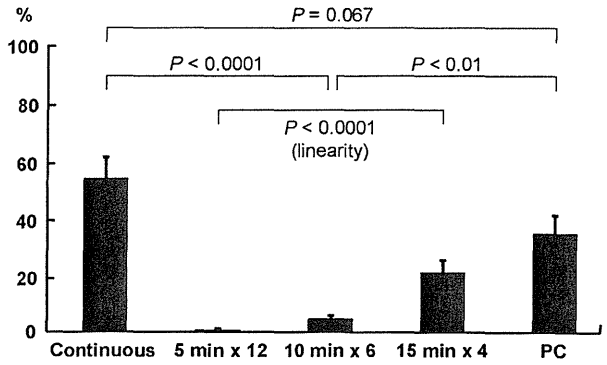

Fig. 6 Degree of hepatocyte apoptosis induction after 3 h of reperfusion. Data (means with SEM) are expressed as the proportions (%) of hepatocytes with TUNEL-positive staining. Data are presented as means with SEM. $N = 8$ in each group

characteristic biochemical marker of apoptosis, was not detected in the groups with shorter IC cycles, but it gradually increased with longer IC cycles and was also increased in the Continuous group. On the other hand, a smear of DNA, but not a DNA ladder, was detected in the PC group, indicating non-specific degradation, i.e., necrosis (Fig. 7).

Histological assessment of the degree of liver necrosis

Figure 8 shows the representative histological appearance of the liver in the five experimental groups. Necrosis occurred typically in the pericentral and midzonal regions of the hepatic lobule and usually in confluent areas of adjacent cells. We counted the areas of grade 2 and grade 3 necrosis semi-quantitatively (Fig. 9). The areas of both grade 2 and 3 necrosis were significantly smaller in the IC groups than in the Continuous group, and these areas became further reduced when the duration of the clamping/reperfusion cycles became shorter. In contrast, the areas of necrosis in the PC group were similar to those in the Continuous group.

Discussion

In the present study, we adopted 15 min of clamping alternated with 5 min of reperfusion as the control IC condition, because this is the most widely used clinical protocol for IC at experienced centers [4, 5, 10, 12, 13], and thus the time ratio of clamping to reperfusion was set at 3:1 in all IC groups. We adopted 10/10 min of preconditioning/reperfusion as the PC protocol because this is the one most widely used clinically [11, 13] and was reportedly most effective in a previous experimental study [23]. We excluded 10 min of PC from the duration of total

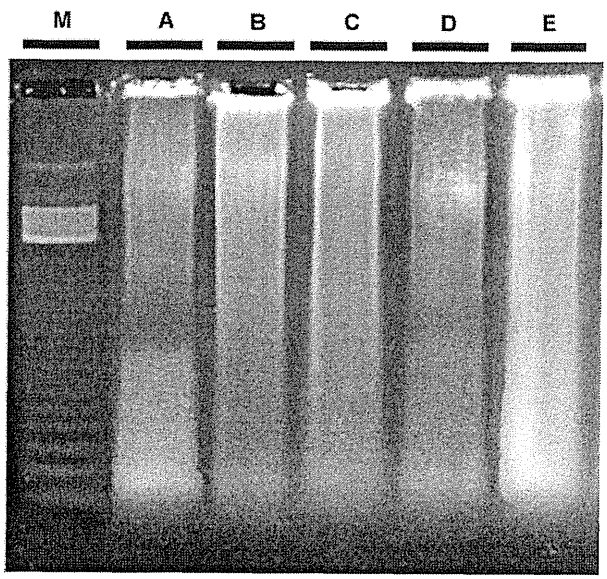

Fig. 7 DNA agarose gel electrophoresis of livers after 3 h of reperfusion. Although significant DNA laddering was not detected for shorter cycles of intermittent inflow occlusion, the level of apoptotic DNA laddering gradually increased with longer cycles of intermittent occlusion, and the level also increased in the continuous group, indicating increasing DNA fragmentation or apoptosis. On the other hand, a smear of DNA, but not DNA laddering, was detected in the preconditioning group. *Lane A* Continuous inflow occlusion for 60 min, *lane B* intermittent inflow occlusion for 5 min alternated with 1.67 min of reperfusion (5 min × 12), *lane C* intermittent inflow occlusion for 10 min alternated with 3.3 min of reperfusion (10 min × 6), *lane D* intermittent inflow occlusion for 15 min alternated with 5 min of reperfusion (15 min × 4), *lane E* ischemic preconditioning for 10 min followed by 10 min of reperfusion (PC)

ischemia on the basis of previous clinical and experimental studies [11–13, 19, 23].

Bile flow is reportedly a reliable indicator of liver and/or graft function, and of viability in hepatic warm as well as cold I/R injury [19, 22, 24]. Koeppel et al. [25] reported that decreased bile flow after cold I/R liver injury was explained by the impaired biliary excretion of GSH, a primary osmotic driving force in the bile flow. Reactive oxygen species are thought to play pivotal roles in liver I/R injury [26]. GSH is an endogenous radical scavenger that reacts spontaneously with nearly all oxidants formed during inflammation [27]. Previous studies have shown that the administration of GSH precursors attenuated liver I/R injury by increasing intracellular GSH [28, 29]. Likewise, Peralta's group (Serafin et al. [30]) have reported that PC conferred resistance against the liver damage induced by reactive oxygen species by preventing the depletion of GSH [30]. In line with these findings, liver GSH content was reported to be a valid indicator of the degree of I/R injury [30–32] and therefore we measured its alteration.

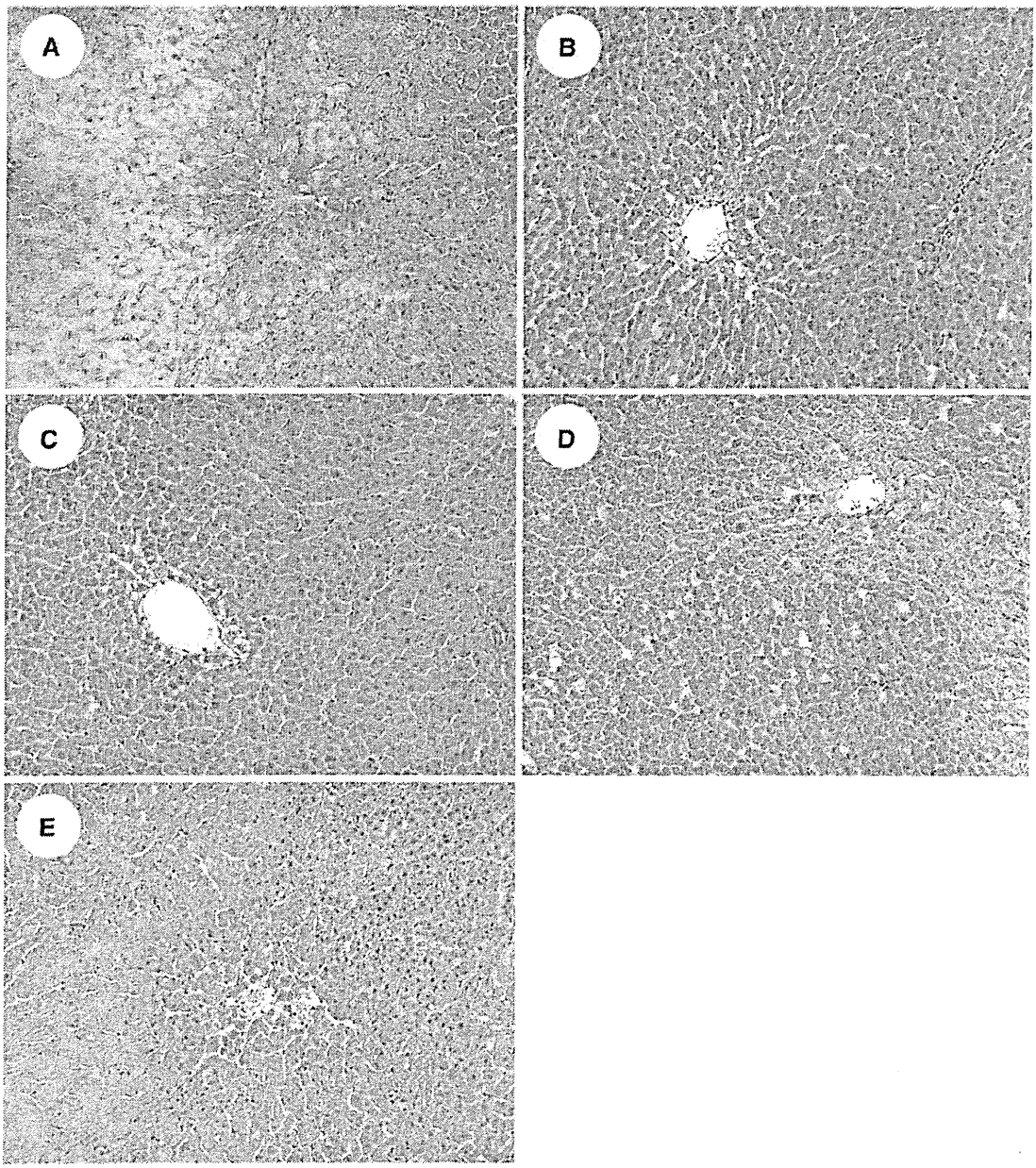

Fig. 8 Tissue sections stained with hematoxylin and eosin. Representative histological appearances after 3 h of reperfusion are shown. **a** Continuous inflow occlusion for 60 min, **b** intermittent inflow occlusion for 5 min alternated with 1.67 min of reperfusion (5 min × 12), **c** intermittent inflow occlusion for 10 min alternated with 3.3 min of reperfusion (10 min × 6), **d** intermittent inflow occlusion for 15 min alternated with 5 min of reperfusion (15 min × 4), **e** ischemic preconditioning for 10 min followed by 10 min of reperfusion (PC) (Original magnification ×200)

In the present study, the alterations in bile flow and liver GSH content showed similar trends among the experimental groups. Of note, the magnitude of the alterations in bile flow was more marked than the alterations in liver GSH content. Although biliary GSH content was not measured, assuming that the decrease in the bile flow in the present work was attributable to the decreased biliary GSH excretion, it could be speculated that the alteration in the GSH content was more profound in the bile than in the liver. As support for this consideration, Accatino et al. [33] have reported that, while bile flow and biliary excretion of GSH was markedly decreased after liver I/R injury, liver GSH content was unchanged.

