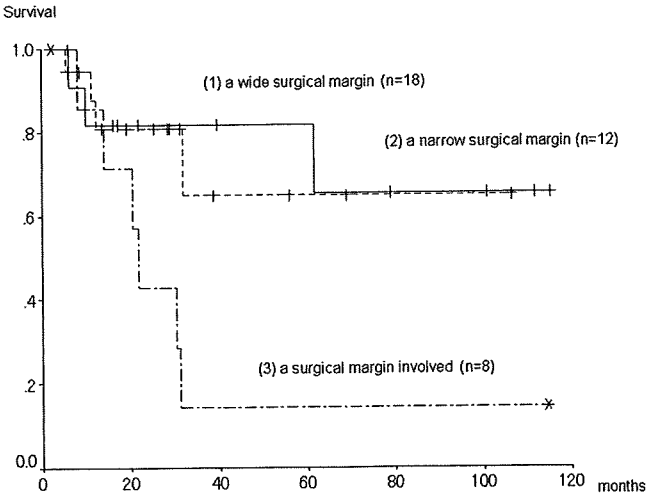

Fig. 2. Overall survival in the 38 patients without lymph node metastasis according to surgical margin status. The statistically significant differences revealed were: (1) versus (2), $P = 0.8252$; (1) versus (3), $P = 0.0396$; (2) versus (3), $P = 0.0373$.

surgical margin group. However, within the group with a negative surgical margin, no difference in survival was observed between the narrow and wide surgical margin subgroups. In contrast in the 19 patients with lymph node metastasis, there was no significant correlation of survival with surgical margin status. In connection with the width of the surgical margin, a narrow resectional margin had no adverse effect on patient survival in cases without either lymph node or intrahepatic metastasis.

Table II shows the relationship between surgical margin status and other clinicopathological factors in the 38 patients with no lymph node metastases. The surgical margin status was significantly associated with intrahepatic metastases ($P = 0.011$) and ICGR15 ($P = 0.036$), respectively.

Table III lists the sites of recurrence in the 38 patients with no lymph node metastases. The leading site of recurrence was the liver, followed by the lymph nodes; there was no significant correlation with the surgical margin status. No local recurrences were observed in patients with a wide surgical margin, and intrahepatic recurrence was more frequently observed in patients with a narrow surgical margin.

DISCUSSION

Previous articles have reported that lymph node involvement and intrahepatic metastasis are significant poor prognostic factors in patients with ICC undergoing macroscopic curative resection [16–21]. These factors represent completely different modes of spreading in cases with the MF type of ICC: lymph node metastasis spread via lymphatic flow along Glisson's sheath might

TABLE II. Relationship Between Surgical Margin Status and Other Clinicopathological Factors in 38 Patients Without Lymph Node Metastases

Factors	Surgical margin	>5 mm (n = 12)	≤5 mm (n = 18)	Involved (n = 8)	*P*-value
Width	Mean ± S D mm	17 ± 9 mm	3 ± 2 mm	—	<0.001
Age (median: 66 years)	<66 (n = 19)	6 (32)	8 (42)	5 (26)	0.697
	≥66 (n = 19)	6 (32)	10 (53)	3 (15)	
Gender	M (n = 24)	7 (29)	13 (54)	4 (17)	0.509
	F (n = 14)	5 (36)	5 (36)	4 (28)	
Size (median: 5.2 cm)	≤5.2 (n = 19)	9 (47)	7 (37)	3 (16)	0.111
	>5.2 (n = 19)	3 (16)	11 (58)	5 (26)	
Intrahepatic metastases	Absent (n = 26)	10 (38)	14 (54)	3 (8)	0.011
	Present (n = 12)	2 (17)	4 (33)	5 (50)	
Portal vein invasion	Absent (n = 34)	12 (35)	14 (41)	8 (24)	0.083
	Present (n = 4)	0	4 (100)	0	
Hepatic vein invasion	Absent (n = 25)	9 (36)	12 (48)	4 (16)	0.511
	Present (n = 13)	3 (23)	6 (46)	4 (31)	
Bile duct invasion	Absent (n = 16)	5 (31)	7 (44)	4 (25)	0.869
	Present (n = 22)	7 (32)	11 (50)	4 (18)	
Histological differentiation	Well (n = 8)	1 (13)	6 (74)	1 (13)	0.207
	Mod/poor (n = 30)	11 (37)	12 (40)	7 (23)	
ICGR15 (median 8%)	≤8 (n = 20)	10 (50)	7 (35)	3 (15)	0.036
	>8 (n = 18)	2 (11)	11 (61)	5 (28)	
Extent of hepatectomy	Segmentectomy and less (n = 10)	3 (30)	4 (40)	3 (30)	0.711
	Lobectomy and more (n = 28)	9 (32)	14 (50)	5 (18)	

ICGR15: Indocyanine green retention rate at 15 min.

