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Identification of Adipophilin as a Potential Plasma Biomarker for Colorectal Cancer using Label-free Quantitative Mass Spectrometry and Protein Microarray

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Abbreviations used in this paper: 2DICAL, 2-dimensional image converted analysis of liquid chromatography and mass spectrometry; **AUC,** area under the curve; **CC,** correlation coefficient; **CEA,** carcinoembryonic antigen; **CI,** confidence interval; **CV,** coefficient of variance; **ESI,** electrospray ionization; **FOB,** fecal occult blood; **HFM,** hollow-fiber membrane; **HFMT,** hollow-fiber membrane-based technique; **(HP)LC,** (high-performance) liquid chromatography; **JMS,** Jichi Medical School Hospital; **LMW,** low-molecular weight; **MS,** mass spectrometry; **MS/MS,** tandem mass spectrometry; ***m/z*,** mass to charge ratio; **NCCH,** National Cancer Center Hospital; **OMC,** Osaka Medical College Hospital; **ONH,** Osaka National Hospital; **Q-TOF,** quadrupole time-of-flight; **ROC,** receiver operating characteristics; **RT,** retention time, **SD,** standard deviation; **TMUH,** Tokyo Medical University Hospital; **UICC,** international union against cancer.

ABSTRACT

Background: The aim of this study was to identify a new plasma biomarker for use in early detection of colorectal cancer.

Methods: Using the combination of hollow-fiber-membrane (HFM)-based low-molecular weight protein enrichment and 2-Dimensional Image Converted Analysis of Liquid chromatography and mass spectrometry (2DICAL), we compared the plasma proteome of 22 colorectal cancer patients to those of 21 healthy controls. An identified biomarker candidate was then validated in two larger cohorts [Validation-1 (n = 210) and Validation-2 (n = 113)] using a high-density reverse-phase protein microarray.

Results: From a total of 53,009 mass peaks, we identified 103 with an area-under-curve (AUC) value of 0.80 or higher that could distinguish cancer patients from healthy controls. A peak that increased in colorectal cancer patients, with an AUC of 0.81 and *P*-value of 0.0004 (Mann-Whitney *U*-test), was identified as a product of the *PLIN2* gene [also known as perilipin-2, adipose differentiation-related protein (ADRP), or adipophilin]. An increase in plasma adipophilin was consistently observed in colorectal cancer patients, including those with stage I or stage II disease ($P < 0.0001$, Welch's *t*-test). Immunohistochemical analysis revealed that adipophilin is expressed primarily in the basal sides of colorectal cancer cells forming polarized tubular structures, and that it is absent from adjacent normal intestinal mucosae.

Conclusions: Adipophilin is a plasma biomarker potentially useful for the detection of early-stage colorectal cancer.

Impact: The combination of HFM and 2DICAL enables the comprehensive analysis of plasma proteins and is ideal for use in all biomarker discovery studies.

Introduction

Colorectal cancer is the second leading cause of cancer deaths in Western countries (1), and is the third leading cause of cancer deaths in Japan, where there were more than 43,000 estimated colorectal cancer deaths in 2008 (2). Treatment of colorectal cancer without metastasis is relatively uncomplicated, and a favorable prognosis can be expected for these patients (3, 4). However, the 5-year survival rate of patients with metastatic colorectal cancer is estimated to be less than 5% (5), underscoring the importance of early detection. The modality used most commonly for colorectal cancer mass screening is fecal occult blood (FOB) test. Three large randomized trials demonstrated that inclusion of FOB in colorectal cancer screening significantly reduces the rates of colorectal cancer mortality (6-8). However, FOB has a relatively high false-positive rate (9, 10), and as a result, a large number of healthy individuals receive radiological or endoscopic re-examination after the FOB test, placing excessive physical and physiological burdens on examiners and examinees, as well as imposing an undue financial burden upon society. The only approved screening alternative to FOB for the diagnosis of colorectal cancer is testing for the tumor marker carcinoembryonic antigen (CEA). Unfortunately, CEA is not useful as a marker for the early detection of colorectal cancer (11). Therefore, it is necessary to identify a new biomarker to supplement these current diagnostic modalities.

Alterations in the protein content of clinical samples reflect the dynamic biological changes of patients more directly than changes in mRNA levels (12). Plasma/serum proteins are thus valuable resources for the discovery of biomarkers with direct clinical application. We previously developed a quantitative proteomics platform, called 2DICAL (13). This technology is especially advantageous in clinical studies in which a large number of patient samples must be compared. We were able to identify a

number of plasma/serum biomarkers with high potential for clinical application using 2DICAL (14-18). However, the direct analysis of plasma/serum proteins using 2DICAL remains technically challenging. Proteins secreted by cancer cells are considerably diluted in the blood circulation and present only in a low concentration (19, 20). The concentration of serum/plasma proteins ranges over more than 10 orders of magnitude, and thus the efficient removal of abundant plasma/serum proteins is essential for the detection of low-abundance cancer-related biomarker proteins (21).

In this study we applied a high-performance hollow-fiber membrane (HFM) technology to the enrichment of low-molecular weight (LMW) proteins (17, 22) and searched for new plasma biomarkers that might be applicable to the early diagnosis of colorectal cancer. The LMW plasma protein fraction is made up of various functional proteins, such as cytokines, chemokines, and peptides, and is considered to be a rich unexplored archive of biologic information (20). The HFM-based technique (HFMT) utilizes a fully automated system that can separate and concentrate low-abundance plasma proteins from relatively high-molecular weight abundant proteins such as albumin, immunoglobulin, transferrin, and apoliporptroteins with high efficiency and reproducibility (22). Here, we report the identification of adipophilin, an adipose-differentiation-related protein, as a novel tumor marker for colorectal cancer through a comprehensive analysis of the LWM plasma proteome of colorectal cancer patients using HFM and 2DICAL technologies.

Patients and Methods

Plasma samples

Plasma samples were collected prospectively from 366 individuals and then split

randomly into three cohorts (Training, Validation-1 [V1], and Validation-2 [V2]) (Table 1). The cohorts were essentially hospital-based, and consisted of healthy volunteers and newcomers (primarily to gastrointestinal services) between August 2006 and October 2008 at the following seven hospitals in Japan: National Cancer Center Hospital (NCCH; Tokyo), Osaka National Hospital (ONH; Osaka), Jichi Medical School Hospital (JMS; Shimotsuke), Osaka Medical Center for Cancer and Cardiovascular Diseases (Osaka), Tokyo Medical University Hospital (TMUH; Tokyo), Osaka Medical College Hospital (OMC; Osaka), and Fukuoka University Hospital (Fukuoka). This multi-institutional collaborative study group was organized by the "Third-Term Comprehensive Control Research for Cancer" conducted by the Ministry of Health, Labour and Welfare of Japan and joined the International Cancer Biomarker Consortium (23). Written informed consent was obtained from every subject.

All patients diagnosed as having cancer had histological or cytological proof of colorectal adenocarcinoma. Demographic and laboratory data for the cases are summarized in Table 1. The staging of cancer was defined according to TNM classification by the International Union against Cancer (UICC). The Training cohort comprised 43 cases, including untreated colorectal cancer patients from TMUH (n = 8), JMS (n = 9), and ONH (n = 5), and healthy controls from NCCH (n = 2), TMUH (n = 9), OMC (n = 6), and ONH (n = 4). The V1 and V2 cohorts comprised 210 and 113 cases, respectively, from the seven hospitals as described above. The V1 cohort included 101 patients with colorectal cancer and 109 healthy controls. The V2 cohort comprised 26 patients with colorectal cancer and 87 healthy controls.

For all the samples used in this study, the same protocol was used for blood collection, storage, and freeze/thawing to ensure absence of any pre-analytical bias caused by differences in sample handling. Blood was collected in a tube with

ethylenediaminetetraacetic acid (EDTA) at the time of diagnosis. Plasma was separated by centrifugation and frozen at -80°C until analysis. Macroscopically hemolyzed samples were excluded from the present analysis. The protocol of this study was reviewed and approved by the institutional ethics committee board of each participating institute.

