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Increased serum levels of complement C3a anaphylatoxin indicate the presence of colorectal tumors

Jens K. Habermann^{1,2,8,*}, Uwe J. Roblick^{2,8,*}, Brian T. Luke⁴, DaRue A. Prieto⁵, William J. J. Finlay⁶, Vladimir N. Podust⁷, John M. Roman⁵, Elisabeth Oevermann², Thomas Schiedeck², Nils Homann³, Michael Duchrow², Thomas P. Conrads⁵, Timothy D. Veenstra⁵, Stanley K. Burt⁴, Hans-Peter Bruch², Gert Auer⁸, and Thomas Ried¹

¹Genetics Branch, National Cancer Institute, NIH, Bethesda, MD, USA ²Department of Surgery, University of Schleswig-Holstein, Campus Lübeck, Germany ³Internal Medicine Department, University of Schleswig-Holstein, Campus Lübeck, Germany ⁴Advanced Biomedical Computing Center, SAIC-Frederick, Inc., NCI Frederick, Frederick, MD, USA ⁵Laboratory of Proteomics and Analytical Technologies, SAIC-Frederick, Inc., NCI Frederick, Frederick, MD, USA ⁶Biomedical Diagnostics Group, Dublin City University, Dublin, Republic Of Ireland ⁷Ciphergen Biosystems, Inc., Fremont, CA, USA ⁸Department of Oncology and Pathology, Cancer Center Karolinska, Karolinska Institutet, Stockholm, Sweden

Abstract

Background & Aims—Late diagnosis of colorectal carcinomas results in a significant reduction of average survival times. Yet, despite screening programs about 70% of tumors are detected at advanced stages (UICC III/IV). We explored whether detection of malignant disease would be possible through identification of tumor specific protein biomarkers in serum samples.

Methods—A discovery set of sera from patients with colorectal malignancy (n=58) and healthy control individuals (n=32) were screened for potential differences using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS). Candidate proteins were identified, and their expression levels validated in independent sample sets using a specific immunoassay (ELISA).

Results—Utilizing class comparison and custom developed algorithms we identified several *m/z* values that were differentially expressed between the malignant samples and the healthy controls of the discovery set. Characterization of the most prominent *m/z* values revealed a member of the complement system, the stable form of C3a anaphylatoxin, i.e., C3a-desArg. Based on a specific ELISA, serum levels of complement C3a-desArg predicted the presence of colorectal malignancy in a blinded validation set (n=59) with a sensitivity of 96.8% and a specificity of 96.2%. Increased serum levels were also detected in 86.1% of independently collected sera from patients with colorectal adenomas (n=36), while only 5.6% were classified as normal.

Conclusion—Complement C3a-desArg is present at significantly higher levels in serum from patients with colorectal adenomas (p<0.0001) and carcinomas (p<0.0001) than in healthy individuals.

Correspondence and reprint requests: Dr. Thomas Ried, M.D., National Institutes of Health, 50 South Drive, Building 50, Room 1408, Bethesda, MD 20892-8010, Tel: (301) 594-3118, Fax: (301) 402-1204, Email: ried@mail.nih.gov.

*Drs. Habermann and Roblick contributed equally to this study.

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This suggests that quantification of C3a-desArg levels could ameliorate existing screening tests for colorectal cancer.

Keywords

Colorectal Cancer; Polyps; Screening; Serum; SELDI-TOF MS; C3a-desArg

INTRODUCTION

Detection of cancer at early stages is critical for curative treatment interventions. While the five-year disease free survival for UICC stage I tumors exceeds 90%, this percentage is reduced to 63% in UICC stage III carcinomas¹. It should therefore be obvious that tools and methodologies for early cancer detection directly impact on survival times. In present clinical practice, screening for cancer and pre-invasive polyps of the colorectum is based on clinical examination, the detection of occult fecal blood (FOBT)², and on sigmoidoscopy or colonoscopy. The successful implementation of these screening procedures has contributed to a reduction of disease-associated mortality of colorectal carcinomas³. However, colorectal tumors still rank among the most common malignancies in the Western world: approximately 140,000 new cases will be diagnosed in the U.S. annually, and about 55,000 patients will die of the disease¹. The persistent delay in diagnosis and the associated high mortality are attributable to a low compliance to some screening tests (e.g., colonoscopy) and to the low sensitivity of others (e.g., FOBT)⁴.

There is reasonable hope and emerging evidence that the presence of malignant disease could be detected by specific changes in the composition of serum proteins. Comprehensive serum proteome profiling for such tumor specific markers has therefore become a field of intensive research^{5–8}. For instance, determination of serum levels of prostate specific antigen (PSA) for the detection of prostate cancer, despite issues regarding specificity and sensitivity, has become routine clinical practice⁹. Other biomarkers indicate the presence of ovarian and prostate carcinomas^{10–13}. However, the use of single or combination of serum markers, including carcinoembryonic antigen (CEA), has so far failed to deliver diagnostic tests of high sensitivity and specificity for colon cancer.