TABLE III. Initial Recurrence Sites and Surgical Margin Status in 38 Patients Without Lymph Node Metastases

Surgical margin	>5 mm	≤5 mm	Involved	*P*-value
Recurrence	6/12 (50%)	8/18 (44%)	6/8 (75%)	0.3460
Sites[a]				
Local recurrence	0	1 (13)	1 (11)	0.7389
Liver	2 (33)	7 (58)	3 (33)	
Lymph nodes	2 (33)	2 (26)	3 (33)	
Peritoneal	0	1 (13)	0	
Lung	1 (16)	1 (13)	1 (11)	
Bone	1 (16)	0	1 (11)	
Skin	1 (16)	0	0	
Solitary recurrence	4 (67)	4 (50)	4 (67)	0.7575
Multiple recurrence	2 (34)	4 (50)	2 (34)	

[a]Sites include multiple organs.

represent non-curable systemic disease, and intrahepatic metastasis might result from spread to the liver via the portal venous system [15,22,23]. In the present study, lymph node metastasis was the only independent prognostic factor predictive of poor survival, and intrahepatic metastasis was revealed to be a significant factor by univariate, but not multivariate analysis.

The prognosis of solitary ICC without lymph node metastases was favorable when microscopic curative resection could be accomplished. In contrast, nine patients who had both lymph node and intrahepatic metastasis survived for no longer than 25 months, with a median survival time of 17 months. If intraoperative ultrasonography confirms the presence of intrahepatic metastases, surgical treatment should be abandoned in patients suspected to have lymph node involvement. As preoperative assessment of lymph node involvement is generally difficult and definitive pathological diagnosis can be obtained only after intraoperative examination, aggressive surgery might be employed with predictable safety even if a small number of metastases are recognized [20,21].

ICCs are usually large and centrally located at the time of diagnosis as compared with other hepatic malignancies, which are initially difficult to detect [1,9,15]. Thirteen of the patients (23%) had a positive surgical margin even after major hepatic resection, which was necessary in 45 patients (79%). One of the major reasons for this might be that a positive surgical margin might not be significantly related with the size of the main tumor itself, but to the presence of intrahepatic metastatic nodules, which might become exposed because of the difficulty in recognizing them during the hepatic dissection. Consistent with this speculation, a positive surgical margin did not function as a negative prognostic factor in patients with intrahepatic metastasis.

Positive surgical margin has previously been reported as another important prognostic factor [12–14], but in the current study, it was not found to be a significant. However, a negative surgical margin may be associated with a significantly better survival rate in patients without lymph node metastases. These results suggest that the role of microscopic curative resection is extremely important in potentially curative condition.

The appropriate width of the surgical margin in patients with ICC has been an important clinical concern among hepatic surgeons. This is because the most frequent modes of the spread of ICC are direct sinusoidal invasion in the absence of a clear tumor capsule and vascular spread with microscopic metastatic deposits in the surrounding liver parenchyma [15]. Cherqui et al. [9] stated that curative resection requires a clear margin of ≥1 cm. Conversely, Valverde et al. [10] and Huang et al. [11] reported that a potentially narrow surgical margin was not a contraindication to resection. The present study indicated that a narrow width of the surgical margin did not adversely influence survival. The presence of possible tiny metastatic deposits around the main tumors might be negligible as compared with the spread of an intrahepatic tumor via the portal venous system in patients with ICC undergoing macroscopic curative hepatectomy. This is because the most frequent site of recurrence was the remnant liver and not local recurrence at the resectional margin.

A negative surgical margin had a definitively favorable impact on survival in patients with a solitary ICC without lymph node metastasis, and careful attention should be paid not to expose tumors at the surgical margin during hepatic dissection. In any event, the surgical outcome in ICC patients without intrahepatic or lymph node metastasis was excellent regardless of the width of the surgical margin.

