

# Undifferentiated pelvic adenocarcinomas: diagnostic potential of protein profiling and multivariate analysis

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## Abstract

**Background and aims** Despite improved techniques, the determination of tumor origin in poorly differentiated adenocarcinomas still remains a challenge for the pathologist. Here we report the use of protein profiling combined with principal component analysis to improve diagnostic

decision-making in tumor samples, in which standard pathologic investigations cannot present reliable results.

**Materials and methods** A poorly differentiated adenocarcinoma of unknown origin located in the pelvis, infiltrating the sigmoid colon as well as the ovary, served as a model to evaluate our proteomic approach. Firstly, we characterized the protein expression profiles from eight advanced colon and seven ovarian adenocarcinomas using two-dimensional gel electrophoresis (2-DE). Qualitative and quantitative patterns were recorded and compared to the tumor of unknown origin. Based on these protein profiles, match sets from the different tumors were created. Finally, a multivariate principal component analysis was applied to the entire 2-DE data to disclose differences in protein patterns between the different tumors.

**Results** Over 89% of the unknown tumor sample spots could be matched with the colon standard gel, whereas only 63% of the spots could be matched with the ovarian standard. In addition, principal component analysis impressively displayed the clustering of the unknown case within the colon cancer samples, whereas this case did not cluster at all within the group of ovarian adenocarcinomas.

**Conclusion** These results show that 2-DE protein expression profiling combined with principal component analysis is a sensitive method for diagnosing undifferentiated adenocarcinomas of unknown origin. The described approach can contribute greatly to diagnostic decision-making and, with further technical improvements and a higher throughput, become a powerful tool in the armamentarium of the pathologist.

UJ Roblick and FG Bader contributed equally to this work and should be recognized as first authors.

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## Introduction

In patients with malignant tumors, physical and radiographic findings combined with histopathological features and ultrastructural characteristics can designate the primary site of the neoplasm in most instances. However, the distinction of poorly differentiated pelvic adenocarcinomas originating from the ovaries or the colorectum still remains a challenge for the pathologist. At an advanced stage, a poor degree of tumor differentiation and a lack of organ-specific biological markers often make an accurate differentiation of such cases impossible. This can lead to a therapeutic dilemma, since improper assessment of neoplasias and false tumor classification prohibit an appropriate choice of treatment. Creating entity-specific expression profile data bases along with data analysis techniques may be a powerful strategy to address such diagnostic difficulties [3].

Two-dimensional gel electrophoresis-based protein analysis focuses on expression profiles at the protein level and represents an efficient approach in separating thousands of different polypeptides at a time. It also enables the investigator to obtain quantitative expression patterns that are characteristic for different tumor entities. Multivariate analysis approaches have previously been reported to make a distinction between entities and histological subtypes possible, if sufficiently large datasets are available [4].

## Materials and methods

### 2-DE tumor bank and sample preparation

Over the last years, an extensive number of tumor samples (including prostate, ovary, breast, lung, and colorectal tissue) of different stages have been collected and analyzed prospectively [1, 2, 5, 13, 16, 33]. All specimens were obtained directly from the operating room after surgical resection. Representative samples were then selected in collaboration with a pathologist. Cells were obtained and enriched by scraping the cut surface of the tumor, followed by tumor cell collections in 2–5 mL of ice-cold RPMI-1640 medium containing 5% fetal calf serum and 0.2 mM phenylmethylsulphonyl fluoride/0.83 mM benzamidine. Using a syringe, the cells were dislodged mechanically. Tissue fragments and connective tissue were removed using a two-phase nylon mesh filter system (250 and 160  $\mu\text{m}$  pore size). The cell suspensions were underlaid with 1–2 mL of ice-cold Percoll (54.7% in phosphate-buffered saline (PBS)) and centrifuged at 1,000 $\times g$  for 10 min at a temperature of 4°C. The cell interface was collected and washed twice with PBS. The obtained tumor cells were stored at  $-80^{\circ}\text{C}$  and further extracted and solubilized as described by Franzen et

al. in 1995 [15]. The representativity of each sample was assessed by a pathologist by comparing the routine histology (H & E section) with a Giemsa-stained smear of the filtrated cells. Only those samples that contained more than 90% tumor cells per smear after enrichment were considered for 2-DE evaluation.

