はほとんどないと考えられる。担当医師の判断で 13 mL でも身体的な影響が予測される場合、当然本研究の対象としては除外する。

本研究で解析するのは血中のペプチドとタンパク質のみであり、「ヒトゲノム・遺伝子解析研究に関する倫理指針」に定められている生殖細胞系のゲノム・遺伝子情報は含まれない。本研究は既に診断された臨床診断・病理診断と血漿、血清のプロテオーム解析結果をレトロスペクティブに比較する研究であり、提供者の診断や治療方針に一切影響を与えない。

解析者には上記にある臨床情報のみが提供されるため、プロテオームの解析結果と姓名などの個人情報が関連付けられることもない。プロテオーム解析を行うのは法的に守秘義務をもつ医師または歯科医師の国立がん研究センター研究所とプロテオームリサーチセンターの職員、その監督・指導下に業務を行う国立がん研究センターとプロテオームリサーチセンターの職員と登録された常勤の研究補助員、癌研究振興財団のリサーチレジデントと医師または歯科医師の研修者に限られる。解析を行う実験室は使用時以外施錠し、個人情報を電子媒体として外部ネットワークに接続されたコンピューターに記憶させることはない。匿名化措置とこのような情報の厳密な管理により、個人情報が遺漏する可能性はないと考えられる。また解析後の検体は適切な方法で破棄する。

以上により提供者に対し、本研究により新たに生じる提供者個人の不利益で明らかに予測されるものはない。

１１．集計表（記録表）とその保管

創薬臨床研究分野には上記にある観察項目、臨床検査項目のみが提供されるため、プロテオームの解析結果と提供者の氏名や ID 番号などの個人情報が関連付けられることはない。解析結果の生データは創薬臨床研究分野解析室のサーバーコンピューターとそのバックアップ以外に保存しない。解析室は作業中以外常時施錠し、関係者以外を入室させない。プロテオーム解析を行うのは法的に守秘義務をもつ医師または歯科医師の国立がん研究センター研究所の職員と、その監督・指導下に業務を行う国立がん研究センターの職員と登録された常勤の研究補助員、癌研究振興財団のリサーチレジデントと医師または歯科医師の研修者に限られる。

１２．予後の追跡方法、期間

本研究には該当しない。

１３．最終集計・解析方法

各種がんについて、対照者に比べ、量や翻訳後修飾が変化するタンパク質を探索し、見出された腫瘍マーカー候補の測定系を構築し、健常者および他の疾患（他臓器のがん、良性疾患）とのあいだで定量比較し、疾患特異性・臓器特異性を検討する。また、同一疾患内で病期別発現を調べ、早期診断マーカーとしての有用性を検討する。実用化にあたっては、既存の腫瘍マーカーとの優劣を比較して判断する。

１４．研究成果の発表方法

本研究の成果は特許申請の可能性を検討後、多施設共同研究として学術論文や学会発表として発表し、その成果を広く医療の進歩のために還元する。これらの情報の公開に際しては、個人情報が含まれることはない。

１５．研究費算定根拠

既に解析に使用する機器は収得済みであるので、採血管などの消耗品と株式会社エスアールエルに委託して行う既存の腫瘍マーカーを測定するための研究費が必要である。委託測定の経費は国立がん研究センター研究所創薬臨床研究分野が負担する。

研究費
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１６．病歴の記載：プロトコール番号、実施年月、後の調査に必要な記録など

実施年月
本研究の承認日から平成２７年３月３１日まで
　ただし、必要に応じて国立がん研究センターと各共同研究施設の倫理審査委員会の審査と承認を経て、研究期間を延長することがある。

病歴の記載：プロトコール番号、後の調査に必要な記録など
該当しない。

１７．モニタリングの内容及び方法

該当しない。

１８．特許に関連したとりきめ（得られたデータの帰属）

本研究で臨床応用の可能性と市場性のある成果が得られた場合に限り、協議し、互いの同意を得た上で、特許を出願する。

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Cancer Epidemiology, Biomarkers & Prevention

Research Article

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

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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 two-dimensional image converted analysis of liquid chromatography and mass spectrometry (2DICAL), we compared the plasma proteome of 22 colorectal cancer patients with 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. *Cancer Epidemiol Biomarkers Prev; 20(10); 2195–203. ©2011 AACR.*

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 showed 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

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Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (http://cebp.aacrjournals.org/).

