Cancer Chemother Pharmacol (2011) 67:911–917
DOI 10.1007/s00280-010-1371-4

ORIGINAL ARTICLE

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

Hiroko Masuda · Norikazu Masuda · Yoshinori Kodama · Masami Ogawa · Michiko Karita · Jun Yamamura · Kazunori Tsukuda · Hiroyoshi Doihara · Shinichiro Miyoshi · Masayuki Mano · Shoji Nakamori · Toshimasa Tsujinaka

Received: 24 January 2010 / Accepted: 14 May 2010 / Published online: 1 July 2010

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/m^2, epirubicin 100 mg/m^2, and cyclophosphamide 500 mg/m^2/q3w, 4 courses) and taxanes (paclitaxel 80 mg/m^2/qw, 12 courses or docetaxel 75 mg/m^2/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 (≥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.

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

H. Masuda · N. Masuda · M. Ogawa · M. Karita · J. Yamamura · S. Nakamori · T. Tsujinaka
Department of Surgery,
National Hospital Organization Osaka National Hospital,
Osaka, Japan

Y. Kodama · M. Mano
Department of Pathology,
National Hospital Organization Osaka National Hospital,
Osaka, Japan

H. Masuda (✉) · K. Tsukuda · H. Doihara · S. Miyoshi
Department of Cancer and Thoracic Surgery,
Okayama University Graduate School of Medicine,
Dentistry and Pharmaceutical Sciences,
2-5-1 Shikatacho Kitaku,
Okayama 700-8558, Japan
e-mail: masuhiro123@hotmail.com

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/m^2, epirubicin 100 mg/m^2, cyclophosphamide 500 mg/m^2/q3w, 4 courses) and taxanes (paclitaxel 80 mg/m^2/qw, 12 courses or docetaxel 75 mg/m^2/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 EnvisionTM + 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 overexpression (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)
T	
1	1 (3)
2	24 (72)
3	6 (18)
4	2 (6)
N	
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 immunohistochemistrical factors

Variables	Number (%)	pCR (%)	*P* 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)		
N				
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 (≥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	*P* value
Non-basal-like	3.9	0.13
HER2 (2+)	10.2	0.12
High Ki-67	8.4	0.03*

* Statistically significant

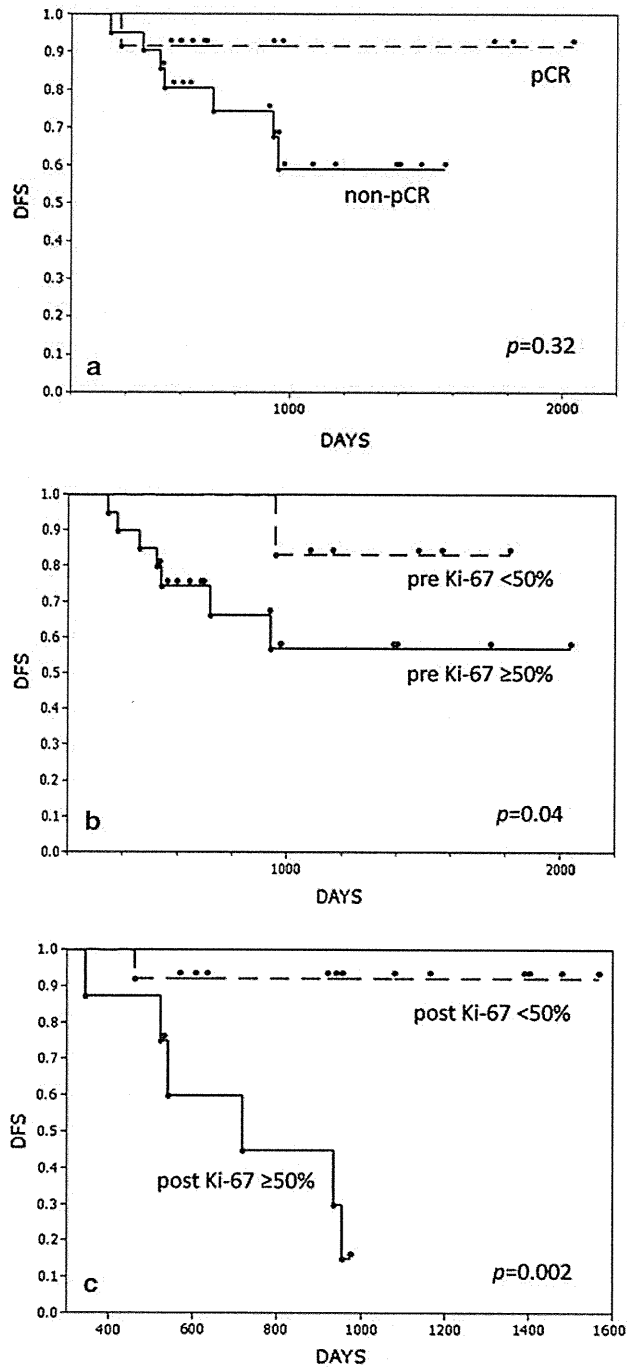

Fig. 1 Disease-free survival (DFS). **a** DFS of pCR and non-pCR patients after NAC. Non-pCR patients showed worse disease-free survival compared with pCR patients, but it was not statistically significant ($P = 0.32$). **b** DFS based on Ki-67 expression of pre-chemotherapy. High Ki-67 (≥50%) patients 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 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).

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

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.

