the treatment groups (χ^2_1=0.7; P=.39; HR, 0.94 [95% CI, 0.81-1.08]).

Progression-Free Survival

Six hundred eighty-eight patients (63%) developed local recurrence, metastases, or both; of these, 597 had died. Two hundred forty-four patients (22%) were alive and progression free. Progression-free survival analysis was based on all patients, of whom 844 (78%) had either progressive disease or died. The median progression-free survival was 14.3 (95% CI, 13.5-15.1) months, with 12-month and 24-month rates of 58.7% (95% CI, 55.7%-61.6%) and 30.1% (95% CI, 27.3%-32.9%), respectively. The median progression-free survival for patients treated with fluorouracil plus folinic acid was 14.1 (95% CI, 12.5-15.3) months and 14.3 (95% CI, 13.5-15.6) months for patients treated with gemcitabine (Figure 2).

Survival estimates at 12 and 24 months were 56.1% (95% CI, 51.8%-60.3%) and 30.7% (95% CI, 26.7%-34.6%), respectively, for the fluorouracil plus folinic acid group and 61.3% (95% CI, 57.1%-65.5%) and 29.6% (95% CI, 25.6%-33.5%) for the gemcitabine group. Log-rank analysis revealed no statistically significant difference in progression-free survival estimates between the treatment groups (χ^2_1=0.40; P=.53; HR, 0.96 [95% CI, 0.84-1.10]).

Toxicity

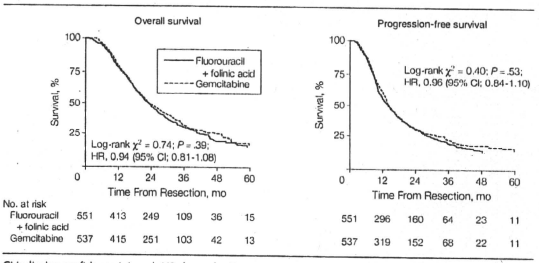
Patients receiving fluorouracil plus folinic acid had significantly increased grade 3/4 stomatitis (P<.001) and diarrhea (P<.001), whereas patients receiving gemcitabine reported significantly increased grade 3/4 hematologic toxicity (P=.003) (TABLE 2). One hundred seventeen patients (11%) reported 149 treatment-related serious adverse events, the majority attributable to inpatient hospitalization. Seventy-seven patients (14%) receiving fluorouracil plus folinic acid reported 97 treatment-related serious adverse events, compared with 40 (7.5%) receiving gemcitabine, who reported 52 events (P<.001).

Table 1. Patient Characteristics at Randomization (continued)

Characteristic	No. (%) Fluorouracil + Folinic Acid (n = 551)	Gemcitabine (n = 537)	Total (N = 1088)
Extent of resection			
Standard	364 (73)	364 (74)	728 (73)
Radical	102 (20)	82 (16)	184 (19)
Extended radical	36 (7)	47 (10)	83 (8)
Venous resection[b]			
No	430 (84)	435 (87)	865 (85)
Yes	83 (16)	67 (13)	150 (15)
Cholecystectomy			
No	122 (24)	117 (23)	239 (23)
Yes	396 (76)	391 (77)	787 (77)
Local invasion			
No	303 (58)	284 (57)	587 (57)
Yes	216 (42)	218 (43)	434 (43)
Other operative finding			
No	442 (85)	432 (87)	874 (86)
Yes	75 (15)	66 (13)	141 (14)
Postoperative complications			
No	405 (78)	372 (74)	777 (76)
Yes	112 (22)	131 (26)	243 (24)

Abbreviations: CA19-9, carbohydrate antigen 19-9; IQR, interquartile range.
[a] International Union Against Cancer (fifth edition, 1997) stages III and IVa are both equivalent to American Joint Committee on Cancer (seventh edition, 2010) stage IIB.
[b] Superior mesenteric vein or hepatic portal vein/superior mesenteric vein confluence.

Figure 2. Survival Results by Randomized Treatment



CI indicates confidence interval; HR, hazard ratio.

Prognostic Factors for Overall Survival

Univariate survival analysis of categorical variables revealed that not smoking, World Health Organization performance status 0, negative resection margins, negative lymph node status, well-differentiated tumors, stage I disease, and tumors with no local invasion were associated with improved survival (TABLE 3 and eFigure 1 and eFigure 2, available at http://www.jama.com). The increased risk of death in patients with positive margins compared with patients with negative margins was 35% (log-rank χ^2_1=16.3; P<.001; HR, 1.35 [95% CI, 1.17-1.56]). There was no significant difference in the effect of treatment across subgroups according to R status (test of heterogeneity, χ^2_1=0.3, P=.56). The continuous covariates of tumor diameter (Wald χ^2_1=10.1, P=.001) and postoperative CA19-9 level (Wald χ^2_1=126.6, P<.001) were also each significantly associated with survival at univariate

Table 2. Reported Toxicity

Toxicity Variable	Reported NCI CTC Version 2 Toxicity[a]				
	Fluorouracil + Folinic Acid (n = 551)		Gemcitabine (n = 537)		
	Grade 1/2, No.	Grade 3/4, No. (%)	Grade 1/2, No.	Grade 3/4, No. (%)	*P* Value[b]
WBC count	154	32 (6)	262	53 (10)	.01
Neutrophils	180	121 (22)	270	119 (22)	.94
Platelets	57	0	170	8 (1.5)	.003
Nausea	292	19 (3.5)	282	13 (2.5)	.37
Vomiting	159	17 (3)	131	11 (2)	.34
Stomatitis	304	54 (10)	96	1 (0)	<.001
Alopecia	189	1 (0)	135	1 (0)	>.99
Tiredness	340	45 (8)	351	32 (6)	.16
Diarrhea	333	72 (13)	194	12 (2)	<.001
Other	262	67 (12)	290	43 (8)	.03

Abbreviations: CTC, Common Terminology Criteria; NCI, National Cancer Institute; WBC, white blood cell.
[a] Toxicity grades defined per CTC Version 2.0.[22]
[b] From Fisher exact test with significance level set to $P<.005$ and with Bonferroni adjustment to account for multiple testing.

analysis but not age (Wald $\chi^2_1=0.7$, $P=.40$).

Factors with a log-rank significance of $P<.10$ were considered for inclusion in the Cox proportional hazards frailty modeling: sex, smoking, performance status, grade of disease, lymph node status, stage (I/II vs III/IV), and local invasion. The continuous covariates tumor size and postoperative CA19-9 level were included under nonlinear transformations. Stratification factors (country [random effect] and resection margin status) and treatment group were included in all models.

A model based on 766 patients with complete data (545 deaths) identified grade of disease (Wald $\chi^2_3=28.8$, $P<.001$), nodal status (Wald $\chi^2_1=19.1$,

Table 3. Univariate Survival Analysis of Categorical Variables[a]

Factor	No.		Survival Rate, %		Survival, Median (95% CI), mo	HR (95% CI)	Log-Rank χ^2	*P* Value
	Patients	Deaths	12 mo	24 mo				
Sex								
Men	598	427	78.7	46.4	21.7 (20.3-24.2)	1 [Reference]	3.4	.06
Women	490	326	80.1	51.3	24.9 (22.7-27.5)	0.87 (0.76-1.01)		
Smoking status								
Never	396	271	82.8	52.6	25.5 (22.6-29.2)	1 [Reference]	8.1	.02
Past	399	281	78.3	48.0	22.9 (21.1-25.9)	1.12 (0.95-1.32)		
Present	165	128	75.8	42.0	20.4 (17.6-23.8)	1.36 (1.10-1.67)		
Performance score								
0	371	243	80.7	54.4	25.8 (23.6-28.6)	1 [Reference]	8.5	.02
1	589	418	79.9	47.1	22.6 (21.1-24.9)	1.20 (1.03-1.41)		
2	128	92	72.1	38.2	19.2 (16.9-22.6)	1.37 (1.08-1.74)		
Resection margins								
Negative	704	460	82.8	51.4	24.7 (22.8-26.9)	1 [Reference]	16.3	<.001
Positive	384	293	73.0	43.4	19.9 (17.7-23.0)	1.35 (1.17-1.56)		
Tumor grade								
Well differentiated	147	86	90.7	57.3	27.9 (23.9-36.1)	1 [Reference]	24.2	<.001
Moderately differentiated	663	457	81.7	51.4	24.7 (22.6-26.4)	1.31 (1.04-1.65)		
Poorly differentiated	260	199	66.6	36.5	17.1 (15.3-20.1)	1.79 (1.39-2.31)		
Lymph nodes								
Negative	307	161	86.1	63.1	35.0 (29.4-40.6)	1 [Reference]	52.3	<.001
Positive	778	589	76.7	43.2	21.0 (19.4-22.3)	1.89 (1.59-2.26)		
Tumor stage[b]								
I	104	53	87.0	57.0	32.8 (22.3-∞)	1 [Reference]	31.8	<.001
II	298	186	83.6	58.0	28.1 (24.8-31.7)	1.31 (0.96-1.77)		
III	622	468	76.2	42.9	20.7 (18.8-22.3)	1.88 (1.41-2.50)		
IVa	42	31	73.2	43.2	22.6 (15.1-27.0)	1.75 (1.13-2.73)		
Local invasion								
No	587	397	80.5	51.5	24.8 (22.3-27.1)	1 [Reference]	6.6	.01
Yes	434	326	77.5	44.7	21.8 (19.9-23.8)	1.21 (1.05-1.40)		
Treatment								
Fluorouracil + folinic acid	551	388	78.5	48.1	23.0 (21.1-25.0)	1 [Reference]	0.74	.39
Gemcitabine	537	365	80.1	49.1	23.6 (21.4-26.4)	0.94 (0.81-1.08)		