Several technologies are available for proteome screening. One approach is based on surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS). SELDI utilizes chromatographic surfaces that retain proteins from a complex sample mixture according to their specific properties (e.g., hydrophobicity and charge), and the molecular weights of the retained proteins are then measured by TOF MS^{14, 15}. We have investigated the potential of this methodology for discovery of features (proteins or protein complexes) in the serum that are characteristic for patients with colorectal malignancy. This discovery phase was followed by protein identification of prominent features at specific *m/z* values and independent experimental verification with an ELISA test using an extended validation set including serum samples from patients with colorectal adenomas.

METHODS

Study population

149 serum samples were collected at the Department of Surgery, University Hospital Schleswig-Holstein, Campus Lübeck, Germany, consisting of a discovery set of 32 healthy controls and 58 patients with colorectal malignancy and an independently collected, non-overlapping, blinded validation set of 59 samples. Peripheral blood samples were collected in adherence with protocols approved by the local Institutional Ethical Review Board as follows: blood from cancer patients was collected from patients during the initial presentation at the

hospital, which in our clinic precedes the day of surgery by about four to five days. These patients were not fasting nor were they at the time of phlebotomy admitted to the hospital and therefore not exposed to specific environmental factors. The healthy control group was comprised of medical personnel that was also not-fasting at time of blood collection. Blood was drawn into serum tubes (S-Monovette®, Sarstedt, Nümbrecht, Germany), immediately stored on ice until serum preparation was performed within two hours after collection. Samples were then stored at -20°C . Clinical data are summarized in Table 1A. In addition to the collection of serum samples for SELDI-TOF MS-based protein profiling, we collected a set of samples from patients with colorectal polyps ($n=36$). These samples were collected at the Department of Internal Medicine at the University Hospital Schleswig-Holstein, Campus Lübeck, prior to an explorative colonoscopy. These samples were used for quantification of serum levels of complement C3a-desArg using an ELISA test only (see below). Therefore, three sets of samples were used in this investigation: a discovery set containing 32 healthy individuals and 58 patients with colorectal malignancy; a validation set containing 59 samples with unknown status (either with or without colon cancer); and a testing set containing 36 patients with colorectal polyps. Mass spectra were acquired for all individuals in the discovery and validation sets; the discovery set was used to identify a putative biomarker and its discriminating ability was tested on the validation set. Serum levels of complement C3a-desArg were then measured using an ELISA test in all three sets of individuals. Serum levels of the discovery set were used to establish thresholds that were applied to the validation set. We also used levels of both, the discovery and validation set (whose status was now known), to determine appropriate serum concentration thresholds that were then applied to the testing set of polyp sera. The clinical data are provided in Table 1B.

Sample preparation

Non-fractionated, total serum samples were processed using two types of ProteinChip® Arrays, immobilized metal affinity capture (IMAC3) and weak cationic exchange (WCX2) arrays, according to protocols provided by the manufacturer (Ciphergen Biosystems, Inc., Fremont, CA). All samples were randomized; duplicates were analyzed on separate ProteinChip® Arrays. Both types of ProteinChip Arrays were analyzed on the ProteinChip Biology System II (PBSII) SELDI-TOF mass spectrometer (Ciphergen). Mass accuracy was assessed daily through external calibration with All-in-1 Peptide and All-in-1 Protein standards (Ciphergen). The arrays were analyzed using the following PBSII automated settings: laser intensities 215 (IMAC3) and 220 (WCX2), detector sensitivity 8, focus mass 5000, m/z range 0–200,000, 130 averaged laser shots per sample spectrum. Data were collected using Ciphergen ProteinChip® software version 3.0.2.

Method of Examination

The ProteinChip® Array data were treated by an initial truncation of the spectra to eliminate m/z values below 1500 Da. After scaling each spectrum in the discovery set to a constant total ion current, the spectra were averaged into a single spectrum to identify peak regions with sufficient intensity. Each region had a total width of 0.3% of m/z and in general contained approximately 15 recorded intensities. A region was only retained if the maximum intensity did not occur in the first or last two recorded m/z values of this region in at least 60% of the samples of a given status (normal or cancer), thereby removing shoulder regions from consideration. This conservative approach dramatically reduced the SELDI-TOF MS data points to 305 significant regions on the IMAC3 array, and 322 significant regions on the WCX2 array, therefore reducing probability of chance fitting of data^{16, 17}. The same scaling was applied to each spectrum in the validation set, and the final set of 305 and 322 significant regions were examined to find the maximum intensity in each region for the IMAC3 and WCX2 spectra, respectively. The spectra of the two array surfaces (IMAC3 and WCX2) were then combined, such that each spectrum in the discovery and validation set presented 627 features.