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reexamination after the FOB test, placing excessive physical and physiologic 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 2-dimensional image converted analysis of liquid chromatography and mass spectrometry (2DICAL; ref. 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 biological 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 apolipoproteins 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 3 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 7 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 histologic or cytologic 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 7 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 preanalytical bias caused by differences in sample handling. Blood was collected in a tube with 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 25 mmol/L ammonium bicarbonate buffer (pH 8.0). The total of 4 mL of the plasma dilution was injected into a HFMT machine (22). After 1 hour of fully automated operation, the solution containing LMW proteins was recovered and lyophilized.

Liquid chromatography mass spectrometry

The HFMT-treated samples were digested with sequencing grade modified trypsin (Promega) and analyzed in duplicate using a nano flow high-performance

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Table 1. Clinicopathologic characteristics of cases in the training ($n = 43$) and validation cohorts (V1: $n = 210$; V2: $n = 113$)

	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[a]			0.782[a]			0.252[a]
Male	14	17		63	70		13	56	
Female	8	4		38	39		13	31	
Age, y			<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	Ð		88	Ð		24	Ð	
Rectum	0	Ð		13	Ð		2	Ð	
Clinical stage			*NA*			*NA*			*NA*
I	3	Ð		19	Ð		12	Ð	
II	6	Ð		31	Ð		5	Ð	
III	8	Ð		32	Ð		8	Ð	
IV	5	Ð		17	Ð		1	Ð	
Unknown	0	Ð		2	Ð		0	Ð	
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[b], mean (SD)	320 (375)	96 (78)	<0.001[c]	Ð	Ð		Ð	Ð	
Protein intensity[d], mean (SD)	Ð	Ð		3.91 (0.06)	3.82 (0.13)	<0.001[e]	3.57 (0.14)	3.42 (0.20)	<0.001[e]

NOTE: Wilcoxon test was applied to assess differences between values.
Abbreviations: NA, not applicable; ULN, upper limit of normal.
[a]Calculated by Fisher's exact test.
[b]Intensity of the corresponding peak as measured using quantitative mass spectrometry.
[c]Calculated using Mann–Whitney *U* test.
[d]Measured using reverse-phase protein microarray (logarithmic variable).
[e]Calculated using Welch's *t*-test.

liquid chromatography (NanoFrontier nLC; Hitachi High-technologies) connected to an electrospray ionization quadrupole time-of-flight (ESI-Q-TOF) mass spectrometer (Q-Tof Ultima; Waters).

Mass spectrometry (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 53,009). The stability of liquid chromatography mass spectrometry (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 ($\pm$ SD) was as high as 0.951 ($\pm$ 0.039) and the mean CV was as low as 0.054 ($\pm$ 0.011).

Protein identification by tandem mass spectrometry

Peak lists were generated using the Mass Navigator software package (version 1.2; Mitsui Knowledge Industry) 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). The search parameters used were as follows: the human protein database was selected; up to 1 missed cleavage was allowed; "none" was designated as the enzyme; mass tolerances for precursor and fragment ions were $\pm$ 0.6 and

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± 0.2 Da, respectively; the score threshold was set to $P < 0.05$ on the basis of 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 (mAb) against adipophilin (LifeSpan Biosciences) and mouse mAb against human complement C3b-α (Progen). Ten microliter of 1:50 diluted plasma sample and 0.3 μg of fully recombinant adipophilin (BioVendor) 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 (HRP)-conjugated anti-mouse IgG as described previously (24, 25). Blots were developed using an enhanced chemiluminescence detection system (GE Healthcare).