Abbreviations: CI, confidence interval; HR, hazard ratio.
[a] Reporting where log-rank $P<.10$.
[b] International Union Against Cancer (fifth ed, 1997) stages III and IVa are both equivalent to American Joint Committee on Cancer (seventh ed, 2010) stage IIB.

$P < .001$), and CA19-9 level (Wald $\chi^2_1 = 110.4$, $P < .001$) as significant independent prognostic factors of overall survival (TABLE 4). To maximize the data for modeling, further analysis excluding CA19-9 level, which was associated with a substantial amount of missing data (321 patients), resulted in a model based on 1030 patients with complete data (715 deaths). This confirmed grade of disease (Wald $\chi^2_3 = 25.2$, $P < .001$), nodal status (Wald $\chi^2_1 = 41.7$, $P < .001$), performance status (Wald $\chi^2_2 = 10.9$, $P = .004$), tumor size (Wald $\chi^2_1 = 8.9$, $P = .003$), and smoking status (Wald $\chi^2_3 = 9.2$, $P = .03$) as significant independent prognostic factors of overall survival.

Tests of heterogeneity within pathological (eFigure 3) or demographic (eFigure 4) subgroups did not reveal any significant findings.

Quality of Life

Five hundred sixty-five patients (280 randomized to receive fluorouracil plus folinic acid and 285 to receive gemcitabine) completed quality-of-life questionnaires, including a baseline questionnaire. The subgroups were representative of patients in the main study based on patient characteristics. Of these, 438 completed 3-month questionnaires, 417 completed 6-month questionnaires, and 307 completed 12-month questionnaires. Standardized AUC scores are based on average standardized scores ranging between 0 and 100. There were no significant differences in mean standardized AUC for global quality-of-life scores across treatment groups conditional on patient survival; mean standardized AUC was 43.6 (SD, 20.1) for patients receiving fluorouracil plus folinic acid, compared with 46.6 (SD, 19.7) for those receiving gemcitabine ($P = .08$).

COMMENT

There have been few large randomized controlled trials of adjuvant treatment following resection in pancreatic cancer. The first of these, the ESPAC-1 trial,[6,12] concluded that chemotherapy with fluorouracil plus folinic acid improved overall survival but chemoradiotherapy did not.[6,12] The failure of adjuvant chemoradiotherapy to enhance survival was also reflected in the results of the EORTC multicenter prospective randomized trial.[5] The Radiation Therapy Oncology Group (RTOG) 9704 trial randomized 538 patients to receive either prechemoradiation and postchemoradiation gemcitabine or prechemoradiation and postchemoradiation fluorouracil.[7] The median survival in the 451 eligible patients was 16.7 and 18.8 months, respectively ($P = .34$), and in the 388 patients with cancer of the pancreatic head was 20.5 months vs 16.9 months, respectively ($P = .09$).[7] The primary end point in the CONKO-001 trial was disease-free survival.[13] This was 13.4 months for gemcitabine and 6.9 months for surgery alone ($P < .001$), while the median overall survival was 22.1 months and 20.5 months, respectively ($P < .06$).[13]

The ESPAC-3 trial found a median survival of 23.0 months for patients treated with fluorouracil plus folinic acid and 23.6 months for those treated with gemcitabine and a median progression-free survival of 14.1 months and 14.3 months, respectively. Tumor

Table 4. Cox Proportional Hazards Models[a]

Factor	HR (95% CI)	Wald χ^2	*P* Value
Including CA19-9			
Country (19 RE)	NA	0.7	.52
Resection margins (negative vs positive)	1.18 (0.99-1.40)	3.3	.07
Treatment (fluorouracil + folinic acid vs gemcitabine)	0.88 (0.75-1.05)	2.1	.15
Tumor grade			
Well differentiated	1 [Reference]	28.8	<.001
Moderately differentiated	1.72 (1.27-2.32)		
Poorly differentiated	2.32 (1.68-3.20)		
Missing	1.12 (0.53-2.36)		
Lymph nodes (negative vs positive)	1.60 (1.29-1.97)	19.1	<.001
CA19-9[b]	NA	110.4	<.001
Excluding CA19-9[c]			
Country (19 RE)	NA	0.8	.41
Resection margins (negative vs positive)	1.17 (1.01-1.37)	4.1	.04
Treatment (fluorouracil + folinic acid vs gemcitabine)	0.90 (0.78-1.04)	1.9	.16
Tumor grade			
Well differentiated	1 [Reference]	25.2	<.001
Moderately differentiated	1.27 (1.00-1.61)		
Poorly differentiated	1.81 (1.39-2.36)		
Missing	1.11 (0.56-2.22)		
Lymph nodes (negative vs positive)	1.82 (1.52-2.18)	41.7	<.001
Performance status			
0	1 [Reference]	10.9	.004
1	1.22 (1.03-1.43)		
2	1.49 (1.16-1.92)		
Maximum tumor size[d]	1.25 (1.08-1.45)	8.9	.003
Smoking			
Never	1 [Reference]	9.2	.03
Past	1.08 (0.91-1.29)		
Present	1.38 (1.11-1.71)		
Missing	1.22 (0.94-1.59)		

Abbreviations: CA19-9, carbohydrate antigen 19-9; CI, confidence interval; HR, hazard ratio; NA, not applicable; RE, random effects.

[a] See Table 3 for numbers of patients, numbers of deaths, and 12-month and 24-month survival rates.

[b] Second-degree fractional polynomial transformation applied: CA19-9^(−0.5) + log(CA19-9).

[c] Patients = 1030; deaths = 715.

[d] Log transformation applied; HR based on a 1-unit increase in log(tumor size).

grade, nodal status, tumor size, postoperative serum CA19-9 levels, performance status, and smoking were all independent prognostic factors of overall survival. Although resection margin status was significant on univariate analysis, this was not so on multivariate analysis, confirming the previous results of ESPAC-1 that primary tumor characteristics dominate outcome.[23]

The prognostic significance of CA19-9 level in ESPAC-1 mirrored that in the RTOG trial, with both studies using postresectional values.[24] This is important: preoperative levels are artificially elevated in the presence of obstructive jaundice, because CA19-9 is excreted in bile and there is no simple correction factor. In the CONKO-001 trial, patients with CA19-9 levels greater than 2.5 times the upper limit of normal were excluded, indicating that in that study there was a bias toward patients with a more favorable prognosis.[13] That tobacco smoking affected long-term outcome was a novel finding and should add further weight against the use of tobacco.

The absence of an overall survival difference between postoperative adjuvant fluorouracil plus folinic acid compared with gemcitabine contrasts with the findings of a much smaller study in patients with nonresected advanced pancreatic cancer that showed a survival benefit with gemcitabine as compared with fluorouracil.[13] The fluorouracil regimen used in that trial (600 mg/m^2 bolus once weekly without folinic acid) was less intensive than that used in ESPAC-3.[13] This fluorouracil regimen may be less efficacious than the Mayo Clinic regimen, but there are no large randomized trials that have directly compared these 2 treatments in pancreatic cancer.

