metastasis did not influence the 5-year DFS. Shiromizu and coworkers [14] reported that patients with pulmonary metastasis alone without pelvic lymph node metastasis showed a good prognosis. However, treatment for metastatic pulmonary foci does not consist of surgery alone, and their subjects included patients with metastatic foci in organs other than the lungs. These factors may have contributed to the difference in results.

Our results showed that DFI did not significantly influence 5-year DFS. Barter and associates [5] reported that the interval between cancer diagnosis and onset of lung metastasis was not prognostic in cervical cancer. Seki and colleagues [16] reported that there were no significant differences between DFIs. However, Anderson and coworkers [10] reported that comparing DFI and survival, there were trends toward increased survival with greater DFI in patients with uterine cancer. Fuller and associates [19] reported that a prolonged time to initial recurrence (latent period) greater than 36 months was associated with improved survival and that there was a 60% survival among patients with latent periods of 60 months or more. Takita and coworkers [20] reported that for many malignancies, the interval between the initial diagnosis and the onset of lung metastasis is prognostic in surgically treated patients. However, that series involved not only cervical cancer but also other sites.

On univariate and multivariate analyses in our series, patients with one or two metastatic pulmonary foci showed a higher 5-year DFS than patients with three or four metastatic pulmonary foci. With respect to overall 5-year survival, Seki and colleagues [16] reported that there were no significant differences in the survival curves between the solitary and the multiple metastasis group.

There are no previous studies that have evaluated surgical therapy only in patients with stage Ib or II cervical cancer in whom pulmonary metastasis was detected after the disease-free period after initial treatment and resection was performed, as determined in this study. The results of this study may provide important information for future surgical therapy for pulmonary metastasis from cervical cancer.

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Secondary cytoreductive surgery for recurrent epithelial ovarian carcinoma: proposal for patients selection

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The value of secondary cytoreductive surgery (SCS) for recurrent ovarian cancer is still controversial. The aim of this study was to clarify candidates for SCS. Between January 1987 and September 2000, we performed SCS in 44 patients with recurrent ovarian cancer, according to our selection criteria, disease-free interval (DFI) >6 months, performance status <3, no apparent multiple diseases, age <75years and no progressive disease during preoperative chemotherapy, if undertaken. The variables were investigated by univariate and multivariate analyses. Of 44 patients, 26 (59.1%) achieved complete removal of all visible tumours at SCS. Secondary cytoreductive surgery outcome, complete or incomplete resection, was significantly related to overall survival ($P=0.0019$). As for variables determined before SCS, DFI >12 months, no liver metastasis, solitary tumour and tumour size <6 cm were independently associated with favourable overall survival after recurrence in the multivariate analysis. Patients with three or all four variables ($n=31$) had significantly better survival compared with the other patients ($n=13$) (47 *vs* 20 months in median survival, $P<0.0001$). In these patients, fairly good median survival (40 months) was obtained even in patients with incomplete resection. Secondary cytoreductive surgery had a large impact on survival of patients with recurrent ovarian cancer when they had three or all of the above-mentioned four factors at recurrence. These patients should be considered as ideal candidates for SCS.
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Since Griffiths (Griffiths, 1975) first demonstrated the inverse relationship between residual tumour size after primary debulking and survival of ovarian cancer patients in 1975, many investigators have reproduced and confirmed this observation (Hacker *et al*, 1983; Vogl *et al*, 1983; Delgado *et al*, 1984; Conte *et al*, 1985; Louie *et al*, 1986; Neijt *et al*, 1987; Hainsworth *et al*, 1988; Sutton *et al*, 1989). Thus, the value of debulking of large tumour masses in the primary surgery of ovarian cancer has been generally accepted, and primary cytoreductive surgery followed by chemotherapy is considered to be a standard treatment procedure for patients with advanced ovarian cancer.

The cytoreduction contributes to removal of the tumour burden and relief of symptoms caused by tumours or massive ascites. In addition, the cytoreduction has another important effect on the sensitivity to postsurgical chemotherapy. By removing bulky tumours, the decreased growth fractions should increase (Norton and Simon, 1977) and poorly perfused anoxic cells should decrease. By reducing the number of cancer cells, the chance for cancer cells to undergo spontaneous mutations resulting in drug resistance should decrease (Goldie and Coldman, 1979). All these effects are believed to enhance the sensitivity to chemotherapy.

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Theoretically, the favourable effects of cytoreduction may also be expected in patients with recurrent ovarian cancer. Recently, several investigators have reported the significant value of secondary cytoreductive surgery (SCS) in a subset of patients with recurrent ovarian cancer (Jänicke *et al*, 1992; Eisenkop *et al*, 1995, 2000; Vaccarello *et al*, 1995; Cormio *et al*, 1999; Zang *et al*, 2000, 2004; Munkarah *et al*, 2001; Scarabelli *et al*, 2001; Tay *et al*, 2002). The value of complete resection at the time of SCS for highly selected patients is in consensus in these recent reports. They reported a considerable number of factors related to good prognosis including longer disease-free interval (DFI), smaller size of residual tumour at primary cytoreductive surgery, good response to first-line chemotherapy, younger age at recurrence and smaller size of maximum tumour at recurrence. However, there is limited information regarding the ideal candidates for SCS. Although only preoperative or intraoperative variables before starting SCS should be analysed for selection of the candidate, these variables have been analysed together with SCS outcome in most previous studies. In addition, the follow-up periods of living patients were rather short (the median or average follow-up periods were between 1 and 4 years) (Jänicke *et al*, 1992; Vaccarello *et al*, 1995; Cormio *et al*, 1999; Zang *et al*, 2000, 2004; Munkarah *et al*, 2001; Scarabelli *et al*, 2001) in most of the previous reports.

Since 1987, we have performed SCS according to our criteria of patient selection in 44 out of 70 ovarian cancer patients who had recurrence after DFI. In the present study, the median follow-up

period of living patients is 60 months after the initiation of treatment, SCS or chemotherapy before SCS, for recurrence. Using univariate and multivariate analyses of variables before starting SCS, we planned to clarify the ideal candidates for SCS among patients with recurrent ovarian cancer.

PATIENTS AND METHODS

Patient selection

Between January 1984 and December 1999, we treated 236 patients with stage I to IV epithelial ovarian cancer at the Department of Obstetrics and Gynecology, University of Tokyo Hospital. Our standard surgical procedures for ovarian cancer consist of total abdominal hysterectomy, bilateral salpingo-oophorectomy, infracolic or total omentectomy, and in advanced cases, debulking of tumour masses with maximum efforts. Patients with no or small intraperitoneal residual tumours (less than 2 cm in diameter) also underwent systematic retroperitoneal lymphadenectomy. The extent of retroperitoneal lymphadenectomy is pelvic lymph nodes only (1984–1986) or both pelvic and aortic lymph nodes (1987–1999). All but stage Ia patients underwent at least six cycles of cisplatin-based chemotherapies following surgery as described previously (Onda *et al*, 1998). Of the 236 patients, 204 (86%) achieved complete clinical remission after primary treatment.

