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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

AFP, AFP-L3, DCP, and GP73 as markers for monitoring treatment response and recurrence and as surrogate markers of clinicopathological variables of HCC

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Abstract
Background Alpha-fetoprotein (AFP), lens culinaris agglutinin-reactive fraction of AFP (AFP-L3), des-γ-carboxy prothrombin (DCP), and Golgi protein-73 (GP73) have been used or proposed as tumor markers for hepatocellular carcinoma (HCC).
Methods They were measured in 96 patients undergoing hepatectomy for HCC to investigate their treatment response and association with variables linked with tumor invasiveness and/or prognosis. Values at 1 month post-surgery in the 77 patients without recurrence within 6 postoperative months were adopted as those after surgery.
Results GP73 levels did not change after hepatectomy, but levels of other markers decreased and areas under receiver operating characteristic curves (95% CI) were: 0.64 (0.56–0.72), 0.63 (0.55–0.71), 0.79 (0.73–0.86), and 0.63 (0.55–0.71) for AFP, AFP-L3, DCP, and combination of AFP and AFP-L3, respectively. Cutoff points giving specificities of 96.1% (sensitivities at these points) were: 124 ng/mL (28.1%), 10% (21.9%), and 60 mAU/mL (52.1%), for AFP, AFP-L3, and DCP, respectively. The combination of AFP and AFP-L3 provided a sensitivity of 26.0% at a specificity of 96.1%. The increased DCP value was, or tended to be, associated with a larger tumor, vascular invasion, intrahepatic metastases, and a lower grade of tumor cell differentiation. Although similar associations were found between AFP and vascular invasion as well as a lower grade of tumor cell differentiation, no such relationship was found with AFP-L3.
Conclusions DCP is a more effective tumor marker than AFP and AFP-L3. AFP-L3 showed comparable accuracy to AFP but no benefit was found in their combination. GP73 did not play a significant role in this context. Indices of tumor invasiveness were most closely associated with DCP.

Keywords AFP · AFP-L3 · DCP · GP73 · HCC

Abbreviations

AFP	Alpha-fetoprotein
AFP-L3	Lens culinaris agglutinin-reactive fraction of AFP
AUROC	Areas under ROC curve
CI	Confidence interval
DCP	Des-γ-carboxy prothrombin
GP73	Golgi protein-73
HCC	Hepatocellular carcinoma
ROC curve	Receiver operating characteristic curve

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Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of death from cancer worldwide and its incidence has been increasing in countries where the incidence of HCV infection is increasing [1]. Serum alpha-fetoprotein (AFP) has been used as a de facto standard biological tumor marker of HCC since the 1970s. However, AFP can be elevated in patients with chronic hepatitis and/or cirrhosis in the absence of HCC, leading to an unreliable role of AFP in surveillance [2].

To date, several other tumor markers have been investigated as complements for AFP. Plasma des-γ-carboxy prothrombin (DCP), also known as protein induced by vitamin K deficiency or antagonist-II (PIVKA-II), was first reported in 1984 [3]; and it has been widely used for two decades in Japan, especially since 1997 when DCP could be measured with a tenfold higher sensitivity [4]. The lens culinaris agglutinin-reactive fraction of alpha-fetoprotein (AFP-L3) has also been proposed as a marker for HCC, and has been commonly used in Japan since the 1990s. AFP-L3 is a fucosylated variant of AFP and the percentage of AFP-L3 over total AFP levels is used as an index of HCC [5]. AFP-L3 is reportedly more specific to HCC than AFP, representing its malignant potential [6]. In the meantime, Golgi protein-73 (GP73) has recently been shown to have a superior diagnostic ability to AFP [7, 8].

In addition to their use as diagnostic tools for surveillance, biological tumor markers play several important roles in the following aspects: monitoring treatment response, as indices of specific clinicopathological variables that provide prognostic information, and detecting disease relapse after curative treatment [9]. However, these aspects have never before been investigated in a comprehensive manner.

In the present study, we investigated the roles of AFP, DCP, AFP-L3, and GP73 as HCC tumor markers, paying particular attention to these unaddressed issues in patients with HCC undergoing liver resection.

Materials and methods

Patients

Between June 2007 and August 2008, 116 consecutive patients who were scheduled to undergo liver resection for suspected HCC were enrolled and followed prospectively at the Hepato-Biliary-Pancreatic Surgery Division of Tokyo University Hospital, Tokyo, Japan (Fig. 1). The

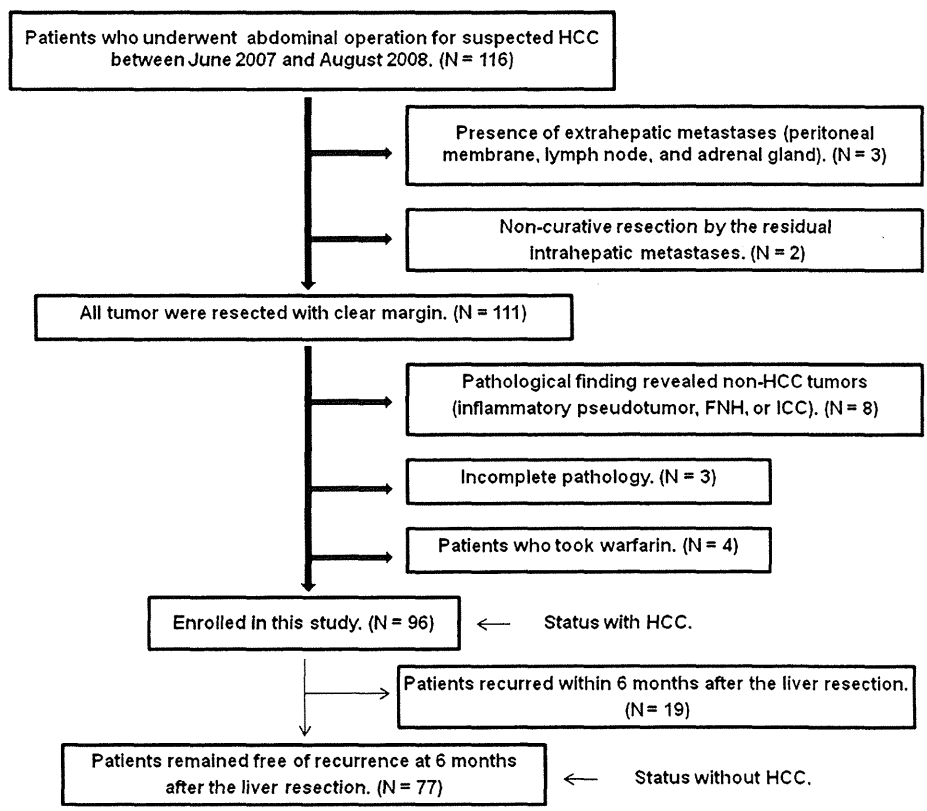

Fig. 1 Schematic flowchart of enrolled patients. *HCC* hepatocellular carcinoma, *FNH* focal nodular hyperplasia, *ICC* intrahepatic cholangiocarcinoma

study protocol was approved by the institutional ethics board and written informed consent was obtained from each subject before treatment. Preoperative diagnosis of HCC was made by using abdominal ultrasonography and dynamic computed tomography (CT) scanning. Other imaging modalities were added if necessary. The indication of liver resection was determined according to previously described criteria [10].

Curative resection was defined as the removal of all recognizable tumors with a clear margin. HCC diagnosis was finally confirmed by pathological examination of the resected specimens in all cases.

From these 116 patients, 96 were finally included. Twenty patients were excluded for the following reasons: three by the presence of extrahepatic metastases found intraoperatively, two due to non-curative liver resection, eight in whom pathological examination revealed non-HCC tumors (intrahepatic cholangiocarcinoma in five, inflammatory pseudotumors in two, focal nodular hyperplasia in one), three due to the incomplete pathological examination as a result of total necrosis of the tumor, and four by the prescription of warfarin, a DCP-inducing agent (Fig. 1). Patients' background characteristics and tumor characteristics are presented in Tables 1 and 2, respectively.

Table 1 Patient background characteristics ($N = 96$)

Variables	N
Age (years)[a]	67.0 (21.0–84.0)
Gender	
Male	80 (83.3%)
Female	16 (16.7%)
Hepatitis B virus infection[b]	
No	70 (72.9%)
Yes	26 (27.1%)
Hepatitis C virus infection[b]	
No	50 (52.1%)
Yes	46 (47.9%)
Indocyanine green retention rate at 15 min (ICG R15)[c]	
<10%	36 (37.5%)
10–19%	46 (47.9%)
20–29%	9 (9.4%)
30–39%	5 (5.2%)
Child–Turcotte–Pugh grade[d]	
A	80 (83.3%)
B	16 (16.7%)
Background liver status[e]	
Normal liver	5 (5.2%)
Chronic hepatitis	57 (59.4%)
Cirrhosis	34 (35.4%)

24 patients were negative for both hepatitis B and C virus infection

[a] Expressed as median with range

[b] No patients were positive for both hepatitis B and C virus infection

[c] No patient was ICG R15 ≥40%

[d] No patient was Child–Turcotte–Pugh grade C

[e] Pathological findings assessed in the resected specimen

Follow-up after hepatectomy

Monthly follow-up was conducted by assessment of tumor markers (AFP, DCP, and AFP-L3) and ultrasound. Dynamic CT scan was conducted at 3 and 6 months post-surgery. We defined recurrence as the appearance of new lesions with radiological features typical of HCC, as confirmed by at least two imaging methods [11].