### Protein quantification

Protein concentrations of samples were determined by the addition of 25 mL concentrated assay reagent (Bio-Rad) to 1 mL solubilized sample diluted in 100 mL Milli-Q water using 96-well microplates [15]. A standard curve was constructed using different concentrations of bovine serum albumin. The plate was read using a Multiscan reader (Labsystems).

Total protein concentration was determined by Bradford protein assay according to the manufacturer's protocol. Bovine serum albumin was used as a standard [11].

### Electrophoresis

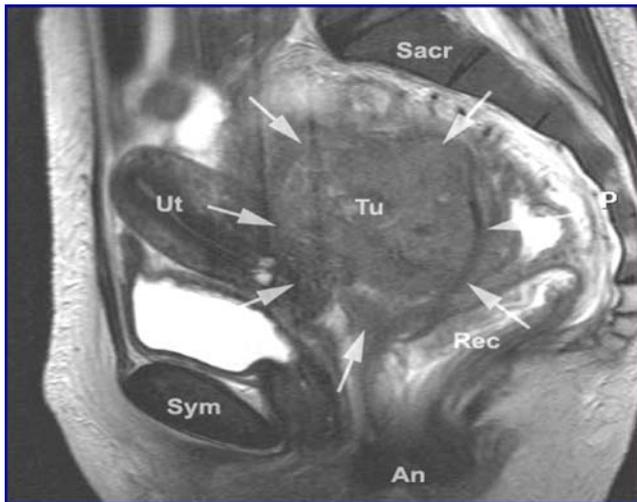
Before application, all 15 samples were diluted to a total volume of 500  $\mu\text{m}$  containing 7 M urea, 2 M thiourea, 1% CHAPS, 0.4% IPG buffer, 0.3% DTT, and a trace of bromphenol blue [6]. The cell extracts were applied on precast immobilized pH-gradient strips (IPG pH 4–7, linear, 17 cm; Bio-Rad) and run for approximately 45,500 Vh using the PROTEAN IEF Cell (Bio-Rad). After IEF separation, the strips were subsequently equilibrated  $2 \times 15$  min in 50 mM Tris-HCl, at pH 8.8 in 6 M urea, 30% glycerol, and 2% SDS. DTT (1%) was included in the first equilibration step and 2.5% iodoacetamide in the second step. SDS-PAGE (10–13% linear gradient) was used for the second dimension.

### Staining methods

After 2-DE separation, we used silver staining for spot visualization prior to match set analysis. The silver staining protocol has been described by Rabilloud et al. [31]. For spot identification by MS, we used Sypro Ruby according to the manufacturer's protocols.

### Colon and ovarian tumor databases

Since the pelvic tumor of unknown origin was classified as a T4 stage malignancy, we extracted cells only from advanced (T3 and T4) adenocarcinomas (colon  $n=8$ ; ovary  $n=7$ ). All samples were prepared and subjected to 2-DE analysis following standard procedures as described earlier [1, 15].



