Protein profiling of non-malignant and malignant ascites by SELDI-TOF MS: Proof of principle

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Abstract. Ascites is a common clinical symptom in liver cirrhosis, inflammatory disorders of the abdomen and a major late manifestation of metastatic malignancies. Standard cytopathological techniques and immunocytochemistry have specificities and sensitivities of ~95 and 60%, respectively for the presence of tumor cells. Development of faster and more accurate screening methods would be of great clinical utility. In this work we examined differential analysis of the unbound proteins in the supernatant of ascites fluid by Protein-Chip SELDI mass spectrometry. There were 21 tumor cell-positive and 34 tumor cell-negative samples. We used principal component analysis coupled with linear regression applied to the mass spectra of the samples to distinguish between the sample groups. Two sample sets for statistical analysis were created after randomization, a training set with 37 samples and a validation set with 18 samples resulting in a specificity of 93% and a sensitivity of 83% on the training set. The validation set yielded a specificity and sensitivity of 75%. This study suggests that SELDI-TOF mass spectrometry appears to have great potential as a surrogate diagnostic tool to evaluate effusion specimens.

Introduction

Ascites as abdominal effusion, is subdivided into transudative ascites due to liver cirrhosis or cardiac insufficiency and exsudative ascites due to metastatic malignancies, inflammatory processes or as a consequence of chemotherapy (1).

Presence of cancer cells in ascites, peritoneal carcinosis, is an indicator of poor prognosis and is a typical first symptom in some types of cancer (e.g. ovarian or pancreatic carcinoma). Next to cellular component excreted proteins, released surface proteins or cytoplasmatic proteins following cell death may be present in ascites. Protein content can range from 8 μg/μl in transudative to 80 μg/μl in exsudative ascites.

In standard cytological diagnostics, frequently activated mesothelial cells mimic adenocarcinoma cells therefore posing major difficulties in diagnosis (Fig. 1). Using standard morphological procedures, cytological examination has a specificity of ~99% and a sensitivity of ~60% respectively (2-5). In critical cases immunocytochemistry (ICC)(6) or immunohistochemistry in cell-blocks (7,8) can be helpful. Various antibodies against cytokeratins, BerEP4 and calretinin are applied and assist in the differentiation of mesothelial and carcinoma cells as described in Fetsch *et al* (6). However, this technique is time-consuming, expensive and does not result in absolute accuracy. A superior, faster and more economical methodology showing a similar or higher level of accuracy, is desirable. SELDI-TOF (surface-enhanced-laser-desorption-ionization time-of flight) was first described in 1998 (9). SELDI is, similar to MALDI, a protein-mass-spectrometrical technique, but uses chromatographic surfaces to select a subset (e.g. via charge) of the proteins to be measured. Protein mass spectrometry is able to test native samples after simple and quick preparation. Protein spectra can be used to detect single marker proteins or, as a profile or 'fingerprint', to distinguish between e.g. malignant and benign ascites samples. Protein-mass-spectrometry has been applied to a variety of samples: fresh, frozen or paraffinized tissue samples, often microdissected (10), as well as to urine (11,12), serum (13-18), fine-needle aspirates (19) and cell lines (20-28) or other body fluids including ascites, for the detection of single known proteins (13,29). To our knowledge, ascites fluid has not been examined by SELDI for profile analysis yet. We believe that mass spectrometry such as SELDI-TOF MS is a promising technology to analyze cytological samples due to the high sensitivity, rapid procedure and low cost.

Materials and methods

All samples were collected and included in this study according to the guidelines and permission of the ethics board of the

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University Clinics of Aachen and Goettingen. Samples were categorized in tumor cell-positive samples with the presence of one tumor cell per low power field. Tumor cell-negative samples were termed negative by lack of presence of any suspect cell or background. We included all samples with definite diagnosis between January and November 2006. We included one sample per patient.

A total of 55 samples, characterized by experienced cytopathologists, was identified from routine samples by applying rigid stratification standards. The final study population had 21 cases positive for cancer and 34 that were non-cancer. Table I shows the various diagnoses for this population.