In conclusion, gemcitabine did not result in improved overall survival compared with fluorouracil plus folinic acid in patients with resected pancreatic cancer. As a logical progression from these data we have designed the ESPAC-4 trial, currently in progress, to compare combination chemotherapy with gemcitabine plus capecitabine, an orally active fluoropyrimidine,[25] with gemcitabine alone.

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Author Contributions: Dr Neoptolemos had full access to all the data in the study and takes full responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Neoptolemos, Stocken, Bassi, Ghaneh, Cunningham, Moore, Friess, Dervenis, Büchler.

Acquisition of data: Neoptolemos, Bassi, Cunningham, Goldstein, Padbury, Gallinger, Mariette, Wente, Izbicki, Lerch, Dervenis, Oláh, Butturini, Doi, Lind, Smith, Palmer, Buckels, Thompson, McKay, Rawcliffe, Büchler.

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Drafting of the manuscript: Neoptolemos, Stocken, Ghaneh, Goldstein, Wente, Dervenis, Oláh, Buckels, Rawcliffe, Büchler.

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Study supervision: Neoptolemos, Bassi, Ghaneh, Padbury, Izbicki, Friess, Dervenis, Oláh, Butturini, Smith, Palmer, Thompson, Büchler.

Ms Rawcliffe was the trial coordinator responsible for central administration ensuring ethical standards for collection and verification of data. The results were interpreted by the ESPAC working party (all of the above). Drs Neoptolemos, Ghaneh, and Stocken prepared the initial draft and were responsible for collating changes proposed by the aforementioned into the final paper before final approval by all participants in the European Study Group for Pancreatic Cancer.

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The Full List of ESPAC Specialists Who Contributed to the Treatment of Patients in the ESPAC-3 Trial is presented in the eAppendix.

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Online-Only Material: eFigures 1 through 4 and the eAppendix are available at http://www.jama.com.

REFERENCES

1. International Agency for Research on Cancer, World Health Organization. Globocan 2008. World Health Organization Web site. http://globocan.iarc.fr/. Accessed July 15, 2010.
2. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010 [published online ahead of print July 7, 2010]. *CA Cancer J Clin*. doi:10.3322/caac.20073.
3. Wagner M, Redaelli C, Lietz M, Seiler CA, Friess H, Büchler MW. Curative resection is the single most important factor determining outcome in patients with pancreatic adenocarcinoma. *Br J Surg*. 2004;91(5):586-594.
4. Kalser MH, Ellenberg SS. Pancreatic cancer: adjuvant combined radiation and chemotherapy following curative resection. *Arch Surg*. 1985;120(8):899-903.
5. Smeenk HG, van Eijck CHJ, Hop WC, et al. Long-term survival and metastatic pattern of pancreatic and periampullary cancer after adjuvant chemoradiation or observation: long-term results of EORTC trial 40891. *Ann Surg*. 2007;246(5):734-740.
6. Neoptolemos JP, Dunn JA, Stocken DD, et al; European Study Group for Pancreatic Cancer. Adjuvant chemoradiotherapy and chemotherapy in resectable pancreatic cancer: a randomised controlled trial. *Lancet*. 2001;358(9293):1576-1585.
7. Regine WF, Winter KA, Abrams RA, et al. Fluorouracil vs gemcitabine chemotherapy before and after fluorouracil-based chemoradiation following resection of pancreatic adenocarcinoma: a randomized controlled trial. *JAMA*. 2008;299(9):1019-1026.
8. Twombly R. Adjuvant chemoradiation for pancreatic cancer: few good data, much debate. *J Natl Cancer Inst*. 2008;100(23):1670-1671.
9. Bakkevold KE, Arnesjo B, Dahl O, Kambestad B. Adjuvant combination chemotherapy (AMF) follow-

ing radical resection of carcinoma of the pancreas and papilla of Vater—results of a controlled, prospective, randomised multicentre study. *Eur J Cancer*. 1993; 29A(5):698-703.
10. Takada T, Amano H, Yasuda H, et al; Study Group of Surgical Adjuvant Therapy for Carcinomas of the Pancreas and Biliary Tract. Is postoperative adjuvant chemotherapy useful for gallbladder carcinoma? a phase III multicenter prospective randomized controlled trial in patients with resected pancreaticobiliary carcinoma. *Cancer*. 2002;95(8):1685-1695.
11. Kosuge T, Kiuchi T, Mukai K, Kakizoe T; Japanese Study Group of Adjuvant Therapy for Pancreatic Cancer (JSAP). A multicenter randomized controlled trial to evaluate the effect of adjuvant cisplatin and 5-fluorouracil therapy after curative resection in cases of pancreatic cancer. *Jpn J Clin Oncol*. 2006; 36(3):159-165.
12. Neoptolemos JP, Stocken DD, Friess H, et al; European Study Group for Pancreatic Cancer. A randomized trial of chemoradiotherapy and chemotherapy after resection of pancreatic cancer. *N Engl J Med*. 2004;350(12):1200-1210.
13. Oettle H, Post S, Neuhaus P, et al. Adjuvant chemotherapy with gemcitabine vs observation in patients undergoing curative-intent resection of pancreatic cancer: a randomized controlled trial. *JAMA*. 2007;297(3):267-277.
14. Ueno H, Kosuge T, Matsuyama Y, et al; Japanese Study Group of Adjuvant Therapy for Pancreatic Cancer. A randomised phase III trial comparing gemcitabine with surgery-only in patients with resected pancreatic cancer. *Br J Cancer*. 2009;101 (6):908-915.
15. Burris HA III, Moore MJ, Andersen J, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol*. 1997;15(6):2403-2413.
16. Pedrazzoli S, Beger HG, Obertop H, et al. A surgical and pathological based classification of resective treatment of pancreatic cancer: summary of an international workshop on surgical procedures in pancreatic cancer. *Dig Surg*. 1999;16(4):337-345.
17. Carter R, Stocken DD, Ghaneh P, et al; European Study Group for Pancreatic Cancer (ESPAC). Longitudinal quality of life data can provide insights on the impact of adjuvant treatment for pancreatic cancer—subset analysis of the ESPAC-1 data. *Int J Cancer*. 2009;124(12):2960-2965.
18. Kaplan EL, Meier P. Non parametric estimation from incomplete observations. *J Am Stat Assoc*. 1958; 53:457-481.
19. Peto R, Pike MC, Armitage P, et al. Design and analysis of randomized clinical trials requiring prolonged observation of each patient, II: analysis and examples. *Br J Cancer*. 1977;35(1):1-39.
20. Cox DR. Regression models and life-tables. *J R Stat Soc [B]*. 1972;34:187-220.
21. Royston P, Altman DG. Regression using fractional polynomials of continuous covariates: parsimonious parametric modelling. *Appl Stat*. 1994;43: 429-467.
22. National Cancer Institute. Common Toxicity Criteria (CTC) Version 2.0. National Cancer Institute Web site. http://ctep.cancer.gov/protocoldevelopment/electronic_applications/docs/ctcv20_4-30-992.pdf. 1999. Accessed August 10, 2010.
23. Butturini G, Stocken DD, Wente MN, et al; Pancreatic Cancer Meta-Analysis Group. Influence of resection margins and treatment on survival in patients with pancreatic cancer: meta-analysis of randomized controlled trials. *Arch Surg*. 2008;143(1):75-83.
24. Berger AC, Garcia M Jr, Hoffman JP, et al. Postresection CA 19-9 predicts overall survival in patients with pancreatic cancer treated with adjuvant chemoradiation: a prospective validation by RTOG 9704. *J Clin Oncol*. 2008;26(36):5918-5922.
25. Cunningham D, Chau I, Stocken DD, et al. Phase III randomized comparison of gemcitabine versus gemcitabine plus capecitabine in patients with advanced pancreatic cancer. *J Clin Oncol*. 2009;27(33):5513-5518.

If we have made obvious mistakes, we should not try, as we generally do, to gloss them over, or to find something to excuse . . . them; we should admit to ourselves that we have committed faults, and open our eyes wide to all their enormity, in order that we may firmly resolve to avoid them in the time to come.