References

1. Dawson SJ, Provenzano E, Caldas C (2009) Triple negative breast cancers: clinical and prognostic implications. Eur J Cancer 45(Suppl 1):27–40
2. Rakha EA, El-Sayed ME, Green AR et al (2007) Prognostic markers in triple-negative breast cancer. Cancer 109:25–32
3. Kreike B, van Kouwenhove M, Horlings H et al (2007) Gene expression profiling and histopathological characterization of triple-negative/basal-like breast carcinomas. Breast Cancer Res 9:R65
4. Kuroda N, Ohara M, Inoue K et al (2009) The majority of triple-negative breast cancer may correspond to basal-like carcinoma, but triple-negative breast cancer is not identical to basal-like carcinoma. Med Mol Morphol 42:128–131
5. Pintens S, Neven P, Drijkoningen M et al (2009) Triple negative breast cancer: a study from the point of view of basal CK5/6 and HER-1. J Clin Pathol 62:624–628
6. Rakha EA, Elsheikh SE, Aleskandarany MA et al (2009) Triple negative Breast cancer: distinguishing between basal and nonbasal subtypes. Clin Cancer Res 15:2301–2310
7. Gluz O, Liedtke C, Gottschalk N et al (2009) Triple-negative breast cancer-current status and future directions. Ann Oncol 20:1913–1927
8. Fisher B, Bryant J, Wolmark N et al (1998) Effect of preoperative chemotherapy on the outcome of women with operable breast cancer. J Clin Oncol 16:2672–2685
9. Scholl SM, Fourquet A, Asselain B et al (1994) Neoadjuvant versus adjuvant chemotherapy in premenopausal patients with tumors considered too large for breast conserving surgery: preliminary results of a randomized trial:S6. Eur J Cancer 30A:645–652
10. Liedtke C, Mazouni C, Hess KR et al (2008) Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer. J Clin Oncol 26:1275–1281
11. Carey LA, Dees EC, Sawyer L et al (2007) The triple negative paradox: Primary tumor chemosensitivity of breast cancer subtypes. Clin Cancer Res 13:2329–2334
12. Abrial SC, Penault-Llorca F, Delva R et al (2005) High prognostic significance of residual disease after neoadjuvant chemotherapy: a retrospective study in 710 patients with operable breast cancer. Breast Cancer Res Treat 94:255–263
13. von Minckwitz G, Sinn HP, Raab G et al (2008) Clinical response after two cycles compared to HER2, Ki-67, p53, and bcl-2 in independently predicting a pathological complete response after preoperative chemotherapy in patients with operable carcinoma of the breast. Breast Cancer Res 10:R30
14. OgawaY Hai E, Matsumoto K et al (2008) Androgen receptor expression in breast cancer: relationship with clinicopathological factors and biomarkers. Int J Clin Oncol 13:431–435
15. Berns EM, Foekens JA, Vossen R et al (2000) Complete sequencing of TP53 predicts poor response to systemic therapy of advanced breast cancer. Cancer Res 60:2155–2162
16. Assersohn L, Salter J, Powles TJ et al (2003) Studies of the potential utility of Ki67 as a predictive molecular marker of clinical response in primary breast cancer. Breast Cancer Res Treat 82:113–123
17. Burcombe RJ, Makris A, Richman PI et al (2005) Evaluation of ER, PgR, HER2 and Ki-67 as predictors of response to neoadjuvant anthracycline chemotherapy for operable breast cancer. Br J Cancer 92:147–155
18. Harvey JM, Clark GM, Osborne K et al (1999) Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. J Clin Oncol 17:1474–1481
19. Goldhirsh A, Glick JH, Gelber RD et al (2005) Meeting highlights: international expert consensus on the primary therapy of early breast cancer 2005. Ann Oncol 16:1569–1583
20. Rouzier R, Perou CM, Symmans WF et al (2005) Breast cancer molecular subtypes respond differently to preoperative chemotherapy. Clin Cancer Res 11:5678–5685
21. Goldhirsch A, Ingle JN, Gelber RD et al (2009) Thresholds for therapies: highlights of the St Gallen International expert consensus on the primary therapy of early breast cancer. Ann Oncol 20:1319–1329
22. Wolff AC, Hammond MEH, Schwartz JN et al (2007) American society of clinical oncology/College of American pathologists guideline recommendations of human epidermal growth factor receptors 2 testing in breast cancer. J Clin Oncol 25:118–145
23. Hammond ME, Hayes DF, Dowsett M et al (2010) American society of clinical oncology/College of American pathologists guideline recommendations for immunohistochemical testing and estrogen and progesterone receptors in breast cancer. J Clin Oncol 2010 (Epub)
24. Pal SK, Mortimer J (2009) Triple-negative breast cancer: novel therapies and new directions. Maturitas 63:269–274
25. Corkery B, Crown J, Clynes M et al (2009) Epidermal growth factor receptor as a potential therapeutic target in triple-negative breast cancer. Ann Oncol 20:862–867
26. Inbar-Rozensal D, Castiel A, Visochek L et al (2009) A selective eradication of human nonhereditary breast cancer cells by phenanthridine-derived polyADP-ribose polymerase inhibitors. Breast Cancer res 11:R78

Published OnlineFirst August 9, 2011; DOI:10.1158/1055-9965.EPI-11-0400

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

Junichi Matsubara[1,6], Kazufumi Honda[1], Masaya Ono[1], Shigeki Sekine[2], Yoshinori Tanaka[7], Michimoto Kobayashi[7], Giman Jung[7], Tomohiro Sakuma[3], Shoji Nakamori[8], Naohiro Sata[11], Hideo Nagai[11], Tatsuya Ioka[9], Takuji Okusaka[4], Tomoo Kosuge[4], Akihiko Tsuchida[5], Masashi Shimahara[10], Yohichi Yasunami[12], Tsutomu Chiba[6], and Tesshi Yamada[1]

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

Authors' Affiliations: Divisions of [1]Chemotherapy and Clinical Research, and [2]Molecular Pathology, National Cancer Center Research Institute; [3]BioBusiness Group, Mitsui Knowledge Industry; [4]Hepatobiliary and Pancreatic Oncology and Hepatobiliary and Pancreatic Surgery Divisions, National Cancer Center Hospital; [5]Third Department of Surgery, Tokyo Medical University, Tokyo; [6]Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, Kyoto; [7]New Frontiers Research Laboratories, Toray Industries, Kamakura; [8]Department of Surgery, Osaka National Hospital, National Hospital Organization; [9]Department of Hepatobiliary and Pancreatic Oncology, Osaka Medical Center for Cancer and Cardiovascular Diseases; [10]Department of Oral Surgery, Osaka Medical College, Osaka; [11]Department of Surgery, Jichi Medical University, Shimotsuke; and [12]Department of Regenerative Medicine and Transplantation, Fukuoka University Faculty of Medicine, Fukuoka, Japan

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

Corresponding Author: Junichi Matsubara, Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, 54 Shogoinkawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan. Phone: 81-75-751-4302; Fax: 81-75-751-4303; E-mail: jmatsuba@kuhp.kyoto-u.ac.jp

doi: 10.1158/1055-9965.EPI-11-0400

Published OnlineFirst August 9, 2011; DOI:10.1158/1055-9965.EPI-11-0400

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

Published OnlineFirst August 9, 2011; DOI:10.1158/1055-9965.EPI-11-0400

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 (± SD) was as high as 0.951 (± 0.039) and the mean CV was as low as 0.054 (± 0.011).