Since validation set spectra were not used for the identification of putative biomarkers, only discovery set spectra were then analyzed as to whether the two technical repeats per serum sample should be averaged or kept as duplicates. Since biomarkers are serum proteins whose blood concentration depends upon whether or not an individual has a disease, it is important to distinguish these peaks from those used in a single classifier to account for variations in peak intensities due to individual and experimental variations. The experimental variation is the difference between the duplicate spectra; if it is too small the spectra should be averaged so that they do not adversely influence the classifiers. If the experimental variation is large, the samples should be kept separate to maintain a realistic spread in peak intensities. While we acknowledge the possibility that averaging of duplicate spectra may be problematic, we submit that our procedure did not adversely affect the qualitative results, as demonstrated through confirmation of serum C3a levels with an independent ELISA test. The 627 peak intensities from both chip surfaces were used to determine the Euclidean distance between each spectrum and its duplicate, and this was compared to the distances between it and the spectra from other samples. If each member of a duplicate pair of spectra had, on average, two or more spectra from other samples that were closer to it than it was to its duplicate, there was no *a priori* way to associated these spectra with the same individual and they were kept separate. Otherwise, the duplicate spectra were averaged. This also has the effect of not allowing a suboptimal spectrum to contaminate its duplicate. Outlier detection identified eight spectra that were excluded from subsequent analysis. The remaining spectra (69 cancerous and 39 controls) composed the discovery set, which was used exclusively to identify features that distinguish malignant sera from control sera. We then applied a total of 11 independent methods with the rationale that a true biomarker will appear not only in one but several analytical algorithms as a strong discriminative feature.

Five of these different methods were used to determine how malignant sera could be separated from healthy control samples based only on individual features. In addition, evolutionary programming in six sets of 16 runs was used to test how well features could separate in a pairwise concerted form, employing average-linkage (ALC) and complete-linkage (CLC) clustering algorithms as well as Distance-Dependent K-Nearest Neighbors (DD-KNN) ¹⁸. Here, the Euclidean distance metric was used with either absolute differences (AD) or relative differences (RD) in the intensities of the chosen set of features. Further information on all of these methods is available in supplementary Methods. Based on all methods, a total of 21 features were selected upon scoring in the top five models by any of the methods that examined individual features, or when appearing in the best model or regular appearance in the top 100 models at least five times in a set of 16 runs (Supplementary Table 1). This set of 21 features was then used to identify representative peaks in the spectrum by finding all features whose intensities have a sufficient correlation to those listed in supplementary Table 1 ($r > 0.70$) and then visually inspecting the raw spectra. This produced a set of 33 peaks (18 from the IMAC3 array and 15 from the WCX2 array) that clustered into 9 groups. The intensities of the peaks in each group are shown in supplementary Figure 1. The results on the IMAC array show that the peaks at 9148.7 and 8941.1 were identified by 10 and eight of the 11 methods, respectively, and appeared to have a high discriminating value. The peak at 8941.1 has a higher intensity than the 9148.7 peak (maximum intensities are 246.3 and 78.8, respectively), suggesting that the former represents the major serum state of this protein product while the latter represents some modified form (which was confirmed after protein identification). All analytical procedures were completed before our clinical collaborators in Lübeck, Germany, decoded patient diagnoses of the validation set.

Protein identification

Serum samples were fractionated on an anion exchange resin (Q HyperD® F, Pall Corporation, East Hill, NY). The resulting fractions were further enriched using YM-30 Microcon filtration

units (Millipore Inc., Bedford, MA) or additionally purified by reverse phase chromatography using RPC Poly-Bio beads (Polymer Laboratories Inc., Amherst, MA). The chromatographic fractions were monitored by SELDI-TOF MS. Enriched fractions were finally purified by SDS-PAGE (Invitrogen, Carlsbad, CA). Colloidal Blue stained bands were excised from gels. Whole bands of interest were extracted from gels with 50% formic acid, 25% acetonitrile, 15% isopropanol, and 10% water¹⁹ and reanalyzed using the SELDI-TOF MS to confirm that masses of proteins from SDS-PAGE bands correspond to masses of selected biomarkers/features. Extracts were evaporated in vacuum and in-solution digested with trypsin¹⁹. Tryptic digests were analyzed using tandem mass spectrometer Q-TOF2 (Waters-Micromass Inc., Milford, MA) equipped with PCI-1000 ProteinChip Interface (Ciphergen). Spectra were collected from 1 to 3 kDa in single MS mode. After reviewing the spectra, specific ions were analyzed by MS/MS. The collision-induced dissociation spectra were submitted to the database-mining tool Mascot (Matrix Science Inc., Boston, MA) for identification.

Identity of biomarkers was confirmed by ProteinChip immunoassay or beads-based immunoassay. In the first case, a specific antibody was cross-linked to the PS20 ProteinChip array. The crude serum was incubated on spots with immobilized antibody, unbound proteins were removed by multiple washes, and the specifically captured proteins were analyzed directly using the ProteinChip Reader^{20, 21}. In the second approach, 2 μ l of Protein A Hyper D beads (Pall Corporation) were loaded with a specific antibody. Beads were washed three times with Phosphate Buffered Saline (PBS) to remove unbound proteins. 2–5 μ l serum samples diluted to 50 μ l in PBS were bound to the beads for 30 min at room temperature. The beads were washed three times with PBS and once with water. Bound proteins were eluted from the beads with 0.1 M acetic acid. Eluted fractions were analyzed by SELDI-TOF MS using NP20 ProteinChip Arrays.

