Table 2 Correlation between the results of cytological examination and presence/absence of peritoneal dissemination

	P0	P1	Total
Cy0	514	27	541
Cy1	101	124	225
Indeterminate	8	5	13
Undone	196	21	217
	819	177	996

P0 without peritoneal dissemination, *P1* with peritoneal dissemination, *Cy0* cytology-negative, *Cy1* cytology-positive

Table 3 Number of patients per peritoneal dissemination and cytology type of tumors

	Type4	Other Types	Total
P0Cy0	53	432	485
P0Cy1	33	55	88
P1Cy0	9	13	22
P1Cy1	61	45	106
	156	545	701

P0 without peritoneal dissemination, *P1* with peritoneal dissemination, *Cy0* cytology-negative, *Cy1* cytology-positive

701 patients were divided into four groups: (1) peritoneal dissemination-negative and cytology-negative (P0Cy0), (2) peritoneal dissemination-negative and cytology-positive (P0Cy1), (3) peritoneal dissemination-positive and cytology-negative (P1Cy0), and (4) peritoneal dissemination-positive and cytology-positive (P1Cy1). The number of patients in each category is given in Table 3.

Survival

The overall survival curves of the four groups are shown in Fig. 1. The prognosis of the patients with P1 and/or Cy1 was worse than that of the patients with P0Cy0. The prognosis of the P0Cy1 patients was better than that of the P1Cy1 patients ($p = 0.0002$, log-rank). The median survival time of the P0Cy1 patients was 12 months. The 2-year and 5-year survival rates in the P0Cy1 patients were 25.3% (95% confidence interval [CI] = 16.2–34.4%), and 7.8% (95% CI = 2.0–13.5%) (Table 4). Five (5.7%) of the 88 P0Cy1 patients survived for more than 5 years without evidence of recurrent disease.

The 88 P0Cy1 patients consisted of 33 patients with type4 gastric cancer and 55 with other types of gastric cancer. The survival of P0Cy1 patients with type 4 gastric cancer was significantly worse than that of the patients with other types of gastric cancer, as shown in Fig. 2 ($p = 0.0072$, log-rank). The median survival time was 10 months. The 2-year survival rate was 12.1% (95% CI = 0.12–22.1%) (Table 4). None of the patients survived for more than 40 months. Among the 88 P0Cy1 patients, 51 patients received postoperative adjuvant chemotherapy, mainly based on fluorouracil, while 35 did not, although this was not randomized. There was no information about adjuvant therapy for two patients who had moved to other hospitals soon after surgery. There was no significant difference in the survival curves between the P0Cy1 patients who received and did not receive adjuvant chemotherapy ($p = 0.1238$, log-rank) (Fig. 3).

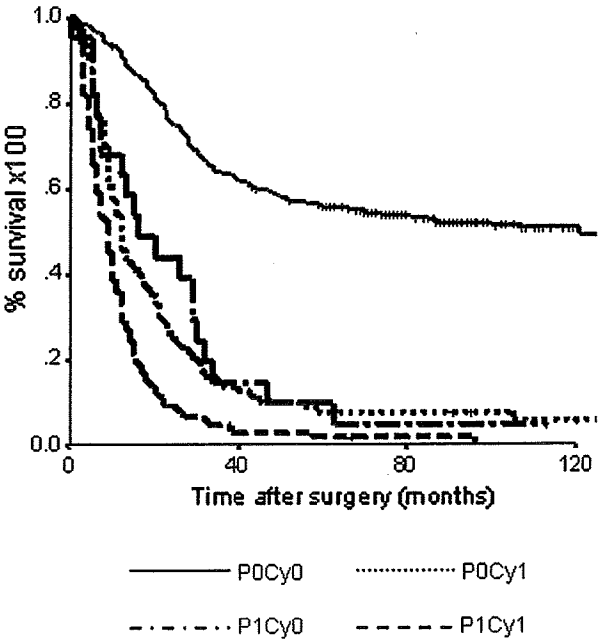

Fig. 1 Overall survival curves of gastric cancer patients (P0Cy0, P0Cy1, P1Cy0, and P1Cy1) are shown. The survival of P0Cy1 patients was poor but better than that of P1Cy1 patients ($p = 0.0002$)

Discussion

Lavage cytology-positive (Cy1) is most commonly encountered among gastric cancer patients with deeply invading tumors that extend outside the gastric wall [9, 15]; therefore, it is thought that the cancer cells escape from the surface of the tumors into the intraperitoneal cavity [16]. This is not clearly supported by some experiments, but Cy1 may reflect systemic spread of the tumor cells via the lymphatic pathway, which can cause retroperitoneal invasion, hydronephrosis, and rectal stenosis [17].