—Arthur Schopenhauer (1788-1860)

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ORIGINAL ARTICLE

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/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

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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

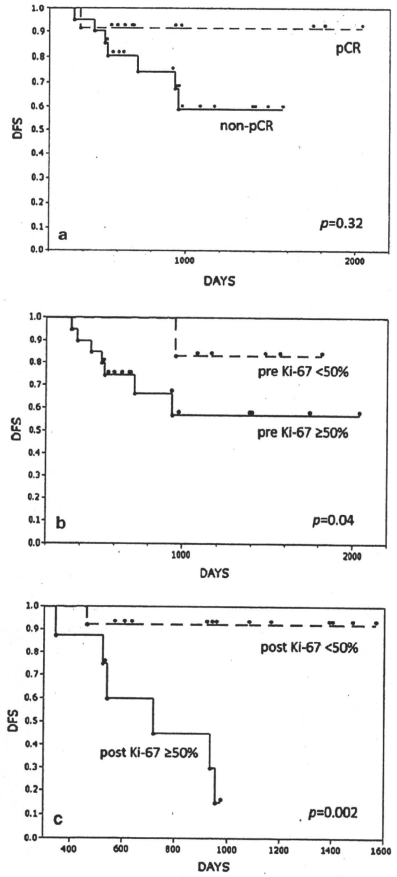

Fig. 1 Disease-free survival (DFS). **a** DFS of pCR and non-pCR patients after NAC. Non-pCR patients showed worse disease-free survival compared with pCR patients, but it was not statistically significant ($P = 0.32$). **b** DFS based on Ki-67 expression of pre-chemotherapy. High Ki-67 (≥50%) patients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

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Plasma biomarker discovery and validation for colorectal cancer by quantitative shotgun mass spectrometry and protein microarray

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The development of a new plasma biomarker for early detection would be necessary to improve the overall outcome of colorectal cancer. Here we report the identification and validation of the ninth component of complement (C9) as a novel plasma biomarker for colorectal cancer by cutting-edge proteomic technologies. Plasma proteins were enzymatically digested into a large array of peptides, and the relative quantity of a total of 94 803 peptide peaks was compared between 31 colorectal cancer patients and 59 age/sex-matched healthy controls using 2D image-converted analysis of liquid chromatography and mass spectrometry. The selected biomarker candidates were validated in 345 subjects (115 colorectal cancer patients and 230 age/sex-matched healthy controls) using high-density reverse-phase protein microarrays. Plasma levels of Apo AI and C9 in colorectal cancer patients significantly differed from healthy controls with P values of 7.94×10^{-4} and 1.43×10^{-12} (Student's t-test), respectively. In particular, C9 was elevated in patients with colorectal cancer, including those with stage-I and -II diseases ($P = 3.01 \times 10^{-3}$ and $P = 1.13 \times 10^{-5}$, respectively). Although the significance of the present study must be validated in an independent clinical study, the increment of plasma C9 may be applicable to the early detection of colorectal cancer. (*Cancer Sci*, doi: 10.1111/j.1349-7006.2010.01818.x, 2010)

Colorectal cancer is the third most common cancer worldwide, with an estimated one and half million newly diagnosed cases every year.[1] In Japan, colorectal cancer is currently the third cause of cancer death in men and the first in women, but its incidence is predicted to increase and become the leading cause by 2015, most likely due to changing dietary habits as well as environmental conditions.[2,3] Successful prevention of death from colorectal cancer depends on its early detection. The surgical management of early stage colorectal cancer without metastasis is relatively uncomplicated. There is a significant level of evidence that the application of the fecal occult blood test to mass screening reduces the risk of colorectal cancer death, but its sensitivity and specificity, especially for early stage colorectal cancer, seem to be insufficient. Barium enema, flexible sigmoidoscopy/colonoscopy and ^{18}F-fluorodeoxyglucose positron emission tomography have higher specificity, but may not be cost- and labor-effective for mass screening of the asymptomatic general population.

The circulating blood proteome holds great promise as a reservoir of disease information, and a large variety of plasma/serum proteins have been used as disease biomarkers. Carcinoembryonic antigen (CEA) is a serum biomarker most widely used for colorectal cancer. However, the serum level of CEA often does not elevate in patients with colorectal cancer in the early stages and cannot be applicable to early detection of the disease.[4] We therefore have been searching for new serum/plasma biomarkers that can be used for mass survey of colorectal cancer.

Recently, various mass spectrometry (MS)-based proteomic technologies have been applied to clinical samples in the hope of identifying new disease biomarkers.[5–9] Among those technologies, shotgun proteomics has been considered the most promising because of its high sensitivity and protein identification capability: protein samples are enzymatically digested into a large array of peptides with uniform physical characteristics, and every peptide fragment is analyzed by liquid chromatography (LC) and MS. However, the number of samples that can be compared by shotgun proteomics is usually limited because isotope labeling is necessary to give a quantitative dimension to shotgun proteomics. The protein contents of each human sample vary significantly among individuals, and biomarker candidates can be distinguished from simple personal heterogeneity only by comparing a sufficient number of cases and controls. To overcome this limitation, we developed a software named 2D image converted analysis of liquid chromatography and MS (2DICAL),[9,10] which enabled accurate quantitative comparison across a theoretically unlimited number of LC-MS data without isotope labeling.[10] Using this powerful software we successfully identified biomarkers that can predict the hematological toxicities and survival of pancreatic cancer patients receiving gemcitabine.[11,12]

In the present study we searched for a biomarker that can be used for the early detection of colorectal cancer using 2DICAL. We carefully selected cases and controls to be compared by matching their age and gender distributions, as well as residential areas. We identified the significance increment of complement component C9 in the sera of patients with colorectal cancer, and its significance was validated in a large cohort using another innovative proteome technology, high-density reverse-phase protein microarray (RPPM).

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Materials and Methods

Patients and plasma samples. Plasma samples (n = 345) were collected from 115 patients diagnosed with colorectal cancer and 230 healthy volunteers matched with cancer patients by sex, age and residential area (two controls for each cancer patient; Table 1) between October 1998 and March 2002 with informed consent, as described previously.[13] Thirty-one cancer patients and 59 controls were randomly selected and subjected to 2DICAL analysis.

Another set of plasma samples were collected prospectively from 378 individuals including healthy volunteers and newcomers mainly to the Department of Gastroenterology at the National Cancer Center Hospital (Tokyo), Osaka National Hospital (Osaka), Jichi Medical School Hospital (Shimotsuke), Osaka Medical College (Osaka), Tokyo Medical University Hospital (Tokyo), Osaka Medical Center for Cancer and Cardiovascular Diseases (Osaka), and Fukuoka University Hospital (Fukuoka). This multi-institutional study was conducted as part of the "Third-Term Comprehensive Control Research for Cancer" conducted by the Ministry of Health, Labour and Welfare of Japan. Written informed consent was obtained from every individual, and the study protocol was approved by the ethics committee of each participating institution.

Sample preparation and LC-MS. Twelve abundant plasma proteins including albumin, IgG, α1-antitrypsin, IgA, IgM, transferrin, haptoglobin, α1-acid glycoprotein, α2-macroglobulin, Apo AI, apolipoprotein AII and fibrinogen were deduced from plasma samples using IgY-12 spin columns (Beckman Coulter, Fullerton, CA, USA) prior to MS analysis.[11] The deduced samples were precipitated with acetonitrile, dried and digested to peptide with modified trypsin. LC-MS and data acquisition were performed as described previously.[14] Briefly, MS spectra were acquired using nano-electrospray ionization (nano-ESI)-quadruple time-of-flight (QqTOF) MS (QTOF Ultima; Waters, Milford, MA, USA) every second for 60 min in the 250–1600 m/z range. The MS peaks of each sample with the same m/z were extracted every 1 m/z and aligned. Peak lists were created using the MassNavigator software package (version 1.2; Mitsui Knowledge Industry, Tokyo, Japan). Targeted tandem MS (MS/MS) data were analyzed with the Mascot software package (version 2.2.1; Matrix Sciences, London, UK) against the NCBInr database (NCBInr_20070419. fast).

Antibodies. Anti-Apo AI rabbit polyclonal antibody was purchased from Calbiochem (Darmstadt, Germany), anti-C9 mouse monoclonal antibody from AntibodyShop (Gentofte, Denmark) and anti-α2-macroglobulin mouse monoclonal antibody from R&D Systems (Minneapolis, MN, USA).

