Table 3
Surgical procedures

Type of operation	No. of patients	(%)
Total	44	(31.2)
Distal	81	(57.4)
Other resection	10	(7.1)
Bypass or exploration	6	(4.3)
Extent of dissection		
<D2	63	(46.7)
≥D2	72	(53.3)
Curability		
Curative resection (R0)	107	(75.9)
Non-curative resection (R≥1)	28	(19.9)
Bypass and exploration	6	(4.3)
ICU stay		
Elective	8	(5.7)
Emergency	1	(0.7)
No	132	(93.6)
Re-operation	2	(1.4)
Operation time (min)	194 (30–357) minutes	
Blood loss (ml)	310 (15–2572) ml	
Postoperative hospital stay (days)	17 (10–79) days	

Other resections includes surgical mucosectomy, wedge resection, and proximal gastrectomy.
Elective intensive care unit (ICU) stay was decided before the operation when the patients had severe co-morbidities.

other distant metastases. The predominant stage was stage I, followed by stages III, IV, and II.

3.4. Surgical procedures (Table 3)

Table 3 More than half of the patients underwent a distal gastrectomy. 53.3% of patients had resection with D2 lymph node dissection. Resection rate was 95.7% (135/141). One hundred and seven patients underwent operation with curative intent. The operation for the patients with positive lavage cytology was regarded as non-curative. The median operation time was 194 min Median blood loss was 310 ml. Postoperative hospital stay period was 17 days.

3.5. Early results (Table 4)

Table 4 Postoperative morbidity rate was 27.0% (38/141) overall, 28.0% (30/107) for the operations with curative intent, and 23.5% (8/34) for the palliative operations. There was no difference between curative and palliative operations. Surgery-related complications were less common. Pancreatic-related abscess was the most common. Pneumonia, regardless of the existence of aspiration, was most frequent postoperative complication. There was only one patient, who required intensive care unit (ICU) management due to post-operative complications.

Table 4
Postoperative complications

	No. of patients	(%)
Surgery-related		
Pancreatic-related abscess	10	(7.1)
Anastomotic leakage	1	(0.7)
Bleeding	0	(0)
Others	0	(0)
Non-surgery-related		
Pneumonia	13	(9.2)
Pulmonary embolism	0	(0)
Cardiac	5	(3.5)
Liver	2	(1.4)
Delirium	4	(2.8)
Empty disturbance	5	(3.5)
Others	2	(1.4)
Overall	38	(27.0)

The operation-related death was zero. The hospital mortality rate was also zero.

3.6. Survival

Fifty-nine patients died during the follow-up period. Forty-three of the deaths were related to gastric cancer. Twelve of the patients died of other causes (20.3%). Six were due to other malignancies (10.1%), six were due to other diseases (10.1%). Four occurred for unknown reasons (6.8%). Twenty-nine patients died within one year of their operation.

The 3-year survival rates were 59.0 (48.2–69.8)% for the whole population, 70.0 (58.3–81.7)% after curative resection and 16.1 (0–33.7)% after non-curative operations. After operations with curative intent, the 3-year survival rate was 80.3 (63.9–96.7)% for early gastric cancer, and 61.8 (45.7–77.9)% for advanced gastric cancer. The 5-year survival rates were 48.8 (36.0–61.6)% for the whole population, 56.6 (41.4–71.8)% after curative resection and 16.1 (0–33.7)% after non-curative operations. After operations with curative intent, the 5-year survival rate was 73.6 (54.0–93.2)% for early gastric cancer, and 41.7 (20.0–63.4)% for advanced gastric cancer.

4. Discussion

The Japanese population is ageing. However, they are still educated enough to be interested in health-checks for gastric cancer. A better public education of the elderly has increased cancer awareness, and thereby decreased the risk of developing symptoms, cases that are traditionally associated with a poor prognosis.

The increased age of the population is accompanied by an increase in age-related diseases. The preoperative surgical risk is often high, as has been reported in

Refs. [2,4,5]. However, the grade of complications were usually not severe in our series. Although we observed a high incidence of hypoalubuminaemia and low BMI, nutritional support via intravenous hyperalimentation was not essential before the operation. The ASA score was II in 80% and they did not have severe complications. They were only classified as score II because of their age i.e. 80 years and older.

The number of patients with stage I disease was 40% and less than that of previous study reported in Ref. [2]. Widespread use of endoscopic treatment has contributed to a decrease in gastrectomy for patients with early gastric cancer [6].

The resection rate of gastric carcinoma in the elderly has reached 95.7%, due to the early detection of disease and the ability to perform extensive resections, as well as the enormous improvements in preoperative staging.

Studies from other countries have reported high morbidity and mortality rates [4,5], especially in emergency cases. However, surgery-related complications were decreased in our study compared with those in previous series and the operation-related death rate was zero.

We previously reported that total gastrectomy and extended nodal dissection were both associated with a high operation-related death rate, especially in patients with preoperative morbidity. Therefore, curative operations were our aim, but at the same time, making efforts to perform limited dissections and to avoid total gastrectomy whilst preserving curability. The proportions of extended dissections was as low as 53% in our series.

There were very few obese patients in our series and these cases have higher morbidity and mortality rates [7]. In addition, the grade of preoperative co-morbidities was not severe in most of our patients. Our operations were all elective. In our institution, operation for gastric carcinoma is carried out only by specialists since 1993. Our stapling technique has improved and reduced the anastomotic leak rate [8]. Abscesses were common in the past after total gastrectomy with splenectomy. However, management of the abscess has been standardised as a result of a careful evaluation of past cases [9]. These factors have contributed to a decrease in our morbidity and mortality rates.

Gastrectomy can be carried out very safely in elderly patients by specialists. The survival rate was better than in the previous series. Life-expectancy for the general population of 80 years and older has increased and is now 8.26 years for males and 11.04 years for females. Therefore, death by other causes has decreased in this study. The 3(5)-year survival rate for early gastric cancer was excellent; 80.3 (73.6)%. Overall, 3(5)-year survival rates for the Japanese general population are 79 (61)%. There was no significant difference in survival between the early gastric cancer group and the general population.

Studies from the literature have reported that even patients with early gastric cancer usually die within 3 years without treatment [10]. Achievement of a curative R0 resection is always important, even for elderly patients.

Survival after non-curative resection is very poor. There is seldom an indication for a palliative distal or total gastrectomy. Preoperative staging, including laparoscopic exploration, is important to find candidates for surgical resection.

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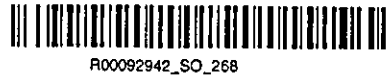

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Original article

Pancreaticoduodenectomy for advanced gastric cancer

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Abstract

Background. **Although pancreaticoduodenectomy has been rarely performed for gastric caner because of frequent morbidity and mortality, some favorable results after this procedure have been reported recently. Our objective was to present our data that might aid in the selection of patients to undergo this procedure.**

Methods. **Between 1970 and 2001, 23 patients who had pancreaticoduodenectomy for gastric cancer with tumor invading the pancreatic head were identified, and they were the subjects of this study. Clinical, operative, and pathological data, and morbidity and mortality rates were collected and analyzed. Survival outcome was also calculated and analyzed.**

Results. **Five patients underwent this procedure for disease in the gastric remnant, 18 undergoing the procedure for primary tumors. Median operating time was 8 h (range, 6–13 h), and median blood loss was 1600 ml (range, 700–16 000 ml). Regarding extent of gastrectomy, all patients with primary cancer ($n = 18$) underwent a distal gastrectomy and patients with disease in the gastric remnant ($n = 5$) underwent a completion gastrectomy. Incurable factors, including paraaortic lymph node metastasis, positive lavage cytology, or peritoneal dissemination were found in 8 patients. The postoperative morbidity rate was 73.9%; however, operation-related death was zero. The overall 5-year survival rate was 34.3%. The 5-year survival rate of the 8 patients with incurable factors was 0%, while that of the 15 patients without incurable factors was 47.4%.**

Conclusion. **If an R0 resection can be achieved by pancreaticoduodenectomy, this procedure should be performed for patients with tumor invading the pancreatic head. Patients with incurable factors should not be considered for pancreaticoduodenectomy.**

Key words Gastric cancer · Pancreaticoduodenectomy · Combined resection of adjacent organs

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Introduction

Complete removal of all evaluable disease, i.e., R0 resection, is vital to a successful outcome in gastric cancer treatment. Extended surgery is occasionally required for advanced gastric cancer with infiltration of adjacent organs to achieve complete tumor clearance. For locally advanced gastric cancer with infiltration of the pancreatic head or duodenum, pancreaticoduodenectomy (PD) is required. However, this procedure has been rarely performed because of substantial morbidity and mortality [1]. Prior to the 1990s, few reports regarding PD for gastric cancer had been published [2]. Only Kishimoto et al. [3] and Scott et al. [4] referred to a long survivor after this procedure in their reports about gastrectomy with combined resection. Recently, with current advances in operative techniques and in nutritional support, some favorable results of the patients undergoing this procedure have been reported [5–7]. However, only a few reports with a large number of cases have been published so far. In the current study, we present our data that might aid in the selection of patients to consider who should undergo this procedure.

Subjects and methods

A retrospective review of our prospective database, spanning from 1970 to 2001 and containing 9349 patients, identified 195 (2.1%) who had locally advanced cancer with macroscopically suspected infiltration of the pancreatic head. We included patients with pancreatic head invasion from metastatic lymph nodes, and excluded type 4, linitis plastica cancer. Of the 195 patients identified, 23 underwent PD with presumed curative intent, and they were the subjects of this study.