Supernatants containing soluble proteins were used for further processing. Samples were stored immediately at -80˚C and repeated freeze/thaw cycles were avoided to protect protein integrity. We used Q10 (former SAX, strong anion exchange)(Ciphergen, Freemont, CA, USA) ProteinChip surfaces for this study. Ready-to-use ion exchange surface lead to a pre-fractionation and therefore to a reproducible reduction of sample complexity. Chips were pre-treated using an optimized protocol: 5 min 10 mM HCl and 5 min 10 mM ammonium acetate with 0.1% Triton X100, pH 6.5. Then 5 μl of peritoneal effusion were diluted with 45 μl of 10 mM ammonium acetate 0.1% Triton X100. A total amount of 50 μl was incubated onto chip surfaces for 20 min at room temperature, humidity controlled air and vigorous shaking. Unbound proteins were removed by stringent washing steps (3x phosphate buffered saline (PBS) pH 7.0, 3x aqua bidest.). After air drying, a repeated application of 0.8 μl matrix solution followed, and finished preparation. Matrix solution consisted of a saturated solution of 4-hydroxi-3,5-dimethoxicinnamic acid in 50% aqueous acetonitrile solution with 0.5% trifluoroacetic acid. Intensive care was taken to standardize each detail of the protocol to avoid artificial results due to biased sample proceeding. In addition, the laboratory was climate controlled (temperature 21˚C ±0.5, humidity 40% ±1) to enable a standardized matrix crystallization throughout the whole study. Air dried chips were measured within 2 h using PBS IIc SELDI-Protein Chipreader (Ciphergen), ProteinChip 3.1 software (Ciphergen) and optimized measuring protocols were empirically determined.

Spectra were randomized and divided into two groups: a training set, consisting of 14 tumor cell-positive and 23 tumor cell-negative samples and a validation set, consisting of 7 tumor cell positive and 11 tumor cell negative samples. Raw spectra were imported into Matlab R14 (Mathworks, Natick, MA, USA) for analysis. Spectra were analyzed from 4000 to 20,000 Da and were renormalized for this range to remove the influence of low molecular weight components using the standard Euclidean 2-norm. Baselines were corrected to remove baseline drifts as a result of intensity variations using a moving window spline approximation. To reduce the number of dimensions, a principle component analysis (PCA) was performed whereby each spectrum was transformed into a set of coefficients ordered by their relative contribution to the variance in the training set. PCA analysis revealed that 95% of the variation in the training set could be explained by 7 components where the original spectra were captured with ~13000 channels. The 7 coefficients were later used in a linear stepwise regression model to differentiate between the presence of tumor cells and samples without tumor cells in the training set. The same linear model was later applied to the validation set to assess the performance of the algorithm on an independent set of data.

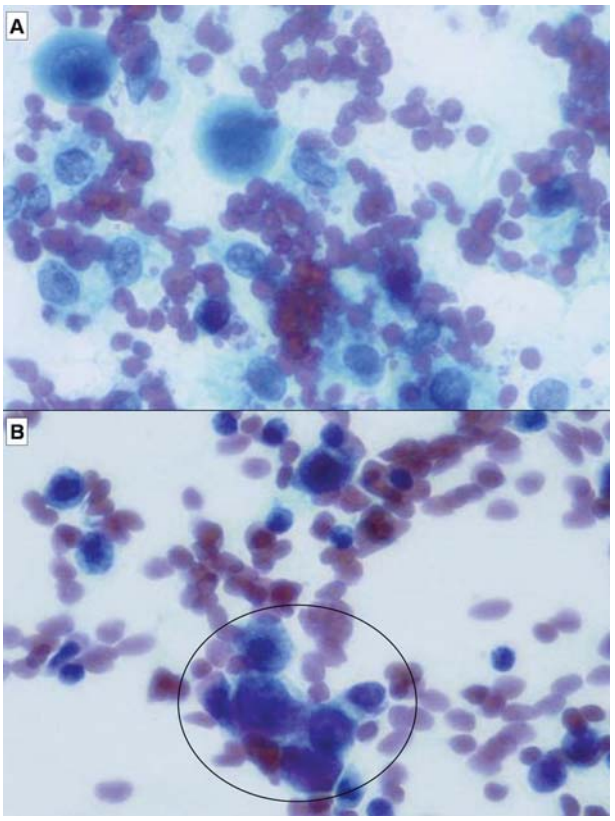

Figure 1. (A) An ascites sample with activated mesothelial cells in e.g. inflammation. (B) A sample of ascites in peritoneal carcinosis is shown. The activated mesothelial cells mimic carcinoma cells causing difficulties in diagnosis.

Results

Valid spectra of each sample within a mass window of 0-20000 m/z were obtained. Mass range from 0 to 4000 Dalton was removed due to matrix interference (16,30). Mass/charge ratio and relative intensity of each peak were included for analysis. In general all spectra demonstrated bundles of high peaks in the range from 4000 to 5000, around 5500, 7000, 9500, 11500, between 13000 and 14500 and around 17000.