ELISA methods

All measurements of serum concentration for complement C3a and complement C3a-desArg were performed using the OptEIA Human C3a ELISA kit (BD Biosciences Pharmingen, San Diego, CA). In accordance with the manufacturer's recommendations, all serum samples were examined at a dilution of 1:10000 to ensure signal in the linear range of the reference standard curve. Using this ELISA kit, physiological serum levels of complement C3a-desArg are in the range of $8,707.2 \pm 1,797.3$ ng/ml. Analyses for each serum sample and reference standard in all ELISA tests were performed in triplicate. The mean coefficient of variation (CV) value for serum analyses of the complement C3a ELISA test was $5.61\% \pm 3.66$. All ELISA tests were performed using the Ultrawash Plus Plate Washer (Dynex, Chantilly, VA) and the VersaMax Microplate Reader (Molecular Devices, Sunnyvale, CA).

RESULTS

Here we report a comprehensive evaluation of serum protein patterns in an effort to identify biomarkers for colon tumors. Figure 1A presents a summary of the experimental setup. In the first step of the experimental procedures we screened sera from 32 healthy controls and 58 sera of patients with colorectal malignancy using SELDI-TOF mass spectrometry. Following truncation of spectra and normalization, SELDI-TOF MS revealed 33 m/z values that were a reflection of nine different serum proteins and their associated adducts. The m/z values on the IMAC3 array at 8941.1 Da and 9148.7 Da appeared to be the strongest discriminative features, while the discriminating ability of the proteins producing the Group 8 and 9 peaks (Supplementary Figure 1) were not as convincing. These findings were corroborated by the identification of a corresponding peak from the WCX2 array surface at 8937.6 Da ($r = 0.811$, $p < 0.0001$). Figure 1B exemplarily shows a SELDI-TOF (IMAC3 array) spectrum from a normal sample and a cancer sample covering the m/z values at 8941.1 and 9148.7 Da. Since

the control sera were collected from significantly younger individuals as compared to the malignant sera (Table 1A) we analyzed each selected m/z value for the possibility that the observed differences might simply be a reflection of age. We could not detect any age-dependent expression of any of these m/z values in the cancer samples of the discovery set; for instance, the m/z value at 8941.1 revealed a Pearson's correlation coefficient of expression levels and age of $r = 0.204$, showing that there is no correlation between expression levels and age (Figure 2A). The analysis of the discovery set therefore suggested that serum profiling using SELDI-TOF MS identifies protein peaks that allow the discernment of patients with colorectal malignancy from control individuals in our collection of sera. To exclude fortuitous separation of the malignant samples from healthy controls in the discovery set, the predictive value of the 8941.1 Da peak was then tested with an independently collected, blinded validation set consisting of 59 samples. Thirteen of the 59 samples (22.0%) received an unknown classification, i.e., the peak values were between the upper and lower thresholds. Forty-five of the remaining 46 samples were correctly classified (sensitivity=96.9% and specificity=100%).

Early detection of cancer is a perceived clinical goal. Sixteen of the tumor samples in the discovery set tested here were UICC stage I and II, i.e., early stage tumors. The independent validation set contained nine such tumors. Figure 2B shows the plot of the intensities of the m/z value at 8941.1 Da according to the UICC stage of the malignant sera compared to the control sera. The figure demonstrates that there is no correlation between peak intensity and tumor stage.

The fact that SELDI-TOF MS based serum proteome profiling revealed distinct m/z values whose discerning power was corroborated in an independent, blinded validation set prompted us to infer that these peaks indeed reflected biomarkers of colorectal malignancy. We therefore proceeded with protein identification of the most prominent features. We identified complement C3a-desArg at the peaks with m/z of 8941.1 and 9148.7 (the peak at 9148.7 is the expected satellite peak of C3a-desArg and reflects the sinapinic acid adduct caused by matrix-assisted ionization). C3a-desArg is the stable form of C3a in serum²². The results are presented in Figure 3A. We next wished to confirm the SELDI-TOF MS based results using an independent method for protein quantification. Serum levels of complement C3a-desArg were assessed using a commercially available ELISA test (this ELISA detects both C3a and its derivative C3a-desArg, which is the stable form of the protein in serum). After SELDI-TOF MS analysis and protein identification, sufficient serum volumes were left for 57 cancer samples and 32 normal samples of the discovery set. Serum levels were also determined for all 38 malignant samples and 21 normal samples in the validation set. The results from both sets were then compared to the intensity values obtained from the SELDI-TOF spectra. The results are presented as a scatter plot in Figure 3B. The regression analysis revealed good correlation between the SELDI-TOF MS derived data and quantification of protein concentration with the immunoassay ($r=0.71$). Using solely the serum levels determined with the ELISA test for C3a-desArg in the discovery set, not taking into consideration any of the additional SELDI-TOF MS peaks, nor any values from the validation set, we determined threshold values for the prediction of malignancy that were then applied to the validation set. The threshold values were determined by the intensity at which the probability of belonging to the malignant or normal group equals 60% in a 6-neighbor DD-KNN model. According to these criteria, the serum threshold for healthy individuals was equal or lower than 11,842 ng/ml and equal or higher than 17,637 ng/ml for individuals with colon malignancy. Applying these thresholds to the samples of the validation set we were able to correctly predict 35 of the malignant samples to be malignant; none was predicted to be normal, while three samples could not be assigned to either group (because the values were between the lower and higher threshold of the serum levels). None of the normal samples was classified as malignant, 19 of 21 were correctly classified as normal, while two samples could not be predicted. When these thresholds

