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CLINICAL–ALIMENTARY TRACT

Increased Serum Levels of Complement C3a Anaphylatoxin Indicate the Presence of Colorectal Tumors

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See CME Quiz on page 1284.

Background & Aims: Late diagnosis of colorectal carcinoma results in a significant reduction of average survival times. Yet despite screening programs, about 70% of tumors are detected at advanced stages (International Union Against Cancer stages 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. Candidate proteins were identified and their expression levels were validated in independent sample sets using a specific immunoassay (enzyme-linked immunosorbent assay). **Results:** By using class comparison and custom-developed algorithms we identified several *m/z* values that were expressed differentially 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 (ie, C3a-desArg). Based on a specific enzyme-linked immunosorbent assay, 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), whereas only 5.6% were classified as normal. **Conclusions:** Complement C3a-desArg is present at significantly higher levels in serum from patients with colorectal adenomas ($P < .0001$) and carcinomas ($P < .0001$) than in healthy individuals. This suggests that quantification of C3a-desArg levels could ameliorate existing screening tests for colorectal cancer.

Detection of cancer at early stages is critical for curative treatment interventions. Although the 5-year disease-free survival for International Union Against Cancer (UICC) stage I tumors exceeds 90%, this percentage is reduced to 63% in UICC stage III carcinomas.[1] It should therefore be obvious that tools and methodologies for early cancer detection directly impact survival times. In present clinical practice, screening for cancer and preinvasive polyps of the colorectum is based on clinical examination, the detection of occult fecal blood,[2] and on sigmoidoscopy or colonoscopy. The successful implementation of these screening procedures has contributed to a reduction of disease-associated mortality of colorectal carcinomas.[3] However, colorectal tumors still rank among the most common malignancies in the Western world: approximately 140,000 new cases will be diagnosed in the United States annually, and about 55,000 patients will die of the disease.[1] The persistent delay in diagnosis and the associated high mortality are attributable to a low compliance to some screening tests (eg, colonoscopy) and to the low sensitivity of other tests (eg, occult fecal blood test).[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 for the detection of prostate cancer, despite issues regarding specificity and sensitivity, has become routine clinical practice.[9] Other biomarkers indicate the presence of ovarian and prostate carcinomas.[10–13] However, the use of single or a combination of serum markers, including carcinoembryonic antigen, 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 uses chromatographic surfaces that retain proteins from a complex sample mixture according to their specific properties

Abbreviations used in this paper: ELISA, enzyme-linked immunosorbent assay; IMAC3, immobilized metal affinity capture; SELDI-TOF MS, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry; UICC, International Union Against Cancer; WCX2, weak cationic exchange.

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Table 1. 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	n = 58	n = 32	n = 38	n = 21
Tumor (T), metastasis (M)	(T = 38, M = 20)		(T = 17, M = 21)	
SELDI-TOF MS	n = 69	n = 39	n = 76	n = 42
Tumor (T), metastasis (M)	(T = 46, M = 23)		(T = 34, M = 42)	
Sex				
Female	26	16	11	12
Male	32	16	27	9
Average age, *y*	63.05	31.34	65.07	37.33
Range	39–81	19–43	42–81	26–61
UICC staging				
I	8		5	
II	11		4	
III	18		3	
IV	21		26	
TNM staging				
T1	2		2	
T2	8		4	
T3	23		10	
T4	5		1	
Localization				
Cecum	1		1	
Ascending	0		2	
Transverse	1		0	
Descending	0		1	
Sigmoid	12		5	
Rectum	24		8	
Metastasis/recurrence				
Liver	8		17	
Lung	5		1	
Liver and lung	3		2	
Recurrence	4		1	

(eg, hydrophobicity and charge), and the molecular weights of the retained proteins then are 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 enzyme-linked immunosorbent assay (ELISA) test using an extended validation set including serum samples from patients with colorectal adenomas.