We measured the serum level of ALT at 3 h after liver I/R to assess the degree of hepatocyte injury. Moreover, we conducted both qualitative and semi-quantitative

histological evaluation of liver specimens obtained at identical time points, paying particular attention to the emergence of necrotic areas. In addition, the induction of hepatocyte apoptosis was assessed in terms of the TUNEL assay and DNA fragmentation. Although hepatocyte apoptosis reportedly plays a central role in models of liver injury induced by FAS [34] and tumor necrosis factor (TNF)-alpha [35], its role in hepatic I/R injury has been questioned [36]. Most previous studies supporting a major role of hepatocyte apoptosis in liver I/R injury used a quantified TUNEL assay [37, 38], but these studies have been criticized on the grounds that the TUNEL assay also detects cells that have succumbed to oncotic death, and is not necessarily specific for apoptosis [26, 36]. For this reason, we also studied DNA laddering by agarose gel electrophoresis and confirmed the results of the TUNEL assay [39]. On the other hand, it has been reported that apoptosis and oncosis may share the same underlying mechanism, and that the outcome depends on the strength of the stimulus, or that excessive parenchymal cell apoptosis itself is a signal for the necrotic reaction, including that in models of liver I/R injury [34, 40]. Indeed, agarose gel electrophoresis revealed signs suggestive of the presence of both apoptosis and oncosis according to the experimental conditions employed (Fig. 7). Overall, the results of the TUNEL assay and DNA laddering are thought to be at least comprehensive indicators of the cell death caused by liver I/R injury.

Necrosis occurred in the pericentral and midzonal regions of the hepatic lobule, probably because these regions are furthest removed from the oxygen supply (Fig. 8). In contrast, TUNEL-positive cells were spread over a wider area, including areas of necrosis and parts of the periportal region. In addition, individual cells, rather than groups of contiguous cells, showed TUNEL positivity, especially in the peripheral midozonal and perioral regions (Fig. 5). Taking into account the non-specific TUNEL staining of necrotic cells, these findings suggested that, at least under the present experimental conditions, the main mode of cell death during warm I/R liver injury was oncotic necrosis and lent support for the contention that pathways leading to oncosis and apoptosis were shared [34, 41]. A warm I/R injury may culminate in either apoptosis or necrosis, depending on other variables such as ATP supply or the extent of hypoxia.

The most straightforward results of the present study are that all of the parameters of liver I/R injury were universally ameliorated in the IC groups in comparison with findings in the continuous inflow occlusion group. Moreover, it was noteworthy that when the time ratio of clamping to alternated reperfusion was kept constant, shorter cycles of IC were more effective. Several studies have examined whether the degree of I/R injury is affected by the duration of the IC cycles, but all them focused on the optimal duration of alternated reperfusion under a fixed inflow occlusion time [21], or vice versa [20, 39]. Horiuchi et al. reported that the extent of liver injury was reduced as the duration of reperfusion increased (15 min compared with 5 and 10 min) if the period of clamping was fixed at 15 min [21]. In contrast, Clavien's group (Jang et al. [20, 39] and Kang et al. [20, 39]) documented that the protective effect of IC was similar for both 15 and 30 min of intermittent inflow occlusion, compared with continuous inflow occlusion, when the duration of alternated reperfusion was fixed at 5 min [20, 39]. However, scrutinization of their data showed that within the IC groups, the magnitude of liver injury appeared to be more marked for 30 min of occlusion, and hence their results were in line with those of Horiuchi et al. [20, 39]. In the present investigation, we studied and clarified, for the first time, the separate effect of IC cycle length on protection against I/R.

In the clinical setting of liver resection, however, 15 min of clamping alternated with 5 min of reperfusion is the most popular and well accepted IC condition, and it may be argued that it is not practical to shorten the cycle length further. Here, we have to bear in mind the allometric law in animals, known as Kleiber's 3/4-power scaling law, which states that an animal's basal metabolic rate is proportional to 3/4 power of its body weight, and thus the metabolic time or physiological time of a species is proportional to 1/4 power of its body weight [42]. If we apply this law to extrapolate the present results to a human setting, the values of 15/5, 10/3.3, and 5/1.7 min in rats weighing 250 g correspond to 60/20, 40/13, and 20/7 min, respectively, for a human weighing 50 kg. The currently most popular human IC protocol (15/5 min) may be optimal, and a longer cycle might induce liver damage, albeit at a subclinical level.

Unexpectedly, PC did not confer a protective effect against liver I/R injury, except for the prevention of apoptosis induction, although all of the experiments were conducted under identical conditions. This result showed a clear contrast to those for IC, which demonstrated universal attenuation of every aspect of liver I/R injury, irrespective of cycle length, and with constant linearity between cycle length and the strength of the effect. Also, these results were contrary to those of previous studies indicating that PC suppressed liver I/R injury to an extent equal to that of IC [8, 19, 20, 39]. A possible explanation for this apparent discrepancy is that, under the present experimental conditions, 10 min of ischemia followed by 10 min of reperfusion did not have a preconditioning effect but worked as an additive ischemia. Peralta et al. [23] evaluated the effects of various types of PC in a rat model of 90 min of warm liver I/R injury. They reported that, for 10 min of reperfusion, 10–15 min of ischemic preconditioning was most

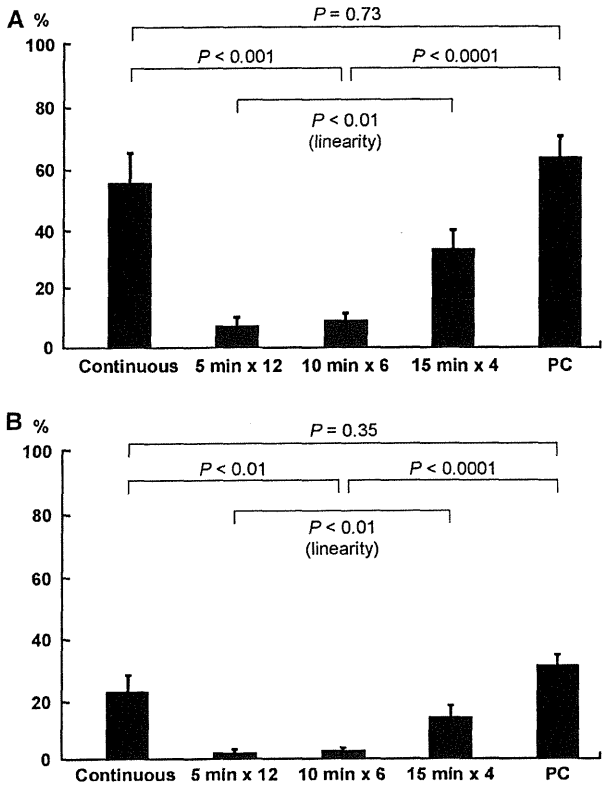

Fig. 9 Percentages of grade 2 (**a**) and grade 3 (**b**) necrosis after 3 h of reperfusion. Data (%) are expressed as means with SEM. $N = 8$ in each group

effective, and that either a shorter or longer period of preconditioning was associated with diminished protection [23]. On the other hand, their group (Serafin et al. [30]) compared three different PC methods in a rat model involving 60 min of liver I/R injury, i.e., 5 min of preconditioning followed by 10 min of reperfusion (5/10), 10 min of preconditioning followed by 15 min of reperfusion (10/15), and 10 min of preconditioning followed by 10 min of reperfusion (10/10) [30]. The group reported that the protective effect became weaker in this order, and was null in the 10/10 group [30]. Therefore, the results in their two studies ([23] and [30]) were contradictory in regard to the optimal length of preconditioning and following reperfusion in PC protocol. Likewise, it has been reported that the effect of preconditioning was obscure in aged [11] patients. Taking all these issues into account, it is possible to conclude that preconditioning may attenuate liver I/R injury, but that the degree of attenuation or the presence/absence of the effect itself is largely dependent on the particular conditions of individuals or ischemia, including the preconditioning protocol.

In conclusion, we have shown, using a rat model of liver warm I/R injury, that IC exerts a universal protective effect against I/R injury. In addition, if the time ratio of clamping to alternated reperfusion is constant, the protective effect increases as the cycle length becomes shorter. By contrast, preconditioning did not work effectively against I/R injury, at least under the present experimental conditions. Overall, IC is a robust method for reducing liver I/R injury that can be applied widely under various conditions; by contrast, preconditioning is a less robust protocol for liver protection.

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Conflict of interest The authors do not have a financial relationship with any organization that sponsored the research. The authors also have full control of all primary data and that they agree to allow the journal to review their data if requested.

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Research Article

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Superselective transarterial chemoembolization for hepatocellular carcinoma. Validation of treatment algorithm proposed by Japanese guidelines

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Background & Aims: Transcatheter arterial chemoembolization with lipiodol (TACE) is widely performed in patients with hepatocellular carcinoma (HCC) unsuitable for curative treatment. It has recently been recommended for patients with 2 or 3 tumors >3 cm or ⩾4 tumors in a treatment algorithm proposed by Japanese guidelines. However, the best indication and appropriateness of the algorithm for TACE are still unclear.

Methods: In 4966 HCC patients who underwent TACE, survival was evaluated based on tumor number, size and liver function; and the adequacy of the algorithm for TACE was validated. Exclusion criteria were: vascular invasion, extrahepatic metastasis, and prior treatment. The mean follow up period was 1.6 years.

Results: The overall median and 5-year survivals were 3.3 years and 34%, respectively. Multivariate analysis revealed that Child–Pugh class, tumor number, size, alpha-fetoprotein, and des-gamma carboxy-prothrombin were independent predictors. The survival rate decreased as the tumor number ($p = 0.0001$) and size increased ($p = 0.04$ to $p = 0.0001$) in all but one subgroup in both Child–Pugh-A and -B. The stratification of these patients to four treatments in the algorithm showed potential ability to discriminate survivals of the resection and ablation (non-TACE) groups from those of the TACE group in Child–Pugh-B and partially in A.