Depletion of high-molecular weight plasma proteins

The plasma samples of the Training cohort were filtered through a 0.22-μm-pore-size filter. Five hundred microliter of the sample was diluted by adding 3.5 mL 25mM ammonium bicarbonate buffer (pH 8.0). The total of 4 mL of the plasma dilution was injected into a HFMT machine (22). After one hour of fully automated operation, the solution containing LMW proteins was recovered and lyophilized.

LC-MS

The HFMT-treated samples were digested with sequencing-grade modified trypsin (Promega, Madison, WI) and analyzed in duplicate using a nano-flow high-performance liquid chromatography (HPLC) (NanoFrontier nLC, Hitachi High-technologies, Tokyo, Japan) connected to an electrospray ionization quadrupole time-of-flight (ESI-Q-TOF) mass spectrometer (Q-Tof Ultima, Waters, Milford, MC).

MS peaks were detected, normalized, and quantified using the in-house 2DICAL software package, as described previously (13). A serial identification (ID) number was applied to each of the MS peaks detected (1 to 53009). The stability of LC-MS was monitored by calculating the correlation coefficient (CC) and coefficient of variance (CV) of every measurement. For all 53,009 peaks observed in the 43 duplicate runs, the mean CC (± SD) was as high as 0.951 (± 0.039) and the mean CV was as low

as 0.054 (± 0.011).

Protein identification by tandem mass spectrometry (MS/MS)

Peak lists were generated using the Mass Navigator software package (version 1.2) (Mitsui Knowledge Industry, Tokyo, Japan) and the peak lists were searched against the SwissProt database (downloaded on April 22, 2009) using the Mascot software package (version 2.2.1) (Matrix Science, London, UK). The search parameters used were as follows: the human protein database was selected; up to one missed cleavage was allowed; "none" was designated as the enzyme; mass tolerances for precursor and fragment ions were ± 0.6 Da and ± 0.2 Da, respectively; the score threshold was set to $P < 0.05$ based on the size of the database used in the search. If a peptide matched multiple proteins, the protein name with the highest Mascot score was selected.

Immunoblot analysis

Primary antibodies used were mouse monoclonal antibody against adipophilin (LifeSpan Biosciences, Seattle, WA) and mouse monoclonal antibody against human complement C3b-α (PROGEN, Heidelberg, Germany). Ten microliter of 1:50 diluted plasma sample and 0.3 μg of fully recombinant adipophilin (BioVendor, Modrice, Czech Republic) as positive control were separated by SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane. The membrane was then incubated with primary antibody followed by horseradish peroxidase-conjugated anti-mouse IgG as described previously (24, 25). Blots were developed using an enhanced chemiluminescence (ECL) detection system (GE Healthcare, Buckinghamshire, UK).

Reverse-phase protein microarray

The plasma samples from the V1 and V2 cohorts were serially diluted 1:32, 1:64, 1:128, and 1:256 using a Biomek 2000 Laboratory Automation Robot (Beckman Coulter, Fullerton, CA), and randomly plotted onto ProteoChip® glass slides (Proteogen, Seoul, Korea) in quadruplicate in a 6144-spot/slide format using a Protein Microarrayer Robot (Kaken Geneqs, Matsudo, Japan). The spotted slides were incubated overnight with the same primary antibody as used in Western blotting. The slides were incubated with biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA) followed by streptavidin-horseradish peroxidase conjugate (GE Healthcare, Buckinghamshire, UK). Peroxidase activity was detected using the Tyramide Signal Amplification (TSA®) Cyanine 5 System (PerkinElmer, Waltham, MA). The slides were counterstained with Alexa Fluor® 546-labeled goat anti-human IgG (Invitrogen, Eugene, OR) (spotting control).

The stained slides were scanned on a microarray scanner (InnoScan® 700AL, Innopsys, Carbonne, France). Fluorescence intensity, determined as mean values of quadruplicate samples, was determined using the Mapix® software (Innopsys, Carbonne, France). All intensity values were transformed into logarithmic variables. The reproducibility of our reverse-phase protein microarray assay was reported previously (18).

Immunohistochemistry

Twenty colorectal cancer cases were selected from the surgical pathology archive panel of the National Cancer Center Hospital, as described previously (24). Sections (4-μm thick) were cut from paraffin blocks of colorectal cancer tissues and mounted on silanized glass slides, and were subsequently stained by the avidin-biotin complex method. The primary antibody was the same as used in immunoblot analysis.

Statistical analysis

The statistical significance of inter-group differences was assessed with the Wilcoxon test, Mann-Whitney *U*-test, Welch's *t*-test, Kruskal-Wallis test, or Fisher's exact test, as appropriate. The area under the curve (AUC) value of the receiver operating characteristics (ROC) analysis was calculated for each marker to evaluate its diagnostic significance using ROCKIT software (version 0.9.1; the Kurt Rossmann Laboratories, Chicago, IL). A composite index of two markers was generated using the result of multivariate logistic regression analysis, which also enabled the calculation of sensitivity, specificity, and ROC curves. Statistical analyses were performed using an open-source statistical language R (version 2.7.0) with the optional module Design package.

Results

Plasma biomarker discovery by quantitative MS

To identify a diagnostic biomarker for patients with colorectal cancer including those with early-stage diseases, we compared the plasma proteomes of 22 colorectal cancer patients with those of 21 healthy controls (Training cohort) using 2DICAL (Table 1). Among a total of 53,009 independent MS peaks detected within the range 250-1,600 *m/z* and within the time range 20-70 minutes, we found 103 peaks with a discriminatory AUC value of > 0.800. A representative 2-dimensional view of all the MS peaks, with the *m/z* displayed along the X-axis and the LC retention time (RT) along the Y-axis, is shown in Figure 1A. The 103 MS peaks which distinguished between colorectal cancer patients and healthy controls with AUC values of > 0.800 are highlighted in red.

Eleven MS/MS spectra acquired from those 103 peaks matched six proteins in the database with Mascot score > 40 (Supplementary Table S1). We focused attention on a MS peak (ID 83) derived from the amino acid sequence of *ADFP* gene product (Supplementary Fig. S1), because the expression level of adipophilin was previously reported to be upregulated in clear cell renal carcinoma, but no such upregulation has been described in colorectal cancer. The adipophilin-derived MS peak (ID 83, at 749 *m/z* and 47.4 minutes) in representative patients from cancer and control groups is shown in Figure 1B. The distribution of the MS peak (ID 83) in patients with colorectal cancer (*red*) and healthy controls (*blue*) in the Training cohort (AUC = 0.814) is shown in Figure 1C. The differential expression and identification of adipophilin was confirmed by denaturing SDS-PAGE and immunoblotting analyses (Fig. 1D).

Protein microarray validation

To further validate the utility of using adipophilin for the diagnosis of colorectal cancer, the relative level of adipophilin in a total of 323 plasma samples was quantified using reverse-phase protein microarrays (Fig. 2). Quadruplicate spots for representative cases with high and low levels of adipophilin are shown in Figure 2. The power of plasma adipophilin level to discriminate colorectal cancer was validated in two larger independent Validation cohorts (V1: n = 210, V2: n = 113) that included early-stage colorectal cancer (Table 1). In the V1 cohort, the adopophilin level was significantly higher in patients with colorectal cancer than in healthy controls (Welch's t-test $P = 5.49\times10^{-10}$, Fig. 3A and Table 1), with an AUC value of 0.767 [95% confidential interval (CI): 0.699-0.825] (Fig. 3B). The colorectal cancer discriminatory power of adipophilin was also apparent in the V2 cohort (P = 0.00009, Fig. 3C and Table 1), with an AUC value of 0.742 [95% CI: 0.625-0.836] (Fig. 3B).

There was no difference in the plasma level of adipophilin among different disease stages (Kruskal-Wallis test $P = 0.280$). Notably, however, the adipophilin level was significantly higher even in patients with stage I or II disease (localized early colorectal cancer without metastasis to lymph nodes) than in healthy controls, while the CEA level in early-stage patients did not significantly differ from that of healthy controls (Table 2).