Patients and Methods

Study Population

A total of 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, nonoverlapping, 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 4–5 days. These patients were not fasting nor were they admitted to the hospital at the time of phlebotomy and therefore were not exposed to specific environmental factors. The healthy control group was composed of medical personnel who also were not fasting at the time of blood collection. Blood was drawn into serum tubes (S-Monovette; Sarstedt, Nümbrecht, Germany) and immediately was stored on ice until serum preparation was performed (within 2 hours after collection). Samples then were stored at −20°C. Clinical data are summarized in Table 1. 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, Germany, before an explorative colonoscopy. These samples were used for quantification of serum levels of complement C3a-desArg using an ELISA test only (see later). Therefore, 3 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 then were measured using an ELISA test in all 3 sets of individuals. Serum levels of the discovery set were used to

Table 2. Clinical Parameters for 36 Serum Samples From Patients With Colorectal Polyps

Patient	Age, *y*	Sex	Polyp size, *mm*	Polyp location	Histology	Dyplasia	Synchronous polyps	ELISA Adjusted Concentration, *ng/mL*	Coefficient of variation *%*	SD
1	63	m	20	3	Hyperplastic	No	0	28,317.853	12.2	0.345
2	75	f	16	3	Tubular	Low grade	0	50,345.413	3.6	0.179
3	63	m	12	2	Tubular	Low grade	0	44,735.147	10.5	0.47
4	68	f	30	NA	Tubulovillous	Low grade	0	33,889.272	4.4	0.149
5	86	f	4	3	Tubulovillous	Low grade	2	22,058.816	4.4	0.098
6	75	m	15	3	Tubulovillous	Low grade	0	5648.808	5.3	0.03
7	80	f	15	3	Tubular	Low grade	3	15,112.206	10.7	0.162
8	55	f	7	3	Tubular	Low grade	3	17,698.282	15.2	0.268
9	89	f	2	3	Tubular	Carcinoma in situ	1	20,257.066	12.8	0.259
10	70	f	10	3	Tubulovillous	Low grade	0	28,649.786	15	0.429
11	53	f	8	0	Tubulovillous	Low grade	0	36,740.409	7.7	0.281
12	78	m	9	0	NA	Carcinoma in situ	0	27,534.233	14.5	0.4
13	74	f	6	3	Tubular	Low grade	0	19,772.251	14.7	0.291
14	73	m	2	0 and 3	Tubular	Low grade	6	24,323.259	1.9	0.046
15	71	m	NA	0 and 3	Tubular	Low grade	5	11,869.255	6.6	0.078
16	75	m	5	NA	NA	NA	0	17,289.496	11.9	0.207
17	67	f	3	0	Hyperplastic	No	0	25,875.368	8.1	0.209
18	61	f	2	3	Hyperplastic	No	0	15,661.015	12.9	0.201
19	76	m	2	0 and 3	Tubular	Low grade	3	15,655.595	1.2	0.02
20	62	m	2	3	Hyperplastic	No	3	21,696.217	11.9	0.257
21	69	m	3	3	Hyperplastic	No	0	32,292.145	7.1	0.228
22	62	m	7	0 and 3	Tubulovillous	Low grade	3	16,996.113	6.7	0.114
23	79	f	3	3	Tubular	Low grade	0	29,859.718	8.3	0.247
24	66	f	6	0 and 3	Tubulovillous	Low grade	60	24,894.408	5.9	0.146
25	81	m	5	0	Tubulovillous	Low grade	3	23,211.359	6.4	0.148
26	47	f	2	3	Hyperplastic	No	5	20,885.996	5.6	0.117
27	71	m	5	3	Hyperplastic	No	2	18,776.944	6.1	0.115
28	88	f	2	0, 2, and 3	Tubular	Low grade	7	9122.904	2.4	0.022
29	62	m	15	0	Tubulovillous	Low grade	0	15,495.583	4.4	0.069
30	71	m	10	3	Tubular	Low grade	4	18,287.728	10	0.183
31	52	f	3	0 and 3	Hyperplastic	No	3	11,967.37	13.5	0.162
32	64	m	22	1 and 3	Tubulovillous	Low grade	4	17,067.89	5.2	0.089
33	54	m	15	1 and 3	Tubular	Low grade	2	13,206.723	5.6	0.074
34	69	m	10	1 and 3	Tubular	Low grade	3	42,076.839	10.9	0.459
35	67	m	15	0 and 3	Tubulovillous	Low grade	4	18,987.849	4	0.075
36	53	f	7	3	Tubulovillous	Low grade	0	29,158.652	4.2	0.122

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

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 then were applied to the testing set of polyp sera. The clinical data are provided in Table 2.