were then applied to the samples in the discovery set, fewer samples could be correctly assigned to the respective groups: three of the 57 cancerous samples in the discovery set were predicted to be normal and three others could not be predicted; however, all 32 normal samples in the discovery set were predicted to be normal (Table 2A).

The convincing performance of C3a-desArg indicating the presence of colorectal carcinomas prompted us to explore whether this marker would also be useful for the detection of colorectal adenomas. Towards this end, we collected sera from 36 patients for which the presence of a polyp was determined by colonoscopy. Sera from these patients were not analyzed using SELDI-TOF MS, but solely by means of ELISA for complement C3a-desArg. The ELISA test results for all polyp sera are included in Figure 3C. The mean serum levels of C3a-desArg in patients with polyps ($22,928.2 \pm 9,901.8$ ng/ml) were lower than the levels observed in patients with invasive carcinomas ($43,646.6 \pm 18,963.7$ ng/ml), however, significantly increased over mean serum levels in healthy controls ($5,139.3 \pm 3,233.1$ ng/ml). The BD OpEIA™ Human C3a ELISA kit that was used here reports a mean C3a-desArg serum concentration in healthy individuals of $8,707.2 \pm 1,797.3$ ng/ml. In analogy to the algorithm described above, we utilized the data from the discovery set of 89 samples to predict the presence of colorectal adenoma (i.e., lower thresholds of 11,842 ng/ml and higher thresholds of 17,637 ng/ml). With these thresholds 24 of the 36 patients with adenomas showed serum C3a-desArg levels above the set threshold, two revealed levels that suggested that they were normal, and 10 samples were positioned between the upper and lower thresholds. This assessment changed when the data from the validation set of 59 patients were included in calculating classification thresholds, which were then below 11,566 ng/ml for normal and above 13,652 ng/ml for cancer samples. Three of 95 cancer samples of the discovery and validation sets were now classified as normal and 92 were correctly classified, while 51 of 53 normal samples were correctly classified and the remaining two were misclassified. There were no non-classifiable samples. The sensitivity is 96.8%, and the specificity 96.2%. We calculated a positive predictive value of 97.8%, and a negative predictive value of 94.4%. In the adenoma serum collection, now 31 samples showed levels above the cutoff, two were characterized as normal, and three showed C3a-desArg serum levels between the cutoff values (see Table 2B for a summary). We did not observe a correlation of serum C3a-desArg levels with the size of the polyps or with the grade of dysplasia. The ELISA values for all 184 samples analyzed here are displayed in Figure 3C.

Individuals in the control groups were younger than those afflicted with cancer or with adenomas. In order to explore whether the serum levels of complement C3a-desArg show a correlation with age, we now plotted all ELISA values for complement C3a-desArg against age in all groups and for all individuals. We did not observe a correlation of serum levels and age in the control groups ($r = 0.134$), in serum samples from patients with adenomas ($r = -0.064$), nor in samples from patients with cancer ($r = -0.044$). These findings support our initial conclusion based on the plot of SELDI-MS peak intensities against age in the cancer patients of the discovery set, shown in Figure 2A.

In summary, the results presented here show that SELDI-TOF MS based serum protein profiling reveals certain m/z values that allow discernment of sera from patients with and without colorectal cancer. The subsequent protein identification revealed complement C3a-desArg as the determining protein that allows prediction of the presence of malignant colorectal disease with a sensitivity of 96.8% and a specificity of 96.2%. The marker also proved useful when applied to an additional independent sample set consisting of sera from patients with colorectal adenomas, in which 86.1% of the adenoma samples revealed serum levels of complement C3a-desArg above the previously determined threshold for cancer samples, 8.3% were undetermined, and only 5.6% were classified as normal.