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), and randomly plotted onto ProteoChip glass slides (Proteogen) in quadruplicate in a 6144-spot/slide format using a Protein Microarrayer Robot (Kaken Geneqs). 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) followed by streptavidin–HRP conjugate (GE Healthcare). Peroxidase activity was detected using the Tyramide Signal Amplification Cyanine 5 System (PerkinElmer). The slides were counterstained with Alexa Fluor 546–labeled goat anti-human IgG (Invitrogen; spotting control).

The stained slides were scanned on a microarray scanner (InnoScan 700AL; Innopsys). Fluorescence intensity, determined as mean values of quadruplicate samples, was determined using the Mapix software (Innopsys). 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 intergroup 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). A composite index of 2 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 done 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 to 1,600 m/z and within the time range 20 to 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 tandem mass spectrometry spectra acquired from those 103 peaks matched 6 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 2 larger independent validation cohorts (V1: $n = 210$, V2: $n = 113$) that included early-stage colorectal cancer (Table 1). In the V1 cohort, the adipophilin level was significantly higher in patients

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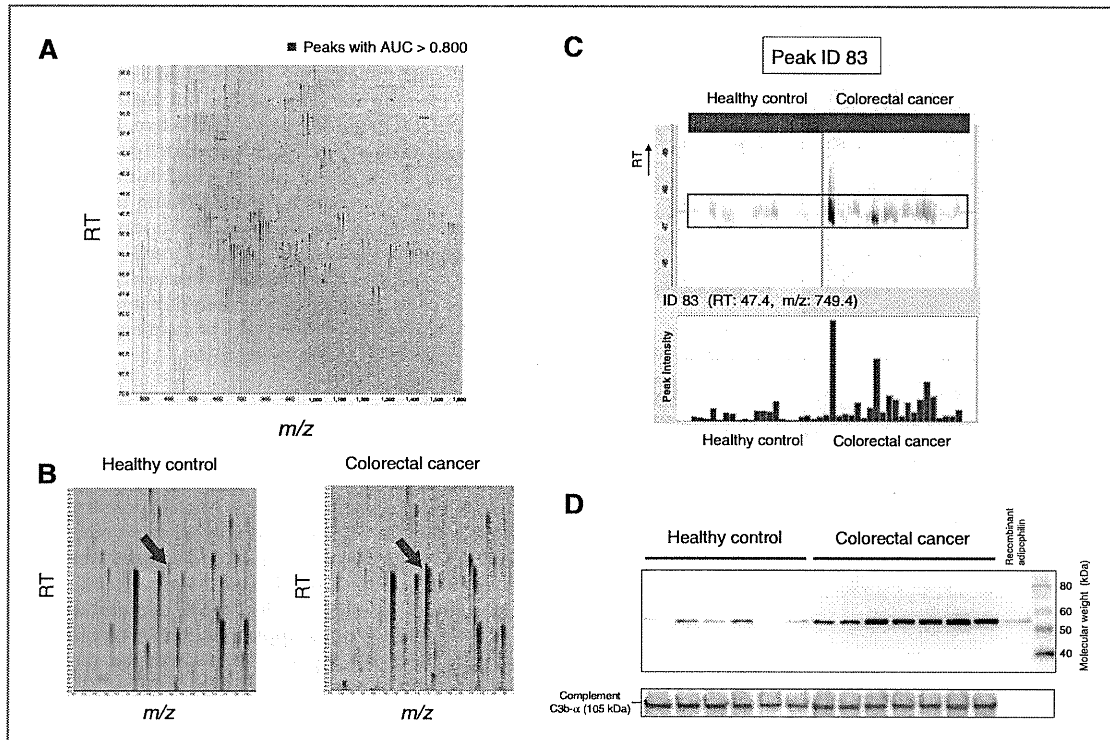

Figure 1. A, two-dimensional display of all (>53,000) MS peaks detected. The 103 MS peaks for which the mean intensity determined in duplicate analyses distinguished between colorectal cancer and healthy control patients (with AUC values >0.800) are highlighted in red. B, adipophilin-derived MS peaks 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).