**Fig. 1** MRI scan of a huge pelvic tumor mass of unclear origin (arrows), including the recto-sigmoidal junction as well as the left ovary. *Tu* tumor, *Sacr* sacrum, *Ut* uterus, *Sym* symphysis, *Rec* rectum, *An* anus

### Pelvic tumor of unknown origin

Directly after resection, we received a sample of a large pelvic tumor attached to the ventral side of the rectosigmoid junction, which could not be distinguished from the left ovary (Fig. 1). An intraoperative frozen section of the tumor mass was classified as a poorly differentiated adenocarcinoma of unknown origin. The carcinoembryonic antigen (CEA) serum level of 3.5 U/mL was within the physiological range (<5 U/mL), whereas the level of the cancer-associated antigen 125 (CA 125) was slightly increased (54.0 U/mL; normal range <35 U/mL). Routine histopathological investigations led to the diagnosis “adenocarcinoma of unknown origin”. Immunohistochemical cytokeratin (CK) 7 and 20 staining pointed in the direction of a colonic malignancy (CK 20+; CK 7–). To differentiate the divergent serological and immunohistochemical results, the sample was considered for 2-DE evaluation. Thus, sample preparation, handling, and storage were performed as described above for the ovarian and colon cancer samples.

### Image analysis

Silver-stained 2-DE gels were scanned at 105  $\mu$ m resolution (12 bits/pixel) using a GS 710-calibrated imaging densitometer (Bio-Rad). Data were analyzed using PC-based PDQuest™ software (Bio-Rad version 8.0) [20]. Two match sets, a colon match set (eight colon gels and unknown tumor gel) and an ovarian match set (seven ovarian gels and unknown tumor gel) were created. The analysis included protein spot detection, background sub-

traction and quantification. Individual polypeptides were quantified as parts per million of the total integrated optical density. Each spot sustained an individual identification number. Gel comparison was performed using the semi-automated gel to gel algorithm provided by the PDQuest software. Matching results were controlled manually for each spot in order to avoid and correct automated matching errors. Individual quantifications of resolved proteins were normalized according to the total intensity of valid spots. All gels were evaluated for degree of similarity including qualitative and quantitative data from the separated proteins.

### Data preprocessing and principal analysis

Protein expression data of both match sets were evaluated by principal component analysis (PCA). The data was exported from the PDQuest™ software into a Microsoft Excel datasheet. The PC software SIMCA- P (version 8.0, Umetri, Umea, Sweden) was used for PCA analysis. PCA reduces the dimensionality of the datasets and extracts the obtained information into principal components (PCs). Only the first three PCs were used in the analysis of our match sets since they capture most of the variation of the datasets. The last few PCs are generally assumed to capture only the “noise” in the data and therefore they were not evaluated. The data was centered based on PCs 1–3. PCA rotates the data in such a way that the highest linear variation is described by the first component axis (t1), the residual variation by the second component axis (t2), and so on.

### In-gel digestion and CD technology for preparation of tryptic digests

Protein spots were excised manually from the gels and in-gel digested [28] using a MassPREP robotic protein-handling system (Micromass). Gel pieces were destained twice with 100 mL 50 mM ammonium bicarbonate (Ambic)/50% (v/v) acetonitrile at 40°C for 10 min. Pieces containing protein were reduced by 10 mM DTT in 100 mM Ambic for 30 min, shrunk in acetonitrile, and the proteins were then alkylated with 55 mM iodoacetamide in 100 mM Ambic for 20 min. Trypsin (25 mL of a 12 ng/mL solution in 50 mM Ambic) was added and incubation was carried out for 4.5 h at 40°C. Peptides were extracted with 30 mL 5% formic acid/2% acetonitrile followed by extraction with 24 mL 2.5% formic acid/50% acetonitrile. The acetonitrile was evaporated under atmospheric pressure overnight at 10°C. For electrospray (ES) ionization MS/MS, the peptide extracts were desalted with C18 ZipTips (Millipore), activated and equilibrated using

