

maximum of two dose reductions was allowed for defined adverse events of grade 3 or 4; the minimum dose for *nab*-paclitaxel was 50 mg/m² and that for carboplatin was an AUC of 3. All patients underwent comprehensive baseline assessments including clinical laboratory tests and imaging studies. Patients also received follow-up assessments and monitoring at regular intervals. Toxicity evaluations were based on CTCAE v3.0. Objective response was determined according to the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.0.

Pharmacokinetics

Blood samples were collected on days 1 and 15 of cycle 1 (before as well as 0.5, 1, 1.5, 2, 4, 6, 8, 24, 48, and 72 h after dosing of *nab*-paclitaxel; before as well as 0.5, 1.0, 1.5, 3.5, 5.5, 7.5, and 23.5 h after dosing of carboplatin) and centrifuged, and the plasma supernatants were stored at -20°C until analysis. The plasma concentration of paclitaxel was measured by validated high-performance liquid chromatography and tandem mass spectrometry, with the lower limit of quantification being 1.00 ng/mL. The plasma concentration of free platinum derived from carboplatin was determined by validated inductively coupled plasma mass spectrometry, with the lower limit of quantification also being 1.00 ng/mL.

The maximum observed concentration (C_{max}) was determined directly from the observed plasma concentrations. The apparent terminal elimination rate constant (λ_z) was estimated by linear regression analysis of the decline from individual plasma concentration–time data. At least three points that resulted in the highest correlation coefficient were used for the λ_z calculation. The terminal elimination half-life ($t_{1/2}$) was calculated as $t_{1/2} = \ln(2) / \lambda_z$ for each patient. The area under the plasma concentration–time curve from time 0 to the last measurable time (AUC_{0-t}) was calculated by the trapezoidal method. Individual AUCs extrapolated to infinity (AUC_{inf}) were calculated from the last measurable concentration (C_{last}) according to the formula $AUC_{inf} = AUC_{0-t} + C_{last} / \lambda_z$. Individual area under the plasma concentration–time curves for free platinum were estimated from the concentration data. All pharmacokinetic parameters for paclitaxel and free platinum were calculated by noncompartmental techniques with the use of WinNonlin software (Professional Network version 5.2; Pharsight, Mountain View, CA).

Results

Patients

Eighteen chemotherapy-naïve patients with advanced NSCLC were enrolled in the study (Table 1). Four patients

Table 1 Characteristics of the study patients

Number of patients	18
Median age, years (range)	64 (37–72)
Sex	
Male	14
Female	4
Performance status	
0	9
1	9
Disease stage	
IIIB	1
IV	17
Histology	
Adenocarcinoma	14
Squamous cell carcinoma	3
Other	1

were female and 14 were male, and the median age was 64 years (range, 37 to 72). Histological analysis revealed that 14 patients had adenocarcinoma and three had squamous cell carcinoma; one patient who had unspecified NSCLC was classified as “other.”

Treatment delivery and safety

All 18 enrolled patients received at least one dose of the study treatment. A total of 82 cycles of treatment was delivered overall, with a median number of cycles per patient of 4 (range, 1 to 11). Dose reductions stipulated by the study protocol were instituted in 12 patients, mainly as

Table 2 Treatment-related toxicities

Adverse event	Toxicity grade			
	1	2	3	4
Hematologic				
Neutropenia	1	4	8	4
Anemia	3	9	4	0
Thrombocytopenia	5	6	0	0
Leukopenia	3	4	8	1
Nonhematologic				
Anorexia	4	5	3	0
Nausea	5	3	1	0
Vomiting	3	3	0	0
Fatigue	9	3	0	0
Arthralgia	8	1	0	0
Myalgia	10	1	0	0
Neuropathy: sensory	9	0	1	0
Alopecia	14	4	–	–
Febrile neutropenia	–	–	2	0

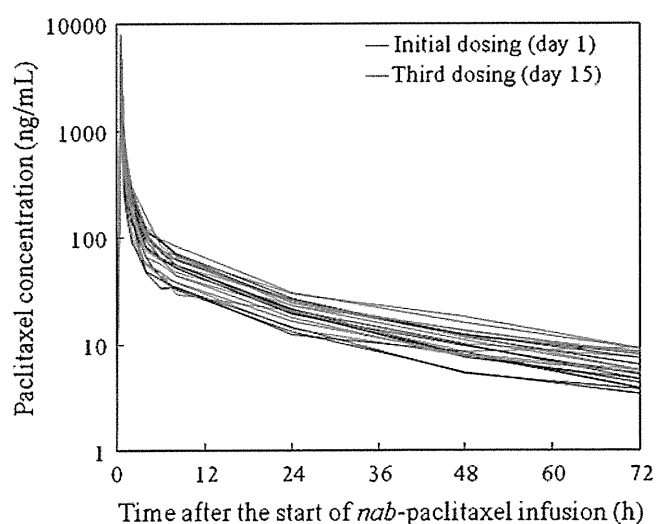


Fig. 1 Individual plasma paclitaxel concentration–time profiles for the initial (day 1) and third (day 15) dosings of *nab*-paclitaxel

a result of the development of neutropenia. The most common reason for treatment discontinuation was disease progression. Treatment was withdrawn because of adverse events (neuropathy or neutropenia) in two patients. All patients were evaluable for safety analysis. The major adverse events during the entire treatment period are shown in Table 2. Hematologic adverse events of grade ≥ 3 included neutropenia (67%), leukopenia (50%), and anemia (22%), with neutropenia of grade 4 being observed in four patients and leukopenia of grade 4 in one patient. In 12 patients with grade 3 or 4 neutropenia, the median time to neutrophil nadirs was 15 days and the median time from nadir to attaining recovery neutrophil level of $1500/\text{mm}^3$ or more was 11 days. Nonhematologic toxicities of grade ≥ 3 included anorexia (17%), febrile neutropenia (11%), nausea (6%), and sensory neuropathy (6%). There were no treatment-related deaths. The

adverse events observed in the present study were predictable from the safety profiles of *nab*-paclitaxel and carboplatin, and all events were well managed.

