The Use of Laser Microdissection and SELDI-TOF MS in Ovarian Cancer Tissue to Identify Protein Profiles

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Abstract. *Background: There is a strong need for prognostic biomarkers in ovarian cancer patients due to the heterogeneous responses on current treatment modalities. Materials and Methods: This study investigates the feasibility of combining laser microdissection (LMD) and surface enhanced laser desorption ionization-time of flight mass spectrometry (SELDI-TOF MS) in ovarian cancer tissue to obtain protein profiles. Results: Ideal conditions for preparing a protein lysate were determined and subsequently analysed on SELDI-TOF MS. Applying these protocols on tissue of 9 ovarian cancer patients showed different protein profiles between platinum sensitive and resistant patients. Conclusion: This shows that combining optimised protocols for LMD with SELDI-TOF MS can be used to obtain discriminatory protein profiles. However, studies with large patient numbers and validation sets are essential to identify reliable biomarkers using this approach.*

Despite recent advances in the understanding of molecular pathways and the introduction of targeted therapies, treatment of ovarian cancer patients remains a challenging task. Apart from the diagnostic challenge (most patients are diagnosed with advanced stage disease), prognostication remains difficult since non-predictable factors, such as stage, residual tumor load after primary surgery and platinum sensitivity, can highly influence the course of the disease.

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Most gene and protein studies trying to unravel the molecular events behind this disease used body fluids such as serum, plasma, ascites, urine or cell culture models as starting material (1, 2). Limitations for these studies are the presence of highly abundant proteins in these fluids or mediums masking the detection of low concentrated peptides or proteins, inter- and intra-individual differences due to *e.g.* hormonal influences and starvation and the need for prospectively and well controlled collected samples. Furthermore, cell culture models have their limitations since manipulation of cells can iatrogenically cause changes at the protein level (3).

Tumor tissue biopsies are an attractive alternative. However, biopsies of ovarian tumor tissue can consist of a mixture of tumor cells and non-tumor cells: oocyte containing follicles, stromal cells, blood vessels or infiltrating lymphocytes. As proteins associated with a specific type of cancer would be ideal for biomarker use or targeted therapy, it is important to work with a pure and homogeneous tumor cell population. Therefore, recent techniques such as laser microdissection can be used. Subsequent peptide and protein information can be gathered using mass spectrometric techniques.

This study aimed to combine the techniques of LMD and SELDI-TOF MS analysis on ovarian cancer tissue biopsies to obtain reliable protein patterns. Therefore, several experimental conditions that could possibly influence these patterns were tested and after defining the ideal settings a small study was performed comparing protein profiles of ovarian cancer patients resistant or sensitive to platinum based chemotherapy.

Materials and Methods

Ovarian cancer patients and tumor specimens. Tumor specimens were obtained from the historical tumor bank of the Department of Obstetrics and Gynecology, University Hospitals Leuven, Belgium.

These were obtained after written informed consent and ethical approval from the local ethical committee and were handled according to protocol. Samples were obtained during primary surgery, snap frozen in liquid nitrogen after prelevation (delay between prelevation and freezing is less than 30 minutes) and stored at –80˚C until further processing.

Medical records were reviewed for clinicopathological and follow-up data. Patients were identified as platinum resistant when the tumor recurred within 6 months after surgery followed by primary platinum based chemotherapy.

Determining optimal conditions for LMD of tissue samples and preparation of cell lysates. Cryostat sections (5 μm) were cut from frozen tissue samples on a Prosan cryostat at –20˚C and mounted on a glass slide. These were subsequently stained with haematoxylin and eosin and were used as a control and orientation for tumor cell localization. Additional serial sections were made for LMD (16 μm thickness) and mounted on nuclease and human nucleic acid free membrane slides (polyethylene terepthlate (PET) membrane, 1.4 μm, MMI), which were stained with haematoxylin only and air dried. LMD was performed using the CellCut plus system (Olympus – MMI, Hamburg, Germany). Areas of necrosis, lymphocytic infiltration and regions with psammoma bodies were avoided.

To determine the number of cells needed to obtain a reliable profile with SELDI-TOF MS several numbers of cells varying from 5,000 to 80,000 were tested.