10 mL 70% acetonitrile/0.1% trifluoroacetic acid (TFA) twice, 10 mL 50% acetonitrile/0.1% TFA twice, and finally 10 mL 0.1% TFA twice. The sample was loaded onto the ZipTip by pipetting 20 times and washed using 10 mL 0.1% TFA twice. The tryptic fragments were eluted with 60% acetonitrile/1% acetic acid. Samples with proteins in low yield were analyzed using a Gyrolab MALDI SP1 Workstation (Gyros AB). In this approach, 96 micro-columns (packed with a C18 resin to a volume of 10 nL) were incorporated into a CD platform and used for desalting by reverse-phase chromatography. The columns were conditioned with 50% acetonitrile in water. The samples were loaded onto the columns, and solvents passed through by the spin of the disc. The wash solution (200 nL 0.1% TFA) was directed to a waste exit. Peptides were eluted from the columns using 200 nL 50% acetonitrile containing 1 mg/mL *a*-cyano-4-hydroxycinnamic acid matrix and 0.1% TFA. The eluate was captured in an open matrix-assisted laser desorption ionization (MALDI) target area of 200 mm×400 mm for solvent evaporation and the peptide/matrix crystallization. For on-CD MALDI analysis, the cut CD was accommodated in the target compartment of the MALDI instrument.

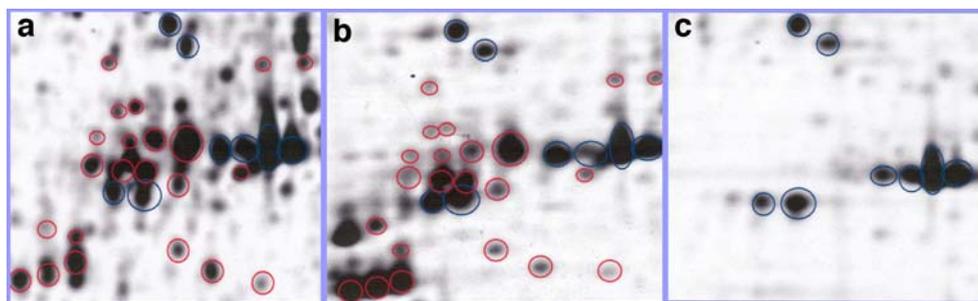
#### Mass spectrometry

The tryptic fragments were mass analyzed by MALDI mass spectrometry (Voyager DE-PRO; Applied Biosystems) and where relevant, ES ionization quadrupole time-of-flight (Q-TOF) tandem MS (Micromass) for sequence information. Samples for MALDI analysis were mixed at a 1:1 (v/v) ratio with a saturated *a*-cyano-4-hydroxycinnamic acid solution in 50% acetonitrile/0.1% TFA. Database searches were carried out using the MS-Fit search program (<http://prospector.ucsf.edu>). Only protein hits with three or more matching peptide masses were considered.

## Results

### 2-DE evaluation

Figure 2 shows small areas of silver-stained 2-DE polyacrylamide gels of a representative colon and ovarian cancer case as well as from the unknown tumor sample. The gels showed an average number of 1,178 separated polypeptide spots (range 986–1,253). It was already apparent during visual inspection that the protein expression pattern of the unknown tumor sample and the colon adenocarcinomas looked fairly similar. In contrast, the ovarian cancers showed obvious differences in protein expression: There were more quantitative similarities between the unknown tumor sample and the colon samples in the database than to the ovarian cancer samples. Overall, 89% (1,073 of 1,206) of the unknown sample spots were matched with the colon standard gel, whereas only 63% (760 of 1,206) of the spots could be matched with the ovarian standard. The degree of similarity was also assessed by correlation coefficient analysis (*r*-value calculation). This analysis was based on the polypeptide quantities (optical densities of matched spots). Analysis of the correlation within the eight colon cancer samples showed an average correlation of 0.71 (0.59–0.78), representing the intertumoral variations. A comparison of all the ovarian samples led to an average *r* value of 0.64 (0.52–0.73). A comparison of both match-set standards (colon vs. ovary) in a higher level match set resulted in a correlation coefficient of *r*=0.41, reflecting the tumor-entity-related differences in polypeptide expression. Correlation of the protein expression of the unknown tumor sample in each match set yielded an average coefficient of 0.70 (0.58–0.73) within the colon match set. In contrast, the *r* value was only 0.36 (0.30–0.41) when the unknown sample was compared with the ovarian cancer samples.