Pharmacokinetics

Twelve and nine patients were evaluable for paclitaxel pharmacokinetics analysis for the initial dosing of *nab*-paclitaxel administered with carboplatin (day 1 of cycle 1) and for the third dosing of *nab*-paclitaxel alone (day 15 of cycle 1), respectively. The individual paclitaxel concentration–time profiles are shown in Fig. 1. Paclitaxel pharmacokinetic profiles showed a multiphasic elimination and appeared similar for the initial and third dosings. A trace level of paclitaxel was detected in the trough samples obtained before the third dosing (Fig. 1), suggesting that paclitaxel accumulates in plasma after repeated administration of *nab*-paclitaxel (on days 1, 8, and 15). The C_{max} , AUC_{0-t} , AUC_{inf} and $t_{1/2}$ values for paclitaxel were all $\sim 20\%$ higher after the third dosing (day 15) of *nab*-paclitaxel compared with those after the initial dosing on day 1 (Table 3).

Pharmacokinetic parameters for carboplatin were calculated from the free platinum concentration after the initial dosing of carboplatin following that of *nab*-paclitaxel (Table 3). The free platinum concentration–time profiles showed monophasic elimination with a $t_{1/2}$ of ~ 4 h. The C_{max} and AUC_{inf} for free platinum were 23,903 ng/mL and 3.89 mg·min/mL, respectively.

Tumor response

All 18 enrolled patients were evaluable for response. There were seven partial responses and no complete responses, yielding an overall response rate of 38.9% (95% confidence interval, 17.3 to 64.3).

Table 3 Pharmacokinetic parameters for paclitaxel and free platinum in plasma

Parameter	Initial dosing (day 1)		Third dosing (day 15)	
	Mean	SD	Mean	SD
Paclitaxel				
C_{max} (ng/mL)	3460	905	4443	1827
AUC_{0-t} (ng·h/mL)	3893	897	4565	1346
AUC_{inf} (ng·h/mL)	4073	929	5060	1325
$t_{1/2}$ (h)	24.2	3.02	29.5	3.18
CL (L/h/ m^2)	25.9	6.61	21.0	5.51
V_z (L/ m^2)	913	292	897	269
Free platinum				
C_{max} (ng/mL)	23,903	3901		
AUC_{0-t} (mg·min/mL)	3.86	0.35		
AUC_{inf} (mg·min/mL)	3.89	0.36		
$t_{1/2}$ (h)	3.97	0.21		

CL total clearance; V_z volume of distribution

Discussion

We have evaluated the feasibility of administering *nab*-paclitaxel (100 mg/m²) weekly as a 30-min uninterrupted intravenous infusion combined with q3w IV administration of carboplatin at an AUC of 6 in Japanese patients with advanced NSCLC. We found that this combination could be safely administered without steroid and antihistamine premedication to prevent the development of hypersensitivity reactions. With regard to nonhematologic toxicities, sensory neuropathy, arthralgia, and myalgia of grade 2 or 3 were each observed in only one patient (5.6%). These results compare favorably with the toxicity profiles described for the standard combination of carboplatin plus solvent-based paclitaxel in previous studies with NSCLC patients, in which higher frequencies (~20%) of sensory neuropathy, arthralgia, and myalgia of grade 2 or 3 were observed [13]. Our findings thus support the notion that *nab*-paclitaxel, a Cremophor EL-free formulation of paclitaxel, has an improved toxicity profile compared with that of conventional paclitaxel formulated with Cremophor EL. This difference in toxicity profiles is important because such toxicities can be debilitating in patients for whom symptom palliation is the primary therapeutic goal.

Although no effect of the sequence of *nab*-paclitaxel and carboplatin administration on the pharmacokinetics of paclitaxel has been described previously [14], possible interactions of *nab*-paclitaxel and concomitantly administered carboplatin have not been specifically investigated. In the present study, the effect of carboplatin coadministration on the pharmacokinetics of paclitaxel was examined in similar patients treated with carboplatin and weekly *nab*-paclitaxel. The pharmacokinetic parameters of paclitaxel after *nab*-paclitaxel administration with or without carboplatin were similar to those described after single administration of *nab*-paclitaxel at 100 mg/m² in a previous study [7]. Whereas the AUC and C_{max} values of paclitaxel were increased ~20% after the third dosing (day 15) of *nab*-paclitaxel compared with those after the first dosing, these increases were likely due to the accumulation of paclitaxel after repeated *nab*-paclitaxel administration because a trace level of paclitaxel in plasma was detected immediately before the third dosing of *nab*-paclitaxel. The pharmacokinetics of carboplatin revealed that the AUC_{inf} calculated from the free platinum concentration was 3.89±0.36 mg·min/mL, corresponding to a range of 24.5 to 45.3% below the target AUC of 6. This finding is consistent with previous results showing that the measured AUC of carboplatin was 30% lower than the target AUC when a modified Calvert formula with creatinine clearance was substituted for glomerular filtration rate [15, 16]. Together, the present data suggest that concomitant administration of *nab*-paclitaxel and carboplatin according to the selected

treatment schedule had no substantial impact on the pharmacokinetics of either drug. However, pharmacokinetic findings remain to be verified in a crossover study designed to examine drug-drug interactions.

The physical nature of the Cremophor EL-paclitaxel suspension may limit access of paclitaxel to tumor cells. Preclinical studies have shown that the Cremophor EL-free formulation of paclitaxel (*nab*-paclitaxel) yielded higher intratumoral levels of paclitaxel, as a result of albumin transportation into tumor cells, as well as higher antitumor activity [7, 8]. Although tumor evaluation was not the primary objective of the present study and the small sample size precluded any definitive conclusion regarding treatment efficacy, the combination of *nab*-paclitaxel and carboplatin yielded promising results, with seven partial responses observed among the 18 evaluable patients, for a response rate of 38.9%. A previous study of patients with advanced NSCLC showed that first-line treatment with weekly *nab*-paclitaxel (100 mg/m²) plus carboplatin (according to the same schedule as that in the present study) similarly obtained a response rate of 48% (95% confidence interval, 28 to 68) [12]. Given its favorable safety profile and promising antitumor activity, this drug combination is currently under evaluation in a large randomized phase III trial (*n*=1050) in comparison with a standard dose of solvent-based paclitaxel plus carboplatin.