Immunoblot analysis. Protein was separated by SDS-PAGE and transferred to polyvinyldene difluoride membrane, as described previously.[15] The membrane was incubated with anti-Apo AI, anti-C9 or anti-α2-macroglobulin (loading control) and then with relevant horseradish peroxidase (HRP)-conjugated secondary antibody. Blots were detected by ECL western blotting detection reagent according to the manufacturer's instruction (Amersham Biosciences, Buckinghamshire, UK).

RPPM. Plasma samples were serially diluted using PBS containing 0.01% triton X with or without 1% bovine serum albumin (BSA). The diluted plasma samples were printed onto slide glasses coated with ProLinker (ProteoChip; Proteogen, Seoul, Korea)[16,17] using a protein microarrayer equipped with 32 stainless steel pins of 100 μm diameter (Kakengeneqs, Matsudo, Japan) at 4°C. Printed microarray slides were incubated overnight at 37°C and stored at 4°C under desiccation. After returning to room temperature, the array slides were blocked with PBS containing 0.5% casein for 30 min and hybridized overnight with the first antibodies at 4°C. After washing, the array slides were hybridized with relevant biotinylated second antibodies (Vector Laboratories Inc. Burlingame, CA, USA) for 1 h and subsequently with avidin–HRP conjugated reagent (Amersham Biosciences) for 30 min. The fluorescent Cy5 signals were amplified using the tyramide signal amplification sys-

Table 1. Clinicopathological characteristics of individuals examined in the present study

	All cases (n = 345)			Cases analyzed by 2DICAL (n = 90)		
	Cancer (n = 115)	Healthy (n = 230)	P value	Cancer (n = 31)	Healthy (n = 59)	P value
Age (mean ± SD) (years)	59.3 ± 8.9	59.4 ± 8.9	0.93‡	56.8 ± 9.9	56.4 ± 9.8	0.79‡
Gender						
Male	71	142	0.92§	20	36	0.75§
Female	44	88		11	23	
Primary site						
Cecum	6			0		
Ascending colon	27			9		
Transverse colon	8			4		
Descending colon	5			3		
Sigmoid colon	18			5		
Rectum	51			10		
Clinical stage†						
0	17			5		
I	35			10		
II	28			7		
III	25			6		
IV	10			3		
Histology						
Well-differentiated adenocarcinoma	74			21		
Moderately differentiated adenocarcinoma	37			8		
Poorly differentiated adenocarcinoma	2			1		
Others	2			1		

†According to TNM Classification of Malignant Tumors (International Union Against Cancer), 6th Edition. ‡Student's t-test. §Chi-square test.

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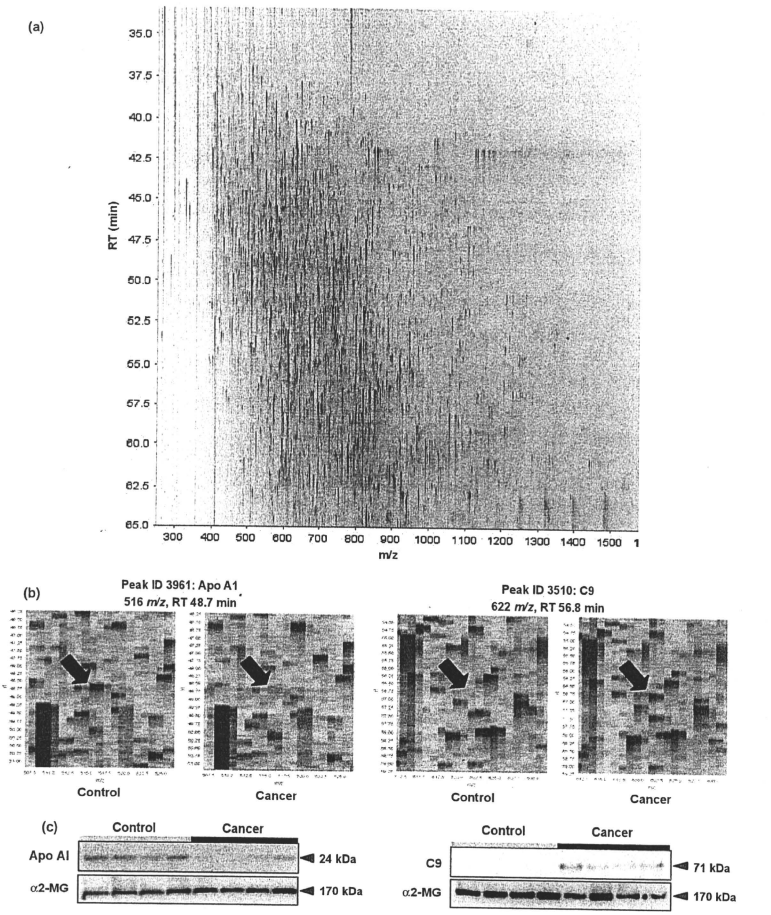

Fig. 1. Plasma biomarker discovery by 2D image converted analysis of liquid chromatography and mass spectrometry (2DICAL). (a) Two-dimensional display of >90 000 peaks of a representative sample with retention time (RT; in minutes) along the vertical (*Y*) axis and with mass to charge (*m/z*) ration value along the horizontal (*Y*) axis. (b) Peptide peaks derived from apolipoprotein AI (Apo AI; left; ID 3961) and complement component C9 (C9; right; ID 3510) in a representative colorectal cancer patient (Cancer) and a representative healthy individual (Control). (c) Detection of Apo AI, C9 and α2-macroglobulin (α2-MG, loading control) in plasma samples of four representative colorectal cancer patients and four healthy controls by immunoblotting.

tem (Perkin Elmer, Boston, MA, USA) according to the manufacturer's instructions. Counterstaining was performed with Alexa 488-labeled anti-human IgG antibody (Invitrogen, Carlsbad, CA, USA). Dual-color fluorescent signals (green and red) were detected with a confocal laser microarray scanner (InnoScan 700 AL; Innopsys, Carbonne, France). The median signal intensity of quadruplicates was calculated using the Mapix software (Innopsys).

Measurement of CEA. The plasma level of CEA was determined using the CEA Enzyme Immunoassay kit (Hope Laboratories, Belmont, CA, USA) according to the manufacturer's instruction.

Statistical analysis. Statistically significant differences were detected using Paired *t*-test and Student's *t*-test. Interquartile range (IQR), receiver operator characteristics (ROC) and area under the curve (AUC) analyses were performed using the

Table 2. Protein identification of peptide peaks with significant difference between colorectal cancer patients and healthy controls