Protein identification by tandem mass spectrometry

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 ± 0.6 and

Published OnlineFirst August 9, 2011; DOI:10.1158/1055-9965.EPI-11-0400

± 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

Published OnlineFirst August 9, 2011; DOI:10.1158/1055-9965.EPI-11-0400

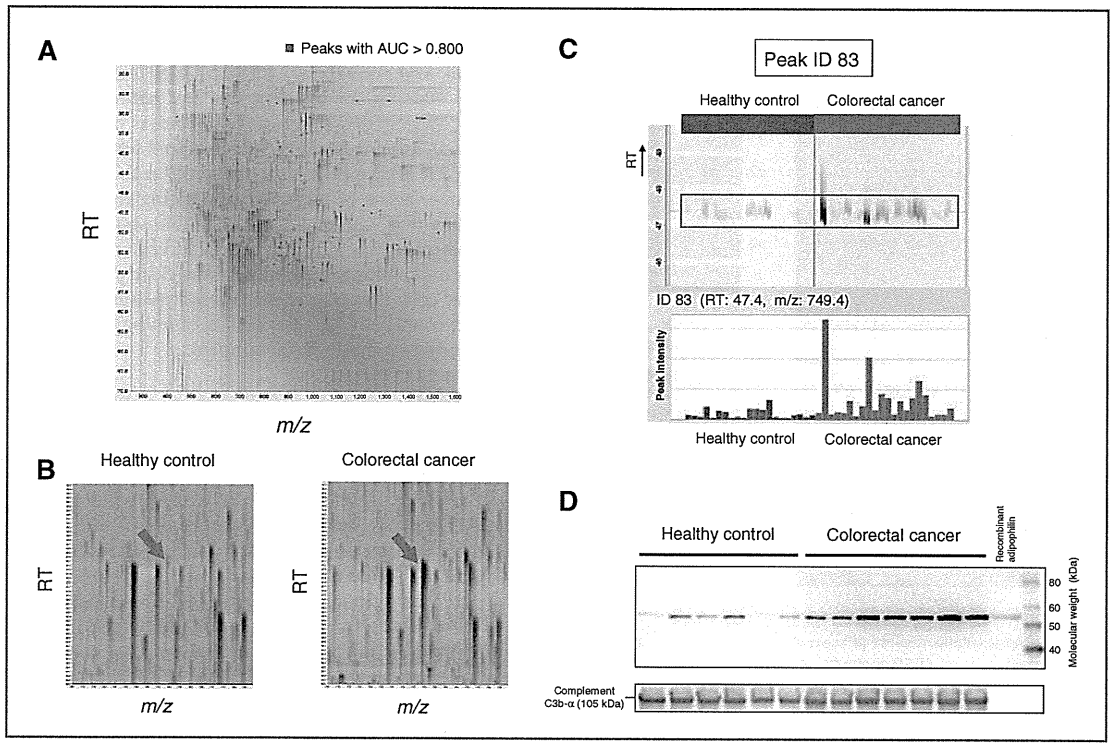

Figure 1. A, two-dimensional display of all (>53,000) MS peaks detected. The 103 MS peaks for which the mean intensity determined in duplicate analyses distinguished between colorectal cancer and healthy control patients (with AUC values >0.800) are highlighted in red. B, adipophilin-derived MS peaks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

Published OnlineFirst August 9, 2011; DOI:10.1158/1055-9965.EPI-11-0400

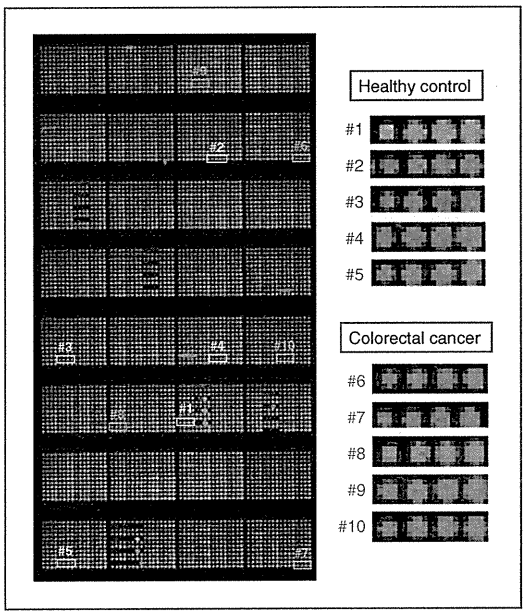

Figure 2. Representative reverse-phase protein microarray slide of the V1 cohort stained with anti-ADFP antibody (left). Magnified images of quadruplicate spots of representative individuals with high and low levels of adipophilin (right).