Adipophilin complements CEA

The levels of adipophilin and CEA were not mutually correlated (Pearson's $r = 0.13$ in the V1 cohort and 0.12 in the V2 cohort), and the AUC values of CEA in both cohorts (Fig. 3D) were comparable to that of a previous report (26). Combining adipophilin and CEA quantitation yielded a significant improvement in the ability to distinguish patients with colorectal cancer from healthy controls compared to quantitating CEA alone; the AUC improved to 0.849 [95% CI: 0.790-0.896] in the V1 cohort ($P = 0.0008$) and 0.787 [0.673-0.874] in the V2 cohort ($P = 0.022$) (Fig. 3D), indicating that plasma adipophilin and CEA have complementary diagnostic utility.

Due to the low prevalence of colorectal cancer among an asymptomatic population, a high specificity is required for a screening biomarker. If we defined the upper limit of the normal range of the composite index (adipophilin plus CEA; Fig. 3D) to include 95% of healthy controls in each validation cohort, the sensitivity of the index was 54% [95% CI: 41-66%] in the V1 cohort and 31% [13-56%] in the V2 cohort.

Adipophilin expression in colorectal cancer

The expression and cellular distribution of adipophilin in colorectal cancer tissues were examined using an immunohistochemical assay of eight well-differentiated, ten

moderately-differentiated, and two poorly-differentiated adenocarcinomas. A total of 14 of 20 cancer tissues from the well- and moderately-differentiated cases showed positive staining for adipophilin, but neither of the two poorly-differentiated samples was positive. In a majority of the well- and moderately-differentiated tumors, strong staining for adipophilin was observed in the cytoplasm or cell membrane of tumor glands facing the basement membrane (Fig. 4A and 4B). Adipophilin was not expressed in normal epithelial cells of the colorectal mucosa (Fig. 4C). The expression of adipophilin was clearly diminished in cancer cells invading in a scattered manner (Fig. 4D), which is consistent with the lack of staining observed in poorly-differentiated tumor samples.

DISCUSSION

In the present study, we first enriched the LMW plasma protein fraction using HFMT, then compared its contents between patients with colorectal cancer and healthy controls using 2DICAL (Fig. 1). The high efficacy of combining HFMT and 2DICAL for plasma biomarker discovery was demonstrated for the first time in our previous study of pancreatic cancer (17), and the present results further strengthened the credible evidence for the applicability of this combination of methods to all types of future plasma biomarker research. Any biomarker candidate identified by proteomic approaches must be validated using a different method in a statistically sufficient number of cases and controls before it can be considered for clinical application. We employed another innovative technology, a reverse-phase protein microarray, for independent validation of our finding that adipophilin discriminates colorectal cancer (Fig. 2). Our high-density protein microarray enabled the high-throughput quantification of one protein in hundreds of clinical samples in one experiment (18), while keeping the required volume

of each sample to a minimum (nanoliter level). Although the availability of clinical samples is often limited, it is often necessary to waste hundreds of microlitters of samples for preliminary experiments involving techniques such as conventional enzyme-linked immunosorbent assay (ELISA). Because of their minimal sample requirements, plasma microarrays are considered to be ideal alternatives to ELISAs for biomarker validation. However, the absolute concentration and optimal cut-off value of adipophilin were not determined in the present study. It may be necessary to establish an ELISA prior to the clinical application of the present results.

Although the expression of adipophilin is known to be induced in various types of pathological and physiological conditions such as lactating mammary epithelial cells, few studies have assessed the significance of its expression in cancer cells (27, 28). We found that adipophilin is expressed in well- or moderately-differentiated adenocarcinomas, but not in the adjacent normal colonic mucosa or poorly-differentiated adenocarcinoma (Fig. 4). The immunohistochemical data suggest that the expression of adipophilin is induced during the process of early colorectal carcinogenesis, but lost during the process of cancer promotion. Consistent with our findings, Yao *et al.* also reported that adipophilin expression correlates well with the differentiation status of clear cell renal carcinoma of the kidney (29). They also reported that adipophilin expression is a prognostic factor for the cancer-specific survival of patients with renal clear cell carcinoma (29). The prognostic significance of adipophilin expression in colorectal cancer, however, remains to be determined.

The expression of adipophilin is known to be regulated by hypoxia inducible factor (HIF) and the peroxisome proliferator-activated receptor (PPAR) family of proteins. Both HIF and PPAR were reported to be closely involved in carcinogenesis, especially in colorectal cancer (30, 31). Moreover, PPARγ may be a molecular target of

anticancer therapy (32). Since the exact nature of the interactions between these proteins (adipophilin, HIF, and PPAR) has not been extensively investigated, further studies are needed to elucidate the biological and clinicopathological significance of adipophilin expression in colorectal cancer. The present findings may provide novel insights into the molecular mechanism of colorectal cancer development and progression and into the development of new anticancer therapeutics.

There are some limitations to our study. First, we have no data regarding the body-mass index of cases included in the present study. The relationship between obesity and an increased risk of colon cancer is now generally accepted (33-35), and alteration of adipocytokine levels can reportedly affect intestinal carcinogenesis (36). Although adipophilin was originally identified as a marker of adipocyte development (27, 37), its relevance to body shape and cachexia remain to be elucidated. Adipophilin is a 50 kDa protein belonging to the PAT family (perilipin, adipophilin, TIP47, S3-12, and OXPAT), which comprises proteins involved in the coating of lipid droplets (27, 38, 39). Secondly, we have no data of FOB test results for the cases used in this study, and thus it was not possible to demonstrate the superiority of adipophilin to FOB. However, a recent large-scale study demonstrated that 11% of patients with negative FOB results had cancers or adenomas that required treatment (40). Since the adipophilin level was significantly elevated even in patients with localized early colorectal cancer (Table 2), adipophilin may supplement or surpass the diagnostic power of FOB. Finally, there was a difference in the age distribution between cancer and control in all cohorts. However, age did not correlate with plasma adipophilin level in the cancer and control group (Pearson's $r = 0.03$ and $r = 0.09$, respectively). We therefore estimate the influence of difference in age to be negligible.

In conclusion, we identified plasma adipophilin as a new tumor marker for

colorectal cancer using LMW protein profiling. The increase of plasma adipophilin level in colorectal cancer was validated in two larger cohorts, and the diagnostic power was revealed to be superior to that of CEA in the detection of early-stage (stages I and II) colorectal cancer. To our knowledge, this is the first study demonstrating the expression of adipophilin in colorectal cancer. While bearing the above limitations in mind, an independent validation study is warranted.

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FIGURE LEGENDS

Figure 1.

(A) Two-dimensional display of all (>53,000) MS peaks detected. The 103 MS peaks for which the mean intensity determined in duplicate analyses distinguished between colorectal cancer and healthy control patients (with AUC values >0.800) are highlighted in red.

(B) Adipophilin-derived MS peaks in representative patients from cancer and control groups. Arrows indicate ID 83, at 749 *m/z* and a RT of 47.4 minutes.

(C) Adipophilin-derived MS peaks (ID 83) in 43 duplicate LC-MS runs aligned according to RT (*top*). Columns represent the mean intensity of duplicate analyses of the 43 individuals in the Training cohort (*bottom*).

(D) Verification of quantitative MS data and protein identification. The levels of plasma adipophilin and complement C3b-α (loading control) were determined using immunoblotting in representative colorectal cancer patients and healthy individuals selected from the Training cohort. Recombinant adipophilin (0.3 μg) was applied as a positive control (lane next to the molecular weight standard ladder).

Figure 2.

Representative reverse-phase protein microarray slide of the V1 cohort stained with anti-ADFP antibody (*left*). Magnified images of quadruplicate spots of representative individuals with high and low levels of adipophilin (*right*).

Figure 3.

(A, C) Plasma adipophilin level in healthy controls and patients with colorectal cancer in the V1 (A) and V2 (C) cohorts. Horizontal lines represent the average adipophilin

level.

(B) ROC analyses illustrating the discriminatory capability of adipophilin in the V1 (*solid line*) and V2 (*dashed line*) cohorts.

(D) ROC analyses illustrating the discriminatory value of CEA and the composite index of adipophilin and CEA in the V1 and V2 cohorts.

Figure 4.