In conclusion, *nab*-paclitaxel administered weekly at 100 mg/m² in combination with carboplatin given q3w at an AUC of 6 was found to be well tolerated in Japanese patients with advanced NSCLC. No clinically relevant pharmacokinetic interaction of the two therapeutic agents was detected, and the observed antitumor activity merits further clinical investigation.

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Conflicts of interest H. Yamaya, K. Ono have been full-time employees of Taiho Pharmaceutical Co. Ltd.

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Predictive factors for the effectiveness of neoadjuvant chemotherapy and prognosis in triple-negative breast cancer patients

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Abstract

Purpose Triple-negative breast cancers (TNBCs) do not derive benefit from molecular-targeted treatments such as endocrine therapy or anti-HER2 therapy because they lack those molecular targets. On the other hand, TNBCs have been shown to respond to neoadjuvant chemotherapy (NAC). In this study, we analyzed TNBC patients who were treated with NAC at Osaka National Hospital over a recent 5-year period to clarify the predictive factors for NAC and prognostic factors.

Patients and methods Thirty-three TNBC patients underwent sequential NAC with anthracycline (FEC100: 5FU 500 mg/m², epirubicin 100 mg/m², and cyclophosphamide 500 mg/m²/q3w, 4 courses) and taxanes (paclitaxel 80 mg/m²/qw, 12 courses or docetaxel 75 mg/m²/q3w, 4 courses)

from May 2003 to July 2008. Pre-therapeutical and surgical specimens were studied for expressions of ER, PgR, HER-2, EGFR, cytokeratin 5/6, Ki-67, p53 and androgen receptor by immunohistochemistry (IHC). We analyzed clinicopathological factors and molecular markers in regard to the response to NAC and prognosis.

Results Pathological complete response (pCR) was achieved in 12 TNBC patients (36%). The pCR rate in the basal-like phenotype was significantly lower than in the non-basal-like phenotype (23 vs. 64%, respectively: $P = 0.02$). High pre-operative expressions of Ki-67 ($\geq 50\%$) and HER-2 (2+) were considered as predictive factors for a better response from NAC. Pre-operative Ki-67 expression showed a significant correlation with disease-free survival (DFS) and a lower expression of Ki-67 ($< 50\%$) after NAC was favorable for DFS among non-pCR patients.

Conclusions A non-basal-like phenotype and higher expressions of Ki-67 and HER-2 (2+) were favorable factors for NAC. However, a higher expression of Ki-67 on the surgical specimen after NAC was also a poor prognostic factor.

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Keywords Triple-negative breast cancer · Neoadjuvant chemotherapy · Pathological complete response · Ki-67 · Basal-like phenotype

Abbreviations

TNBC Triple negative breast cancer
NAC Neoadjuvant chemotherapy
pCR Pathological complete response
ER Estrogen receptor
PgR Progesterone receptor
AR Androgen receptor
EGFR Epidermal growth factor receptor
CK Cytokeratin

Introduction

Triple-negative breast cancers (TNBCs) are characterized by the lack of expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER-2). These cancers occur in ~20–25% of all breast cancers and are associated with an unfavorable prognosis. They derive no benefit from molecularly targeted treatments such as endocrine therapy or trastuzumab [1]. Therefore, identifying appropriate treatments for TNBC is an important issue.

Recent precise gene expression analysis revealed that TNBC is a heterogeneous group of tumors. One of the subgroups is a basal-like subtype, which is characterized by similar gene expression as the basal/myoepithelial cells of the normal breast [1–5]. Basal-like breast cancer has also been identified with immunohistochemical (IHC) staining of basal markers, such as cytokeratins (CKs) and epithelial growth factor receptor (EGFR). TNBCs without these basal markers are classified as non-basal-like subtypes, which are rare breast cancers, and classifications based on gene expression have not been clarified yet. Non-basal-like tumors are also reported to have a better prognosis than basal-like phenotypes [6, 7]. Because of the lack of targeted therapies and their aggressive clinical behaviors, TNBCs are relevant groups to be investigated for their characteristics. Though TNBCs are considered to have poor prognosis generally, TNBCs have been shown to be chemosensitive.

Neoadjuvant chemotherapy (NAC) in primary breast cancers has been shown to produce an outcome equivalent to that of adjuvant chemotherapy [8, 9]. Patients who show a pathological complete response (pCR) in the primary tumors after NAC have a better prognosis [10]. The pathological responses are important prognostic parameters and can be used as surrogate parameters for clinical outcome, so we analyzed the effects of clinicopathological factors as well as immunohistochemical factors on pathological responses after NAC. However, the paradox that TNBC and HER-2 positive subtypes showed higher chemosensitivity but worse survival due to higher relapse after chemotherapy is also known well [10, 11].

Several biological markers have been proposed as prognostic characteristics in breast cancers. ER, PR and HER-2 are such biological markers as well as being therapeutic markers and Ki-67, p53 and androgen receptor (AR) are shown to be associated with prognosis [12–16]. AR is known to be present in the majority of primary and metastatic invasive breast tumors and is often co-expressed with ER and PR in these tumors. Though little is known about the role of AR in hormonal response, AR expression has been shown to be associated with a better outcome for untreated breast cancer patients [14]. Ki-67 is a nuclear antigen expressed in the G1, S, and G2 phases but not in the

G0 or resting phase of the cell cycle. Ki-67 has been established as a proliferation marker in breast cancers and high proliferation activity has been found to have predictive value for the response to NAC [17]. Also p53 expression status has been used as a predictive factor for response to systemic therapy, because tumor cells with non-functional p53 do not respond to systemic therapy due to a failure in apoptosis [13, 15].

