are now investigating the association of morphine-related adverse events with polymorphisms in *COMT*, together with polymorphisms in *UGT2B7*, *ABCB1*, and *OPRM1* which we have not yet examined.

Large prospective studies are needed to determine whether genetic testing for *UGT2B7*, *ABCB1*, and *OPRM1* helps to predict the risk of morphine-induced adverse reactions and to elucidate the detailed mechanisms of morphine-related adverse events, taking into account medical aspects as well as cost effectiveness.

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Conflict of interest statement None.

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Original article

Prognostic significance of peritoneal tumour cells identified at surgery for colorectal cancer

H. Katoh, K. Yamashita, T. Sato, H. Ozawa, T. Nakamura and M. Watanabe

Department of Surgery, Kitasato University Hospital, Kitasato 1-15-1, Sagamihara 228-8555, Kanagawa, Japan
Correspondence to: Professor M. Watanabe (e-mail: gekaw@med.kitasato-u.ac.jp)

Background: The prognostic significance of intraperitoneal tumour cells (IPCs) in colorectal cancer is not clear. This study aimed to determine whether detection of IPCs could be used a prognostic marker for selecting patients at high risk of recurrence.
Methods: The study included 226 patients with colorectal cancer who underwent elective resection. Clinical variables, including the presence of IPCs, were analysed for their prognostic significance.
Results: Thirty-three patients (14·6 per cent) were positive for IPCs. Univariable analysis indicated that the presence of IPCs was a significant prognostic factor in patients with stage III colorectal cancer; the 5-year disease-specific survival rate was 14 per cent in IPC-positive patients *versus* 79 per cent in those without IPCs ($P < 0·001$). Multivariable analysis showed that IPC positivity was the most robust prognostic factor in stage III disease (hazard ratio 2·2; $P = 0·003$), whereas nodal category (N1 or N2) showed no significant association with prognosis. In addition, IPCs were associated with haematogenous recurrence ($P = 0·004$) rather than peritoneal or local recurrence ($P = 0·077$) in patients with stage III disease.
Conclusion: The presence of IPCs is a significant prognostic factor in patients with stage III colorectal cancer.

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Introduction

Intraperitoneal tumour cells (IPCs) have been recognized as carrying prognostic implications in colorectal cancer[1–4]. The presence of IPCs is a robust prognostic factor in gastric cancer[5], resulting in the use of more sensitive detection methods employing polymerase chain reaction (PCR) to identify patients at high risk of peritoneal dissemination[6,7]. The detection of IPCs has also been reported to be useful in predicting recurrence, particularly peritoneal recurrence, in patients with colorectal cancer[2,4]. IPCs can be detected by several techniques including simple cytological examination of peritoneal lavage fluid (conventional cytology)[8], immunocytology[9], imprint cytology of the peritoneal surface[10] and reverse transcriptase–PCR for carcinoembryonic antigen (CEA) and cytokeratin 20[11,12]. However, compared with gastric cancer, the prognostic value of conventional cytology for IPCs remains controversial in colorectal cancer[13,14].

As there are no reports in the literature that have considered the effect of IPCs in patients with different stages of colorectal cancer, the authors undertook the present study to evaluate the prognostic impact of IPCs in colorectal cancer by stage with conventional cytology of peritoneal lavage fluid, and to determine whether the presence of IPCs might be used instead of tumour node metastasis (TNM) stage to identify patients with a high risk of recurrence or metastasis, and therefore requiring adjuvant chemotherapy.

Methods

Between 1 April 1991 and 31 March 2005, 226 patients with sporadic colorectal cancer who underwent elective resection of the colon or intraperitoneal rectum, and who had a clinical diagnosis of advanced disease (TNM stage II, III or IV), were eligible for entry into the study as long as cytology results were available. Other eligibility criteria included age 20–75 years, life expectancy greater than 3 months, no previous chemoimmunotherapy or radiotherapy, and the absence of severe liver dysfunction, heart failure, renal dysfunction or other severe systemic complications. The study was performed in accordance

with the clinical research guidelines of the ethics committee of Kitasato University School of Medicine. All patients gave written informed consent.

All patients underwent potentially curative surgery, involving a histologically complete resection of the primary tumour with sufficient margins and regional

Table 1 Analysis of clinicopathological variables in 226 patients with stage II–IV colorectal cancer in terms of survival and intraperitoneal tumour cell status

		5-year DSS		IPC status			
	No. of patients	%	*P*‡	Positive	Negative	Positivity rate (%)	*P*§
Sex			0·949				0·189
M	127 (56·2)	46·0		15	112	11·8	
F	99 (43·8)	43		18	81	18	
Age (years)			0·135				0·246
< 60	82 (36·3)	48		15	67	18	
≥ 60	144 (63·7)	43·0		18	126	12·5	
Tumour position			0·178				0·688
Colon	156 (69·0)	41·2		24	132	15·4	
Rectum	70 (31·0)	52		9	61	13	
Differentiation			0·285				0·054
Well or moderate	196 (86·7)	45·9		25	171	12·8	
Poor*	30 (13·3)	37		8	22	27	
TNM stage			< 0·001				
II	27 (11·9)	85		2	25	7	0·731¶
III	91 (40·3)	72		10	81	11	0·119#
IV	108 (47·8)	8·1		21	87	19·4	0·164**
T category			0·005				0·365
T1–2	10 (4·4)	100		0	10	0	
T3–4	216 (95·6)	42·1		33	183	15·3	
pN category			< 0·001				
N0	38 (16·8)	65		5	33	13	0·769††
N1	122 (54·0)	50·1		13	109	10·7	0·033‡‡
N2	66 (29·2)	21		15	51	23	0·305§§
Lymphatic invasion			0·147				> 0·999
No	6 (2·7)	83		1	5	17	
Yes	220 (97·3)	43·7		32	188	14·5	
Vascular invasion			0·001				0·386
No	26 (11·5)	80		2	24	8	
Yes	200 (88·5)	40·1		31	169	15·5	
Preop. CEA level (ng/ml)			< 0·001				0·084
Normal (≤ 2.5)	86 (38·1)	72		8	78	9	
Raised (> 2.5)	140 (61·9)	26·5		25	115	17·9	
Preop. CA19-9 level (ng/ml)			< 0·001				0·005
Normal (≤ 37)	147 (65·0)	59·3		14	133	9·5	
Raised (> 37)	79 (35·0)	17		19	60	24	
IPCs			0·001				—
Present	33 (14·6)	16		—	—	—	
Absent	193 (85·4)	49·1		—	—	—	
Peritoneal dissemination			< 0·001				< 0·001
Yes	29 (12·8)	17		16	13	55	
No	197 (87·2)	49·0		17	180	8·6	
Distant metastasis†			< 0·001				0·163
Yes	72 (31·9)	19		14	58	19	
No	154 (68·1)	56·3		19	135	12·3	
Postop. chemotherapy			0·006				0·833
Yes	164 (72·6)	46·7		25	139	15·2	
No	62 (27·4)	40		8	54	13	

Table 1 (Continued)

		5-year DSS		IPC status			
	No.of patients	%	*P*‡	Positive	Negative	Positivity rate (%)	*P*§
Periop. transfusion			< 0·001				0·655
Yes	49 (21·7)	29·8		8	41	16·3	
No	177 (78·3)	48·7		25	152	14·1	

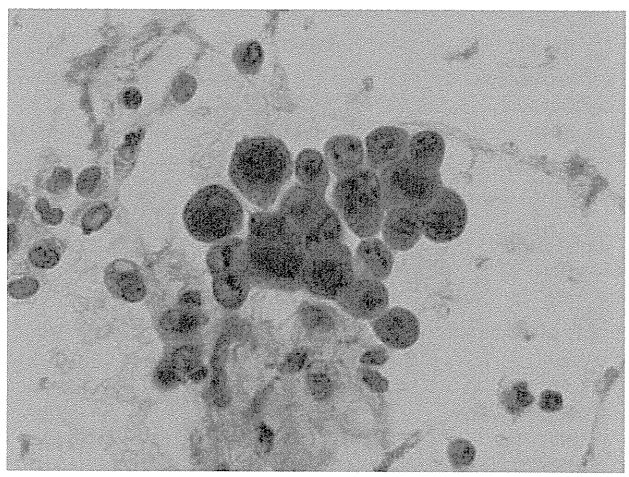
Values in parentheses are percentages. *Includes poorly differentiated, mucinous and undifferentiated types; †does not include peritoneal dissemination. DSS, disease-specific survival, IPC, intraperitoneal tumour cell; (p)TNM, (pathological) tumour node metastasis stage according to International Union Against Cancer (UICC) classification; CEA, carcinoembryonic antigen. ‡Log rank test; §Fisher's exact test; ¶stage II *versus* stage III; #stage III *versus* stage IV; **stage IV *versus* stage II; ††N0 *versus* N1; ‡‡N1 *versus* N2; §§N2 *versus* N0.

lymphadenectomy. More than six lymph nodes were harvested in all patients (mean 21·1 nodes), as examination of six or fewer nodes is related to a poor prognosis[15]. Fourteen patients underwent laparoscopic surgery and 212 had an open procedure. Patients were followed up until cancer-related death or to the endpoint of the study, 31 March 2007. Those who survived for more than 5 years were censored at 5 years. None of these patients was censored as a result of death related to another disease or to surgery, and none had undergone emergency surgery for obstruction or perforation. Preoperative chemotherapy or radiotherapy was not performed in this patient cohort.