with colorectal cancer than in healthy controls (Welch's t test $P = 5.49 \times 10^{-10}$, Fig. 3A and Table 1), with an AUC value of 0.767 (95% 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, whereas 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 with 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 with 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 8 well differentiated, 10

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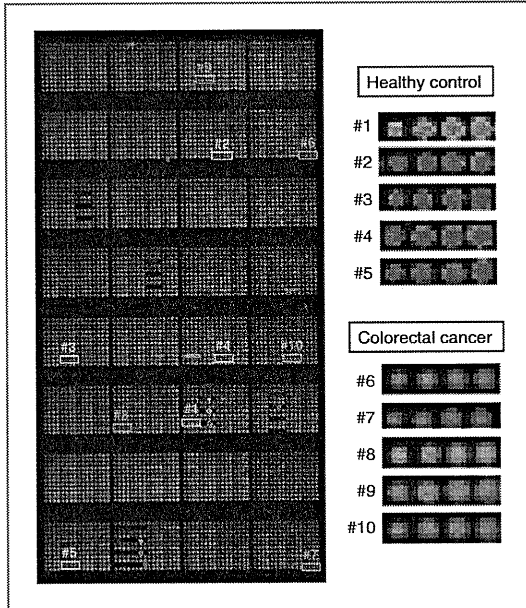

Figure 2. Representative reverse-phase protein microarray slide of the V1 cohort stained with anti-ADFP antibody (left). Magnified images of quadruplicate spots of representative individuals with high and low levels of adipophilin (right).

moderately differentiated, and 2 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 2 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 B). 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 this 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 shown 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

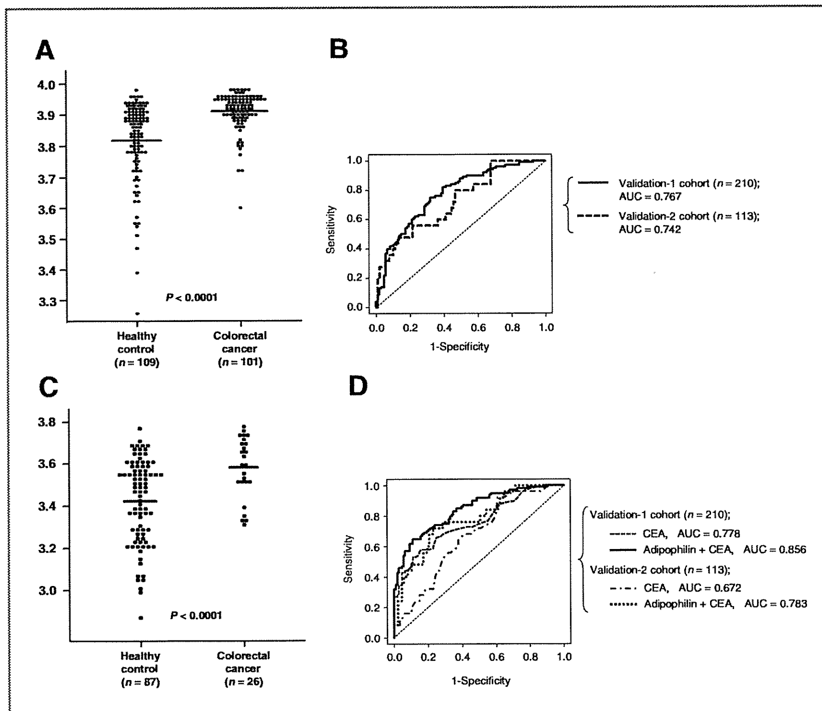

Figure 3. A and C, plasma adipophilin level in healthy controls and patients with colorectal cancer in the V1 (A) and V2 (C) cohorts. Horizontal lines represent the average adipophilin level. B, ROC analyses illustrating the discriminatory capability of adipophilin in the V1 (solid line) and V2 (dashed 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.