Regression analysis of principal component analysis (PCA) coefficients revealed that components 2 and 3 were diagnostically relevant (p=0.007 and p=0.0001, respectively). Component 1 represented the average of all spectra. Since the spectra were normalized, the contribution of this component was similar for all spectra, indicating that they were normalized. In Fig. 2 the bandwidth of variation is shown within the test set and validation set. Combining PCAs 2 and 3 (Fig. 3) into the model yielded a sensitivity of 83%, a specificity of 93% and an accuracy of 86.5% with a p-value of <6e-6 on the training set. A scatter plot demonstrates a

Table I. Samples of this study, divided in subgroups of cytological classification and clinical diagnosis.

Cytological classification	No. of cases Val (test)	Diagnosis
Negative	14	Cardiac insufficiency or liver cirrhosis
	5	Inflammation
	5	With history of a malignancy
	6	No clinical diagnosis given
Positive	3	Adenocarcinoma (no detail on origin)
	10	Ovarian carcinoma
	3	Breast carcinoma
	1	Lung carcinoma

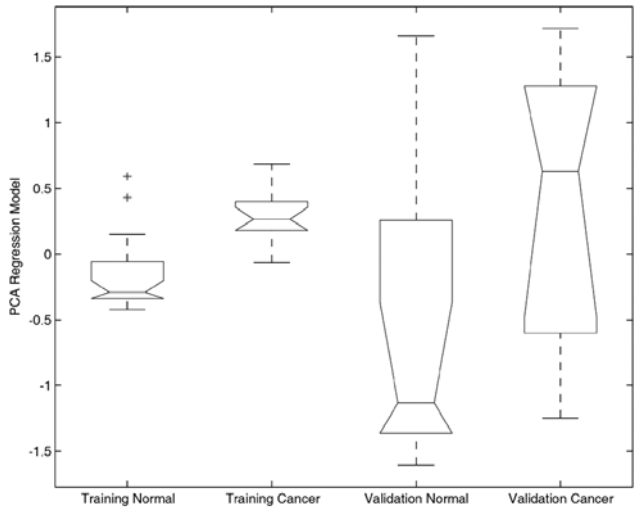

Figure 2. Box plot of training set and validation set showing the bandwidth of variation. The training set shows very low variation and a small box, overlapping only in the area of the bars. In contrast, the validation set shows a much larger box and bars, overlapping in a large proportion.

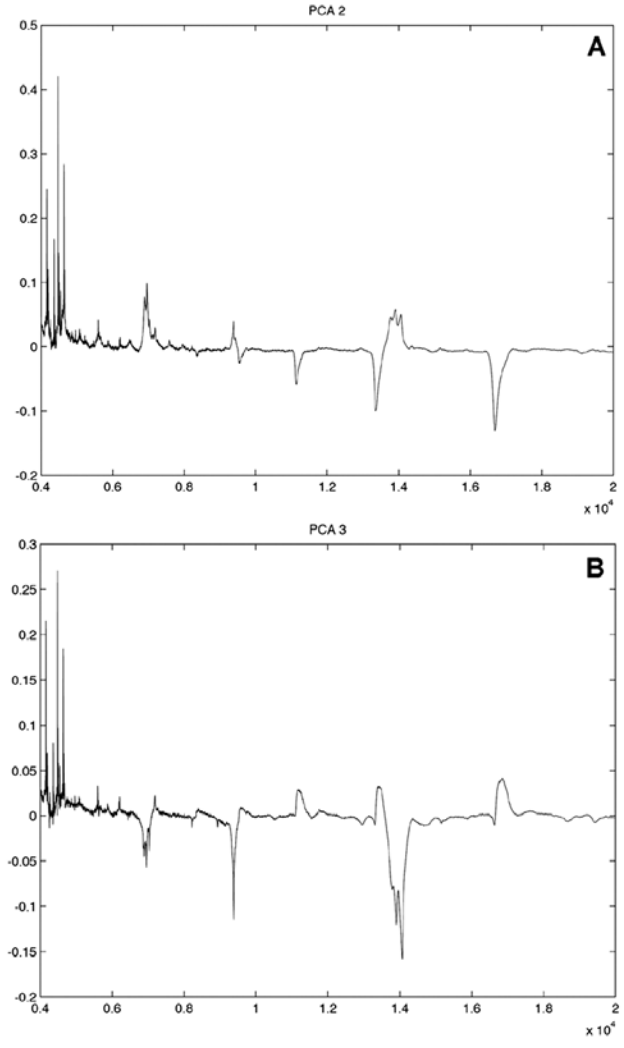

Figure 3. Principal component 2 (A) and 3 (B), giving the x and y coordinates for the scatter plot. The two principal components 2 and 3 describe the coefficients of the second and third highest variation of their relative contributions to the variance in the training set.

separation of the two sample groups of the training set (Fig. 4). The validation set had a sensitivity of 72% and a specificity of 73% using the same model coefficients.