**Fig. 2** Corresponding close-up gel segments representing colon cancer (a), tumor of unknown origin (b), and ovarian cancer (c). A comparison of 2-DE patterns shows qualitative and quantitative differences between the pelvic tumor and the ovarian adenocarcinomas,

whereas a high degree of similarity is seen when compared with the colonic adenocarcinomas. *Blue encirclement*: spots detected in all members; *red encirclement*: spots unique in the tumor of unknown origin; and the colon cancer

Principal component analysis of colon vs. unknown tumor and ovary vs. unknown tumor

PCA was applied separately to the entire data of the two match sets, each including the tumor sample of unknown origin. Normalized spot data were imported from an Excel datasheet into the Simca software including the entire density data of about 1,200 spots/gel (about 10,800 and 9,600 respective density values per plot). When using the first two PCs (t1 against t2), a clustering of the eight colon samples and the pelvic tumor of unknown origin could be clearly shown (Fig. 3a). In contrast, visualization of the two first components of the ovarian match set showed the unknown sample as a clear outlier, whereas the seven ovarian adenocarcinomas clustered together (Fig. 3b).

Principal component analysis of unknown tumor vs. colon subentities

In a secondary analytical step, we applied a PCA to the entire database of colorectal subentities comprising of normal mucosa, adenomas, and carcinomas of different stages and metastases to prove whether or not the unknown tumor sample would cluster with the other primary colon cancers. Using spotfire statistical analysis, the relationship of the four colonic histological subtypes to the unknown sample could be visualized in a three-dimensional PCA plot

(Fig. 4) The PCA results showed a close clustering of the unknown pelvic tumor to the colon cancer cohort.