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Table 2. Plasma adipophilin and CEA levels according to clinical stage of colorectal cancer [UICC TNM classification of malignant tumors, 6th edition (2002)] in the V1 cohort

	Colorectal cancer patients				Healthy controls
	Stage I	Stage II	Stage III	Stage IV	
No. of cases	19	31	32	17	109
Adipophilin[a], mean (SD)	3.90 (0.05)	3.91 (0.07)	3.91 (0.07)	3.93 (0.03)	3.82 (0.13)
P^b (vs. healthy controls)	1.07×10^{-5}	3.31×10^{-6}	1.65×10^{-6}	2.27×10^{-11}	Đ
CEA, mean (SD), ng/mL	2.63 (1.71)	13.7 (36.2)	224 (1,068)	200 (579)	2.07 (1.74)
P^b (vs. healthy controls)	0.20	0.09	0.25	0.18	Đ

[a]Measured using a reverse-phase protein microarray (values were transformed into logarithmic variables).
[b]Welch's *t* test (comparison with healthy controls).

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 1 protein in hundreds of clinical samples in 1 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 microliters of samples for preliminary experiments involving techniques such as conventional 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 this study. It may be necessary to establish an ELISA prior to the clinical application of the present results.

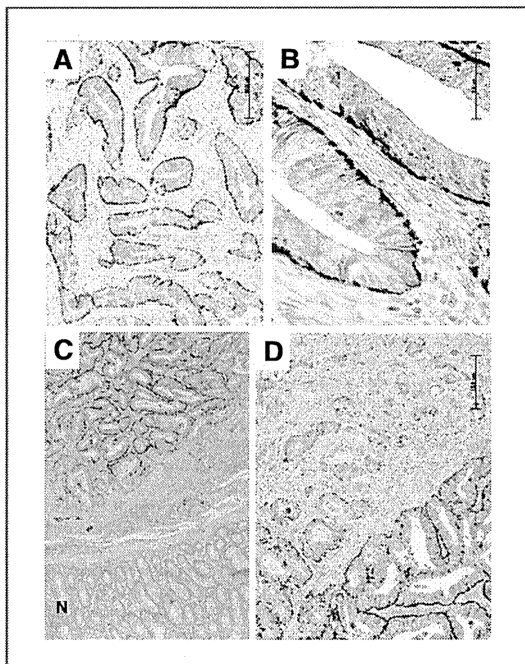

Figure 4. Immunohistochemical analysis of adipophilin in colorectal cancer (A–D) and adjacent normal colonic mucosa (designated by N; C). Original magnification; A and D = 100×; B = 400×; C = 40×.

Although the expression of adipophilin is known to be induced in various types of pathologic and physiologic 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 and colleagues 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). Because 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 clinicopathologic significance of adipophilin expression in colorectal

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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 about the body mass index of cases included in this 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). Second, we have no data of FOB test results for the cases used in this study and thus it was not possible to show the superiority of adipophilin to FOB. However, a recent large-scale study showed that 11% of patients with negative FOB results had cancers or adenomas that required treatment (40). Because 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 2 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 showing the expression of adipophilin in colorectal cancer. While bearing the above limitations in mind, an independent validation study is warranted.

Disclosure of Potential Conflicts of Interest

The sponsors of the study had no role in the design of the study, data collection, data analysis and interpretation, the decision to submit the manuscript for publication, or the writing of the manuscript.