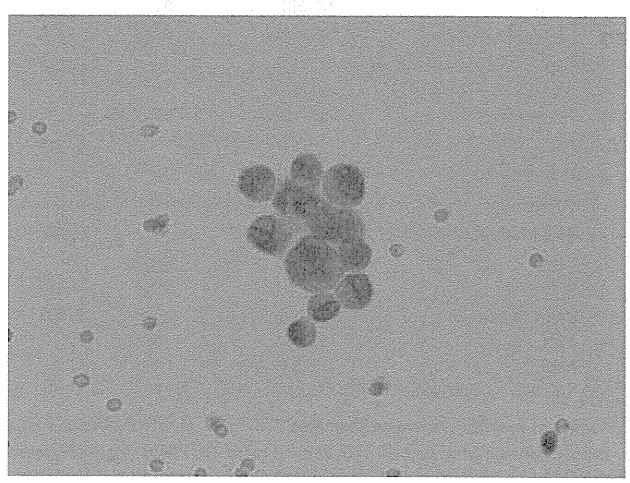
The site of the primary lesion was the colon in 156 patients (caecum, 23; ascending colon, 36; transverse colon, 22; descending colon, eight; sigmoid colon, 67) and the intraperitoneal rectum in 70. Clinical details for the 226 patients are shown in *Table 1*. Patient demographics, tumour characteristics and postoperative course were recorded and analysed. Perioperative transfusion was defined as transfusion of allogeneic blood during surgery or the first 2 days after operation[16–18]. The TNM classification was used according to the International Union Against Cancer (UICC) staging system.

Samples

Immediately the abdomen had been opened, and before manipulation of the tumour, 100 ml warm saline was instilled into the peritoneal cavity over the tumour site and 20 ml was reaspirated for cytological examination. The lavage fluid was centrifuged (1500 r.p.m. for 3 min) and smeared on to slides. Cytological examination was performed after Papanicolaou and periodic acid–Schiff staining. Morphological evidence of malignancy by microscopy resulted in a diagnosis of IPC positivity (*Fig. 1*). The cytologists were not aware of the clinical findings.



a Papanicolaou staining



b PAS staining

Fig. 1 **a** Typical Papanicolaou stain of intraperitoneal tumour cells (IPCs), showing a loose cluster of large and variously sized cells, eccentric nuclei and large prominent nucleoli (original magnification ×40). **b** Typical periodic acid–Schiff (PAS) stain of IPCs, showing red-stained mucin in the cytoplasm (original magnification ×40)

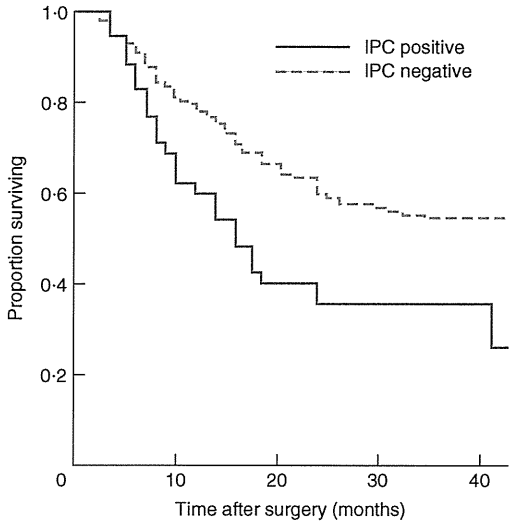
Table 2 Analysis of clinicopathological variables in 91 patients with stage III colorectal cancer in terms of survival and intraperitoneal tumour cell status

		5-year DSS				IPC status			
		Univariable analysis		Multivariable analysis					
	No.of patients	%	P†	HR	P‡	Positive	Negative	Positivity rate (%)	P§
Sex			0·476						0·189
M	47 (52)	76		—	—	3	44	6	
F	44 (48)	66				7	37	16	
Age (years)			0·643						0·487
< 60	33 (36)	67		—	—	5	28	15	
≥ 60	58 (64)	74				5	53	9	
Tumour position			0·689						> 0·999
Colon	63 (69)	69		—	—	7	56	11	
Rectum	28 (31)	77				3	25	11	
Differentiation			0·560						0·169
Not poor	84 (92)	72		—	—	8	76	10	
Poor*	7 (8)	69				2	5	29	
T category			0·099						0·590
T1–2	9 (10)	100		665·0	0·086	0	9	0	
T3–4	82 (90)	69				10	72	12	
pN category			0·118						0·105
N1	73 (80)	76		1·8	0·201	6	67	8	
N2	18 (20)	54				4	14	22	
Lymphatic invasion			0·483						> 0·999
No	3 (3)	100		—	—	0	3	0	
Yes	88 (97)	71				10	78	11	
Vascular invasion			0·319						0·681
No	17 (19)	88		—	—	1	16	6	
Yes	74 (81)	69				9	65	12	
Preop. CEA level (ng/ml)			0·010						0·325
Normal (≤ 2.5)	51 (56)	81		1·6	0·026	4	47	8	
Raised (> 2.5)	40 (44)	57				6	34	15	
Preop. CA19-9 level (ng/ml)			0·258						0·590
Normal (≤ 37)	82 (90)	73		—	—	10	72	12	
Raised (> 37)	9 (10)	57				0	9	0	
IPCs			< 0·001						—
Present	10 (11)	14		2·2	0·003	—	—	—	
Absent	81 (89)	79				—	—	—	
Adjuvant chemotherapy			0·452						0·718
Yes	63 (69)	73		—	—	8	55	13	
No	28 (31)	66				2	26	7	
Periop. transfusion			0·765						0·591
Yes	8 (9)	73		—	—	0	8	0	
No	83 (91)	72				10	73	12	

Values in parentheses are percentages. *Includes poorly differentiated, mucinous and undifferentiated types. DSS, disease-specific survival, IPC, intraperitoneal tumour cell; HR, hazard ratio; (p)TNM, (pathological) tumour node metastasis stage according to International Union Against Cancer (UICC) classification; CEA, carcinoembryonic antigen. †Log rank test; ‡Cox proportional hazard model; §Fisher's exact test.

Treatment and follow-up after surgery

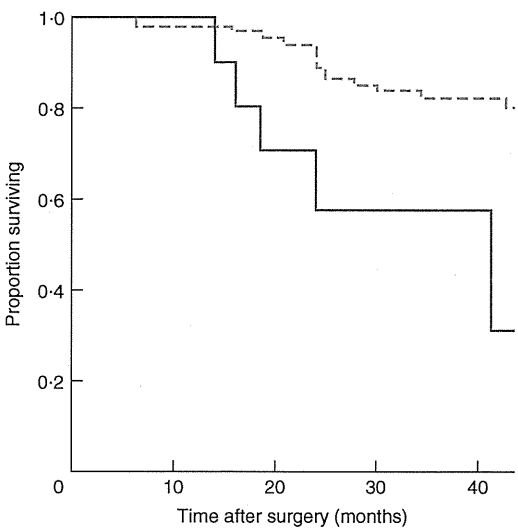
Postoperative therapy was recommended to all patients with stage III or IV colorectal cancer. Adjuvant chemotherapy, started within 7 weeks of operation, was received by 24 of 27 patients with stage II and by 63 of 91 with stage III disease. Regimens consisted of 5-fluorouracil (5-FU) alone (infusion or oral administration), 5-FU plus protein-bound polysaccharide K, 5-FU plus leucovorin, 5-FU plus leucovorin plus irinotecan, 5-FU plus irinotecan and other chemotherapeutic drugs for at least 3 months or three cycles.