Sample Preparation

Nonfractionated, total serum samples were processed using 2 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 (Ciphergen Biosystems, Inc.). Both types of ProteinChip Arrays were analyzed on the ProteinChip Biology System II 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 ProteinChip Biology System II automated settings: laser intensities 215 (IMAC3) and 220 (WCX2), detector sensitivity 8, focus mass 5000, m/z range 0–200,000, and 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 less than 1500 daltons. 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 retained only if the maximum intensity did not occur in the

first or last 2 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 2 array surfaces (IMAC3 and WCX2) then were combined, such that each spectrum in the discovery and validation set presented 627 features. Because validation set spectra were not used for the identification of putative biomarkers, only discovery set spectra then were analyzed as to whether the 2 technical repeats per serum sample should be averaged or kept as duplicates. Because biomarkers are serum proteins whose blood concentration depends on 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 caused by 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. Although 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 shown 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 with the distances between it and the spectra from other samples. If each member of a duplicate pair of spectra had, on average, 2 or more spectra from other samples that were closer to it than it was to its duplicate, there was no a priori way to associate 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 8 spectra that were excluded from subsequent analysis. The remaining spectra (69 cancerous, 39 controls) comprised 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 analytic 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 6 sets of 16 runs was used to test how well features could separate in a pair-wise concerted form, using average-linkage and complete-linkage clustering algorithms and distance-dependent K-nearest neighbors.[18] Here, the Euclidian distance metric was used with either absolute differences or relative differences in the intensities of the chosen set of features. Further information on all of these methods is available in the Supplementary Methods section (see supplementary materials online at www.gastrojournal.org). Based on all methods, a total of 21 features were selected based on scoring in the top 5 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 5 times in a set of 16 runs (Supplementary Table 1; see supplementary materials online at www.gastrojournal.org). This set of 21 features then was 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 (see supplementary materials online at www.gastrojournal.org). The results on the IMAC array show that the peaks at 9148.7 and 8941.1 were identified by 10 and 8 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, 246.3 and 78.8, respectively), suggesting that the former represents the major serum state of this protein product and the latter represents some modified form (which was confirmed after protein identification). All analytic 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 enriched further 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 sodium dodecyl sulfate–polyacrylamide gel electrophoresis (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% water,[19] and reanalyzed using the SELDI-TOF MS to confirm that masses of proteins from sodium dodecyl sulfate–polyacrylamide gel electrophoresis bands corresponded to masses of selected biomarkers/features. Extracts were evaporated in vacuum and in solution digested with trypsin.[19] 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 kilodaltons 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.

The identity of biomarkers was confirmed by ProteinChip immunoassay or a 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 μL of Protein A Hyper D beads (Pall Corporation) were loaded with a specific antibody. Beads were washed 3 times with phosphate-buffered saline (PBS) to remove unbound proteins. A total of 2- to 5-μL serum samples diluted to 50 μL in PBS were bound to the beads for 30 minutes at room temperature. The beads were washed 3 times with PBS and once with