Tumor marker measurement

Blood samples for tumor markers were taken both 7 days prior to and 1 month after liver resection. Serum AFP levels were measured by an immunometric assay (ST AIA-PACK AFP, Tosoh, Tokyo, Japan). Serum AFP-L3 levels were measured by lectin-affinity electrophoresis coupled with antibody-affinity blotting (LBA AFP-L3, Wako Pure Chemical Industries, Osaka, Japan), and were expressed by the ratio of AFP-L3 to total AFP (%) [5, 12]. AFP-L3 levels were not detected when AFP concentrations were <10 ng/mL, thus AFP-L3 values were defined as 0% in this range [13]. Plasma DCP levels were measured by the two-step enzyme immunoassay (Picolumi PIVKA-II, Eizai, Tokyo, Japan) [4]. Serum GP73 autoantigen and GP73 autoantibody levels were measured by prototype enzyme-linked immunosorbent assays (Quanta Lite™ GP73 Antigen ELISA and Quanta Lite™ GP73 Antibody ELISA, INOVA Diagnostics Inc., San Diego, USA). Assay results were assessed spectrophotometrically and expressed as optical densities (OD).

Relationship between tumor markers

In the measurement of multiple tumor markers, marker values should ideally be independent to each other. With this in mind, we assessed the relationship between each tumor marker before liver resection.

Ability of tumor markers to assess therapeutic response

The marker values of 96 patients before liver resection were defined as those with HCC. Of these, 77 patients remained free of recurrence 6 months after liver resection. Marker values of these 77 patients 1 month post-surgery were defined as values at complete tumor remission. To assess the ability of tumor markers to reflect the therapeutic response after curative resection, we constructed receiver operating characteristic (ROC) curves, and calculated the

Table 2 Characteristics of tumor-related variables and correlations with tumor marker values ($N = 96$)

Variables	*N* (%)	AFP (ng/mL)[a]	AFP-L3 (%)[a]	DCP (mAU/mL)[a]
Tumor size (mm)				
≤20	33 (34.4)	5.0 (3.0–32.0)	0.0 (0.0–1.0)	20.0 (16.0–29.0)
20–50	39 (40.6)	7.0 (4.0–384.0)	0.0 (0.0–9.4)	74.0 (22.0–203.0)
>50	24 (25.0)	17.5 (5.3–811.3)	0.5 (0.0–14.0)	924.0 (220.5–10286.0)
		$r_S = 0.25$, $P < 0.05$	$r_S = 0.14$, $P = 0.17$	$r_S = 0.66$, $P < 0.0001$
Tumor number				
1	64 (66.7)	7.5 (4.0–142.8)	0.0 (0.0–2.3)	70.0 (19.0–414.8)
2	23 (24.0)	10.0 (4.0–126.0)	0.3 (0.0–9.4)	36.0 (23.0–288.0)
≥3	9 (9.3)	37.0 (3.0–1998.0)	0.5 (0.0–7.5)	164.0 (17.0–459.0)
		$P = 0.88$	$P = 0.76$	$P = 0.93$
Capsular formation				
No	7 (7.3)	11.0 (4.0–53.0)	0.0 (0.0–0.5)	22.0 (11.0–288.0)
Yes	89 (92.7)	8.0 (4.0–152.0)	0.0 (0.0–6.8)	69.0 (20.5–384.0)
		$P = 0.85$	$P = 0.62$	$P = 0.19$
Capsular infiltration[b]				
No	21 (23.6)	6.0 (3.0–114.0)	0.0 (0.0–2.3)	27.0 (17.5–173.5)
Yes	68 (76.4)	9.5 (4.0–357.3)	0.0 (0.0–10.0)	80.0 (21.5–414.8)
		$P = 0.26$	$P = 0.70$	$P = 0.14$
Vascular invasion				
No	58 (60.4)	6.0 (3.0–53.5)	0.0 (0.0–3.5)	31.5 (18.5–224.3)
Yes	38 (39.6)	17.5 (5.0–795.8)	0.5 (0.0–10.4)	132.5 (27.3–789.8)
		$P < 0.05$	$P = 0.23$	$P < 0.05$
Intrahepatic metastases				
No	84 (87.5)	7.5 (4.0–119.5)	0.0 (0.0–6.1)	45.5 (20.0–200.5)
Yes	12 (12.5)	78.5 (4.0–687.0)	0.3 (0.0–3.2)	377.0 (58.3–12143.0)
		$P = 0.38$	$P = 0.92$	$P = 0.06$
Tumor differentiation				
Well	11 (11.5)	4.0 (2.0–14.0)	0.0 (0.0–0.5)	22.0 (16.0–108.0)
Moderate	70 (72.9)	7.0 (4.0–143.5)	0.0 (0.0–5.3)	67.5 (19.8–312.0)
Poor	15 (15.6)	34.0 (7.0–1072.0)	0.5 (0.0–21.8)	173.0 (32.0–9888.0)
		$P = 0.10$	$P = 0.10$	$P < 0.05$

AFP alpha-fetoprotein, *AFP-L3* lens culinaris agglutinin-reactive fraction of AFP, *DCP* des-γ-carboxy prothrombin

[a] Median with inter-quartile range

[b] We assessed 89 patients with tumor capsular formation

areas under ROC curves (AUROCs). The sensitivity/specificity at several cutoff points which were conventionally used and of specific interest in the present study were also calculated.

AFP-L3 is always measured simultaneously with AFP and its significance depends on that of AFP [6]. Similarly, AFP-L3 is thought to play a role in patients with intermediately elevated AFP values, because of its high specificity [6, 14]. With this in mind, the significance of the AFP-L3 measurement in addition to AFP was investigated through the ROC curve constructed by combining the two assays. In the combination assays, three different cutoff ranges were set as follows: in the low (AFP value <20 ng/mL) and the high (AFP value ≥400 ng/mL) cutoff ranges, cutoff points were varied according to AFP values; whereas in the intermediate cutoff range (20 ng/mL ≤ AFP value < 400 ng/mL), cutoff points were varied according to AFP-L3 values where AFP values <20 ng/mL were always classified into marker negative while AFP values ≥400 ng/mL were classified into marker positive. For example, when the AFP-L3 value of 15% was adopted as the cutoff value in the intermediate cutoff range, a patient with AFP of 800 ng/mL was classified as marker positive even when the AFP-L3 value was 5%. Here, the transition point of AFP at 400 ng/mL was adopted according to the EASL 2000 criteria [15].

Association of tumor marker values with clinicopathological variables representative of tumor invasiveness and prognosis

We assessed the association of respective marker values with clinicopathological variables that have been reported as being representative of tumor invasiveness and/or poor prognosis. Variables were assessed pathologically on the resected specimens (Table 2). Vascular invasion was defined as the presence of portal vein invasion, venous invasion, or biliary invasion. Multiple primary tumor nodules and intrahepatic metastases were differentiated by using the guidelines proposed by the Liver Cancer Study Group of Japan [16].

Alteration of marker positive/negative status through hepatectomy and postoperative marker positive status as an early indicator of tumor recurrence

We examined the alteration in the marker positive/negative status through treatment in a patient-by-patient manner. Then, we assessed the association between marker positive/negative status and tumor recurrence during the early postoperative phase, recurrence within 6 months of liver resection.

Statistical analysis

Marker values were expressed as medians with inter-quartile ranges. Correlations between marker values were analyzed by Spearman's rank correlation (r_S). AUROCs for markers were compared by Wilcoxon's rank sum test [17]. Associations between marker values and clinicopathological variables were analyzed by Wilcoxon's rank sum test or by the Kruskal–Wallis test, as appropriate. P values <0.05 were accepted as statistically significant. All statistical analyses were performed using the GraphPad Prism® computer software, version 5 (GraphPad Software Inc., San Diego, CA, USA).

Results

Relationship between tumor markers

The values of AFP and AFP-L3 showed a close association ($r_S = 0.83$), and those of GP73 autoantigen and GP73 autoantibody were moderately related ($r_S = 0.48$). No significant correlation was found in any of the other combinations of tumor marker values (Table 3).

Ability of tumor markers to assess therapeutic response

Tumor marker values of 96 patients before liver resection and those of 77 patients 1 month post-surgery in whom no recurrence was detected until 6 months post-surgery are depicted in Fig. 2.

Since GP73 did not appear to be a tumor marker representing tumor status, the following analyses exclude GP73 autoantigen and GP73 autoantibody results. The overall abilities of these tumor markers and the combination of AFP and AFP-L3 to reflect the therapeutic response after curative resection are depicted in Fig. 3.

Sensitivity/specificity at various cutoff points for these tumor markers is demonstrated in Table 4. The sensitivity of DCP was higher than those of other markers at a specificity of 96.1%, while sensitivities of other markers were of a similar extent. At a specificity of 97.4%, sensitivities of AFP-L3 and the combination of AFP and AFP-L3 were 16.7 and 26.0%, respectively ($P = 0.12$).

Association of tumor marker values with clinicopathological variables representative of tumor invasiveness and prognosis

Correlations between AFP, AFP-L3, and DCP values and clinicopathological variables are shown in Table 2. Increased DCP value was associated with the indices representing tumor growth and invasiveness such as tumor size, presence of vascular invasion, and lower grade of tumor cell differentiation. Although similar, albeit moderate, tendency was observed in the relationship between AFP value and these variables, no apparent association was found between AFP-L3 value and these indices.