No. at risk

IPC positive	33	20	12	8	7
IPC negative	193	151	115	91	72

a Stage II–IV

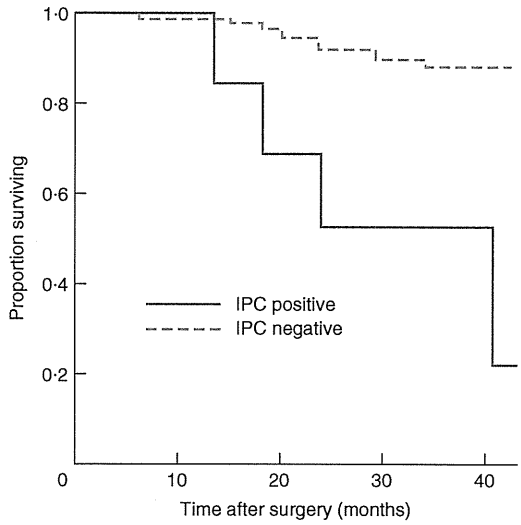


No. at risk

IPC positive	10	10	7	4	4
IPC negative	81	80	74	60	46

b Stage III

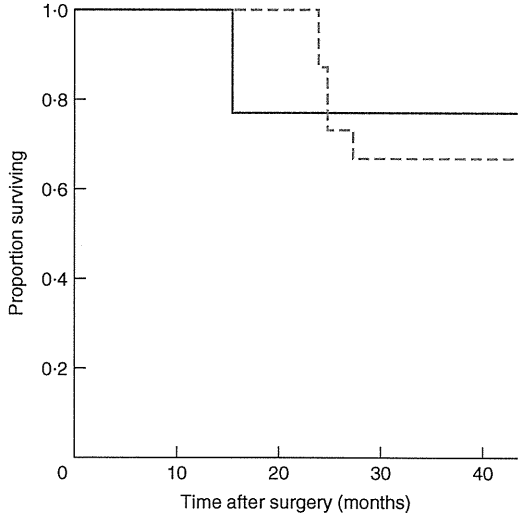
Fig. 2 Kaplan–Meier analysis of 5-year disease-specific survival in patients with colorectal cancer according to intraperitoneal tumour cell (IPC) status. **a** Patients with stage II–IV colorectal cancer. **b** Patients with stage III colorectal cancer. **a** $P = 0{\cdot}001$, **b** $P < 0{\cdot}001$ (log rank test)



No. at risk

IPC positive	6	6	4	3	3
IPC negative	67	66	60	52	38

a N1



No. at risk

IPC positive	4	4	3	1	1
IPC negative	14	14	14	8	8

b N2

Fig. 3 Kaplan–Meier analysis of 5-year disease-specific survival in patients with stage III colorectal cancer according to intraperitoneal tumour cell (IPC) status. **a** Patients with N1 disease. **b** Patients with N2 disease. **a** $P < 0{\cdot}001$, **b** $P = 0{\cdot}112$ (log rank test)

Patients were followed up for at least 3 (range 3–15) years, or until death. Follow-up was at least 3 monthly during the first year, and then every 6 months. Assessment included medical history-taking; physical examination; haematological tests; determination of serum CEA and CA19-9 levels (evaluated at every visit); colonoscopy, chest radiography and chest computed tomography (CT) (once yearly); and

abdominal ultrasonography and abdominal CT (every 6 months).

The diagnosis of recurrence was made on the basis of imaging and, if necessary, either cytological analysis or biopsy findings. Treatment of recurrence or metastasis included surgical resection (if possible), or 5-FU-based chemotherapy or radiotherapy. Thirty-six patients with stage IV colorectal cancer and four with stage III disease chose not to undergo postoperative treatment for recurrence.

Statistical analysis

Length of follow-up was determined from the date of resection of the primary lesion. Clinicopathological characteristics and follow-up data were analysed in terms of the 5-year disease-specific survival (DSS) rate. DSS was estimated by means of the Kaplan–Meier method and compared with the log rank test[19]. A multivariable proportional hazard model was employed using factors for which univariable analysis had suggested prognostic potential ($P < 0{\cdot}150$)[20]. Fisher's exact test was used to analyse the association between IPCs and other tumour factors. Statistical calculations were performed with StatView® version 5.0 (SAS Institute, Cary, North Carolina, USA). $P < 0{\cdot}050$ was considered statistically significant.

Results

Table 1 shows the association of clinicopathological parameters and IPC status in the whole cohort of 226 patients with colorectal cancer. There was a significant association of IPCs with N2 disease (N2 *versus* N1, $P = 0{\cdot}033$), increased preoperative serum CA19-9 concentration ($P = 0{\cdot}005$) and synchronous peritoneal dissemination ($P < 0{\cdot}001$). Poor differentiation ($P = 0{\cdot}054$) and increased preoperative serum CEA levels ($P = 0{\cdot}084$) were only marginally related to IPC positivity.

Table 3 Association of intraperitoneal tumour cell status and first recurrence site in 91 patients with stage III colorectal cancer

	IPC positive	IPC negative	P‡
Local or peritoneal recurrence*			0·077
Yes	3	7	
No	7	74	
Haematogenous recurrence†			0·004
Yes	7	18	
No	3	63	

*Sensitivity 30 per cent; specificity 91 per cent; accuracy 85 per cent.
†Sensitivity 28 per cent; specificity 96 per cent; accuracy 77 per cent.
‡Fisher's exact test.

The IPC positivity rate tended to increase in line with TNM stage (*Table 1*).

IPCs were found in 33 (14·6 per cent) of the 226 patients with stage II–IV colorectal cancer; these patients had a poor prognosis in comparison with patients without IPCs ($P = 0{\cdot}001$) (*Fig. 2a*). However, comparison of the different stages indicated that IPC positivity was strongly associated with a poor outcome only in patients with stage III colorectal cancer, as shown in *Fig. 2b* and *Table 2* ($P < 0{\cdot}001$), but not in stages II or IV (data not shown). The value of IPC detection was therefore analysed subsequently for patients with stage III colorectal cancer.

On univariable analysis, preoperative CEA concentration was the factor with the next most significant association with poor outcome in patients with stage III disease ($P = 0{\cdot}010$) (*Table 2*). IPCs were found in ten (11 per cent) of the 91 patients, and their 5-year DSS rate was 14 per cent compared with 79 per cent for IPC-negative patients. Even when the analysis was confined to the 63 patients who received adjuvant chemotherapy, eight patients were found to have IPCs; their 5-year DSS rate was significantly worse than that of patients without IPCs (19 *versus* 81 per cent respectively; $P < 0{\cdot}001$).

Multivariable analysis revealed IPC positivity to be the most robust independent prognostic factor in patients

Table 4 Association of tumour depth and lymph node metastasis with first recurrence site in 118 patients who had curative surgery

	Invasion of serosa/adventitia			No invasion of serosa/adventitia		
	No LN metastasis	LN metastasis	P*	No LN metastasis	LN metastasis	P*
Local or peritoneal recurrence			> 0·999			0·602
Yes	0	2		2	3	
No	6	30		19	56	
Haematogenous recurrence			0·644			0·058
Yes	2	8		0	11	
No	4	24		21	48	

LN, lymph node. *Fisher's exact test.

with stage III colorectal cancer (hazard ratio 2·2; $P = 0·003$) (*Table 2*), ahead of preoperative CEA concentration ($P = 0·026$); T and N categories were both eliminated. *Table 2* also shows the association between IPC positivity and clinicopathological factors in patients with stage III colorectal cancer. In the correlation analysis, the IPC positivity rate was 8 per cent for N1 and 22 per cent for N2 disease, and only N category approached significance ($P = 0·105$). After adjustment for TNM substage, the 5-year DSS rate for IPC-positive patients was 17 and 0 per cent for N1 and N2 respectively, compared with 82 and 64 per cent for IPC-negative patients ($P < 0·001$ and $P = 0·112$) (*Fig. 3*).

Sites of first recurrence in patients with stage III disease were then analysed in terms of IPC positivity. Interestingly, the presence of IPCs correlated with haematogenous recurrence ($P = 0·004$) rather than local recurrence or peritoneal dissemination ($P = 0·077$) (*Table 3*). Although the sensitivity was about 30 per cent for both local/peritoneal and haematogenous recurrence, the specificity was 91 and 96 per cent respectively. Thus, IPCs may not only denote cancer cell shedding from the primary tumour but also indicate systemic micrometastasis. Resection of haematogenous recurrences was performed in two of the seven patients with IPC positivity, and five of the 18 patients without IPCs ($P > 0·999$).

In addition, an analysis of recurrence was performed for patients from the entire cohort with involvement of the serosa or adventitia, with or without lymph node metastasis, who had undergone curative surgery (*Table 4*). Again, haematogenous recurrence was more common than local or peritoneal dissemination in these patients, whether or not there was nodal metastasis; local or peritoneal recurrence was seen in none of six patients (0/6) without lymph node metastasis and 2/32 patients with lymph node metastasis, while haematogenous recurrence was seen in 2/6 patients without lymph node metastasis and 8/32 patients with lymph node metastasis. In patients without serosa/adventitia involvement, haematogenous recurrence occurred more frequently in the presence than in the absence of lymph node metastasis ($P = 0·058$) (*Table 4*).

Discussion

In this study, the presence of IPC positivity was analysed to determine its prognostic value in patients with advanced colorectal cancer and to select patients with a poor prognosis in each stage. The multivariable analysis showed that IPC positivity was the most robust prognostic indicator in patients with stage III disease, but not in those with stage II or stage IV colorectal cancer. Moreover, it is of interest that, with regard to site of first recurrence, IPC positivity was associated more strongly with haematogenous recurrence than with peritoneal dissemination or local recurrence, a finding that to the authors' knowledge has not been published previously.