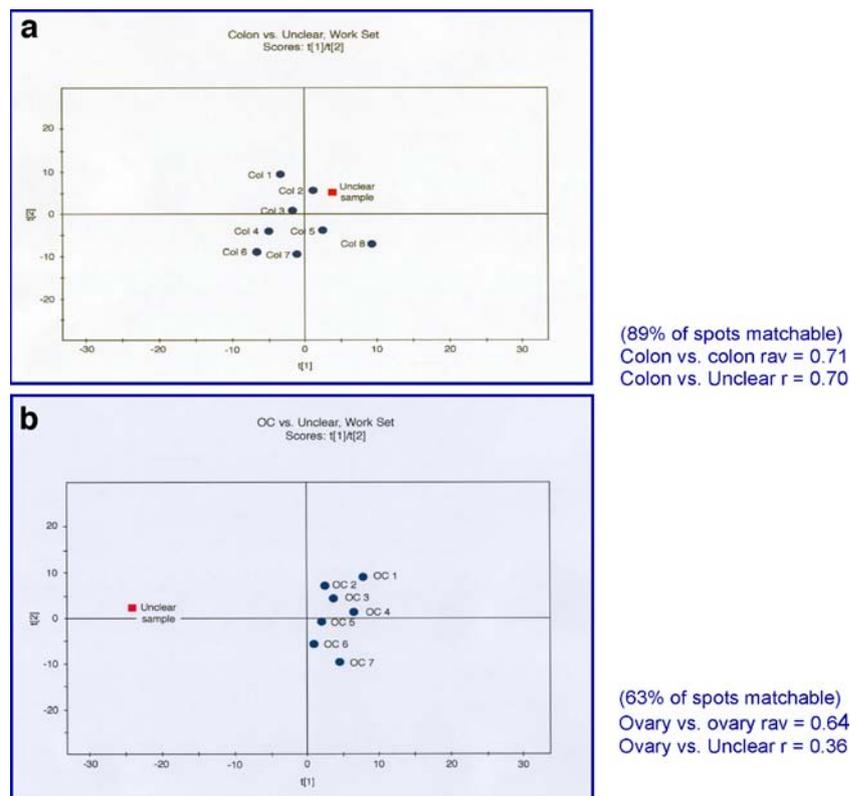
Protein identification

Based on the match-set data and the deviated protein expression intensities we identified, ten polypeptides that were at least 1.5-fold differentially expressed showed statistically significant differences in their relative parts per million value (Mann–Whitney test,  $p < 0.05$ ). As controls we used five equally expressed spots throughout all match-set members. Proteins upregulated in the colon cancer and the tumor of unknown origin remained unchanged in ovarian cancer. Furthermore, proteins upregulated in ovarian cancer remained unchanged in colon cancer as well as the unknown tumor sample. Thirdly, proteins both upregulated in all samples compared to their correlating normal tissue. Data are shown in Table 1.

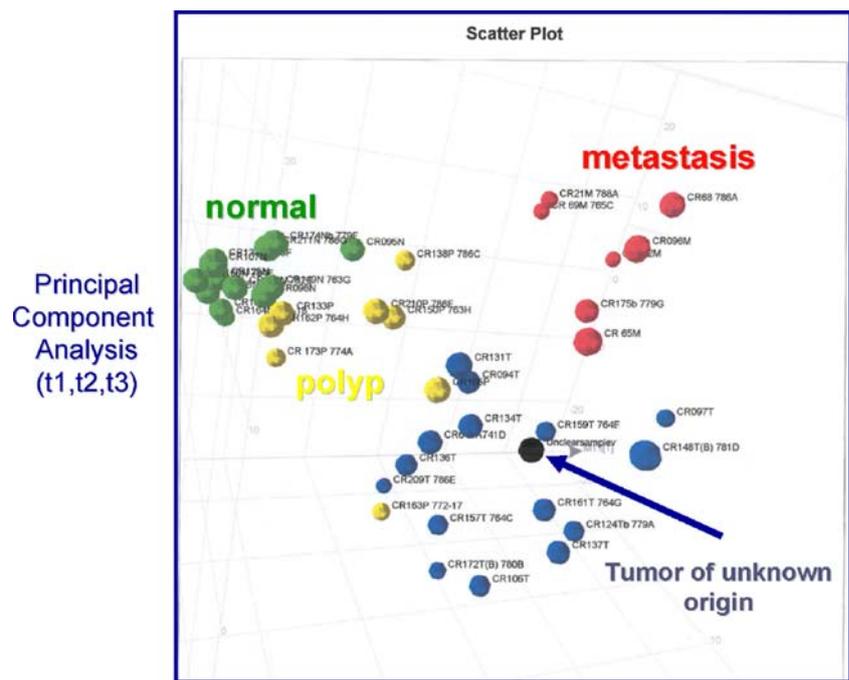
## Discussion

Advanced and undifferentiated malignancies are often onerous to distinguish for the pathologist, especially when the anatomical region is of close relation as it is in colorectal and ovarian cancer [21, 30]. Before an appropriate radio- and/or chemotherapy can be planned, malignan-

**Fig. 3** **a** PCA of the match-set “colon–tumor of unknown origin” data. The clustering of the unclear tumor sample within the eight colonic adenocarcinomas shows the similarity of these cases. *Blue circles*: colonic adenocarcinomas (Col 1–8). *Red square*: tumor of unknown origin (*unclear sample*). **b** PCA of the match-set “ovary–tumor of unknown origin” data demonstrating the unrelatedness of the unclear tumor sample to the ovarian malignancies. The first two components are plotted. *Blue circles*: ovarian adenocarcinomas (OC 1–7). *Red square*: tumor of unknown origin (*unclear sample*)