DISCUSSION

Disease associated mortality rates of colorectal carcinomas remain disturbingly high¹. This is mainly attributable to late detection. The gap between the general possibility of early detection and the persistent high mortality rates is due to limited sensitivity or specificity of existing tests, such as screening for fecal occult blood (FOBT)², or due to an unfortunate lack of compliance for others, for instance colonoscopy^{3, 4}. Therefore, several additional approaches for early detection are being pursued, such as the detection of genetically or epigenetically altered genes in stool samples^{23, 24}, and the presence of cancer cells or abnormal proteins in the peripheral blood²⁵. While promising, none of these approaches has resulted in the implementation of complementary screening tests to digital rectal examination, colonoscopy, and FOBT.

Proteomic technologies have developed rapidly over the past few years and approaches for the parallel interrogation of multiple proteins in tissue or body fluids have become possible^{7, 8, 25}. Comparable to the developments in genomics, such technologies now allow screening for differential patterns in normal and diseased states without *a priori* knowledge of specific alterations. One such screening platform is based on a protein array or biochip technology, where multiple proteins are attached to solid surfaces¹⁴. For instance, SELDI-TOF MS based screening enables the separation and at least partial characterization of multiple proteins in tissue and serum samples. The results can then be used to derive at patterns of spectra of multiple proteins that are specific for a certain disease state. Such an approach has been applied to the identification of SELDI-TOF MS patterns that are indicative of the presence of ovarian or prostate carcinoma^{10, 11, 13, 26}. Here we have used SELDI-TOF MS to verify or falsify our hypotheses that, firstly, sera from patients with colorectal malignancy are different from normal healthy controls, and that, secondly, these differentially expressed m/z values point to relevant biomarkers. Indeed, several peaks were prominent enough to allow very good separation of the two groups. However, tumor prediction in our sample set did not rely on classifiers based on SELDI-TOF spectra. Instead, and in contrast to many previous applications of SELDI-TOF MS-based serum proteome profiling, we were exclusively interested in using these SELDI-TOF spectra for the detection, characterization, and independent validation of proteins that constitute the discerning m/z values. These steps were followed by the validation of serum levels of the detected proteins using a specific immunoassay (ELISA) in an independent validation set, and in sera from patients with colorectal polyps. The initial analysis of the discovery set allowed identification of 21 discriminative features that could distinguish colorectal malignancy-associated sera from healthy control sera. Prior to protein identification, the assumption that these features are indeed bona fide biomarkers was tested using an independently collected and blinded validation set: indeed, the separation into healthy individuals and patients with cancer was possible for more than 95% of the unknown samples. Identification of proteins at the most prominent m/z values revealed complement C3a-desArg, the stable derivative of complement C3a in serum and plasma²². Complement C3a-desArg, also referred to as ASP (acylation-stimulating protein)²⁷, is an acute phase reactant and mainly produced in the liver and in adipocytes. It is involved in triglyceride storage, and associated with obesity, cardiovascular disease, diabetes, and dyslipidemia²². The complement system can also be activated through the exposure to tumor antigens²⁸. One could therefore speculate that perhaps the presence of even relatively small adenomas can trigger a systematic reaction. The mechanistic link, however, between complement C3a activation and colorectal tumors remains to be established. Possibly, the observed complement activation could be at least partially involved in the paraneoplastic phenomenon of an increased thrombosis risk. We are not aware of data that support the interpretation that protein levels of complement C3a are upregulated in primary tumor samples similar to serum levels²⁹. This would be consistent with the interpretation that we measure changes that are a reflection of a systematic reaction of the organism to the presence of neoplastic growth. This hypothesis could be potentially

validated in animal models of colon cancer. In recently published papers, Li and colleagues had reported, among other proteins, overexpression of C3a-desArg in serum from patients with breast cancer, even though the discriminative power was lower than in our collection of sera^{30, 31}. We can therefore not exclude the possibility that complement activation, as measured by increased serum levels of C3a-desArg reflect a more generalized reaction to the presence of malignant disease, rather than specifically to colon cancer. Limited previous reports on serum levels of members of the complement system are not conclusive in this regard³².

Here we have explored the value of SELDI-TOF MS based serum proteome profiling for the detection of *m/z* values specific for malignant colorectal disease. The differentially expressed features were then successfully validated, which in turn prompted protein identification. We are obviously aware of potential pitfalls of serum proteome profiling, which could include sample bias, underlying conditions other than cancer, and, in particular, analytical approaches regarding experimental procedures and data interpretation, which inspired considerable controversy^{16, 17, 33}. Therefore, we feel it necessary to emphasize that the algorithms that we developed and applied were designed for the identification of potential biomarkers only, and not for the generation of the best possible classifier based on SELDI-TOF spectra. Most importantly, all SELDI-TOF MS derived results reported here were confirmed with an established, specific immunoassay for complement C3a-desArg. This is different from many previous studies that employed SELDI-TOF MS for detection of, e.g., ovarian or colon carcinomas^{10, 33, 34}. The fact that the SELDI-TOF MS data were confirmed with a specific immunoassay attests to the robustness and reproducibility of the data. However, we realize that the sera from patients with colorectal cancers and polyps were from individuals that were older than those included in the control group. The mean age of cancer patients in the discovery set was 63 years (range 39–81) and in the validation set 65 years (42–81), compared to a mean age in the control group of 31 years (19–43) in the discovery set and 37 years (26–61) in the validation set (the mean age of patients with polyps was 68 years (47–89)). We would therefore like to emphasize the potential caveat that the significant differences in the expression levels of complement C3a-desArg could, at least partially, be a reflection of the probands' age. However, when plotting age against serum levels in the individual groups (or plotting the SELDI-TOF MS peaks as shown in Figure 2A), such a correlation did not become evident.