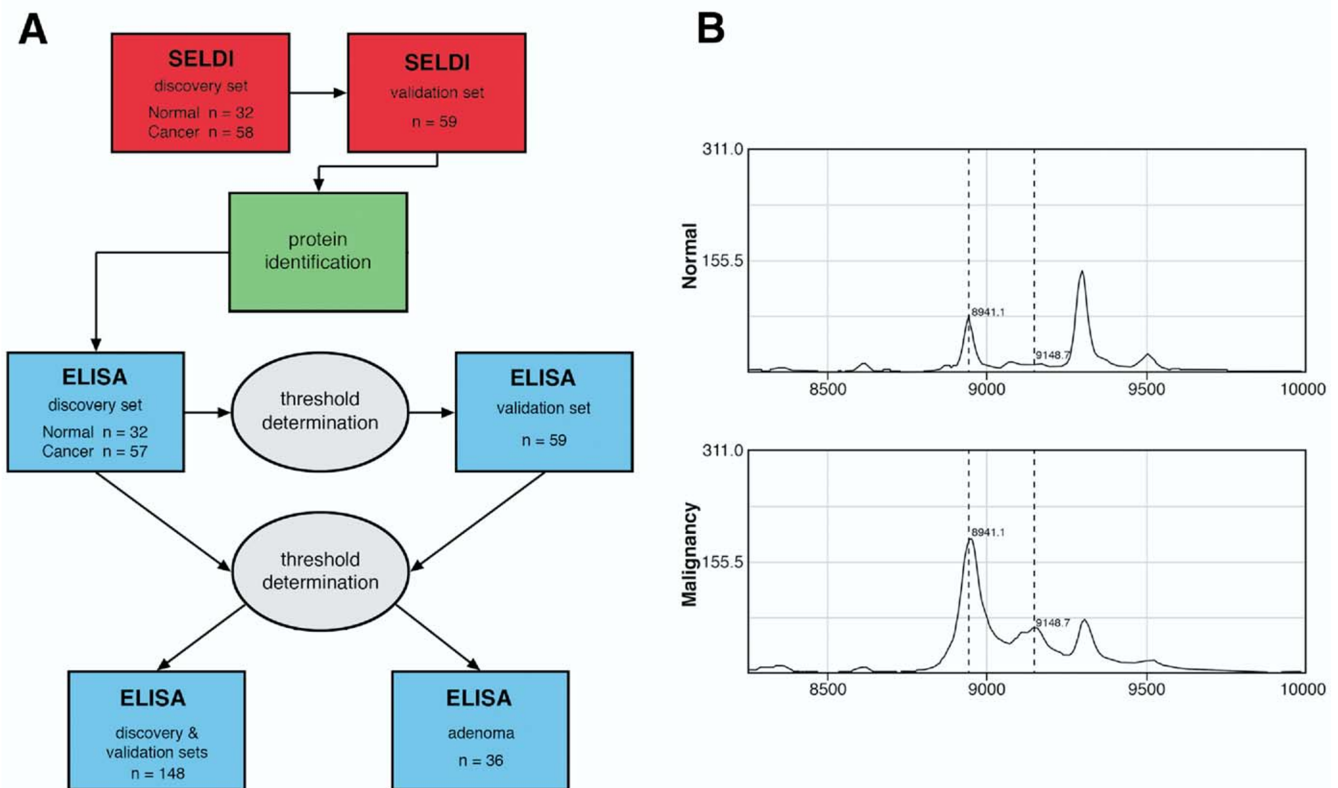

Figure 1. (*A*) Flow-chart of experimental and analytic procedures for identification of colorectal cancer–specific serum markers. The first step focused on SELDI-TOF MS–based profiling of a discovery set (*red*). The reproducibility of the data set was explored with an independent validation set (*red*). 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 ProteinChip 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.

water. Bound proteins were eluted from the beads with 0.1 mol/L 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 1:10,000 to ensure signal in the linear range of the reference standard curve. With the use of this ELISA kit, physiologic serum levels of complement C3a-desArg are in the range of 8707.2 ± 1797.3 ng/mL. Analyses for each serum sample and reference standard in all ELISA tests were performed in triplicate. The mean coefficient of variation value for serum analyses of the complement C3a ELISA test was 5.61% ± 3.66%. All ELISA tests were performed using the Ultrawash Plus Plate Washer (Dynex, Chantilly, VA) and the VersaMax Mircoplate 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 1*A* presents a summary of the experimental set-up. 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 MS. After truncation of spectra and normalization, SELDI-TOF MS revealed 33 *m/z* values that were a reflection of 9 different serum proteins and their associated adducts. The *m/z* values on the IMAC3 array at 8941.1 and 9148.7 daltons appeared to be the strongest discriminative features, although 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 daltons ($r = 0.811$, $P < .0001$). Figure 1*B* 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 daltons. Because the control sera were collected from significantly younger individuals as compared with the malignant sera (Table 1) 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 $r = 0.204$,