ID	M/Z	RT	prot_acc	prot_desc	Cancer†	Control†	pep_score	AUC	*P*-value‡	pep_seq
664	651.33	46.18	APOA1_HUMAN	Apolipoprotein A-I precursor (Apo-AI) (ApoA-I) (contains: apolipoprotein A-I [1-242])	34.01 ± 18.34	60.26 ± 41.89	58.87	0.77	0.00038	THLAPYSDELR
3961	516.78	48.71	APOA1_HUMAN	Apolipoprotein A-I precursor (Apo-AI) (ApoA-I) (contains: apolipoprotein A-I [1-242])	11.40 ± 3.85	17.32 ± 7.43	49.08	0.81	0.00006	LSPLGEEMR
2384	632.03	63.40	A2GL_HUMAN	Leucine-rich alpha-2-glycoprotein precursor (LRG)	42.66 ± 23.89	24.61 ± 10.75	46.04	0.79	0.00094	ENQLEVLEVSWLHGLK
2819	506.81	45.11	APOA1_HUMAN	Apolipoprotein A-I precursor (Apo-AI) (ApoA-I) (contains: apolipoprotein A-I [1-242])	16.56 ± 7.20	26.31 ± 14.57	45.5	0.77	0.00063	AKPALEDLR
516	642.31	52.07	APOA1_HUMAN	Apolipoprotein A-I precursor (Apo-AI) (ApoA-I) (contains: apolipoprotein A-I [1-242])	41.38 ± 21.38	79.15 ± 64.65	33.51	0.76	0.00040	WQEEMELYR
5489	728.37	57.22	CO9_HUMAN	Complement component C9 precursor (contains: complement component C9a; complement component C9b)	22.00 ± 8.48	15.71 ± 4.73	32.3	0.75	0.00000	AIEDYINEFSVR
3510	621.88	56.75	CO9_HUMAN	Complement component C9 precursor (contains: complement component C9a; complement component C9b)	34.36 ± 18.88	18.48 ± 8.90	26.47	0.77	0.00003	LSPIYNLVPVK
3401	618.36	59.06	APOA1_HUMAN	Apolipoprotein A-I precursor (Apo-AI) (ApoA-I) (contains: apolipoprotein A-I [1-242])	15.13 ± 4.59	25.00 ± 13.78	25.32	0.77	0.00022	DLATVYVDVLK
393	626.82	50.77	APOA1_HUMAN	Apolipoprotein A-I precursor (Apo-AI) (ApoA-I) (contains: apolipoprotein A-I [1-242])	45.28 ± 25.93	88.60 ± 65.21	25.1	0.79	0.00005	VQPYLDDFQK
3615	743.04	48.25	STX16_HUMAN	Syntaxin-16 (Syn16)	16.95 ± 14.15	9.81 ± 3.18	24.74	0.75	0.02199	RPPPKWVDGVDEIQYDVGR
348	615.86	61.43	APOA1_HUMAN	Apolipoprotein A-I precursor (Apo-AI) (ApoA-I) (contains: apolipoprotein A-I [1-242])	55.73 ± 34.80	109.93 ± 75.99	23.96	0.77	0.00008	QGLLPVLESFK
5664	622.33	57.48	PDGFD_HUMAN	Platelet-derived growth factor D precursor (PDGF D)	25.96 ± 9.12	21.18 ± 7.81	21.52	0.66	0.00001	LIFVYTLICANFCSCR
11415	1057.01	48.26	EP400_HUMAN	E1A-binding protein p400 (EC 3.6.1.-) (p400 kDa SWI2/SNF2-related protein)	11.57 ± 4.37	8.79 ± 2.29	18.02	0.75	0.01037	GRPPIATFSANPEAKAAAAPFQTSQASASAPR
2259	941.45	60.01	KCD10_HUMAN	BTB/POZ domain-containing protein KCTD10	71.39 ± 18.04	61.47 ± 17.33	16.49	0.66	0.00010	EEMSGESVVSSAVPAAATR
6778	804.81	46.45	DIP2A_HUMAN	Disco-interacting protein 2 homolog A	10.73 ± 5.51	7.57 ± 1.59	16.21	0.77	0.00017	KAVLSMNGLSYGVIR
7297	804.98	46.47	K0460_HUMAN	Uncharacterized protein KIAA0460	9.99 ± 5.47	6.87 ± 1.49	16.02	0.78	0.00005	AAGGGGGSSKASSSSASSAGALESSLDR
7749	662.33	46.17	ADPPT_HUMAN	L-Aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase (EC 2.7.8.-)	10.70 ± 2.16	14.59 ± 5.05	15.92	0.77	0.00206	FTNKEWETIR

†Data are presented as mean ± SD. ‡Paired *t*-test. AUC, area under curve; RT, retention time.

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components available in R-project (http://www.r-project.org/).[11] A composite index of two markers was generated using the results of multivariate logistic regression analysis, which also enabled calculation of the ROC curve.

Results

Identification of biomarker candidates. We compared plasma proteome data between 31 colorectal cancer patients and 59 healthy volunteers (Table 1) using 2DICAI. A total of 94 803 independent peptide peaks were detected across the 90 cases (Fig. 1a) and numbered from ID1 to ID94803. Ninety MS peaks showed a statistically significant difference between healthy controls and colorectal cancer patients (maximum mass peak intensity > 10 [arbitrary unit] and $P \leq 0.0001$ [Paired t-test] or AUC ≥ 0.75). Twenty-nine peaks were further selected by visual inspection (Fig. 1b) and subjected to MS/MS analysis. MS/MS spectra obtained from 17 peptide peaks matched 10 proteins deposited in the NCBInr database (Table 2). We selected two proteins, Apo AI and C9, for further analyses because the same protein annotation was obtained from at least two independent peaks.

Identification and differential expression of Apo AI and C9 were confirmed by immunoblotting plasma samples of representative cases (Fig. 1c). Apo AI was downregulated in cancer patients, while C9 was upregulated.

Establishment of RPPM. For the rapid selection and validation of plasma biomarker candidates, we established a high-density protein microarray platform. Plasma samples were serially diluted and printed in quadruplicate onto a hydrophobic glass surface in a format of 6144 spots within an area of 17.65 × 34.57 mm. The location of each spot was visualized by staining human IgG (green), and the relative amounts of Apo A1 and C9 proteins were quantified by hybridization with antibodies (red). Fluorescent signal intensity showed linearity in the plasma dilution range of ×32 to ×4096 in a quality control experiment (Fig. 2a) and was highly reproducible among four independent experiments (Fig. 2b). Over 78% of spots showed coefficients of variation (CV) values of < 0.1 (Fig. 2c).

Validation of biomarker candidates. In order to examine the significance of Apo AI and C9 in a larger cohort, we used RPPM, onto which plasma samples were spotted in a high-density manner. The plasma samples were serially diluted and randomly printed four times onto a microarray. Figure 3a depicts the entire image of RPPM stained with anti-C9 antibody. Representative blots of colorectal cancer and control samples are shown in Figure 3b. The RPPM revealed that Apo AI was downregulated in colorectal cancer patients compared with healthy controls, and the difference between colorectal cancer and healthy controls was statistically significant ($P = 0.000794$; Fig. 3c). C9 was significantly upregulated in colorectal cancer ($P = 1.43 \times 10^{-12}$; Fig. 3d). The results of RPPM were well correlated with those of immunoblot assay (Fig. S1), thus confirming the preciseness of RPPM.

The AUC values of Apo AI and C9 were 0.621 and 0.730, respectively (Fig. 3e,f). Although the level of plasma Apo AI was significantly different between colorectal cancer patients and healthy controls, the utility of Apo AI as a biomarker of colorectal cancer seems to be limited due to its relatively low AUC value. Statistically significant differences for upregulation of C9 were observed in patients with stage I, II, III and IV[18] colorectal cancer ($P = 3.02 \times 10^{-3}$, 1.13×10^{-5}, 5.22×10^{-8} and 3.45×10^{-4}, respectively; Fig. 3g). The AUC values of C9 for the early (stages 0–II) and advanced (stages III and IV) colorectal cancer patients were 0.667 and 0.862, respectively.

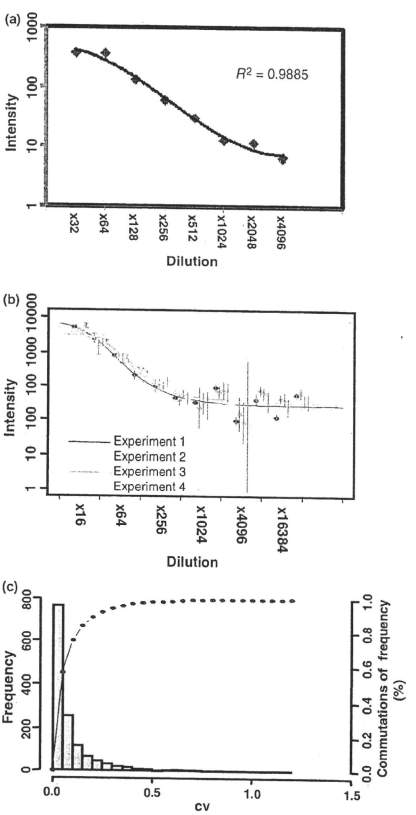

Fig. 2. Reproducibility of reverse-phase plasma microarray (RPPM). (a) Linearity of RPPM. A plasma sample was serially diluted from 32 to 4096 folds and spotted onto a ProteoChip glass. The glass was stained with anti-apolipoprotein AI antibody as described in the Materials and Methods. The mean fluorescence intensity of quadruplicates (vertical axis in arbitrary units) is plotted against plasma dilution (horizontal axis). (b) Reproducibility of RPPM. A plasma sample was serially diluted from 16 to 32 768 folds and processed as described above. The same experiment was conducted four times (Experiments 1–4), and their dilution curves overlapped. The dots represent the median intensity of quadruplicates. Bars represent interquartile ranges (IQR). (c) Distribution of the coefficients of variation (CV) values among quadruplicates in the 6144 spots (1536 quadruplicates) of RPPM stained with anti-Apo AI antibody. The dotted line represents cumulative frequency (%; right vertical axis label).