While we attempted to standardize serum collection as much as possible, including the times from phlebotomy to storage at -20°C , our samples were collected at a single clinical institution and we can therefore not predict how expansion to a multicenter setting would affect the sensitivity and specificity of our test, and it is possible, yet unlikely, that other conditions than colorectal cancer, such as inflammatory disease systematically influenced the observed serum levels of C3a-desArg. However, the data presented in this proof of principle study of some 165 individuals warrant further exploration in the general population. If confirmed, screening of serum levels of complement C3a-desArg could contribute to a reduction of the incidence of colorectal carcinomas, and to a shift towards the diagnosis of cancer at earlier stages.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A: Flow-chart of experimental and analytical procedures for identification of colorectal cancer specific serum markers. The first step focused on SELDI-TOF MS-based profiling of a discovery set (red). The reproducibility of the data set was explored with an independent validation set (red). These steps were followed by protein characterization of features in prominent m/z values (green). An ELISA test for complement C3a-desArg was used to validate all SELDI-TOF MS-based results (blue) and to predict samples from a validation set and a set of sera from patients with colorectal adenomas (blue) based on serum level thresholds derived from the discovery set, or a combination of the discovery and training set (gray).

B: Examples of SELDI-TOF spectra (IMAC3 ProetinChip Array) from a healthy individual (normal) and a patient with colorectal carcinoma (malignancy). The dotted lines indicate peaks at prominent m/z values at 8941.1 and 9148.7. These peaks show higher expression in the cancer sample.

Figure 2.

A: Scatter plot of SELDI-TOF MS-based m/z intensities at 8941.1 and age of patients. A Pearson's correlation coefficient of expression levels and age of $r = 0.204$ indicated that there is no correlation.

B: Summary of the SELDI-TOF MS-based values for the peak intensity at 8941.1 of the discovery and the validation set. Note that the peak intensities do not correlate with the UICC stage in either of the samples. SELDI-TOF MS values for 8941.1 are lower in healthy individuals (N).

Figure 3.

A: Immunoassay with an antibody against complement C3a-desArg reveals the identity of this protein at the prominent SELDI-TOF MS-derived m/z values of 8941.1 and 9148.7. The analysis confirms increased expression of complement C3a-desArg (8933.28) in serum from cancer patients compared to serum from healthy individuals (Normal). The peak at 9141.19 is the expected matrix-induced adduct.

B: Scatter plot of SELDI-TOF MS-based measured intensities for the m/z intensities at 8941.1 and ELISA data for C3a-desArg. The regression analysis revealed good correlation between the SELDI-TOF MS-derived data and quantification of protein concentration with the immunoassay ($r=0.71$). The green squares indicate values from normal individuals, while the red squares those from patients with colorectal cancer.

C: Summary of all ELISA values for complement C3a-desArg for the healthy individuals (N), sera from patients with colorectal adenomas (P), and with colorectal carcinomas according to UICC stage.

Table 1A: Clinical parameter of samples in the discovery set and the blinded validation set.

	Discovery Set (n=90)		Validation Set (n=59)	
	Malignancy	Control	Malignancy	Control
Patients				
Tumor (T), Metastasis (M)	n=58 (T=38, M=20)	n=32	n=38 (T=17, M=21)	n=21
SELDI-TOF MS				
Tumor (T), Metastasis (M)	n=69 (T=46, M=23)	n=39	n=76 (T=34, M=42)	n=42
Sex	Female Male	26 32	11 27	12 9
Age	(average in years) (range in years)	63.05 (39-81)	65.07 (42-81)	37.33 (26-61)
UICC Staging	UICC I UICC II UICC III UICC IV	8 11 18 21	5 4 3 26	
TNM Staging	T1 T2 T3 T4	2 8 23 5	2 4 10 1	
Localization	Cecum Ascending Transverse Descending Sigmoid Rectum	1 0 1 0 12 24	1 2 0 1 5 8	
Metastasis/Recurrence	Liver Lung Liver&Lung Recurrence	8 5 3 4	17 1 2 1	