The prognosis of the patients who are found at the time of surgery to show peritoneal dissemination is expectedly very poor. The indication of mass reductive or palliative surgery should be evaluated by clinical trial [18], but it is regarded, by consensus, that gastric cancer patients with

Table 4 Survival rate and median survival time of POCy1 gastric cancer patients per type of tumor

	1 year	2 years	3 years	5 years	MST
POCy1					
All ($n = 88$)	46.0 (35.5–56.5)	25.3 (16.2–34.4)	13.8 (6.5–21.0)	7.8 (2.0–13.5)	12 (9.7–14.3)
Type 4 ($n = 33$)	45.5 (28.5–62.4)	12.1 (0.1–22.1)	0	0	10 (6.8–13.2)
Others ($n = 55$)	51.9 (38.5–65.2)	33.3 (20.8–45.9)	22.2 (11.1–33.3)	12.5 (3.5–21.5)	13 (7.6–18.4)

MST median survival time in months (95% confidence interval)

Values are % (95% confidence interval)

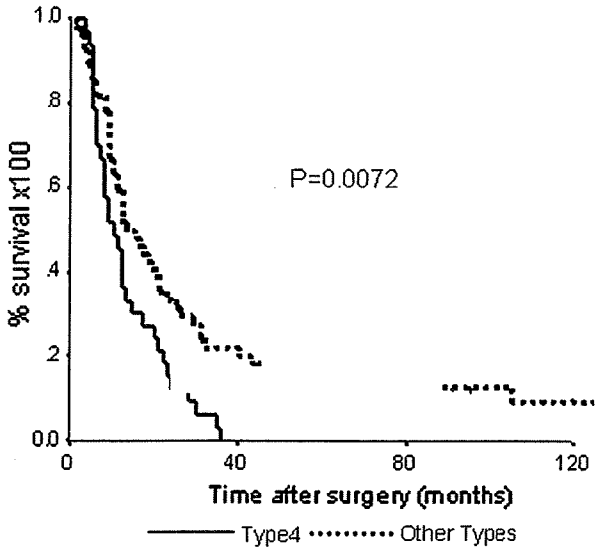

Fig. 2 The survival of POCy1 patients with type 4 advanced gastric cancer was significantly worse than that of patients with other types of advanced gastric cancer ($p = 0.0072$)

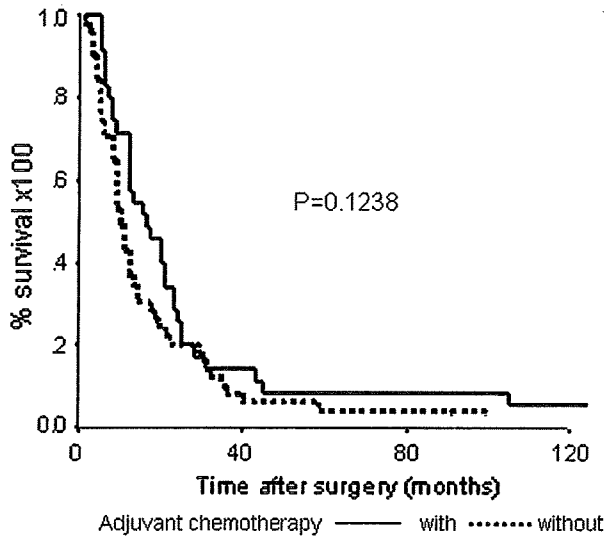

Fig. 3 There was no significant difference in the survival curves between POCy1 patients treated/not treated by adjuvant chemotherapy ($p = 0.1238$)

definite peritoneal dissemination are not suitable candidates for gastrectomy.

Cytological examination of intraperitoneal lavage fluid is performed in many institutions in Japan. In some institutions the result is confirmed intraoperatively, while in others it is confirmed on the following day. Cy1 is now included as one of the factors defining Stage IV in the Japanese classification of gastric carcinoma [19] because the prognosis of these patients with Cy1 is poor. However, the knowledge of a patient being Cy1 alone does not seem to be sufficient to decide on the therapeutic procedure [20]. The current consensus is that gastric cancer patients with intraoperatively confirmed Cy1 undergo standard gastrectomy and postoperative adjuvant chemotherapy [21]. Extended lymph node dissection and resection of other organs have gradually become less frequent in these patients. The efficacy of adjuvant chemotherapy with S-1 (1 M tegafur-0.4 M gimestat-1 M otastat potassium) after curative surgery has been reported [3]; however, no satisfactory postoperative adjuvant chemotherapy regimen for gastric cancer patients with Cy1 has been established. In our study, adjuvant chemotherapy using agents other than S-1 yielded no survival benefit. At our institution, S-1 was given as adjuvant chemotherapy to the patients, mainly after the end of the study period. In a future article we shall report on the efficacy of adjuvant chemotherapy with S-1 in gastric cancer patients with Cy1 compared with that in the subjects of this study as the historical control.