By September 2000, 70 of the 204 (34%) patients had recurrence and, from January 1987 to September 2000, 44 of the 70 (63%) patients underwent SCS prior to or following chemotherapy. Administration of chemotherapy before SCS was decided based on various clinical factors including short DFI (DFI <12 months) and poor performance status (PS 3) defined by ECOG (Eastern Cooperative Oncology Group). Our selection criteria for SCS were as follows: (1) DFI >6 months, (2) age at recurrence <75 years, (3) PS 0–2 just before the surgery, (4) absence of apparent extensive intraperitoneal dissemination or multiple distant metastases and (5) no progressive disease during presurgical chemotherapy, if undertaken. There were three exceptions to the above-mentioned criteria for SCS. One patient with DFI <6 months (5 months) underwent SCS, because the recurrent site was expected to be limited to a solitary aortic lymph node by CT. The other two patients had PS 3 at surgery. One patient with three metastatic brain tumours underwent emergent brain surgery followed by γ-knife radiosurgery to one residual tumour (Kawana *et al*, 1997), and one patient underwent ileocaecal resection because of acute bowel obstruction. Before the treatment, informed consent was obtained from all of the patients.

Chemotherapy

Of 44 patients, 21 (47.7%) received chemotherapy before SCS and all of 44 patients were treated with chemotherapy after SCS. In all, one to eight (median: 2) cycles of presurgical chemotherapy were performed in eight of 13 (61.5%) patients with DFI <12 months and 13 of 31 (41.9%) patients with DFI >12 months. In total, 44 patients received two to nine (median: 4) cycles of postsurgical chemotherapy.

In all, two to four cycles of presurgical chemotherapy were generally administered until beneficial response (partial or minor response) was observed. In two patients, second-line chemotherapy showed no beneficial response, and SCS was performed after successful third-line chemotherapy (seven and eight cycles in total). One patient received only a cycle of presurgical chemotherapy, because SCS could not be scheduled immediately after diagnosis of recurrence.

The number of postsurgical chemotherapy given was determined by SCS outcome and response to chemotherapy, evaluated by CT scan and serum level of CA125. Generally, three to four cycles of chemotherapy were planned for patients with no residual tumour and five to six cycles of chemotherapy were planned for patients with any residual disease. In principle, we gave at least two cycles of chemotherapy after the serum level of CA125 was normalised. Thus, three patients were treated with more than six cycles of chemotherapy after SCS. On the contrary, chemotherapy was discontinued before accomplishment of the planned cycles in five patients because rapid disease progression or severe adverse effects were observed during the planned cycles.

In presurgical and postsurgical chemotherapies, a platinum-based combination, CAP, EP or TJ, was used. The CAP regimen consisted of 600 mg m^{-2} of cyclophosphamide, 30 mg m^{-2} of doxorubicin and 50–75 mg m^{-2} of cisplatin. The EP regimen consisted of 80 mg m^{-2} of etoposide during days 1–5 and 75 mg m^{-2} of cisplatin. Paclitaxel was introduced in Japan in 1998 and, thereafter, a TJ regimen consisting of paclitaxel (175 mg m^{-2} over 3-h infusion) and AUC 5 of carboplatin was used as second-line chemotherapy.

Statistical methods

Survival was measured from the day of starting treatment for recurrence, that is, the day of starting presurgical chemotherapy or the day of performing SCS. The survival curves were determined by the Kaplan–Meier product limit method (Kaplan and Meier, 1958). Factors influencing survival were analysed using the log-rank test (univariate) and Cox's proportional-hazards regression analysis (multivariate). These analyses were performed using a JMP program (SAS Institute Inc., USA). Contingency table analysis was performed using the χ^2 test or χ^2 test for trend.

RESULTS

Patient characteristics

The number of patients was three in stage I, two in stage II, 36 in stage III and three in stage IV according to the International Federation of Gynecology and Obstetrics (FIGO). Histology was serous type in 35, clear-cell type in three, endometrioid type in three, transitional cell type in two and mixed epithelial type in one. Median DFI was 18.5 months with a range of 5–58 months: one patient (2.3%) had 5 months, 12 (27.3%) had 6–12 months and 31 (70.5%) had >12 months. Median age at recurrence was 52 years with a range of 37–74 years. Median follow-up period of patients, excluding those who died, was 60 months with a range of 17–199 months from the initiation of treatment for recurrence.

Surgery

Our attempt to perform SCS resulted in exploratory laparotomy in four patients (9.1%) due to the presence of unexpected extensive peritoneal tumours. Various debulking surgeries classified into four categories such as (1) gastrointestinal resection, (2) resection of other organs, (3) lymph node dissection and (4) other tumour debulking was performed with maximum efforts in the remaining 40 patients (90.9%). Among these patients, gastrointestinal resection (category 1) was required in 11 patients (25.0%), large bowel resection in nine patients (20.5%), small bowel resection in three patients (6.8%), partial gastrectomy in one patient and ileocaecal resection in one patient (2.3%), and one of the patients (2.3%) underwent sigmoid colostomy. Three patients had category 1 surgeries at two sites. Resection of other organs (category 2) was required in six patients (13.6%), splenectomy in three patients (6.8%), distal pancreatectomy in two patients (4.5%), partial liver resection in one patient, hysterectomy in one patient and brain tumour resection in one patient (2.3%). Two patients had category 2 surgeries at two sites. Regional or distant lymph node dissection (category 3) was performed in 12 patients (27.3%). Five patients

(11.4%) underwent systematic aortic lymphadenectomy and one (2.3%) underwent both systematic pelvic and aortic lymphadenectomies. Selective dissections of the following lymph nodes were performed in six patients: aortic nodes in one patient, pelvic nodes in one patient, axillary nodes in one patient, portal nodes in one patient, inguinal nodes in one patient and mesenteric nodes in one patient (2.3%). Other tumour debulking (category 4) including removal of tumours in the remnant omentum, the diaphragmatic muscles and vaginal stump, and tumours on the visceral or parietal peritoneum including the under surface of the diaphragm, was performed in 22 patients (50.0%); omentectomy in seven patients; partial full-thickness diaphragm resection in one patient; resection of tumours around the vaginal stump in four patients (9.1%); peritoneum resection of disseminated tumours on the under surface of the diaphragm; and other peritoneal surfaces in 16 patients (36.4%). Six patients were counted twice because they underwent two types of category 4 surgeries. In all, 10 patients underwent two or three out of the above four categories of debulking surgery. No patients died within a month following SCS.

Cytoreductive outcome and survival of patients

Among a total of 44 patients, complete resection of visible tumours was achieved in 26 patients (59.1%), largest residual tumours <1 cm in diameter were left in 11 patients (25.0%) and largest residual tumours $\geqslant 1$ cm in diameter were left in seven patients (15.9%). The median survival and 5-year survival of all patients who underwent cytoreductive surgery were 32 months and 33.2% (Figure 1), whereas the median survival and 5-year survival of 26 patients who had recurrence after complete remission achieved by primary treatment and did not undergo the surgery were 11 months and 3.9%. Figure 2 shows the survival of patients after the initiation of treatment for recurrence according to the outcome of SCS (SCS outcome). The median survival and 5-year survival after recurrence of the patients with largest residual tumours 0, <1 and $\geqslant 1$ cm were 52 months and 47.6%, 23 months and 18.2% and 20 months and 0%, respectively ($P=0.0007$, log rank). The overall survival of patients with no residual tumour was much better than that of patients with residual tumours (22 months in median survival and 12.0% in 5-year survival, figure not shown) with statistical significance ($P=0.0019$). There was no statistical difference in overall survival between patients with residual tumours <1 and $\geqslant 1$ cm ($P=0.1314$).