In these 23 patients, clinical data, including age, sex, symptoms, and primary tumor or tumor in the gastric remnant, were collected and analyzed, using the appro-

Table 1. Patients undergoing pancreaticoduodenectomy

		Disease	Stage	pT	pN	P	CY	Adjuvant Chemo.	Combined resection	Recurrence	FUT (months)	Status
1	63/F	Primary	IV	4	1	0	0	−	Liver	N	13	DOD
2	42/M	Primary	IIIB	3	1	0	ND	−		—	157	DOC
3	64/M	Primary	IIIB	2	2	0	0	−		—	182	NED
4	67/M	Primary	IV	3	2	0	ND	−		—	87	DOC
5	76/M	Primary	IV	4	3	0	0	−	Colon	Unclear	4	DOD
6	67/M	Primary	IIIB	4	0	0	0	+		—	26	DOC
7	65/M	Primary	IV	4	3	0	1	+		N	6	DOD
8	74/F	Primary	IV	2	3	0	0	−	Colon	H	34	AWD
9	70/M	Primary	IV	4	2	0	0	−	Colon	N, H	14	DOD
10	62/M	Primary	II	2	0	0	0	−	Colon	—	52	NED
11	65/M	Primary	IV	4	2	0	0	−		N	36	AWD
12	65/F	Primary	IV	4	2	0	0	−		N, H, spleen	12	DOD
13	58/M	Primary	IV	4	3	0	0	−	Colon	N	6	DOD
14	60/M	Primary	IIIB	2	2	0	0	−	Colon	—	12	NED
15	64/M	Primary	IV	4	2	1	1	−	Colon	Unclear	19	DOD
16	51/F	Primary	IIIB	2	2	0	0	−		H	11	DOD
17	61/M	Primary	IV	4	1	0	ND	−		H	4	DOD
18	70/M	Primary	IV	4	3	0	1	−		N, lung	4	DOD
19	60/M	Remnant	IV	4	2	1	1	−		N	13	DOD
20	57/M	Remnant	IV	4	1	0	0	−	Liver, colon	N, H	26	DOD
21	64/F	Remnant	IIIB	4	0	0	0	−		N	64	DOD
22	47/M	Remnant	IV	4	3	0	0	−		N	17	DOD
23	60/M	Remnant	IIIB	4	0	0	0	−	Colon	P	4	AWD

Primary, Primary tumor; remnant, tumor of the gastric remnant; P, peritoneal dissemination; CY, lavage cytology; ND, not done; N, lymph node; H, liver; FUT, follow-up time; NED, no evidence of disease; AWD, alive with disease; DOC, dead of other cause; DOD, dead of disease; unclear, site of recurrence unclear

priate nonparametric tests. Operative data, including operating time, blood loss, hospital stay, extent of gastrectomy, extent of lymphadenectomy, and combined resection with PD, were also evaluated. Pathological data, including pT, pN stage, site of tumor, and incurable factors, such as paraaortic lymph node metastasis (pN3), peritoneal dissemination, and positive lavage cytology, were analyzed according to the Japanese classification. Perioperative morbidity and mortality were also investigated.

The survival data of the 195 patients with tumors invading the pancreatic head, including the 23 PD patients, were calculated by the Kaplan-Meier method and analyzed by the log-rank method.

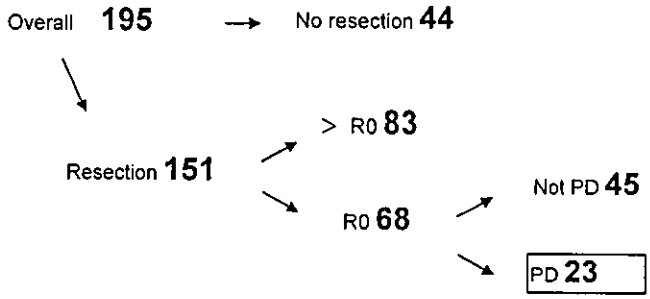

Fig. 1. Patients with tumors invading the pancreatic head. *No resection*, patients undergoing only exploration or bypass operation. *Not PD*, patients undergoing R0 resection, but with a lesser pancreatic resection than pancreaticoduodenectomy (*PD*)

Results

Demographics

Of the 195 patients with tumors invading the pancreatic head, 151 (77%) underwent resection, and the remaining 44 underwent only an exploration or a bypass surgery. In 68 patients, an R0 resection was carried out. In 45 patients with R0 resections, a lesser pancreatic resection (not PD) was performed because of a slight degree of tumor infiltration. The remaining 23 patients (12%) underwent PD (Fig. 1).

In the 23 patients undergoing PD, the median age at the time of resection was 64 years (range, 42–76 years), with a male-to-female ratio of 18:5 (Table 1). Twenty-one patients (91.3%) were symptomatic, most commonly with abdominal pain (n = 13) and symptoms due to obstruction, including fullness and vomiting (n = 11).

Eighteen patients underwent the PD procedure for primary cancer and 5 for gastric remnant cancer following previous Billroth I gastrectomy. Of the 5 patients with gastric remnant cancer, 4 had undergone distal partial gastrectomy for gastric cancer. Two of these

patients had early cancers, and the other 2 had advanced disease. The disease-free intervals were 1.5 and 6 years for those with advanced cancers and 8 and 10 years in those with early cancers. The fifth patient had had a partial gastrectomy for a benign gastric ulcer 30 years previously.

Operative data

The median operating time for PD was 8h (range, 6–13h), with a blood loss of 1600ml (700–16000ml). The median length of postoperative hospital stay was 37 days (range, 25–92 days). Regarding extent of gastrectomy, patients with primary cancer (n = 18) underwent a distal gastrectomy and those with gastric remnant cancer (n = 5) underwent a completion gastrectomy. As to extent of lymph node dissection, 14 patients underwent D2 lymphadenectomy and 9 underwent D3. In 9 patients, a combined resection of the colon was performed because of direct infiltration of the mesocolon (Table 1). Two patients underwent a partial hepatectomy because of a direct invasion of the liver. Modified Child's method was selected for a reconstruction for all patients. Two patients received postoperative adjuvant chemotherapy of 5-fluoruracil (5-FU) after surgery.

Table 2. Postoperative morbidity

	n
Postoperative morbidity	17 (73.9%)
Pancreatic fistula	10 (43.5%)
Abdominal abscess	3 (13.0%)
Anastomotic or jejunal stenosis	3 (13.0%)
Cholangitic infection	3 (13.0%)
Anastomotic leakage	2 (8.7%)

Table 3. Survival of patients with tumor invading the pancreatic head

	n	Median survival (months)	5-Year survival rate (%)
Overall	195	10	13.6
No resection	44	7	0
Resection	151	12	17.7
>R0	83	8	7.9
R0	68	21	29.3
Not PD	45	22	28.1
PD	23	17	34.3

No resection, Patients who underwent only exploration or bypass operation; not PD, patients who underwent R0 resection but received a lesser pancreatic resection than PD

Pathology

Resection specimens from all patients revealed adenocarcinoma of gastric origin. In 7 patients, infiltration of the pancreatic head could not be confirmed histopathologically. Regarding site of tumor, 18 primary tumors involved the antrum, and 11 of these tumors extended into the duodenum.

Incurable factors, including pN3, peritoneal dissemination, and positive lavage cytology were found in eight patients (Table 1). No patient in this series had a visceral metastasis. In 6 patients, pN3 was found. These patients had been considered as negative for pN3 intraoperatively, but the finding was changed to positive by pathological examination postoperatively. Of these 6 patients, 2 also had positive lavage cytology. Two patients had positive lavage cytology and peritoneal dissemination synchronously; the peritoneal dissemination was a single nodule that was removed easily at operation.

Seventeen patients developed recurrences. The most common recurrence sites were nodal, in 11 patients, followed by liver, in 6; peritoneum in 1; lung in 1, spleen in 1, and unclear, in 2.

Morbidity and mortality

Postoperative complications were seen in 17 patients (73.9%; Table 2). Pancreatic fistula was the most common. All patients who developed this complication recovered, after receiving drainage and continuous irrigation, using double-lumen drainage tubes. No operation-related death occurred in this series.

Regarding the long-term postoperative morbidity, body weight at 12 months was maintained within 10% of the preoperative weight in all patients who lived for more than 1 year. Serum albumin levels were not decreased. However, two patients who underwent PD with completion gastrectomy required total parenteral nutrition (TPN) at home, for 1 and 3 years, respectively, after discharge from hospital, because of malnutrition. Postoperative pancreatic endocrine function was adequate in all patients, but three patients required pancreatic exocrine enzyme support postoperatively.

Survival

In the 195 patients with tumors invading the pancreatic head, the 5-year survival rate was 13.6%. Of these 195 patients, the 68 patients who underwent an R0 resection showed a better survival outcome, with a 5-year survival of 29.3%. In patients who had R0 resections, there was no significant difference in survival between patients who underwent PD and those not receiving PD (Table 3).