Conclusions: TACE showed higher survival rates in patients with fewer tumor numbers, smaller tumor size, and better liver function. The treatment algorithm proposed by the Japanese guidelines might be appropriate to discriminate the survival of patients with non-TACE from TACE therapy.

Cancer

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide with 626,000 new cases every year, and is the third most common cause of death from cancer [1]. The frequency of curative treatment, such as resection, local ablation, and/or liver transplantation is low (only 30%) due to advanced cancer stage and associated liver cirrhosis at the time of diagnosis [2]. Among several treatments, transcatheter arterial chemoembolization with lipiodol (TACE) is widely performed in patients with unresectable HCC at an initial and recurrent time, which accounts for 32% and 58% of all treatment modalities, respectively, in Japan [3]. Superselective TACE is indispensable to maximize the effect in targeted tumors and to minimize liver injury [4].

Recently, two treatment algorithms for HCC were proposed: the Barcelona Clinic Liver Cancer (BCLC) classification, in 2001 [5] and the Japanese guidelines, in 2005 [6]. The first one recommends TACE in patients with multi-nodular HCC in Child–Pugh A or B in the intermediate stage, while the second recommends TACE in patients with 2 or 3 tumors, >3 cm in diameter or ⩾4 tumors in liver damage A or B. In both guidelines, vascular invasion and/or extrahepatic spread are excluded. However, the survival rate of TACE-stratified to recommended treatment of the Japanese guidelines algorithm and its appropriateness have not

Keywords: Hepatocellular carcinoma (HCC); Transcatheter arterial chemoembolization (TACE); Prognostic factor; Validation of treatment algorithm; Japanese guidelines.

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been yet determined. Thus, we conducted this research with a large scale of samples.

Patients and methods

During 6 years, from January 2000 to December 2005, a total of 60,773 patients with primary liver cancer were prospectively registered bi-annually by the Liver Cancer Study Group of Japan (LCSGJ) throughout 800 medical institutions using a registration/questionnaire sheet with more than 180 questions. Among them, 53,008 patients were clinically diagnosed with HCC with multiple imaging modalities, tumor markers, and/or needle biopsy. Four thousand nine hundred sixty-six patients were selected in the current cohort study. Inclusion criteria were the following: TACE was performed in naïve patients as an initial treatment and any other therapy such as resection and local ablation was not performed during the first investigation period within at least 2 years. Exclusion criteria were: vascular invasion of the portal and hepatic veins, invasion of the biliary duct, extrahepatic spread and history of previous treatment for HCC.

HCC was diagnosed using ultrasonography (US), dynamic computed tomography (CT), magnetic resonance imaging (MRI), and/or pathologically by biopsy specimens (3.2%). Abnormal elevation of tumor markers was also referred: alpha-fetoprotein (AFP) >400 ng/ml (normal, <20) and des-gamma carboxyl prothrombin (DCP) >100 mAU/ml (normal, <40). Typical HCC was depicted as hyper-attenuation in arterial phase and hypo-attenuation or wash-out in delayed phase (around 3 min after the beginning of contrast injection) of dynamic CT and on dynamic MRI. If the tumor showed an atypical profile and was larger than 2 cm in diameter, further examination was recommended as follows: angiography, combination of CT and angiography, MRI with super-paramagnetic iron oxide, CE-US with micro-bubble (Levovist, Bayer Schering Pharma, Germany), and/or needle biopsy. If the tumor was less than 2 cm, a follow up study with US was recommended [6]. The extrahepatic metastases were routinely examined by CT, US, and chest X-ray.

The distribution of background factors of patients with TACE is shown in Table 1. The study population predominantly consisted of patients older than 60 years [n = 4205 (85%)] and among them 3369 were male patients (68%). The proportion of Child–Pugh A/B/C was 69% (n = 3229 patients), 28% (n = 1296), and 4% (n = 167), respectively. 3479 patients (73%) were positive for hepatitis C virus antibody and 449 were positive for hepatitis B virus surface antigen. The maximum tumor size was ⩽2 cm in diameter for 32% and ⩽3 cm for 56% of tumors. The mean diameter was 3.8 ± 3.5 (standard deviation, SD) cm. The tumor number was one in 2252 patients (46%), two in 1003, three in 565, and more than four in 1092. 1868 patients (40%) had a normal AFP value and 900 had more than 401 ng/ml. 2128 patients (52%) had a DCP value ⩽100 mAU/ml.

According to the TNM stage revised by the LCSGJ in 2000 [7], 836 patients were in stage I, 2070 (43%) in stage II, and 1887 in stage III. The embolization area was less than one segment in 1589 patients (33%), equal to or more than one segment to less than one lobe in 2134 (44%), and the whole liver in only 247 patients (5%). Hypervascular HCC accounted for 98% (n = 4787 patients) and non-hypervascular HCC for 2% (n = 100). Mean bilirubin value was 1.1 ± 0.9 mg/dl (SD). Performance status (PS) according to Eastern Cooperative Oncology Group scale was PS0 in 1485 (80%) patients, PS1 in 298, PS2 in 48, PS3 in 23, and PS4 in 2 out of 1856 patients, namely 99% of patients, which were available during the last two years (January, 2004 to December, 2005) of the present study, were in PS0–2.

In most patients, the catheter tip was advanced at the nearest site of the feeding artery as possible. The emulsion of the anticancer agent and lipiodol followed by gelatin sponge particles was injected under X-ray monitoring. The dose of emulsion and particles of embolic materials was determined mainly based on the tumor size and extension. The anticancer agent used was epirubicin hydrochloride in 1490 patients (74%), doxorubicin hydrochloride in 191 patients, mitomycin C in 190 patients, and cisplatin and zinostatin stimalamer (SMANCS) in 72 patients each, for a total of 2015 patients with a mean dose of lipiodol of 4.8 ± 3.0 ml (SD), which data were available during the last two years (January, 2004 to December, 2005). The patients underwent dynamic CT or MRI with AFP and DCP measurement every three to four months, and repeated TACE was determined when local recurrence, intrahepatic metastases and/or de novo HCC was found.

To analyze the survival rate, all patients in Child–Pugh A or B were divided in four groups depending on tumor number (single, two, three, and more than four lesions). Each group was subsequently subdivided in four subgroups based on tumor size; ⩽2, 2.1 to 3.0, 3.1 to 5.0, and ⩾5.1 cm in diameter. Patients in Child–Pugh C were excluded from this analysis due to their small number (n = 167). The survival rate was calculated from the date of TACE to December 31, 2005. Patient's death was the endpoint irrespective of the cause of death. The mean follow up period was 1.6 ± 1.3 years (SD). TACE-related death was designated as death within 30 days after the initial TACE.

Treatment algorithm proposed by Japanese guidelines

The treatment algorithm proposed by Japanese guidelines [6] has six treatments determined by three factors: degree of liver damage [7], number of tumors, and tumor diameter (Fig. 1). For patients with liver damage A or B, four treatments are recommended: resection for single tumor or local ablation for single tumor ⩽2 cm and liver damage B; resection or ablation for 2 or 3 tumors ⩽3 cm; resection or TACE for 2 or 3 tumors >3 cm; TACE or hepatic arterial infusion chemotherapy for more than 4 tumors. For patients with liver damage C, liver transplantation for 1 to 3 tumors ⩽3 cm or single tumor ⩽5 cm as indicated by the Milan criteria [8], and palliative care for ⩾4 tumors are recommended. In the present study, Child–Pugh class was adopted instead of degree of liver damage because the former is widely used to evaluate liver function, especially for candidates to TACE.

The executing rate of TACE was calculated with the following formula: number of patients stratified to TACE in treatment algorithm divided by a total number of patients who actually received TACE × 100 (%). The adequacy of treatment algorithm for TACE was validated when the survivals of patients stratified to TACE group (for 2 or 3 lesions >3 cm or more than 4 lesions) and those of patients stratified to non-TACE group (such as resection and ablation for single lesion or 2 or 3 lesions ⩽3 cm) could be discriminated.

Statistical analysis

The survival rate was obtained by the Kaplan–Meier method and compared by the log-rank test in Tables 1, 2A and B, and 3. The multivariate analysis was performed with the Cox's proportional hazard model. All variables, except for one of the embolization area of the liver due to the factor obtained following TACE therapy, with p value less than 0.05 on univariate analysis, were subjected to multivariate analysis. All significance tests were two-tailed, and p value less than 0.05 was considered statistically significant. All statistical analyses were carried out with the Statistical Analysis System (SAS) version 8.02 (SAS Inc., Cary, NC).

Results

Survival rates

For overall survival of the 4966 patients who underwent TACE, the median, and 1-, 2-, 3-, 4- and 5-year survival rates were 3.3 years (40 months) and 87%, 70%, 55%, 42%, and 34%, respectively (Fig. 2). The 3- and 5-year survival in Child–Pugh A, B, and C was 61% and 40%; 43% and 22%; 23% and 0%, respectively (Table 1).

Patient characteristics analyzed by univariate and multivariate analyses

The univariate analysis revealed that there was a significant difference between the following seven variables ($p = 0.0001$); Child–Pugh class, maximum tumor size, number of lesions, AFP, DCP, TNM stage, and extent of embolization area (Table 1).

The multivariate analysis showed that the following five variables were independent predictors in trial 1; Child–Pugh class, tumor size, number of lesions, AFP, and DCP (Supplementary Table 1). In trial 2, where tumor size and number of lesions in trial 1 were replaced by TNM stage, four variables were independent predictors: Child–Pugh class, TNM stage, AFP, and DCP.

Survival rates of patients stratified to four groups divided by lesion number and to four subgroups subdivided by lesion size

In Child–Pugh A patients (n = 3194), the overall median and 3-year survival rate in four groups divided by tumor number: single, two, three, and more than 4 lesions, were 5.4 years and 73%, 3.8 years and 59%, 3.1 years and 52%, and 2.8 years and

Table 1. Distribution of background factors and results of the univariate analysis in 4966 patients with hepatocellular carcinoma who underwent transcatheter arterial chemoembolization with lipiodol.