Intraperitoneal chemotherapy has been reported to be effective in the treatment of peritoneal dissemination[21] and it has been suggested to be useful in preventing peritoneal recurrence[22,23]. However, with regard to metachronous recurrence, the association of IPC positivity with haematogenous rather than local or peritoneal dissemination suggests that intraperitoneal chemotherapy alone may be insufficient in IPC-positive patients after curative surgery. Tanaka and colleagues[24] reported a correlation between extranodal invasion of lymph nodes and peritoneal dissemination in gastric cancer, and a previous experimental study by the same authors showed that lymphatic invasion and obstruction may cause peritoneal dissemination via the lymphatic route[25]. In accordance with these findings, in the present study IPC positivity showed an association with advanced N category that approached significance in patients with stage III colorectal cancer. On the other hand, lymphatic obstruction by cancer cells has been shown to cause lymphaticovenous communication in the mesentery[26], suggesting that lymph node obstruction may play a role in the mechanism of both peritoneal dissemination and haematogenous metastasis.

In a previous study of stage IV colorectal cancer, a significant relationship was also found between peritoneal dissemination and hepatic metastasis[27]. Extensive intraoperative lavage with 10 litres of saline provided successful prophylaxis by reducing the number of viable cancer cells that spread from opened lymphatic channels following curative surgery with lymph node dissection[28]. It would be of interest to compare the rates of peritoneal and haematogenous recurrence with or without such extensive intraoperative lavage.

Several parameters have been reported as independent prognostic factors in stage III colorectal cancer. In terms of clinicopathological factors, these included the number of metastatic lymph nodes[29,30], negative lymph node count[31], metastatic lymph node ratio[32–35], the number of evaluated lymph nodes[36,37] and preoperative CEA levels[38]. However, of these, only the number of metastatic nodes is available in clinical practice at present for TNM substaging[39], and confirmed in a prospective study[30]. Interestingly, of the various clinicopathological factors studied here, IPC positivity was the best prognostic discriminator in stage III disease, and was better than N category. A negative lymph node count, metastatic lymph node ratio (or node density

factor) and the number of evaluated lymph nodes did not have prognostic significance (data not shown). Preoperative CEA level has been considered insufficient as a prognostic marker[40], and the present authors recently concluded that, with the advancement of adjuvant chemotherapy and diagnostic tools, it has a lesser place prognostically in stage III colorectal cancer[16]. With regard to molecular and genetic markers, DNA ploidy alone has been confirmed prospectively in stage III colorectal cancer, although its value is limited in patients with proximal colonic cancer[41]. A glycine to valine mutation in codon 12 of the Ki-*ras* gene was also reported to be a risk factor for relapse or cancer-related death[42]. In addition, there is a report that *TP53* gene mutation status is a significant prognostic factor in stage III colorectal cancer[43]. Numerous other molecular and genetic markers have also been reported to indicate a poor prognosis, but none is suitable for routine application at present. IPC status, however, is suitable and easily determined, and may have potential in selecting patients at high risk of recurrence after surgery with curative intent.

In conclusion, conventional cytological examination for IPCs has potential as a robust independent prognostic factor in patients with stage III colorectal cancer. Moreover, IPC positivity was associated with distant recurrence rather than peritoneal dissemination and local recurrence. However, because of the small size of the study, further work is needed to confirm the value of IPC status as a prognostic factor in stage III colorectal cancer.

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High Ki67, Bax, and thymidylate synthase expression well correlates with response to chemoradiation therapy in locally advanced rectal cancers: proposal of a logistic model for prediction

M Kikuchi*[,1,2], T Mikami[1], T Sato[3], W Tokuyama[1], K Araki[1], M Watanabe[3], K Saigenji[2] and I Okayasu[1]

[1]Department of Pathology, Kitasato University School of Medicine, Sagamihara, Kanagawa, Japan; [2]Department of Gastroenterology, Kitasato University School of Medicine, Sagamihara, Kanagawa, Japan; [3]Department of Surgery, Kitasato University School of Medicine, Sagamihara, Kanagawa, Japan

BACKGROUND: Recently, preoperative chemoradiation therapy (CRT) for rectal cancer has been increasingly used as a neoadjuvant treatment. In the present study, the relation between histological response to CRT and immunohistochemical markers in biopsy specimens was investigated.

METHODS: Biopsy specimens from a total of 60 patients were collected before preoperative CRT with S-1 and irinotecan, and liniac 45 Gy. Immunohistochemical staining for Ki67, Mcm3, Bax, Bcl-2, ssDNA, Grp78, thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD), CD34, vascular endothelial growth factor, nestin, and L-type amino-acid transporter 1 was performed to allow comparison of the Ki67 labelling index (LI), Bax score, TS score, DPD score, microvessel density by CD34, and Grp78 score with cancer regression.

RESULTS: When the cases were divided into responders (Dworak grades 3 and 4) and non-responders (grades 1 and 2) groups, good correlations were evident with Ki67 LI, Bax, Grp78, and TS expression. On multiple logistic regression analysis, Ki67 LI, Bax, and TS scores were found to be independent factors. With their use in a logistic model, *P*-values could predict responder cases with a sensitivity of 82.8% and a specificity of 83.9%.

CONCLUSION: Using this system, treatment strategy for locally advanced rectal cancers can be determined before chemoradiation.

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Chemoradiation therapy (CRT) is increasingly used in neoadjuvant approaches for rectal cancer. Although in locally advanced cases there is generally no improvement of overall survival, local control is better than with postoperative CRT (Sauer *et al*, 2004). Combining irinotecan with 5-fluorouracil (5-FU) and leucovorin (LV) chemotherapy can provide higher rates of tumour regression, progression-free survival, and overall survival for metastatic colorectal cancer (Saltz *et al*, 2000). Recently, our group has developed a novel protocol for neoadjuvant CRT combining S-1 with irinotecan and radiation, allowing a complete pathological response rate of 31.6% to be achieved (Sato *et al*, 2007). It is well established that a pathological complete response (CR) or near CR (>95% pathological response) is significantly linked with improved patient survival (Ruo *et al*, 2002; Guillem *et al*, 2005).

S-1 is a novel oral fluoropyrimidine, combining tegafur (FT), 5-chloro-2,4-dihydroxypyridine (gimeracil or CDHP), and potassium oxonate (oteracil potassium or Oxo). FT is a prodrug for 5-FU that acts as an effector. CDHP reversibly inhibits the degradation of 5-FU by dihydropyrimidine dehydrogenase (DPD), resulting in prolonged high concentrations of 5-FU in the blood (Sato *et al*, 2007). Irinotecan (CPT-11) inactivates topoisomerase I through the formation of stable topoisomerase I–DNA cleavable complexes (Hsiang *et al*, 1985; Hsiang and Liu, 1988; Hertzberg *et al*, 1989). Interaction of the trapped cleavable complex with a replication fork results in replication arrest and fork breakage, finally leading to cell death (D'Arpa and Liu, 1989).

High proliferative activity examined with Ki67 and proliferating cell nuclear antigen (PCNA) staining (Willett *et al*, 1995), high Bax expression (Chang *et al*, 2005), and high thymidylate synthase (TS) (Negri *et al*, 2008) have been demonstrated to predict regression. However, these factors have been treated as univariate factors. No clinically applicable system for prediction of response of CRT has been proposed. We therefore have investigated cell proliferation, apoptosis, apoptosis-associated protein, expression of glucose-regulated protein 78 (Grp78), TS, DPD, and angiogenesis in biopsy samples in an attempt to develop a predictive system. Selection of these parameters was for the following reasons.

Thymidylate synthase provides *de novo* thymidylate for DNA synthesis, catalysing the methylation of deoxyuridine monophosphate to deoxythymidine monophosphate (Danenberg, 1977). The activity of 5-FU mainly depends on intracellular

*Correspondence: Dr M Kikuchi, Department of Pathology, Kitasato University School of Medicine, 1-15-1, Kitasato, Sagamihara, Kanagawa 228-8555, Japan; E-mail: masaomi@kitasato-u.ac.jp
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conversion to its active metabolite, 5-fluoro-2′-deoxyuridine-5′-monophosphate, which inhibits DNA synthesis by forming a stable complex with TS in presence of folates (Pinedo and Peters, 1988), and then initiates cell-cycle arrest or cell death. In general, high expression of thymidine phosphorylase and low expression of DPD in tumours are considered to result in higher intratumoural concentration of 5-FU (Jakob *et al*, 2005).

Glucose-regulated protein 78 is a member of the Hsp70 superfamily of heat-shock proteins whose increased expression is part of a coordinated protein response required to alleviate endoplasmic reticulum stress (Misra *et al*, 2005), maintain endoplasmic reticulum function, and protect cells against cell death. Glucose-regulated protein 78 may confer resistance against adriamycin- and etoposide-mediated apoptosis in cancer cells through inhibition of Bax and caspase-7 activation (Reddy *et al*, 2003; Davidson *et al*, 2005; Ermakova *et al*, 2006; Lee *et al*, 2006; Ranganathan *et al*, 2006).

Recently, the concept of cancer stem cells has attracted increasing attention with regard to human cancers (Wang and Dick, 2005). Minichromosome maintenance (Mcm) proteins 2–7 are present through all phases of the proliferative cell cycle, but are absent in 'out-of-cycle' states, suggesting functions as replication licensing factors (Stoeber *et al*, 2001), and Dudderidge *et al* (2005) have proposed that the Mcm2-Ki67 labelling index (LI) reflects the presence of non-proliferating dormant 'cancer stem' cells, associated with reduced disease-free survival in renal cell carcinoma cases.