In order to extract a maximum amount of proteins several lysis conditions were investigated. Chemical lysis of these cells was performed comparing 3 different lysis buffers: (i) U9 buffer (urea 9M, CHAPS 2%, tris-HCl 50 mM, pH 9; Bio-Rad, Nazareth, Belgium) +/– complete protease inhibitor (Roche, Vilvoorde, Belgium); (ii) N-octyl glucoside 0.1%, urea 9 M, EDTA 1 mM (except for IMAC30 arrays), EGTA 1 mM, sodium orthovanadate 5 mM, sodium fluoride 10 mM and (iii) Complete lysis-M kit (Roche, Vilvoorde, Belgium). Temperature during lysis was tested ranging from lysis on ice, 4˚C, room temperature to 70˚C and duration of lysis ranged from 60 to 120 min. Subsequently several volumes of lysis buffer, ranging from 35 to 200 μL were tested.

LMD of 9 ovarian cancer tissue samples and preparation of cell lysates. For this study approximately 30,000 cells were dissected in quadruplicate, each in 30-60 minutes with adjusted settings for cutting speed, focus and laser energy to obtain a clear cut. Dissected cells were lysed and proteins extracted using 50 μL U9 buffer per 30.000 cells for 60 min at 4˚C with shaking on a MicroMix 5 (DPC, UK). This mixture was then centrifuged for 5 min at 5,000 rpm and 4˚C, diluted in the appropriate binding buffer according to the used ProteinChip (0.1M sodium phosphate, 0.5 M sodium chlorate for IMAC30; 0.1M sodium acetate pH 4.0 for CM10; 10% acetonitrile (ACN), 0.1% tri-fluoroacetic acid (TFA) for H50 and Tris-HCl 10-100 mM, pH 7.5-9 for Q10; Bio-Rad, Nazareth, Belgium) and filtered through a Nanosep MF device (0.2 μm; PALL inc, Haasrode, Belgium) to remove gross debris. This lysate was collected and stored at –80˚C until MS analysis.

Protein profiling of laser microdissected cells with SELDI-TOF MS in 9 ovarian cancer patients. Protein lysates were analysed on copper-coated IMAC30 (immobilized metal affinity capture array), CM10 (weak cation exchanger), H50 (hydrophobic or reversed phase array) and Q10 (strong anion exchanger) arrays (Bio-Rad, Nazareth, Belgium). For the IMAC30 arrays, spots were pre-incubated twice with 50 μL of 0.1 M copper sulphate for 5 min at room temperature followed by a wash step with 0.1 M sodium acetate buffer pH 4 for 5 min at room temperature. For the H50 arrays spots were pre-washed twice with 50 μL of 50% ACN in ultrapure LC-MS grade water (Biosolve, Valkenswaard, The Netherlands) for 5 min at room temperature. Following these wash steps, and for CM10 and Q10 arrays immediately, spots were pre-incubated twice with array specific binding buffer followed by application of 100 μL of protein lysate and incubated for 60 min at 4˚C with shaking on a MicroMix 5. After three additional wash steps with the same binding buffer and two final washes with water, 2×1 μL of 20% α-cyano-4-hydroxy cinnamic acid (CHCA, Bio-Rad, Nazareth, Belgium) dissolved in 1% TFA/100% ACN were applied.

Mass analysis was performed using SELDI-TOF MS (PCS 4000 Enterprise, Ciphergen ProteinChip Reader Inc., Fremont, CA, USA) according to an automated data collection protocol for a molecular weight range of 0-20,000 Da. The following settings were used: (a) laser intensity of 3,500 nJ; (b) focus mass 10,000 Da; (c) matrix attenuation 500 Da; (d) sampling rate 400 MHz; (e) 2 warming shots (not included in analysis), 10 data shots per point and (f) total number of points evaluated equal to 12.5% of the spot surface. Mass accuracy was calibrated externally using the all-in-one peptide standard according to the manufacturer's protocol (Bio-Rad, Nazareth, Belgium). A quality control sample (pooled serum) was analyzed weekly to validate the output of the system.