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

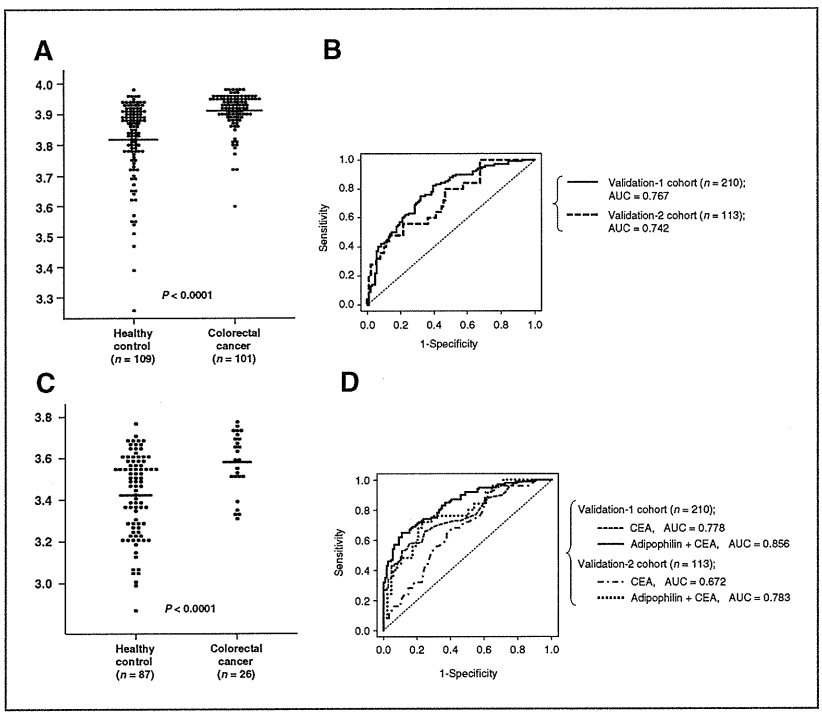

Figure 3. A and C, plasma adipophilin level in healthy controls and patients with colorectal cancer in the V1 (A) and V2 (C) cohorts. Horizontal lines represent the average adipophilin level. B, ROC analyses illustrating the discriminatory capability of adipophilin in the V1 (solid line) and V2 (dashed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

Published OnlineFirst August 9, 2011; DOI:10.1158/1055-9965.EPI-11-0400

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}	Ð
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	Ð

[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.

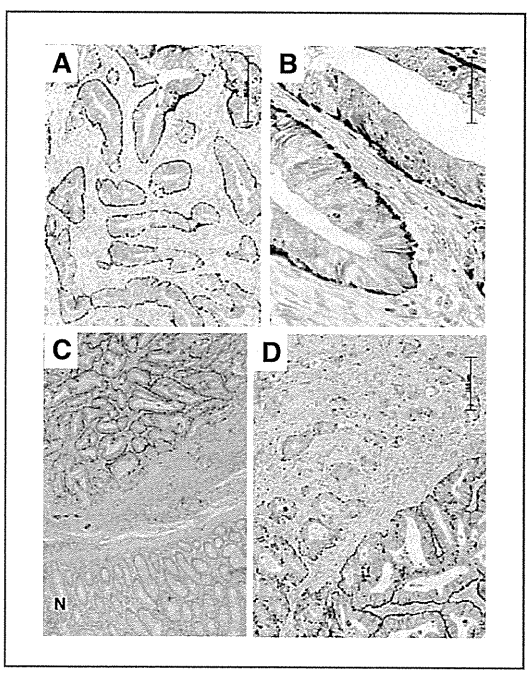

Figure 4. Immunohistochemical analysis of adipophilin in colorectal cancer (A–D) and adjacent normal colonic mucosa (designated by N; C). Original magnification; A and D = 100×; B = 400×; C = 40×.

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

Published OnlineFirst August 9, 2011; DOI:10.1158/1055-9965.EPI-11-0400

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

We thank Ms. Ayako Igarashi, Ms. Tomoko Umaki, and Ms. Yuka Nakamura for their technical assistance.

Grant Support

This study was supported by the "Program for Promotion of Fundamental Studies in Health Sciences" conducted by the National Institute of Biomedical Innovation of Japan, the "Third-Term Comprehensive Control Research for Cancer" and "Research on Biological Markers for New Drug Development" conducted by the Ministry of Health and Labor of Japan.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 27, 2011; revised July 12, 2011; accepted August 1, 2011; published OnlineFirst August 9, 2011.

References

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, et al. Cancer statistics, 2008. CA Cancer J Clin 2008;58:71–96.
2. Ministry of Health, Labour and Welfare. Japanese Government: Vital Statistics of Japan. 2009; http://ganjoho.ncc.go.jp/professional/statistics/odjrh3000000hwsa-att/cancer_mortality(1958-2008).xls.
3. Andre T, Boni C, Navarro M, Tabernero J, Hickish T, Topham C, et al. Improved overall survival with oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment in stage II or III colon cancer in the MOSAIC trial. J Clin Oncol 2009;27:3109–16.
4. Wolmark N, Yothers G, O'Connell MJ, Sharif S, Atkins JN, Seay TE, et al. A phase III trial comparing mFOLFOX6 to mFOLFOX6 plus bevacizumab in stage II or III carcinoma of the colon: Results of NSABP Protocol C-08. J Clin Oncol 2009;27: abstr LBA4.
5. Tol J, Koopman M, Cats A, Rodenburg CJ, Creemers GJ, Schrama JG, et al. Chemotherapy, bevacizumab, and cetuximab in metastatic colorectal cancer. N Engl J Med 2009;360:563–72.
6. Mandel JS, Bond JH, Church TR, Snover DC, Bradley GM, Schuman LM, et al. Reducing mortality from colorectal cancer by screening for fecal occult blood. Minnesota Colon Cancer Control Study. N Engl J Med 1993;328:1365–71.
7. Kronborg O, Fenger C, Olsen J, Jorgensen OD, Sondergaard O. Randomised study of screening for colorectal cancer with faecal-occult-blood test. Lancet 1996;348:1467–71.
8. Hardcastle JD, Chamberlain JO, Robinson MH, Moss SM, Amar SS, Balfour TW, et al. Randomised controlled trial of faecal-occult-blood screening for colorectal cancer. Lancet 1996;348:1472–7.
9. Greenberg PD, Bertario L, Gnauck R, Kronborg O, Hardcastle JD, Epstein MS, et al. A prospective multicenter evaluation of new fecal occult blood tests in patients undergoing colonoscopy. Am J Gastroenterol 2000;95:1331–8.
10. van Rossum LG, van Rijn AF, Laheij RJ, van Oijen MG, Fockens P, van Krieken HH, et al. Random comparison of guaiac and immunochemical fecal occult blood tests for colorectal cancer in a screening population. Gastroenterology 2008;135:82–90.
11. Locker GY, Hamilton S, Harris J, Jessup JM, Kemeny N, Macdonald JS, et al. ASCO 2006 update of recommendations for the use of tumor markers in gastrointestinal cancer. J Clin Oncol 2006;24:5313–27.
12. Yamaguchi U, Nakayama R, Honda K, Ichikawa H, Hasegawa T, Shitashige M, et al. Distinct gene expression-defined classes of gastrointestinal stromal tumor. J Clin Oncol 2008;26:4100–8.
13. Ono M, Shitashige M, Honda K, Isobe T, Kuwabara H, Matsuzuki H, et al. Label-free quantitative proteomics using large peptide data sets generated by nanoflow liquid chromatography and mass spectrometry. Mol Cell Proteomics 2006;5:1338–47.
14. Matsubara J, Ono M, Negishi A, Ueno H, Okusaka T, Furuse J, et al. Identification of a predictive biomarker for hematologic toxicities of gemcitabine. J Clin Oncol 2009;27:2261–8.
15. Negishi A, Ono M, Handa Y, Kato H, Yamashita K, Honda K, et al. Large-scale quantitative clinical proteomics by label-free liquid chromatography and mass spectrometry. Cancer Sci 2009;100:514–9.
16. Ono M, Matsubara J, Honda K, Sakuma T, Hashiguchi T, Nose H, et al. Prolyl 4-hydroxylation of alpha-fibrinogen: a novel protein modification revealed by plasma proteomics. J Biol Chem 2009;284:29041–9.
17. Matsubara J, Honda K, Ono M, Tanaka Y, Kobayashi M, Jung G, et al. Reduced plasma level of CXC chemokine ligand 7 in patients with