It was reported that high intratumoural microvessel density (MVD) and vascular endothelial growth factor (VEGF) were correlated with poor prognosis of colorectal cancer (Des Guetz *et al*, 2006). It has been also reported that VEGF-positive rectal cancer was resistant to radiotherapy (Zlobec *et al*, 2008). Nestin, a class VI intermediate filament protein, has recently received attention as a marker for detecting newly formed endothelial cells (Teranishi *et al*, 2007).

As for apoptosis, cancer cell apoptosis in biopsy before CRT was correlated with tumour regression whereas apoptosis inhibitory protein Bcl-2 expression indicated no correlation with regression (Rödel *et al*, 2002). In addition, L-type amino-acid transporter 1 (LAT1) is highly expressed in malignant tumours to support growth and proliferation, and the inhibition of LAT1 activity led to cancer cell apoptosis (Kim *et al*, 2008).

Using these parameters, multiple logistic regression analysis was adopted to generate a model for predicting response to pre-operative CRT.

MATERIALS AND METHODS

A total of 60 cases of rectal cancer treated with preoperative CRT were collected. The patients' clinical criteria were previously reported (Sato *et al*, 2007). Briefly, all had previously untreated locally advanced distal rectal cancer T3 or T4, N0-2, and M0 (UICC classification) (Sobin and Wittekind, 2002), with an Eastern Cooperative Oncology Group performance status of 0-2 (Oken *et al*, 1982). Ages were 20–80 years at enrolment.

Biopsy materials of the 60 cases were endoscopically obtained from the rectal cancers before the initiation of therapy, at least two pieces of carcinoma being sampled for each case. The histological typing was in accordance with the WHO classification (Hamilton and Aaltonen, 2000). Tumour size was measured using double-contrast barium enema in 56 cases, but no X-ray photographs were available for 4 cases. Clinical tumour node metastasis (TNM) stage was judged with computed tomographic scans and/or magnetic resonance images. Because images were not available for two cases, clinical TNM stage could not be determined for these. A summary of clinical data of the cases is shown in Table 1. All patients received preoperative chemoradiation as follows: radiotherapy was

Table 1 Characteristics of the patients

Characteristic	
Age (year) (mean ± s.d.) ($n=60$)	63.9 ± 10.6 (range 32–81)
Sex (male/female) ($n=60$)	44 (73.3%)/16 (26.7%)
Tumour size (mm) (mean ± s.d.) ($n=56$)	47.4 ± 17.2 (range 20–95)
Clinical T stage ($n=58$)	
cT3	56
cT4	2
Clinical N stage ($n=58$)	
cN0	32
cN1/cN2	26
Histological type (biopsy) ($n=60$)	
Well	36
Moderate	23
Poor	1
CEA (mg/100 ml) (mean ± s.d.) ($n=60$)	8.9 ± 12.7
CA19-9 (ng/ml) (mean ± s.d.) ($n=60$)	19.7 ± 27.2

s.d., standard deviation. Normal ranges of CEA and CA19-9 were <5 mg/100 ml and <37 ng/ml, respectively. Tumour size (based on double-contrast barium enema), clinical TN stage, and tumour markers were evaluated before chemoradiation therapy.

administered in liniac fractions of 1.8 Gy per day, 5 days per week. The total dose of radiation was 45 Gy. S-1 (80 mg m^{-2} per day) and was given orally after breakfast and dinner on days 1–5, 8–12, 22–26, and 29–33. Irinotecan (80 mg m^{-2}) was administered as a continuous i.v. infusion for 90 min on days 0, 8, 22, and 29. Radical surgery was performed at least 4–6 weeks after the completion of 5 weeks of chemoradiation. The dose of S-1 was in accordance with the manufacturer's guideline (Taiho Pharmaceuticals Co. Ltd, Tokyo, Japan). The recommended dose of irinotecan was examined in our previous study (Sato *et al*, 2007). This protocol was started in 2004 with approval of the ethics committee of Kitasato University Hospital. All patients gave written informed consent.

Pathological evaluation

Therapeutic responses to preoperative CRT were evaluated with the surgically resected specimens. The excised tissues were fixed in buffered formalin and embedded in paraffin. In each case, the entire lesion was serially sliced at 4 mm for routine processing and embedding in paraffin. Then, 4-μm-thick sections were cut, stained with haematoxylin and eosin, and examined by light microscopy. Amounts of residual tumour mass, fibrotic changes, radiation vasculopathy, and peritumoural inflammatory reaction were checked, and therapeutic effects were assessed using Dworak grades (Dworak *et al*, 1997) as follows:

grade 0: no regression;
grade 1: dominant tumour mass with obvious fibrosis and/or vasculopathy;
grade 2: dominant fibrotic changes with few tumour cells or groups (easy to find);
grade 3: very few tumour cells in fibrotic tissue with or without mucous substance;
grade 4: no tumour cells, only fibrotic mass (total regression).

Immunohistochemistry of Ki67, Mcm3, Bax, Bcl-2, ssDNA, Grp78, TS, DPD, CD34, VEGF, nestin, and LAT1

Formalin fixed, paraffin-embedded histological sections (4 μm in thickness) in tumour biopsies before CRT were immunostained for 12 antigens (Ki67, Mcm3, Bax, Bcl-2, ssDNA, Grp78, TS, DPD, CD34, VEGF, nestin, and LAT1). The antibodies used and methods

Table 2 Antibodies used for the immunohistochemical study

Antibody	Clone	Source	Dilution	Antigen retrieval
Ki67	Monoclonal MIB-1	DakoCytomation, Glostrup, Denmark	1 : 50	Treatment in hot bath (95–98°C) for 40 min (Dako Target Retrieval Solution (pH 9.0))
Mcm3	Monoclonal 3A2	MBL, Nagoya, Japan	1 : 400	Microwave treatment for 15 min (Dako Target Retrieval Solution (pH 9.0))
Bax	Monoclonal B-9	Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA	1 : 100	Microwave treatment for 15 min (citrate buffer (pH 6.0, 0.01 mol/l))
Bcl-2	Monoclonal 124	DakoCytomation	1 : 50	Microwave treatment for 15 min (Dako Target Retrieval Solution (pH 9.0))
ssDNA	Polyclonal A4506	DakoCytomation	1 : 400	Not applied
Grp78	Polyclonal H-129	Santa Cruz Biotechnology, Inc.	1 : 100	Microwave treatment for 30 min (citrate buffer (pH 6.0, 0.01 mol/l))
TS	Monoclonal TS106	DakoCytomation	1 : 50	Autoclave (121°C) treatment for 15 min (citrate buffer (pH 6.0, 0.01 mol/l))
DPD	Polyclonal RDPDPA	Taiho Pharmaceuticals Co., Ltd., Tokyo, Japan	1 : 400	Autoclave (121°C) treatment for 10 min (EDTA (pH 8.0, 1 mmol/l))
CD34	Monoclonal QBEnd 10	DakoCytomation	1 : 500	Not applied
VEGF	Polyclonal A-20	Santa Cruz Biotechnology, Inc.	1 : 100	Not applied
Nestin	Polyclonal N1602	IBL, Takasaki, Japan	1 : 500	Not applied
LAT1	Monoclonal	Fuji Biomedix, Tokyo, Japan	Prediluted	Microwave treatment for 5 min (in the buffer supplied by kit)

Mcm, minichromosome maintenance; Grp78, glucose-regulated protein 78; TS, thymidylate synthase; DPD, dihydropyrimidine dehydrogenase; VEGF, vascular endothelial growth factor; LAT1, L-type amino-acid transporter 1.

for antigen retrieval are listed in Table 2. Endogenous peroxidase was blocked with 3.0% hydrogen peroxide for 10 min, and incubation with Protein block serum-free solution (DakoCytomation, Glostrup, Denmark) for 10 min. Sections were incubated with the anti-Ki67, Bax, Grp78, TS, DPD, and CD34 primary antibodies for 60 min at room temperature, and with the anti-Mcm3, Bcl-2, ssDNA, VEGF, and nestin antibodies overnight at 4°C. After incubation with either labelled polymer, anti-mouse, or anti-rabbit (EnVision+ System HPR; DakoCytomation) for 60 min at room temperature, 3,3′-diaminobenzidine was used as the chromogen. Nuclei were counterstained with methyl green solution to facilitate histopathological assessment. The immunohistochemical protocol with the LAT1 staining kit (Fuji Biomedix, Tokyo, Japan) was according to the manufacturer's manual.

Evaluation of immunohistochemical staining

Ki67 and Mcm3 LI were determined as percentage values counting at least 1000 tumour cells in high-power fields ($\times 400$). With ssDNA staining, immunohistochemically positive cells were so few in number that at least 5000 nuclei were counted. ssDNA indices also were determined as percentage values.