Acknowledgments

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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

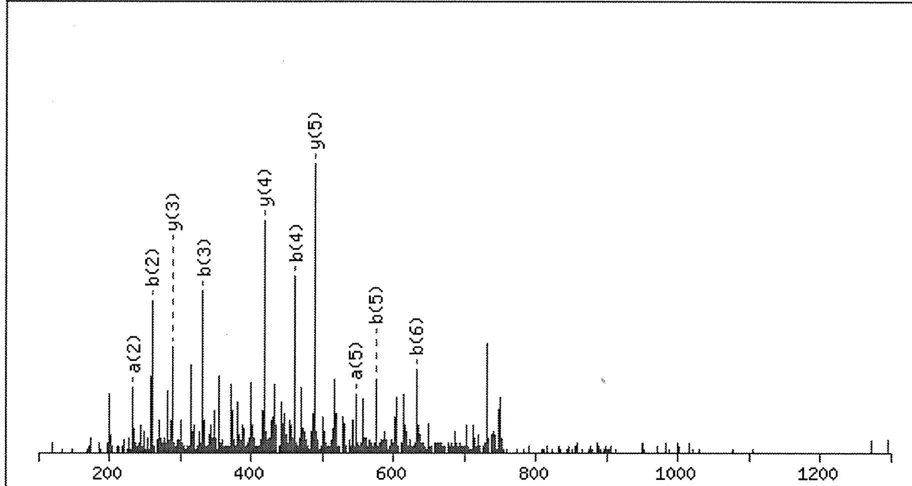
Supplementary Table S1 (online).

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.

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Cancer Epidemiology, Biomarkers & Prevention

Research Article

Reduced Plasma Level of CXC Chemokine Ligand 7 in Patients with Pancreatic Cancer

Junichi Matsubara[1,2], Kazufumi Honda[1], Masaya Ono[1], Yoshinori Tanaka[3], Michimoto Kobayashi[3], Giman Jung[3], Koji Yanagisawa[4], Tomohiro Sakuma[4], Shoji Nakamori[5], Naohiro Sata[6], Hideo Nagai[6], Tatsuya Ioka[7], Takuji Okusaka[8], Tomoo Kosuge[8], Akihiko Tsuchida[9], Masashi Shimahara[10], Yohichi Yasunami[11], Tsutomu Chiba[2], Setsuo Hirohashi[1], and Tesshi Yamada[1]

Abstract

Background: Early detection is essential to improve the outcome of patients with pancreatic cancer. A noninvasive and cost-effective diagnostic test using plasma/serum biomarkers would facilitate the detection of pancreatic cancer at the early stage.

Methods: Using a novel combination of hollow fiber membrane–based low-molecular-weight protein enrichment and LC-MS-based quantitative shotgun proteomics, we compared the plasma proteome between 24 patients with pancreatic cancer and 21 healthy controls (training cohort). An identified biomarker candidate was then subjected to a large blinded independent validation ($n = 237$, validation cohort) using a high-density reverse-phase protein microarray.

Results: Among a total of 53,009 MS peaks, we identified a peptide derived from CXC chemokine ligand 7 (CXCL7) that was significantly reduced in pancreatic cancer patients, showing an area under curve (AUC) value of 0.84 and a P value of 0.00005 (Mann–Whitney U test). Reduction of the CXCL7 protein was consistently observed in pancreatic cancer patients including those with stage I and II disease in the validation cohort ($P < 0.0001$). The plasma level of CXCL7 was independent from that of CA19-9 (Pearson's $r = 0.289$), and combination with CXCL7 significantly improved the AUC value of CA19-9 to 0.961 ($P = 0.002$).

Conclusions: We identified a significant decrease of the plasma CXCL7 level in patients with pancreatic cancer, and combination of CA19-9 with CXCL7 improved the discriminatory power of the former for pancreatic cancer.

Impact: The present findings may provide a new diagnostic option for pancreatic cancer and facilitate early detection of the disease. *Cancer Epidemiol Biomarkers Prev; 20(1); 160–71. ©2011 AACR.*

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Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (http://cebp.aacrjournals.org/).