Alteration of marker positive/negative status through hepatectomy and postoperative marker positive status as an early indicator of tumor recurrence

In this analysis, the cutoff points for various marker values were set at those which gave the equivalent specificities

Table 3 Correlation (r_S) between the levels of respective tumor marker values in 96 patients with HCC before liver resection

	AFP (ng/mL)	AFP-L3 (%)	DCP (mAU/mL)	GP73 autoantigen (OD)	GP73 autoantibody (OD)
AFP (ng/mL)	–	0.83	0.14	0.07	0.05
AFP-L3 (%)	–	–	0.11	0.05	0.04
DCP (mAU/mL)	–	–	–	−0.18	−0.17
GP73 autoantigen (OD)	–	–	–	–	0.48

AFP alpha-fetoprotein, *AFP-L3* lens culinaris agglutinin-reactive fraction of AFP, *DCP* des-γ-carboxy prothrombin, *GP73* Golgi protein-73

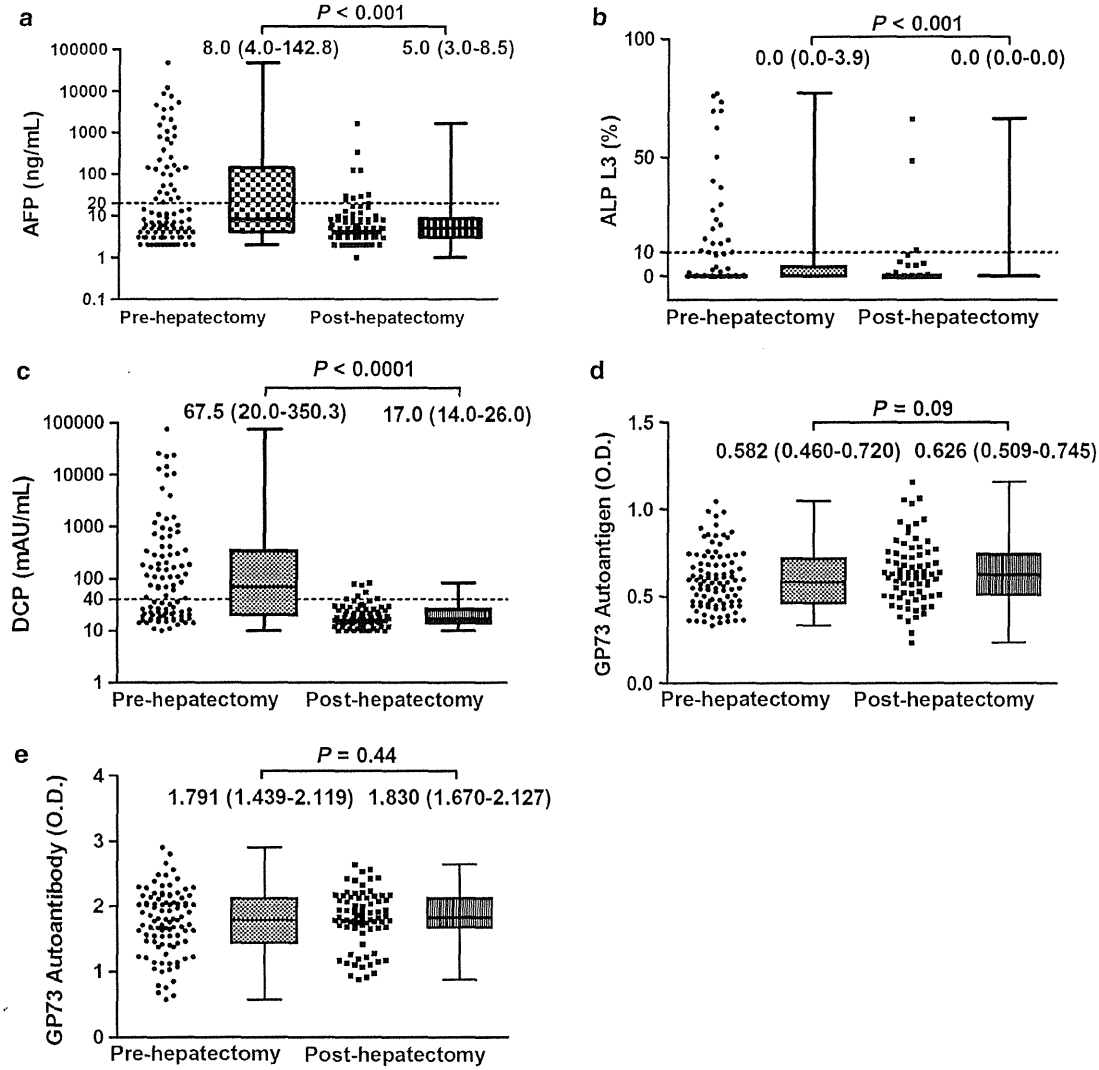

Fig. 2 Tumor marker values before hepatectomy ($N = 96$) and 1 month after hepatectomy ($N = 77$). Values after hepatectomy exclude the 19 patients whose tumor recurred within six postoperative months. **a** AFP, **b** AFP-L3, **c** DCP, **d** GP73 autoantigen, **e** GP73 autoantibody. Marker distributions are expressed by *scatter dot plots* and *box* and *whiskers*. *Top* and *bottom of boxes* are first and third quartiles, respectively. *Length of box* represents inter-quartile range within which 50% values were located. *Line through middle of each box* represents median. *Error bars* show minimum and maximum values (range). *Figures above box* and *whiskers* represent medians (inter-quartile ranges). *Dashed lines* represent cutoff values most frequently used in clinical settings and as follows: AFP, 20 ng/mL; AFP-L3, 10%; DCP, 40 mAU/mL

taking into account conventionally used values [18]. They were 200 ng/mL for AFP, 10% for AFP-L3, and 60 mAU/mL for DCP (Table 4). Specificities at these points were 97.4% for AFP, 96.1% for AFP-L3, 96.1% for DCP, and 96.1% for the combination of AFP and AFP-L3. Analysis was conducted in patients with positive preoperative marker status. The rates of patients who still had marker positive status 1 month post-surgery were as follows: 6/21 (28.6%) for AFP, 6/21 (28.6%) for AFP-L3, 4/50 (8.0%) for DCP, and 8/25 (32.0%) for the combination of AFP and AFP-L3. In these patients, imaging-proven recurrences within six postoperative months were detected with the following incidences: 4/6 (66.7%), 4/6 (66.7%), 2/4 (50.0%), and 5/8 (62.5%) for AFP, AFP-L3, DCP, and the combination of AFP and AFP-L3, respectively. Whereas, recurrence rates within six postoperative months for those patients whose preoperative positive marker status turned negative 1 month post-surgery were as follows: 4/15 (26.7%), 2/15 (13.3%), 11/46 (23.9%), and 3/17 (17.7%) for AFP, AFP-L3, DCP, and the combination of AFP and AFP-L3, respectively. Therefore, the risk of developing early postoperative recurrence in patients whose marker status remained positive 1 month post-surgery was higher than in patients whose marker values changed to negative