In this study, the 5-year survival rate of gastric cancer patients with POCy1 was 7.8%. This poor result must be interpreted as suggesting that previously used treatment, including surgery alone, was not suitable for these patients [22]. If those patients undergo surgery first, more intensive adjuvant chemotherapy would be needed. Currently, S-1 is given to these patients as adjuvant therapy [21, 23], but is S-1 monotherapy sufficient? A feasibility study of S-1 plus platinum as adjuvant therapy is ongoing (data not published); however, compliance with this therapy may not be favorable due to the unstable postoperative status of the gastric cancer patients. It is quite natural to expect that preoperative chemotherapy might be useful for those patients [24].

In order to carry out preoperative chemotherapy, information on Cy1 must be confirmed by staging laparoscopy [25]. In Japan, staging laparoscopy has been popular, but it may be difficult for it to be routinely performed in every advanced gastric cancer patient at every institution. Definitive evidence on the efficacy of preoperative chemotherapy, such as that from the MAGIC trial [26], is mandatory for encouraging the use of this therapy in Japan.

When only type 4 advanced gastric cancer patients are included in the analysis, the prognosis of those with Cy1 is extremely poor. No patient survived for more than 40 months after surgery in this study. The survival curve of the patients with P0Cy1 was almost the same as that of the patients who were found to have peritoneal dissemination (P1Cy1) at the time of the surgery (data not shown). The indication for gastrectomy for these patients must be discussed [27]. No surgeon performs gastrectomy for linitis plastica with peritoneal dissemination, except for palliating stenosis or bleeding. The former therapeutic strategy of immediate surgery and adjuvant chemotherapy has a less curative power for these patients with such a poor prognosis, and preoperative chemotherapy should be tried. Controlled arm may be the chemotherapy without surgery [28]. Information on Cy1 is necessary for determining the therapeutic strategy in patients with type 4 advanced gastric cancer, therefore, staging laparoscopy must be carried out first.

The patients with peritoneal dissemination are not always cytology-positive. The survival of P1Cy0 patients is better than that of P1Cy1 patients (Fig. 1) ($P = 0.0028$, log-rank). When the analysis is limited to type 4 gastric cancer, the survival of P1Cy0 patients is also better than that of P0Cy1 and P1Cy1patients (not shown), but the sample size (P1Cy0: $n = 9$) is too small for statistical evaluation. The P1Cy0 patients with local disseminated nodules may be the subset that can benefit from intraoperative chemotherapy.

In conclusion, curative treatment has been scarce for gastric cancer patients with Cy1 until now. The prognostic benefit of adjuvant chemotherapy with S-1 has been expected for years, but more intensive adjuvant chemotherapy, preoperative chemotherapy, and intraperitoneal chemotherapy [29] also warrant trials. The prognosis of type 4 gastric cancer patients with Cy1 is especially poor; therefore, it is recommended that such patients be treated at large-volume institutions with new therapeutic strategies developed based on clinical trials.

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TEACHING CASES

Solid-pseudopapillary neoplasm of the pancreas with massive central calcification in an old man

Ikuo Matsuda [a], Hiroyuki Hao [a], Masataka Zozumi [a], Kenji Koishi [b], Tomohiro Matsumoto [b], Nobuaki Kaibe [b], Yoshinori Fujiwara [b], Mitsuru Sasako [b], Seiichi Hirota [a,*]

[a] Department of Surgical Pathology, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan
[b] Department of Surgery, Hyogo College of Medicine, Nishinomiya, Japan

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ABSTRACT

Solid-pseudopapillary neoplasm (SPN) is a rare pancreatic tumor primarily affecting women in their twenties. It is characterized by a well-demarcated or encapsulated mass, indolent behavior and favorable prognosis. Capsular or punctate calcification is occasionally observed. Reported herein is a case of SPN of the pancreas with massive calcification in a 76-year-old Japanese man. Macroscopically, the pancreatic tumor appeared to be a simple calcified nodule, but histological examination revealed that it was an epithelioid tumor with massive calcification. The tumor cells, forming nests and cords, had eosinophilic cytoplasm and small eccentric nuclei. They were immunohistochemically positive for vimentin, CD56 and neuron-specific enolase. Nuclear accumulation of β-catenin protein and a point mutation of the *β-catenin* gene by genomic DNA sequencing confirmed that the tumor was SPN. This is a very rare case of pancreatic SPN with massive calcification in an old man.