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Introduction

Pancreatic adenocarcinoma is one of the most aggressive and lethal of diseases. The overall 5-year survival rate of patients with pancreatic cancer is less than 5%, which is the lowest among the more common cancers (1, 2), and the disease is the fifth leading cause of cancer death in Japan and the fourth in the United States, with greater than 23,000 estimated annual deaths in Japan and greater than 33,000 in the United States (3, 4). The 5-year survival rate of patients who were able to undergo surgical resection reaches 20% to 40% (5, 6), but the majority of pancreatic cancer patients have already developed lymph node and/or distant organ metastasis at their first clinical presentation, and only about 20% of patients are able to undergo radical resection (7, 8). The introduction of gemcitabine has significantly improved the overall survival of patients with unresectable pancreatic cancer, but their median survival period still remains about 6 months (9–11). These statistics

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demonstrate that early detection is essential for improving the outcome of patients with pancreatic cancer.

Computed tomography (CT), magnetic resonance imaging (MRI), and positron emission tomography (PET) are not cost-effective for the screening of pancreatic cancer because of the relatively low incidence of the disease. If a noninvasive and cost-effective screening test employing plasma/serum markers could be devised, it would significantly facilitate the early detection of pancreatic cancer. However, no biomarker suitable for screening of pancreatic cancer is currently available (12). CA19-9 is an established biomarker useful for the follow-up of pancreatic cancer patients receiving treatment, but has not been recommended for cancer screening because of its insufficient sensitivity and specificity (7, 13). Therefore, the discovery of a new biomarker that would be able to supplement CA19-9 has been anticipated.

Recently, advanced proteomic technologies based on mass spectrometry (MS) have been increasingly applied to studies of clinical samples to identify new biomarkers of various diseases (14) including pancreatic cancer (12, 15). It is anticipated that alterations in the protein content of clinical samples reflect the biological status of patients more directly than those in mRNA (16). We previously developed a new shotgun proteome platform, 2-Dimensional Image Converted Analysis of Liquid chromatography and mass spectrometry (2DICAL; ref. 17). 2DICAL is highly advantageous for clinical proteomics because of its high quantification accuracy and throughput. Using 2DICAL, we have been able to identify several plasma/serum biomarkers useful for cancer detection and therapy tailoring (18–20).

The serum/plasma proteome accumulates a large variety of disease-related alterations and is considered to be a rich source of biomarkers. However, for proteomic analysis of blood samples, the efficient depletion of a handful of particularly abundant proteins, such as albumin and immunoglobulin, has been challenging (21). Recently, we developed a novel method for the pretreatment of serum/plasma using the high-performance hollow fiber membrane (HFM) filtration technique (22). This method employs multistage filtration and cascaded cross-flow processes, enabling fully automated separation of proteins below a predetermined molecular weight (22). As the more abundant plasma proteins generally have relatively large molecular weights, they can be efficiently eliminated using the HFM technique.

To identify new biomarkers that might be useful for the early detection of patients with pancreatic cancer, we performed a comprehensive analysis of low-molecular-weight (LMW) plasma proteins in these patients using a combination of the HFM and 2DICAL techniques. A large variety of LMW proteins are known to be secreted from diseased tissues and can serve as good diagnostic biomarkers for various diseases (23, 24). Here, we report the identification and validation of an LMW chemotactic cytokine, CXC chemokine ligand 7 (CXCL7), as a novel biomarker for pancreatic cancer.

Patients and Methods

Plasma samples

Plasma samples were collected prospectively from 282 individuals (K. Honda, T. Okusaka, K. Felix, S. Nakamori, N. Sata, H. Nagai, et al., manuscript submitted) including healthy volunteers and newcomers to mainly departments of gastroenterology between August 2006 and October 2008 at the following 7 hospitals in Japan: National Cancer Center Hospital (NCCH), Osaka National Hospital (ONH), Jichi Medical School Hospital, Osaka Medical College (OMC), Tokyo Medical University Hospital (TMUH), Osaka Medical Center for Cancer and Cardiovascular Diseases, and Fukuoka University Hospital. 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 as part of the International Cancer Biomarker Consortium (25). The procedures used for collection and storage were kept uniform for all plasma samples.