Published OnlineFirst August 9, 2011; DOI:10.1158/1055-9965.EPI-11-0400

pancreatic cancer. Cancer Epidemiol Biomarkers Prev 2011;20:160–71.
18. Matsubara J, Ono M, Honda K, Negishi A, Ueno H, Okusaka T, et al. Survival prediction for pancreatic cancer patients receiving gemcitabine treatment. Mol Cell Proteomics 2010;9:695–704.
19. Kennedy S. The role of proteomics in toxicology: identification of biomarkers of toxicity by protein expression analysis. Biomarkers 2002;7:269–90.
20. Tirumalai RS, Chan KC, Prieto DA, Issaq HJ, Conrads TP, Veenstra TD. Characterization of the low molecular weight human serum proteome. Mol Cell Proteomics 2003;2:1096–103.
21. Anderson NL, Anderson NG. The human plasma proteome: history, character, and diagnostic prospects. Mol Cell Proteomics 2002;1:845–67.
22. Tanaka Y, Akiyama H, Kuroda T, Jung G, Tanahashi K, Sugaya H, et al. A novel approach and protocol for discovering extremely low-abundance proteins in serum. Proteomics 2006;6:4845–55.
23. http://www.fhcrc.org/science/international_biomarker/.
24. Honda K, Yamada T, Hayashida Y, Idogawa M, Sato S, Hasegawa F, et al. Actinin-4 increases cell motility and promotes lymph node metastasis of colorectal cancer. Gastroenterology 2005;128:51–62.
25. Idogawa M, Yamada T, Honda K, Sato S, Imai K, Hirohashi S. Poly (ADP-ribose) polymerase-1 is a component of the oncogenic T-cell factor-4/beta-catenin complex. Gastroenterology 2005;128:1919–36.
26. Carpelan-Holmstrom M, Louhimo J, Stenman UH, Alfthan H, Jarvinen H, Haglund C. Estimating the probability of cancer with several tumor markers in patients with colorectal disease. Oncology 2004;66:296–302.
27. Heid HW, Moll R, Schwetlick I, Rackwitz HR, Keenan TW. Adipophilin is a specific marker of lipid accumulation in diverse cell types and diseases. Cell Tissue Res 1998;294:309–21.
28. de Wilde J, Smit E, Snepvangers FJ, de Wit NW, Mohren R, Hulshof MF, et al. Adipophilin protein expression in muscle–a possible protective role against insulin resistance. FEBS J 2010;277:761–73.
29. Yao M, Huang Y, Shioi K, Hattori K, Murakami T, Nakaigawa N, et al. Expression of adipose differentiation-related protein: a predictor of cancer-specific survival in clear cell renal carcinoma. Clin Cancer Res 2007;13:152–60.
30. Paul SA, Simons JW, Mabjeesh NJ. HIF at the crossroads between ischemia and carcinogenesis. J Cell Physiol 2004;200:20–30.
31. Osawa E, Nakajima A, Wada K, Ishimine S, Fujisawa N, Kawamori T, et al. Peroxisome proliferator-activated receptor gamma ligands suppress colon carcinogenesis induced by azoxymethane in mice. Gastroenterology 2003;124:361–7.
32. Grommes C, Landreth GE, Heneka MT. Antineoplastic effects of peroxisome proliferator-activated receptor gamma agonists. Lancet Oncol 2004;5:419–29.
33. Pischon T, Lahmann PH, Boeing H, Friedenreich C, Norat T, Tjonneland A, et al. Body size and risk of colon and rectal cancer in the European Prospective Investigation Into Cancer and Nutrition (EPIC). J Natl Cancer Inst 2006;98:920–31.
34. Giovannucci E, Michaud D. The role of obesity and related metabolic disturbances in cancers of the colon, prostate, and pancreas. Gastroenterology 2007;132:2208–25.
35. Renehan AG, Tyson M, Egger M, Heller RF, Zwahlen M. Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies. Lancet 2008;371:569–78.
36. Mutoh M, Teraoka N, Takasu S, Takahashi M, Onuma K, Yamamoto M, et al. Loss of adiponectin promotes intestinal carcinogenesis in Min and wild-type mice. Gastroenterology 2011;140:2000–8.
37. Brasaemle DL. Thematic review series: adipocyte biology. The perilipin family of structural lipid droplet proteins: stabilization of lipid droplets and control of lipolysis. J Lipid Res 2007;48:2547–59.
38. Jiang HP, Serrero G. Isolation and characterization of a full-length cDNA coding for an adipose differentiation-related protein. Proc Natl Acad Sci U S A 1992;89:7856–60.
39. Martin S, Parton RG. Lipid droplets: a unified view of a dynamic organelle. Nat Rev Mol Cell Biol 2006;7:373–8.
40. Johnson CD, Chen MH, Toledano AY, Heiken JP, Dachman A, Kuo MD, et al. Accuracy of CT colonography for detection of large adenomas and cancers. N Engl J Med 2008;359:1207–17.