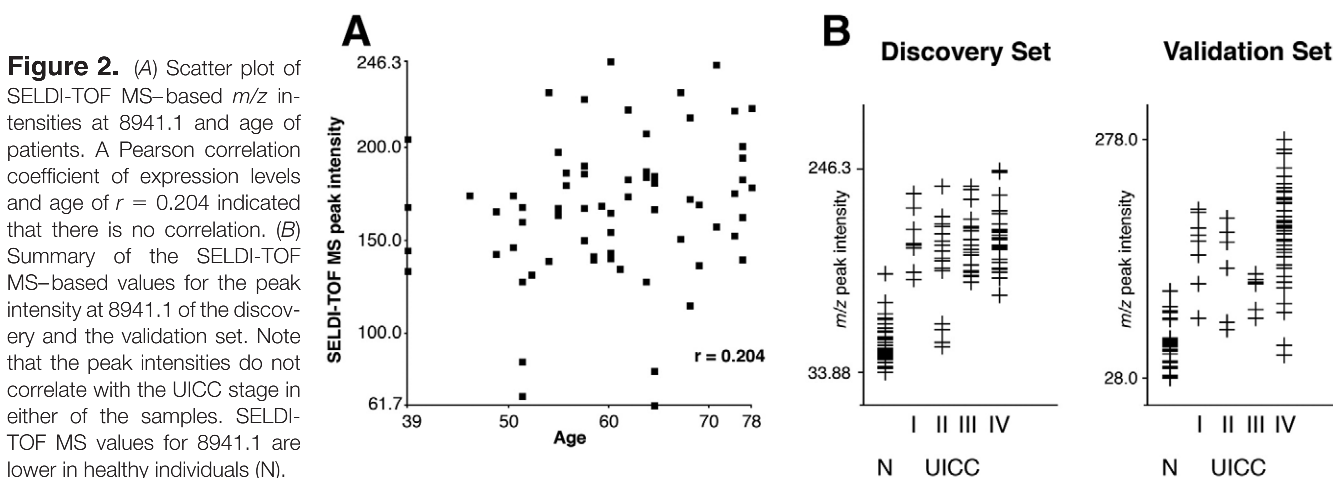

Figure 2. (*A*) Scatter plot of SELDI-TOF MS–based *m/z* intensities at 8941.1 and age of patients. A Pearson correlation coefficient of expression levels and age of $r = 0.204$ indicated that there is no correlation. (*B*) Summary of the SELDI-TOF MS–based values for the peak intensity at 8941.1 of 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).

showing that there is no correlation between expression levels and age (Figure 2*A*). 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 dalton peak then was tested with an independently collected, blinded validation set consisting of 59 samples. Thirteen of the 59 samples (22.0%) received an unknown classification (ie, the peak values were between the upper and lower thresholds). Forty-five of the remaining 46 samples were classified correctly (sensitivity, 96.9%; specificity, 100%).

Early detection of cancer is a perceived clinical goal. Sixteen of the tumor samples in the discovery set tested here were UICC stages I and II (ie, early stage tumors). The independent validation set contained 9 such tumors. Figure 2*B* shows the plot of the intensities of the *m/z* value at 8941.1 daltons according to the UICC stage of the malignant sera compared with the control sera. Figure 2*B* shows 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 serum.[22] The results are presented in Figure 3*A*. 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 also were determined for all 38 malignant samples and 21 normal samples in the validation set. The results from both sets then were compared with the intensity values obtained from the SELDI-TOF spectra. The results are presented as a scatter plot in Figure 3*B*. The regression analysis revealed good correlation between the SELDI-TOF MS–derived data and quantification of protein concentration with the immunoassay ($r = 0.71$). By 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 then were 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 distance-dependent K-nearest neighbors model. According to these criteria, the serum threshold for healthy individuals was 11,842 ng/mL or less, and 17,637 or more ng/mL for individuals with colon malignancy. By 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, although 3 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 classified correctly as normal, although 2 samples could not be predicted. When these thresholds then were applied to the samples in the discovery set, fewer samples could be assigned correctly to the respective groups: 3 of the 57 cancerous samples in the discovery set were predicted to be normal and 3 others could not be predicted; however, all 32 normal samples in the discovery set were predicted to be normal (Table 3).