Because chemotherapy is the only treatment other than surgery for TNBC, the definition of clinical markers in regard to chemotherapeutic response and prognosis is very important. However, there are still few studies focusing on TNBC. In this study, we analyzed clinicopathological factors, phenotypes, and molecular markers of TNBC in regard to the response to NAC and prognosis.

Patients and methods

Patients and neoadjuvant chemotherapy

One hundred and 63 breast cancer patients underwent NAC with a sequential regimen containing anthracycline (FEC100: 5FU 500 mg/m², epirubicin 100 mg/m², cyclophosphamide 500 mg/m²/q3w, 4 courses) and taxanes (paclitaxel 80 mg/m²/qw, 12 courses or docetaxel 75 mg/m²/q3w, 4 courses) at Osaka National Hospital (Osaka, Japan) from May 2003 to July 2008. The criteria for entry were invasive breast cancer patients from 20 to 70 years old with any T and N0-2 disease, who were diagnosed histologically, were absent from distant metastasis and with normal organ functions. Thirty-three patients (20%) among 163 breast cancer patients were identified as TNBCs. The clinical evaluation of the response to NAC was determined by clinical findings, CT and MRI examinations according to RECIST. All patients were included in clinical trials approved by an institutional review board and asked for written informed consent.

Immunohistochemistry

Pre-therapeutical specimens were obtained by the 14G-needle biopsy in all cases and pathological examinations using standard hematoxylin and eosin staining were carried out. Immunohistochemical evaluation for ER, PgR, HER-2, EGFR, CK5/6, Ki-67, p53 and AR in tissue sections were detected using antibodies (ER:Cat.No. 760-2596I, PgR: 760-2816, HER-2:760-2901, EGFR:790-2988, CK5/6:960-4253, Ki-67:760-2910, p53:760-2912, Ventana Japan, Yokohama, Japan, AR:M3562, Dako Japan, Tokyo, Japan). Visualization of the bound antibodies was performed using a DAKO Envision™ + System (Dako Japan Inc., Tokyo, Japan) according to the manufacturer's instructions. Positive

cell rates (%) of ER and PgR were determined as a ratio of positive cells to total cancer cells and a value of 10% or higher were rated as positive [18, 19]. HER-2 expression was defined as (0) to (3+) based on positive cell rates and the intensity of IHC staining. Tumors showing weak over-expression (2+) of HER-2 were also tested by the fluorescence in situ hybridization (FISH) method to clarify the gene amplification of the *HER-2* gene. The *HER-2* gene is visualized as green fluorescent grains and a control of centromere 17 is visualized as orange fluorescent grains (Path Vysion, Abbott, IL, USA). Thus, HER-2 positives were either strong positives (3+) from IHC or positive for gene amplification from FISH analysis.

TNBCs are negative for ER, PgR and HER-2 as described earlier. Among TNBCs with 1–9% of ER and/or PgR expression were defined as hormone receptor (HR) weak and analyzed separately. TNBCs with HER-2 (2+) and that were FISH negative were also analyzed separately.

Proliferative activity was determined by IHC for the Ki-67 antibody. Ki-67 values were expressed as the percentage of positive cell counts among at least 100 tumor cells in each case. Patients with positive staining of Ki-67 at 50% or more were defined as high Ki-67 patients. AR and p53 were defined as positive if tumor cells showed positive staining regardless of rate. Basal-like subtype was defined as CK5/6 positive and/or EGFR positive in 5% or more cells.

Surgical treatment

All patients underwent surgical treatment after NAC. Breast conservative therapy or a mastectomy with or without axillary dissection was performed according to the decision of the surgeons' conference. Surgical specimens were histologically analyzed again, and the pathological response for NAC was evaluated. When no residual invasive tumor cells were found, tumors were identified as pathological complete response (pCR). Surgical specimens from non-pCR patients were analyzed for expressions of Ki-67, p53 and AR as described earlier.

Statistics

A univariate analysis of the pCR rate was carried out by the χ^2 test, and a multivariate analysis was done by multiple logistic regression analysis. The patients' survival was calculated from the first date of treatment until the date of death or the end of follow-up. A univariate analysis of disease-free survival (DFS) was done using the Kaplan–Meier method with a log-rank test, and a multivariate disease survival analysis was carried out under the Cox proportional hazards model. All data were analyzed with JMP for Windows (SAS Institute, Tokyo, Japan).

Results

Relationship between pCR and clinicopathological factors

Thirty-three patients were identified as TNBCs, and the patients' data are shown in Table 1. The age of the patients ranged from 30 to 68 years old (median 50.0) and 21 patients had clinically positive nodes. Clinical response after NAC was rated as clinical complete response for 14 patients (42%), a clinical partial response for 14 patients (42%), a clinical stable disease for 3 patients (9%), and as a clinical progress disease for 2 patients (6%). Also pCR was achieved in only 12 patients (36%).

The correlations between clinicopathological factors such as tumor size, lymph nodal metastasis, age, histological grade, and pCR rate were analyzed (Table 2). However,

Table 1 Patients' characteristics

Variables	No (%)
Total	33
Age: years-old	30–68 (50 ± 11.1)
Histology	
Papillo-tubular	4 (12)
Solid tubular	14 (42)
Schirrous	11 (33)
Special type	4 (13)
<i>T</i>	
1	1 (3)
2	24 (72)
3	6 (18)
4	2 (6)
<i>N</i>	
0	12 (36)
1	17 (52)
2	4 (12)
Histological grade	
1	1 (3)
2	4 (12)
3	27 (81)
Unknown	1 (3)
HER-2	
0	18 (55)
1+	11 (33)
2+	4 (12)
HR (hormone receptor)	
Negative	26 (79)
Weak	7 (21)

T and *N* were defined by the criteria of UICC-breast

HR weak is a tumor with low levels of ER and/or PgR determined by IHC (1–9% weakly positive cells)