Using the Ciphergen Express Software, baseline subtraction and noise reduction were completed before peak intensities were normalized to the total ion current. Outlier spectra were identified and removed from analysis when the normalisation factor deviated more than 2 standard deviations. Numeric data were exported to Excel files for further biostatistical processing.

Background correction was performed for each sample separately and peaks were identified on the average of all samples (independent of platinum sensitivity) including all peaks with an absolute value ≥5.

Results

Determining optimal conditions for LMD of tissue samples and preparation of cell lysates. (a) Number of cells. A protein profile could be obtained with 10,000 cells though improvement was observed when the amount of cells was increased to 30,000. Further increase in the amount of cells did not result in any additional peaks or improvement of the spectrum (Figure 1).

(b) Lysis conditions. Three different lysis buffers were used to extract proteins and the best results were seen with U9 buffer. The homemade lysis buffer and the complete lysis-M buffer could not be used with the protein chip arrays since only noise was detected. Furthermore, analysing laser microdissected cells lysed with buffer and complete protease inhibitor tablets showed several peaks in the peptide range possibly due to the protease inhibitors (Figure 2). Therefore, protease inhibitors were omitted in further experiments.

Subsequently, several temperature conditions were tested during lysis with U9 lysis buffer. When lysis was performed

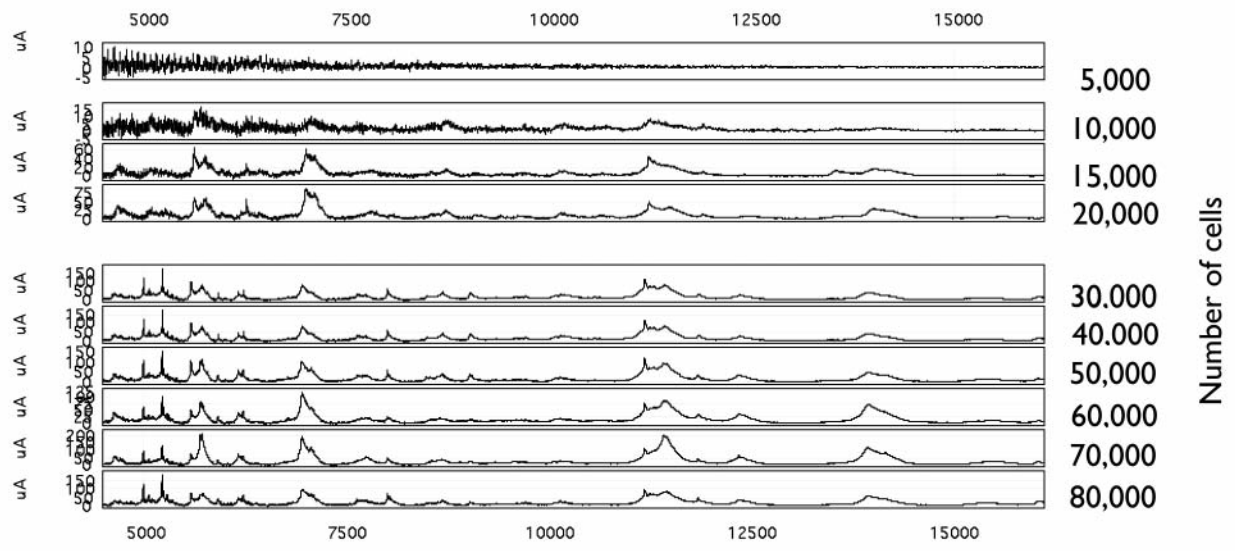

Figure 1. *Protein profile of laser microdissected ovarian cancer tumor cells with increasing amount of cells (5,000-80,000 cells) on IMAC30 array. X-axis shows the mass/charge (m/z) ratio and Y-axis the relative intensity (uA).*