The convincing performance of C3a-desArg indicating the presence of colorectal carcinomas prompted us to explore whether this marker also would be useful for the detection of colorectal adenomas. Toward this end, we collected sera from 36 patients for whom 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 3*C*. The mean serum levels of C3a-desArg in patients with polyps (22,928.2 ± 9901.8 ng/mL) were lower than the levels observed in patients with invasive carcinomas (43,646.6 ± 18,963.7 ng/mL), however, the levels were increased significantly compared with the mean serum levels in healthy controls (5139.3 ± 3233.1 ng/mL). The OptEIA Human C3a ELISA kit (BD Biosciences) that was used

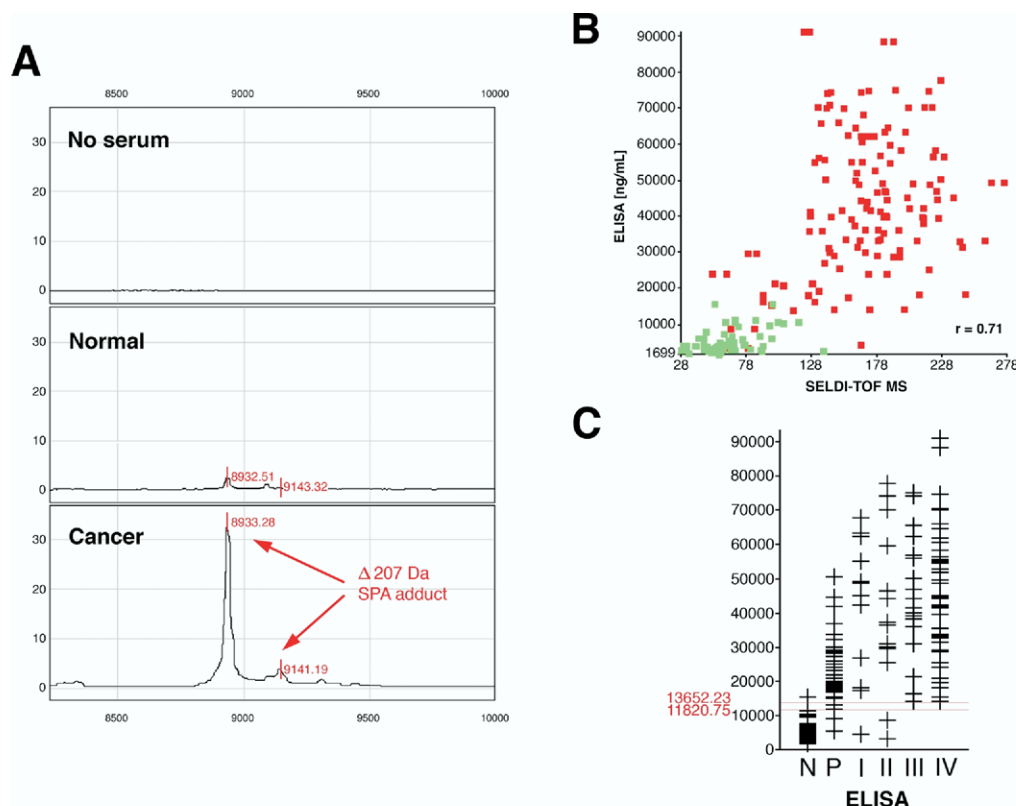

Figure 3. (*A*) Immunoassay with an antibody against complement C3a-desArg reveals the identity of this protein at the prominent SELDI-TOF MS–derived *m/z* values of 8941.1 and 9148.7. The analysis confirms increased expression of complement C3a-desArg (8933.28) in serum from cancer patients compared with 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, and the red squares indicate values 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.