Background factors	No. of patients	Proportion (%)	Survivals (%)				*p*	Hazard ratio (95% CI)
			1-yr	3-yr	4-yr	5-yr		
Age, yr							0.88	
<60	756	15	85	56	43	39		Ref.
≥60	4205	85	87	55	42	33		1.01 (0.88, 1.16)
Gender							0.40	
M	3369	68	86	54	42	35		1.05 (0.93, 1.18)
F	1597	32	89	56	42	33		Ref.
Child-Pugh classification							0.0001	
A	3229	69	90	61	49	40		Ref.
B	1296	28	82	43	27	22		1.81 (1.62, 2.04)
C	167	4	69	23	12	-		3.05 (2.44, 3.81)
HBV and HCV							0.50	
HCV Ab positive	3479	73	87	55	41	34		1.01 (0.87, 1.18)
HBs Ag positive	449	9	84	53	37	35		1.11 (0.90, 1.38)
Both positive	89	2	89	58	54	43		0.83 (0.56, 1.25)
Both negative	768	16	86	56	44	32		Ref.
Maximum tumor size (cm)							0.0001	
≤2	1549	32	93	65	50	42		Ref.
2.1-3	1178	24	89	53	42	35		1.38 (1.19, 1.60)
3.1-5	1291	27	85	52	37	29		1.62 (1.41, 1.87)
≥5.1	811	17	77	44	34	23		2.19 (1.88, 2.56)
No. of lesions							0.0001	
1	2252	46	91	66	53	45		Ref.
2	1003	20	88	55	42	34		1.35 (1.17, 1.56)
3	565	12	86	45	27	20		1.77 (1.50, 2.08)
≥4	1092	22	79	39	30	20		2.18 (1.91, 2.48)
Alpha-fetoprotein (ng/ml)							0.0001	
≤20	1868	40	92	64	50	44		Ref.
21-200	1613	34	89	55	40	30		1.38 (1.21, 1.57)
201-400	311	7	82	45	33	29		1.73 (1.40, 2.14)
401-1000	309	7	81	43	32	21		2.07 (1.68, 2.55)
≥1001	591	13	72	38	32	23		2.49 (2.13, 2.92)
Des-gamma carboxy-prothrombin (mAU/ml)							0.0001	
≤100	2128	52	92	65	52	40		Ref.
101-299	599	15	88	52	41	32		1.50 (1.26, 1.78)
300-499	245	6	84	49	27	24		1.93 (1.54, 2.42)
500-999	294	7	82	50	33	20		1.89 (1.51, 2.36)
≥1000	794	20	76	38	26	18		2.52 (2.18, 2.91)
TNM stage							0.0001	
I (T1N0M0)	836	17	93	72	59	51		Ref.
II (T2N0M0)	2070	43	90	60	46	37		1.51 (1.27, 1.80)
III (T3N0M0)	1887	39	81	42	30	22		2.60 (2.19, 3.09)
Extent of embolization							0.0001	
<one segment	1589	33	90	64	51	44		Ref.
1 seg. ≤ to <1 lobe	2134	44	87	55	41	32		1.33 (1.17, 1.51)
1 lobe ≤ to <whole liver	873	18	85	47	32	23		1.67 (1.43, 1.94)
Whole liver	247	5	74	37	29	-		2.27 (1.85, 2.80)

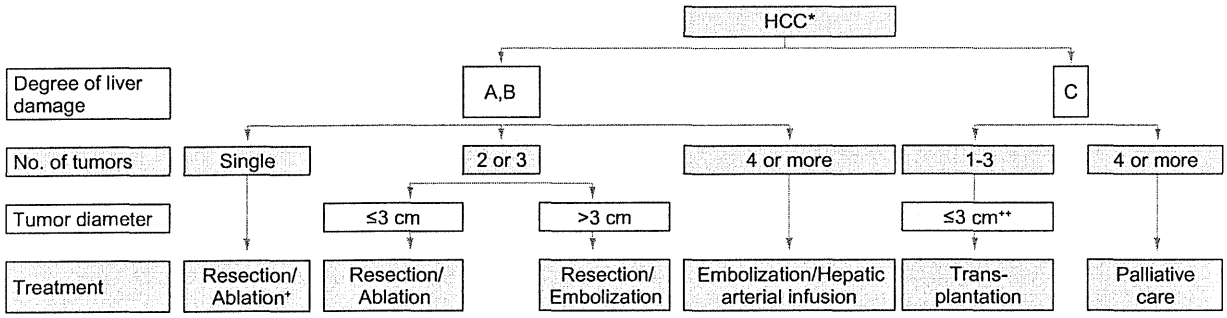

Fig. 1. Treatment algorithm for HCC proposed by Japanese guidelines. (+) shows local ablation for single lesion ≤2 cm in patients with liver damage B. (++) means liver transplantation for no more than 3 lesions ≤3 cm or single lesion ≤5 cm. The asterisk shows that for patients with vascular invasion and liver damage A, hepatectomy, TACE or hepatic arterial infusion chemotherapy may be recommended, while chemotherapy is an option for patients with extrahepatic metastasis.

46%, respectively ($p = 0.0001$, Table 2A). The survival rate of four subgroups subdivided by tumor size from ≤2 cm to ≥5.1 cm decreased as the lesion size increased in all ($p = 0.04$ to $p = 0.0001$) but one group with 3 lesions ($p = 0.07$). The highest 3-year survival was 80% in patients with single lesion ≤2 cm, and the lowest 3-year survival was 30% in patients with more than 4 lesions ≥5.1 cm.

In Child–Pugh B (n = 1284), the overall median and 3-year survival rate of four groups were 3.1 years and 53%, 2.8 years and 49%, 2.0 years and 24%, and 1.9 years and 22%, respectively ($p = 0.0001$, Table 2B). The survival rate of four subgroups divided by tumor size in each group decreased as the lesion size increased in all ($p = 0.01$ to $p = 0.0004$) but one group with single lesion ($p = 0.49$). The highest 3-year survival was 65%, found in patients with 2 lesions ≤2 cm, and the lowest was 0% in patients with three lesions ≥5.1 cm.

Validation of the treatment algorithm proposed by the Japanese guidelines

Of 3168 patients with TACE in Child–Pugh A, 1475 were stratified to resection or ablation therapy for single lesion in the treatment algorithm (Fig. 1), 506 to resection or ablation for 2 or 3 lesions ≤3 cm, 463 to resection or TACE for 2 or 3 lesions >3 cm, and 724 to TACE or hepatic arterial infusion chemotherapy for ≥4 lesions (Table 3). The median and 3-year survival rates of the corresponding four treatments were 5.4 years and 73%, 3.5 years and 59%, 3.4 years and 55%, and 2.8 years and 46%, respectively, with a significant difference ($p = 0.0001$). The comparisons of the survival curves between two treatment groups showed a significant difference in all ($p = 0.013$ to $p = 0.0001$) but one comparison between treatments for 2 or 3 lesions ≤3 cm and for 2 or 3 lesions >3 cm ($p = 0.06$) (Fig. 3). Namely, survival discrimination was feasible between one of two TACE treatments for >4 lesions and non-TACE therapies such as resection or ablation.

Similarly, 1274 patients with Child–Pugh B were stratified to four treatment categories (Table 3). The median and 3-year survivals of these treatments from single to ≥4 lesions were 3.1 years and 53%, 2.8 years and 49%, 1.7 years and 30%, and 1.9 years and 22%, respectively, with a significant difference ($p = 0.0001$). The comparisons of survival curves between two treatment groups showed a significant difference in all ($p = 0.0001$) but two comparisons; single lesion *vs.* 2 or 3 lesions ≤3 cm ($p = 0.79$) and 2 or 3 lesions >3 cm *vs.* ≥4 lesions ($p = 0.84$) (Fig. 4). Namely, survival discrimination was feasible between two TACE therapy groups, i.e., 2 or 3 lesions >3 cm and ≥4 lesions and two non-TACE groups.

The executing rate of TACE was 37% in both Child–Pugh A (1187/3168 patients) and B (467/1274).

TACE-related mortality rate

After the initial TACE, treatment-related death occurred in 19 (0.38%) out of 4966 patients. The breakdown of the cause of death was cancer in 5 patients (26%), hepatic failure in 3 (16%), rupture of esophago-gastric varices in one patient, intra-peritoneal rupture of HCC in another patient, and other causes in 9 patients. Ten patients were in Child–Pugh A, 8 were in class B and one was in class C.

Discussion

The present study demonstrates that the overall median and 3-, and 5-year survival rates of TACE were 3.3 years (40 months), 55%, and 34%, respectively, and were better than those previously reported by the LCSGJ (34 months, 47%, and 26% [9]), mainly due to exclusion criteria of vascular invasion in the current study. The multivariate analysis revealed that five variables were independent predictors in trial 1: Child–Pugh class, tumor size, tumor number, AFP, and DCP; and four variables in trial 2, where tumor size and tumor number were replaced by TNM stage. These results are similar to those of a previous study [9] other than Child–Pugh class instead of degree of liver damage and DCP value were newly adopted.

There was an inverse correlation between tumor number and overall survival of patients with TACE therapy ($p = 0.0001$) in both Child–Pugh A and B (Tables 2A and B) as well as between tumor diameter and survival in all but one group, each in Child–Pugh A and B. Namely, the fewer the tumor number and the smaller the tumor size, the better the survival rates. The best 3-year survival (80%) was found in patients with a single HCC ≤2 cm in Child–Pugh A, and the worst 3-year survival (0%) in patients with three lesions ≥5.1 cm in class B. However, in clinical practice, the best survivor with TACE is not recommended to TACE but to resection or local ablation due to relatively higher 3-year survival rates, 90% and 85%, respectively [3]. The current study has revealed a wide range of survival rates for patients with

Table 2. The overall survivals of four groups divided by tumor number and survivals of four subgroups divided by tumor size in patients who underwent TACE. (A) Child-Pugh A (n = 3194 patients). (B) Child-Pugh B (n = 1284).