A heat map of the spectra demonstrates the differences in peaks visually; a distinct pattern is observed between samples with or without cancer cells with several extra peaks observed in the samples with tumor cells (see Fig. 5).

In order to compare our results with automated programs, we performed four different automated statistical program types as supervised tests (Table II). Here, a hierarchical cluster analysis gave the best results in specificity and sensitivity of 95 and 80% respectively.

Discussion

The technique of analyzing ascites via the protein content has been studied since at least the 1950s (31). Several methods, such as measuring the amount of total protein and albumin, lead to an accuracy of 88% to separate non-cirrhotic (exsudative) and cirrhotic (transudative) ascites. However, this technique was unable to differentiate malignant ascites (32). Single serum components such as albumin were found to be of potential use for differentiation of cardiac peritoneal effusion versus portal hypertension (33) as well as its

Table II. Overview of applied automated statistical programs and methods showing a specificity of up to 98% and a sensitivity of up to 93% with a training set, using hierarchical clustering.[a]

Software	Method	Peak (m/z)	Specificity (%)	Sensitivity (%)
ProteinChip	Univariate	6445 14050	60	70
ClinProTools	Quick classifier (univariate)	16679	85	67
ClinProTools	Genetic algorithm (GA)	1244 3824 3900 9257	57	80
	(multivariate)	19095		
ClinProTools	Support vector machine (SVM)	1422 5113 6445 10978 16679	49	93
	(multivariate)	18645		
XLMiner	Hierarchical clustering (multivariate)	-	98	78

[a]Hierarchical clustering (XLMiner, BioControl, Arlington, VA, USA). All automated programs must be performed by normalized and extracted data, holding the peak information, given by the ProteinChip. The exact algorithms for data extraction and normalization are subject to possibile adjustments. Loss of data by normalization and peak identification is probable. In the first univariate analysis in the table, made with ProteinChip, peaks were chosen by sight and the values calculated by hand. The three following analyses are combined within one program, ClinProTools (Bruker, Bremen, Germany). The used algorithms are not published.

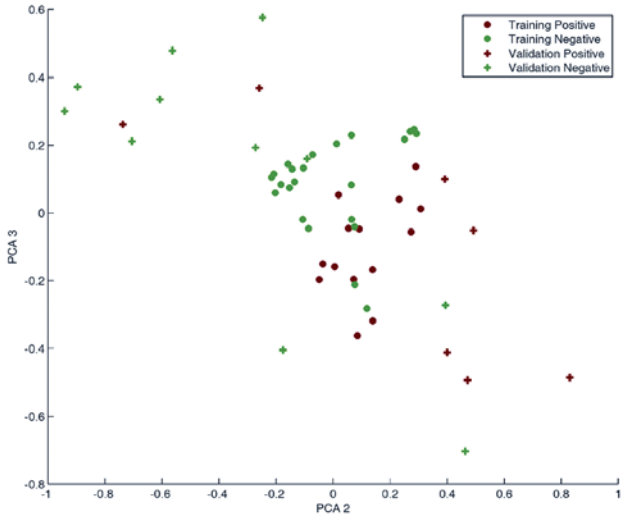

Figure 4. Scatter plot of training set and validation set. The samples of the training set are highly concentrated in certain areas, showing only two samples with a position in the positive group. The samples of the validation set are more dispersed, showing a concentration within the right grouping, but with three samples of the negative group in the wrong area, respectively two in the positive group.

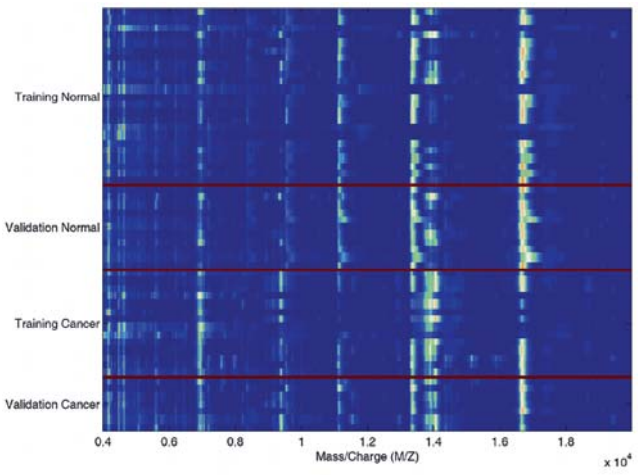

Figure 5. Heat map of peaks, sorted by training set and test set. The peaks around 7000 and 14000 appear to show more and stronger peaks in cancer samples, whereas in the region around 11000 the negative group shows a slightly higher width and brightness.

gradient to serum concentration for diagnosis of peritoneal carcinosis (34).