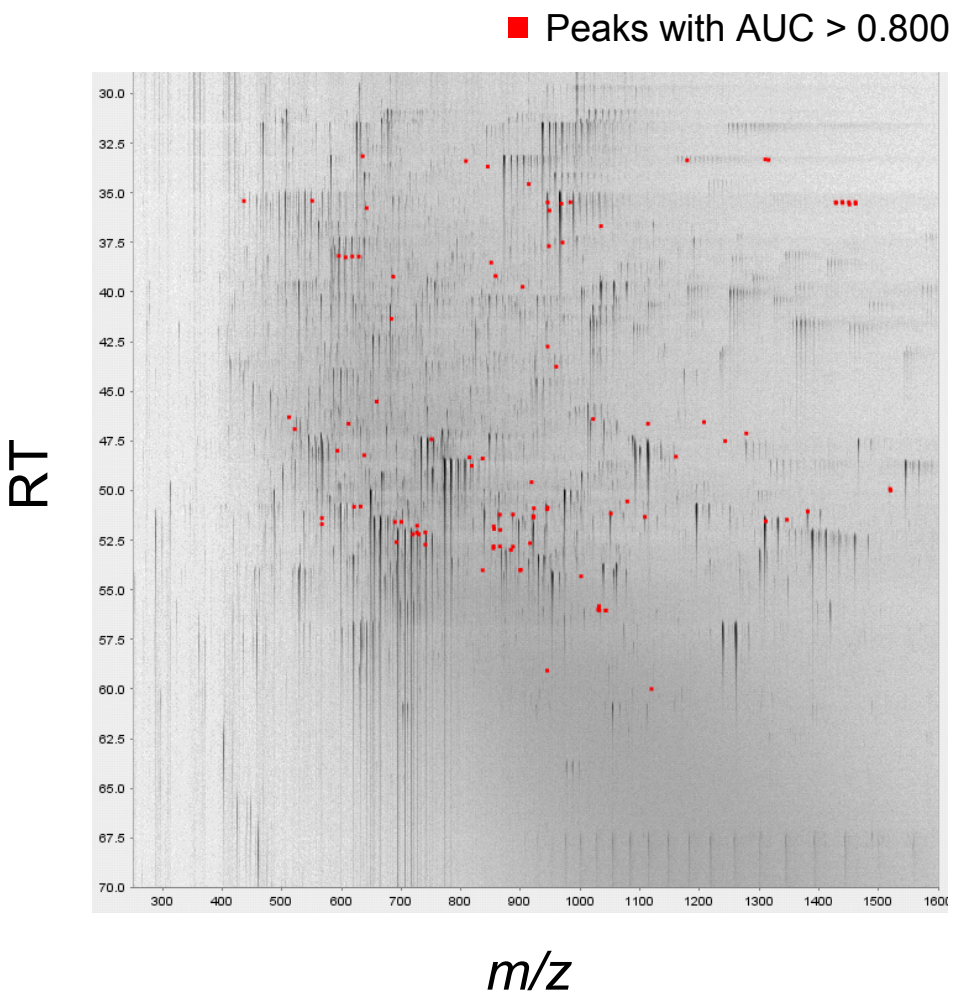
Immunohistochemical analysis of adipophilin in colorectal cancer (A-D) and adjacent normal colonic mucosa (designated by N) (C).

Original magnification; A and D = 100×; B = 400×; C = 40×.

LIST OF TABLES

Figure 1

A


Peaks with AUC > 0.800
RT
30.0
32.5
35.0
37.5
40.0
42.5
45.0
47.5
50.0
52.5
55.0
57.5
60.0
62.5
65.0
67.5
70.0
300
400
500
600
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800
900
1000
1100
1200
1300
1400
1500
m/z

B

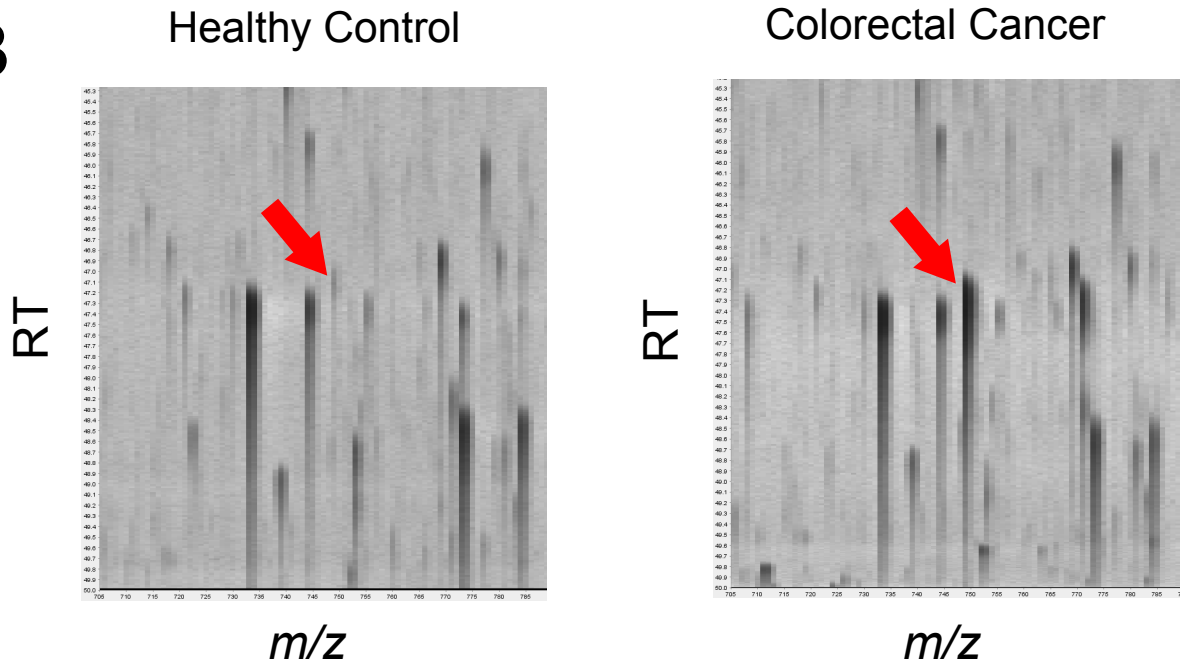
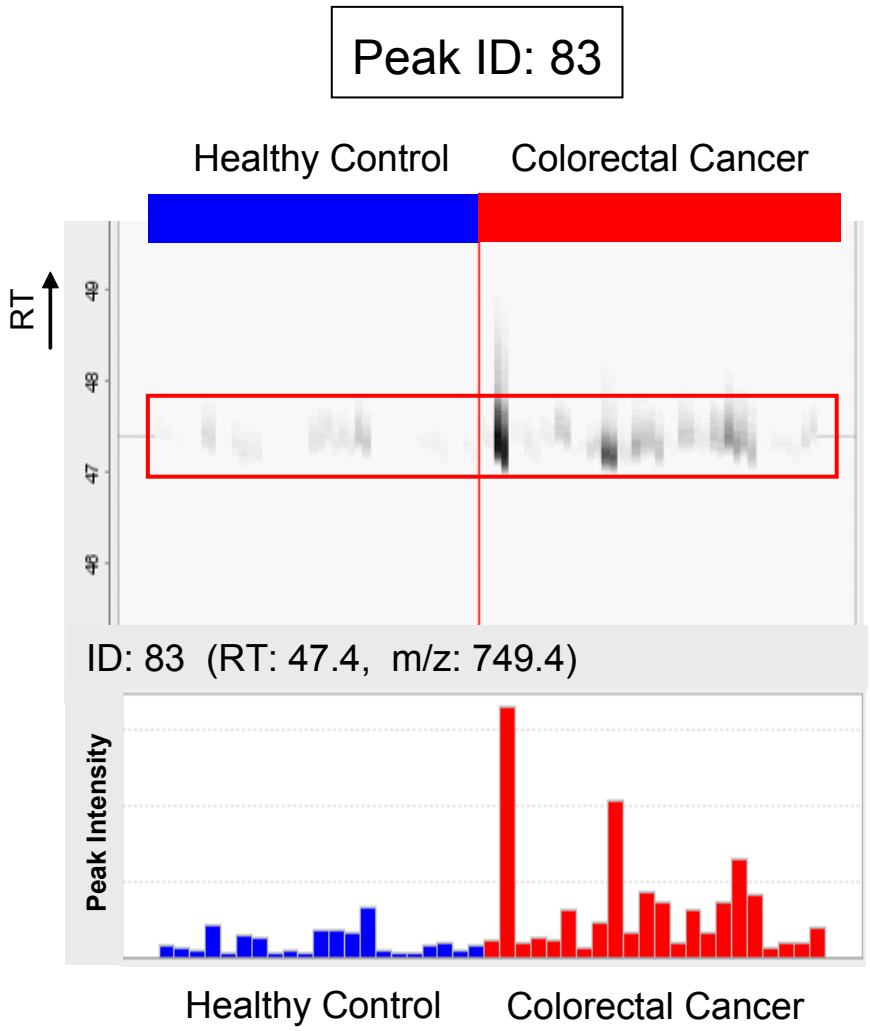