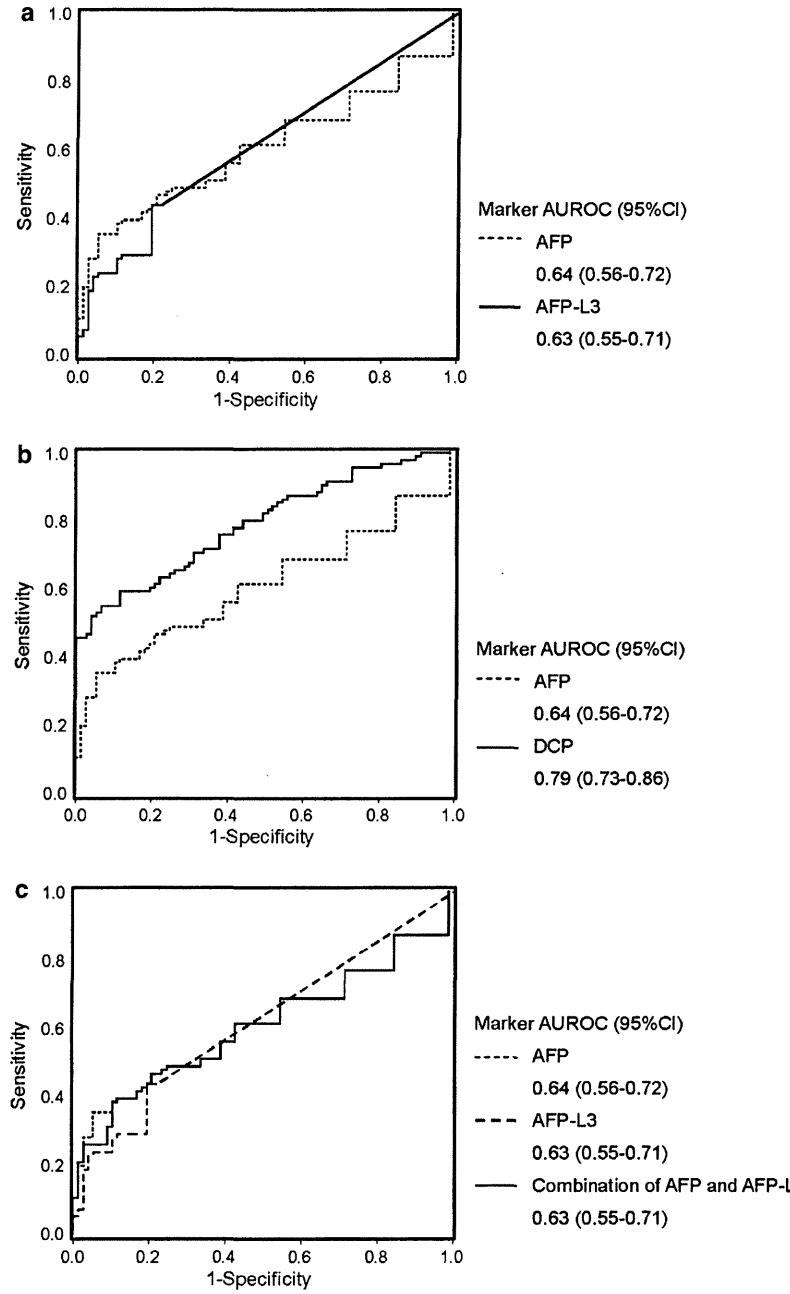

Fig. 3 Receiver operating characteristic (ROC) curves comparing tumor markers and corresponding area under ROCs (AUROCs) (95% CI). **a** AFP versus AFP-L3. $P = 0.73$. **b** AFP versus DCP. $P < 0.005$. **c** AFP or AFP-L3 versus combination of AFP and AFL-L3. $P = 0.16$ (AFP vs. combination of AFP and AFP-L3) and $P = 0.27$ (AFP-L3 vs. combination of AFP and AFP-L3), respectively

status. This was expressed as the risk ratio (95% CI) in Table 5: 2.5 (0.9–6.9), 5.0 (1.2–20.5), 2.1 (0.4–25.3), 3.5 (1.1–11.3) for AFP, AFP-L3, DCP, and the combination of AFP and AFP-L3, respectively.

Discussion

AFP, AFP-L3, and DCP have been widely used in Japan for screening and monitoring treatment response and/or relapse [19, 20]. Ideally, levels of tumor markers should fall to within a normal range after effective treatment and rise before the tumor relapse is detected by imaging studies. This aspect is especially important in the case of transcatheter arterial embolization and chemotherapy, because radiological findings do not necessarily reflect the degree of biological remission achieved by necrosis or fibrosis [21].

HCC biomarkers have also been reported to substitute as markers of specific clinicopathological variables representing the malignant potential of the tumor. In cases of

Table 4 Sensitivity/specificity at various cutoff points for tumor markers

AFP (ng/mL)	15	20	124‡	200
Sensitivity (%)	39.6	39.0	28.1	21.9
Specificity (%)	83.1	87.0	96.1	97.4
AFP-L3 (%)	5	10‡	15§	20
Sensitivity (%)	24.0	21.9	16.7	14.6
Specificity (%)	92.2	96.1	97.4	97.4
DCP (mAU/mL)	20	30	40	60‡
Sensitivity (%)	77.1	59.4	55.2	52.1
Specificity (%)	58.4	80.5	90.9	96.1
Combination of AFP (ng/mL) and AFP-L3 (%)	20[a] (AFP)	10[b,‡] (AFP-L3)	15[b,§] (AFP-L3)	400[a] (AFP)
Sensitivity (%)	39.6	26.0	26.0	17.7
Specificity (%)	87	96.1	97.4	98.7
Combination of AFP (ng/mL) or DCP (mAU/mL)	20 (AFP) or 40 (DCP)	20 (AFP) or 60 (DCP)	400 (AFP) or 40 (DCP)	400 (AFP) or 60 (DCP)
Sensitivity (%)	68.8	67.7	59.4	57.3
Specificity (%)	79.2	83.1	89.6	94.8

AFP alpha-fetoprotein, *AFP-L3* lens culinaris agglutinin-reactive fraction of AFP, *DCP* des-γ-carboxy prothrombin, *CI* confidence interval

[a] AFP values of 20 or 400 ng/mL was adopted as cutoff value irrespective of AFP-L3 value

[b] Patients with AFP value <20 ng/mL classified as marker negative; AFP value ≥400 ng/mL as marker positive. For patients with 20 ng/mL ≤ AFP < 400 ng/mL, AFP-L3 values of 10 or 15% adopted as cutoff value

‡ Comparisons of sensitivities: AFP versus AFP-L3, $P = 0.33$ (95% CI −0.06 to 0.18); AFP versus DCP, $P < 0.001$ (95% CI −0.37 to −0.11); AFP versus combination of AFP and AFP-L3, $P = 0.73$ (95% CI −0.10 to 0.14); AFP-L3 versus DCP, $P < 0.001$ (95% CI −0.43 to −0.17); AFP-L3 versus combination of AFP and AFP-L3, $P = 0.51$ (95% CI −0.16 to 0.07)

§ AFP-L3 versus combination of AFP and AFP-L3, $P = 0.12$ (95% CI −0.20 to 0.02)

non-surgical therapy, markers could therefore provide prognostic data when pathological information are unobtainable; and in cases of liver resection and transplantation, they may do so prior to the treatment [22]. In the present investigation, we evaluated these aspects of AFP, AFP-L3, and DCP as well as GP73.

GP73 antigen expression is barely detectable in normal subjects, but is strongly upregulated in the hepatocytes of patients with acute hepatitis, cirrhosis, and during the progression of chronic liver disease. GP73 was also a promising serum marker for HCC in preliminary studies [7, 8]. In the present study, however, neither GP73 autoantibody nor GP73 autoantigen levels appear to reflect the tumor status (Fig. 2). Other studies also reported the insufficiency of serum GP73 as an HCC-specific marker, although they confirmed that it may be a marker for chronic liver diseases or hepatitis C virus-related HCC [23, 24]. It should be noted that GP73 was increased in patients with liver disease, in particular, with the advancement of disease; HCC usually develops in the later stages of hepatitis C virus infection; and all previous studies suggesting the significance of GP73 as an HCC tumor marker were cross-sectional. Hence, it is more appropriate to consider that high levels of GP73 in patients with HCC reflect the fact that HCC develops at the advanced stage of chronic liver diseases.

To date, several studies have assessed the diagnostic accuracies of AFP-L3 and/or DCP in comparison with that of AFP through ROC curves. Two studies examined the significance of AFP-L3 and two of them reported that it was comparable to that of AFP [25, 26]. Five studies evaluated DCP [27–31], and four of these reported the superiority of DCP over AFP [27, 28, 30, 31]. Two studies examined the accuracies of AFP-L3 and DCP simultaneously in comparison with AFP [14, 32]. The former appeared to rank diagnostic accuracies in the decreasing order of DCP, AFP, and AFP-L3 [14], although no statistical comparison was done. The latter reported that AFP,

Table 5 Alteration of marker positive/negative status through hepatectomy and association with early phase of recurrence

Pre-hepatectomy		Post-hepatectomy			
Marker status	No. patients (rate)	Marker status	No. patients (rate)	No. patients with tumor recurrence ≤6 months	Risk ratio (95% CI)
Cutoff values set at 200 ng/mL					
AFP (−)	75/96 (78.1%)	AFP (−)	75/75 (100%)	11/75 (14.7%)	
		AFP (+)	0/75 (0.0%)	0/0 (0.0%)	
AFP (+)	21/96 (21.9%)	AFP (−)	15/21 (71.4%)	4/15 (26.7%)	2.50 (0.91–6.88)
		AFP (+)	6/21 (28.6%)	4/6 (66.7%)	
Cutoff values set at 10%					
AFP-L3 (−)	75/96 (78.1%)	AFP-L3 (−)	74/75 (98.7%)	13/74 (17.6%)	
		AFP-L3 (+)	1/75 (1.3%)	0/1 (0.0%)	
AFP-L3 (+)	21/96 (21.9%)	AFP-L3 (−)	15/21 (71.4%)	2/15 (13.3%)	5.00 (1.22–20.46)
		AFP-L3 (+)	6/21 (28.6%)	4/6 (66.7%)	
Cutoff values set at 60 mAU/mL					
DCP (−)	46/96 (47.9%)	DCP (−)	44/46 (95.7%)	5/44 (11.4%)	
		DCP (+)	2/46 (4.3%)	1/2 (50.0%)	
DCP (+)	50/96 (52.1%)	DCP (−)	46/50 (92.0%)	11/46 (23.9%)	2.09 (0.40–25.32)
		DCP (+)	4/50 (8.0%)	2/4 (50.0%)	
Cutoff values set accordingly[a]					
Combination (−)	71/96 (74.0%)	Combination (−)	70/71 (98.6%)	11/70 (15.7%)	
		Combination (+)	1/71 (1.4%)	0/1 (0.0%)	
Combination (+)	25/96 (26.0%)	Combination (−)	17/25 (68.0%)	3/17 (17.7%)	3.54 (1.11–11.29)
		Combination (+)	8/25 (32.0%)	5/8 (62.5%)	

AFP alpha-fetoprotein, *AFP-L3* lens culinaris agglutinin-reactive fraction of AFP, *DCP* des-γ-carboxy prothrombin, *CI* confidence interval

[a] Patients with AFP value <20 ng/mL classified as marker negative; AFP value ≥400 ng/mL as marker positive. For patients with 20 ng/mL ≤ AFP < 400 ng/mL, AFP-L3 values of 10% were adopted as cutoff value

which showed similar accuracy to DCP, was superior to AFP-L3 [32].