here reported a mean C3a-desArg serum concentration in healthy individuals of 8707.2 ± 1797.3 ng/mL. In analogy to the algorithm described earlier, we used the data from the discovery set of 89 samples to predict the presence of colorectal adenoma (ie, 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, 2 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 then were less than 11,566 ng/mL for normal and greater than 13,652 ng/mL for cancer samples. Three of 95 cancer samples of the discovery and validation sets now were classified as normal and 92 were classified correctly, whereas 51 of 53 normal samples were classified correctly and the remaining 2 were misclassified. There were no nonclassifiable samples. The sensitivity was 96.8%, and the specificity was 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 greater than the cut-off level, 2 were characterized as normal, and 3 showed C3a-desArg serum levels between the cut-off values (see Table 4 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 3*C*.

Table 3. 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		
	Malignant (n = 38)	Normal (n = 21)	Malignant (n = 57)	Normal (n = 32)	Polyps (n = 36)
Predicted as malignant	35	0	51	0	24
Predicted as normal	0	19	3	32	2
Not predictable	3	2	3	0	10

Individuals in the control groups were younger than those afflicted with cancer or with adenomas. 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

Table 4. 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			
	Malignant (n = 95)	Normal (n = 53)		Polyps (n = 36)
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%		

did not observe a correlation of serum levels and age in the control groups ($r = 0.134$), in serum samples from patients with adenomas ($r = -0.064$), or 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 2*A*.

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 greater than 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 high.[1] This is attributable mainly to late detection. The gap between the general possibility of early detection and the persistent high mortality rates is caused by limited sensitivity or specificity of existing tests, such as screening for fecal occult blood,[2] or by an unfortunate lack of compliance for others (eg, 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 blood.[25] Although promising, none of these approaches has resulted in the implementation of complementary screening tests to digital rectal examination, colonoscopy, and fecal occult blood testing.

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 with 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, in which multiple proteins are attached to solid surfaces.[14] 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 then can be used to derive 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, first, sera from patients with colorectal malignancy are different from normal healthy controls, and that, second, these differentially expressed *m/z* values point to relevant biomarkers. Indeed, several peaks were prominent enough to allow very good separation of the 2 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 interested exclusively 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 up 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. Before protein identification, the assumption that these features are indeed bona fide biomarkers was tested by 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 plasma.[22] Complement C3a-desArg, also referred to as *acylation-stimulating protein*,[27] is an acute phase reactant and is produced mainly in the liver and in adipocytes. It is involved in triglyceride storage and is associated with obesity, cardiovascular disease, diabetes, and dyslipidemia.[22] The complement system also can be activated through exposure to tumor antigens.[28] 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 involved at least partially in the paraneoplastic phenomenon of an increased thrombosis risk. Our previous data do not support the interpretation that protein levels of complement C3a are up-regulated in primary tumor samples similar to serum levels.[29] 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 potentially could be validated in animal models of colon cancer. In recently published reports, Li et al[30,31] 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. We therefore cannot 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 regard.[32]

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 then were 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, analytic approaches regarding experimental procedures and data interpretation, which inspired considerable controversy.[16,17,33] Therefore, we believe 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 used SELDI-TOF MS for detection of carcinomas (eg, ovarian or colon).[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 who 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 y) and in the validation set was 65 years (range, 42–81 y), compared with a mean age in the control group of 31 years (range, 19–43 y) in the discovery set and 37 years (range, 26–61 y) in the validation set (mean age of patients with polyps, 68 y; range, 47–89 y). We therefore would 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 2*A*), such a correlation did not become evident.

Although 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 therefore cannot predict how expansion to a multicenter setting would affect the sensitivity and specificity of our test, and it is possible, yet unlikely, that conditions other 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 toward the diagnosis of cancer at earlier stages.

Appendix

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1053/j.gastro.2005.06.065.

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