Biochemical methods such as enzyme activity assays or serum analysis methodologies did not prove to be of great value. Tangkijvanich *et al* showed a telomerase activity assay in peritoneal effusion with a sensitivity and specificity of up to 76 and 95% respectivly (35). Sialic acid was described as having an accuracy of 82% in patients with malignant peritoneal effusion (36). ßHCG has been described as

relatively positive in malignant peritoneal effusion (37). Senger *et al* found a 34-42 Da protein secreted by tumor cell lines that causes increased microvascular permeability (VPF) (38,39). Even mass-spectrometry (MALDI with immunoprecipitation) has been used in testing peritoneal effusion for heterogeneity of transthyretin in patients with ovarian cancer (13). Other ICC markers used for serum diagnostics in clinical pathology have been the subject of studies, e.g. CEA (4,34,40), BG I and Ca19.9 (41).

None of the above publications have been implemented for routine clinical diagnostic procedures to date, due to difficult standardization and lack of considerable improvement concerning diagnostic accuracy (sensitivity and specificity), speed and costs.

Tumor identification via SELDI-TOF analysis has already been shown to be of potential use for a variety of specimens (14,15,17,42-44). SELDI in serum revealed to be especially difficult due to high concentrations of albumin, that binds small proteins and causes loss of information (26). Ascites fluid contains many proteins as well as albumin but in a much lower concentration than serum (32,45,46).

Proper analysis of SELDI has shown to be crucial. Similar to the early usage of cDNA analysis, automated software programs have been first used to separate sample subgroups (17,18,47). Currently, custom made data analysis by statisticians is needed to stratify subgroups by means of reproducibility (20,48).

The comparison of different algorithms is often included in a single publication (20). Without the opportunity to utilize an approved technique, univariate statistical approaches are replaced by a large number of multivariate analyses. Such analyses include genetic algorithms, cluster analysis, regression analysis or principal component analysis (20,42). In addition, other basic problems such as the correct evaluation of the signal-to-noise ratio have not yet resulted in an adequate general guideline (48). At this point it appears that pattern recognition is an adequate technique to analyze comparative proteomic data sets. It is, however, imperative to ascertain a careful study design, unbiased sample processing, standardized mass spectrometric protocols and accurate analysis in order to obtain significant and reproducible results (48-52).

Numerous studies have been published to evaluate protein signatures by SELDI-TOF for malignancies, after Petricoin *et al* (16) reported signature peaks in serum of patients with ovarian cancer. These include gastric cancer (53), pancreatic carcinoma (43,54), cholangiocarcinoma (55), prostate neoplasms (14) and breast cancer (15). SELDI-TOF MS has, however, not previously been applied to free proteins in thoracic or peritoneal effusions.

To our knowledge results, presented in this study are the first description of generation and application protein patterns in peritoneal effusion supernatant. With current cytological methods, diagnostic accuracy of a single sample of malignant peritoneal effusion is reported to range up to 95% (1,56).

Different automated supervised methods were tested and revealed a variety of results, the greatest at 98% specificity and a sensitivity of 78% with cluster analysis. With this new approach using principal component analysis, we were able to show a specificity and sensitivity of up to 73%. Compared to standard cytological examination, this method does not show any initial advantage. In sensitivity it gives slightly better results than in classical cytopathological examination, but in specificity it is less accurate.

As with authors described earlier, we were able to detect differences in the selective surfaces of the chips, depending on the batch and age. To our knowledge, industrial efforts are being undertaken to overhaul this problem.

However, we strongly believe that after the validation of larger cohorts, MS-TOF technologies in conjunction with elaborate bioinformatics may help as an initial stringent, relatively low priced stratification tool for effusion diagnostics as presorting and/or reassurance in conjunction with common cytological examination. In the laboratory, SELDI could be performed as a parallel analysis, with the need of only 5 μl supernatant. In the case of positive SELDI results, but negative cytology, this method could lead to an additional microscopical examination.

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