**Fig. 4** PCA plot of the protein expression database for colorectal tumors, metastases, polyps, and normal mucosa showing the distribution of the subentities in the tumor of unknown origin (arrow) in a three-dimensional space. Clusters within all other tumor samples demonstrating the close relatedness and thus its colorectal origin



cies have to be accurately classified. In this context, misdiagnosis may lead to delayed identification and misguided clinical interventions. CEA was proposed as a marker to distinguish between ovarian carcinoma and colon cancer lesions. Unfortunately, it can be found to be positive in both tumor entities [22–24]. Routine histopathology, even combined with additional immunohistochemical procedures, is occasionally of limited value in distinguishing

between poorly differentiated tumors, for example, of ovarian and colorectal origin. The immunoreactivity of different cytokeratins has been described as being relatively organ specific. Normally, colon cancer expresses a CK 20-positive and CK 7-negative pattern compared to ovarian malignancies that are mainly CK 7 positive and CK 20 negative [12, 34]. Thus, CK 7 and 20 antibodies are often used in attempts to determine the nature of doubtful pelvic

**Table 1** Identified proteins using MALDI-TOF and Swiss Prot/ExPASy database

| Protein identity                              | Regulation pattern |               |                | Accession number        |
|-----------------------------------------------|--------------------|---------------|----------------|-------------------------|
|                                               | Colon cancer       | Unknown tumor | Ovarian cancer |                         |
| <i>PCNA</i>                                   | ↑                  | ↑             | ↑              | <i>NP_872590</i>        |
| <i>NM-23 H1</i>                               | ↑                  | ↑             | ↑              | <i>P15531</i>           |
| <i>Elongation factor 1</i>                    | ↑                  | ↑             | ↑              | <i>P29692</i>           |
| <i>Proliferation-associated protein 2G4</i>   | ↑                  | ↑             | ↑              | <i>Q9UQ80</i>           |
| <i>HSP 27</i>                                 | ↑                  | ↑             | ↑              | <i>P04792</i>           |
| <b><i>Oncoprotein 18</i></b>                  | ↔                  | ↔             | ↑              | <b><i>NP_981946</i></b> |
| <b><i>Glutathione-S-transferase π</i></b>     | ↔                  | ↔             | ↑              | <b><i>AAH10915</i></b>  |
| <b><i>Triose-phosphate isomerase</i></b>      | ↔                  | ↔             | ↑              | <b><i>NP_000356</i></b> |
| <b><i>Calreticulin</i></b>                    | ↔                  | ↔             | ↑              | <b><i>AAB51176</i></b>  |
| <b><i>Tropomyosin 5</i></b>                   | ↔                  | ↔             | ↑              | <b><i>P06753</i></b>    |
| <b><i>3,2trans-enoyl-CoA isomerase</i></b>    | ↑                  | ↑             | ↔              | <b><i>P42126</i></b>    |
| <b><i>Succinate dehydrogenase</i></b>         | ↑                  | ↑             | ↔              | <b><i>P31040</i></b>    |
| <b><i>5 lipoxygenase</i></b>                  | ↑                  | ↑             | ↔              | <b><i>P09917</i></b>    |
| <b><i>Keratin, type I cytoskeletal 20</i></b> | ↑                  | ↑             | ↔              | <b><i>P35900</i></b>    |
| <b><i>Prohibitin</i></b>                      | ↑                  | ↑             | ↔              | <b><i>P35232</i></b>    |

Proteins upregulated in both the colon and the ovarian cancer samples are in *italics* and can therefore be described as “markers of malignancy”. Proteins differentially expressed in the unknown cancer sample and the ovarian cancer sample are in ***bold italics***. Proteins equally expressed in the colon cancer sample and the tumor of unknown origin are in ***bold***.

adenocarcinomas [35]. However, positive CK 7 expression is occasionally observed also in colorectal carcinomas [24]. In addition, rectal tumors show CK 7 reactivity in up to 53% and both CK 7 and CK 20 positivity have been reported in 71% of all cases [7]. This is of great importance since the close vicinity of the ovaries and the rectosigmoid junction may lead to overgrowth in both directions. Furthermore, mucinous ovarian adenocarcinomas have been shown to express CEA, CK 7, CK 20, and CA 125 which frequently makes a precise distinction from colonic adenocarcinomas impossible [8, 12, 24]. Therefore, in pelvic tumors of unknown origin, CK 7 and 20 immunohistochemistry has to be interpreted with caution [7].