We measured the plasma level of CEA in 42 samples (20 healthy controls and 22 colorectal cancer patients: stage 0 [$n = 2$], stage I [$n = 5$], stage II [$n = 5$], stage III [$n = 5$] and stage IV [$n = 5$]), for which the residual sample volume was sufficient for the measurement with enzyme-linked immunosorbent assay (ELISA). The AUC values of CEA for the early

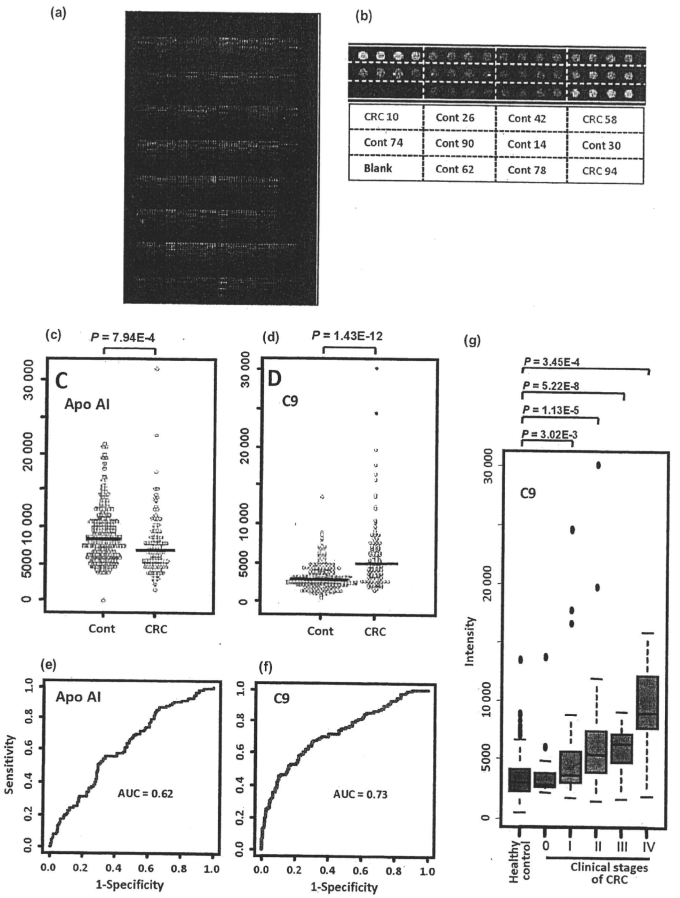

Fig. 3. Validation of altered plasma apolipoprotein AI (Apo AI) and C9 in colorectal cancer by reverse-phase plasma microarray (RPPM). (a) Dual-color scanning image of RPPM, on which serially (64- to 512-fold) diluted plasma samples of colorectal cancer patients (n = 115) and healthy controls (n = 230) were randomly spotted in quadruplicate. The RPPM was stained with anti-complement component C9 (red) and anti-human IgG (green) antibodies, as described in the Materials and Methods. (b) Representative spots of colorectal cancer patients (CRC) and healthy controls (Cont). (c) Distribution and median values (vertical bars) of the plasma Apo AI level (in arbitrary units) measured by RPPM. A statistically significant difference was recognized between healthy controls and colorectal patients (P = 0.00794, Student's t-test). (d) Distribution and median values (vertical bars) of plasma C9 level (in arbitrary units) measured by RPPM. Statistical significance was recognized between healthy controls and colorectal patients ($P = 1.43 \times 10^{-12}$, Student's t-test). (e) Receiver operator characteristic (ROC) analysis of Apo AI. AUC, area under the curve. (f) ROC analysis of C9. (g) Box-and-whisker diagram showing the different plasma levels of C9, determined by RPPM for healthy controls and each clinical stage of colorectal cancer. Boxes represent the median values and the 25–75 percentile ranges. Whiskers indicate the most extreme data point, which are no more than 1.5 times the interquartile ranges from the boxes.

(stages 0–II) and advanced (stages III and IV) colorectal cancer patients over healthy individuals were 0.594 and 0.810, respectively, indicating its inferiority to C9 in detecting early stage colorectal cancer.

Alterations of Apo AI and C9 in other cancers. Finally, we measured the level of Apo AI and C9 in 378 plasma samples collected prospectively from different medical institutions using RPPM. The observed alterations of plasma Apo AI and C9

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Table 3. Alterations of plasma Apo AI and C9 in various diseases

	n	Apo AI			C9		
		Average†	SD	P value‡	Average†	SD	P value‡
Healthy control	109	4679.5	3265.6		527.2	674.7	
Colorectal cancer	100	2318.4	2015.0	1.71×10^{-9}	1792.7	1628.1	3.82×10^{-11}
Gastric cancer	105	2812.0	2357.5	3.00×10^{-6}	1629.6	1533.9	3.31×10^{-10}
Hepatocellular carcinoma	14	2621.6	2260.8	0.007	477.0	332.4	0.651
Esophageal cancer	10	3074.6	1572.6	0.014	1639.1	1036.7	0.008
Pancreatic cancer	14	2934.3	2214.8	0.016	1436.8	1125.6	0.010
Cholangiocarcinoma	18	1674.6.	1377.0	1.26×10^{-8}	2519.6	2086.8	0.001
Pancreatitis	8	1925.9	1970.3	0.005	1564.4	1502.5	0.093

†Determined by RPPM (in arbitrary units). ‡Compared with healthy controls (student's *t*-test).

proteins in colorectal cancer patients were reproducible, even in this independent cohort (Table 3). The reduction of plasma Apo AI protein seems not to be specific to colorectal cancer, and was observed in patients with various cancers as well as chronic pancreatitis. The increment of plasma C9 protein was also not specific to colorectal cancer patients; patients with gastric cancer, esophageal cancer, pancreatic cancer and cholangiocarcinoma also showed a statistically significant increase of plasma C9 protein. The AUC value of C9 for colorectal cancer patients over healthy individuals (0.796) was higher than that of CEA (0.762; Fig. 4). The combination with C9 improved the AUC value of CEA from 0.762 to 0.852.

Discussion

The context of circulating blood proteins may represent underlying physiological and pathogenic conditions. Therefore, the blood proteome is considered an ample source of biomarker discovery. In order to identify a new biomarker that can be used for a non-invasive and inexpensive blood test of colorectal cancer, we first compared plasma proteome data using 2DICAL. We found that 10 proteins showed statistically significant differences between colorectal cancer patients and controls (Table 2). The differences of Apo AI and C9 were then verified by immunoblotting with relevant antibodies (Fig. 1c). Apo AI is the major protein component of plasma high density lipoprotein.[19] Apo AI has been repeatedly reported to be downregulated in the plasma samples of patients with various cancers including ovarian and pancreatic cancers.[20,21]

Any biomarker candidates identified by genomic or proteomic approaches must be validated in a statistically sufficient number of cases and controls using a different quantitative method before being considered for clinical application.[22,23] Accordingly, we determined the relative plasma levels of Apo AI and C9 in 345 individuals using RPPM (Fig. 3) and confirmed the results in an independent cohort consisting of 378 plasma samples collected from healthy controls and patients with various diseases (Table 3). The collection and storage of all the plasma samples were performed under the same protocol to exclude any sampling biases. Conventionally, ELISA has been used for such validation, but the standard sandwich ELISA assay requires two antibodies that do not interfere with each other. As a result, the development of ELISA usually takes several months for every biomarker candidate protein. And more importantly, ELISA requires a relative large volume (~100 μL) of samples. Because the supply of clinical materials is often limited, it may be unfavorable to use hundreds of microlitters of precise samples for preliminary experiments. Our high-density RPPM requires a minimal sample volume of the nanoliter order and one antibody. RPPM is an alternative validation method that can determine the clinical utility of candidate biomarker protein in a single experiment.[12]

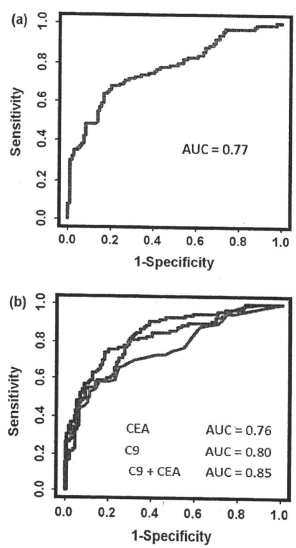

Fig. 4. Confirmation in an independent cohort. (a) Receiver operator characteristic (ROC) analysis of apolipoprotein AI (Apo AI; colorectal cancer patients [$n = 100$] over healthy controls [$n = 109$]). AUC, area under the curve. (b) ROC analysis of carcinoembryonic antigen (CEA), plasma complement component-9 (C9) and their combination (colorectal cancer patients [$n = 100$] over healthy controls [$n = 109$]).