The present analyses revealed that DCP was superior to AFP while AFP-L3 was comparable to AFP (Fig. 3; Table 4). This finding agrees with the general conclusion from previous studies. We must bear in mind, however, that although AFP is inferior to DCP as a single tumor marker, they are independent markers and thus thought to be complementary to each other (Table 3). Furthermore, we sought the utility of additional measurements of AFP-L3 given known AFP values based on the considerations described earlier. It is of interest that the diagnostic accuracy of the combination of AFP with AFP-L3 was equivalent to AFP alone but superior to AFP-L3 alone (Fig. 3; Table 4). These results argue that the additional measurement of AFP-L3 to AFP is not mandatory, and that to the contrary, AFP-L3 data should always be interpreted in reference to AFP.

A high specificity has been reported as a feature of AFP-L3 [6]. The apparent discrepancy of the present results from reported characteristics can not be explained straightforwardly. One possible explanation may be related to the limitation of this study, that is, the present cohort comprised those undergoing hepatectomy. The majority of patients had one or two HCC nodules and the tumor diameter was relatively larger than that of the non-surgical cohort. The significance of AFP-L3 may be more marked in patients with multiple and/or small HCC nodules. Recently, a newly developed micro-total analysis system (μ-TAS) was reported not only to have higher analytical sensitivity than current methods in the determination of AFP-L3 but also to have the ability to measure AFP-L3 at a lower total AFP concentration [33]. Further studies by use of the μ-TAS system are expected to answer the question unaddressed in the present study.

Although the association of tumor markers with clinicopathological variables has been evaluated in many studies, the majority of these works only assessed associations with variables of interest and/or exclusively for AFP, AFP-L3, or DCP. Variables were also usually assessed by radiological findings or in specimens obtained by biopsy. In the present study, we investigated these associations in a comprehensive manner using pathological findings of resected specimens (Table 2). An elevated DCP value was broadly associated with variables representing tumor invasiveness and/or poor prognosis (Table 2). A similar, moderate association was confirmed for AFP but not

AFP-L3, although a similar trend was observed. This agrees in part with previous studies, which showed that respective tumor markers stood for specific pathological indices, for example, AFP for poor degree of tumor cell differentiation [34, 35], AFP-L3 for poor degree of tumor cell differentiation and presence of vascular invasion [36–38], and DCP for the presence of vascular invasion and/or intrahepatic metastasis [34, 39, 40].

One of the unique features of the present study is that we were able to follow the alterations in tumor marker values before and after the hepatectomy in a patient-by-patient manner. At cutoff points giving fairly high specificities (96.1–97.4%), almost all patients who had been negative for respective tumor markers before the hepatectomy also remained marker negative after the operation. By contrast, a considerable proportion of patients who had been marker positive did not attain marker negative status even after the curative liver resection (Table 5). Later follow-up revealed that this had been the unidentified sign of recurrence. This prediction ability appeared to be most prominent in AFP-L3 compared with AFP or DCP. This observation is in line with previous reports that suggested the significance of AFP-L3 lies in the early recognition of HCC in the follow-up of patients with cirrhosis [6].

In conclusion, DCP was shown to be a better tumor marker than AFP and AFP-L3 in monitoring the treatment response in patients with HCC, but AFP was a useful complementary marker to DCP. The accuracy of AFP-L3 was comparable to that of AFP, but no benefit was found in the additional measurement of AFP-L3 to AFP. Conversely, AFP-L3 values should be interpreted in reference of those of AFP. GP73 was not an HCC marker under the present clinical conditions. Correlation of tumor marker values with clinicopathological variables representing the malignant potential of HCC and/or poor prognosis was strongest for DCP, followed by AFP and AFP-L3 in this order. The significance of the AFP-L3 measurement may lie in the early recognition of tumor recurrence after treatment.

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Population Pharmacokinetics of Gemcitabine and Its Metabolite in Japanese Cancer Patients

Impact of Genetic Polymorphisms

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Abstract

Background and Objective: Gemcitabine (2′,2′-difluorodeoxycytidine) is an anticancer drug, which is effective against solid tumours, including non-small-cell lung cancer and pancreatic cancer. After gemcitabine is transported into cells by equilibrative and concentrative nucleoside transporters, it is phosphorylated by deoxycytidine kinase (DCK) and further phosphorylated to its active diphosphorylated and triphosphorylated forms. Gemcitabine is rapidly metabolized by cytidine deaminase (CDA) to an inactive metabolite, 2′,2′-difluorodeoxyuridine (dFdU), which is excreted into the urine. Toxicities of gemcitabine are generally mild, but unpredictable severe toxicities such as myelosuppression and interstitial pneumonia are occasionally encountered. The aim of this study was to determine the factors, including genetic polymorphisms of *CDA*, *DCK* and solute carrier family 29A1 (*SLC29A1* [*hENT1*]), that alter the pharmacokinetics of gemcitabine in Japanese cancer patients.

Patients and Methods: 250 Japanese cancer patients who received 30-minute intravenous infusions of gemcitabine at 800 or 1000 mg/m^2 in the period between September 2002 and July 2004 were recruited for this study. However, four patients were excluded from the final model built in this study because they showed bimodal concentration-time curves. Two patients who experienced gemcitabine-derived life-threatening toxicities in October 2006 and January 2008 were added to this analysis. One of these patients received 30-minute intravenous infusions of gemcitabine at 454 mg/m^2 instead of the usual dose (1000 mg/m^2).

Plasma concentrations of gemcitabine and dFdU were measured by high-performance liquid chromatography-photodiode array/mass spectrometry. In total, 1973 and 1975 plasma concentrations of gemcitabine and dFdU, respectively, were used to build population pharmacokinetic models using nonlinear mixed-effects modelling software (NONMEM® version V level 1.1).

Results and Discussion: Two-compartment models fitted well to plasma concentration-time curves for both gemcitabine and dFdU. Major contributing factors for gemcitabine clearance were genetic polymorphisms of *CDA*, including homozygous *CDA**3 [208G>A (Ala70Thr)] (64% decrease), heterozygous *3 (17% decrease) and *CDA* –31delC (an approximate 7% increase per deletion), which has a strong association with *CDA**2 [79A>C (Lys27Gln)], and coadministered S-1, an oral, multicomponent anti-cancer drug mixture consisting of tegafur, gimeracil and oteracil (an approximate 19% increase). The estimated contribution of homozygous *CDA**3 to gemcitabine clearance provides an explanation for the life-threatening severe adverse reactions, including grade 4 neutropenia observed in three Japanese patients with homozygous

*CDA**3. Genetic polymorphisms of *DCK* and *SLC29A1* (*hENT1*) had no significant correlation with gemcitabine pharmacokinetic parameters. Aging and increased serum creatinine levels correlated with decreased dFdU clearance.

Conclusion: A population pharmacokinetic model that included *CDA* genotypes as a covariate for gemcitabine and dFdU in Japanese cancer patients was successfully constructed. The model confirms the clinical importance of the *CDA**3 genotype.

Background

Gemcitabine (2′,2′-difluorodeoxycytidine, dFdC) is an anticancer drug, which is effective against solid tumours, including non-small-cell lung cancer and pancreatic cancer.[1] After gemcitabine is transported into cells by equilibrative and concentrative nucleoside transporters (ENTs and CNTs),[2,3] it is initially phosphorylated by deoxycytidine kinase (DCK) to 2′,2′-difluorodeoxycytidine monophosphate and then is further phosphorylated to its active diphosphorylated and triphosphorylated forms, dFdCDP and dFdCTP.[4,5] Gemcitabine is rapidly metabolized by cytidine deaminase (CDA) to an inactive metabolite, 2′,2′-difluorodeoxyuridine (dFdU), which is excreted into the urine.[6]

Toxicities of gemcitabine are generally mild,[7,8] but unpredictable severe toxicities such as myelosuppression and interstitial pneumonia are occasionally encountered.[9-11] In a previous paper, we reported that a single nucleotide polymorphism (SNP) of the *CDA* gene, *CDA* 208G>A (Ala70Thr), designated as *CDA**3, caused reduced CDA activity and led to increased incidences of severe neutropenia in patients receiving gemcitabine-based combination chemotherapy.[12] Moreover, all three patients who were homozygous for *CDA**3 encountered life-threatening gemcitabine-mediated toxicities, including grade 4 neutropenia.[13,14] Pharmacokinetic data available from two of these patients revealed a gemcitabine clearance of about 20% of the median clearance rate.[12,14]

In this study, we performed a population pharmacokinetic analysis of gemcitabine in Japanese cancer patients to determine which factors, including genetic factors, affect the pharmacokinetics of gemcitabine and to facilitate individualized gemcitabine-based chemotherapies.

Methods

Patients

The ethics committees of the National Cancer Center (Tokyo, Japan) and the National Institute of Health Sciences (Tokyo, Japan) approved this study. Written informed consent was obtained from all participants. 250 patients, who received 30-minute intravenous infusions of gemcitabine 800 or 1000 mg/m^2 from September 2002 to July 2004, participated in this study. Two patients[14] who experienced gemcitabine-mediated life-threatening toxicities in October 2006 and January 2008 were added to the study. One of these patients, who carried a homozygous *CDA**3 gene, received 30-minute intravenous infusions of gemcitabine 454 mg/m^2 instead of the usual dose (1000 mg/m^2). All patients in this study have been previously reported,[12,14] and their characteristics are summarized in table I.