Introduction

Solid-pseudopapillary neoplasm (SPN), synonymously designated as soild-pseudopapillary tumor, is a rare pancreatic neoplasm (0.9–2.7% of all pancreatic malignancies), primarily affecting women in their twenties [6]. It is characterized by indolent behavior and a favorable prognosis. The typical SPN shows a well-demarcated or encapsulated mass consisting of various amounts of solid and cystic components. Capsular, peripheral or punctate calcification is occasionally noted in SPN [2,4], which is helpful in the differential diagnosis between SPN and pancreatic adenocarcinoma. Here we present a case of SPN of the pancreas with a massive central calcification in a 76-year-old Japanese man.

Case report

A 76-year-old Japanese man was admitted to hospital with the chief complaint of hematemesis. Endoscopic examination of the stomach revealed a Borrmann type 2 advanced gastric cancer of 6 cm in diameter in the middle body of the posterior wall of the greater curvature. Plain abdominal X-ray film revealed a calcified shadow in the left subphrenic region (Fig. 1a), and computed tomography for exploring metastasis of the gastric cancer demonstrated no apparent lesion suspicious of metastasis but a calcified mass at the pancreatic tail (Fig. 1b). The mass was suspected of being pancreatolithiasis for his history of alcohol intake (60 g/day × 60 years), and was excised when the patient underwent total gastrectomy for the gastric cancer. Histopathological examination of the resected stomach revealed poorly differentiated adenocarcinoma infiltrating subserosal tissue (data not shown). The resected pancreatic mass, 20 mm in diameter, without apparent fibrous capsule, was hard and appeared to be a simple nodular calcification (Fig. 2a).

Materials and methods

The tissue was fixed in 10% buffered formalin and embedded in paraffin. Three-micrometer-thick sections were stained with hematoxylin and eosin (HE). Immunohistochemistry was performed on representative sections employing an appropriate antigen retrieval and a standard two-step indirect method of Envision System (Dako, Glostrup, Denmark). The following antibodies were used: vimentin (Dako), neuron-specific enolase (Shandon Immunon, Pittsburgh, PA, USA), CD56 (Novocastra Laboratories, Newcastle, UK), α1-antitrypsin (Shandon Immunon), and α1-antichymotrypsin (Shandon Immunon), pancytokeratin (AE1/AE3; Dako), synaptophysin (Dako), chromogranin (Shandon Immunon), insulin (NICHIREI, Tokyo, Japan),

* Corresponding author. Tel.: +81 798 45 6666; fax: +81 798 45 6671.
E-mail address: hiros@hyo-med.ac.jp (S. Hirota).

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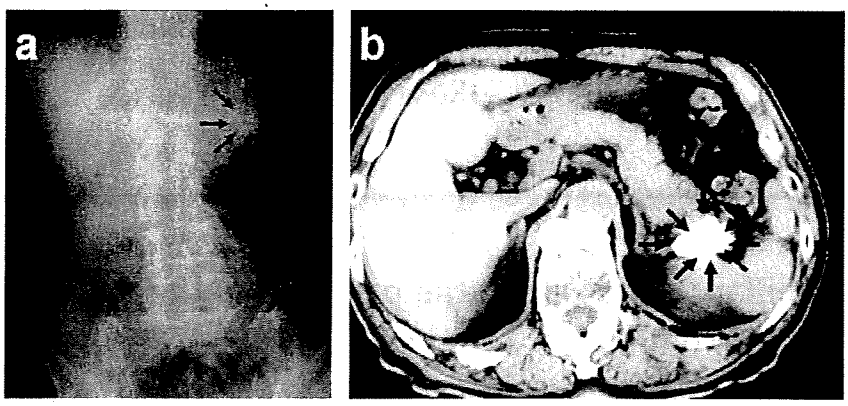

Fig. 1. Preoperative abdominal X-ray film (a) shows a calcified shadow in the left subphrenic region, and preoperative abdominal computed tomography (b) reveals a calcified mass at the pancreatic tail (arrows).

