomarker candidates are shown in Figure 4. The DNA-directed RNA polymerase 1 was up regulated 1.79 fold, histone H2B Type 1 was up regulated 3.39 and the Cu/Zn SOD was up regulated 1.86 fold in the chemoresistant group, the thioredoxin domain-containing protein 17 was up regulated 2.68 fold in the chemosensitive group.

Exemplary Validation of Biomarker Candidates

The Cu/Zn SOD has previously been found to be up regulated in cisplatin-resistant ovarian cancer cell lines [11] and has to our knowledge not yet been described to be involved in detoxification processes related to anticancer drug treatment in colorectal cancer. Therefore, we choose this biomarker candidate for an exemplary further validation. The technical validation of results from the LC-MALDI workflow was carried out using western blotting. Whole cell lysates of six cell cultures from the study were blotted against an anti-Cu/Zn SOD polyclonal antibody. Signals were detected at a molecular weight of approx. 16kDa. The results from the western blotting experiments confirmed the LC-MALDI data and showed elevated expression of the Cu/Zn SOD in chemoresistant compared with chemosensitive cell cultures as shown in Figure 5. In the next step of validation, we were interested in whether the basal enzyme activity of the Cu/Zn SOD shows any kind of correlation to the expression data or chemosensitivity. Therefore, we measured the Cu/Zn SOD enzyme activity in thirteen of fourteen cell cultures from the study and found no correlations to any parameters (data not shown).

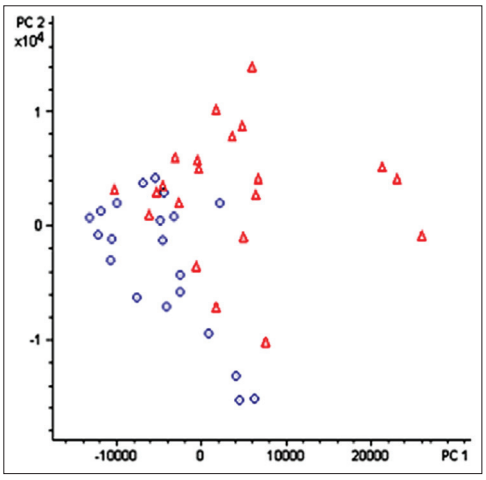

Figure 3: Principal component analysis analysis of the generated proteomic profiles. Chemoresistant cell lines (red) cluster against chemosensitive cell lines (blue), overlapping slightly

DISCUSSION

The need for new biomarkers in the clinical development of anticancer agents is mandatory; furthermore the current clinical situation needs better patient stratification for first line therapy with conventional chemotherapeutic regiments. Predictive biomarkers are useful in selecting patients who will more likely benefit from therapy and thus increase their overall survival with minimized side-effects. Ideally, besides being informative, biomarker assays should also preferably be able to use tissue samples that are readily accessible in patients. This is the case in the therapy of colorectal cancers were surgical removal of the tumor is mostly the first step in therapy. This tumor material is, if properly collected, a good basis for molecular analysis. In consequence, there is a great need for molecular techniques that enable a robust discovery, validation and integration of predictive biomarkers into the clinical situation.

In this study, we have shown that the newly established top down LC-MALDI workflow is applicable to the proteomic analysis of the intact, low molecular proteome. This enables the discovery of low molecular weight biomarker candidates, which can potentially not be detected in proteomic workflows applying

Table 2: List of biomarker candidates identified by in gel digestion. Expression differences are displayed as fold change in relation to S=chemosensitive versus R=chemoresistant group