Factors influencing survival in univariate analyses

Factors influencing overall survival after recurrence were analysed using univariate analyses. Factors analysed and the results of univariate analyses are listed in Tables 1 and 2. As for prognostic factors determined during primary therapy, univariate analyses revealed that peritoneal tumour spread ($P=0.039$), FIGO stage ($P=0.045$) and aortic lymph node metastasis ($P=0.009$) were significantly associated with overall survival after recurrence. Regarding prognostic factors determined at recurrence, univariate analyses revealed that DFI ($P=0.002$), presence of liver metastasis ($P=0.005$), number of recurrent tumours ($P=0.007$), size of maximum tumour ($P<0.001$) and SCS outcome ($P=0.002$) had significant associations with overall survival after recurrence.

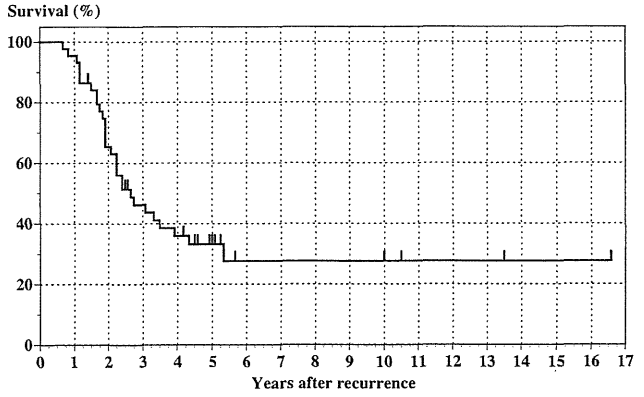

Figure 1 Survival of all 44 patients who underwent SCS.

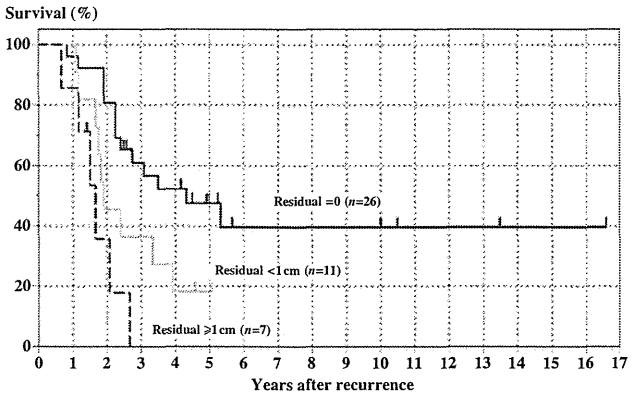

Figure 2 Outcome of SCS and survival. Survival of the patients with largest residual tumours 0, <1 and $\geqslant 1$ cm is shown in solid black, solid grey and dotted black line, respectively. The difference of survival is statistically significant ($P=0.0007$, log rank). There is no statistical difference in survival between patients with residual tumours <1 and $\geqslant 1$ cm ($P=0.1314$, log rank).

Table 1 Univariate analyses for variables during primary treatment

Variables	Number	Median survival (months)	*P*-value
Peritoneal tumour spread			
Localised to the pelvis	10	NA	
Extended beyond the pelvis	34	29	0.039
Stage			
I/II	5	NA	
III/IV	39	29	0.045
Aortic lymph node metastases			
Absent	25	64	
Present	14	27	
Not assessed	5	25	0.009
Pelvic lymph node metastases			
Absent	20	47	
Present	21	32	
Not assessed	3	25	0.126
Systematic lymphadenectomy			
Not performed	3	25	
Pelvic only	7	29	
Pelvic and aortic	34	33	0.296
Histology			
Serous	35	37	
Others	9	23	0.197
Residual tumour at PCS			
0	34	32	
Any	10	40	0.961

PCS = primary cytoreductive surgery; NA = not applicable.

Table 2 Univariate analyses for variables at recurrence

Variables	Number	Median survival (months)	P-value
Age at recurrence (years)			
<50	17	29	
⩾50	27	40	0.860
Disease-free interval (months)			
⩾12	31	47	
<12	13	23	0.002
Intraperitoneal tumour			
Absent	12	64	
Present	32	27	0.117
Pelvic or aortic lymph node metastases			
Absent	34	32	
Present	10	37	0.419
Distant metastasis			
Absent	38	32	
Present	6	40	0.496
Liver metastasis			
Absent	42	33	
Present	2	20	0.005
No. of recurrent tumours			
Solitary	16	64	
Multiple	28	27	0.007
Size of maximum tumour (cm)			
<6	38	40	
⩾6	6	14	<0.001
Massive ascites (>500 ml)			
Absent	41	33	
Present	3	32	0.318
PS			
0–2	42	29	
3	2	42	0.746
Presurgical chemotherapy			
Not done	23	33	
Done	21	29	0.677
Bowel resection			
Not done	33	33	
Done	11	27	0.650
Residual tumour at SCS			
0	26	52	
Any	18	22	0.002

PS = performance status; SCS = secondary cytoreductive surgery.

Factors influencing survival in multivariate analysis

To determine patient selection for the surgery, we performed multivariate analysis using statistically significant prognostic factors in univariate analyses. Out of eight significant factors, SCS outcome was omitted in the multivariate analysis because SCS outcome is not yet known on considering indications for the surgery, although SCS outcome had a statistically significant correlation with the number of recurrent tumours ($P<0.001$, χ^2 test). The multivariate analysis using the remaining seven factors revealed that four factors determined at recurrence, specifically DFI, presence of liver metastasis, number of recurrent tumour and size of maximum tumour, were independently and significantly associated with survival after recurrence (Table 3). Additionally, the multivariate analysis using only these four factors confirmed

Table 3 Multivariate analysis using the seven prognostic variables in the univariate analyses

	Multivariate analysis	
Variables	Risk ratio (95% CI)	P-value
Peritoneal tumour spread at PCS		
Localised to the pelvis	1.00	
Extended beyond the pelvis	0.80 (0.42–1.76)	0.540
Stage		
I/II	1.00	
III/IV	0.90 (0.22–5.60)	0.893
Aortic lymph node metastases at PCS		
Absent	1.00	
Present	1.23 (0.56–2.64)	
Not assessed	1.78 (0.61–5.33)	0.088
Disease-free interval (months)		
⩾12	1.00	
<12	2.45 (1.11–5.39)	0.027
Liver metastasis		
Absent	1.00	
Present	4.00 (1.40–10.03)	0.013
No. of recurrent tumours		
Solitary	1.00	
Multiple	3.73 (1.79–9.58)	<0.001
Size of maximum tumour (cm)		
<6	1.00	
⩾6	7.43 (3.12–18.92)	<0.001

PCS = primary cytoreductive surgery.

that all four factors were independently and significantly associated with survival after recurrence. The relative risk (95% confidence interval) was 0.37 (0.20–0.68) for DFI >12 months, 0.23 (0.10–0.65) for absence of liver metastasis, 0.26 (0.12–0.48) for a solitary tumour and 0.20 (0.09–0.42) for size of maximum tumour <6 cm.