A

Group/ subgroup	No. of patients	Survival (%) 1-yr	3-yr	4-yr	5-yr	Median (yr)	p	Hazard ratio (95% CI)
Single lesion								
Overall*	1475	93	73	62	52	5.4		
≤2 cm	546	97	80	73	65	-	0.0001	Ref.
2.1-3.0	353	92	71	56	36	4.5		1.88 (1.36, 2.62)
3.1-5.0	328	92	66	53	46	4.6		1.97 (1.42, 2.75)
≥5.1	219	86	66	48	-	4.2		2.38 (1.64, 3.46)
Two lesions								
Overall*	634	91	59	47	40	3.8		
≤2 cm	178	97	64	49	42	3.9	0.04	Ref.
2.1-3.0	144	91	50	39	-	2.8		1.57 (1.01, 2.44)
3.1-5.0	190	90	66	47	39	4.0		1.23 (0.80, 1.90)
≥5.1	104	84	53	45	38	4.1		1.94 (1.19, 3.15)
Three lesions								
Overall*	361	90	52	33	24	3.1		
≤2 cm	102	92	65	30	-	3.6	0.07	Ref.
2.1-3.0	82	95	51	32	-	3.1		1.16 (0.68, 1.99)
3.1-5.0	111	94	48	38	-	3.0		1.30 (0.80, 2.10)
≥5.1	58	73	35	-	-	2.2		2.02 (1.19, 3.45)
More than 4 lesions								
Overall*	724	82	46	37	25	2.8		
≤2 cm	168	92	59	54	44	4.4	0.0001	Ref.
2.1-3.0	137	83	54	51	32	4.0		1.47 (0.97, 2.25)
3.1-5.0	207	82	43	25	16	2.5		2.00 (1.38, 2.90)
≥5.1	190	74	30	18	-	1.7		2.89 (2.01, 4.17)

B

Group/ subgroup	No. of patients	Survival (%) 1-yr	3-yr	4-yr	5-yr	Median (yr)	p	Hazard ratio (95% CI)
Single lesion								
Overall*	568	87	53	34	30	3.1		
≤2 cm	213	89	56	33	24	3.3	0.49	Ref.
2.1-3.0	169	88	50	44	39	2.9		1.00 (0.69, 1.44)
3.1-5.0	132	87	54	20	-	3.1		1.24 (0.83, 1.86)
≥5.1	47	78	49	-	-	2.5		1.40 (0.80, 2.47)
Two lesions								
Overall*	276	83	49	34	-	2.8		
≤2 cm	86	93	65	48	-	4.0	0.0004	Ref.
2.1-3.0	70	93	43	21	-	2.3		1.86 (1.04, 3.34)
3.1-5.0	82	69	41	27	-	2.1		2.49 (1.47, 4.21)
≥5.1	31	58	-	-	-	1.5		3.66 (1.83, 7.32)
Three lesions								
Overall*	150	77	24	14	-	2.0		
≤2 cm	40	89	28	19	-	2.0	0.005	Ref.
2.1-3.0	43	76	30	-	-	2.3		1.09 (0.50, 2.35)
3.1-5.0	41	73	34	17	-	1.8		1.31 (0.65, 2.64)
≥5.1	23	62	-	-	-	1.4		3.16 (1.49, 6.72)
More than 4 lesions								
Overall*	290	72	22	10	-	1.9		
≤2 cm	57	90	32	24	-	2.0	0.01	Ref.
2.1-3.0	68	75	17	8	-	2.1		1.53 (0.85, 2.76)
3.1-5.0	89	73	25	-	-	2.0		1.54 (0.88, 2.69)
≥5.1	65	54	-	-	-	1.2		2.55 (1.43, 4.56)

*A significant difference was demonstrated in overall survival among four groups ($p = 0.0001$).

TACE, mainly because of the heterogeneity of the population, therefore it would be helpful for candidates to determine chemoembolization as tailor-made treatment of choice; this is particularly suitable for patients averse to curative therapy and with severely associated diseases, or elderly patients.

The executing rate of patients who actually had undergone TACE and were stratified to TACE in the treatment algorithm was 37% in both Child-Pugh A and B. Namely, the remaining 63% of patients satisfied the criteria of resection or local ablation (non-TACE therapy), which could suggest the possible increase of survival in these patients, if they underwent resection or local ablation. The reason for the lower executing rate might be the less publicity in which the guideline was published one year after the completion of this 6-year study.

The discrimination of patients' survival was feasible in this treatment algorithm between TACE and non-TACE therapies in Child-Pugh B and in part in class A. Further studies are needed to validate the suitability of these guidelines using patients who underwent resection or local ablation and are stratified to four treatments like in the TACE study.

To our knowledge, the present study is the first report to clarify the median and 3- and 4-year survivals of patients treated by TACE and stratified in the four treatments recommended by the Japanese guidelines in Child-Pugh A and B, separately. Interestingly, Llovet *et al.* [10] stated that chemoembolization improved median survival up to 19-20 months in intermediate stage of BCLC classification, which is similar to our results; 1.7 years (20 months) in patients with TACE for 2 or 3 lesions >3 cm and

Table 3. Survival rates of patients treated with TACE stratified to four treatment categories recommended by Japanese guidelines in Child-Pugh A and B.

Criteria of treatment	No. of patients	Survival (%)				Median	*p*	Hazard ratio (95% CI)
		1-yr	3-yr	4-yr	5-yr	(yr)		
Child-Pugh A	3168							
Single lesion	1475	93	73	62	52	5.4	0.0001	Ref.
2-3 lesions, ≤3 cm	506	94	59	40	36	3.5		1.45 (1.18, 1.78)
2-3 lesions, >3 cm	463	87	55	44	31	3.4		1.82 (1.48, 2.25)
≥4 lesions	724	82	46	37	25	2.8		2.39 (2.01, 2.84)
Child-Pugh B	1274							
Single lesion	568	87	53	34	30	3.1	0.0001	Ref.
2-3 lesions, ≤3 cm	239	90	49	33	-	2.8		1.04 (0.79, 1.36)
2-3 lesions, >3 cm	177	68	30	19	-	1.7		2.11 (1.62, 2.75)
≥4 lesions	290	72	22	10	-	1.9		2.17 (1.72, 2.74)

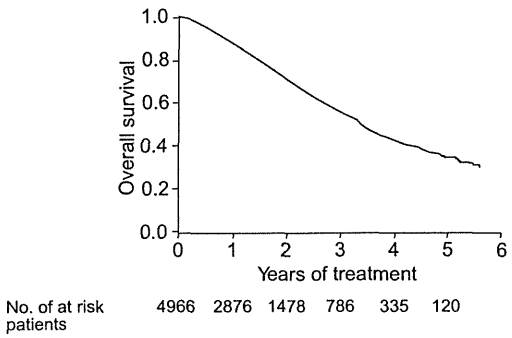

Fig. 2. Overall survival rate of 4966 HCC patients who underwent TACE.

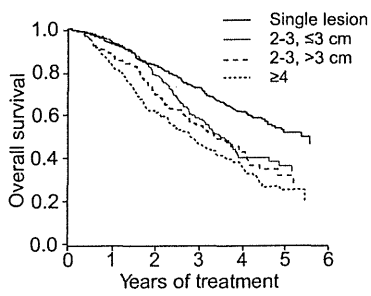

Fig. 3. The survival curves of 3168 patients in Child-Pugh A stratified to four treatment groups according to Japanese guidelines. Overall, there was a significant difference ($p = 0.0001$). There was also a significant difference between two treatment groups except for one; 2 or 3 lesions ≤3 cm *vs.* 2 or 3 lesions >3 cm ($p = 0.06$).

1.9 years (23 months) in those for ≥4 lesions in Child-Pugh B (Table 3). If the criteria for TACE are similar in the intermediate stage of the BCLC staging system [5] and in the treatment algorithm of the Japanese guidelines: equal to or more than 4 lesions and/or 2 or 3 lesions >3 cm [11], the current data will be useful to compare the survival outcomes of TACE in the East and West. The survival rates of our study will be also used as reference data

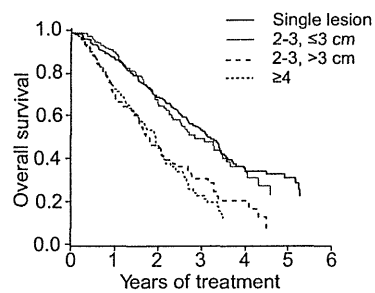

Fig. 4. The survival curves of 1274 patients in Child-Pugh B stratified to four treatment groups. Overall, a significant difference was seen ($p = 0.0001$). A significant difference was observed between two groups except for two; single lesion *vs.* 2 or 3 lesions ≤3 cm, and 2 or 3 lesions >3 cm *vs.* ≥4 lesions.

when clinical trials of TACE with or without anti-angiogenic drugs are newly designed [12,13].

The treatment-related mortality rate was 0.38%, which was slightly improved compared to that of our previous study of 0.5% [9], and much better than that of 2.4% reported by a systematic review [14]. The improvement is mainly attributable to the exclusion criteria of vascular or biliary duct invasion and the decreased proportion of Child-Pugh C patients, from 10% [9] to 4%.

As a limitation of this study, the session numbers of TACE per patient and dosage of anticancer agent used at initial TACE were not available due to lack of inclusion in the questionnaire sheet. Our patients received different TACE protocols for anticancer agent. Given that the large majority of patients were treated with epirubicin or doxorubicin, it could be worth limiting the analysis to these patient cohorts.

In conclusion, the overall median and 3- and 5-year survival of TACE were 3.3 years, 55% and 34% in 4966 HCC patients without vascular invasion and extrahepatic spread. The tumor number, size, liver function, AFP, and DCP were independent predictors. These results will be helpful for physicians to select chemoembolization as optimal therapy for their patients, especially when curative treatment is contraindicated due to severely associated disease and/or aging. The treatment algorithm of the Japanese guidelines might be appropriate to discriminate patient survival

with non-TACE from TACE therapy in Child-Pugh B and in part in A. The survival rates of patients stratified to TACE in these guidelines will be useful for comparing the outcome of TACE in the East and West, and for designing new clinical trials for TACE with and without a novel molecular targeted agent as reference data.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2011.10.021.