Healthy Control
Colorectal Cancer
RT
RT
m/z
m/z

Figure 1

C


Peak ID: 83
Healthy Control
Colorectal Cancer
RT
49
48
47
46
ID: 83 (RT: 47.4, m/z: 749.4)
Peak Intensity
Healthy Control
Colorectal Cancer

D

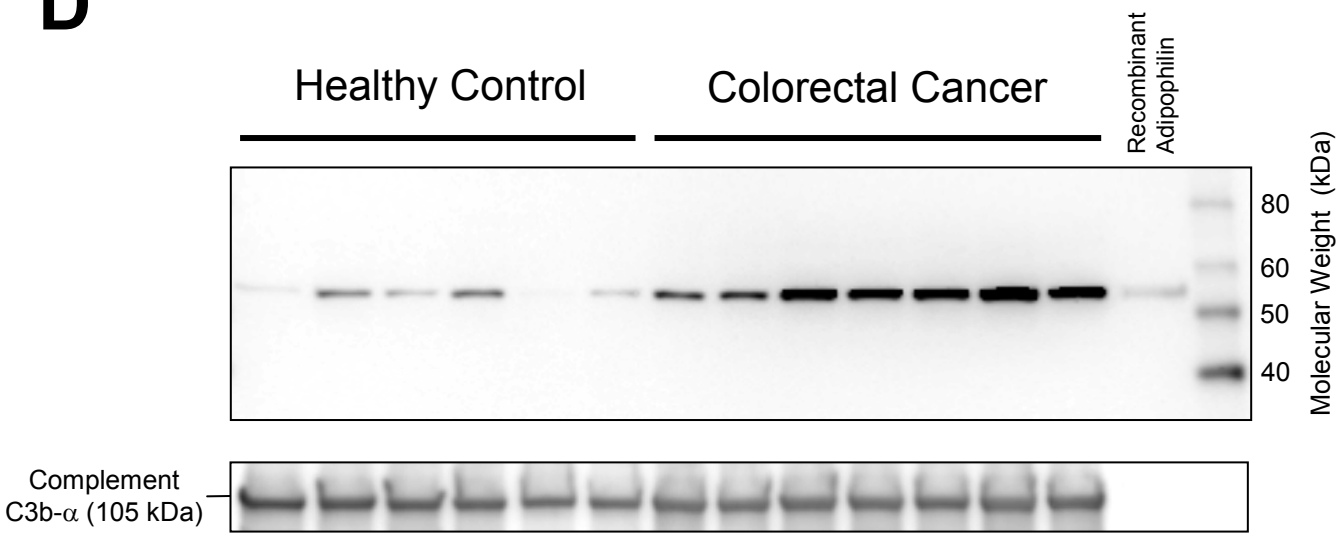
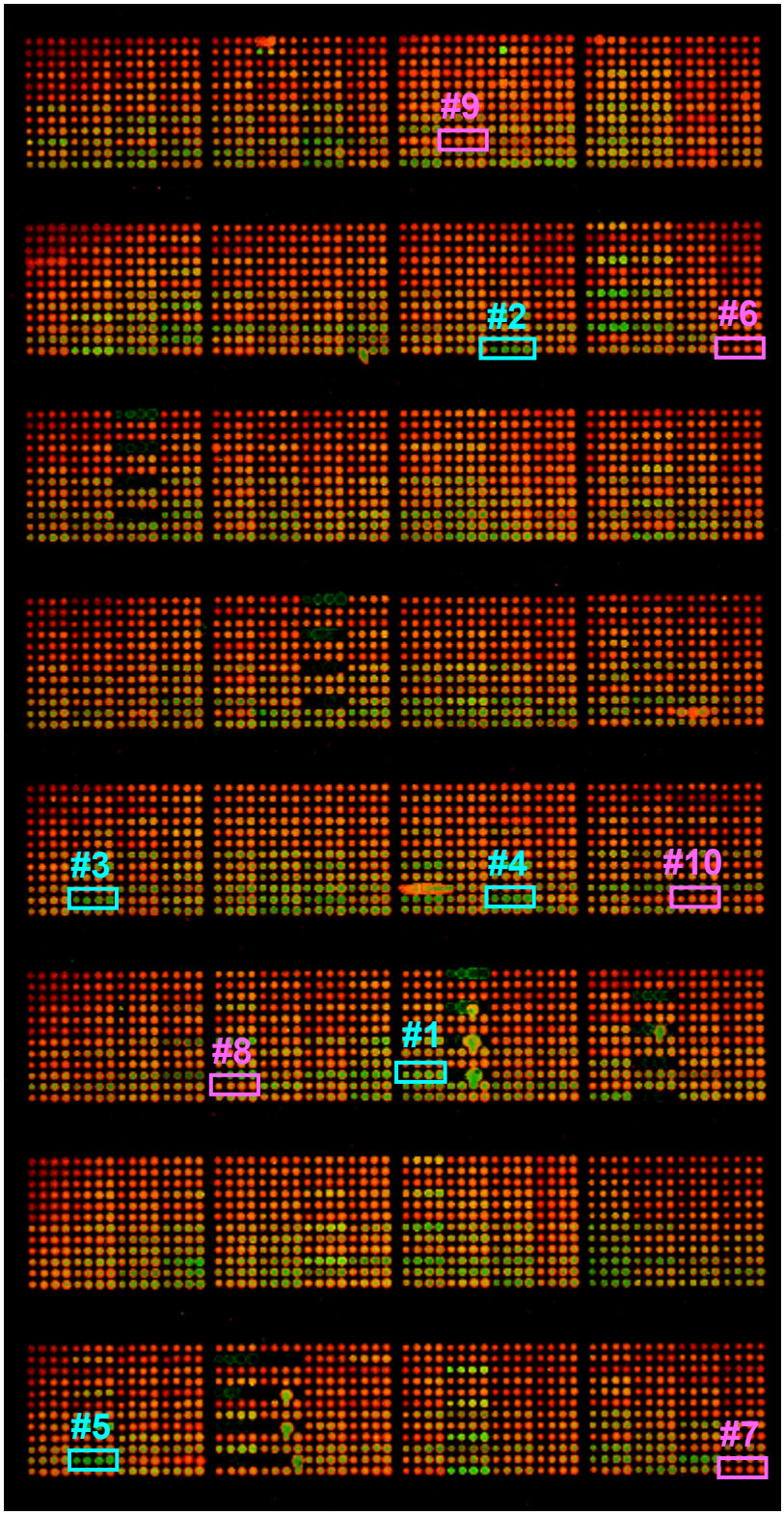

Healthy Control
Colorectal Cancer
Recombinant Adipophilin
80
60
50
40
Molecular Weight (kDa)
Complement C3b-α (105 kDa)