In the 23 PD patients, the median follow-up time was 13 months (range, 4–182 months). The status of the

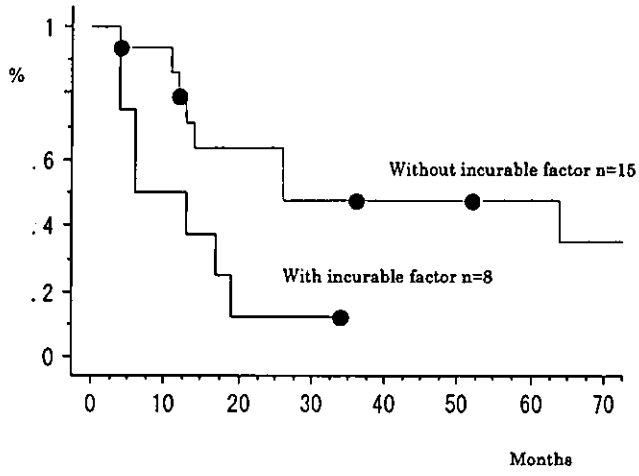

Fig. 2. Survival curves of patients undergoing pancreaticoduodenectomy (*PD*). The overall 5-year survival rate and the median survival of the 8 patients with incurable factors were 0% and 6 months, respectively, and these values in the 15 patients without incurable factors were 47.4% and 26 months ($P = 0.035$)

patients was as follows: no evidence of disease, 3; alive with disease, 3; dead of other causes, 3; and dead of disease, 14. The overall 5-year survival rate was 34.3%. The 5-year survival rate and the median survival of the 8 patients with incurable factors (pN3, positive lavage cytology, and peritoneal dissemination) were 0% and 6 months respectively, while these values in the 15 patients without incurable factors were 47.4% and 26 months (Fig. 2). Four patients have survived for more than 5 years.

Discussion

In our data, of 195 patients with tumors invading the pancreatic head, 23 (12%) underwent PD. This procedure has been rarely performed because of high morbidity and mortality rates. Prior to the 1990s, there had been only a few reports about this procedure [2–4]. Recently, with current advances in operative techniques, nutritional support, and antibiotics, some favorable results have been reported [5–11]. Ohashi [9] reported a large number of patients (145) undergoing this procedure. The 5-year survival rate of patients undergoing PD in that study was 6%, and it was approximately equal to the result for patients undergoing more than R0 resection in our data. Thus, it is inferred that Ohashi's subjects included patients with far-advanced tumors that could not be removed by this procedure. With proper indications, PD could account for 10% of surgeries for tumors invading the pancreatic head, and the number of patients who would have this procedure would be around 30, even at a large institution.

In our study, tumor infiltration of the pancreatic head could not be confirmed in 7 patients (30%) histopathologically. Such patients, theoretically, could have avoided this procedure; however, inconsistency between macroscopic and microscopic findings of infiltration has been reported to be 30%–50%, often because of inflammatory reactions surrounding the tumor [8,12]. Even if the latest diagnostic modalities, such as computed tomography (CT), magnetic resonance imaging (MRI), and endoscopic ultrasound (EUS) are used, it is very difficult to distinguish between inflammatory reactions and tumor infiltration before operation. Intraoperative ultrasound could be more helpful than these modalities, but it was not used in any patients in the present series. It seems that inconsistency at a level of around 30% is unavoidable at present.

Morbidity after PD was in Ohashi's study [9] 51.6% and 37.8% in that of Shchepotin et al. [11]. Regarding mortality, these authors reported rates of 6.3%, and 10.8%, respectively. Buchholtz et al. [1] recommended that PD should not be performed for gastric cancer because of an unacceptable risk, with no greater degree of palliation. The morbidity rate in our series (73.9%) was higher than the rates in these previous reports [9,11], to be sure. However, the operative mortality rate was 0% and all surviving patients could resume a regular life. Pancreatic fistula was the most common complication in this series. This is critical, as it may lead to intraabdominal abscess and rupture of arterial aneurysm. This complication was diagnosed by the detection of infectious drain discharge with a high concentration of amylase (>10000IU/l). For the early detection of pancreatic fistula, the concentration of amylase in the drain discharge is checked routinely after PD. When pancreatic fistula has developed, continuous drainage is performed, initially. If there is infection, continuous irrigation, using double-lumen drainage tubes, is done. To achieve better control of this complication, the medical staff including not only the surgeon but also nursing staff, have to be skilled at careful drain management. Therefore, this procedure should be performed only at institutions where PD for pancreatic cancer is frequently performed.

No patient in our series developed diabetes mellitus after PD, and only three required pancreatic exocrine enzyme support postoperatively. However, after PD with completion gastrectomy, two patients required TPN at home for a long period because of malnutrition. Total gastrectomy combined with PD should be considered very carefully, as nutritional problems may be severe.

The overall prognosis of patients with tumors invading the pancreatic head was poor; however the 5-year survival rate of patients undergoing R0 resection was about 30% in this series. In the patients with R0 resec-

tions, there was no significant difference in survival between those requiring PD and those not requiring PD. Thus, to achieve R0 resection is an important objective, irrespective of whether or not PD is performed.

Ajisaka et al. [5] and Shchepotin et al. [11] reported that the 5-year survival rates of patients undergoing PD were 35% and 17%, respectively. In a study of 26 patients undergoing PD combined with right hemicolectomy, Yonemura et al. [10] reported that the 5-year survival rate of 13 patients with tumors infiltrating the pancreatic head was 55%. In our series, the 5-year survival rate for such patients was 34.3%. In PD patients without incurable factors, the 5-year survival rate was higher, at 47%, and 4 patients have survived for more than 5 years. Careful application of the PD procedure can achieve improved survival outcome. Kodama et al. [13] and Habu et al. [14] mentioned that a small amount of peritoneal dissemination and limited liver metastasis, respectively, were not contraindications for PD. However, most patients in the present series who had incurable factors died of the disease soon after operation. Incurable factors, such as pN3, positive lavage cytology, peritoneal dissemination, and visceral metastasis, should be regarded as a contraindication for PD.

In summary, the results after PD for patients with advanced gastric cancer with tumors invading the pancreatic head were acceptable from the aspects of morbidity, mortality, and survival benefit. If an R0 resection can be achieved by PD in such patients, this procedure should be performed. Patients with incurable factors should not be considered for PD. The combination of PD and total gastrectomy should be considered with caution.

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Expression of thymidylate synthase, thymidine phosphorylase, dihydropyrimidine dehydrogenase, E2F-1, Bak, Bcl-X, and Bcl-2, and clinical outcomes for gastric cancer patients treated with bolus 5-fluorouracil

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Abstract. Few studies have investigated the biological factors associated with sensitivity to bolus infusions of 5-fluorouracil (5FU), including sequential methotrexate (MTX)/5FU therapy. We investigated the relationship between the expression of thymidylate synthase (TS), thymidine phosphorylase (TP), dihydropyrimidine dehydrogenase (DPD), E2F-1, Bcl-2, Bak, and Bcl-X, and the chemotherapeutic effects of sequential MTX/5FU. We studied 38 patients with unresectable or recurrent gastric cancer, treated weekly with sequential MTX/5FU (MTX 100 mg/m^2, 5FU 600 mg/m^2, by bolus infusions, with a three-hour interval). Expression of the above proteins was examined in initial biopsy samples with immunohistochemical methods. Immunohistochemical reactivity was defined as positive when over 25% of cancer cells showed strong staining in the cytoplasm for TS, TP, DPD, Bak, Bcl-2, and Bcl-X, and in the nucleus for E2F-1. The overall response rate was 28% in the 29 patients who had measurable lesions. Bak-negative patients showed a higher response rate than Bak-positive patients (39% versus 9%, respectively; p=0.1096), although expression of the other proteins was not associated with chemosensitivity. The median survival time (MST) of all patients was 256 days. Bak-negative patients survived significantly longer than Bak-positive patients (MST, 302 days versus 134 days, respectively; p=0.0044). Bcl-X-negative patients survived significantly longer than Bcl-X-positive patients (MST, 302 days versus 215 days, respectively; p=0.0080). Furthermore, patients negative for both Bak and Bcl-X had significantly better prognoses than other patients (MST, 373 days; p<0.0001). Within the limits of the small patient population, multivariate analysis using the Cox proportional hazards model showed that Bak, Bcl-X, and histological type were independent variables predicting survival (p=0.0008, 0.0081, and 0.0082, respectively). Although previously described predictive markers for protracted infusion of 5FU, including TS, TP, and DPD, might not be associated with clinical outcome in patients treated with sequential MTX/5FU, Bak may be a useful marker for chemoresponse and survival. Furthermore, both Bcl-X expression and the coupled expression of Bak and Bcl-X, as well as histological type, may be useful predictive markers for survival.

Introduction

A large number of molecular factors are implicated in a patient's sensitivity to anti-cancer drugs, including 5-fluorouracil (5FU). In many recent studies, thymidylate synthase (TS) expression has been identified as a predictor of response to 5FU (1-3). Thymidine phosphorylase (TP) catalyzes the reversible phosphorylation of thymidine to thymine and 2-deoxyribose-1-phosphate (4), and increases the conversion of 5FU to its active metabolites, which play an important role in the inhibition of TS. Overexpression of TP enhances the patient's sensitivity to protracted infusional 5FU regimens (5). The E2F family are transcription factors that regulate the transcription of genes that encode proteins required for DNA synthesis, such as TS (6). A recent study has reported that overexpression of E2F-1 in a human

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fibrosarcoma cell line resulted in increased resistance to 5FU via the up-regulation of TS (7). Kasahara *et al* (8) reported that overexpression of TS might be due to the enhanced expression of E2F-1 in colon cancer specimens. Dihydropyrimidine dehydrogenase (DPD) is the first and rate-limiting enzyme of 5FU catabolism, and its activity is potentially a factor controlling 5FU responsiveness (9,10).