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Tolerability of adjuvant chemotherapy with S-1 after curative resection in patients with stage II/III gastric cancer

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Abstract. The results of the Japan Clinical Oncology Group trial demonstrated that adjuvant chemotherapy with S-1 for stage II/III gastric cancer is effective and suggested that this therapy should be adopted as the standard treatment following curative D2 gastric dissection. We reviewed treatment outcomes in 58 consecutive patients who received adjuvant therapy with S-1 for stage II/III gastric cancer following curative D2 dissection; the standard dosage used was determined on the basis of the patient body surface area. Twenty-four patients (41.3%) discontinued treatment before 12 months. Patients who completed 12 months of adjuvant therapy with S-1 were younger and more frequently treated by senior doctors (>15 years of experience) than those who did not. However, no differences existed in pathological features and surgical procedures between groups. Overall survival and relapse-free survival were better in patients who completed 12 months of adjuvant therapy with S-1. Fatigue and nausea were associated with discontinuation of S-1 treatment. In conclusion, immediately after surgery, fatigue and gastrointestinal symptoms of ≤ grade 2 may have a major impact on treatment compliance. Prior to the commencement of S-1 administration, both patients and doctors should be made completely aware of the toxicity, compliance and efficacy issues associated with this adjuvant therapy.

Introduction

S-1 is an oral anticancer preparation composed of a mixture of tegafur [FT, a prodrug of 5-fluorouracil (5-FU)], 5-chloro-2,4-dihydroxypyridine (CDHP, a biochemical modulator that inhibits 5-FU biodegradation) and potassium oxonate (Oxo, added to reduce the gastrointestinal toxicity of 5-FU) (1-3). In the two registration phase II studies in Japan, the rate of response to treatment with S-1 alone exceeded 40% in patients with advanced or recurrent gastric cancer (4,5). The Japan Clinical Oncology Group (JCOG) conducted a randomized prospective controlled study to evaluate the efficacy of single-agent S-1 as adjuvant therapy for patients with stage II/III (Japanese Classification of Gastric Carcinoma, JCGC) (6) gastric cancer following curative D2 dissection (7). When the final analysis was performed in September 2006, 3-year overall survival (OS) was 80.5% for S-1 treated patients and 70.1% for patients who underwent surgery alone. The hazard ratio for death in S-1 treated patients was 0.68 (P=0.0024). The results of this trial demonstrated that adjuvant chemotherapy with S-1 for stage II/III gastric cancer is effective and suggested that this therapy should be adopted as the standard treatment following curative D2 gastric dissection (8).

To investigate the tolerability of adjuvant chemotherapy with S-1 for stage II/III gastric cancer following curative D2 dissection, we reviewed treatment outcomes in patients receiving this adjuvant therapy.

Materials and methods

Patients. Between August 2007 and July 2010, 283 patients underwent gastrectomy for adenocarcinoma of the stomach with curative intent at the National Defense Medical College Hospital (Tokorozawa, Saitama, Japan). Of these, 64 patients (41-84 years old) had pathological stage II/III disease according to the JCGC (6). All patients were informed of the efficacy of the adjuvant chemotherapy trial of S-1 for gastric cancer (ACTS-GC) and provided their consent to the study (7).

Treatment regimen. S-1 was orally administered twice daily for 4 weeks, followed by a 2-week rest. This schedule was repeated every 6 weeks for 12 months until tumor recurrence, observation of unacceptable toxicity levels or refusal by the patient to undergo further treatment. Dosages were assigned according to the patient body surface area: <1.25 m^2, 80 mg/day; 1.25-1.5 m^2, 100 mg/day; and ≥1.5 m^2, 120 mg/day. Dosage modification and treatment interruption were performed according to the protocol in the registration trial (5,7). The dose or treatment schedule was modified at the physician's discretion according to the toxicity profiles. In

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Key words: gastric cancer, adjuvant therapy, tolerability

Table I. Demographic and clinicopathological data.

Clinicopathological data	Total	Completed	Discontinued	P-value
Number of patients	58	34	24	
Age (years)	63.4±8.0	61.4±83.4	66.3±6.5	0.02
Gender				
Male	43 (74.1%)	24 (70.6%)	19 (79.2%)	0.42
Female	15 (25.9%)	10 (29.4%)	5 (20.8%)	
Histological classification				
Intestinal	23 (39.7%)	12 (35.3%)	11 (45.8%)	0.52
Diffuse	32 (55.2%)	19 (55.9%)	13 (54.2%)	
Adenosquamous	2 (3.4%)	2 (5.9%)	0 (0.0%)	
Tumor depth				
T2	24 (41.4%)	15 (44.1%)	9 (37.5%)	0.83
T3	32 (55.2%)	18 (52.9%)	14 (58.3%)	
T4	2 (3.4%)	1 (2.9%)	1 (4.2%)	
Lymph node metastasis				
N0	10 (17.2%)	6 (17.6%)	4 (16.7%)	0.85
N1	27 (46.6%)	15 (44.1%)	12 (50.0%)	
N2	21 (36.2%)	13 (38.2%)	8 (33.3%)	
Stage				
II	25 (43.1%)	15 (44.1%)	10 (41.7%)	0.18
IIIA	18 (31.0%)	8 (23.5%)	10 (41.7%)	
IIIB	15 (25.9%)	11 (32.4%)	4 (16.7%)	
Type of gastrectomy				
Total	30 (51.7%)	20 (58.8%)	10 (41.7%)	0.24
Distal	28 (48.3%)	14 (41.2%)	14 (58.3%)	
Reconstruction				
Billroth I	22 (37.9%)	11 (32.4%)	11 (45.8%)	0.48
Billroth II	3 (5.2%)	2 (5.9%)	1 (4.2%)	
Roux en Y	33 (56.9%)	21 (61.8%)	12 (50.0%)	
Cholecystectomy				
Yes	24 (41.4%)	14 (41.2%)	10 (41.7%)	0.95
No	34 (58.6%)	19 (55.9%)	14 (58.3%)	
Splenectomy				
Yes	20 (34.5%)	12 (35.3%)	8 (33.3%)	0.81
No	38 (65.5%)	22 (64.7%)	16 (66.7%)	
Doctor in charge				
Junior (≤15 yrs)	25 (43.1%)	11 (32.4%)	14 (58.3%)	0.04
Senior (>15 yrs)	33 (56.9%)	23 (67.6%)	10 (41.7%)	
Total amount of S-1 (mg)	16495.4±8851.9	23146.7±3335.6	7350.0±4954.9	<0.0001

principle, if patients had hematological toxic effects of grade 3 or 4 or non-hematological toxic effects of ≥ grade 2, their daily dose was reduced and/or their schedule was changed from a 4-week administration followed by a 2-week rest, to a 2-week administration followed by a 1-week rest.

Measures. If no gross residual disease was evident at the time of surgery and the resection margins were tumor-free on histological examination, surgery was considered curative. Pathological findings in gastric cancer patients were described on the basis of the JCGC (6). Adverse reactions were evaluated according to the common toxicity criteria of the National Cancer Institute, version 3.0 (http://ctep.cancer.gov).

OS was measured from the date of resection to the date of mortality from any cause. Relapse-free survival was measured from the date of resection to the date when relapse was evident by computed tomography, gastrointestinal endoscopic examination, abdominal ultrasonography, upper gastrointestinal series and/or positron emission tomography. Data for the patients who survived were censored in our survival analyses.

Table II. Adverse reactions to adjuvant therapy with S-1 among the 58 patients included in this study.

	No. of patients				Percentage (%)	
Adverse reaction	Grade 1	Grade 2	Grade 3	Grade 4	Grade 1 or 2	Grade 3 or 4
Leukopenia	5	3	3	-	14.0	5.3
Anemia	26	3	-	-	50.9	0
Elevated t-bil level	4	2	-	-	10.5	0
Stomatitis	5	2	-	-	12.3	0
Anorexia	20	1	1	-	36.8	1.8
Nausea	6	2	-	-	14.0	0
Diarrhea	8	6	1	-	24.1	1.8
Skin lesions	8	1	-	-	15.5	0
Fatigue	8	3	-	-	19.3	0
Watering or dry eye	7	6	-	-	22.8	0

t-bil, total bilirubin.

The medication completion rate was measured from the date of treatment commencement to the date of treatment discontinuation. Data for patients in whom S-1 treatment was discontinued due to tumor recurrence or mortality were censored in this analysis. All patients were observed at our hospital or outpatient clinic at 2- to 4-week intervals up to 12 months after surgery, 3- to 4-month intervals during the 2 years of the study and every 6 or 12 months thereafter for 3 years.

Statistical analysis. Statistical calculations were performed using StatView version 5.0 (SAS Institute, Inc., Cary, NC, USA). Data are expressed as the means ± SEM. Statistical analyses were performed using the Mann-Whitney U test or Chi-square test with Fisher's exact test, as appropriate. Survival and medication completion rates were calculated using the Kaplan-Meier method and the significance of the difference was determined by a log-rank test. $P<0.05$ was considered to indicate a statistically significant result.

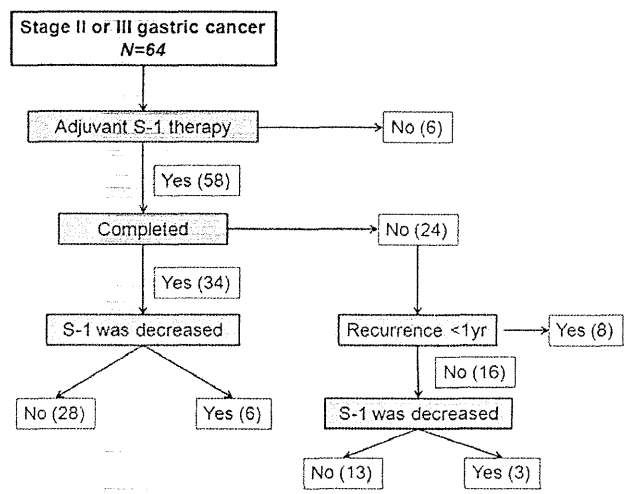

Figure 1. Flowchart of the treatment outcomes of adjuvant therapy with S-1.