Immunoreactivity for Bax, Bcl-2, Grp78, TS, DPD, and VEGF was evaluated using a score based on the classification of Sinicrope *et al* (1995). The staining intensity was scored as follows: none, 0; weak, 1; moderate, 2; intense, 3. If heterogeneity of staining intensity existed in a section, the staining intensity was scored based on that which was predominantly observed. The percentages of positive cells were assigned to one of five categories of protein expression: 0, $\leqslant$5%; 1, 5–25%; 2, 25–50%; 3, 50–75%; 4, $\geqslant$75%. The two scores were then multiplied to produce a weighted score for each tumour specimen. Two pathologists (MK and TM) independently scored the lesions and determined the final scores by discussion when they differed.

CD34-expressing capillaries were counted to give the MVD. Nestin-examined capillaries were considered as capillaries consisting of newly formed endothelial cells (Teranishi *et al*, 2007). Areas of highest neovascularisation were found by scanning tumour sections at low power ($\times 100$). The highest vascular counts of two different fields were averaged and used to calculate numbers of microvessels per mm^2, defined as MVD with both stainings.

For LAT1, a biomarker for high-grade malignancy, staining intensity was scored according to a previous report (Sakata *et al*, 2009): none, 0; weak, 1; moderate, 2; intense, 3. The percentages of positive cells were assigned to one of four categories: 0, $<$0%; 1, 1–10%; 2, 10–30%; 3, $>$30%. The values for the two variables were then multiplied, resulting in a scoring from 0 to 9. Two pathologists (MK and TM) independently scored the lesions.

Statistical analysis

Data were analysed using Dr. SPSS II (SPSS, Chicago, IL, USA) and Statview 5.0 (SAS Institute Inc., Cary, NC, USA) software. Immunohistochemical labelling and scores were compared using the Kruskal–Wallis test and the Mann–Whitney *U*-test. Logistic regression analysis was performed with a stepwise method. $P<0.05$ was considered as statistically significant.

RESULTS

Pathological response to CRT

Pathological evaluation of responses to preoperative CRT in resected rectum revealed radiation effects in all cases, with fibrosis and vascular changes. All 60 cases were classified into Dworak regression grades 1–4. Of these, 15 (25.0%) showed complete pathological responses (regression grade 4) and 14 (23.3%) showed microscopic residual tumours (regression grade 3), whereas 21 (35.0%) and 10 (16.7%) showed moderate (regression grade 2) or minimal (regression grade 1) responses to preoperative CRT, respectively.

Ki67, Mcm3, and ssDNA expression was confined to tumour cell nuclei, and Bax, Bcl-2, Grp78, and VEGF immunoreactivity to the tumour cell cytoplasm. TS and DPD were expressed in both nuclei and cytoplasm. LAT1 was confined to cell membranes, and CD34 and nestin were expressed in endothelium of intratumoural microvessels (Figure 1).

Figure 2 demonstrates the relationship between each immunohistochemical marker of the tumour biopsies before CRT and the pathological tumour response. A high Ki67 LI, Bax score, TS score, DPD score, MVD by CD34, and a low Grp78 score correlated with regression on univariate analysis. Recent studies have revealed that

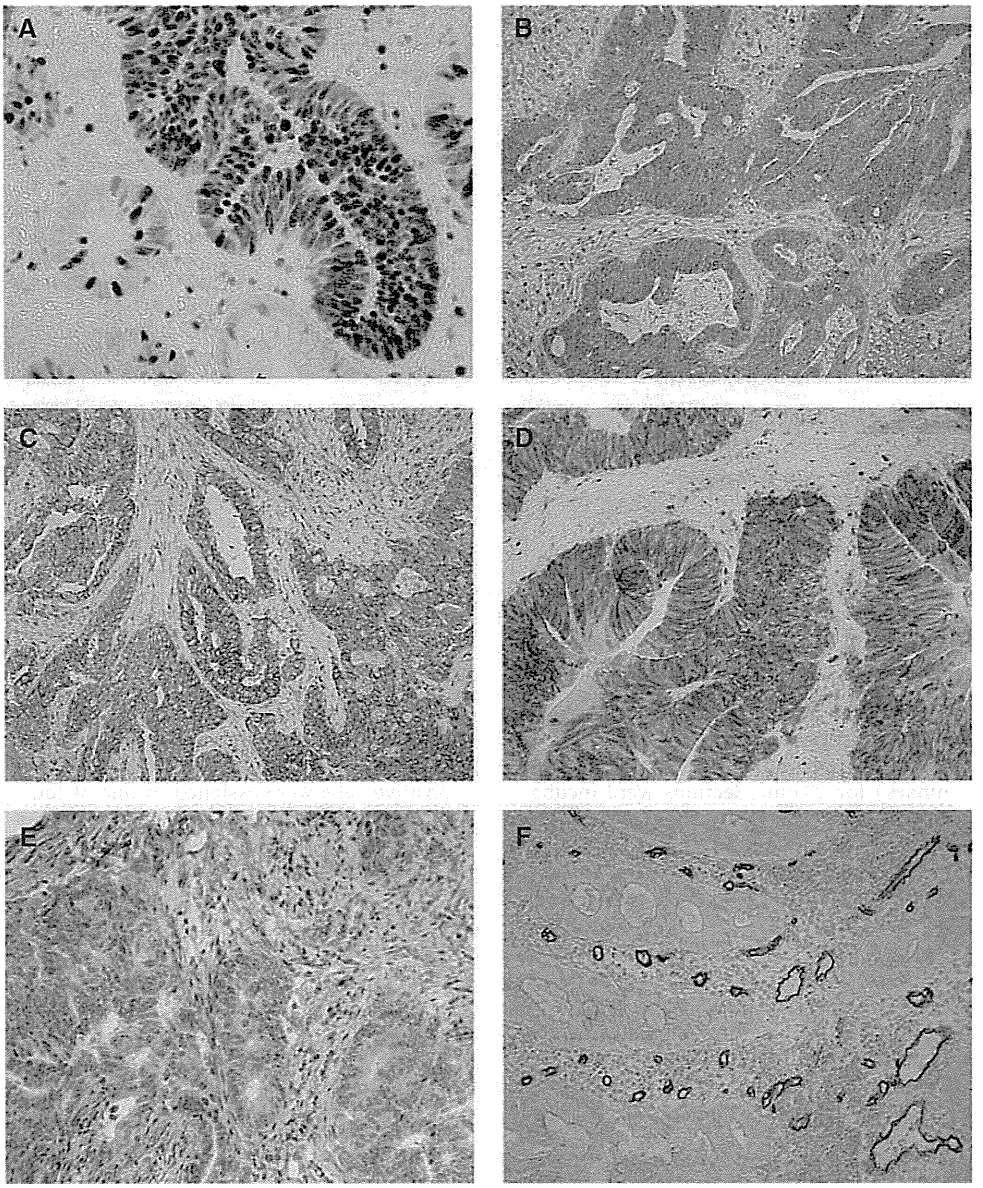

Figure 1 Immunohistochemical staining of pre-treatment rectal biopsy specimens from locally advanced rectal cancers. (**A**) Ki67 immunoreactivity, (**B**) Bax immunoreactivity, (**C**) Grp78 immunoreactivity, (**D**) TS immunoreactivity, (**E**) DPD immunoreactivity, and (**F**) CD34 immunoreactivity. Note Ki67 immunoreactivity confined to the tumour cell nuclei, Bax, and Grp78 to the tumour cell cytoplasm, TS and DPD to the tumour cell nucleus and cytoplasm, and CD34 to the endothelium of intratumoural microvessels.

pathological CR and greater than 95% pathological response groups achieve a significantly improved overall survival and recurrence-free survival when compared with less than 95% pathological response groups (Ruo *et al*, 2002; Guillem *et al*, 2005). Therefore, we divided the cases into two groups: Dworak grades 1 and 2, and grades 3 and 4 (Gavioli *et al*, 2005). The latter were considered as responders to CRT. A high Ki67, Bax score, and TS score and a low Grp78 score were well correlated with response. On the other hand, there were no associations with the other immunohistochemical factors, as well as clinicopathological factors (Table 3).