Evidence has accumulated in the last few years that many and perhaps all agents of cancer chemotherapy affect tumor-cell killing *in vitro* and *in vivo* by inducing apoptosis (11). Tumors intrinsically resistant to chemotherapy are unable to activate the apoptotic machinery and may be fundamentally resistant to chemotherapeutic cell death. The Bcl-2 family plays a central role in the regulation of apoptotic cell death. Bcl-2 is the prototype of this family, and inhibits the induction of apoptosis. Some other family members (e.g., Bcl-X_L) are also anti-apoptotic, whereas others (e.g., Bax, Bak, Bcl-X_S, Bik, and Bid) display pro-apoptotic functions (12). Many of these proteins physically bind to each other, forming a complex network of homo- and hetero-dimers. The relative ratios of anti- and pro-apoptotic Bcl-2 family proteins dictate the ultimate sensitivity or resistance of cells to various apoptotic stimuli, including to anti-cancer drugs (13). Kondo *et al* (14) reported that the administration of bcl-X-antisense oligonucleotides or the overexpression of Bak, which binds Bcl-X_L and inhibits the anti-apoptotic effects of Bcl-X_L (15), caused an increase in apoptotic cell death and also induced high sensitivity to 5FU in a human gastric cancer cell line.

A number of synergistic interactions have been demonstrated between 5FU and other antineoplastic drugs in clinical investigations. The sequential use of the drugs methotrexate (MTX) and 5FU (sequential MTX/5FU) was the first regimen for which clinical efficacy against malignancies of the gastrointestinal tract was demonstrated (16). This regimen has also shown clinical benefits for patients with peritoneally disseminated gastric cancer (17,18). The therapy consists of a weekly schedule of MTX given as a bolus infusion three hours before a bolus infusion of 5FU.

Many studies have investigated the relationship between molecular factors and patient sensitivity to protracted infusions of 5FU. Despite the different mechanisms of cytotoxicity associated with bolus versus infusional 5FU, few studies have investigated the biological factors associated with sensitivity to bolus infusions of 5FU, including sequential MTX/5FU. The objective of this study was to clarify the relationship between the expression of TS, TP, DPD, E2F-1, Bak, Bcl-X, and Bcl-2, and clinical outcome in patients with advanced gastric cancer treated with sequential MTX/5FU.

Materials and methods

Patients and tissue samples. A total of 44 patients with advanced or recurrent gastric cancer were treated with sequential MTX/5FU therapy at the National Cancer Center Hospital East, Kashiwa, Japan, between August 1993 and December 1997. Paraffin-embedded biopsy specimens were collected from 38 patients who fulfilled the following recruitment criteria: i) no prior chemotherapy; ii) age of ≤75 years; iii) an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of ≥2, adequate bone-marrow, renal, and hepatic functions; iv) no serious medical complications, apart from intestinal obstruction or ascites; v) biopsy specimens from the primary tumors available for immunohistochemical analysis.

Treatment schedule. The treatment schedule consisted of weekly administration of MTX (100 mg/m², i.v. bolus) followed three hours later by 5FU (600 mg/m², i.v. bolus). From 24 h after the administration of MTX, calcium leucovorin (10 mg/m², p.o. or i.v.) was administered every six hours, for a total of six times. This treatment was continued until the disease progressed or the patient refused further treatment.

Evaluation of anti-tumor effects. Objective responses in measurable metastatic lesions were evaluated according to standard World Health Organization (WHO) criteria (19). The response at primary sites was not considered in the overall response. Survival time was estimated from the start of the first course to the date of death or the final date of confirmed survival.

Immunohistochemistry. The avidin-biotin peroxidase staining technique (Ventana Medical Systems, Tucson, AZ) was used for immunohistochemical analysis. Paraffin-embedded biopsy specimens collected at the time of initial presentation were cut into 5-μm sections and mounted on glass slides pretreated with aminopropyltriethoxy silane (Sigma Chemical Co., St. Louis, MO). Specimens were deparaffinized and hydrated through xylene and a graded alcohol series. Endogenous peroxidase activity was neutralized with a solution of 3% hydrogen peroxidase in methanol for 15 min. Sections were washed three times in phosphate-buffered saline (PBS), then heated twice in citrate buffer (pH 7.6) in a microwave oven for 5 min at 700 W to retrieve antigenicity. Samples were then washed in PBS, and incubated for 30 min in 10% normal horse serum. The slides were incubated overnight at 4°C with the following antibodies: anti-TS, 1:100 (mouse monoclonal antibody; Taiho) (20), anti-TP, 1:200 (mouse monoclonal antibody 654-1; Roche) (21), anti-DPD, 1:540 (rabbit polyclonal antibody; Taiho) (22), anti-E2F-1, 1:20 (mouse monoclonal antibody; Santa Cruz Biotechnology) (7), anti-Bak, 1:20 (mouse monoclonal antisera; Oncogene Research Products) (23), anti-Bcl-X, 1:20 (rabbit polyclonal antisera; Oncogene Research Products) (23), or Bcl-2, 1:20 (mouse monoclonal antibody; Oncogene Research Products) (23). All these antibodies have been described previously, in detail (7,21-23).

Immunohistological scoring. Pathologists (A. Ochiai and W. Yasui) blind to the clinical outcomes scored the immunohistochemical staining independently. In the immunohistochemical analysis of TS, TP, E2F-1, DPD, Bak, Bcl-X, and Bcl-2 expression, the degree of immunohistochemical reactivity was defined as positive when >25% of cancer cells showed strong staining in the cytoplasm (Fig. 1A). Because E2F-1 is a transcriptional factor, E2F-1 immuno-reactivity was judged to be positive when >25% of cancer cells showed strong staining in the nucleus (Fig. 1B). Although the Bcl-X gene encodes two proteins, a long form (Bcl-X_L) and a short

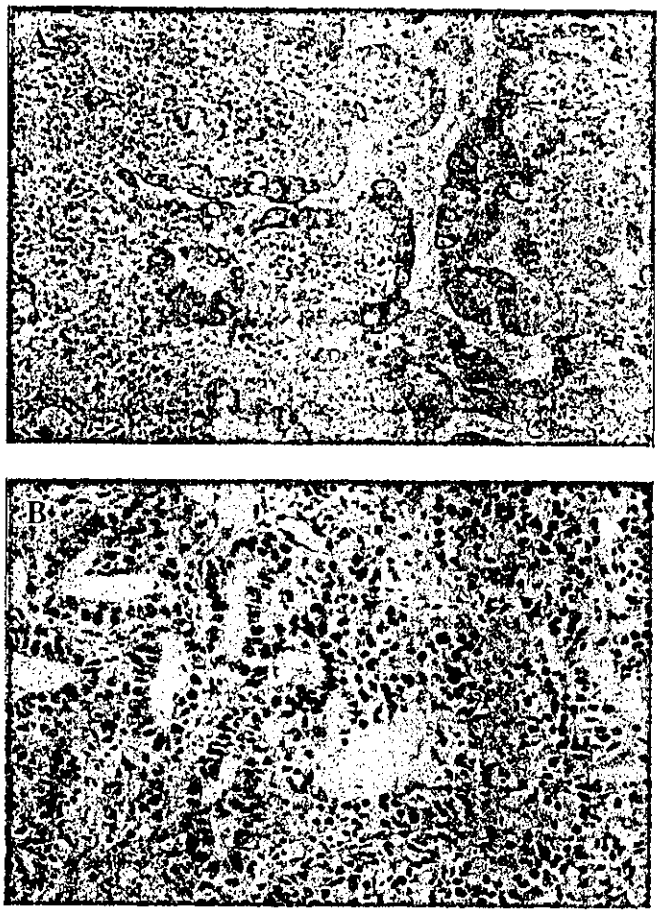

Figure 1. A, In the immunohistochemical analysis of TS, TP, E2F-1, DPD, Bak, Bcl-X, and Bcl-2 expression, the degree of immunohistochemical reactivity was defined as positive when >25% of cancer cells showed strong staining in the cytoplasm. B, Because E2F-1 is a transcriptional factor, E2F-1 immunoreactivity was judged to be positive when >25% of cancer cells showed strong staining in the nucleus.

Table I. Patient characteristics.

	No. of patients	%
Total no.	38	
Age, year		
Median	56	
Range	30-74	
Sex		
Male	28	74
Female	10	26
Performance status		
0	22	58
1	13	34
2	3	8
Histology		
Intestinal	8	21
Diffuse	30	79
Surgical resection		
No	15	39
Yes	23	61
Metastatic site		
Liver	9	24
Abdominal lymph no.	19	50
Neck lymph node	2	6
Peritoneum	18	47
No. of courses		
Median	11	
Range	2-34	

form (Bcl-X_S), via an alternative splicing mechanism (24), the Bcl-X polyclonal antibody used in this study did not cross-react with Bcl-X_S.

Statistical analysis. Survival analysis was performed using the method of Kaplan and Meier (25). We used an unpaired t-test to analyze the differences in the expression levels of proteins. Pearson's correlation test was performed to examine this relationship. The influence of each biological variable on patients' survival was assessed by the Cox proportional hazards model.

Results

Characteristics of the patient population. Patient characteristics are listed in Table I. The characteristics of the 38 patients were: median age of 56 years (range, 30-74 years); and PS of 0, 1, or 2 in 22, 13, and three patients, respectively. In histological terms, 30 patients (79%) had diffuse-type carcinoma and eight patients (21%) had intestinal-type carcinoma. Gastrectomy had been performed in 23 patients (61%), 29 patients (76%) had measurable lesions, 19 patients (50%) had abdominal lymph node metastasis, and 18 patients (47%) had peritoneal metastasis.