Ann Surg Oncol (2011) 18:1891–1898
DOI 10.1245/s10434-010-1539-5

Annals of
SURGICAL ONCOLOGY
OFFICIAL JOURNAL OF THE SOCIETY OF SURGICAL ONCOLOGY

ORIGINAL ARTICLE – COLORECTAL CANCER

OSNA-Based Novel Molecular Testing for Lymph Node Metastases in Colorectal Cancer Patients: Results from a Multicenter Clinical Performance Study in Japan

Hirofumi Yamamoto, MD, PhD[1], Mitsugu Sekimoto, MD, PhD[1], Masatoshi Oya, MD, PhD[2], Noriko Yamamoto, MD, PhD[3], Fumio Konishi, MD, PhD[4], Junichi Sasaki, MD, PhD[4], Shigeki Yamada, MD[5], Kiyomi Taniyama, MD, PhD[6], Harumi Tominaga, MD[7], Masahiko Tsujimoto, MD, PhD[8], Hiroki Akamatsu, MD, PhD[9], Akio Yanagisawa, MD, PhD[10], Chouhei Sakakura, MD, PhD[11], Yo Kato, MD, PhD[3], and Nariaki Matsuura, MD, PhD[12]

[1]Department of Surgery, Gastroenterological Surgery, Graduate School of Medicine, Osaka University, Osaka, Japan; [2]Gastroenterological Center, Department of Gastroenterological Surgery, Cancer Institute Ariake Hospital, Tokyo, Japan; [3]Department of Pathology, The Cancer Institute of the Japanese Foundation for Cancer Research, Tokyo, Japan; [4]Department of Surgery, Saitama Medical Center, Jichi Medical University, Saitama, Japan; [5]Department of Pathology, Saitama Medical Center, Jichi Medical University, Saitama, Japan; [6]Institute for Clinical Research, National Hospital Organization Kure Medical Center and Chugoku Cancer Center, Hiroshima, Japan; [7]Department of Surgery, National Hospital Organization Kure Medical Center and Chugoku Cancer Center, Hiroshima, Japan; [8]Department of Pathology, Osaka Police Hospital, Osaka, Japan; [9]Department of Surgery, Osaka Police Hospital, Osaka, Japan; [10]Department of Surgical Pathology, Kyoto Prefectural University of Medicine, Kyoto, Japan; [11]Department of Surgery, Kyoto Prefectural University of Medicine, Kyoto, Japan; [12]Department of Molecular Pathology, Graduate School of Medicine and Health Science, Osaka University, Osaka, Japan

ABSTRACT

Background. Lymph node (LN) metastasis in colorectal cancer (CRC) is a critical factor in making accurate prognoses and therapeutic decisions. This study evaluated the clinical performance of the one-step nucleic acid amplification (OSNA) assay in accurately diagnosing LN metastases in CRC patients through the specific detection of cytokeratin 19 mRNA levels in LNs.

Methods. The OSNA assay was performed on 121 LNs dissected from early-stage CRC patients (pStage 0 or I) or from patients with benign colorectal disease (study 1). Separately, 385 LNs were dissected from 85 CRC patients (any stage); the OSNA assay was performed on half of each LN, and the results were compared with histopathological examination in 2-mm intervals of the other LN half (study 2).

Results. In study 1, all 121 histopathologically negative LNs were also negative by the OSNA assay (concordance rate for metastasis negative: 1.0, 95% confidence interval [95% CI]: 0.976–1.0). In study 2, the concordance rate between the OSNA assay and the 2-mm-interval histopathological examination was 0.971 (95% CI: 0.950–0.984), with a sensitivity of 0.952 (95% CI: 0.881–0.987) and a specificity of 0.977 (95% CI: 0.953–0.991).

Conclusions. The OSNA assay provided a judgment performance equivalent to a 2-mm-interval histopathological examination, a more detailed assay than the common pathological examination. Therefore, the OSNA assay is considered a new molecular examination method for the diagnosis of LN metastases in CRC patients in clinical settings.

Lymph node (LN) metastasis in colorectal cancer (CRC) is a critical factor in predicting the prognosis of patients lacking distant metastases; it is also a key factor in determining the applicability of postoperative adjuvant chemotherapy.[1–4] The postoperative LN metastasis examination commonly used for CRC patients relies on microscopic examination of hematoxylin and eosin (H&E)

First Received: 11 August 2010;
Published Online: 3 February 2011

H. Yamamoto, MD, PhD
e-mail: hyamamoto@gesurg.med.osaka-u.ac.jp

stained histopathological specimens prepared from the LN section with the largest cutting surface. In this method, however, some of the metastases are unavoidably overlooked because of their localization in the LN. In 2004, the 5-year survival rate of stage IIB (T4N0M0; negative for LN metastases but the tumor directly invades other organs) patients was inferior to that of stage IIIA (T1 or 2N1M0; positive for fewer than 4 LN metastases) patients (72.2% vs 83.4%, respectively), and it is suggested that the high recurrence rate for stage IIB patients could be due to the nondetection of metastases during histopathological examination.[5–8] Accordingly, a new, highly accurate, clinically relevant method to detect LN metastasis is needed to ensure accurate diagnosis in CRC patients following surgery.