Case number	Cell line name	Gender	Age (years)	Tumor localisation	TNM classification	Stage	Chemosensitivity
A845	A845MK	Male	64	Colorectal adenocarcinoma	T2 N2 M0	Dukes' type C	Resistant
A609	A609MK	Female	86	Colorectal adenocarcinoma	T3 N1 M1	Dukes' type D	Sensitive
B352	B352clone3	Male	47	Colorectal metastasis site	M1	Dukes' type D	Sensitive
B429	B429clone8	Male	44	Colorectal adenocarcinoma	T3 N0 M0	Dukes' type B	Sensitive
A806	A806clonel	Female	65	Colorectal adenocarcinoma	T4 N2 M0	Dukes' type C	Resistant
Secondary cell line	SW480	Male	51	Colorectal adenocarcinoma	-	Dukes' type B	Resistant
Secondary cell line	LS174T	Female	58	Colorectal adenocarcinoma	-	Dukes' type B	Resistant
Secondary cell line	LS513	Male	63	Colorectal carcinoma	-	Dukes' type C	Sensitive
Secondary cell line	HCT-15	Male	67	Colorectal adenocarcinoma	-	Dukes' type C	Sensitive
Secondary cell line	HCT-8	Male	67	Colorectal adenocarcinoma	-	Dukes' type C	Sensitive
Secondary cell line	Colo320	Female	55	Colorectal adenocarcinoma	-	Dukes' type C	Resistant
Secondary cell line	Colo678	Male	69	Colorectal metastasis site	-	-	Resistant
Secondary cell line	Caco-2	Male	72	Colorectal adenocarcinoma	-	-	Resistant
Secondary cell line	Lovo	Male	56	Colorectal metastasis site	-	Dukes' type C	Sensitive

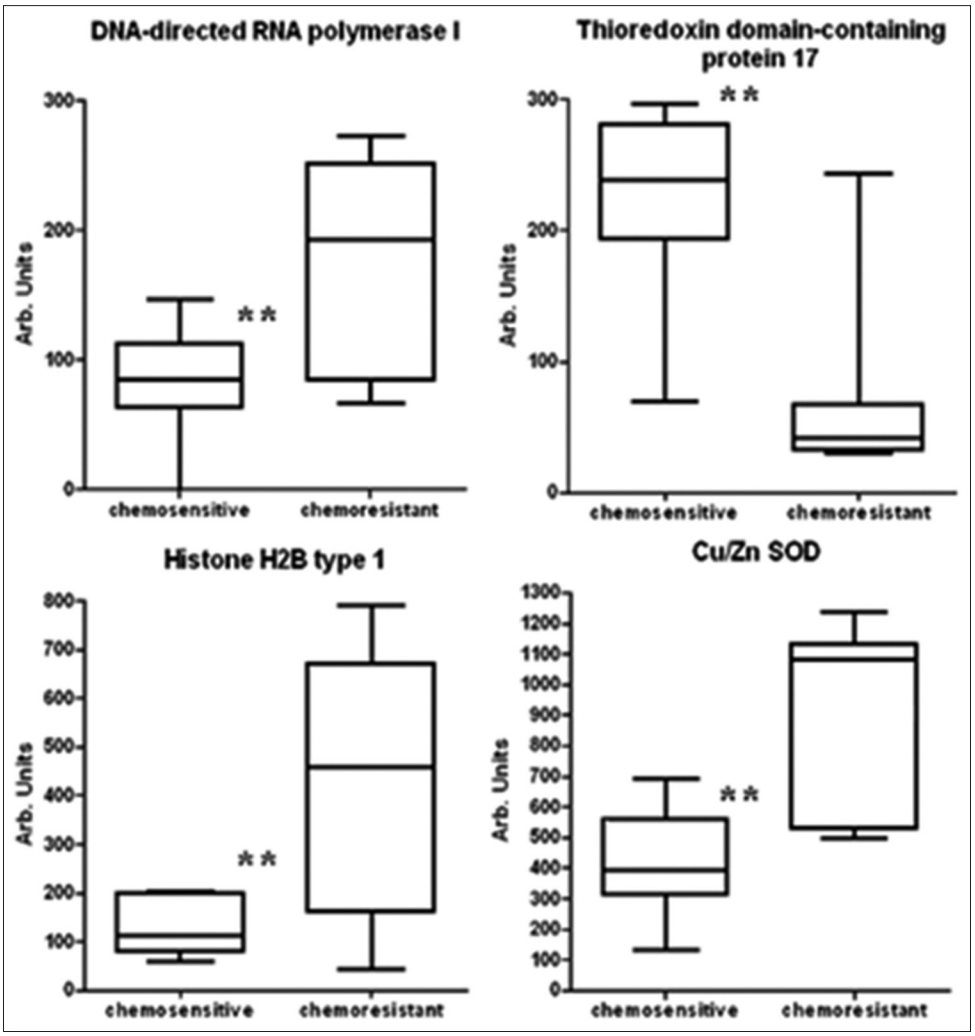

Figure 4: Expression differences of exemplary biomarker candidates between groups revealed by the LC-MALDI workflow. The biomarkers were identified by using an in gel digestion approach