Table I. Demographic and clinical profiles of the gemcitabine (dFdC)-treated population

Variable	Value
Sex (n; male/female)	162/86
Dose (n; mg/m^2)	
1000	243
800	4
454	1
Pancreatic cancer (n)	207
Lung cancer (n)	35
Methothelium cancer (n)	6
Monotherapy (n)	182
Combination therapy (n)	66
cisplatin	26
carboplatin	16
fluorouracil	4
S-1	10
vinorelbine	10
Age (y)[a]	62.67±9.04 [35–80]
BSA (m^2)[a]	1.56±0.17 [1.14–1.97]
Bodyweight (kg)[a]	54.56±9.76 [30–80.3]
Serum creatinine (mg/dL)[a]	0.70±0.19 [0.4–1.5]

a The values are expressed as mean ± SD [range].

BSA = body surface area; **S-1** = an oral product of tegafur with gimeracil and oteracil.

Table II. Allele frequencies of the genetic polymorphisms of *CDA*, *DCK* and *SLC29A1* investigated in this study

Gene	Single nucleotide polymorphism ID	Location	Nucleotide change	Amino acid change	Subjects (n)			Allele frequency
					wild-type	heterozygous	homozygous	
CDA	MPJ6_CDA007	Exon1 (5′-UTR)	-31delC		83	110	55	0.444
	MPJ6_CDA009	Exon1	79A>C	Lys27Gln	160	73	15	0.208
	MPJ6_CDA010	Intron1	IVS1+37G>A		175	59	14	0.175
	MPJ6_CDA011	Exon2	208G>A	Ala70Thr	230	16	2	0.040
DCK	MPJ6_DCK008	5′-Flanking	-360C>G		187	58	3	0.129
	MPJ6_DCK016	Exon3	364C>T	Pro122Ser	219	28	1	0.060
SLC29A1	MPJ6_ET1005	5′-Flanking	-5851G>A		215	31	0	0.063
	MPJ6_ET1008	5′-Flanking	-3797A>G		84	128	34	0.398
	MPJ6_ET1011	5′-Flanking	-3268_-3249del AGGCTCGCGAGCGGAGGTGC		15	114	117	0.707
	MPJ6_ET1026	Intron8	IVS8+169G>A		222	23	1	0.051
	MPJ6_ET1029	Intron10	IVS10+160A>C		154	73	19	0.226
	MPJ6_ET1036	Exon12 (3′-UTR)	1840(*469)C>A		161	69	16	0.205
	MPJ6_ET1039	3′-Flanking	1984+69 (*682)A>C		139	83	24	0.266

CDA = cytidine deaminase; **DCK** = deoxycytidine kinase; **SLC** = solute carrier family; **UTR** = upper translational region.

Plasma Gemcitabine and dFdU Concentrations and Genotypes

Plasma concentrations of gemcitabine and dFdU and genotypes of *CDA*, *DCK* and solute carrier family 29A1 (*SLC29A1* [coding *hENT1*]) have been previously reported.[12,15,16] Blood samples for determining the plasma concentrations were collected before and at timepoints 0, 15, 30, 60, 90, 120 and 240 minutes after completion of the infusion. Table II summarizes the allele frequencies of *CDA*, *DCK* and *hENT1* genotypes for which effects on the pharmacokinetic parameters of gemcitabine were investigated in this study.

Population Pharmacokinetic Model Development

Population pharmacokinetics of gemcitabine and dFdU were analysed using nonlinear mixed-effects modelling software (NONMEM® version V level 1.1; ICON Plc, Dublin, Ireland). The first-order method was applied during the building of population pharmacokinetic models, and the first-order conditional estimation (FOCE) method was applied to obtain estimations in the final model. Selection of covariates was carried out by the forward stepwise inclusion and deletion method at a threshold p-value of 0.001 by a χ^2 test.

De Pas et al.[17] reported linear pharmacokinetics of gemcitabine up to 1500 mg/m^2, after which nonlinear pharmacokinetic behaviour and higher interpatient variability in the maximum plasma concentration (C_{max}) and the area under the plasma concentration-time curve (AUC) were reported. Since all patients received gemcitabine at doses ≤1000 mg/m^2, linear compartment models were selected as gemcitabine population pharmacokinetic models.

To develop a basic population pharmacokinetic model for gemcitabine (selection of a compartment model and description of interindividual and residual error variability), we fitted a one-compartment or two-compartment linear model to plasma concentrations of gemcitabine. The estimated population parameters for a one-compartment model were the volume of distribution (V_d) and clearance (CL), and those for a two-compartment model were the volume of distribution in the central compartment (V_1), clearance (CL_1), the volume of distribution in the peripheral compartment (V_2) and intercompartmental clearance (Q) [step 1]. As previously reported, two patients carrying homozygous *CDA**3 showed unexpectedly high plasma concentrations of gemcitabine;[12,14] therefore, these patients were excluded from this step. Four patients who showed bimodal concentration-time curves for gemcitabine were also excluded from the analysis because an extraordinarily large apparent V_1 for gemcitabine was estimated when they were included. Next, after the data obtained from the two *CDA**3 homozygous patients were added, the contribution of *CDA**3 to the population pharmacokinetics of

gemcitabine was determined (step 2). Similarly, additional candidate covariates, most of which had previously been shown to have univariate correlations with model-independent pharmacokinetic parameters,[12] were examined for their contributions; they included other genetic polymorphisms of *CDA*,[12] *DCK*[15] and *SLC29A1* (*hENT1*),[16] regimens of chemotherapies and patients' characteristics (step 3).

In order to consider the metabolic pathway from gemcitabine to dFdU and dFdU pharmacokinetics, we used subroutines provided by NONMEM® (ADVAN5 and MODEL); the former is prepared for general linear models and the latter defines compartment attributes and rate constants. We examined whether dFdU followed a one-compartment or two-compartment model. The estimated population parameters of dFdU for a one-compartment model were total dFdU clearance (CL_m) and the dFdU volume of distribution (V_m), and those for a two-compartment model were CL_m, the dFdU volume of distribution in the central compartment (V_{m1}), the dFdU volume of distribution in the peripheral compartment (V_{m2}) and intercompartmental dFdU clearance (Q_m). Since only the subroutine TRANS1 (in which micropharmacokinetic parameters such as intercompartmental rate constants [k_{12} or k_{21}] are used) is provided by NONMEM® for ADVAN5, we adequately transformed micropharmacokinetic parameters to macropharmacokinetic ones such as the V_1, V_2, CL and Q. The metabolic fraction of dFdU (f_m) was assumed to be 1 because more than 90% of the administered gemcitabine was recovered as dFdU in the urine.[6] Compartment and error models for dFdU were determined (step 4). Subsequently, selection of candidate covariates for dFdU, which had previously been reported to have correlations with model-independent pharmacokinetic parameters of dFdU,[12] was carried out (step 5). Finally, pharmacokinetic parameters of the model constructed in step 5 were re-estimated using the FOCE method. The final model was evaluated by plots of the observed and predicted individual plasma concentrations of gemcitabine and dFdU, and by plots of the observed plasma concentrations and population conditional weighted residuals, which were calculated using Xpose 4.0 software (Uppsala University, Uppsala, Sweden).[18]

Results

Development of a Population Pharmacokinetic Model for Gemcitabine

We used 1973 plasma concentrations of gemcitabine obtained from 248 patients (figure 1a) for the following analysis. As described in the Methods, the two patients with homozygous *CDA**3 (*CDA* 208G>A) were excluded from the first step analysis because of their extraordinarily different pharmacokinetic profiles (figure 1). Pharmacokinetics of gemcitabine in Japanese cancer patients, excluding the *CDA**3-homozygous patients, were estimated using a two-compartment model. The objective function value (OFV) was at a minimum when interindividual variabilities of pharmacokinetic parameters were assumed to distribute log-normally, and when the variation in the residual errors was assumed to have both proportional and additive components (model 1).

In order to build a model taking into account the *3/*3 patients with extremely low clearance,[12] we added a

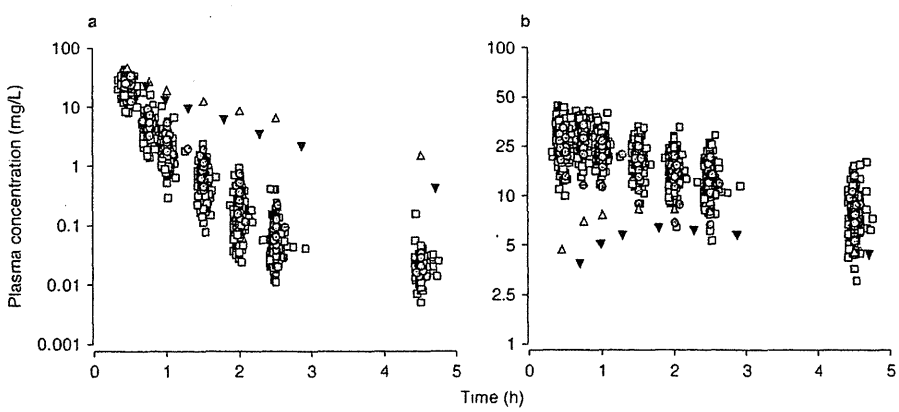

Fig. 1. Plasma concentration-time plots of (a) gemcitabine (dFdC) and (b) 2',2'-difluorodeoxyuridine (dFdU). The light grey squares represent patients without *CDA**3 and the dark grey circles represent patients with heterozygous *CDA**3. The white triangles and black inverted triangles represent patients with homozygous *CDA**3 receiving 1000 and 454 mg/m^2 of gemcitabine, respectively.