The reverse-transcription polymerase chain reaction (RT-PCR) has been used to analyze tumor-specific mRNA as molecular biology based techniques are reportedly shown to be more accurate for detecting LN metastases.[9,10] However, these methods have not yet come into clinical practice, possibly due to the complexity and time-consuming nature of the tests. The one-step nucleic acid amplification (OSNA) assay is a novel technique using the reverse-transcription loop-mediated isothermal amplification (RT-LAMP) method for gene amplification. OSNA is already in clinical use for the diagnosis of LN metastases in breast cancer and is in research use for LN metastases in CRC, using cytokeratin 19 (CK19) mRNA as a molecular marker.[11–15] In this method, the supernatant of a homogenized LN solution is directly analyzed without the mRNA purification process that is usually required in RT-PCR. The use of an automated gene amplification detector (the RD-100i) offers rapidity and simplicity of detection of LN metastases.

The objective of the present study was to determine whether the OSNA assay for CK19 mRNA provided sufficient diagnoses of LN metastases in CRC patients. The multicenter clinical study should clarify the accurate diagnostic power provided by the pathological practice and by the OSNA method.

MATERIALS AND METHODS

Study Design

The present study was conducted in 2 phases, study 1 and study 2. Study 1 was designed to test (through concordance) whether the OSNA assay would yield false positives for histopathologically negative LNs in which no tumor cells were detected by the extensive 0.1-mm-interval histopathological examination with H&E staining and immunohistochemistry (IHC). The goal of study 2 was to investigate (through concordance) whether the OSNA assay would exhibit judgment performance equivalent to the 2-mm-interval histopathological examination. The entire study was conducted with the approval of the institutional review boards and independent ethics committees of each of the 6 institutes between October 22, 2007 and November 5, 2008.

Patient Samples

In preparation for study 1, 173 LNs with a minor axis 4 mm or smaller were dissected from 29 patients who had provided written informed consent for participating in the study. Patients who had been diagnosed as clinical stage 0/I or benign colorectal disease at age 18 or older were enrolled. In study 2, 434 LNs with a minor axis 8 mm or smaller were dissected from 91 CRC patients aged 18 years or older who were enrolled in the study.

Patients who had received preoperative or intraoperative adjuvant therapy and who were suffering or had suffered from any other cancer were excluded from this study, since no basic assessment had been performed for these patients using the OSNA assay.

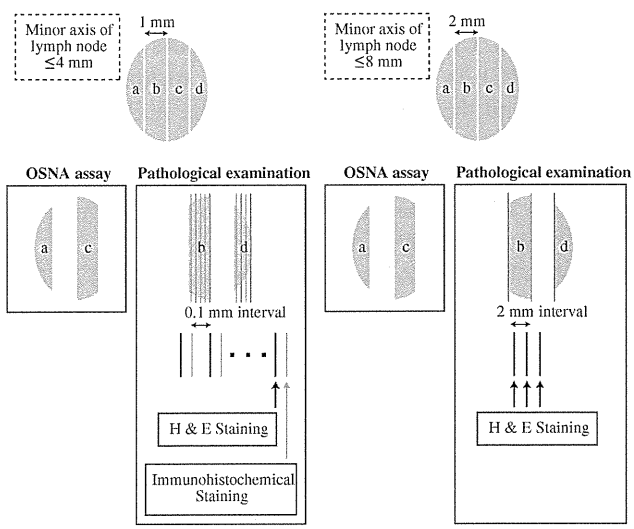

FIG. 1 Lymph node processing in study 1 and study 2. Lymph nodes were divided at 1-mm intervals (study 1) or 2-mm intervals (study 2), and nonadjacent blocks were alternatively subjected to histopathological examination or the OSNA assay. In study 1, the blocks for histopathological examination were processed as pairs of serial sections taken entirely at 0.1-mm intervals. Each pair was stained with hematoxylin and eosin (H&E) and anticytokeratin antibody. In study 2, a subset of the sections prepared from the cut surfaces were stained with H&E

LN Processing

To determine inclusion for analysis in study 1, 173 LNs were divided at 1-mm intervals, and nonadjacent blocks were alternatively subjected to histopathological examination and the OSNA assay (Fig. 1). The blocks for histopathological examination (blocks *b* and *d* in Fig. 1) were processed as pairs of 5-μm thick serial sections taken at 0.1-mm intervals. Subsequently, 1 section of each pair was stained with H&E and the other with anticytokeratin antibody (DAKO AE1/AE3); LNs in which all sections were found to be free of tumor cells by microscopy were selected as analytical samples. The remaining blocks (blocks *a* and *c* in Fig. 1) were subjected to the OSNA assay. Of the 173 LNs, 40 (23%) were derived from pathological stage II or III patients diagnosed by postoperative pathology and were excluded from the analysis, and 12 samples that were not confirmed as LNs were also excluded. The remaining 121 LNs derived from 18 patients (Table 1) were finally designated as histopathologically negative LNs for study 1 and were used in the concordance analysis with the OSNA assay.

TABLE 1 Patient demographics and baseline characteristics

	Study 1 (n = 18)		Study 2 (n = 85)	
	No. of patients	%	No. of patients	%
Age				
Mean	60		66	
Standard deviation	12.0		11.9	
Median	62		64	
Range	36–80		40–93	
Sex				
Male	11	61.1	44	51.8
Female	7	38.8	41	48.2
Tumor site				
Colon	11	61.1	62	72.9
Rectum	3	16.6	23	27.1
(benign colorectal disease)	4	22.2	–	–
Histological types				
Well/moderately	14	77.7	76	89.4
Poor/mucinous	0	0	8	9.4
Adenosquamous carcinoma	0	0	1	1.2
(benign colorectal disease)	4	22.2	–	–
Pathological Stage; TNM 6th edition				
0	4	22.2	1	1.1
I	10	55.5	14	16.4
IIA	–	–	16	18.8
IIB	–	–	0	0
IIIA	–	–	8	9.4
IIIB	–	–	21	24.7
IIIC	–	–	22	25.8
IV	–	–	3	3.5
(benign colorectal disease)	4	22.2	–	–

To determine inclusion for analysis in study 2, 434 LNs were divided into blocks at equal 2-mm intervals (Fig. 1). The 5-μm thick sections that had been prepared from the cut surfaces of nonadjacent alternating blocks (blocks *b* and *d*) were subjected to histopathological examination and stained with H&E. The remaining blocks (*a* and *c*) were subjected to the OSNA assay. There were 49 LNs excluded from the study, because 40 of the samples were not confirmed as LNs, and OSNA data for 9 LNs were not available because of quality-control errors. A total of 385 LNs from 85 patients (Table 1) were finally evaluated by comparing judgments resulting from the OSNA assay with those resulting from the 2-mm-interval histopathological examination. For blinding in both study 1 and study 2, identification codes were assigned separately to LN blocks analyzed by either histopathological examination or OSNA assay, so that the judgment results of one method would not influence those of the other.