Grouping of patients determined by the number of favourable prognostic factors

According to the number of favourable statuses among the above-mentioned four prognostic factors, that is, DFI >12 months, no liver metastasis, solitary tumour and tumour size <6 cm, patients were divided into four groups as follows: patients with all four favourable factors (Group 4, $n=10$), patients with three favourable factors (Group 3, $n=21$), patients with two favourable factors (Group 2, $n=11$) and patients with only one favourable factor (Group 1, $n=2$). There were no patients with zero favourable factors. Complete resection of visible tumours was achieved in 100% (10 of 10), 62% (13 of 21), 18% (two of 11) and 50% (one of two) of patients in Group 4, Group 3, Group 2 and Group 1, respectively. Apparently, a higher rate of complete surgical resection was achieved in patients with a larger number of favourable factors, and the distribution was statistically significant by contingency table analysis ($P<0.001$, χ^2 test for trend). The 5-year survival of Group 4 was 88.9% and median survival was not reached. The 5-year survivals and median survivals of Group 3, Group 2 and Group 1 were 26.0, 0 and 0%, and 37, 20 and 10 months, respectively (figure not shown). The differences of overall survival were also statistically significant among the four groups ($P<0.001$, log rank) and between them (e.g. $P<0.007$ in Group 1 *vs* Group 2, $P<0.001$ in Group 2 *vs* Group 3 and $P<0.001$ in Group

3 *vs* Group 4, log rank). Figure 3 shows the combined survival of Group 4 and Group 3 and that of Group 2 and Group 1. Patients with three or all four favourable factors (Group 3/4) ($n=31$) had significantly better survival compared with those with less than three favourable factors (Group 1/2) ($n=13$) (median and 5-year survival; 47 months and 45.9% *vs* 20 months and 0%, $P<0.001$).

Survival of patients determined by the number of favourable prognostic factors and SCS outcome

Patients with three or all four favourable prognostic factors (Group 3/4) had better survival when complete surgical resection was achieved at the time of SCS ($n=23$) (64 months in median survival, 53.8% in 5-year survival). However, even when SCS left residual tumours, survival of the Group 3/4 patients ($n=8$) was fairly good (40 months in median survival, 25% in 5-year survival). On the other hand, Group 1/2 patients had poorer survival both in completely resected cases ($n=3$) and in incompletely resected cases ($n=10$) (23 and 18 months in median survival, and 0 and 0% in 5-year survival) (Figure 4).

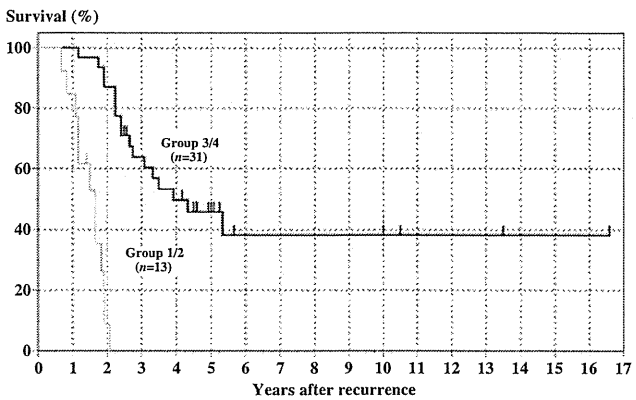

Figure 3 Comparison in survival between patients having one or two favourable prognostic factors (Group 1/2) and three or four favourable factors (Group 3/4). Survival of patients in Group 3/4 and Group 1/2 is shown as a solid black or solid grey line, respectively. Patients in Group 3/4 had significantly better survival compared with patients in Group 1/2 ($P<0.001$, log rank).

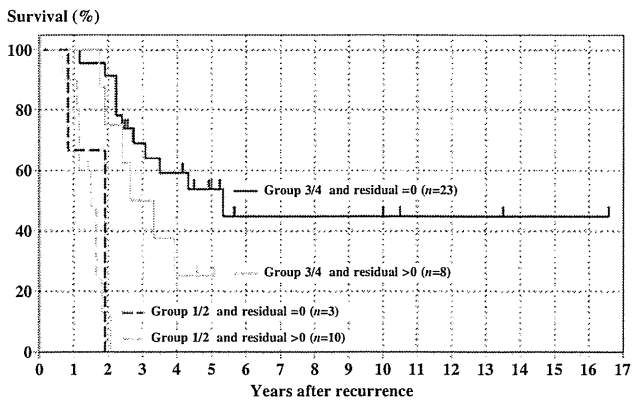

Figure 4 Survival in relation to SCS outcome and number of favourable prognostic factors. Survival of patients in Group 3/4 are shown as solid lines. Solid black line and solid grey line show the survival of patients with no residual tumour and residual tumour at SCS, respectively. Survival of patients in Group 1/2 are shown as dotted lines. Dotted black line and dotted grey line show the survival of patients with no residual tumour and any residual tumour at SCS, respectively.

DISCUSSION

We achieved surgical removal of all visible tumours in 59.1% of patients at the time of SCS. Residual tumours <1 or $\geqslant 1$ cm in diameter were present in 25.0 and 15.9%, respectively. In line with previous reports, removal of all visible tumours at SCS contributed to long-term survival (Figure 2). The rate of complete resection (59.1%) in our series was a little lower than the rates reported by Eisenkop *et al* (2000), Landoni *et al* (1998) and Cormio *et al* (1999). However, in Landoni's study, the subjects were restricted to those patients who were sensitive to first-line chemotherapy and chemotherapy before SCS. Cormio *et al* also restricted the subjects to patients with apparently isolated and resectable tumours and without ascites. Our criteria for patient selection were similar to those of Eisenkop *et al*, and their subjects were patients with DFI >6 months and without liver metastases. They achieved an 82% complete resection rate by using argon beam laser to remove disseminated cancer foci and reported 44 months in median survival and approximately 35% in 5-year survival in the completely resected cases. In our experience, median survival and 5-year survival in completely resected cases were 52 months and 47.6%, respectively, being much better than previous reports. Our rate of optimal cytoreduction, 84.1% (if defined as residual tumour <1 cm), was similar to the rate of complete resection in Eisenkop's report. In our series, optimally resected cases had 40 months in median survival and 38.6% in 5-year survival (figure not shown), in keeping with the survival of completely resected cases in Eisenkop's study. These findings suggest that the debulking efforts performed at SCS in our cases are comparable to those of previous reports.