Table 2 pCR ratio based on clinicopathologic and immunohistochemical factors

Variables	Number (%)	pCR (%)	<i>P</i> volume	Odd
Age (years old)				
<50	18 (55)	6 (33)	0.69	
50≤	15 (45)	6 (40)		
Size (cm)				
<5	25 (76)	11 (44)	0.09	5.5
5≤	8 (24)	1 (13)		
<i>N</i>				
Positive	21 (64)	8 (38)	0.78	
Negative	12 (36)	4 (33)		
Histological grade				
1–2	5 (15)	3 (60)	0.26	
3	27 (84)	9 (33)		
HR				
Negative	26 (79)	10 (38)	0.95	
Weak	7 (21)	2 (28)		
HER-2				
0, 1+	29 (88)	9 (31)	0.08	6.67
2+	4 (12)	3 (75)		
p53				
Positive	21 (64)	8 (38)	0.78	
Negative	12 (36)	4 (33)		
Ki-67				
50≤ (high)	20 (61)	10 (50)	*0.04	5.5
<50 (low)	13 (39)	2 (15)		
AR				
Positive	6 (18)	3 (50)	0.45	
Negative	27 (82)	9 (33)		
Basal-like [#]				
Positive	22 (67)	5 (23)	*0.02	5.9
Negative	11 (33)	7 (64)		
CK5/6				
Positive	14 (42)	2 (14)	*0.02	
Negative	19 (58)	10 (53)		
EGFR				
Positive	18 (55)	4 (22)	0.06	
Negative	15 (45)	8 (53)		

* Statistically significant

[#] Basal-like subtype is defined as CK5/4 positive and/or EGFR positive. Thus, CK5/6 was not used for multivariate analysis

these clinicopathological factors did not show any correlation with the pCR rate.

Relationship between pCR, and molecular markers

Next, the correlation between molecular markers and the pCR rate was also analyzed. HER-2 (2+) tended to show a

higher pCR rate than HER-2 negative (0 or 1+; 75 and 31%, respectively). In this study, basal markers of CK5/6 and EGFR were evaluated with 22 of 33 patients (67%) diagnosed with basal-like phenotype, and eleven patients (33%) diagnosed with the non-basal-like phenotype. The pCR rate for the basal-like phenotype was significantly lower than in the non-basal-like phenotype (23 and 64%, respectively; $P = 0.02$; Table 2). Ki-67 was also considered as a predictive factor for NAC response, because the pCR rate reaches 50% among high Ki-67 ($\geq 50\%$) patients, while it was 15% in low Ki-67 patients ($P = 0.04$). The expressions of HR, p53 and AR were not correlated with pCR in this study. Multivariate analysis showed that only high Ki-67 was a significant factor for the prediction of pCR (Table 3). The classification of basal-like or non-basal-like phenotypes was negative for multivariate analysis, probably because high Ki-67 and non-basal-like were strongly correlated with each other; high Ki-67 accounted for 33% in the basal-like and 75% in the non-basal-like phenotype.

Relationship between pCR and disease-free survival

All patients underwent surgical resection after NAC and non-pCR patients were histologically evaluated. The average observation period after surgery was 2 years and eight patients (24%) showed distant metastasis during the observation period. Seven out of 8 patients had been defined as non-pCR and only one patient obtained pCR after NAC. Non-pCR patients showed a worse DFS compared with pCR patients, but it was not statistically significant (Fig. 1a). Basal-like phenotype and other clinicopathological factors such as age, tumor size and lymph nodal involvement failed to show a correlation with DFS (Table 4). Ki-67 before NAC showed a significant correlation with DFS and high Ki-67 patients showed a poor prognosis (Fig. 1b).

Disease-free survival among non-pCR patients

Among non-pCR patients, only 7 patients (29%) showed a recurrence. We analyzed clinicopathological and IHC factors for better prognosis among non-pCR patients. The immunohistological changes of tumors after NAC were

Table 3 Multivariate analysis of pCR and immunopathological factors

Variables	Odds	<i>P</i> value
Non-basal-like	3.9	0.13
HER2 (2+)	10.2	0.12
High Ki-67	8.4	0.03*