Figure 2



Healthy Control

#1

#2

#3

#4

#5

Colorectal Cancer

#6

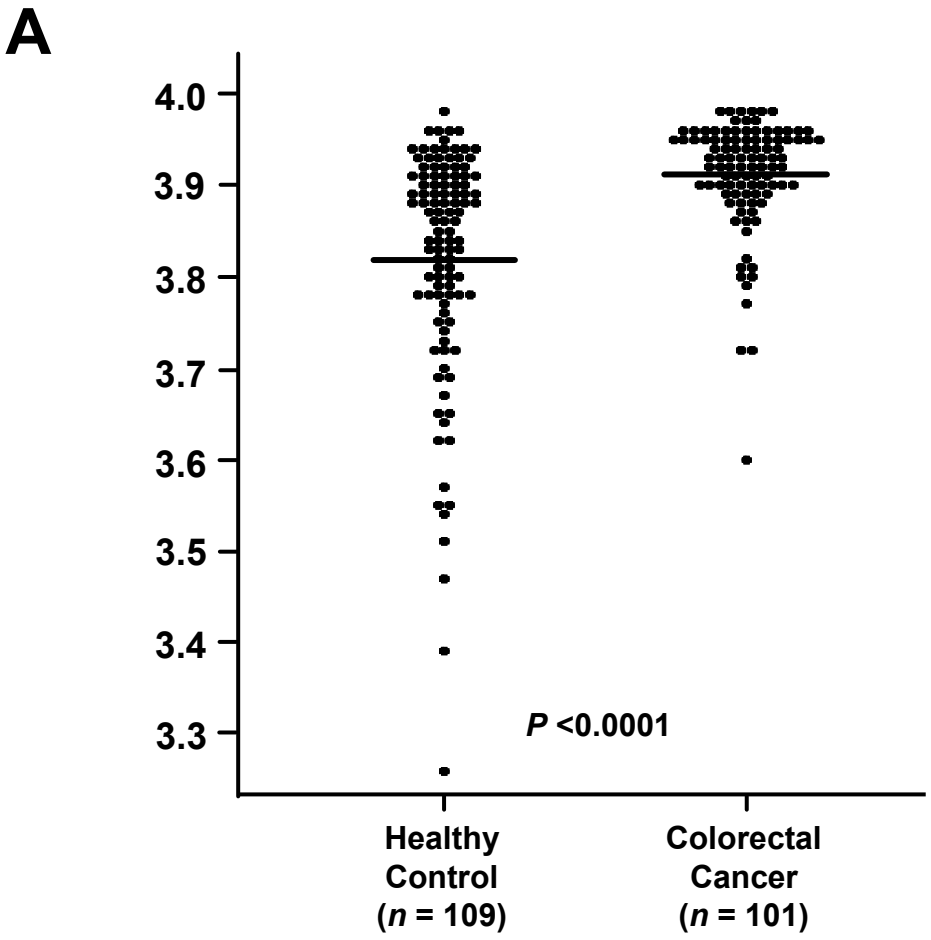
#7

#8

#9

#10

Figure 3


A
4.0
3.9
3.8
3.7
3.6
3.5
3.4
3.3
P <0.0001
Healthy Control (n = 109)
Colorectal Cancer (n = 101)

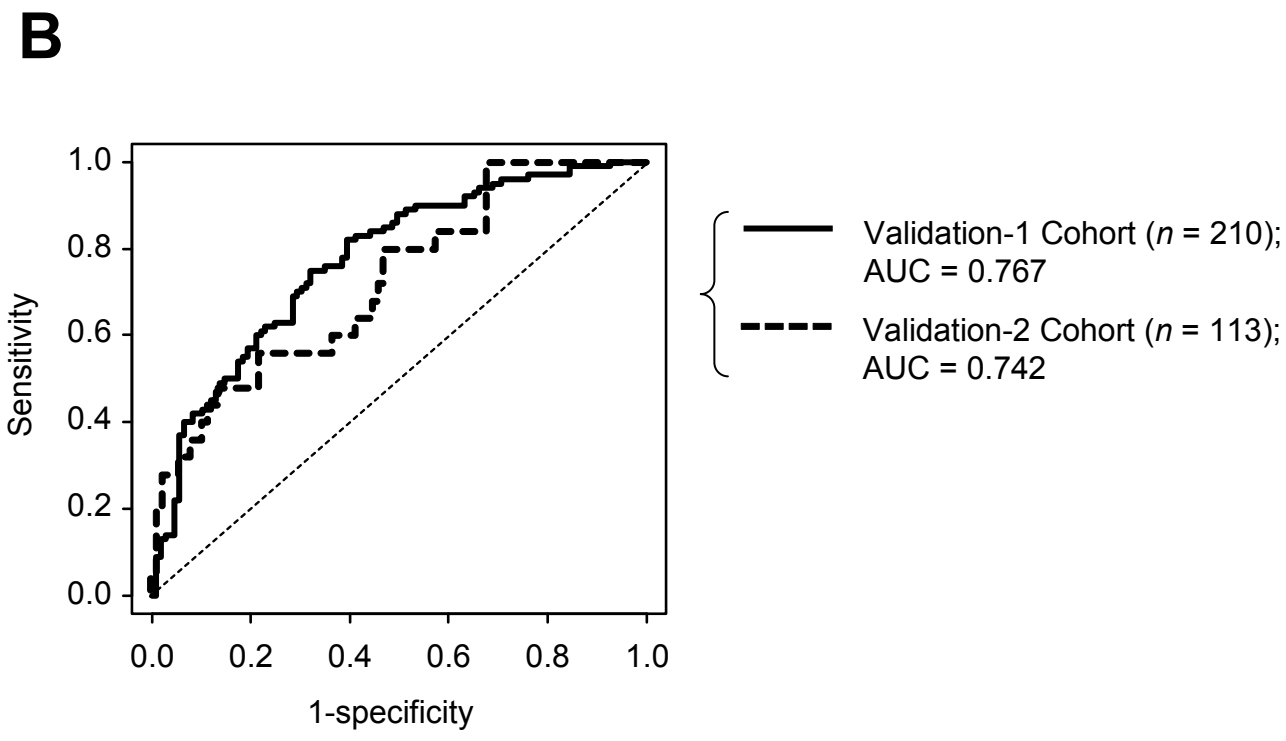
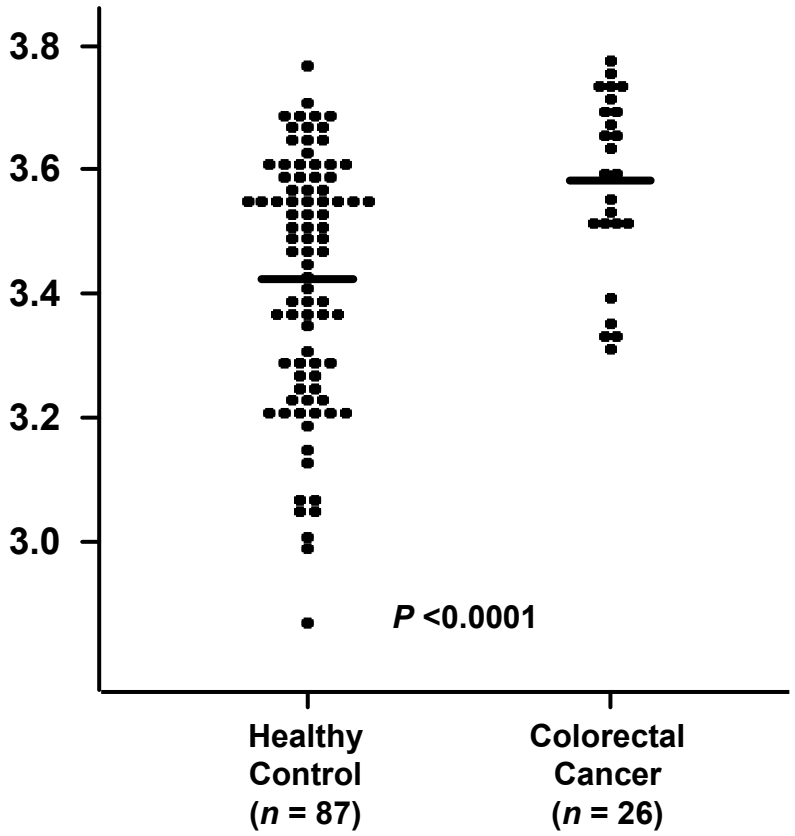

B
1.0
0.8
0.6
0.4
0.2
0.0
Sensitivity
0.0
0.2
0.4
0.6
0.8
1.0
1-specificity
Validation-1 Cohort (n = 210); AUC = 0.767
Validation-2 Cohort (n = 113); AUC = 0.742

Figure 3

C


3.8
3.6
3.4
3.2
3.0
P <0.0001
Healthy Control (n = 87)
Colorectal Cancer (n = 26)