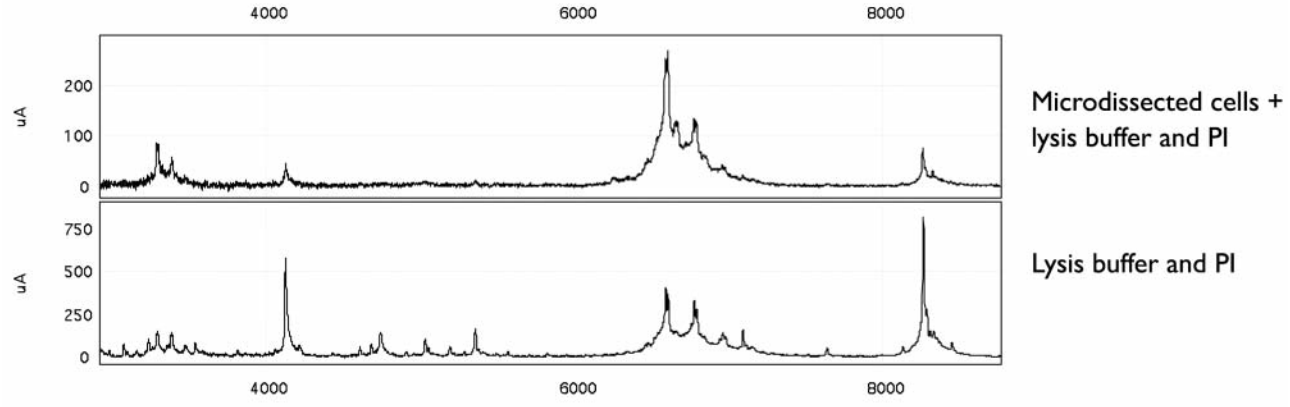

Figure 2. *Protein profile on a IMAC30 array of (a) laser microdissected cells lysed with buffer and complete protease inhibitor (PI) tablets (b) the lysis buffer and PI. X-axis shows the mass/charge (m/z) ratio and Y-axis the relative intensity (uA). Note that virtually all peaks in the upper profile are actually caused by the PI as similar peaks can be detected when the PI is spotted on the chips.*

on ice, crystallisation of urea occurred leading to inappropriate lysis and protein profiles. Lysis at 4˚C gave the best results in relation to intensity of the protein peak compared with room temperature and 70˚C (Figure 3). Prolonging the time of lysis at 4˚C from 60 min to 120 min did not improve detection of protein peaks nor did it deteriorate the profile (Figure 4). Varying the volume of lysis buffer added to the microdissected cells did not alter the protein profile substantially. However, because of the intensity and the signal to noise ratio of the peaks observed, it was concluded that a volume of 50 µL gave the best results (Figure 5).

In conclusion, the ideal conditions for preparing a protein lysate from laser microdissected ovarian cancer cells were determined to be a dissection of 30,000 cells subsequently lysed with the addition of 50 µL U9 lysis buffer at 4˚C over 60 min.

Protein profiling of 9 ovarian cancer patients. (a) Tumor tissue biopsies. Nine patients were identified with a history of ovarian cancer of which 5 were platinum resistant and 4 were platinum sensitive. Patient characteristics are given in Table I.