We present a proteomic approach as an objective method to differentiate samples with diagnostic difficulties based on their characteristic protein profiles. Proteomic techniques have been used to study neoplasms of different organs, such as the prostate, bladder, kidney, breast, lung, stomach, ovary, and colon [1, 5, 9, 10, 13, 14, 16–18, 25–27, 29, 32]. Bloom and colleagues described an approximation of a tumor classifier entirely based on protein profiles using a 2-DE approach. Over 70 samples of six different but histomorphological similar appearing adenocarcinomas could be distinguished with an average predictive accuracy of over 82%. In this study, a discriminating set of proteins were identified and used to train an artificial neural network (ANN) [10].

A multistep approach based on cell lines was undertaken by Nishizuka et al. Among different genomic techniques, they used reversed-phase protein microarray platforms to quantify the protein expression of ovarian and colorectal cancers. They were able to identify vilin as a potential marker for colon and moesin as a potential marker for ovarian cancer [25].

The clear need for additional proteomics-based biomarkers not only for ovarian and colorectal cancers was addressed by Frohlich and coworkers. They identified the disintegrin and metalloproteinase ADAM12 as a stage- and grade-specific biomarker for bladder cancer. The presence and relative amount of ADAM12 in the urine of cancer patients were determined by Western blotting and densitometric measurements. ADAM12 mRNA expression was significantly upregulated in bladder cancer, as determined by microarray analysis, and the level of ADAM12 mRNA correlated with disease stage [19].

Apart from screening for disease-specific markers, the major potential of the approach presented is the digital mapping of 2-DE-based entity-specific protein expression patterns in a database. We could show that not only variations in expression between, e.g. tumor and normal control tissue, but also differences between advanced colon and ovarian tumors can be visualized. Combining those databases with multivariate statistical analysis tools can greatly contribute to diagnostic decision-making, e.g., in

poorly differentiated or undifferentiated tumor tissue. PCA is a useful and established statistical technique that has found application in fields such as face recognition and image compression, and is a common technique for finding patterns in data of high dimension.

Combining PCA with tumor-stage-related databases enables to unravel entity-specific as well as malignancy-specific polypeptides, which in the future may be further developed to diagnostic chips for histopathological and serum diagnosis.

As shown in Table 1, we identified a total of 15 marker proteins upregulated in advanced colon cancer and normally expressed in advanced ovarian cancer and vice versa. Among those, five polypeptides upregulated in both cancer samples compared to their normal tissues could be classified as “markers of malignancy”. Those proteins include cytoskeletal, cell cycle, and proliferation-associated proteins as well as stress proteins belonging to the heat-shock family. These data are in accordance to the paper published by Alaiya et al., in which they compared the polypeptide expression in benign, borderline, and malignant tumors with a classical proteomic approach [2].

However, despite improvements in the 2-DE methodology, the technique has not yet been included in the daily clinical routine. This is at least in part due to its labor-intensive nature including time-consuming image analysis and the relatively high cost of processing samples.

Today, many laboratories are employing proteomic approaches in their basic research studies. However, the art of running 2-DE is still a high-tech procedure that requires a very high level of skills. Efforts have been directed at simplifying 2-DE, such as the availability of commercial first and second dimension precast gels in various pH ranges and sizes. The possibility of running samples on minigels is a step forward since it requires a considerably smaller amount of sample load enabling work with the biopsy material. New developments in biopsy techniques, combined with proteomic approaches like SELDI and others, open the field for analysis of relatively small clinical specimens. In summary, our data clearly show that 2-DE protein expression profiling combined with principal component analysis is a highly sensitive method for diagnosing, e.g., undifferentiated pelvic adenocarcinomas of unknown origin.

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