D

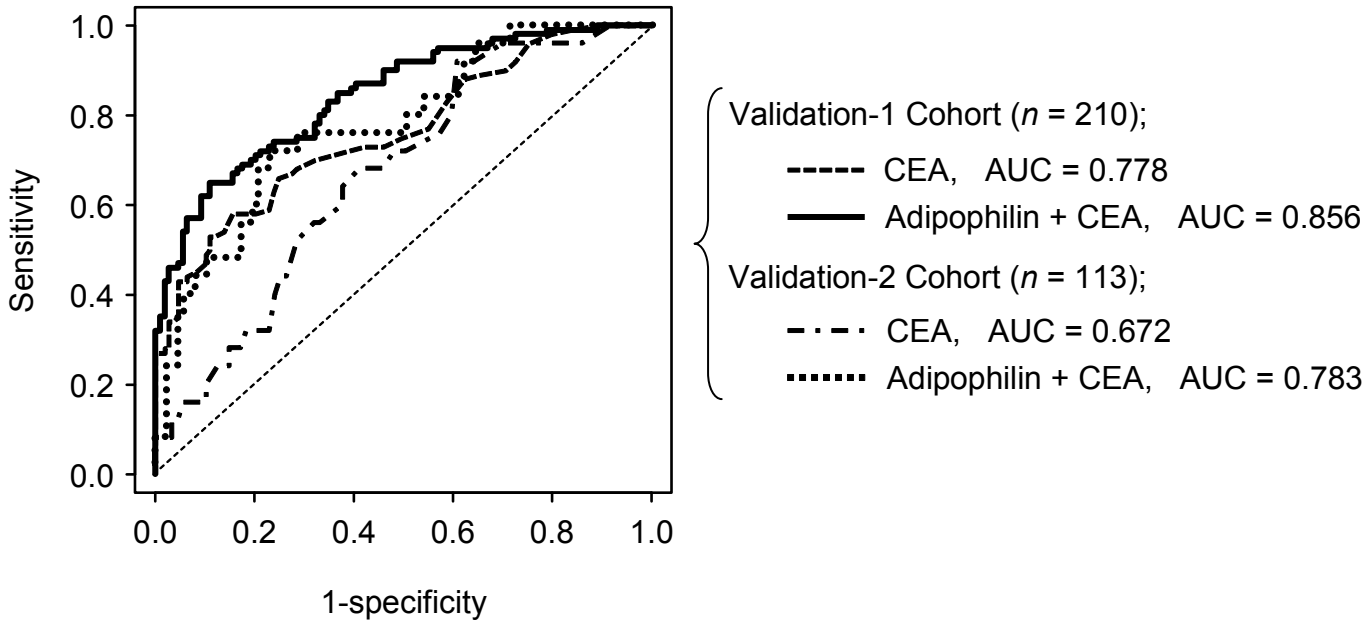

1.0
0.8
0.6
0.4
0.2
0.0
Sensitivity
0.0
0.2
0.4
0.6
0.8
1.0
1-specificity
Validation-1 Cohort (n = 210);
CEA, AUC = 0.778
Adipophilin + CEA, AUC = 0.856
Validation-2 Cohort (n = 113);
CEA, AUC = 0.672
Adipophilin + CEA, AUC = 0.783

Figure 4

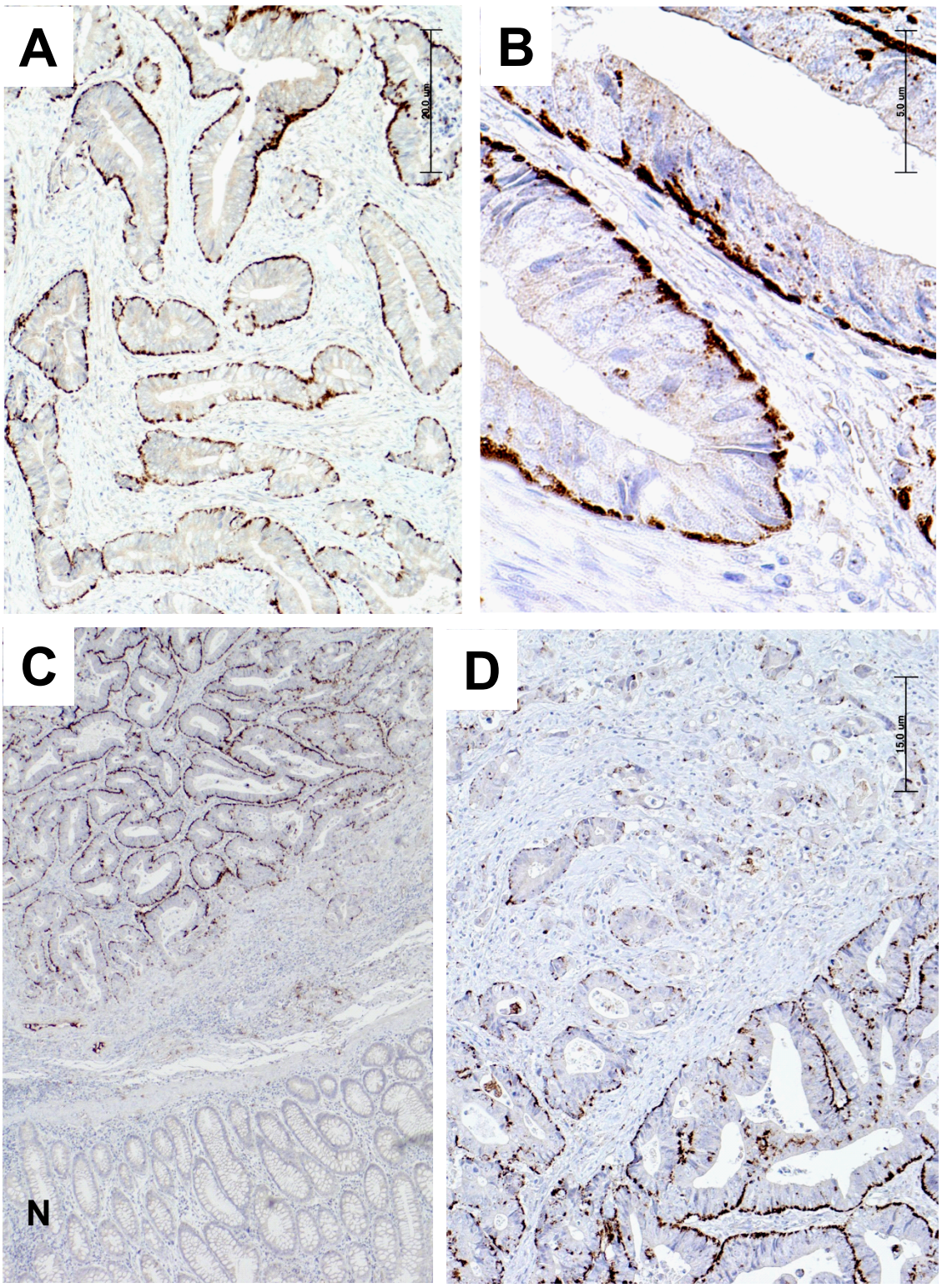


Table 1. Clinicopathologic Characteristics of Cases in the Training and Validation Cohorts

	Training Cohort (*n* = 43)			Validation-1 Cohort (*n* = 210)			Validation-2 Cohort (*n* = 113)		
	Cancer	**Healthy**	***P***	**Cancer**	**Healthy**	***P***	**Cancer**	**Healthy**	***P***
No. of patients	22	21		101	109		26	87	
Sex, no. of patients			0.310*			0.782*			0.252*
Male	14	17		63	70		13	56	
Female	8	4		38	39		13	31	
Age, years			<0.001			<0.001			<0.001
mean (SD)	62 (12)	40 (13)		64 (11)	42 (14)		63 (12)	43 (16)	
Tumor location			*NA*			*NA*			*NA*
Colon	22	—		88	—		24	—	
Rectum	0	—		13	—		2	—	
Clinical stage			*NA*			*NA*			*NA*
I	3	—		19	—		12	—	
II	6	—		31	—		5	—	
III	8	—		32	—		8	—	
IV	5	—		17	—		1	—	
unknown	0	—		2	—		0	—	
CA19-9									
median, U/mL	14.7	5.5	0.010	4	1.6	<0.001	9.4	10.2	0.680
>37.0 (ULN), no. of patients	6	2		39	5		2	4	
CEA									
median, ng/mL	3.5	1.7	0.002	11.8	7.6	0.001	2.6	1.7	0.008
>5.0 (ULN), no. of patients	9	1		24	5		4	5	
Total bilirubin									
median, mg/dL	0.4	0.5	0.114	0.4	0.5	<0.001	0.4	0.5	<0.001
>1.2 (ULN), no. of patients	0	0		1	3		0	4	
Adipophilin									
Mass spectrometry peak intensity†, mean (SD)	320 (375)	96 (78)	<0.001§	—	—		—	—	
Protein intensity‡, mean (SD)	—	—		3.91 (0.06)	3.82 (0.13)	<0.001‖	3.57 (0.14)	3.42 (0.20)	<0.001‖

NOTE. Wilcoxon test was applied to assess differences between values. *Calculated by Fisher's exact test. †Intensity of the corresponding peak as measured using quantitative mass spectrometry. ‡Measured using reverse-phase protein microarray (logarithmic variable). §Calculated using Mann-Whitney *U*-test. ‖Calculated using Welch's *t*-test.