* Statistically significant

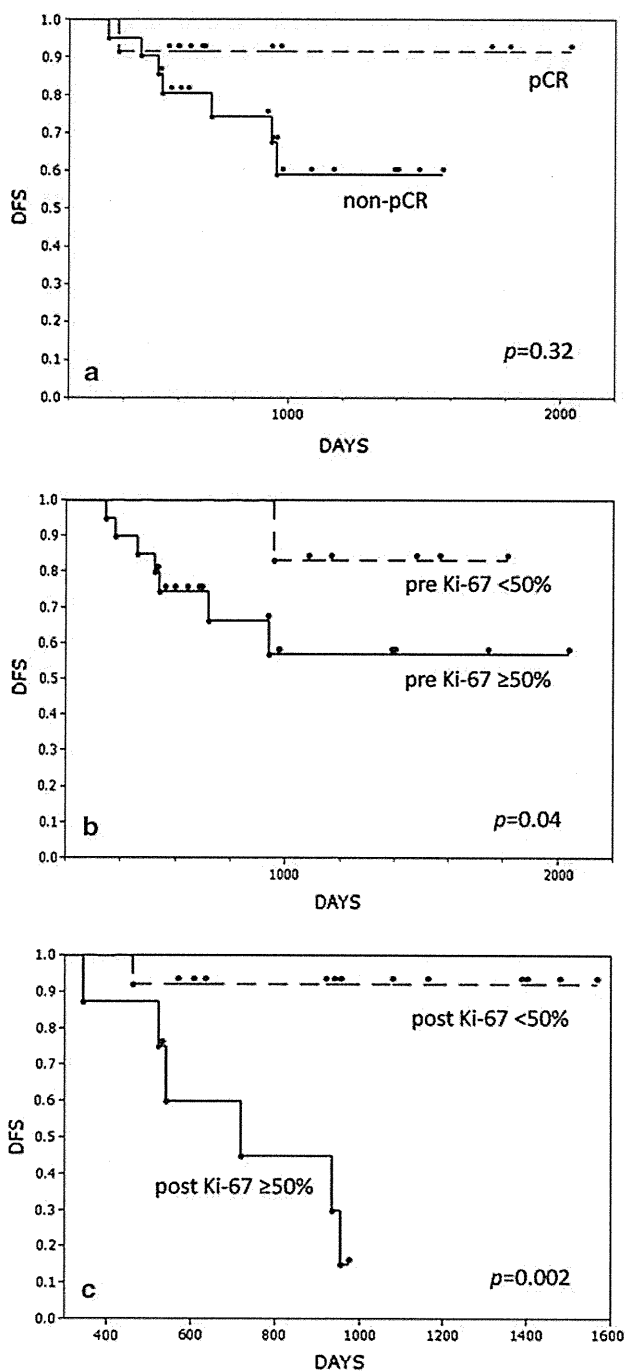


Fig. 1 Disease-free survival (DFS). **a** DFS of pCR and non-pCR patients after NAC. Non-pCR patients showed worse disease-free survival compared with pCR patients, but it was not statistically significant ($P = 0.32$). **b** DFS based on Ki-67 expression of pre-chemotherapy. High Ki-67 ($\geq 50\%$) patients showed significantly worse disease-free survival than low Ki-67 ($< 50\%$) patients ($P = 0.04$). **c** DFS based on Ki-67 expression of post-NAC among non-pCR patients. Non-pCR patients who had high Ki-67 expression after NAC showed a poor prognosis ($P = 0.002$)

evaluated. Among non-pCR patients, 10 patients showed high Ki-67 before chemotherapy and 7 patients still showed high Ki-67 after NAC (Table 5). Among these patients, 6

Table 4 Multivariate analysis of disease-free survival and patients' characteristics

Variables	Hazard ratio	P value
≥ 50 years-old	0.39	0.2
≥ 5 cm	2.2	0.3
N positive	4.2	0.11
HR positive	3.2	0.1
HER-2 (2+)	3.2	0.56
Non-basal	1.4	0.6
High Ki-67	5.95	0.04*
p53 positive	0.48	0.3
AR positive	0.000	0.054
Non-pCR	3.7	0.16
High Ki-67 post-NAC [#]	13.2	0.0029*

[#] Data among non-pCR patients

* Statistically significant

Table 5 The correlation between Ki-67 expression, pCR and the change of Ki-67 expression among non-pCR patients

TNBC (n = 33)	Non-pCR		pCR
	Post-NAC Ki-67		
	High	Low	
Pre-NAC Ki-67			
High	7	3	10
Low	1	10	2

showed a recurrence and Ki-67 values after NAC were significantly correlated with DFS (Fig. 1c). The expressions of p53 and AR after NAC were not correlated with DFS (data not shown).

Discussion

TNBC is defined by the lack of ER, PgR and HER-2 expression. Because targeted therapies are not useful, chemotherapy is the only systemic treatment option for TNBC [1–5]. Thus, a comprehensive examination of the clinical phenotypes of TNBCs which respond to chemotherapy is important. TNBCs are a heterogeneous group and generally divided into two subtypes; basal-like phenotype and non-basal-like phenotype [6]. The basal-like phenotype is characterized as having a high expression of keratins, laminin, and EGFR.

Many data indicated that the pCR rate is higher in TNBC compared with other phenotypes [10]. A pathological evaluation after NAC is very important because pCR after NAC indicates better survival [8, 9]. Our data showed the pCR rate in TNBCs was 36%, which is consistent with previous

reports which stated 22–45% [10, 20]. This study hypothesized that non-basal-like phenotype, HER-2 (2+), and high Ki-67 could be predictive factors for pCR achievement, but multivariate analysis revealed that only Ki-67 was a significant factor for the prediction of pCR. This is probably because the non-basal-like phenotype showed a significantly higher Ki-67 expression compared with the basal-like phenotype. This study is consistent with previous studies which showed that Ki-67 indicates proliferation and high level of proliferation activity are associated with chemosensitivity [14]. Additionally, there are many reports that showed that the basal-like phenotype has a positive correlation with pCR [20]. Rouzier et al. reported that basal-like subtypes were more sensitive to NAC than luminal and normal-like cancers, but normal-like subtypes classified based on gene expression profiles are quite different from non-basal-like phenotypes based on IHC, because normal-like subtypes involved 60% of ER positive samples. Because classification based on gene expression is difficult for clinical use, our data based on IHC classification are quite useful. There are some reports that non-basal-like tumors showed better prognosis than basal-like phenotypes [6, 7]. Though the pCR rate was significantly higher in non-basal-like tumors, there was no difference in DFS between the two groups in this study.

Our study failed to show the significant benefit of pCR on DFS. That is probably because of the small number of the patients included or the short duration after surgical treatment in this study. Most cases which showed a recurrence in such a short period were non-pCR patients, and the only recurrent case in the pCR group was a patient with an intraductal residual after NAC and who showed brain metastasis within a year. In this study, Ki-67 was the only significant factor which was proved to affect DFS. Pre-NAC high Ki-67 was a poor prognostic factor in spite of the positive correlation with pCR. The post-NAC status of Ki-67 was also correlated with recurrence. High Ki-67 expression post-NAC showed a very poor prognosis and low Ki-67 post-NAC showed better survival even in the non-pCR group. The contradiction of high Ki-67 tumors, which showed a high chemosensitivity and high pCR rate but poor prognosis, may indicate the diversity of these tumors. As shown in Table 4, most high Ki-67 patients who could not achieve pCR kept a high expression of Ki-67 after NAC. Tumors which maintained high Ki-67 expression may indicate that the cellular activity is not suppressed by NAC. All of these facts showed that high Ki-67 tumors should be divided into two groups: tumors which show a high sensitivity to current chemo-drugs and a good prognosis and the tumors which continue to have high cellular activity after NAC and show a poor prognosis. Further study is needed to find other treatments for the latter.