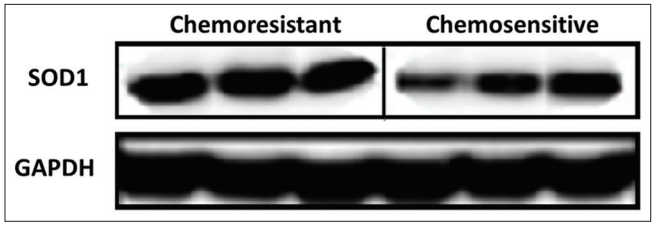

Figure 5: Western blotting results of three chemosensitive and three chemoresistant cell lines for basal Cu/Zn superoxide dismutase expression

tryptic digestion of proteins. Another feature of this workflow is the possibility to detect post translational modifications of the native protein, as well as cleaving products of proteins and degraded proteins. In terms of resolution and reproducibility the workflow seems to be robust. Furthermore, the comprehensive quantification of signal intensities and subsequent generation of non-redundant compound lists facilitates data analysis. In this pilot study, using a panel of colorectal cancer cell cultures, we were able to identify low molecular weight protein biomarker candidates that were already described in literature [11-13], supporting our findings in general, as well as undescribed candidates being even more interesting. Subsequently, we conducted an exemplary validation of one biomarker candidate to illustrate further possibilities for a validation process. Following a first technical validation of data from the discovery study we initially analyzed the biological background and molecular mechanisms of a biomarker candidate in order to prove his causative involvement in chemoresistance. Since changes in protein expression can just be responses to other causative changes, this is also important information in order to analyze the value of a biomarker as drug target.

The standard therapy in colorectal cancer the FOLFOX treatment consists of a combination of 5-FU/LV and Oxa, which are compounds with different modes of action [14]. 5-FU is an antimetabolite, which is mainly incorporated in the RNA but also in DNA; furthermore it has inhibitory effects on enzymes such as the thymidilate synthase [15]. Oxa is a third generation platinum compound. It shares similar mechanisms with other platinum compounds such as cisplatin and carboplatin, in causing mono-adducts and intra-strand or inter-strand cross-links in the double DNA helix that block DNA and mRNA

synthesis [16,17]. Platinum compounds are also known to generate reactive oxygen species during the crosslinking reaction that potentially induces single strand breaks [18]. In regard to these multifaceted effects, we expect a combination of biomarker candidates to be predictive for response to therapy, rather than a single biomarker. The here described regulation of the Cu/Zn SOD, which is known to be involved in detoxification processes may be a first parameter of a protein biomarker combination predicting intrinsic chemoresistance to FOLFOX combination therapy.

In a next step, expression status of a biomarker candidates or combinations should be analyzed in an independent set of clinical specimen. A main obstacle in this step is the classification of patients into responders and non-responder. Here, a comprehensive record of follow up data from patients and the guidance of e.g., RECIST or other criteria are recommended. Furthermore, the micro dissection of cancer cells from the heterogeneous microenvironment of a tumor is mandatory for the analysis of protein expression or cell signaling in cancer cell populations [19]. Further biomarker candidates from this study are in the validation process and an analysis of predictive strength of biomarker combinations will be next steps.

In summary, the newly established top down LC-MALDI discovery workflow is suitable for a comprehensive analysis of the low molecular proteome. This enables the generation of complex protein expression patterns and the discovery single or combinations of biomarkers predicting and explaining chemo resistance. This may potentially improve the understanding of chemoresistance and their individual mechanisms and causes.

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