Univariate analyses revealed that three factors during primary treatment (peritoneal spread, aortic lymph node metastasis, FIGO stage) and five factors at recurrence (DFI, liver metastasis, number of tumours, size of maximum tumour, SCS outcome) were significantly related to overall survival after recurrence. In the multivariate analysis excluding SCS outcome, the significance of all the three factors during primary treatment disappeared. Four factors determined at recurrence, that is, DFI, presence of liver metastasis, number of tumours and size of maximum tumour, were revealed to be independent prognostic factors.

DFI is the most important prognostic factor after recurrence, as described in many previous reports. In most studies, the cutoff period of DFI was set to 12 months. Two cutoff periods were set in Eisenkop's study (Eisenkop *et al*, 2000) (12 and 36 months) and in Tay's study (Tay *et al*, 2002) (12 and 24 months), and patients were divided into three groups. Although we also analysed our patients with DFI >12 months using cutoff periods such as 24 and 36 months, there were no significant differences between patients with and without DFI >24 or 36 months (data not shown). Recently, Zang *et al* (2004) performed SCS even in patients with DFI of 3 months and reported negative influence of DFI on overall survival. However, their follow-up period was only 16 months. This might be too short to detect a statistical difference.

Size of maximum tumour was also identified by Eisenkop *et al* (2000) as an independent prognostic factor. Eisenkop *et al* used 10 cm as the cutoff size, whereas we used 6 cm. The difference may be due to our earlier detection of recurrent tumours by using ultrasonography or CT scan within a 3-month interval. In our cases, there were only two patients in whom maximum tumour size exceeded 10 cm in diameter. At all events, tumour size seems to be an important factor reflecting biological aggressiveness of recurrent tumours.

The number of recurrent tumours has not been previously highlighted as a prognostic determinant. One reason is that some studies restricted the subjects for SCS to patients with isolated tumours or a solitary tumour (Cormio *et al*, 1999; Munkarah *et al*, 2001; Scarabelli *et al*, 2001). Another possible reason is that Eisenkop *et al* (2000) and Tay *et al* (2002) did not analyse the

number of recurrent tumours as a factor influencing survival, although they pointed out that this factor may influence SCS outcome. In concordance with our results, Zang *et al* (2004) reported that the number of recurrent tumours influenced both overall survival and SCS outcome.

The current study revealed that liver metastasis is another important prognostic determinant. Vaccarello *et al* (1995) examined the relationship between site of recurrence and survival, and reported that liver metastasis had a negative influence on survival. In most studies, patients with liver metastasis were excluded from subjects for SCS. In our series, two patients with solitary liver metastasis were included: one patient underwent hepatic resection and the other patient did not undergo hepatic resection because of the presence of unresectable metastatic portal lymph nodes. They did not achieve good survival (20 and 14 months, respectively).

From the results of the multivariate analysis, we propose the following criteria for patient selection for SCS. Patients with recurrent ovarian cancer should be considered as ideal candidates for SCS when they have three or all of the following four factors at recurrence: (1) DFI >12 months, (2) no liver metastasis, (3) a solitary tumour and (4) tumour size <6 cm. Considering our original patient selection, we should propose exclusion criteria including (1) age at recurrence $\geqslant 75$ years, (2) PS 3 or 4 just before SCS and (3) progressive disease during presurgical chemotherapy, if undertaken. Although we used intraoperative findings for the number and size of tumours, size of maximum tumour was consistent between intraoperative findings and imaging in available cases. Therefore, we can accurately evaluate all these factors, except the number of tumours, before SCS. As for the number of tumours, ultrasonography or CT scan before SCS cannot always identify multiple peritoneal disseminated tumours. When the patient meets the criteria for SCS preoperatively, it is recommended to decide whether SCS should be accomplished after reconfirming the criteria at the time of laparotomy.

In the previous studies, several prognostic factors were shown to have significant correlation with overall survival of the patients. However, these factors were obtained from SCS in selected patients in most of the previous studies. In addition, how to use several significant prognostic factors to select good candidates for SCS was not fully analysed. To our knowledge, generally accepted or recommended selection criteria are 'patients with longer DFI' (Bristow *et al*, 1996; Roberts, 1996; Rose, 2000; Sijmons and Heintz, 2000). Thus, it was sometimes difficult to decide whether or not SCS should be performed in patients who have some favourable factors and a few unfavourable factors. We believe that our selection criteria for SCS should be helpful in deciding whether SCS should be performed.

In conclusion, our data suggest that patients with three or all four of the above-mentioned favourable factors are ideal candidates for SCS, and that the final decision should be made at laparotomy in borderline cases. It seems that SCS has a large impact on survival of patients with recurrent ovarian cancer when the patients are selected by the new criteria (47 months in median survival and 45.9% in 5-year survival). However, these patients were likely to have good sensitivity to chemotherapy, because they had DFI >6 months. In a recent trial of recurrent ovarian cancer with DFI >6 months, patients who received platinum-based chemotherapy with or without paclitaxel had a favourable prognosis: 29 and 24 months in median survival and around 20% in 5-year survival, respectively (Parmar *et al*, 2003). Although patients undergoing SCS using the new criteria of patient selection seem to have much better survival than patients receiving chemotherapy alone, our study was retrospective and noncomparative, and our data were based on a relatively small number of strictly selected patients. To provide solid evidence for the therapeutic benefit of SCS and to find better selection criteria for the surgery, further studies including randomised controlled studies are required.

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CLINICAL INVESTIGATION **Cervix**

EARLY DETERMINATION OF UTERINE CERVICAL SQUAMOUS CELL CARCINOMA RADIORESPONSE IDENTIFIES HIGH- AND LOW-RESPONSE TUMORS

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Purpose: To investigate whether early-assessed radioresponse of tumors corresponds with late-assessed radioresponse, which is associated with local disease control in radiotherapy (RT) for cervical cancer.
Methods and Materials: This prospective study included 12 patients with cervical squamous cell carcinoma treated by RT with or without concurrent cisplatin. Tumor volume was estimated by scheduled magnetic resonance imaging before (preRT), 3 to 4 weeks after (early assessment), and 6 to 7 weeks after (late assessment) RT initiation. Radioresponse was assessed with tumor shrinkage curves based on these volumes. Radioresponse for each tumor was calculated as the slope (day^{-1}) of the shrinkage curve by fitting to an exponential equation.
Results: Early-assessed radioresponse ranged from 0.001 to 0.106 day^{-1} (median, 0.021 day^{-1}) and late-assessed radioresponse from 0.009 to 0.091 day^{-1} (median, 0.021 day^{-1}), with no significant difference between them ($p = 0.1191$). The early-assessed radioresponse correlated with the late-assessed radioresponse ($R^2 = 0.714$, $p = 0.0005$).
Conclusions: Correspondence between early- and late-assessed radioresponse in a series of tumors showing a wide range of radioresponse was not particularly close overall. However, early assessment of radioresponsiveness did seem to be useful for characterizing those tumors with high or low radioresponsiveness. © 2006 Elsevier Inc.

Radiosensitivity, Intracavitary radiotherapy, Minimum target dose, Chemoradiotherapy.