Though many reports defined 20–30% of Ki-67 labeling index as a threshold [21], 50% was used for categorization in this study because most TNBCs are positive for Ki-67 and a 50% threshold at 50% was shown to be useful to predict both chemosensitivity and prognosis in TNBC patients.

The prognosis of HER-2 positive breast cancer has been proved by the usage of trastuzumab. The criteria of HER-2 positive are defined as a strong positive IHC or gene amplification in FISH [22]. HER-2 (2+) breast cancers without gene amplification are generally included in TNBC but HER-2 (2+) breast cancers showed higher chemosensitivity in this study and HER-2 (3+) breast cancers have been reported to be chemosensitive. The criteria of HER-2 positivity might be a moot point if TNBCs with HER-2 (2+) show a different cancer biology from TNBCs with negative HER-2.

Less than 10% of hormone receptor positivity had been considered as uncertain endocrine responsiveness or potential resistance [18, 19]. Though tumors with less than 10% hormone receptor positivity were included in TNBCs, we classified those with 0% staining both ER and PgR as HR negative and those with 1–9% as HR weak in this study. But the expressions of HR were not correlated with pCR. Moreover, tumors with any ER positive staining of at least 1% are recommended to be treated with endocrine therapy in latest reports [21, 23]. The categories of highly endocrine responsive and incompletely endocrine responsive are not relevant to the decision for endocrine therapy, but those categories are still important for the decision of chemotherapy.

In this study, we found that the pCR rate for the non-basal-like phenotype was significantly higher than that in the basal-like phenotype, though that difference was negative for multivariate analysis. This is because the positivity of Ki-67 was higher in the non-basal-like phenotype tumors. These data based on classification by IHC are very interesting and informative in a clinical setting because there are some discrepancy between criteria by gene expression profiling and those by IHC. Some previous papers were confused about classification by gene expression and by IHC. Non-basal-like subtype is a term correlated with IHC classification and difficult to adapt to criteria of gene expression. There are few reports focused on the non-basal-like phenotype. Our data may insinuate that non-basal-like subtypes are well adaptive to current chemotherapy and basal-like subtypes need another therapeutic agent. Because our data was based on a small number of patients, further examinations based on IHC classification are needed.

Our study indicated that TNBCs which were found to be non-pCR with high Ki-67 expression after NAC had a poor prognosis. How to treat these TNBCs will be a most important subject for future study. Only chemotherapy is a

proven treatment for TNBCs, but chemotherapy based on anthracyclins and taxanes has not been shown to be enough. There are several studies which showed the efficacy of new chemotherapeutic agents such as carboplatin, bavasituzumab and poly (ADP-ribose) polymerase-1 (PARP-1) inhibitor in TNBCs [24–26]. Studies of NAC with these agents are expected to improve the treatment of TNBCs.

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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.

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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

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 $0.22\text{-}\mu\text{m}$ 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

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	<i>P</i>	Cancer	Healthy	<i>P</i>	Cancer	Healthy	<i>P</i>
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	∅		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 ^b , mean (SD)	320 (375)	96 (78)	<0.001 ^c	∅	∅		∅	∅	
Protein intensity ^d , mean (SD)	∅	∅		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.

^aCalculated by Fisher's exact test.^bIntensity of the corresponding peak as measured using quantitative mass spectrometry.^cCalculated using Mann-Whitney *U* test.^dMeasured using reverse-phase protein microarray (logarithmic variable).^eCalculated 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

± 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 μ g 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- μ m 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

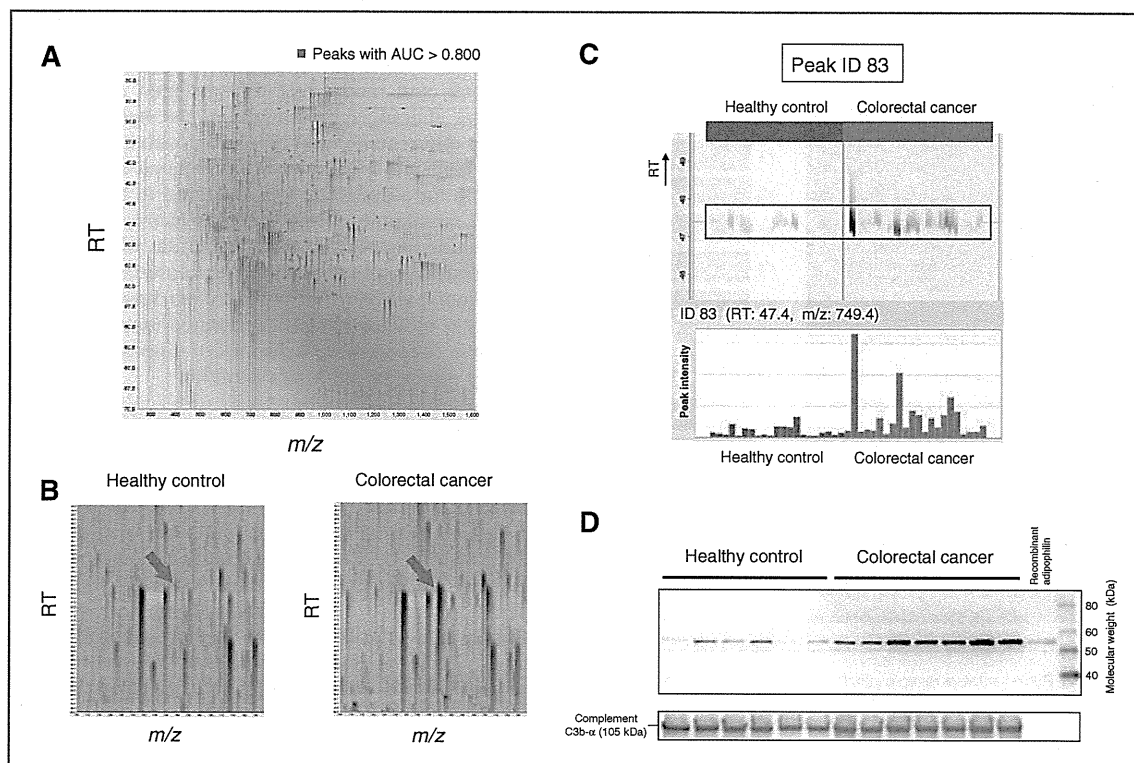


Figure 1. A, two-dimensional display of all (>53,000) MS peaks detected. The 103 MS peaks for which the mean intensity determined in duplicate analyses distinguished between colorectal cancer and healthy control patients (with AUC values >0.800) are highlighted in red. B, adipophilin-derived MS peaks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