Clinical outcomes after sequential MTX/5FU therapy. The median number of treatments with sequential MTX/5FU therapy was 11 (range 2-34). In 29 patients with measurable lesions, the overall response rate was 28% (8/29). With a median follow-up time of 22 months, the MST of all 38 patients was 256 days.

Expression of TS, TP, DPD, and E2F-1, and clinical outcome. The proportion of cases positive for TS, TP, DPD, or E2F-1 was 76%, 37%, 66%, and 37%, respectively. The relationships between the expression of TS, TP, DPD, and E2F-1 and clinical outcome are shown in Table II. Expression of these proteins showed no significant correlation with response or survival. Moreover, no correlation existed between the expression of TS and E2F-1.

Expression of Bcl-2 family proteins and clinical outcome. Of 38 specimens, four (11%) were immunopositive for Bcl-2, 12 (32%) for Bak, and seven (18%) for Bcl-X. The relationships between the expression of Bcl-2, Bak, and Bcl-X, and

Table II. Expression of TS, TP, DPD and E2F-1 and clinical outcomes.

	No. of patients	RR	p-value	MST (days)	p-value
TS					
(+)	29	25 (5/20)	0.67	289	0.59
(-)	9	33 (3/9)		129	
TP					
(+)	14	33 (4/12)	0.68	358	0.36
(-)	24	24 (4/17)		215	
DPD					
(+)	25	30 (6/20)	>0.99	273	0.28
(-)	13	22 (2/9)		256	
E2F-1					
(+)	14	25 (3/12)	>0.99	225	0.44
(-)	24	29 (5/17)		298	

RR, Response rate in 29 patients who had measurable lesions; MST, Median survival time.

Table III. Expression of Bcl-2, Bak and Bcl-X and clinical outcomes.

	No. of patients	RR	p-value	MST (days)	p-value
Bcl-2					
(+)	4	0 (0/3)	>0.99	298	0.4
(-)	34	31 (8/26)		244	
Bak					
(+)	12	9 (1/11)	'0.1096	134	0.0044
(-)	26	39 (7/18)		302	
Bcl-X					
(+)	7	40 (2/5)	0.63	215	0.008
(-)	31	25 (6/24)		302	

RR, Response rate in 29 patients who had measurable lesions; MST, Median survival time.

clinical outcome are shown in Table III. Bak-positive patients showed a higher response rate than Bak-negative patients (39% versus 9%, respectively; p=0.1096). Furthermore, Bak-negative patients survived significantly longer than Bak-positive patients (MST, 302 days versus 134 days, respectively; p=0.0044) (Fig. 2A). Although there was no relationship between the expression of Bcl-X and chemoresponse, Bcl-X-negative patients survived significantly longer than Bcl-X positive patients (MST, 302 days versus 215 days, respectively; p=0.0080) (Fig. 2B). Furthermore,

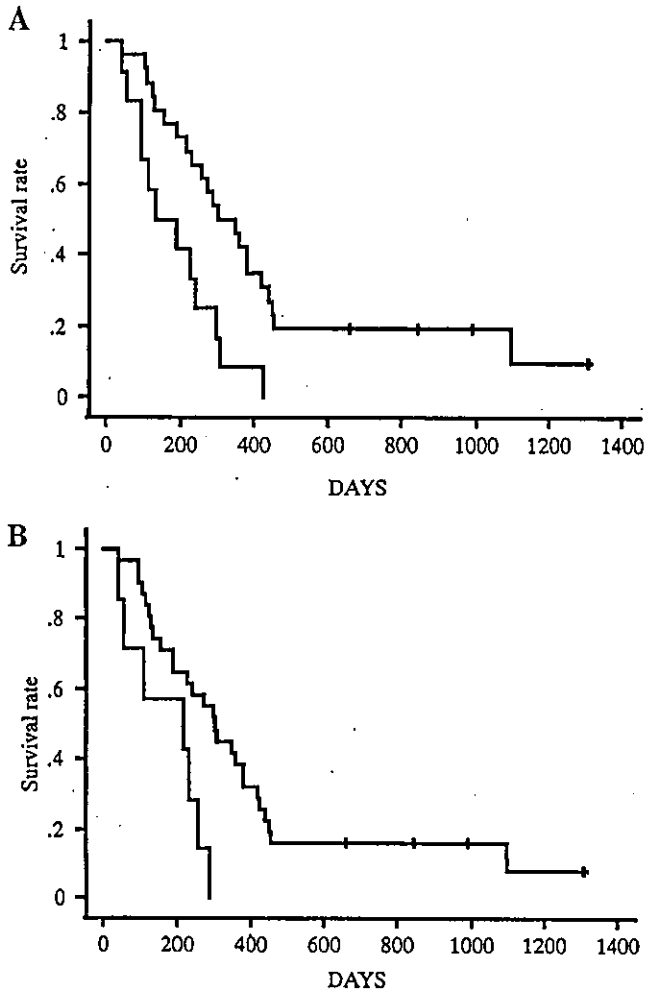

Figure 2. A, Survival of the patients according to the expression of Bak. Continuous line, the Bak-negative patients (n=26); dashed line, the Bak-positive patients (n=12). Bak-negative patients survived significantly longer than Bak-positive patients (MST, 302 days versus 134 days, respectively; p=0.0044). B, Survival of the patients according to the expression of Bcl-X. Continuous line, the Bcl-X-negative patients (n=31); dashed line, the Bcl-X-positive patients (n=7). Bcl-X-negative patients survived significantly longer than Bcl-X positive patients (MST, 302 days versus 215 days, respectively; p=0.0080).

there was no correlation between the expression of Bak or Bcl-X and any clinicopathological feature, including age, sex, histological type, metastatic site, or the presence of a primary site (data not shown).

Coupled expression of Bak and Bcl-X and clinical outcome. The relationships between the coupled expression of Bak and Bcl-X and clinical outcome are shown in Table IV. Although there was no relationship between the coupled expression of Bak and Bcl-X and chemoresponse, Bak-positive Bcl-X-negative patients had poor prognoses (MST, 193 days). Furthermore, Bak-negative Bcl-X-negative patients had significantly better prognoses than the other patients (MST, 373 days; p<0.0001) (Fig. 3).

Relationships between clinicopathological markers and survival. Table V presents the relationships between clinicopathological markers and survival. Histological type was

Table IV. Couple expression of Bak and Bcl-X and clinical outcomes.

	No. of patients	RR	p-value	MST (days)	p-value
Bak (-)/Bcl-X (+)	6	50 (2/4)[a]	0.2	215[b]	0.0001
Bak (+)/Bcl-X (-)	11	11 (1/9)[a]		193[b]	
Bak (-)/Bcl-X (-)	20	36 (5/14)[a]		373[b]	
Bak (+)/Bcl-X (+)	1	0		57	

[a]0.2; [b]0.0001; RR, Response rate in 29 patients who had measurable lesions; MST, Median survival time.

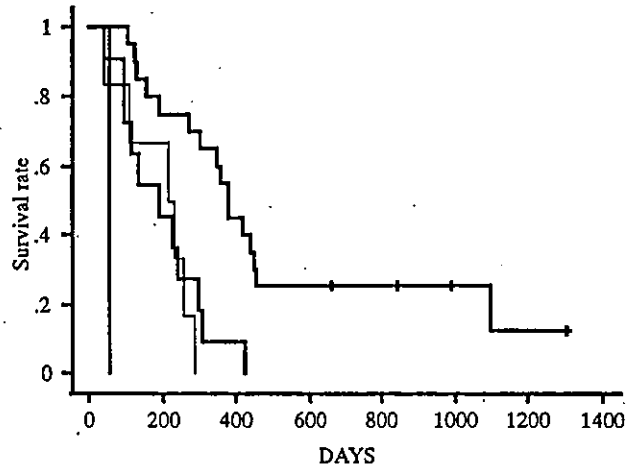

Figure 3. Survival of the patients according to couple expression of Bak and Bcl-X. Continuous line, the Bak-negative Bcl-X-negative patients (n=20); dashed line, the Bak-positive Bcl-X-negative patients (n=11); narrow line, the Bak-negative Bcl-X-positive patients (n=6); dot line, the Bak-positive and Bcl-X-positive patients (n=1). Bak-positive Bcl-X-negative patients had poor prognoses (MST, 193 days). Furthermore, Bak-negative Bcl-X-negative patients had significantly better prognoses than the other patients (MST, 373 days; p<0.0001).

significantly associated with survival (p=0.022), and PS was weakly associated with survival (p=0.056). The relationships of other clinicopathological markers, including age, sex, presence of primary sites, and metastatic site, with survival were negligible.

Multivariate analysis of survival. Within the limits of the small patient population, the effects of clinicopathological and biological variables, including PS, histological type, age, metastatic site, Bak expression, and Bcl-X expression, were examined by multivariate analysis using the Cox proportional hazards model. Results showed that Bak, Bcl-X, and histological type were independent prognostic factors for survival (p=0.0008, 0.0081, and 0.0082, respectively) (Table VI).

Table V. Clinicopathological markers and survival.

	No. of patients	MST (days)	p-value
Age			
≥56	24	234	0.33
<56	14	289	
PS			
0	22	308	0.056
1, 2	16	156	
Histological type			
Intestinal	8	427	0.022
Diffuse	30	234	
Presence of primary site			
Yes	23	289	0.55
No	15	256	
Metastatic liver site			
Yes	9	192	0.21
No	29	298	
Peritoneum			
Yes	18	256	0.47
No	20	244	

MST, Median survival time.