INTRODUCTION

In radiotherapy (RT) for uterine cervical cancer, significant predictors of local disease control include not only clinical stage but also pretreatment tumor size and tumor radioresponse (1–5). Of the latter two, radioresponse is of greater practical importance because whereas pretreatment tumor size is deterministic, radioresponse is subject to modification, for example by concurrent chemotherapy. The degree of tumor shrinkage is commonly used as an index of radioresponse (6, 7)—for example, complete response (disappearance, 100% decrease in volume), partial response (≥65% decrease), and stable disease (<65% decrease). A complete response at the end of RT, which is assessed by subjective pelvic examination, is usually associated with local disease control (3–5). It would therefore be valuable to be able to predict early in the course of RT whether a tumor is to achieve a complete response; if not, intensification of treatment or the use of additional treatment could be considered earlier than otherwise possible. However, because the degree of tumor shrinkage is categorical and independent of time, it is not suitable as an index for the early estimation of radioresponse. In contrast, the speed of tumor shrinkage, another expression of radioresponse, is continuous and a function of time and pretreatment tumor size and should therefore serve as a useful index for prediction of posttreatment size.

Here, we prospectively investigated whether the speed of tumor shrinkage as assessed in the early phase of RT corresponds with that assessed in the late phase of RT, under conditions of standard clinical practice for concurrent chemoradiotherapy as proposed by the U.S. National Cancer Institute (8).

METHODS AND MATERIALS

Patients

The study group consisted of 12 patients with cervical squamous cell carcinoma selected from 19 consecutive cervical squamous cell carcinoma patients treated primarily by RT with or without concurrent cisplatin chemotherapy between December 2003 and

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December 2004. Following normal clinical practice, patients were scheduled to undergo magnetic resonance imaging (MRI) of the pelvis in three phases of RT, namely before and at 3 to 4 weeks (early phase) and 6 to 7 weeks (late phase) after the start of RT. The accuracy and clarity of MRI in demonstrating cervical tumors has been confirmed (9, 10). Seven patients were excluded from the study because not all MR images were available or because the images did not clearly identify the tumor. Clinical disease stages according to the International Federation of Gynecology and Obstetrics staging system were IB1 (n = 1), IIB (n = 1), and IIIB (n = 10). Patients ranged in age from 37 to 81 years (median, 51 years).

Treatment

Radiotherapy consisted of external and intracavitary RT. External RT was performed with a 10-MV X-ray in 1.8-Gy fractions at 5 fractions per week. Clinical target volume was the pelvis (n = 5) or the pelvis plus para-aortic nodes (n = 7), with para-aortic nodes treated prophylactically. A conformal box-field technique was used for all but 1 patient, in whom anterior–posterior opposing portals were used. A central block was placed in the pelvic RT field for the start of intracavitary RT after a total dose of 45.0 Gy (stage IIIB) or 36.0 Gy (stages IB1 and IIB) was reached. Total dose to the pelvis ranged from 50.4 to 66.6 Gy (median, 54.0 Gy), including boost doses to parametrial induration or lymphadenopathy, and total dose to the para-aortic nodes was 45.0 Gy. Intracavitary RT was performed with a high-dose-rate remote afterloading system. The prescribed dosage to reference point A was 6.0 Gy per insertion at three (n = 10) or four (n = 2) weekly insertions per patient. One patient underwent an interstitial implant after three intracavitary insertions. Thus, overall RT treatment duration ranged from 42 to 63 days (n = 11; median, 50 days) and was 70 days for the patient treated by interstitial implant.

Ten patients were treated by concurrent chemotherapy with cisplatin, and 2 (both aged 81 years) were treated by RT alone. Cisplatin was given by single weekly i.v. administration at 35 mg/m^2 (n = 3), 30 mg/m^2 (n = 6), or 20 mg/m^2 (n = 1, aged 72 years) for 3–6 weeks, starting from the first (n = 5), second (n = 4), or third week (n = 1) of RT. Delayed chemotherapy (n = 5) was due to renal dysfunction caused by hydronephrosis, which was managed by nephrostomy.

Tumor measurement with MR images

Magnetic resonance imaging was performed with 1.5-T units. The preRT images were obtained from 1 to 26 days (median, 11 days) before RT, with early-phase images obtained from 18 to 34 days (median, 24 days) and late-phase images obtained from 36 to 59 days (median, 46 days) after the start of RT, the latter being before (n = 1) or during (n = 11) the intracavitary RT course. Tumors identified as high-intensity lesions on T2-weighted images were measured three-dimensionally by width, thickness, and length for each tumor, and tumor volume was calculated on the assumption that the tumor mass was ellipsoid. The volume of tumors that disappeared or were recognized as only a remnant was regarded as 0.01 cm^3, whereas that of those remaining as a small, high-intensity "scar" that was difficult to measure was regarded as 0.05 cm^3.

Radioresponse assessment

Estimated tumor volumes were plotted on a semilogarithmic graph, with the start of RT set as Day 0. The early-phase shrinkage curve was calculated from the preRT and early-phase volumes, the late-phase shrinkage curve from the early-phase and late-phase volumes, and the through-phase shrinkage curve from the preRT and late-phase volumes. The slope of the curve (day^{-1}) (i.e., the speed of shrinkage per day) was determined by fitting an exponential regression equation to the respective curve. Radioresponse was defined as the speed of shrinkage, with radioresponsive tumors thus characterized by steep slopes. With the equation of the through-phase shrinkage curve, the tumor volume at the end of RT (postRT volume) was duly calculated for each tumor and categorized according to the degree of shrinkage. For this, either shrinkage to $\leq$0.05 cm^3 or to $<$1% of the preRT volume was regarded as complete response, whereas shrinkage to $<$35% of the preRT volume and shrinkage confined to $\geq$35% of the preRT volume were defined as partial response and stable disease, respectively.

Statistical analysis

The early-assessed radioresponse was compared with the late-assessed and with the through-assessed radioresponse. Differences in response between phases were analyzed by the Wilcoxon signed rank test. Correlation between the early-assessed and through-assessed radioresponses was analyzed by regression analysis. Radioresponse was compared between the speed of shrinkage (through-assessed radioresponse) and the degree of shrinkage. StatView 5.0 (SAS Institute, Cary, NC) was used for all analyses. P values of $<$0.05 were considered statistically significant.

RESULTS

The preRT volume ranged from 2.3 to 301.6 cm^3 (median, 95.5 cm^3). Complete response was observed in the early phase in one tumor and in the late phase in two (Fig. 1). Radioresponse ranged from 0.001 to 0.106 day^{-1} (median, 0.021 day^{-1}) in the early phase, from 0.013 to 0.121 day^{-1} (median, 0.025 day^{-1}) in the late phase, and from 0.009 to 0.091 day^{-1} (median, 0.021 day^{-1}) in the through phase. Radioresponse did not differ significantly between the early and late phases or between the early and through phases (p = 0.1361 for both). When the tumor that achieved a complete response in the early phase was excluded, however, the difference in response between the early and late

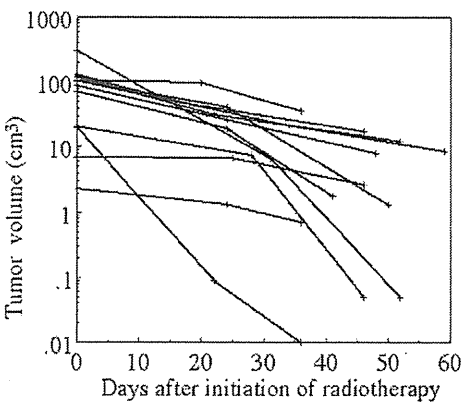

Fig. 1. Tumor shrinkage curves composed of three-phase volumes of preradiotherapy, early phase (3 to 4 weeks), and late phase (6 to 7 weeks) (n = 12).