Results

Of the 64 patients included in the study, 6 refused adjuvant therapy with S-1 due to age (n=4) or financial concerns (n=2). The remaining 58 patients received S-1 within 8 weeks of surgery (Fig. 1). Twenty-four patients (41.3%) discontinued treatment within 12 months as a result of disease relapse (n=8) and intolerable adverse events (n=16). The S-1 dose was decreased in 9 of the 58 patients (15.5%). Of the 34 patients who underwent treatment for 12 months, the S-1 dose was decreased in 6 (17.6%), and of the 24 patients who discontinued treatment, the S-1 dose was decreased in 3 (12.5%). Among the 58 patients who received S-1 therapy, treatment was continued for at least 3 months in 49 patients (84.5%), at least 6 months in 45 patients (77.6%), at least 9 months in 37 patients (63.8%) and 12 months in 34 patients (58.6%).

Demographic and clinicopathological data of patients are shown in Table I. Patients who discontinued S-1 treatment within 12 months were older than those who completed 12 months of adjuvant therapy. However, no differences were observed in tumor stage and surgery type (gastrectomy, reconstruction or resection of other organs) between the two groups. Patients who completed 12 months of adjuvant therapy with S-1 were more frequently treated by senior doctors (>15 years of experience). More favorable outcomes in OS and relapse-free survival were observed in these patients than in those who discontinued treatment (Fig. 2).

Table II summarizes the data concerning the adverse reactions observed among the 58 patients in this study. No patient had ≥ grade 4 adverse events; however, 3 patients had grade 3 leukopenia. In terms of non-hematological adverse events, grade 3 anorexia was observed in 1 patient and grade 3 diarrhea was observed in 1 patient. The most frequent cause of S-1 treatment discontinuation was tumor recurrence. Non-hematological adverse events such as diarrhea and nausea were also associated with treatment discontinuation (Table III). Fig. 3 shows the medication completion rates. S-1 treatment time was significantly shorter in patients who

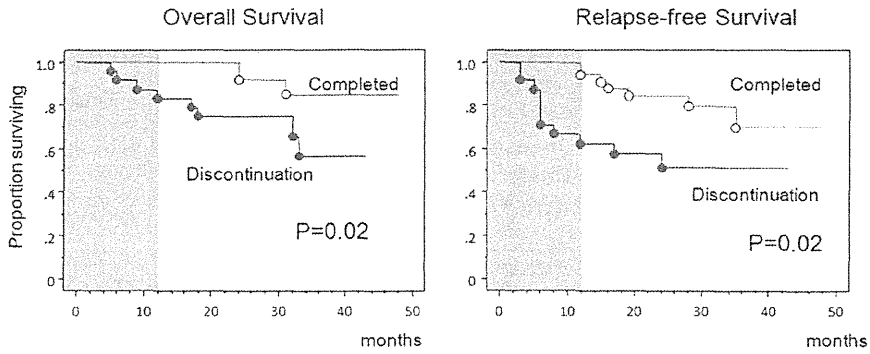

Figure 2. Overall and relapse-free survival rates following curative D2 gastric dissection. Completed, patients who completed 12 months of adjuvant therapy with S-1; Discontinuation, patients who discontinued treatment before 12 months.

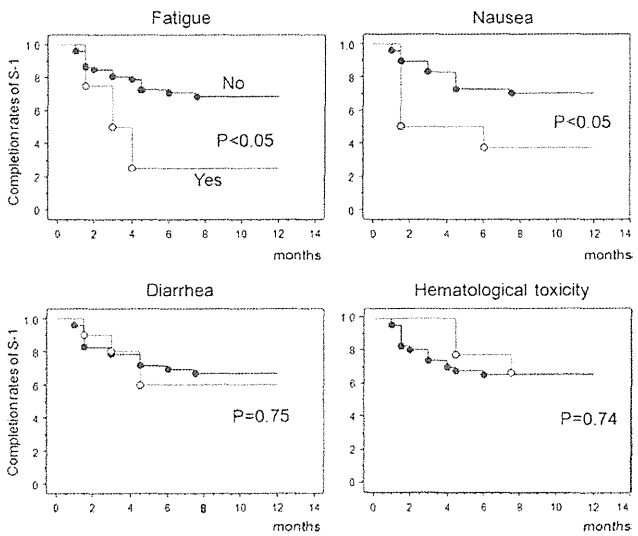

Figure 3. Medication completion rates of adjuvant therapy with S-1 calculated by the Kaplan-Meier method. Discontinuation of S-1 administration due to adverse events was considered as an event.

Table III. Chief causes of S-1 treatment discontinuation.

Adverse reaction	No. of patients	Percentage (%)
Recurrence	8	33.3
Diarrhea	3	12.5
Nausea	3	12.5
Elevated t-bil level	2	8.3
Intestinal obstruction	2	8.3
Fatigue	2	8.3
Neutropenia	1	4.2
Ascites	1	4.2
Stroke	1	4.2
Death of another cause	1	4.2
Total	24	100.0

t-bil, total bilirubin.

experienced fatigue or nausea as an adverse event, whereas diarrhea and hematological toxicity did not significantly affect the period of treatment.

Discussion

This study demonstrated that patients who completed 12 months of adjuvant therapy with S-1 were younger and more frequently treated by doctors with >15 years of experience than those who did not. Non-hematological adverse events such as nausea and fatigue were frequent causes of S-1 treatment discontinuation. Adverse events of ≥ grade 3 were significant causes of treatment discontinuation in a small number of patients.

In a postmarketing survey of S-1 (9), including 3,294 patients with advanced or recurrent gastric cancer, the incidence of adverse reactions following administration of the drug at the usual dose level according to the patient body surface area was 74.1%, which was approximately equal to that obtained in premarketing trials. The major reasons for drug discontinuation during the first and second course of therapy were exacerbation of symptoms (43%) and adverse drug reactions (33%). Therefore, to facilitate S-1 administration for prolonged time periods, the incidence of adverse reactions should be reduced. To accomplish this goal, several regimens have been established (10). Kimura *et al* developed a new S-1 dosing regimen in which S-1 is administered for a 2-week period separated by 1-week drug-free intervals, as adverse reactions due to S-1 therapy begin to appear 2-3 weeks after initial dosing (11). Sakuma *et al* also proposed alternate-day treatment with S-1 as a strategy for reducing toxicity, although the total dose of this regimen was 75% that of standard treatment (12). Both regimens decreased the incidence of adverse reactions and improved treatment compliance when compared with the conventional 4-week administration followed by a 2-week rest regimen.

In the ACTS-GC trial, 143 of 517 (27.7%) patients discontinued S-1 treatment due to adverse events, which was consistent with our results (27.6%). Only 5% patients in the ACTS-GC trial had metastasis or relapse of gastric cancer. Our study, which includes potentially more cases of advanced

stage disease than the ACTS-GC trial, involved relatively shorter time periods of the treatment than the ACTS-GC trial.

Patients who experienced fatigue or nausea as adverse events continued S-1 treatment for significantly shorter time periods. However, diarrhea and hematological toxicity did not significantly affect the treatment period. Following gastrectomy, fatigue and gastrointestinal symptoms such as nausea and appetite loss, even of ≤ grade 2, appeared to have a major impact on treatment compliance.

In conclusion, the completion rate of S-1 treatment did not depend on the type of surgical procedures, i.e., gastrectomy, reconstruction or resection of other organs. Fatigue and gastrointestinal symptoms affected the period of treatment continuation. In addition, patients who completed 12 months of adjuvant therapy with S-1 were more frequently treated by doctors with ≥15 years of experience. Thus, to facilitate the continuation of adjuvant therapy with S-1, patients and doctors must be made completely aware of the issues of toxicity, compliance and efficacy associated with this therapy.

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Membranous and cytoplasmic expression of epidermal growth factor receptor in metastatic pancreatic ductal adenocarcinoma

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Abstract. Recent studies indicate the clinical significance of the cellular localization of epidermal growth factor receptor (EGFR) in a variety of cancer types. Internalization of activated EGFR is reported to be closely associated with patient prognosis. This study investigated the clinical significance of the immunohistochemical localization of EGFR in patients with metastatic pancreatic cancers compared to those with surgically resected pancreatic cancers. Using 44 surgically resected primary pancreatic cancers and 40 primary or metastatic tumors from 20 autopsied patients with far advanced pancreatic cancers, the incidence of membranous and cytoplasmic EGFR overexpression was compared between primary tumors and far advanced tumors by immunohistochemistry using the Dako EGFR pharmDx™ kit, a global standard kit for EGFR assay. In the 44 surgically resected cancers, 13 (30%) exhibited membranous overexpression of EGFR, comprising 1 case (2%) with score 3+ and 12 cases (27%) with score 2+ and 10 (23%) exhibited cytoplasmic overexpression of EGFR. In the 40 tumors at a far advanced stage, the percentage of samples exhibiting positivity for membranous and cytoplasmic EGFR overexpression was 48% (19 of 40) comprising 7 (18%) with score 2+ and 12 (30%) with score 3+ and 33% (13 of 40), respectively. The far advanced tumors tended to show membranous and cytoplasmic EGFR overexpression more frequently than the surgically resected tumors, although the difference was not significant. These findings suggest that membranous and cytoplasmic overexpression of EGFR may be indicative of the potential aggressiveness of pancreatic cancers.

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Key words: epidermal growth factor receptor, metastatic pancreatic cancer, cytoplasmic expression

Introduction

Despite recent advances in diagnostic and therapeutic techniques, pancreatic carcinoma is one of the most lethal malignancies among cancers. The 5-year survival rate of patients having primary pancreatic cancer after complete resection does not reach 15% (1), while the overall 5-year survival rates in patients having inoperable pancreatic cancer are desperately low, ranging from 0.4 to 4% (2,3).

Currently, gemcitabine is a key drug not only for treating advanced pancreatic cancer (4) but also as an adjuvant chemotherapy regimen for resectable pancreatic cancer (5,6). Furthermore, molecular targeting of epidermal growth factor receptor (EGFR) or vascular endothelial growth factor (VEGF) has recently been developed to treat these lesions (7,8). Moore *et al* reported in a phase III trial of patients with advanced pancreatic cancer, that erlotinib, a tyrosine kinase inhibitor of EGFR, in combination with gemcitabine was superior to gemcitabine alone when progression-free and overall survival were compared between the two groups (8).