Table VI. Multivariate analysis of clinicopathological and biological markers.

Variable	Category	RR	95% CI	p-value
Bak	- vs. +	0.19	(0.074, 0.507)	0.0008
Bcl-X	- vs. +	0.22	(0.070, 0.673)	0.0081
Histology	Intestinal vs. diffuse	0.2	(0.063, 0.663)	0.0082
Age	<56 vs. ≥56	0.54	(0.218, 1.334)	0.1817
Liver meta	- vs. +	0.53	(0.187, 1.500)	0.2312
PS	0 vs. 1-2	0.71	(0.070, 0.673)	0.46

RR, Relative risk; CI, Confidence interval.

Discussion

Two primary mechanisms for cell injury by 5FU have been reported: i) inhibition of TS and ii) incorporation into RNA. Bolus infusions of 5FU result predominantly in the disturbance of RNA function, whereas protracted infusions of 5FU are DNA-directed via TS inhibition (26-28). Sequential MTX/

5FU has been considered to inhibit *de novo* purine synthesis, causing an increase in the intracellular pool of phosphoribosylpyrophosphate, increased formation of fluorouridine triphosphate (FUTP), and increased incorporation of FUTP into RNA (26). Molecular factors TS, TP, and E2F are considered to be associated with tumor sensitivity to 5FU through TS inhibition. The present study showed no significant relationship between TS, TP, or E2F status and the clinical outcomes, response and survival. These results are compatible with a sequential MTX/5FU anti-tumor mechanism that is independent of TS.

DPD is the rate-limiting enzyme for 5FU catabolism (29). Because inactivation of 5FU by DPD appeared to be a mechanism underlying clinical resistance to 5FU, strenuous efforts have been made to design inhibitors of DPD. Although recent studies have demonstrated that the expression of DPD is inversely correlated with patient response to protracted infusion of 5FU (10), the present study indicates that the expression of DPD is not significantly correlated with response or survival.

Gastric and colorectal tumors display reduced Bak protein levels compared with normal mucosa (30,31). Furthermore, mutations in the bak gene have been identified in human gastrointestinal cancers (32), suggesting that perturbation of Bak-mediated apoptosis may contribute to the pathogenesis of these tumors.

We found that Bak expression is associated with poor prognoses. Our results seem contrary to the conclusions reached in *in vitro* studies which demonstrated that the overexpression of Bak induces sensitivity to 5FU (14). Bairey *et al* (33) reported that the expression of the pro-apoptotic protein, Bax, was strongly associated with short survival times in patients with diffuse large B-cell lymphomas. This is similar to our finding. There may be an explanation for these discrepancies. Because the anti-Bak antibody does not distinguish mutated Bak protein, it is possible that the overexpressed Bak contained a mutation abrogating its ability to induce cell death.

Because the relative ratios of anti- and pro-apoptotic Bcl-2 family proteins dictate the ultimate sensitivity or resistance of cells to anti-cancer drugs, we investigated the relationships between the coupled expression of these Bcl-2 family proteins and clinical outcome. In the present study, Bak-negative Bcl-X-negative patients had significantly better prognoses than other patients. Within the limits of the small patient population, multivariate analysis showed that Bak and Bcl-X, as well as histological type, were independent variables predicting for survival.

It has recently been demonstrated that Bcl-2 and Bcl-X_L not only inhibit apoptosis, but also inhibit entry into the cell cycle (34-40). In tumor models, high Bcl-2 expression is correlated with anti-apoptosis and a low proliferative rate (41). Therefore, the cell-cycle delay functions of Bcl-2 and Bcl-X_L may also play a role in tumorigenesis. Chattopadhyay *et al* (42) reported that the presence of Bad/Bcl-X_L heterodimers, rather than the absence of Bcl-X_L or Bad, allowed the G_0/G_1 checkpoint to be overcome. Another theory for the paradoxical association of higher levels of Bak with poor outcomes is that high levels of Bak are associated with high levels of Bak/Bcl-X_L heterodimers, which lead to the bypassing of G_0/G_1 arrest without causing significant apoptosis.

Recent studies indicate that Bcl-2 and Bcl-X_L regulate apoptosis by different mechanisms. Simonian *et al* (43) reported that Bcl-X_L may either replace or potentiate the anti-apoptotic effects of Bcl-2. Although the tumors of only four patients were Bcl-2-positive, all Bcl-2-positive tumors were Bcl-X-negative, suggesting that in gastric cancer, Bcl-2 may replace rather than potentiate the effects of Bcl-X_L.

In summary, our findings demonstrate that the expression of Bak protein might be a useful predictive marker for chemoresponse and survival in patients with advanced gastric cancer treated with sequential MTX/5FU. Furthermore, both Bcl-X expression and the coupled expression of Bak and Bcl-X, as well as histological type, might be useful predictive markers for survival. On the other hand, there was no relationship in the present study between clinical outcome and the predictive markers reported in previous studies of regimens involving the protracted infusion of 5FU, including TS, TP, DPD, and E2F-1. This suggests that these markers might not correlate with chemosensitivity to the regimen of bolus infusions of 5FU. However, the number of patients investigated here is too small to draw definite statistical conclusions. Future confirmation is necessary, with prospective analysis of a larger cohort of uniform patients.

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PHARMACOGENETICS AND GENOMICS

UGT1A1 haplotypes associated with reduced glucuronidation and increased serum bilirubin in irinotecan-administered Japanese patients with cancer

Purpose: A comprehensive haplotype analysis of *UGT1A1* in the Japanese population was conducted, and the effects of these haplotypes were investigated with respect to *UGT1A1*-related phenotypic parameters in patients with cancer who received irinotecan.

Methods: The *UGT1A1* gene, including the enhancer, the promoter, and all 5 exons and their flanking regions, was sequenced from 195 Japanese subjects. The gene was divided into 2 blocks, and the haplotypes of each block were assigned. The association of these haplotypes with area under the concentration-time curve (AUC) ratios (7-ethyl-10-hydroxycamptothecin glucuronide [SN-38G]/7-ethyl-10-hydroxycamptothecin [SN-38]) and pretreatment levels of serum total bilirubin was investigated in 85 cancer patients who received irinotecan.

Results: Four haplotype groups (**1*, **60*, **28*, and **6*) were assigned in block 1, and 2 haplotype groups (**1A* and **1B*) were in block 2. The majority of the **1B* haplotypes in block 2 were linked to either the **1* or the **60* haplotype but not to **28* in block 1. Highly significant associations were obtained between the **28* haplotypes and both a reduced AUC ratio ($P = .0014$, Jonckheere-Terpstra [JT] test) and an increased total bilirubin level ($P = .0007$, JT test). Increased total bilirubin levels in the **60* ($P = .0048$, JT test) and **1B* groups ($P = .0224$, JT test) were also observed. The reduction in the AUC ratio by the **6* group was

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moderate ($P = .0372$, JT test) but was remarkable in combination with **60* (**6/*60*) or **28* (**6/*28*) as compared with the **1* group (**1/*1*) ($P = .049$ and $P = .0071$, respectively; nonparametric Dunnett test). *Conclusion:* This study identified several *UGT1A1* haplotypes significantly associated with the reduced AUC ratio (**28* and **6*) and with the increased total bilirubin level (**28*, **60*, and **1B*) and suggested that the novel haplotype **1B* might be functionally important. These findings will be useful for further pharmacogenetic studies on adverse reactions to irinotecan. (Clin Pharmacol Ther 2004;75:501-15.)

Irinotecan, a camptothecin derivative, is a prodrug with strong antitumor activity. The active metabolite SN-38 (7-ethyl-10-hydroxycamptothecin), a topoisomerase I inhibitor,[1,2] is formed by hydrolysis of the parent compound with carboxylesterases.[3] SN-38 is subsequently conjugated by uridine diphosphate–glucuronosyltransferases (UGTs) to form an inactive metabolite, SN-38 glucuronide (SN-38G), in the liver,[4] which is excreted into bile and urine (Fig 1). The principal dose-limiting toxicities of irinotecan therapy are severe diarrhea and leukopenia.[5] Because the biliary SN-38G excreted into the small intestine could be cleaved by bacterial glucuronidases in the colon, the regenerated SN-38 is assumed to be one of the mechanisms of late-onset diarrhea.[6]

Pharmacokinetic studies of irinotecan have shown that there are large variations between individuals in the area under the concentration-time curves (AUCs) of SN-38 and SN-38G.[7,8] Among the UGT isoforms, UGT1A1 is thought to contribute predominantly to SN-38G formation,[4,9] and the wide interindividual variability in SN-38G formation in hepatic tissues was shown to correlate with a *UGT1A1* genetic factor.[4,10] Therefore *UGT1A1* polymorphisms seem to be one of the most important factors for irinotecan efficacy and toxicity.