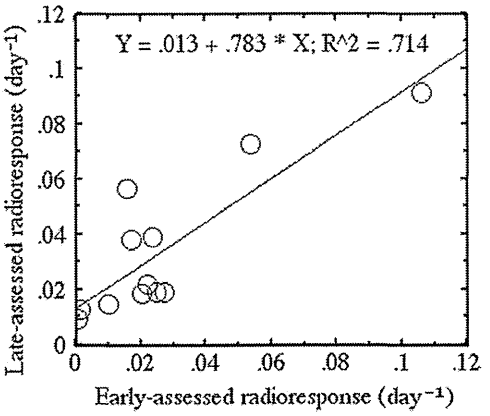

Fig. 2. Correlation between early-assessed and late-assessed radioresponse (n = 12, p = 0.0005).

phases approached significance, with radioresponse greater in the late (range, 0.013–0.121 day^{-1}; median, 0.022 day^{-1}) than in the early phase (range, 0.001–0.054 day^{-1}; median, 0.021 day^{-1}) (n = 11, p = 0.0505).

The early-assessed radioresponse correlated with the late-assessed radioresponse (Fig. 2; R^2 = 0.714, p = 0.0005). This correlation remained significant even when the tumor that achieved a near-complete response was excluded (n = 11, R^2 = 0.496, p = 0.0155).

The postRT volume ranged from 0.01 to 21.95 cm^3 (median, 0.41 cm^3) and was ≤0.05 cm^3 in three tumors. The postRT volume as a percentage ranged from 0 to 17.8% (median, 4.5%) of the preRT volume. Response category was complete response for five tumors and partial response for the remaining seven (Fig. 3). None was categorized as stable disease.

DISCUSSION

Characterization of radioresponse is particularly important for large tumors, from the standpoint of not only radiosensitivity but also dose delivery by intracavitary RT, which is characterized by steep dose fall-off within the tumor. Given that radioresponse normally implies generic radiosensitivity of tumor cells, tumors with low radioresponsiveness require larger doses for local disease control than those with high radioresponsiveness. Nevertheless, large tumors with low radioresponsiveness receive smaller target doses at the tumor periphery (minimum target doses) by intracavitary RT than large tumors with high radioresponsiveness, because the latter undergo significant shrinkage subsequent to the preceding external RT (11). Compared with large tumors, small tumors receive substantially higher minimum target doses irrespective of tumor shrinkage induced by external RT, and these high doses are considered to effectively overcome any radioresistance.

Tumors were categorized by the degree of shrinkage into either complete response or partial response only. Whereas complete response is characterized by shrinkage within a very narrow range (99–100% decrease), partial response is characterized by a wide range of shrinkage (65%–99% decrease) and is therefore not suitable for differentiating tumors at the respective ends of this range. In contrast, the speed of shrinkage is shown as a variable specific to the individual tumor and is therefore useful for differentiating partial response tumors by calculation, if the shrinkage is fitted well by a regression equation.

Our results showed that the early-assessed radioresponse corresponded with the late-assessed radioresponse, although not particularly closely. In contrast, Gong *et al.* (12), who used frequent, rigidly scheduled MRI (four to eight times per patient) and sophisticated tumor measurement methods, reported that the radioresponse of cervical tumors is exponential. Several possible reasons for this apparent discrepancy can be suggested.

First, Gong *et al.* investigated radioresponse during simple treatment with external RT alone, whereas our study involved complex treatment. Second, most of our tumors were treated by concurrent chemotherapy that was nevertheless not always simultaneous with the start of RT and by intracavitary RT that was performed in the late phase. The impact of our treatment might therefore have differed between phases, or even by week. In fact, we previously showed that the use of concurrent chemoradiotherapy tends to increase radioresponse over that achieved with RT alone (13). Further, radioresponse might have been underestimated in our three tumors that achieved a complete response because the response might have occurred before the time of observation. On these bases, we suggest that the lack of a clear exponential radioresponse in the present study was likely due to the complex treatment given, in addition to differences in the accuracy and frequency of tumor measurement.

Although exact correspondence was not obtained, our response assessment, conducted under conditions of stan-

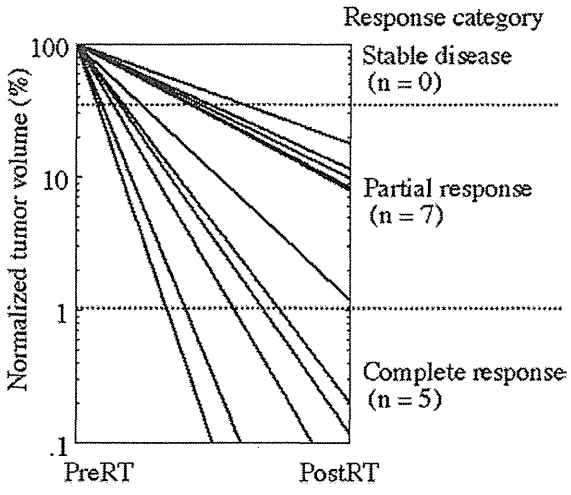

Fig. 3. Comparison of calculated radioresponse at the end of radiotherapy (RT) between the speed of shrinkage (curves) and the degree of shrinkage (response category). The postRT volume was calculated with the regression equation for each tumor at the end of RT for each individual (42–63 days from the start of RT).

dard clinical practice, is considered effective in the differentiation of highly (e.g., >0.05 day^{-1}) and poorly radioresponsive (e.g., <0.02 day^{-1}) tumors, which here represented the upper and lower quartiles of tumors by response, from those moderately radioresponsive, which made up the middle half of tumors. This is because the wide radioresponse seen facilitates the recognition of tumors at the respective ends of radioresponsiveness. Moreover, this finding is consistent between our results and those of Gong *et al.*: radioresponse range from 0.001 to 0.106 day^{-1} (early phase, 106-fold variation) and from 0.009 to 0.091 day^{-1} (through phase, 10-fold variation) in the present study and from 0.007 to 0.182 day^{-1} (26-fold variation, by planimetry) in Gong *et al.* (12).