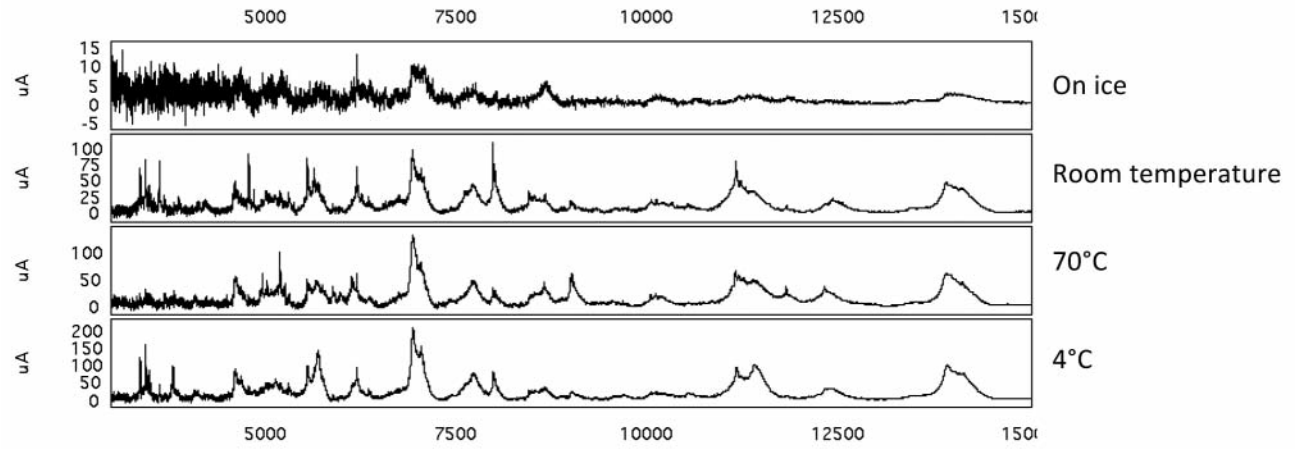

Figure 3. *Protein profiles on a IMAC30 array of laser microdissected ovarian tumor cells with several temperature conditions during lysis with U9 buffer. X-axis shows the mass/charge (m/z) ratio and Y-axis the relative intensity (uA).*

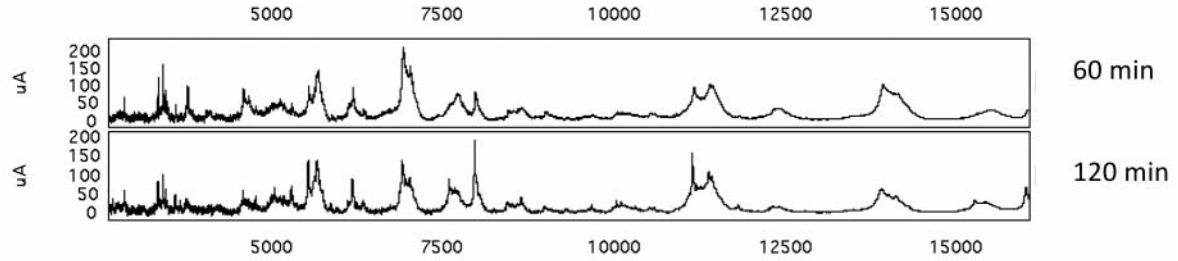

Figure 4. *Protein profiles on a IMAC30 array of laser microdissected ovarian tumor cells after lysis with U9 lysis buffer during 60 or 120 min. X-axis shows the mass/charge (m/z) ratio and Y-axis the relative intensity (uA).*

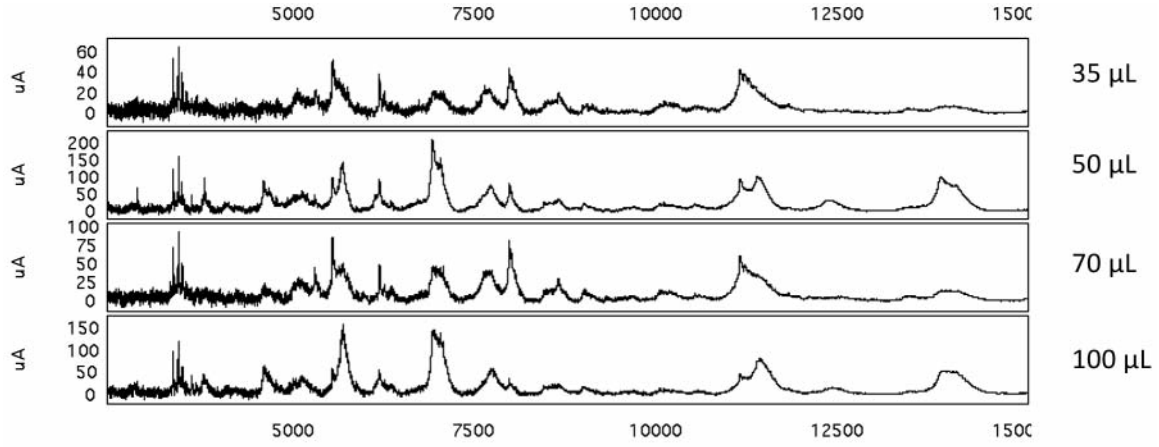

Figure 5. *Protein profiles on a IMAC30 array of laser microdissected ovarian tumor cells after lysis with different volumes of U9 lysis buffer. X-axis shows the mass/charge (m/z) ratio and Y-axis the relative intensity.*

(b) Protein expression and cluster analysis of differentially expressed proteins in platinum sensitive and resistant ovarian cancer tissue. Protein profiles could be obtained on the 4 different arrays used. In comparison to IMAC30, CM10 and H50 arrays, no additional peaks were found on Q10 arrays and therefore this array was not used for further analysis. The average spectrum of the platinum sensitive and resistant samples on IMAC30, CM10 and H50 arrays is shown in Figure 6.