The most extensively studied polymorphism seen was a variation of the TATA box [$A(TA)_6TAA > A(TA)_{5/7/8}TAA$], which is associated with enhanced [$(TA)_5$] or reduced [$(TA)_{7/8}$] *UGT1A1* transcription. The pathogenesis of Gilbert syndrome is associated with the variant $(TA)_7$ (**28*),[11,12] and reduced glucuronidation of SN-38 and bilirubin in hepatic tissues from the **28* patients has also been shown.[13] A substitution of T with G at nucleotide −3279 (the adenine of the translational start codon in GenBank Accession No. AF297093.1 is numbered +1) in the phenobarbital-responsive enhancer module of *UGT1A1* (**60*) was shown to reduce its transcriptional activity and to be associated with an increase in plasma bilirubin levels in the Japanese population.[14] However, this nucleotide change was associated with only a marginal reduction in SN-38G formation in hepatic samples from white subjects.[15] Reduced SN-38 glucuronidation in vitro was also shown by the exonic nonsynonymous single nucleotide polymorphisms (SNPs) 211G>A (G71R, **6*), 686C>A (P229Q, **27*), and 1456T>G (Y486D, **7*).[16,17] Several clinical studies have shown an association of **28* with reduced SN-38G formation[18] and with severe adverse reactions.[19]

Recent pharmacogenomic studies have suggested an advantage to the use of haplotypes, linked combinations of SNPs rather than individual SNPs, to investigate the associations between genotypes and phenotypes.[20] Innocenti et al[15] conducted haplotyping of the *UGT1A1* enhancer/promoter region on hepatic samples from white and black subjects, and this study showed a significant reduction in the SN-38 glucuronidation activity in the **28* variants. We have previously determined the allelic frequencies of the common marker SNPs, namely, *UGT1A1*6*, **7*, **27*, **28*, **60*, and **62* alleles, using 48 samples from Japanese individuals.[21] However, no haplotype analysis has been done on the entire *UGT1A1* gene, including SNPs located in the exons, such as 211G>A (G71R, **6*), which is more common in Asian populations. In this study with 195 Japanese subjects, we sequenced the *UGT1A1* gene, including the enhancer/promoter regions, all exons and their flanking regions, and assigned haplotypes using the detected SNPs. Next the association of these haplotypes with *UGT1A1*-related phenotypes, AUC ratios (SN-38G to SN-38), and pretreatment levels of serum total bilirubin was investigated in 85 patients with cancer who received irinotecan.

METHODS

Materials. SN-38 and SN-38G were kindly supplied by Yakult Honsha Co Ltd (Tokyo, Japan).

Patients. The 195 Japanese subjects in this study consisted of 88 patients with various cancers who were administered irinotecan and 107 patients with cardiovascular disease who were administered β-blockers (the 48 patients described in the previous report[21] were included). The sample size of 195 subjects was estimated to be sufficient to detect SNPs with rare frequencies (>0.01) and to determine allelic frequencies of SNPs accurately with confidence intervals of less than ±5%. Deoxyribonucleic acid was extracted from blood

nificance levels of .25 and .05, respectively, by use of JMP version 5.0 software (SAS Institute, Inc).

On the basis of the results obtained in this study, we confirmed the adequacy of the sample size with power higher than 80% as explained later. We assumed that the 50% decrease in the mean AUC ratio was a minimum detectable difference that might be responsible for a reduction in neutrophil count.[18] We also assumed that the difference between the upper limit of the normal value for total bilirubin level (1.0 mg/dL) and the mean value for total bilirubin level in a control group (0.52 mg/dL in block 1 or 0.53 mg/dL in block 2) was a minimum detectable difference. Then we estimated the required total number of subjects necessary for detecting the specified minimum detectable differences at $\alpha = .05$ and $\beta = .20$ by use of JMP version 5.0 software (SAS Institute, Inc). We obtained the required total numbers of 20 and 18 for AUC ratios in blocks 1 and 2, respectively, and the numbers of 15 and 9 for total bilirubin levels in blocks 1 and 2, respectively. Regarding the total number required, this study (85 and 58 subjects in blocks 1 and 2, respectively) meets the estimated total sample size.

RESULTS

UGT1A1 polymorphisms detected in a Japanese population. The *UGT1A1* enhancer region, promoter region, and all exons and their flanking introns were sequenced for 195 Japanese subjects. All of the allele frequencies were in Hardy-Weinberg equilibrium ($P = .527$ or higher). A total of 25 polymorphisms were detected in this study. Among them, 10 SNPs were novel, including a nonsynonymous SNP (1598A>C [H533P] in exon 5) and 2 SNPs in the 3'-UTR (untranslated region) (1813C>T and 2021T>C). In addition, we detected the known SNPs causing reduced enzymatic activity, −3279T>G (**60*), −40_−39insTA (**28*), 211G>A (**6*, G71R), and 686C>A (**27*, P229Q) with frequencies of 0.262, 0.131, 0.151, and 0.005, respectively. These frequencies were similar to those previously reported in Asian populations.[14,26-28] A previously reported nonsynonymous SNP, 1091C>T (P364L),[28] was also found. Because each SNP frequency between the cancer and arrhythmic patients was similar, the combined data were used for the following LD and haplotype analyses.

LD analysis. Using the detected SNPs, we next performed LD analysis and obtained the pairwise values of ρ^2 and χ^2. As the data for χ^2 and ρ^2 were almost equivalent, the data for ρ^2 are depicted in Fig 2. A strong LD was seen among −3279T>G (**60*), −3156G>A, −364C>T, and −40_−39insTA (**28*). Another close linkage in the enhancer/promoter regions was found among −3177C>G, −3175A>G, and −64G>C. A close association among 1813C>T (211 bases downstream of the stop codon), 1941C>G (339 bases from the stop codon), and 2042C>G (440 bases from the stop codon) was also prominent, and they showed a perfect linkage. IVS2+15T>C was also weakly associated with the 3 SNPs. Thus the *UGT1A1* gene can be divided into at least 2 blocks, each having closely associated SNP groups. Considering that exons 2 to 5 are common among *UGT1A* families and that exons 1 and 2 are fairly distant from each other, we divided the gene into the following 2 blocks: block 1, corresponding to the enhancer and promoter regions and exon 1, and block 2, corresponding to exons 2 to 5.

Haplotype analysis. First, haplotyping was done within each block (in-block haplotyping). In block 1 the haplotypes **1b*, **6a*, **60a*, **60b*, and **28b* were unambiguously assigned. We separately estimated the

Table II. Demographic profiles of patients with cancer receiving irinotecan therapy

	No.
Age (y)	
62 (56-69)*	85
Sex	
Male	65
Female	20
Performance status	
0	29
1	55
2	1
Tumor type	
Small cell lung	34
Non-small cell lung	12
Colon	19
Stomach	16
Others	4
Previous treatment	
1. None	27
2. Other chemotherapy	12
3. Surgery	9
4. Radiotherapy	1
5. 2 and 3	26
6. 2 and 4	8
7. 2, 3, and 4	2
Combination therapy and dose of irinotecan (mg/m^2)*	
Irinotecan alone, 140 (100-150)	24
With platinum antineoplastics, 60 (60)	43
With 5-fluorouracil, 100 (82-100)	7
With mitomycin C, 150 (150)	9
With amrubicin, 60 (60)	2

*The values are expressed as the median with the interquartile range in parentheses.

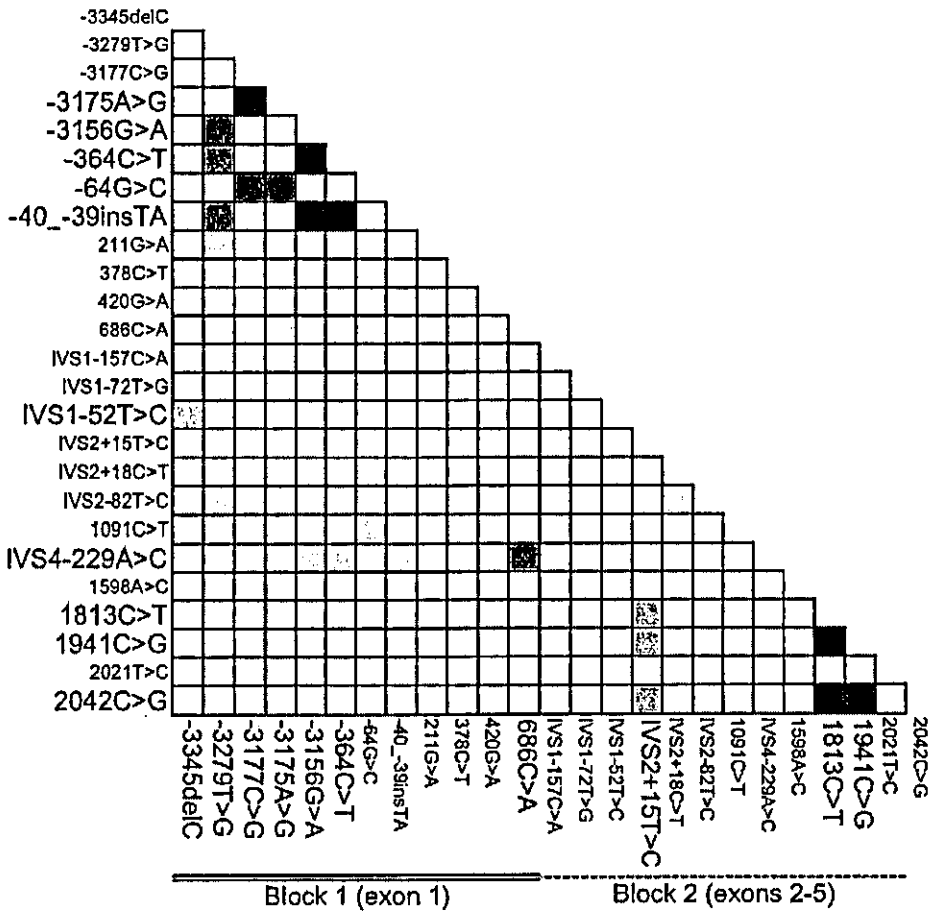

Fig 2. Linkage disequilibrium (LD) analysis for *UGT1A1* single nucleotide polymorphisms (SNPs). Pairwise LD as ρ^2 (from 0 to 1) is expressed as a 10-graded blue color. The denser color represents a higher linkage.

diplotype configuration (a combination of haplotypes) for each subject by using LDSUPPORT software. On the basis of the known key SNPs, the haplotypes inferred in block 1 were classified into 4 groups (**1*, **6*, **28*, and **60*). The additionally assigned haplotypes were 2 **6* subtypes (**6b* and **6c*), 2 **28* subtypes (**28c* and **28d*), and 1 **60* subtype (**60c*) (Fig 3). We classified the haplotypes having both **28* and **60* into the **28* group (**28b* and **28c* in Fig 3). The **28c* haplotype was inferred to have −3279T>G (**60*), −40_−39insTA (**28*), and 686C>A (**27*) on the same chromosome. The most frequent haplotype was **1a* (wild type, frequency = 0.564), followed by **6a* (0.146) and **28b* (0.121). It must be noted that 96% of the subjects with **28* also had **60* (−3279T>G), namely, in **28b* and **28c*.