Table III. Building of covariate population pharmacokinetic models for gemcitabine (dFdC) and 2',2'-difluorodeoxyuridine (dFdU)

Model name	Parameter	Factor[a]	OFV	Model used for comparison	ΔOFV	p-Value
Gemcitabine						
1		Basic model	−532.19			
	CL_1	*CDA**3 (linear)	−2206.79	1	−1674.60	<0.000001
2	CL_1	***CDA**3 homozygous**	−2260.86	1	−1728.68	<0.000001
3	CL_1	***CDA**3 heterozygous**	−2276.14	2	−15.28	9.28E−05
4	CL_1	BSA	−2405.26	3	−129.12	
	V_1	BSA	−2204.71	3	71.43	
	CL_1	Bodyweight	−2276.05	3	0.094	
	V_1	Bodyweight	−2176.05	3	100.09	
	CL_1	Age	−2409.77	4	−4.51	0.034
	V_1	Age	−2410.98	4	−5.72	0.017
	CL_1	Sex	−2406.40	4	−1.14	0.29
	V_1	Sex	−2406.52	4	−1.26	0.26
	CL_1	Cisplatin	−2407.248	4	−1.99	0.16
5	CL_1	**S-1**	−2427.66	4	−22.40	2.21E−06
6	CL_1	***CDA*−31delC**	−2464.89	5	−37.23	1.05E−09
	CL_1	*CDA**2	−2441.21	5	−13.55	0.00023
	CL_1	*CDA* IVS1+37G>A	−2441.84	5	−14.18	0.00017
dFdU						
7		Basic model	91.694			
8	CL_m	**BSA**	45.958	7	−45.736	
	V_{m1}	BSA	10.795	7	−80.899	
9	V_{m1}	**BSA**	−31.64	8	−77.598	
	CL_m	Bodyweight	163.251	7	71.557	
	V_{m1}	Bodyweight	2.496	7	−89.198	
10	CL_m	**Creatinine**	−166.798	9	−135.158	3.05E−31
11	CL_m	**Age**	−197.342	10	−30.544	3.26E−08
12	V_{m1}	**Age**	−212.069	11	−14.73	0.000124
13	V_{m1}	**Sex**	−243.914	12	−31.845	1.67E−08
	CL_m	Sex	−253.677	13	−9.763	0.00178

a The factors indicated in bold type were selected as covariates for the final model.

BSA = body surface area; **CL_1** = clearance of gemcitabine; **CL_m** = clearance of the metabolite dFdU; **OFV** = objective function value; **S-1** = an oral product of tegafur with gimeracil and oteracil; **V_1** = apparent volume of distribution of the central compartment of gemcitabine; **V_{m1}** = apparent volume of distribution of the central compartment of dFdU.

covariate to the basic model to account for the effect of homozygosity of *3 (θ_{*3homo}) on the clearance of gemcitabine (equation 1):

$$CL = \theta_1 \times (1 - \theta_{*3homo} \times CDA*3homo) \quad \text{(Eq. 1)}$$

where CL is total gemcitabine clearance in a patient of interest; θ_1 is gemcitabine clearance in patients without *3/*3; and *CDA**3homo is 1 for *3/*3 and 0 for other patients (*3/non-*3 or non-*3/non-*3). This modification significantly reduced the OFV, as shown in table III (model 2).

Next, the effect of heterozygous *3 on gemcitabine clearance was examined by comparing equations 2 and 3:

$$CL = \theta_2 \times (1 - \theta_{*3homo} \times CDA*3) \quad \text{(Eq. 2)}$$

$$CL = \theta_2 \times (1 - \theta_{*3hetero} \times CDA*3hetero) \times (1 - \theta_{*3homo} \times CDA*3homo) \quad \text{(Eq. 3)}$$

where θ_2 is gemcitabine clearance for patients without *3, *CDA**3 is 0 for non-*3/non-*3, ½ for *3/non-*3 and 1 for *3/*3; $\theta_{*3hetero}$ is a parameter related to the effect of heterozygous *3 but independent of θ_{*3homo}; and *CDA**3hetero is 1 for *3/non-*3 and 0 for *3/*3 or non-*3/non-*3. Equation 3 assumes a nonlinear gene-dose effect of *CDA**3 on CL. The OFV of equation 3 (model 3) was slightly but significantly smaller than that of equation 2, which indicates that the *CDA**3 gene-dose effect is not linear.

The effects of the body surface area (BSA), bodyweight, age and sex on the CL and V_1 of gemcitabine were investigated. As shown in table III, while consideration of an effect of size on the V_1 did not improve the OFV, examination of proportionality between the CL and BSA (model 4) considerably reduced the OFV. Age and sex did not significantly affect the CL and V_1 of gemcitabine (table III), although they were significantly correlated with these parameters in our previous univariate analyses.[12] As shown in table I, 66 patients received a gemcitabine-based combination chemotherapy with either cisplatin, carboplatin, fluorouracil, S-1 (an oral anti-cancer multicomponent drug containing tegafur, gimeracil and oteracil) or vinorelbine. Among the coadministered drugs, only S-1 significantly increased CL (model 5).

The effects of genetic polymorphisms of *CDA* other than *3 on the pharmacokinetics of gemcitabine were also examined. *CDA*–31delC (rs3215400; previously described as *CDA*–33_–31delC [precisely *CDA*–33_–31 C3>C2]), *CDA* 79A>C (Lys27Gln, *2) and *CDA* IVS1+37G>A increased gemcitabine clearance, and their effects were all statistically significant (table III). A delC factor was adopted in the final model for gemcitabine because it gave the smallest p-value and OFV (model 6 in table III).

Although we previously reported that 29 genetic variations of *DCK* were detected in our patients, they were very rare except for *DCK*–360C>G and 364C>T (Pro122Ser) [the allele frequencies were 0.131 and 0.061, respectively, as shown in table II],[15] and their functions were reported to be altered.[19,20] We analysed the effects of *DCK*–360C>G and 364C>T (Pro122Ser) on gemcitabine population pharmacokinetics, but no effects were detected. Thirty-nine genetic polymorphisms of *SLC29A1* (*hENT1*), including two nonsynonymous ones, were also previously reported.[16] Although we analysed the effects of genetic polymorphisms of *hENT1* whose allele frequencies were higher than 0.05 (table II), no effects were observed in univariate analyses (data not shown).

Development of a Combined Population Pharmacokinetic Model for Gemcitabine and dFdU

Next, we added compartments for dFdU where its central compartment was connected with the central compartment of gemcitabine with a first-order metabolic rate constant (CL/V_1) (figure 2). The f_m was assumed to be 1 because >90% of administered gemcitabine was recovered in the urine as dFdU.[6] Since an extraordinarily large V_m for dFdU was obtained if the V_1 for gemcitabine was not fixed, the V_1 was fixed to the value estimated in the previous section (12.60 L). Although the sampling duration in this study was not sufficiently long for pharmacokinetic analysis of dFdU (which has a longer half-life than that of gemcitabine, as shown in figure 1b), a two-compartment model (model 7, the combined basic model for gemcitabine and dFdU) provided a better fit for the data than a one-compartment model (the ΔOFV was −3402.44). Inclusion of covariates such as the BSA, age, serum creatinine level and sex in the model significantly reduced the OFV, as shown in table III.

All covariates selected by the inclusion steps remained after the stepwise exclusion/deletion process. The final population pharmacokinetic model (model 13) for Japanese cancer patients is shown in table IV. This model indicated that gemcitabine clearance was decreased by 64% and 17% in the *3-homozygotes and heterozygotes, respectively, compared with patients without *CDA**3. The increases in gemcitabine clearance by delC were 7.5% for heterozygotes and 15% for homozygotes. If S-1 was coadministered, gemcitabine clearance increased by 19%. CL_m was reduced by 8.6% if a patient was 10 years older than the average age (62.67 years in our patient group) and by about 7.3% if the creatinine level of a patient was 0.1 mg/dL higher than the average level (0.7 mg/dL in our patient group). The V_{m1} for dFdU was decreased by 8.1%

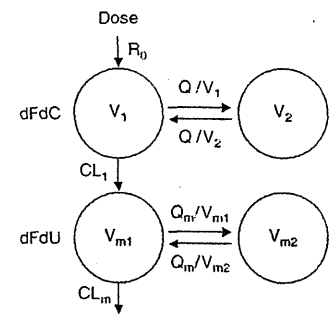

Fig. 2. Compartmental representation of gemcitabine (dFdC) and 2′,2′-difluorodeoxyuridine (dFdU) pharmacokinetics. CL_1 = clearance of gemcitabine; CL_m = clearance of the metabolite dFdU; Q = intercompartmental clearance between the central and peripheral compartments of gemcitabine; Q_m = intercompartmental clearance between the central and peripheral compartments of dFdU; R_0 = zero-order infusion rate constant; V_1 = apparent volume of distribution of the central compartment of gemcitabine; V_2 = apparent volume of distribution of the peripheral compartment of gemcitabine; V_{m1} = apparent volume of distribution of the central compartment of dFdU; V_{m2} = apparent volume of distribution of the peripheral compartment of dFdU.