Histopathological Evaluation

LNs with at least 1 observed tumor cell were judged to be positive, and LNs lacking 1 observed tumor cell were judged to be negative. The final histopathological results were determined by the judgments of 2 independent pathologists to ensure objectivity.

OSNA Assay

The OSNA assay used CK19 mRNA as a marker. The cutoff value between positive and negative LN for metastases in CRC was set at 250 copies/μl based on the logarithmic midpoint between the maximum value of the CK19 mRNA copy number in LNs from pN0 patients and −2SD value from the average of CK19 mRNA copy number in histopathologically positive LNs (our unpublished observation).

Surgically excised and divided LN blocks (*a* and *c*, Fig. 1) were homogenized using LYNORHAG lysis buffer (Sysmex Corp.). CK19 mRNA in each lysate was amplified using the LYNOAMP BC gene amplification reagent (Sysmex Corp.) and detected by measuring the rise time required to exceed a predetermined threshold turbidity caused by the by-product magnesium pyrophosphate. The rise times were analyzed using a previously generated standard curve, and CK19 mRNA concentrations in the LN

were calculated using the RD-100i automated gene amplification detector (Sysmex Corp.).[11]

Statistical Analysis

In study 1, the target value for the negative concordance rate of the OSNA assay was set at 0.99 on the assumption that 1% localization of metastases was the most unlikely level. The 95% confidence interval (95% CI) for the negative concordance rate was calculated from the results of study 1; study 1 was judged to be effective if its lower limit was below 0.89 (target value 0.99–Δ0.1). In study 2, the target value for the concordance rate of the 2 methods was established at 0.95; this value was obtained by defining the 2 methods as sufficiently equivalent if the discordance rate between the OSNA assay and the 2-mm-interval histopathological examination was not more than 5%. The 95% CI for the concordance rate was calculated from the result of study 2; study 2 was judged to be effective if its lower limit was not below 0.85 (target value 0.95–Δ0.1).

The diagnostic ability for LN metastasis by 2-mm-interval pathology and 1-level pathology was assessed by McNemar's test.

CK19 Protein Levels in Primary Tumors

Primary tumors from the 85 patients in study 2 (Table 1) were evaluated by staining with the anti-CK19 antibody (DAKO RCK108). For the primary tumors in which the stained area accounted for less than 10% of the total area, CK19 mRNA copy numbers were assessed by the OSNA assay if the LNs had been judged positive by histopathological examination.

Further Analysis of Discordant LN Samples

In study 2, to confirm the localization of tumor cells in the LNs, the remaining LN blocks (*b* and *d*) used in the histopathological examination were prepared as pairs of 5-μm thick serial sections taken at 0.1-mm intervals and stained with H&E and the anti-CK19 antibody. The samples were examined for the presence or absence of tumor cells and their distribution. When the OSNA assay was negative but the pathological method was positive, CK19 protein expression in tumor cells was confirmed by staining the IHC specimen with the anti-CK19 antibody.

RESULTS

Study 1

Based on the results of the 0.1-mm-interval histopathological examination with H&E staining and IHC with the anticytokeratin antibody, 121 LNs were selected as histopathologically negative (see the section Materials and Methods). For each of these samples, the copy number of CK19 mRNA was less than 250 copies/μl as assayed by OSNA, with no amplification observed during the reaction time in 115 of the 121 LN samples (95.0%) (Fig. 2).

The concordance rate of the OSNA assay (cutoff value 250 copies/μl) for judgment of histopathologically negative LNs was 1.0, providing a sufficiently lower limit of 0.976

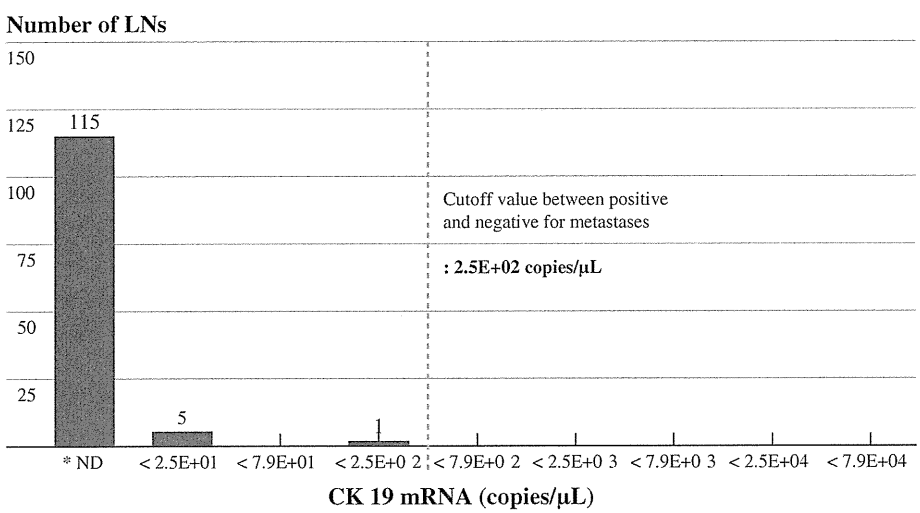

FIG. 2 CK19 mRNA copies/μl detected in histopathologically negative lymph nodes (LNs). Among the LNs derived from patients with benign colorectal disease or colorectal cancer (pStage 0/I), LNs that were found to be tumor-free by examination at 0.1-mm intervals with hematoxylin and eosin and immunohistochemistry were defined as "histopathologically negative." The CK19 mRNA cutoff level to separate "positive for metastasis" and "negative for metastasis" was 250 (2.5E+02) copies/μl (see the section Materials and Methods). *ND, the CK19 mRNA amplification reaction was not detected