Abbreviations: SD, standard deviation; NA, not applicable; ULN, upper limit of normal.

Table 2. Plasma Adipophilin and CEA Levels According to Clinical Stage of Colorectal Cancer (V1 Cohort)

	Colorectal Cancer Patients				Healthy Controls
	Stage I	**Stage II**	**Stage III**	**Stage IV**	
No. of cases	19	31	32	17	109
Adipophilin*, mean (SD)	3.90 (0.05)	3.91 (0.07)	3.91 (0.07)	3.93 (0.03)	3.82 (0.13)
P † (vs. healthy controls)	1.07×10^{-5}	3.31×10^{-6}	1.65×10^{-6}	2.27×10^{-11}	—
CEA, mean (SD), ng/mL	2.63 (1.71)	13.7 (36.2)	224 (1068)	200 (579)	2.07 (1.74)
P † (vs. healthy controls)	0.20	0.09	0.25	0.18	—

*Measured using a reverse-phase protein microarray (values were transformed into logarithmic variables).

†Welch's t-test (comparison to healthy controls).

Abbreviation: SD, standard deviation.

LEGEND FOR SUPPLEMENTARY FIGURE

Supplementary Figure S1 (online).

MS/MS spectra and database search result for a single MS peak (ID 83) derived from adipophilin. The adipophilin peptide matching the amino acid sequence in the database is highlighted in red (*bottom*).

LIST OF TABLES

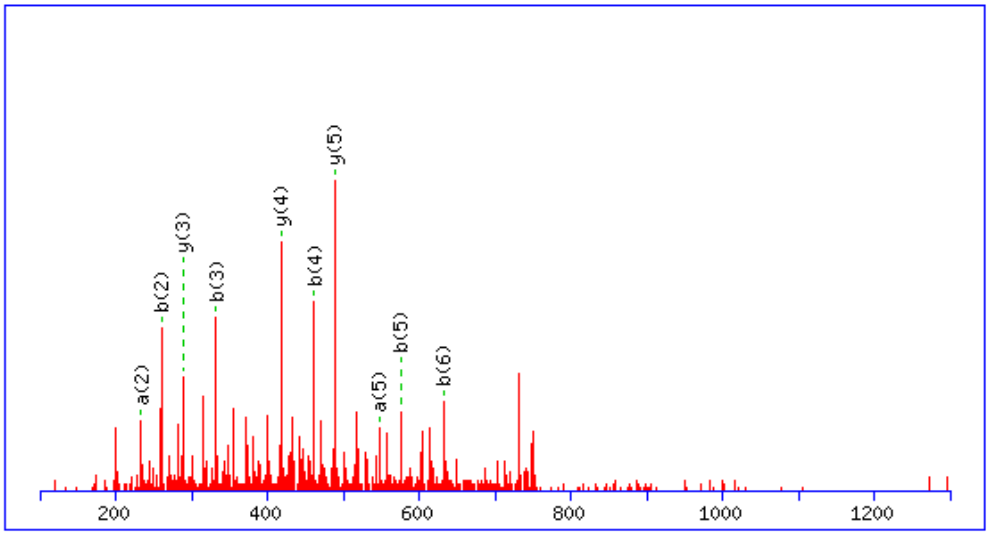
Supplementary Table S1 (online).

Plasma Proteins for which the MS Peak Intensity Differed Significantly between Healthy Controls and Patients with Colorectal Cancer.

Supplementary Figure S1

Peak ID: 83

MS/MS Fragmentation of **EMAENGV**
Found in **ADFP_HUMAN**, Adipophilin (Adipose differentiation-related protein) (ADRP) - Homo sapiens (Human)
Match to Query 2: 748.347984 from(749.355260,1+)



Monoisotopic mass of neutral peptide Mr(calc): 748.3061
Ions Score: 44 Expect: 0.21
Matches (Bold Red): 10/26 fragment ions using 25 most intense peaks

#	a	a*	b	b*	Seq.	y	y*	#
1	102.0550		130.0499		E			7
2	**233.0954**		**261.0904**		M	620.2708	603.2443	6
3	304.1326		**332.1275**		A	**489.2304**	472.2038	5
4	433.1751		**461.1701**		E	**418.1932**	401.1667	4
5	**547.2181**	530.1915	**575.2130**	558.1864	N	**289.1506**	272.1241	3
6	604.2395	587.2130	**632.2345**	615.2079	G	175.1077		2
7					V	118.0863		1

Matched peptides shown in Bold Red

```
  1 MASVAVDPQP SVVTRVVNLP LVSSTYDLMS SAYLSTKDQY PYLKSVCEMA
 51 ENGVKTITSV AMTSALPIIQ KLEPQIAVAN TYACKGLDRI EERLPILNQP
101 STQIVANAKG AVTGAKDAVT TTVTGAKDSV ASTITGVMDK TKGAVTGSVE
151 KTKSVVSGSI NTVLGSRMMQ LVSSGVENAL TKSELLVEQY LPLTEEELEK
201 EAKKVEGFDL VQKPSYYVRL GSLSTKLHSR AYQQALSRVK EAKQKSQQTI
251 SQLHSTVHLI EFARKNVYSA NQKIQDAQDK LYLSWVEWKR SIGYDDTDES
301 HCAEHIESRT LAIARNLTQQ LQTTCHTLLS NIQGVPQNIQ DQAKHMGVMA
351 GDIYSVFRNA ASFKEVSDSL LTSSKGQLQK MKESLDDVMD YLVNNTPLNW
401 LVGPFYPQLT ESQNAQDQGA EMDKSSQETQ RSEHKTH
```

Supplementary Table S1. Plasma Proteins for which the MS Peak Intensity Differed Significantly Between Healthy Controls and Patients with Colorectal Cancer

Gene locus	Protein identification	Mascot score	AUC	Matched peptide
HBD_HUMAN	Hemoglobin subunit delta (Hemoglobin delta chain) (Delta-globin) - Homo sapiens (Human)	90.18	0.86	1
HBB_HUMAN	Hemoglobin subunit beta (Hemoglobin beta chain) (Beta-globin) - Homo sapiens (Human)	74.50	0.82	5
RETBP_HUMAN	Plasma retinol-binding protein precursor (PRBP) (RBP) [Contains: Plasma retinol-binding protein (1-182); Plasma retinol-binding protein (1-181); Plasma retinol-binding protein (1-179); Plasma retinol-binding protein (1-176)] - Homo sapiens (Human)	66.52	0.82	1
HBA_HUMAN	Hemoglobin subunit alpha (Hemoglobin alpha chain) (Alpha-globin) - Homo sapiens (Human)	63.75	0.81	2
ADFP_HUMAN	Adipophilin (Adipose differentiation-related protein) (ADRP) - Homo sapiens (Human)	43.67	0.81	1
PKDRE_HUMAN	Polycystic kidney disease and receptor for egg jelly-related protein precursor (PKD and REJ homolog) - Homo sapiens (Human)	41.62	0.81	1

NOTE. *Peaks with a Mascot score >40.