Multiple logistic regression analysis

Multiple logistic regression analysis was performed with a stepwise method (Tanaka *et al*, 1999). Independent variables were the data for Ki67 LI, Bax score, TS score, and Grp78 score, and dependent variables were no-response (0; Dworak regression grades 1 and 2) or response (1; Dworak regression grades 3 and 4). Other immunohistochemical markers and clinicopathological factors were not used. By the logistic regression analysis, we detected the Ki67 LI, Bax score, and TS score as independent factors (Table 4). The Bax score (odds ratio 18.1) had the strongest influence. The logistic regression formula was as follows:

$$\log_e (p/1-p) = -24 + 0.15 \times [\mathrm{Ki}_{67}\ \mathrm{LI}] + 2.90 \times [\mathrm{Bax\ score}] + 0.60 \times [\mathrm{TS\ score}].$$

Receiver-operating characteristic curve

A receiver-operating characteristic curve was generated by plotting the true-positive rate (sensitivity) on the y axis and the

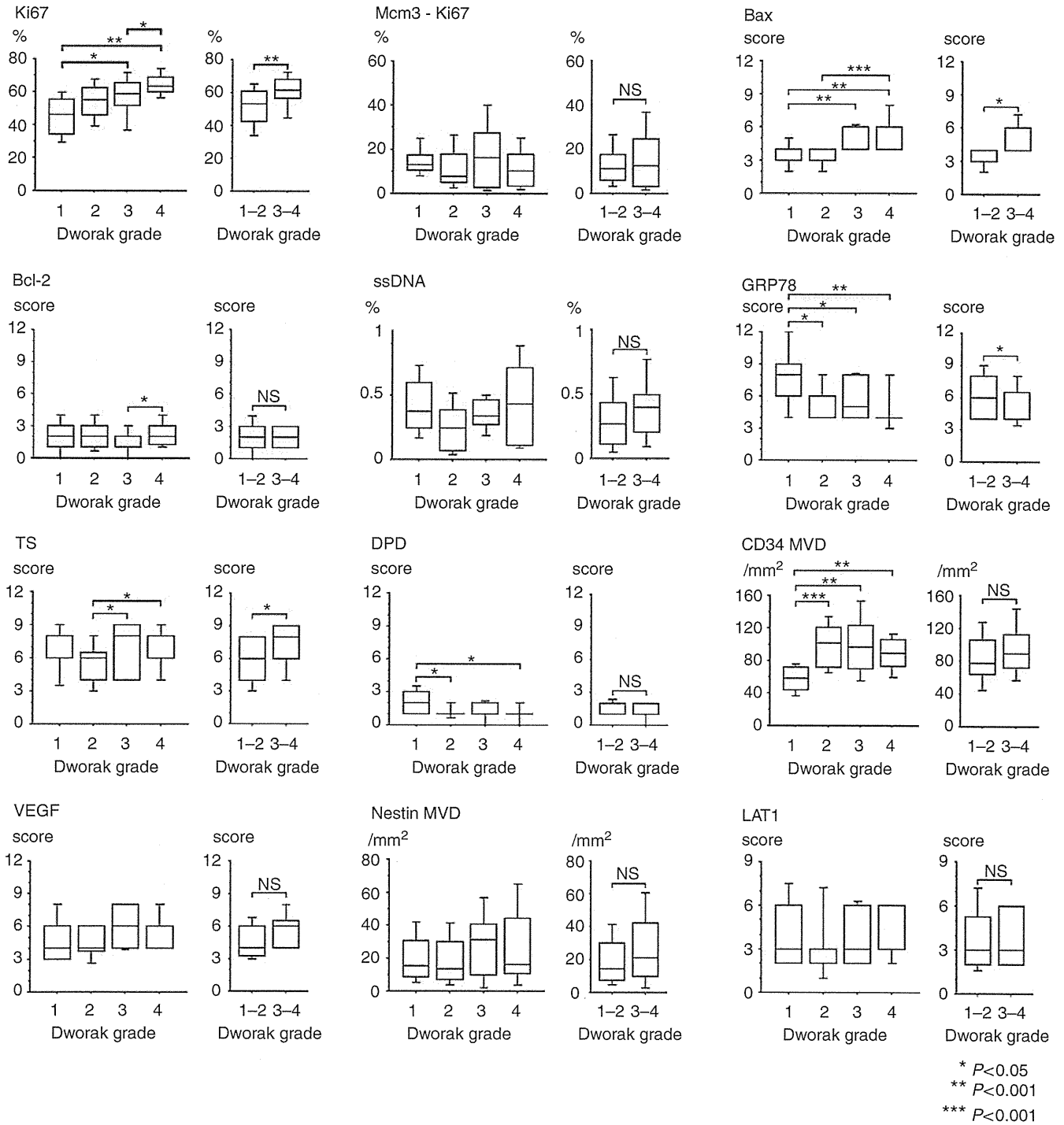

Figure 2 Ki67, Bax, Grp78, TS, DPD, and CD34 (MVD) were significantly related to chemoradiosensitivity ($P<0.05$). High Ki67 LI, Bax score, TS score, and low Grp78 were significantly correlated with tumour regression when responders were defined as having Dworak regression grades 3 and 4.

false-positive rate (1−specificity) on the *x* axis (Figure 3) (Tanaka *et al*, 1999).

Although the *P*-value at the point closest to the left upper corner on the curve is generally considered to represent the best balance of both sensitivity and specificity in distinguishing between response and no-response, we determined four points of *P* as the cut-off values (0.90, 0.50, 0.40, and 0.20) to construct practical criteria for the five categories 'responder', 'probable responder', 'unknown', 'probable non-responder', and 'non-responder' (Table 5). The points of $P=0.90$ and 0.20 meant the points of specificity 100% and sensitivity 100%, respectively. The point of $P=0.50$ meant the point at which the specificity was maximum and the sensitivity was more than 80%. The point of $P=0.40$ meant the point at which the specificity for prediction of non-responder was maximum and the sensitivity more than 80%.

Sensitivity and specificity

A *P*-value for each case was calculated with three immunohistochemical markers examined in 60 sets of biopsy specimens. Using

the calculated *P*-value, we classified the 60 patients into one of the above five categories with criteria distinguishing between responder and non-responder. Sensitivities and specificities of the criteria are shown in Table 5.

DISCUSSION

In this study, we sought clinicopathological factors and immunohistochemical markers that might contribute to prediction of chemoradiation effects on locally advanced rectal cancer. Our conclusion is that it is possible to predict a responder to preoperative CRT, with 82.8% sensitivity and 83.9% specificity, using the value calculated with the three elements of the Ki67 LI, the Bax score, and the TS score in biopsy specimens before CRT. In fact, high expression of Ki67, Bax, and TS was positively correlated with therapeutic effects.

The first factor, high proliferative activity with Ki67 as the marker, was earlier found to correlate with PCNA immunostaining, and mitotic counts after radiation of rectal cancer (Willett *et al*, 1995). Later, beneficial effects of radiotherapy for patients with various carcinoma with high Ki67 LIs were reported (Nakano *et al*, 1997; Raybaud-Diogene *et al*, 1997). However, in other reports, no relation was noted between Ki67 values in biopsy specimens before radiation and response rate in rectal cancers (Suzuki *et al*, 2004; Debucquoy *et al*, 2008). Suzuki *et al* (2004) performed preoperative radiotherapy only. Debucquoy *et al* (2008) combined preoperative radiotherapy and/or 5-FU/LV. Because we adopted CRT for all patients, the response may be more influenced by chemotherapy than radiation.

The second factor, Bax expression, was also reported by Chang *et al* (2005) to correlate well with chemoradiation therapeutic effects, and the authors considered that apoptosis may be important in rectal carcinoma response to CRT. Similarly, Bax overexpression has been found to correlate with anticancer drug sensitivity in a variety of human cancers, through enhanced induction of apoptosis (Krajewski *et al*, 1995; Guo *et al*, 2000; Teranishi *et al*, 2007). However, Gosens *et al* (2008) did not find any link between Bax expression and rectal cancer regression for neoadjuvant chemoradiation. They evaluated the regression grading system described by Rödel *et al* (2005): (1) no regression or <25% of tumour mass, (2) 25 to >50% tumour regression, and

Table 3 Clinicopathological characteristics of the patients separated by Dworak grades 1, 2 *vs* 3, 4

	Dworak grade 3, 4 (responder) ($n=29$)	Dworak grade 1, 2 (non-responder) ($n=31$)	
Age (year) (mean ± s.d.)	63.5 ± 11.4	63.5 ± 9.8	$P=0.11$
Sex			
Male	21	24	
Female	8	7	$P=0.65$
Tumor size (mm) (mean ± s.d.)	46.7 ± 14.4	48.0 ± 19.7	$P=0.98$
Histological type (biopsy)			
Well	17	20	
mod/por	12	11	$P=0.64$
CEA (mg/100 ml) (mean ± s.d.)	8.5 ± 12.7	9.4 ± 8.5	$P=0.23$
CA19-9 (ng/ml) (mean ± s.d.)	17 ± 25	22 ± 29	$P=0.054$

Well, well-differentiated adenocarcinoma; mod/por, moderately to poorly differentiated adenocarcinoma; s.d., standard deviation.

Table 4 Results of multiple logistic regression analysis

	Regression coefficient	*P*-value	Odds ratio	95% CI
Variable				
Ki67 LI	0.15	0.002	1.17	1.06–1.29
Bax score	2.90	0.001	18.1	3.11–105.7
TS score	0.60	0.019	1.83	1.11–3.03
Constant	−24.47	<0.001		

LI, labelling index; CI, confidence interval.