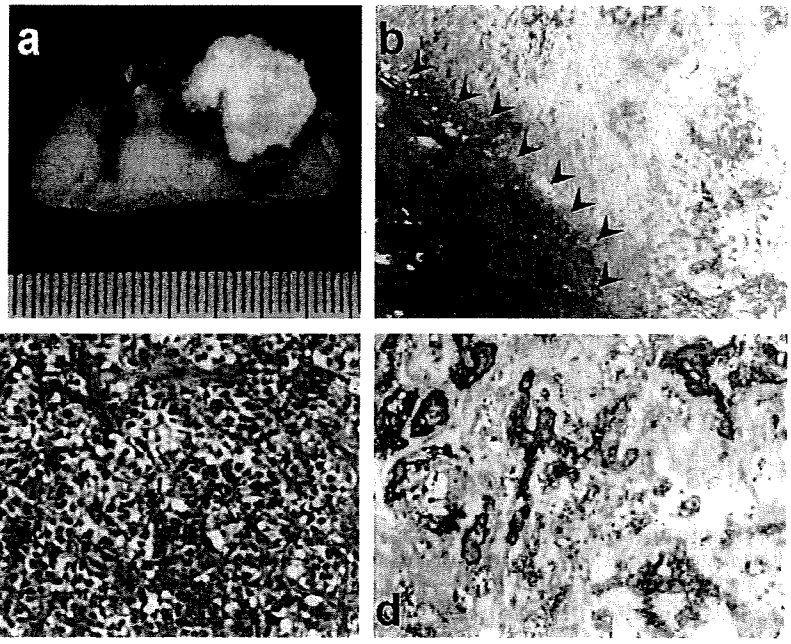

Fig. 2. Cut surface of the resected mass after formalin fixation shows an extensively calcified whitish nodule that does not have a clear fibrous capsule (a). Most of the tumor cells (between arrowheads) are present at the periphery of the central hyalinized and calcified lesion (upper right), and the tumor cells exhibit a mildly infiltrative growth at the boundary with the surrounding pancreatic tissue (b); HE stain, original magnification ×20. Each tumor cell has an eccentric nucleus and eosinophilic cytoplasm (c); HE stain, ×200. At the calcified core of the tumor, degenerated tumor cell nests are sparse (d); HE stain, ×200.

somatostatin (Shandon Immunon), pancreatic polypetide (Shandon Immunon), glucagon (Signet Pathology Systems, Dedham, MA, USA), estrogen receptor α (DAKO), progesterone receptor (DAKO), and β-catenin (Dako). For mutational analysis, genomic DNA was extracted from formalin-fixed, paraffin-embedded tissue sections using Genomic DNA Extraction Kit (Qiagen, Hilden, Germany). DNA sequencing for *β-catenin* gene mutation was performed as described [14] with minor modifications.

Results

Histological examination of the pancreatic mass revealed that it was an epithelioid tumor with extensive central hyaline degeneration and calcification. Most of the tumor cells were present at the periphery of the hyalinized and calcified lesion, and showed an infiltrating pattern at the border with normal pancreatic parenchyma (Fig. 2b). The tumor cells grew, forming nests and cords, and had eosinophilic cytoplasm and small eccentric nuclei (Fig. 2c). They were morphologically distinct from the poorly differentiated gastric adenocarcinoma cells, indicating that the pancreatic tumor was not a metastatic gastric cancer. From these HE images of the tumor, the differential diagnosis for the pancreatic tumor included pancreatic neuroendocrine tumors and SPN. At the calcified center of the tumor, degenerated tumor nests were found to be sparse with abundant calcified material (Fig. 2d).

Immunohistochemically, the tumor cells were positive for vimentin (Fig. 3a), neuron-specific enolase (Fig. 3b), CD56, α1-antitrypsin and α1-antichymotrypsin, and negative for pancytokeratin, synaptophysin and chromogranin (data not shown). With regard to hormonal markers, they were negative for insulin, somatostatin and pancreatic polypeptide, and were faintly and focally positive for glucagon (data not shown). Estrogen receptor α (Fig. 3c) and progesterone receptor (Fig. 3d) were negative in the tumor cells.