In block 2 the haplotypes **1b* to **1i* and **364L* (the haplotype bearing P364L was tentatively named **364L*) were unambiguously identified. The additionally assigned haplotypes by the program were 3 **1* subtypes (**1j*, **1k*, and **1L*) and **533P* (H533P-bearing haplotype) (Fig 4). The most frequent haplotype was **1a* (frequency = 0.810), followed by **1b* (0.079) and **1c* (0.036). As shown in Fig 2, 1813C>T, 1941C>G, and 2042C>G (consisting of **1b*) showed a strong linkage. As described later, the **1* subtypes having the 3 SNPs were named **1B* as a group, and the others were named **1A*.

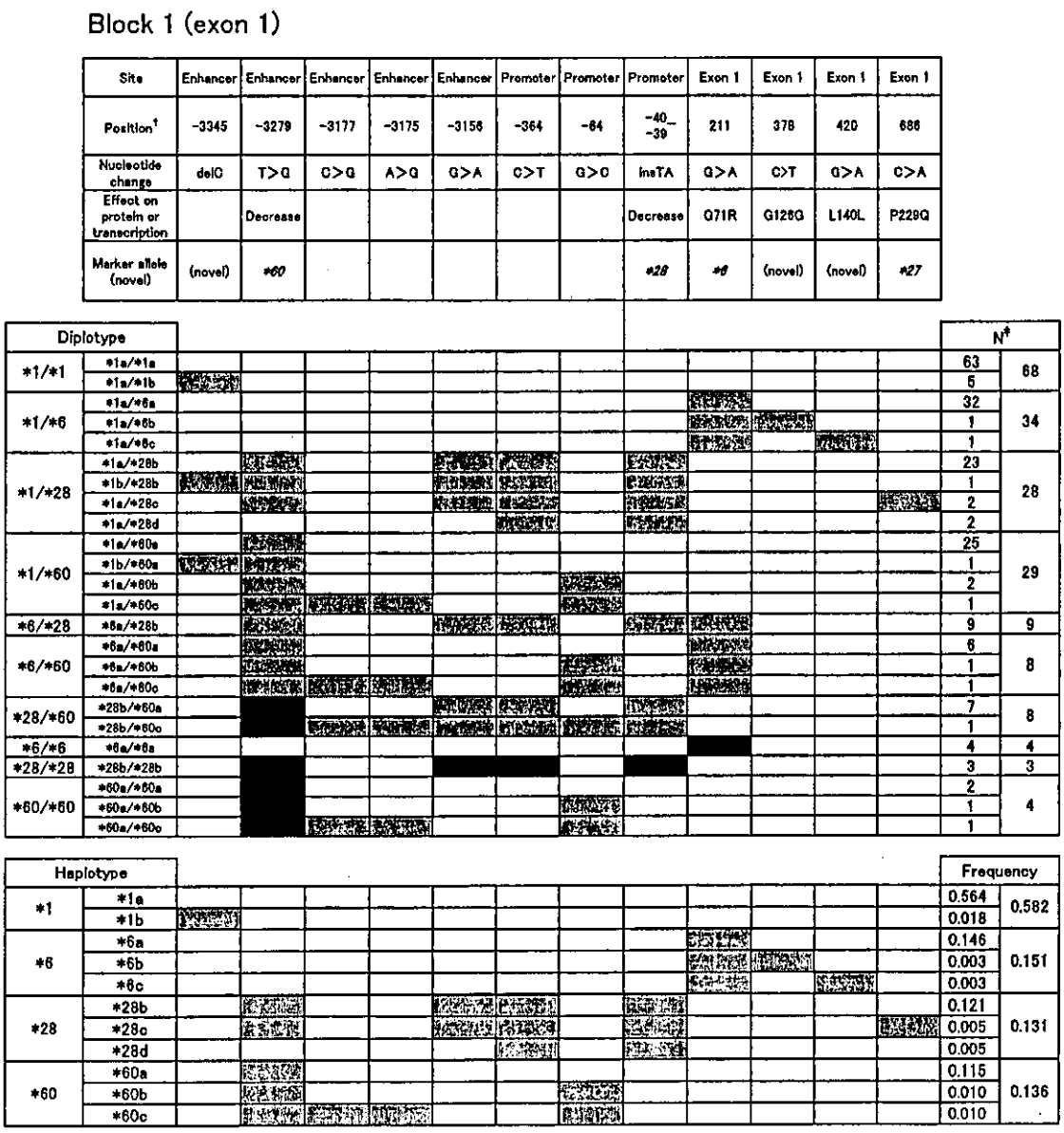

Fig 3. Diplotypes and haplotypes in block 1 (the enhancer/promoter regions and exon 1) of *UGT1A1* for 195 Japanese subjects. The haplotype groups are named according to previous reports, and individual haplotypes are described with small alphabetic letters. *Dagger*, Position in complementary deoxyribonucleic acid (cDNA) or from adenine of the translational initiation codon ATG; *double dagger*, number of subjects.

In addition, the combination of haplotypes was determined with both blocks 1 and 2 covered on the same chromosome (whole-gene haplotyping) (data not shown). Interestingly, **1B* in block 2 existed only on the chromosome having either **1a* or **60a* (or probably **6a*) in block 1 but not **28*. The haplotype combinations (block 1–block 2) were limited to **1a-*1b(*1c)* and **60a-*1b(*1j)* (and rarely **6a-*1c*). For 10 subjects with heterozygous block 1 **60a* and block 2 **1b* (or **533P*) and 5 subjects with heterozygous block 1

Block 2 (exons 2–5)

Site	Intron 1	Intron 1	Intron 1	Intron 2	Intron 2	Intron 2	Exon 4	Intron 4	Exon 5	3'-UTR	3'-UTR	3'-UTR	3'-UTR
Position†	IVS1 −157	IVS1 −72	IVS1 −52	IVS2 +15	IVS2 +18	IVS2 −82	1091	IVS4 −229	1588	1813 (211)	1941 (339)	2021 (419)	2042 (440)
Nucleotide change	C>A	T>G	T>C	T>C	C>T	T>C	C>T	A>C	A>C	C>T	C>G	T>C	C>G
Effect on protein							P364L		H533P				
Marker allele (novel)		(novel)	(novel)		(novel)		*364L*	(novel)	*533P* (novel)	(novel)		(novel)	

Diplotype		N‡	
*1A/*1A	*1a/*1a	126	149
	*1a/*1d	11	
	*1a/*1e	3	
	*1a/*1f	3	
	*1a/*1g	1	
	*1a/*1h	1	
	*1a/*1i	1	
	*1a/*1L	1	
	*1d/*1e	1	
	*1a/*364L	1	
*1A/*1B	*1a/*1b	27	44
	*1a/*1c	12	
	*1a/*1j	1	
	*1a/*1k	1	
	*1c/*1e	1	
	*1b/*1d	1	
	*1a/*533P	1	
*1B/*1B	*1b/*1b	1	2
	*1b/*1c	1	

Haplotype		Frequency	
*1A	*1a	0.810	0.877
	*1d	0.033	
	*1e	0.013	
	*1f	0.008	
	*1g	0.003	
	*1h	0.003	
	*1i	0.003	
	*1L	0.003	
	*364L	0.003	
*1B	*1b	0.079	0.123
	*1c	0.036	
	*1j	0.003	
	*1k	0.003	
	*533P	0.003	

Fig 4. Diplotypes and haplotypes in block 2 (exons 2 to 5) of *UGT1A1* for 195 Japanese subjects. The individual haplotypes were divided into either **1A* or **1B*. *Dagger*, Position in cDNA or from the nearest exon (with the *number in parentheses* indicating the position from the termination codon); *double dagger*, number of subjects.

**6a* and block 2 **1c*, it could not be determined whether both haplotypes were or were not on the same chromosome. However, most of the block 1 **6* haplotypes were linked to block 2 **1a* (data not shown).

UGT1A1 genotype–dependent SN-38 glucuronidation and serum bilirubin levels in irinotecan-administered patients. Next, we investigated the relationship between *UGT1A1* genotypes and AUC ratios (SN-38G/SN-38) or pretreatment levels of serum total bilirubin in cancer patients who were administered irinotecan. The median values of the AUC ratios and the total bilirubin levels in irinotecan-administered patients were 4.35 (interquartile range, 3.16-6.30) and 0.6 mg/dL (interquartile range, 0.5-0.8 mg/dL), respectively. We first assessed a possible influence of patient background factors listed in Table II and irinotecan