EGFR, one of the tyrosine kinase receptors of the ErbB family, is reported to be expressed immunohistochemically in 10-30% of patients with solid tumors including pancreatic carcinoma (9,10). Tyrosine phosphorylation in EGFR protein in cancer cells leads to activation of several downstream intracellular substrates and plays a pivotal role in tumor proliferation, invasion and metastasis (11). Recent studies have suggested that the EGFR gene copy number and expression obtained by fluorescence *in situ* hybridization (FISH) and immunohistochemistry (IHC) predict the clinical response of a tumor to gefitinib, a tyrosine kinase inhibitor of EGFR, in patients with non-small cell lung cancer (12-14). Furthermore, recent studies have found that mutations of the EGFR gene at the restricted region, e.g., exon 19 and exon 21, were closely correlated with response to gefitinib therapy (15-20). However, the relevance of EGFR expression in pancreatic cancer with therapeutic response has remained to be verified (8).

Although immunohistochemical expression of EGFR can also be recognized as positive membranous staining,

Table I. Clinicopathological characteristics of the patients and tumors.

	Surgically resected cancers (n=44)	Far advanced cancers (n=20)	
Parameter	n (%)	n (%)	
Age (mean ± SD, years)	63.3±3.7	57.3±5.7	
<65	23 (52)	13 (65)	
≥65	21 (47)	7 (35)	
Gender			
Male	34 (77)	16 (80)	
Female	10 (23)	4 (20)	
Tumor site			
Head	36 (82)	12 (60)	
Body and/or tail	8 (18)	8 (40)	
Stage			
I	1 (2)		
II	32 (73)		
III	8 (18)		
IV	3 (7)		
Grade			
1	12 (27)	5 (25)[a]	2 (10)[b]
2	28 (64)	3 (15)[a]	9 (45)[b]
3	4 (9)	12 (60)[a]	9 (45)[b]
Median survival (mean ± SD, month)	24.5±10.3		

[a]Primary cancers. [b]Hepatic metastases. SD, standard deviation.

cytoplasmic expression of EGFR can frequently be observed in cancer cells of the pancreas. We previously reported that high cytoplasmic expression of EGFR in primary pancreatic cancer was significantly correlated with higher histological grade and poorer survival (10), suggesting that cytoplasmic EGFR expression could indicate a potentially aggressive or metastatic feature of pancreatic cancer. However, it is unclear whether localization of EGFR expression differs between primary and metastatic sites of pancreatic cancers at surgically resectable stages and those at inoperable far advanced stages.

The present study compared immunohistochemically the levels and localization of EGFR expression between surgically resected primary pancreatic cancers and far advanced cancers obtained at autopsy, in order to clarify the clinical impact of membranous and cytoplasmic EGFR overexpressions in far advanced pancreatic cancers.

Materials and methods

Patients and tumor specimens. This study was performed with approval by the Internal Review Board on Ethical Issues of the National Defense Medical College, Japan. The subjects of this study were 44 patients who underwent surgery with curative intent for primary pancreatic cancers between 1987 and 2000 at the National Defense Medical College Hospital, Tokorozawa, Japan. The clinicopathological characteristics of these cases are summarized in Table I.

The mean patient age was 63.3 years [±3.7 standard deviation (SD)]. Thirty-four (77.3%) were men and 10 (22.7%) were women. More than 80% of tumors were located in the head of the pancreas. As for stage, approximately 90% of the patients were assigned to stage II or stage III (21). Histologically, all 44 patients had invasive ductal adenocarcinoma of the pancreas, and the majority of the patients had moderately differentiated tubular adenocarcinoma. The median survival time was 24.5 months (±10.3 SD).

In addition, a total of 40 tumor specimens from primary sites and hepatic metastatic sites were obtained at autopsy from 20 patients who had died of inoperable far advanced pancreatic cancer between 1980 and 2001 at the same hospital (Table I).

Using these tumor specimens from a total of 64 patients, formalin-fixed paraffin-embedded tissue blocks were prepared, and sections were cut and stained with hematoxylin and eosin (H&E) for routine histopathological examination. Because surgically resected specimens had been cut routinely for pathology specimens once a weak periodically, the duration of formalin fixation of the surgically resected specimens varied from 1 to 6 days. Likewise, the duration of formalin fixation of the autopsied tissues varied from 1 to 6 days. All specimens were diagnosed as ductal adenocarcinomas of the pancreas. After a histological review of the sections by three observers (T.E., H.T. and S.U.), a representative tissue block was selected from each surgically resected primary tumor, each primary tumor obtained by autopsy, and each metastatic tumor obtained

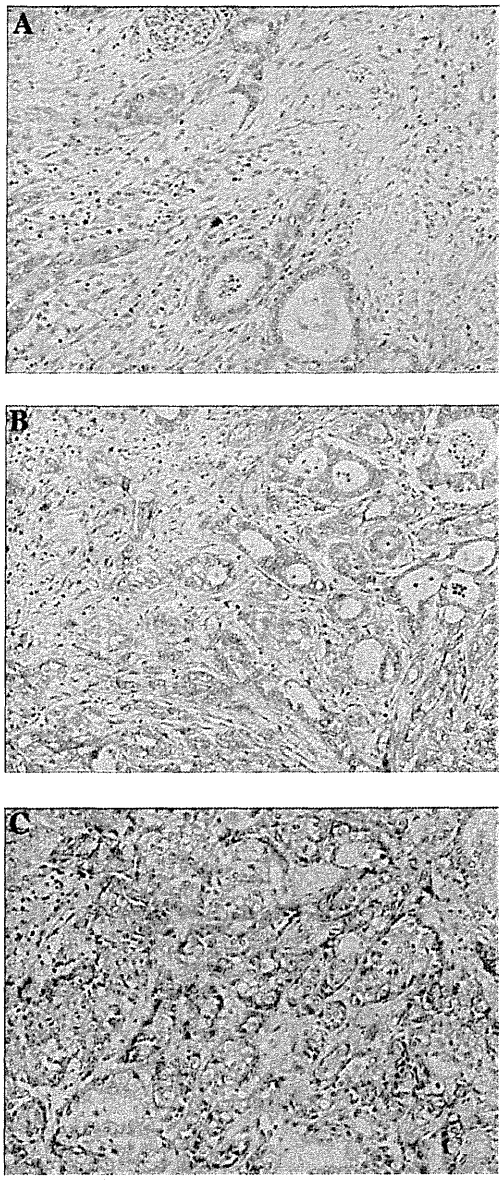

Figure 1. Representative cases of pancreatic ductal adenocarcinoma showing scores of 1+, 2+ and 3+ for membranous EGFR expression. (A) Score 1+, incomplete membrane staining is weakly visible. (B) Score 2+, the entire circumference of the cell membrane is weakly stained. (C) Score 3+, the entire circumference of the cell membrane is heavily stained. Immunoperoxidase stain, x200.

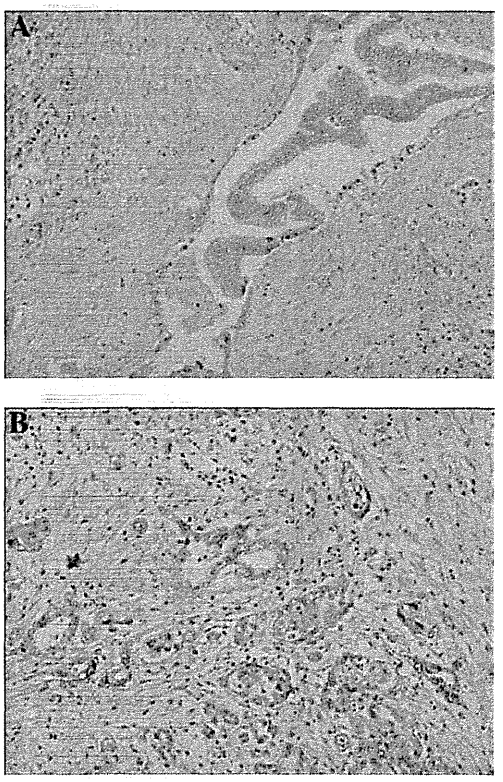

Figure 2. Representative cases of pancreatic ductal adenocarcinoma showing scores of 1+ and 2+ for EGFR cytoplasmic expression. (A) Score 1+, faint diffuse cytoplasmic staining is detected. (B) Score 2+, moderate to strong cytoplasmic staining and strong granular staining is observed. Immunoperoxidase stain, x200.

by autopsy. These tumor tissue blocks were subjected to immunohistochemical studies.

Histological classification. The three observers graded the degree of tumor differentiation. Tumor differentiation was classified into Grade 1 (well-differentiated type), Grade 2 (moderately differentiated type) and Grade 3 (poorly differentiated type), according to the degree of tubular formation (21). The grade of each primary cancer was defined according to the findings in the widest area of the representative section of the cancer.

Immunohistochemistry. Immunohistochemical staining for EGFR was performed using the EGFR pharmDx™ kit (Dako, Carpinteria, CA, USA), a global standard kit for EGFR assay approved by the US Food and Drug Administration (US FDA). Sections were deparaffinized in two sequential xylene baths (5 min), 100% ethanol (3 min) and 95% ethanol (3 min), followed by a 5-min single wash in wash-buffer solution (Dako). Subsequently, at room temperature, the section was rinsed in wash-buffer for 5 min, incubated in proteinase K solution (Dako) for 5 min, rinsed again in the wash-buffer for 5 min, incubated in peroxidase blocking agent for 5 min, rinsed, incubated with the primary EGFR antibody or negative control reagent for 30 min, rinsed, incubated with visualization reagent for 30 min, rinsed twice with the buffer, incubated with substrate chromogen solution for 5 min and finally rinsed again with the buffer. Slides were counterstained with hematoxylin and rinsed gently in reagent quality water. The positive and negative controls used were formalin-fixed, paraffin-embedded pellets of HT-29 and CAMA-1 cell lines, which expressed and did not express EGFR, respectively (Dako).

Immunohistochemical evaluation. Immunohistochemical evaluation was performed for both the cell membrane and cytoplasm, separately, for the primary or metastatic carcinoma samples. The level of membranous EGFR expression was stratified into 4 groups (scores 0, 1+, 2+ and 3+) according to the criteria for the HER2 test (HercepTest) (22). In detail, when membranous staining was observed in <10% of the tumor