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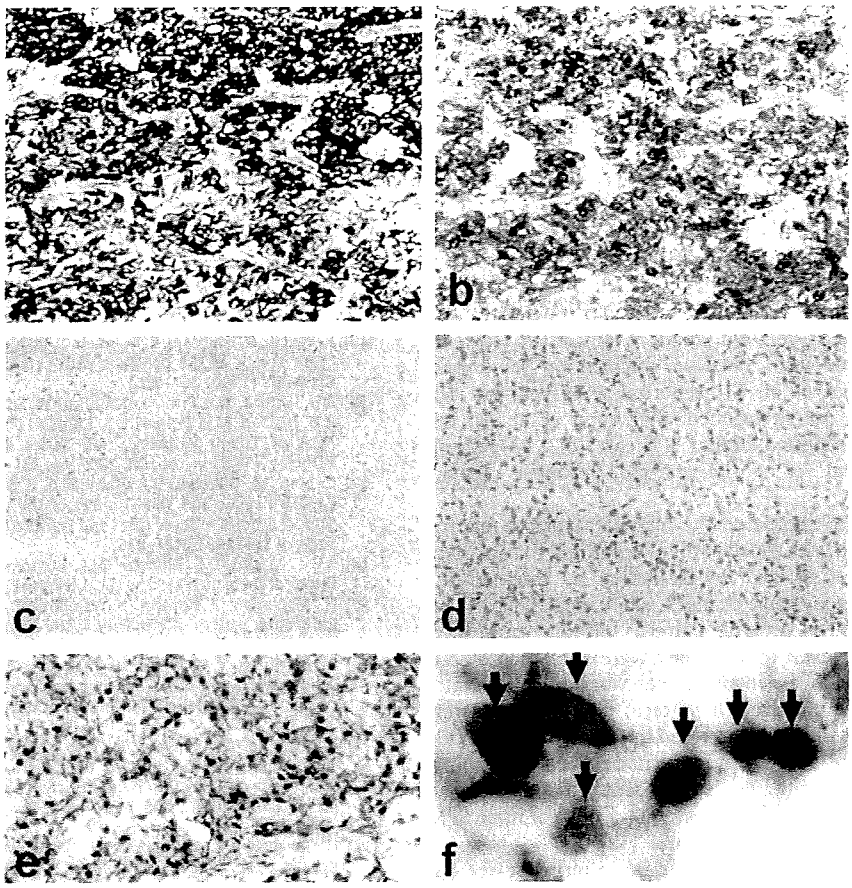

Fig. 3. Tumor cells are positive for vimentin (a); immunohistochemical staining (original magnification × 200) and neuron-specific enolase (b) (immunohistochemical staining, × 200), but negative for estrogen receptor α (c); immunohistochemical staining × 200 and progesterone receptor (d); immunohistochemical staining × 200. They are β-catenin-positive (e); immunohistochemical staining, × 200 and (f); immunohistochemical staining, higher magnification of (e). Arrows in (f) indicate nuclear staining of β-catenin.

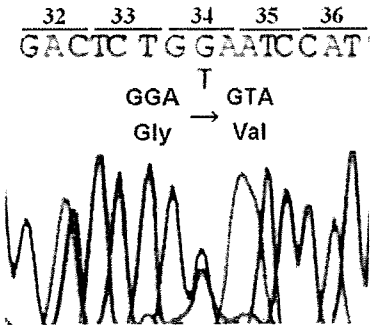

Fig. 4. Genomic DNA sequencing of the *β-catenin* gene of the pancreatic tumor reveals single G to T substitution of the *β-catenin* gene, resulting in Gly (GGA) to Val (GTA) mutation at amino acid residue 34 of *β-catenin* gene.

These histological and immunohistochemical findings of the tumor cells were consistent with those of SPN. The diagnosis of SPN was confirmed by the findings of nuclear accumulation of β-catenin protein by immunohistochemistry (Fig. 3e and f) and the point mutation at exon 3 of the *β-catenin* gene (Gly to Val substitution at the amino acid residue 34, Fig. 4) by the sequencing of genomic DNA. Mutations at exon 3 of the *β-catenin* gene are reported to occur in almost all cases of SPN [1,6,13,14], and the residue 34 is a hot spot of the mutation.

The patient is healthy without evidence of recurrence of gastric cancer and pancreatic SPN five months after the operation.

Discussion

Here we report a rare case of pancreatic SPN under consideration of the following two aspects. First, SPN predominantly affects young women, while the present tumor was observed in a 76-year-old man. Secondly, capsular calcification is occasionally observed in SPN [4] but the SPN of our case showed massive central calcification.

In routine HE staining, SPNs may mimic neuroendocrine tumors in its nested organization and cytological features with eccentric nuclei. Moreover, in immunohistochemistry, SPNs are frequently positive for neuroendocrine markers, including CD56 and neuron-specific enolase [6]. In fact, the pancreatic tumor cells of the present case were arranged in neuroendocrine-like structure and were positive for neuron-specific enolase (Fig. 3b) and CD56 (data not shown). However, SPNs essentially differ from neuroendocrine tumors in the following two points: β-catenin protein accumulates in the nuclei of the SPN tumor cells, and mutations of *β-catenin* gene occur in high percentage (83–90%) of SPN [6]. In our case, the nuclear accumulation of *β-catenin* protein was immunohistochemically detected in the tumor cells (Fig. 3e and f). Furthermore, genomic DNA sequencing of the tumor confirmed single G to T mutation at exon 3 of the *β-catenin* gene, resulting in Gly (GGA) to Val (GTA) substitution at the amino acid residue 34 (Fig. 4). Thus, the pancreatic tumor of our case was definitely diagnosed as SPN.