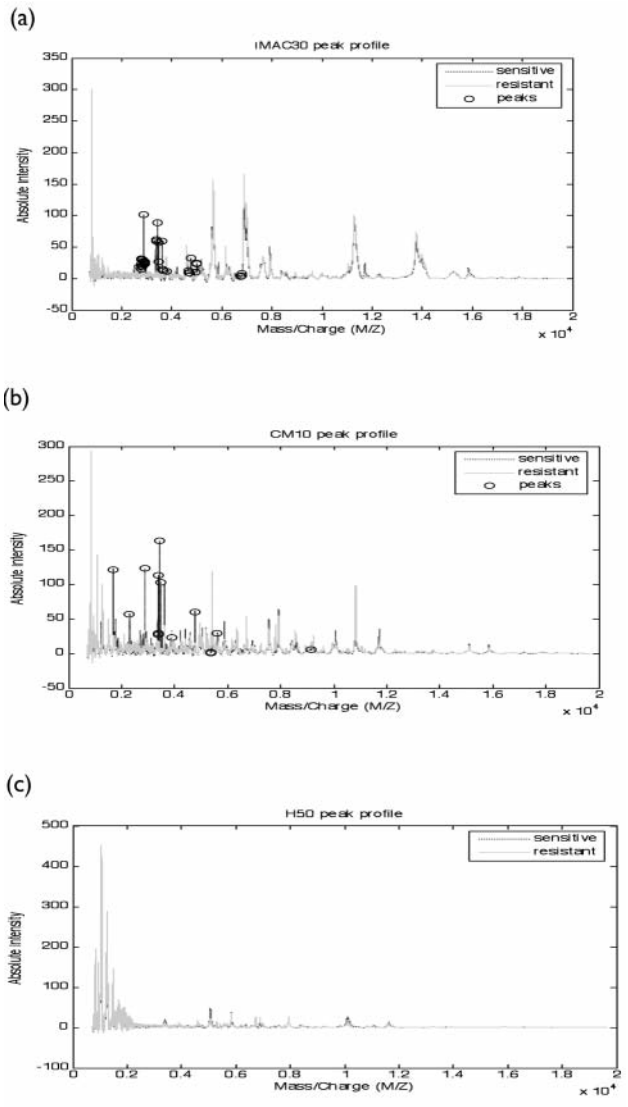

Figure 6. *Average spectrum of the platinum sensitive (blue) and resistant (red) samples on (a) IMAC30, (b) CM10 and (c) H50 arrays. X-axis shows the mass/charge (m/z) ratio and Y-axis the relative intensity.*

Table I. *Clinical and pathological characteristics of ovarian cancer patients (n=9).*

	Platinum sensitive (n=4)	Platinum resistance (n=5)
Median Age, years (range)	77 (75-79)	55.8 (39-73)
Residual tumor load after debulking surgery		
R0	4	4
>2 cm	0	1
FIGO Stage		
IIIc	4	4
IV	0	1
Histology		
Serous papillary	4	4
Mixed (serous papillary – clear cell)	0	1
Tumor grade		
Moderate – Poor	2	1
Poor	2	4
Chemotherapy scheme		
6 x (paclitaxel – carboplatin)	4	2
4 x (topotecan – cisplatin) + 4 x (paclitaxel – carboplatin)	0	3
Median PFI, months (range)	14 (9-31)	2.5 (0-6)

PFI: progression-free interval.

On the IMAC30 array, 1053 peaks could be detected on the average profile (independent of platinum sensitivity) of which 297 were differentially expressed between the platinum sensitive and resistant group. On CM10 arrays, 1023 peaks were detected of which 314 were differentially expressed and on H50 arrays, no differentially expressed peaks between the two study groups could be found.