The U.S. National Cancer Institute has recommended the concurrent use of RT and chemotherapy with cisplatin or cisplatin plus fluorouracil (as radiosensitizers) in place of the conventional use of RT alone to improve survival in patients with locally advanced cervical cancer (8), and the efficacy of this treatment has been confirmed by systematic review and meta-analysis (14). However, this recommendation is based on the assumption that the radioresponse of tumors is unknown. Early knowledge of the radioresponsiveness of tumors during treatment would allow the individualization of treatment. Given that a substantial proportion of patients have been cured by conventional RT treatment alone, those with highly radioresponsive tumors, so-called radiosensitive tumors, might not necessarily require concurrent chemotherapy. Conversely, patients with poorly radioresponsive tumors, so-called radioresistant tumors, might benefit from the intensification of treatment, such as the planned use of interstitial implants and the incorporation of a potent new radiosensitizer (gemcitabine) into concurrent chemotherapy (15).

In conclusion, the early-assessed radioresponse of uterine cervical squamous cell carcinoma corresponded with the late-assessed radioresponse, albeit not particularly strongly. Although it would be premature to incorporate these findings directly into local disease control, early determination might nevertheless be useful for identifying tumors at either extremity of the wide radioresponse range seen here.

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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.

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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

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/m^2, i.v. bolus) followed three hours later by 5FU (600 mg/m^2, i.v. bolus). From 24 h after the administration of MTX, calcium leucovorin (10 mg/m^2, 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

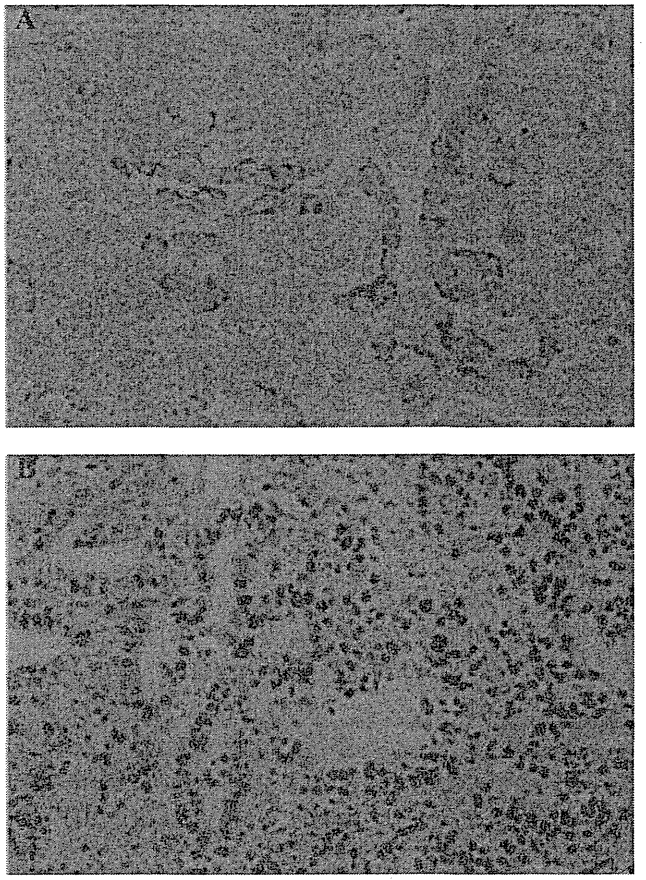

Figure 1. A, In the immunohistochemical analysis of TS, TP, E2F-1, DPD, Bak, Bcl-X, and Bcl-2 expression, the degree of immunohistochemical reactivity was defined as positive when >25% of cancer cells showed strong staining in the cytoplasm. B, Because E2F-1 is a transcriptional factor, E2F-1 immunoreactivity 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 ($Bcl\text{-}X_S$), via an alternative splicing mechanism (24), the Bcl-X polyclonal antibody used in this study did not cross-react with $Bcl\text{-}X_S$.

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,

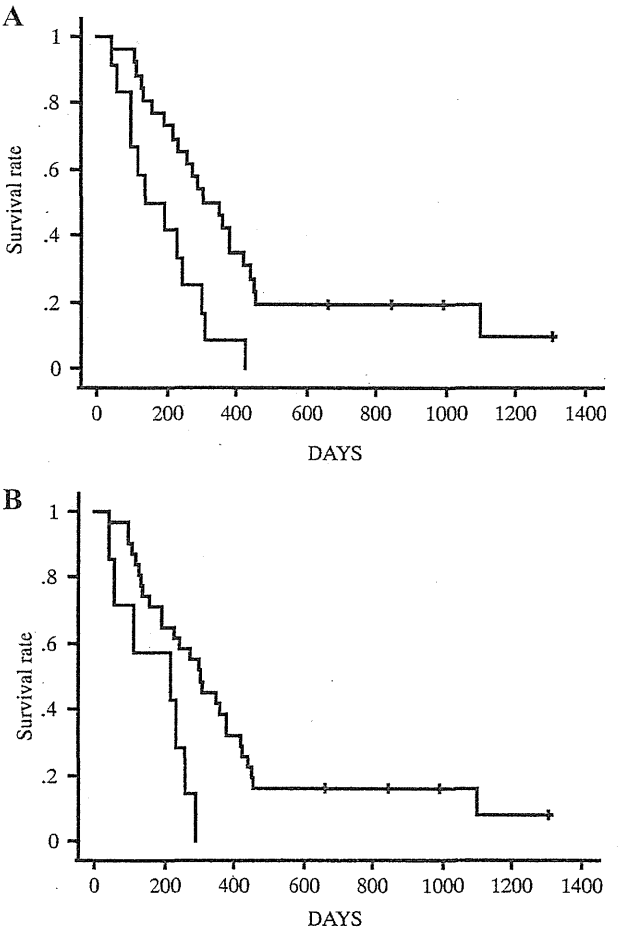

Figure 2. A, Survival of the patients according to the expression of Bak. Continuous line, the Bak-negative patients (n=26); dashed line, the Bak-positive patients (n=12). Bak-negative patients survived significantly longer than Bak-positive patients (MST, 302 days versus 134 days, respectively; p=0.0044).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

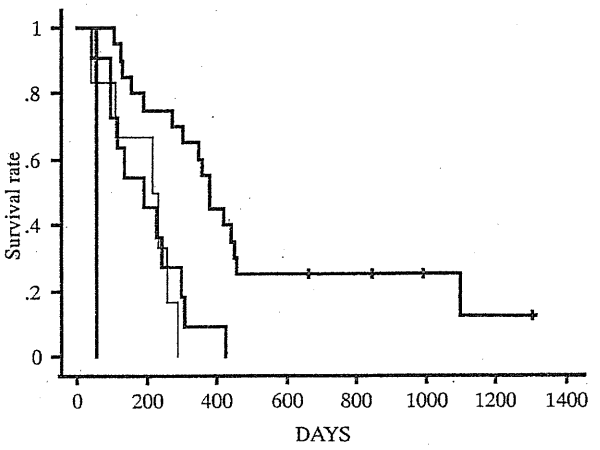

Figure 3. Survival of the patients according to couple expression of Bak and Bcl-X. Continuous line, the Bak-negative Bcl-X-negative patients (n=20); dashed line, the Bak-positive Bcl-X-negative patients (n=11); narrow line, the Bak-negative Bcl-X-positive patients (n=6); dot line, the Bak-positive and 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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