SPNs occur primarily in women in their twenties [2,8,11]. The mean age at diagnosis of the over 750 cases reported to date is 28

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years (range, 7–79 years), and 89% of the patients are female [6–8]. Martin et al. [8] reported an SPN case in a 79-year-old patient without referring to the sex of the patient and to the association with calcification. Takahashi et al. [12] reported an SPN case in a 75-year-old Japanese male without central calcification.

Capsular or punctate calcification is occasionally observed in SPN [4]. For example, in CT imaging studies, Buetow et al. [2] described peripheral calcification in 16 out of 52 SPN cases. This may be due to dystrophic calcification following intratumoral hemorrhage or cystic change, both of which are typically associated with SPN [6]. Prominent capsular calcification of SPN was reported in a 51-year-old Japanese female [5]. However, only some SPN cases with massive calcification have been reported [3,9,10]. Choi et al. [3] reported a 46-year-old SPN case whose sex was not mentioned. Matsunou et al. [9] and Nakamura et al. [10] reported similar SPN cases of a 44-year-old Japanese female and of a 34-year-old Japanese female, respectively.

Takahashi et al. [13] compared the histopathological features of SPN cases in women with those in men. According to their data, most SPNs in women showed encapsulation by thick fibrous tissue and massive degenerative changes, while most SPNs in men exhibited an infiltrative growth pattern without prominent degenerative changes, even though they were similar in size to those in women. Our SPN case in an old man was not encapsulated and showed an infiltrative growth pattern, which is consistent with the features of male SPNs described by Takahashi et al. [13]. However, our SPN case showed massive nodular calcification in the tumor center, presumably subsequent to degenerative changes. These degenerative changes are not typical of male SPNs according to the report by Takahashi et al. [13].

Taken together, our SPN case is very rare in terms of age and sex of the patient and in terms of the degree of the calcification, and it is considered to be the first reported SPN case with massive central calcification in an old male. The differential diagnosis for nodular calcification in the pancreas should include not only pancreatolithiasis but also SPN even in old males.

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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: gckaw@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	

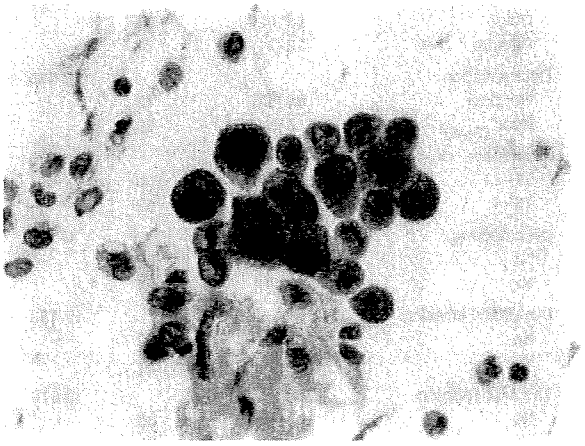
Values in parentheses are percentages. *Includes poorly differentiated, mucinous and undifferentiated types; †does not include peritoneal dissemination. DSS, disease-specific survival, IPC, intraperitoneal tumour cell; (p)TNM, (pathological) tumour node metastasis stage according to International Union 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.

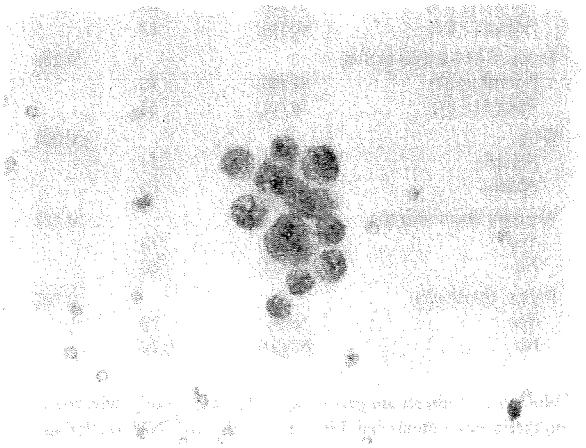
The site of the primary lesion was the colon in 156 patients (caecum, 23; ascending colon, 36; transverse colon, 22; descending colon, eight; sigmoid colon, 67) and the intraperitoneal rectum in 70. Clinical details for the 226 patients are shown in *Table 1*. Patient demographics, tumour characteristics 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)