The 282 plasma samples were split into 2 study sets (referred to as the training and validation cohorts). The training cohort comprised 45 individuals including patients with untreated pancreatic cancer at NCCH ($n = 19$) and TMUH ($n = 5$), and healthy controls at NCCH ($n = 2$), TMUH ($n = 9$), OMC ($n = 6$), and ONH ($n = 4$). The validation cohort comprised 237 individuals including 140 patients with pancreatic cancer, 10 patients with chronic pancreatitis, and 87 healthy controls. All patients diagnosed as having pancreatic cancer had histologically or cytologically proven ductal adenocarcinoma. Demographic and laboratory data are summarized in Table 1. The staging of pancreatic cancer was in accordance with the TNM classification of the International Union against Cancer (UICC).

Blood was collected in a tube with EDTA at the time of diagnosis. The plasma was separated by centrifugation and frozen at $-80°C$ until analysis. Samples showing macroscopic evidence of hemolysis were excluded from the current analysis. Written informed consent was obtained from every subject before blood collection. The protocol of this study was reviewed and approved by the institutional ethics committee boards of each participating institution.

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 microliters of the sample was diluted by adding 3.5 mL of 25 mmol/L of ammonium bicarbonate buffer (pH 8.0). The total 4 mL of the diluted plasma was processed as previously described (22). After 1 hour of fully automated operation, LMW proteins with molecular weights smaller than 60 kDa were recovered (Supplementary Fig. S1) and lyophilized.

The concentration of β2-microglobulin before and after HFM treatment was measured using an ELISA kit (Human Beta-2 Microglobulin ELISA Kit: Alpha Diagnostic Intl. Inc.) to ensure consistent recovery.

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Table 1. Clinicopathologic characteristics of individuals in training and validation cohorts

	Training cohort ($n = 45$)			Validation cohort ($n = 237$)				
	Healthy control	Cancer	*P*	Healthy control	Cancer	P^f	Chronic pancreatitis	P^f
No. of patients	21	24		87	140		10	
Sex, *n*			0.205[a]			0.485[a]		0.487[a]
Male	17	15		56	83		8	
Female	4	9		31	57		2	
Age, y			<0.001			<0.001		0
mean (SD)	40 (13)	64 (7)		43 (16)	66 (10)		58 (13)	
Tumor location			NA			NA		NA
Head	–	14		–	59		–	
Body or tail	–	10		–	76		–	
Unknown	–	0		–	5		–	
Clinical stage			NA			NA		NA
I	–	1		–	5		–	
II	–	6		–	25		–	
III	–	4		–	40		–	
IV	–	13		–	70		–	
CA19-9 median, U/mL	5.5	1,109	<0.001	10.2	476	<0.001	5.2	0.06
>37.0 (ULN), no. of patients	2	19		4	110		0	
DUPAN-2 median, U/mL	12	540	<0.001	12	375	<0.001	25	<0.001
>150.0 (ULN), no. of patients	1	19		0	92		1	
CEA median, ng/mL	1.7	6.0	<0.001	1.7	3.5	<0.001	2.6	0.110
>5.0 (ULN), no. of patients	1	12		5	49		1	
Total bilirubin median, mg/dL	0.5	0.4	0.688	0.5	0.5	0.574	0.5	0.4
>1.2 (ULN), no. of patients	0	2		4	18		0	
CXCL7								
Mass spectrometry peak intensity[b], mean (SD)	332 (240)	138 (346)	<0.001[d]	–	–			
Protein intensity[c], mean (SD)	4.14 (0.18)	3.83 (0.28)	<0.001[e]	4.18 (0.14)	3.92 (0.28)	<0.001[e]	3.99 (0.10)	<0.001[e]

NOTE. Wilcoxon test was applied to assess differences in values.

Abbreviations: CEA, carcinoembryonic antigen; NA, not applicable; ULN, upper limit of normal.

[a]Calculated by Fisher's exact test.

[b]Intensity of the corresponding peak measured by quantitative mass spectrometry.

[c]Measured using reverse-phase protein microarrays (logarithmic variable).

[d]Calculated by Mann-Whitney U-test.

[e]Calculated by Welch's *t* test.

[f]Compared with healthy controls.

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