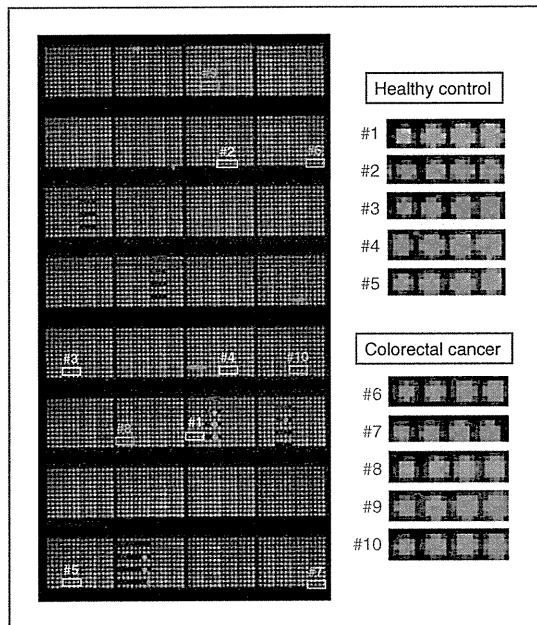


Figure 2. Representative reverse-phase protein microarray slide of the V1 cohort stained with anti-ADFP antibody (left). Magnified images of quadruplicate spots of representative individuals with high and low levels of adipophilin (right).

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

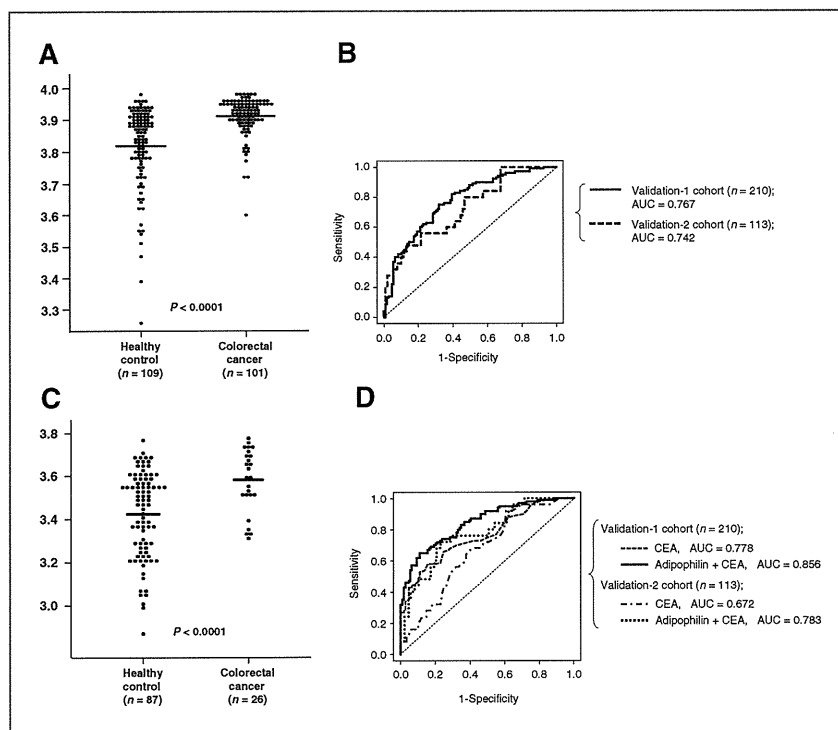


Figure 3. A and C, plasma adipophilin level in healthy controls and patients with colorectal cancer in the V1 (A) and V2 (C) cohorts. Horizontal lines represent the average adipophilin level. B, ROC analyses illustrating the discriminatory capability of adipophilin in the V1 (solid line) and V2 (dashed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

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)
<i>P</i> ^b (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 (1,068)	200 (579)	2.07 (1.74)
<i>P</i> ^b (vs. healthy controls)	0.20	0.09	0.25	0.18	∅

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

^bWelch'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.

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, PPAR γ 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

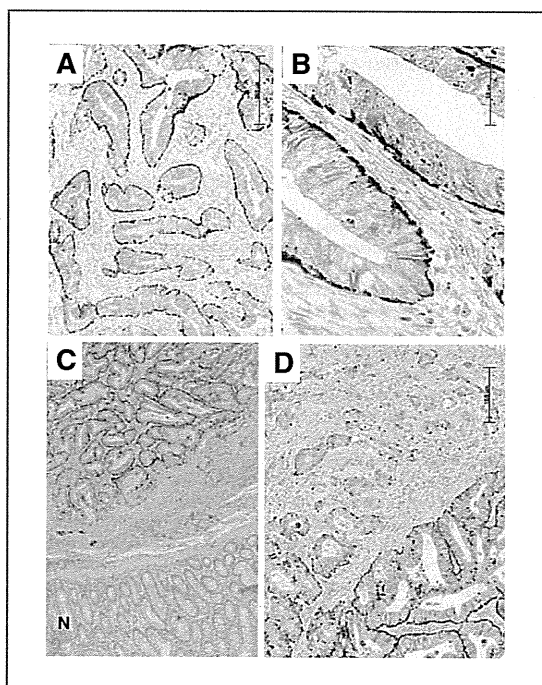


Figure 4. Immunohistochemical analysis of adipophilin in colorectal cancer (A–D) and adjacent normal colonic mucosa (designated by N; C). Original magnification; A and D = 100 \times ; B = 400 \times ; C = 40 \times .

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.

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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.

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