Although protein microarray is a newly established technique and still requires improvement regarding validity and standardization,[24] it has been successfully used for analyzing clinical specimens of prostate cancer,[25] breast cancer,[26] rhabdomyosarcoma[27] and acute myeloid leukemia.[28,29] More recently, Grote *et al.*[30] used protein microarrays for the measurement of serum and plasma CA19-9. They printed a total of 149 sera and plasma samples obtained from pancreatic cancer patients, patients with chronic pancreatitis and healthy controls onto

nitrocellulose-coated slide glasses and obtained results comparable to conventional ELISA. They used 200-μm pins, and signals were detected using diaminobenzidine as a chromogen. We were able to spot as many as 6144 protein samples into a glass slide using a 100-μm innovative screw-shaped pin and hydrophobic surface technologies. The hydrophobic surface of microarrays mediates tight interaction with proteins and prevents protein spot diffusion.[16,17] Only with all these cutting-edge technologies was this level of high-density spotting of adhesive protein samples possible. Fluorescence immunostaining of our RPPM provided wide dynamic range and high reproducibility (Fig. 2). The linearity of fluorescence intensity was obtained in a wide range, and over 78% of quadruplicate showed a CV of < 0.1 (Fig. 2c). All these make our RPPM a reliable tool for biomarker validation.

The ninth component of complement (C9) is one of five component proteins (C5b, C6, C7, C8 and C9) of the membrane attack complex (MAC).[31] The MAC attaches to the surface of target cells and forms a pore across the cell membrane resulting in complement-dependent cytotoxicity (CDC). Aberrant activation of MAC has been implicated in the pathogenesis of various autoimmune and infectious diseases. We previously identified the significant increase of complement components C3 and C4A in the sera of endometrial cancer patients.[9] In the present study we found the plasma level of C9 was significantly elevated in colorectal cancer patients, including those with stage I and II diseases. The expression of membrane-bound CD46, CD55 and CD59 protects cancer cells from CDC,[32] but the precise role of complements and these modifiers in the process of carcinogenesis has not been fully established. Further efforts will be necessary to clarify the biological significance of increased circulating C9 in patients with colorectal cancer.

In the present study we identified and validated C9 as a plasma biomarker potentially useful for the detection of early stage colorectal cancer using the combination of innovative proteomic technologies. Although the clinical significance of C9 must be clarified in an independent clinical study, we were able to demonstrate the utility of the combination of 2DICAL and RPPM in biomarker discovery and validation. The combination is a rapid approach that could be applicable to the discovery of biomarkers for any types of human malignancy.

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Disclosure Statement

No potential conflict of interest relevant to this paper is declared.

References

1 Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005; **55**: 74–108.
2 Okuno K. Surgical treatment for digestive cancer. Current issues – colon cancer. *Dig Surg* 2007; **24**: 108–14.
3 Wakai K, Hirose K, Matsuo K *et al.* Dietary risk factors for colon and rectal cancers: a comparative case–control study. *J Epidemiol* 2006; **16**: 125–35.
4 Duffy MJ, van Dalen A, Haglund C *et al.* Tumour markers in colorectal cancer: European Group on Tumour Markers (EGTM) guidelines for clinical use. *Eur J Cancer* 2007; **43**: 1348–60.
5 Liotta LA, Petricoin EF. Serum peptidome for cancer detection: spinning biologic trash into diagnostic gold. *J Clin Invest* 2006; **116**: 26–30.
6 Omenn GS, States DJ, Adamski M *et al.* Overview of the HUPO Plasma Proteome Project: results from the pilot phase with 35 collaborating laboratories and multiple analytical groups, generating a core dataset of 3020 proteins and a publicly-available database. *Proteomics* 2005; **5**: 3226–45.
7 Honda K, Hayashida Y, Umaki T *et al.* Possible detection of pancreatic cancer by plasma protein profiling. *Cancer Res* 2005; **65**: 10613–22.
8 States DJ, Omenn GS, Blackwell TW *et al.* Challenges in deriving high-confidence protein identifications from data gathered by a HUPO plasma proteome collaborative study. *Nat Biotechnol* 2006; **24**: 333–8.
9 Negishi A, Ono M, Handa Y *et al.* Large-scale quantitative clinical proteomics by label-free liquid chromatography and mass spectrometry. *Cancer Sci* 2009; **100**: 514–9.
10 Ono M, Shitashige M, Honda K *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.
11 Matsubara J, Ono M, Negishi A *et al.* Identification of a predictive biomarker for hematologic toxicities of gemcitabine. *J Clin Oncol* 2009; **27**: 2261–8.
12 Matsubara J, Ono M, Honda K *et al.* Survival prediction for pancreatic cancer patients receiving gemcitabine treatment. *Mol Cell Proteomics* 2010; **9**: 695–704.
13 Machida-Montani A, Sasazuki S, Inoue M *et al.* Atrophic gastritis, *Helicobacter pylori*, and colorectal cancer risk: a case-control study. *Helicobacter* 2007; **12**: 328–32.
14 Ono M, Matsubara J, Honda K *et al.* Prolyl 4-hydroxylation of α-fibrinogen: a novel protein modification revealed by plasma proteomics. *J Biol Chem* 2009; **284**: 29041–9.
15 Honda K, Yamada T, Hayashida Y *et al.* Actinin-4 increases cell motility and promotes lymph node metastasis of colorectal cancer. *Gastroenterology* 2005; **128**: 51–62.
16 Lee Y, Lee EK, Cho YW *et al.* ProteoChip: a highly sensitive protein microarray prepared by a novel method of protein immobilization for application of protein–protein interaction studies. *Proteomics* 2003; **3**: 2289–304.
17 Sasakura Y, Kanda K, Yoshimura-Suzuki T *et al.* Protein microarray system for detecting protein–protein interactions using an anti-His-tag antibody and fluorescence scanning: effects of the heme redox state on protein-protein interactions of heme-regulated phosphodiesterase from *Escherichia coli*. *Anal Chem* 2004; **76**: 6521–7.
18 Greene FL. TNM staging for malignancies of the digestive tract: 2003 changes and beyond. *Semin Surg Oncol* 2003; **21**: 23–9.
19 Breslow JL, Ross D, McPherson J *et al.* Isolation and characterization of cDNA clones for human apolipoprotein A-I. *Proc Natl Acad Sci U S A* 1982; **79**: 6861–5.
20 Zhang Z, Bast RC Jr, Yu Y *et al.* Three biomarkers identified from serum proteomic analysis for the detection of early stage ovarian cancer. *Cancer Res* 2004; **64**: 5882–90.
21 Kozak KR, Su F, Whitelegge JP, Faull K, Reddy S, Farias-Eisner R. Characterization of serum biomarkers for detection of early stage ovarian cancer. *Proteomics* 2005; **5**: 4589–96.
22 Ransohoff DF. Rules of evidence for cancer molecular-marker discovery and validation. *Nat Rev Cancer* 2004; **4**: 309–14.
23 Ludwig JA, Weinstein JN. Biomarkers in cancer staging, prognosis and treatment selection. *Nat Rev Cancer* 2005; **5**: 845–56.
24 Wulfkuhle JD, Edmiston KH, Liotta LA, Petricoin EF 3rd. Technology insight: pharmacoproteomics for cancer – promises of patient-tailored medicine using protein microarrays. *Nat Clin Pract Oncol* 2006; **3**: 256–68.
25 Grubb RL, Calvert VS, Wulkuhle JD *et al.* Signal pathway profiling of prostate cancer using reverse phase protein arrays. *Proteomics* 2003; **3**: 2142–6.
26 Rapkiewicz A, Espina V, Zujewski JA *et al.* The needle in the haystack: application of breast fine-needle aspirate samples to quantitative protein microarray technology. *Cancer* 2007; **111**: 173–84.
27 Petricoin EF 3rd, Espina V, Araujo RP *et al.* Phosphoprotein pathway mapping: Akt/mammalian target of rapamycin activation is negatively associated with childhood rhabdomyosarcoma survival. *Cancer Res* 2007; **67**: 3431–40.

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