Discussion

The combination of LMD and SELDI-TOF MS analysis has been used in several cancer studies (4-8). However, to the best of the authors' knowledge this is the first study focusing on the methods and sample preparation for the combination of these techniques. Manipulation of tissue causes activation of proteases with subsequent degradation of peptides and proteins making short handling times an absolute necessity. On the other hand, crude tissue biopsies consist of all kinds of cells which are not all evenly contributing in the tumor process leading to discovery of false biomarkers which are more related to general inflammation than to tumor activity. Although increasing evidence exists that the surrounding stroma is important in the growth and invasion of ovarian cancer, tumor specific proteins which are of interest for biomarker discovery and targeted therapy, are more likely to be encountered in tumor cells. This has lead to adaptations in the isolation procedures for tumor cells causing possible variability. To minimise this, proteomics studies require the use of strict protocols from sample collection onwards. As a study of Timms *et al*. (9) proved for serum samples, it is very important to collect samples under the same experimental conditions to obtain reliable and comparable results. This observation can also be extrapolated to the collection of tissue samples stressing the need for a strict protocol known to the whole team of the theatre and laboratory.

LMD can be used to identify cells of interest and obtain a homogeneous tumor cell population for further analysis. Several studies showed the feasibility of this technique in cancer research without any negative effect on protein profiles. Recent developments in these instruments have

facilitated handling, and with some practice, dissections of large amount of cells can be performed within acceptable time limits for further protein or even RNA analysis. Previously, concerns were expressed about the use of staining methods influencing protein profiles. Several staining methods have been tested in our own group (data not shown) and the results confirmed current opinions that single haematoxylin staining is preferred regarding minimal loss of protein peaks without any deleterious effect on the protein profiles irrespective of the length of staining (10-13).

Nevertheless, some questions regarding processing of these laser microdissected cells remained unclear for which some experimental conditions were tested in this study. First, the optimal number of laser microdissected cells needed to obtain a reliable protein profile on SELDI-TOF MS was determined and subsequently different lysis protocols were followed to extract a maximum amount of proteins. The use of complete protease inhibitor tablets is commonly used in proteomic studies. This routine was not performed as some of these protease inhibiting peptides can mask peptides of interest in the study sample and compete with binding to the SELDI-surface. The volume of lysis buffer added to the sample, temperature during lysis and time of lysis did not alter the protein profiles dramatically in relation to the amount of peaks detected though some influences on the amplitude of the intensity and the signal to noise ratio were observed. This confirms the need to follow strict protocols to obtain comparable profiles between different study samples. The ideal conditions for preparing a protein lysate from laser microdissected ovarian cancer cells were determined as a dissection of 30,000 cells subsequently lysed with the addition of 50 μL U9 lysis buffer at 4˚C over 60 min.

When applying these determined settings on a small study sample of 9 ovarian cancer patients, it was possible to distinguish differentially expressed proteins between platinum sensitive and resistant patients. Seventy five percent of ovarian cancer patients are diagnosed with advanced stage disease necessitating extensive debulking surgery followed by platinum containing chemotherapy. Despite this, 25% of these patients will relapse within 6 months after primary therapy (14). If these platinum resistant patients could be identified at the time of primary diagnosis, current therapy could be tailored according to the tumor biology. Furthermore, this could give new insights into the pathways of platinum resistance improving efficacy of further research.

Hitherto, only studies on ovarian cell culture models were able to identify platinum resistant associated proteins (15-19) of which the up- or down regulation was responsible for (a) an accelerated detoxification of drug substrates, (b) inhibition of apoptotic cell death through *e.g.* modulation of the actin cytoskeleton, or (c) inhibiting pathways leading to a decreased basal metabolism of energy and glucide which helps cells to live through the duration of drug therapy. These findings still need to be validated in *in vivo* studies with large patient numbers.

In conclusion, optimal settings were identified for combining LMD and SELDI-TOF MS to study protein expression profiles in ovarian cancer tissue and these protocols were applied to tumor tissue of 9 ovarian cancer patients. The results showed differentially expressed proteins between platinum resistant and sensitive ovarian cancer on IMAC30 and CM10 arrays.

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