Table IV. Population pharmacokinetic parameters for gemcitabine (dFdC) and 2′,2′-difluorodeoxyuridine (dFdU) in the final model

Pharmacokinetic parameter	Estimated value	CV%
Gemcitabine		
CL_1 (L/h/m^2)	$73.70 \times BSA \times (1-0.639 \times$ *3homo[a]$) \times (1-0.171 \times$ *3hetero[b]$) \times (1+0.0749 \times$ delC[c]$) \times (1+0.191 \times$ S-1[d]$)$	17.1
V_1 (L)	12.60 (Fixed)	58.9
Q (L/h)	37.50	Not estimated
V_2 (L)	9.54	25.3
dFdU		
CL_{m1} (L/h/m^2)	$11.00 \times BSA \times (1-0.00855 \times (AGE-62.67)) \times (1-0.732 \times (Cre-0.70))$	20.5
V_{m1} (L)	$15.00 \times BSA \times (1-0.00806 \times (AGE-62.67)) \times (1+0.239 \times$ Sex[e]$)$	27.9
Q_m (L/h)	58.0	22.7
V_{m2} (L)	31.7	26.4
Residual error	SD (ε_3); 0.0844	
	CV (ε_1) and CV (ε_2); 0.200 and 0.0412, respectively	

a *3homo: 1 for homozygous *CDA**3 and 0 for others.

b *3hetero: 1 for heterozygous *CDA**3 and 0 for others.

c delC: number of *CDA*–31delC in a patient (delC=0, 1 or 2).

d S-1: 1 for S-1 coadministered to patients and 0 for others.

e Sex: 1 for male and 0 for female.

ε=variance; **AGE**=age (years); **BSA**=body surface area (m^2); **CL_1**=clearance of gemcitabine; **CL_m**=clearance of the metabolite dFdU; **CL_{m1}**=clearance of the metabolite dFdU from central compartment; **Cre**=serum creatinine (mg/dL); **CV**=coefficient of variation (interindividual); **Q**=intercompartmental clearance between the central and peripheral compartments of gemcitabine; **Q_m**=intercompartmental clearance between the central and peripheral compartments of dFdU; **S-1**=an oral product of tegafur with gimeracil and oteracil; **V_1**=apparent volume of distribution of the central compartment of gemcitabine; **V_2**=apparent volume of distribution of the peripheral compartment of gemcitabine; **V_{m1}**=apparent volume of distribution of the central compartment of dFdU; **V_{m2}**=apparent volume of distribution of the peripheral compartment of dFdU.

if a patient was 10 years older than the average age, and was increased by 24% in males compared with females.

Evaluation of the Goodness of Fit

The observed plasma concentrations of gemcitabine and dFdU were plotted against concentrations predicted by the final model, as shown in figure 3a and b, respectively. Most gemcitabine concentrations distributed into two peaks: one peak with scattering around 25 mg/L (collected at the end of the gemcitabine infusion [30 minutes after initiation of the infusion]) and a second peak with scattering close to the point of origin. This dual peak plot was the result of very rapid gemcitabine metabolism. One point at an extremely high concentration represented the C_{max} obtained from a *3/*3 patient, who was administered 1000 mg/m^2 of gemcitabine.[12,13] For both gemcitabine and dFdU, higher plasma concentrations gave more widely scattered plots, indicating that the variation in the residual errors was proportional to the measured concentration (a constant coefficient of variation type). The slopes of the regression lines for gemcitabine and dFdU were very close to 1.0 (1.007 and 0.9908, respectively). Conditional weighted residuals (CWRES) were recently reported as a diagnostic tool for the FOCE approximation.[18] The slopes of the regression lines of CWRES for gemcitabine and dFdU against predicted plasma concentrations were very close to 0.0 (–0.00482 and –0.00926, respectively), indicating a very good fit for the constructed model. Further validation of the model by a visual predictive check or bootstrapping was not performed, because the distribution of some covariates, such as diplotypes of *CDA**3 (non-*3/non-*3 : non-*3/*3 : *3/*3 = 230 : 16 : 2), and coadministration of S-1 (in only 10 of the 248 patients) were unevenly distributed.

Discussion

Recently, Jiang et al.[21] performed population pharmacokinetic analyses on gemcitabine and dFdU, and they adopted two-compartment models for both plasma gemcitabine and dFdU pharmacokinetics. Likewise, in our study, the

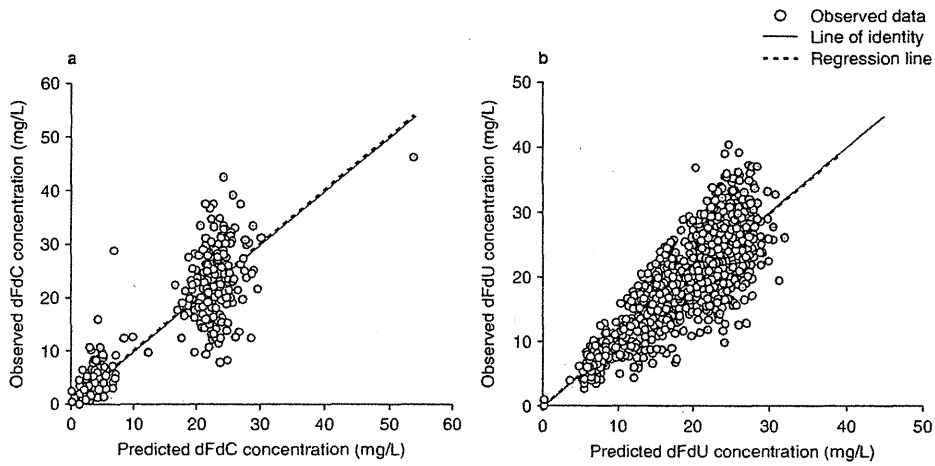

Fig. 3. Plots of observed concentrations against predicted concentrations of (a) gemcitabine (dFdC) and (b) 2′,2′-difluorodeoxyuridine (dFdU).

pharmacokinetics of gemcitabine and dFdU were effectively described by two-compartment models. The values of the estimated CL ([115.0 L/h] from a typical patient with an average BSA of 1.56 m^2), V_1 (12.60 L) and V_2 (9.54 L) were comparable to the values reported by Jiang et al.[21] (162 L/h, 15 L and 15 L, respectively). The estimated CL was slightly smaller and the V_1 was slightly larger than the values reported by Tham et al.[22] (222.8 L/h and 2.96 L, respectively). Although the reasons for these discrepancies are unknown, it should be noted that the population pharmacokinetic analyses performed by Tham et al.[22] included gemcitabine triphosphate (dFdCTP, an active form of gemcitabine) in addition to gemcitabine and dFdU, and the pharmacokinetic models applied in their study were completely different from ours.

The gemcitabine clearance in the *3/*3 patients, obtained from the model-independent analysis, was 80% less than the average clearance in patients without *3.[12,13] The effect of homozygous *3 on gemcitabine clearance, as estimated by the final population pharmacokinetic model, was a 64% decrease. This value, although slightly less than 80%, was the most significant among the covariates. Our current study also confirmed a finding from our previous report that the gene-dose effect of *CDA* was not linear. So far, we have encountered three patients with *3/*3, and all of them experienced life-threatening toxicities, including prolonged severe neutropenia.[12-14] Some of the non-*3/non-*3 and non-*3/*3 patients experienced transient grade 4 neutropenia, but only one patient required supportive treatment.[14] Thus, special attention to *3 homozygotes is advisable.

The effects of –31delC, 79A>C and IVS1+37G>A of *CDA* on gemcitabine clearance were found to be small but significant in this study (table III). All of these genotypes had slightly increased gemcitabine clearance (by <10%). The single nucleotide deletion –31delC is simultaneously present in both the haplotype *2 harbouring 79A>C and several *1 haplotypes (*1b, *1d, etc.) harbouring IVS1+37G>A in the Japanese population.[12] Thus it is reasonable that –31delC, rather than 79A>C or IVS1+37G>A, was selected as the covariate in the final model. This finding suggests that –31delC may be a functional SNP.

The haplotype analysis in our previous report[12] indicated that 208G>A, the tagging SNP of *CDA**3, is not present on a chromosome carrying –31delC, 79A>C or IVS1+37G>A. However, some patients simultaneously carried both haplotypes *2 and *3 (*2/*3). The median value of gemcitabine clearance observed in patients with *2/*3 was slightly higher than that observed in patients with *1/*3, although the difference was not statistically significant.[12]

The SNP 79A>C, a tagging SNP of the haplotype *2, results in the amino acid substitution, Lys27Gln.[12] A recent study[23] has suggested that the average enzymatic activity of CDA was significantly lower in cytoplasmic extracts of red blood cells obtained from patients with homozygous 79A (Lys27) than in those from patients with 79C (Gln27). Furthermore, it was reported that *CDA* 79A, the major allele, was a predictive marker of better response, more severe toxicity, longer time to disease progression and overall survival in Caucasian patients with advanced non-small-cell lung cancer who were treated with cisplatin and gemcitabine.[24] Haplotype *2 harbouring 79A>C also harbours –31delC, which has an incomplete association with the intron SNP IVS1+37. Our findings may explain the effects of 79A>C observed in Caucasian patients, since 79A>C is closely linked with –31delC, and the single nucleotide deletion –31delC in the 5′-untranslated region is responsible for increased clearance, a decreased AUC and less response to gemcitabine. This speculation warrants further study.