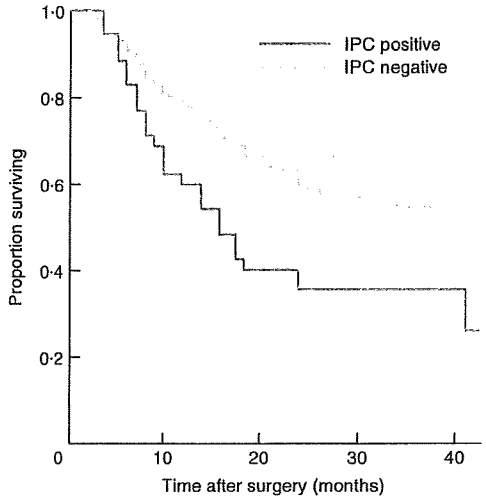
Table 2 Analysis of clinicopathological variables in 91 patients with stage III colorectal cancer in terms of survival and intraperitoneal tumour cell status

		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

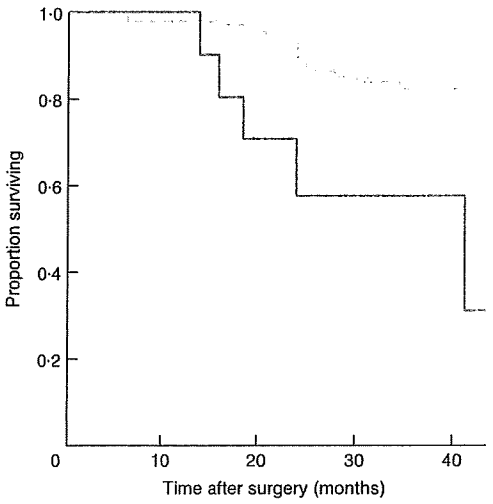
Postoperative therapy was recommended to all patients with stage III or IV colorectal cancer. Adjuvant chemotherapy, started within 7 weeks of operation, was received by 24 of 27 patients with stage II and by 63 of 91 with stage III disease. Regimens consisted of 5-fluorouracil (5-FU) alone (infusion 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

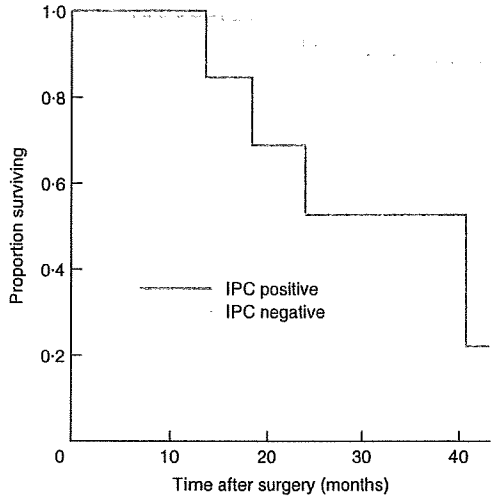


No. at risk

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

b Stage III

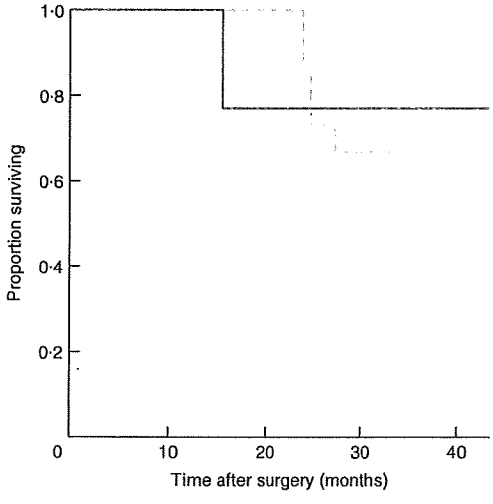
Fig. 2 Kaplan–Meier analysis of 5-year disease-specific survival in patients with colorectal cancer according to intraperitoneal tumour cell (IPC) status. **a** Patients with stage II–IV colorectal cancer. **b** Patients with stage III colorectal cancer. **a** $P = 0{\cdot}001$, **b** $P < 0{\cdot}001$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

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.

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 (×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, ⩽5%; 1, 5–25%; 2, 25–50%; 3, 50–75%; 4, ⩾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 (×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