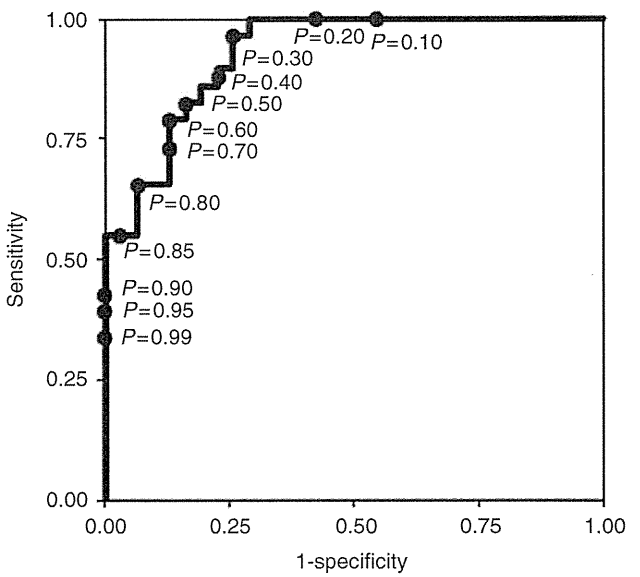

Figure 3 Receiver-operating characteristic curve with the logistic regression model. The area under the curve is 0.928 (95% confidence interval; 0.867–0.988).

Table 5 Criteria for Dworak grades 1, 2 *vs* 3, 4, and their validities tested among the 60 patients

			Pathological response		Validity			
			DG 3, 4	DG1, 2	DG 3, 4		DG 1, 2	
Category	Definition (*P*)	Definition (Π)	Responder ($n=29$)	Non-responder ($n=31$)	Se	Sp	Se	Sp
Responder	$0.90\leqslant P$	$2.20\leqslant \Pi$	13	0	44.8	100		
Probable responder	$0.50\leqslant P<0.90$	$0\leqslant \Pi<2.20$	11	5	82.8	83.9		
Unknown	$0.40\leqslant P<0.50$	$-0.41<\Pi<0$	1	1				
Probable no-responder	$0.20<P\leqslant 0.40$	$-1.39<\Pi\leqslant -0.41$	4	7			80.6	86.2
No-responder	$P\leqslant 0.20$	$\Pi\leqslant -1.39$	0	18			58.1	100

P, probability; DG, Dworak grade; Se, sensitivity; Sp, specificity; $\Pi=\log_e(P/1-P)$.

(3) complete regression. In addition, Bax immunohistochemical values were only intensity of cytoplasmic staining 0–3. Differences in grading systems and immunohistochemical expression scoring could clearly influence the results.

Rau *et al* (2003) immunohistochemically investigated the expression of p53, Bax, p21, Ki67, hMSH2 in pre- and post-therapeutic rectal carcinoma with preoperative radiotherapy. Only low p21 expression in tumour samples was significant in no-response to neoadjuvant chemoradiation. They reported no relation with Bax expression but classified responders as CR or partial response, histopathologically defined with resected post-therapeutic rectum, again differing from our definition as Dworak grades 3 or 4.

The third factor, TS, is important in pyrimidine nucleotide synthesis and represents an important chemotherapeutic target for 5-FU chemotherapy. Immunohistochemically, high TS expression in pre-treatment locally advanced rectal cancer biopsies was earlier shown to be predictive of a higher pathological response in the fluorouracil/oxaliplatin-base chemotherapy (Negri *et al*, 2008). A trend toward a direct correlation between the level of TS expression and response of 5-FU/LV treatment in patients with metastatic colon cancer has been noted (Johnston *et al*, 2003). Similar results have also been reported by Edler *et al* (2002) and Kornmann *et al* (2003).

However, low TS expression was a predictor of response to 5-FU chemotherapy for colorectal cancer metastases (Aschele *et al*, 1999) and advanced colorectal cancer (Cascinu *et al*, 1999). Aschele *et al* (1999) used a regimen of schedule-specific biochemical modulation of 5-FU plus methotrexate, and Cascinu *et al* (1999) applied 5-FU/LV. In both studies, cases with metastases and/or recurrence were included, and TS expression was evaluated as intensity 0 (undetectable staining) to 4 (very high intensity of staining), and then intensity levels 0–2 were considered as low, and 3 and 4 as high expression. We examined both cytoplasmic TS expression intensity and percentage of positive cells, as well as the Bax value. In another study, by Liersch *et al* (2006), TS expression was examined in surgically resected rectal cancer. In the reports, high TS expression correlated with cancer relapse. The clinical meaning of evaluation of TS expression needs further clarification.

The multiple logistic regression analysis revealed Ki67 LI, Bax score, and TS score to be independent factors, with a sensitivity and specificity for prediction of responder cases of 82.8 and 83.9%, respectively. Although the logarithm model is difficult to calculate for daily use, it can be easily converted to a linear model. It is sufficient for users to know the values of $\log_e (P/1-P)$ at the point of criteria. Practically, users can directly substitute the Ki67 LI, Bax score, and TS score into the formula:

$$\prod = \log_e(p/1-p) = -24 + 0.15\times[\text{Ki67 LI}] + 2.90 \times[\text{Bax score}] + 0.60\times[\text{TS score}].$$

If this value Π ($\log_e (P/1-P)$) is larger than 0.00, it indicates a responder case. If it is smaller than -0.41, it indicates a non-responder case (Table 5).

At present, CRT with subsequent surgical resection is performed without selection of cases. However, with our approach, likely responder cases can be chosen before therapy. In the future, our multivariate model should be revised using new factors to improve the sensitivity and specificity. The treatment strategy for locally advanced rectal cancer should be further developed toward so-called tailor-made therapy including such evaluation before preoperative therapy and/or surgical resection.

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ORIGINAL REPORT

Relapse-Related Molecular Signature in Lung Adenocarcinomas Identifies Patients With Dismal Prognosis

Shuta Tomida, Toshiyuki Takeuchi, Yukako Shimada, Chinatsu Arima, Keitaro Matsuo, Tetsuya Mitsudomi, Yasushi Yatabe, and Takashi Takahashi

From the Division of Molecular Carcinogenesis, Center for Neurological Diseases and Cancer, Nagoya University Graduate School of Medicine; Division of Research and Development, Oncomics Co, Ltd; and Departments of Thoracic Surgery, Pathology and Molecular Diagnostics, Aichi Cancer Center Hospital; and the Division of Epidemiology and Prevention, Aichi Cancer Center Research Institute, Nagoya, Japan.

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Corresponding author: Takashi Takahashi, MD, PhD, Division of Molecular Carcinogenesis, Center for Neurological Diseases and Cancer, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan; e-mail: tak@med.nagoya-u.ac.jp.

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

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ABSTRACT

Purpose
In order to aid the development of patient-tailored therapeutics, we attempted to identify a relapse-related signature that allows selection of a group of adenocarcinoma patients with a high probability of relapse.

Patients and Methods
Whole-genome expression profiles were analyzed in 117 lung adenocarcinoma samples using microarrays consisting of 41,000 probes. A weighted voting classifier for identifying patients with a relapse-related signature was constructed with an approach that allowed no information leakage during each training step, using 10-fold cross-validation and 100 random partitioning procedures.

Results
We identified a relapse-related molecular signature represented by 82 probes (RRS-82) through genome-wide expression profiling analysis of a training set of 60 patients. The robustness of RRS-82 in the selection of patients with a high probability of relapse was then validated with a completely blinded test set of 27 adenocarcinoma patients, showing a clear association of high risk RRS-82 with very poor patient prognosis regardless of disease stage. The discriminatory power of RRS-82 was further validated using an additional independent cohort of 30 stage I patients who underwent surgery at a distinct period of time as well as with the Duke data set on a different platform. Furthermore, completely separate training and validation procedures using another data set recently reported by the Director's Challenge Consortium also successfully confirmed the predictive power of the genes comprising RRS-82.

Conclusion
RRS-82 may be useful for identifying adenocarcinoma patients at very high risk for relapse, even those with cancer in the early stage.

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INTRODUCTION

Lung cancer remains the leading cause of cancer death in industrialized countries, including Japan and the United States.[1,2] Adenocarcinomas, which account for more than 50% of non–small-cell lung cancer (NSCLC) cases, are the most frequent type of NSCLC with a heterogeneous nature in various aspects, including clinicopathologic and molecular features, and are showing an increasing trend.[3] The TNM clinical staging system has become the standard for predicting prognoses, however, the best hope for cure relies on surgical resection, which is considered as standard treatment for operable adenocarcinoma patients.[4] Nevertheless, 30% to 35% of surgically treated stage I patients eventually face relapse after the initial surgery, indicating the existence of a subgroup of patients clinically diagnosed as having early-stage disease, who actually have residual cancer cells undetectable by currently available imaging techniques used for staging.[4]

Although a number of prognostic biomarkers, such as altered expressions of oncogenes, and tumor suppressor genes have been proposed, the TNM staging system remains the standard method for predicting patient prognosis, indicating that such prediction may require information derived from the expression status of multiple genes and molecules. At the same time, the advent of microarray technology and completion of the genome project has made it possible to carry out genome-wide profiling of gene expressions.[5] These developments have provided an opportunity for establishing patient-tailored therapeutic strategies, leading to the identification of gene-expression profiles that are associated with the prognosis of individuals with lung cancer.[6-12] However, few prognostic prediction