Table 1B: Clinical parameters for 36 serum samples from patients with colorectal polyps.										
Case	Age	Sex	Polyp size	Polyp location	Histology	Dysplasia	Synchr. polyps	ELISA Adj. Conc. ng/ml	CV%	Std. Dev.
1	63	m	20 mm	3	Hyperplastic	No	0	28317.853	12.2	0.345
2	75	f	16 mm	3	Tubular	Lowgrade	0	50345.413	3.6	0.179
3	63	m	12 mm	2	Tubular	Lowgrade	0	44735.147	10.5	0.47
4	68	f	30 mm	n.a.	Tubulovillous	Lowgrade	0	33889.272	4.4	0.149
5	86	f	4 mm	3	Tubulovillous	Lowgrade	2	22058.816	4.4	0.098
6	75	m	15 mm	3	Tubulovillous	Lowgrade	0	5648.808	5.3	0.03
7	80	f	15 mm	3	Tubular	Lowgrade	3	15112.206	10.7	0.162
8	55	f	7 mm	3	Tubular	Lowgrade	3	17698.282	15.2	0.268
9	89	f	2 mm	3	Tubular	Cis	1	20257.066	12.8	0.259
10	70	f	10 mm	3	Tubulovillous	Lowgrade	0	28649.786	15	0.429
11	53	f	8 mm	0	Tubulovillous	Lowgrade	0	36740.409	7.7	0.281
12	78	m	9 mm	0	n.a.	Cis	0	27534.233	14.5	0.4
13	74	f	6 mm	3	Tubular	Lowgrade	0	19772.251	14.7	0.291
14	73	m	2 mm	0 and 3	Tubular	Lowgrade	6	24323.259	1.9	0.046
15	71	m	n.a.	0 and 3	Tubular	Lowgrade	5	11869.255	6.6	0.078

Table 1B: Clinical parameters for 36 serum samples from patients with colorectal polyps.

Case	Age	Sex	Polyp size	Polyp location	Histology	Dysplasia	Synchr. polyps	ELISA Adj. Conc. ng/ml	CV%	Std. Dev.
16	75	m	5 mm	n.a.	n.a.	n.a.	0	17289.496	11.9	0.207
17	67	f	3 mm	0	Hyperplastic	No	0	25875.368	8.1	0.209
18	61	f	2 mm	3	Hyperplastic	No	0	15661.015	12.9	0.201
19	76	m	2 mm	0 and 3	Tubular	Lowgrade	3	15655.595	1.2	0.02
20	62	m	2 mm	3	Hyperplastic	No	3	21696.217	11.9	0.257
21	69	m	3 mm	3	Hyperplastic	No	0	32292.145	7.1	0.228
22	62	m	7 mm	0 and 3	Tubulovillous	Lowgrade	3	16996.113	6.7	0.114
23	79	f	3 mm	3	Tubular	Lowgrade	0	29859.718	8.3	0.247
24	66	f	6 mm	0 and 3	Tubulovillous	Lowgrade	60	24894.408	5.9	0.146
25	81	m	5 mm	0	Tubulovillous	Lowgrade	3	23211.359	6.4	0.148
26	47	f	2 mm	3	Hyperplastic	No	5	20885.996	5.6	0.117
27	71	m	5 mm	3	Hyperplastic	No	2	18776.944	6.1	0.115
28	88	f	2 mm	0, 2 and 3	Tubular	Lowgrade	7	9122.904	2.4	0.022
29	62	m	15 mm	0	Tubulovillous	Lowgrade	0	15495.583	4.4	0.069
30	71	m	10 mm	3	Tubular	Lowgrade	4	18287.728	10	0.183
31	52	f	3 mm	0 and 3	Hyperplastic	No	3	11967.37	13.5	0.162
32	64	m	22 mm	1 and 3	Tubulovillous	Lowgrade	4	17067.89	5.2	0.089
33	54	m	15 mm	1 and 3	Tubular	Lowgrade	2	13206.723	5.6	0.074
34	69	m	10 mm	1 and 3	Tubular	Lowgrade	3	42076.839	10.9	0.459
35	67	m	15 mm	0 and 3	Tubulovillous	Lowgrade	4	18987.849	4	0.075
36	53	f	7 mm	3	Tubulovillous	Lowgrade	0	29158.652	4.2	0.122

0 = cecum, ascending colon, right flexure; 1 = transverse colon and left flexure; 2 = descending colon; 3 = sigmoid colon and rectum. n.a. = not analyzed.

Table 2

Table 2A: Prediction results of samples in the validation set						
	Thresholds based on discovery set ELISA values only 11,842 and 17,637 ng/ml					
	Validation set		Discovery set		Polyps n=36	
	Malignant n=38	Normal n=21	Malignant n=57	Normal n=32		
Predicted as malignant	35	0	51	0		24
Predicted as normal	0	19	3	32		2
Not predictable	3	2	3	0		10
Table 2B: Prediction results of combined samples						
	Thresholds based on combined discovery and validation set ELISA values 11,566 and 13,652 ng/ml					
	Validation and discovery set combined		Polyps n=36			
	Malignant n=95	Normal n=53				
Predicted as malignant	92	2	Positive predictive value 97.8%			31
Predicted as normal	3	51	Negative predictive value 94.4%			2
Not predictable	0	0	Not predictable 0%			3
	Sensitivity 96.8%	Specificity 96.2%				