ACKNOWLEDGMENTS

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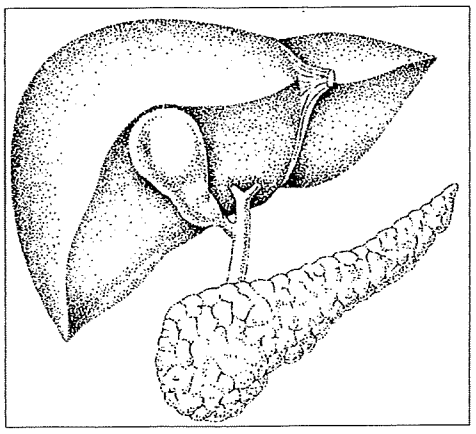

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Changing trends in surgical outcomes after major hepatobiliary resection for hilar cholangiocarcinoma: a single-center experience over 25 years

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Abstract

Background/Purpose. Hepatobiliary resection (HBR) for hilar cholangiocarcinoma (HCCa) remains a technically demanding procedure and is still associated with significant rates of morbidity and mortality. The aim of this study was to characterize changes in surgical outcomes following major HBR for HCCa at a single center over a 25-year period.

Methods. Between 1980 and 2004, 126 patients undergoing preoperative biliary drainage, portal vein embolization, and major HBR were enrolled in this study. The patients were divided into two groups according to the chronological treatment period; i.e., patients who underwent surgery during the initial 20-year period (1980–1999; early group [EG]) and those who underwent surgery during the most recent 5-year period (2000–2004; late group [LG]). Clinicopathological variables were compared retrospectively between the two groups.

Results. The mortality rate improved from 7.9% in the EG to 0% in the LG, but this difference did not reach the level of statistical significance ($P = 0.058$). The overall survival rate at 1, 3, and 5 years was 82.4%, 43.9%, and 35.2%, respectively. The overall survival rate was similar in the two groups ($P = 0.153$). Morbidity was documented in 57.1% of all the patients, and was comparable in the two groups ($P = 0.471$), but the rate of major morbidity was significantly higher in the EG ($P = 0.031$). Red blood cell and fresh frozen plasma transfusion requirements were significantly reduced in the LG, both in regard to the number of patients and the amount of blood product administered. The mean length of postoperative hospital stay was significantly reduced, from 74.4 + −56.3 days in the EG to 29.0 + −11.8 days in the LG ($P < 0.001$). Sixty-nine patients (54.8%) had stage III or IV disease (according to the *General rules for surgical and pathological studies on cancer of the biliary tract* of the Japanese Society of Biliary Surgery), and 55 patients (43.7%) showed positive surgical margins. There were no differences between the two groups in terms of surgical margins or pathological staging.

Conclusions. Improvements were documented in rates of major morbidity, length of hospital stay, and the mortality rate in the LG when compared with the EG. The overall survival rate was similar in the two groups. Blood transfusion requirements were significantly reduced in the LG when compared with the EG. However, the high proportion of patients with positive surgical margins remains a significant problem.

Key words Hilar cholangiocarcinoma · Hepatobiliary resection · Mortality · Blood transfusion · Fresh frozen plasma

Introduction

Although the use of hepatectomy with caudate lobe resection[1] has increased the resection rate of hilar cholangiocarcinoma (HCCa), major hepatobiliary resection (HBR) for HCCa remains a technically demanding procedure and carries a considerable risk of mortality and serious postoperative morbidity, such as liver failure.[2] Most reports of outcomes following major HBR for HCCa are based on small numbers of patients (i.e., <100 patients),[3–13] and the mortality rate after major HBR is often more than 5% even at high-volume centers.[14–17]

However, recent reports suggest that surgical mortality rates are lower at high-volume centers when compared with low-volume centers.[18–21] At our institution, the number of patients undergoing surgical management of biliopancreatic malignancies has increased markedly since 2000. The standard management strategy for patients with potentially resectable HCCa at our institution consists of appropriate preoperative biliary drainage, preoperative portal vein embolization (PVE),[22–24] and major HBR with caudate lobectomy. The aim of this study was to characterize changes in surgical outcomes following major HBR for HCCa at a single center over a 25-year period.

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Patients and methods

Patients

Between January 1980 and December 2004, 175 patients were admitted to our department with a diagnosis of HCCa, including diffuse bile duct carcinoma. Exclusion criteria included patients with intrahepatic cholangiocarcinoma with hilar invasion; patients who did not undergo laparotomy because of highly advanced disease or poor hepatic functional reserve during the preoperative workup; those in whom resection was not possible due to locally advanced status or dissemination; patients who underwent hilar bile duct resection alone; and patients who underwent minor hepatectomy alone. Thus, 126 patients who underwent HBR were enrolled in this study. Medical records, including the hospital charts, operation records, and pathology reports, were retrospectively analyzed.

The patient population consisted of 36 women and 90 men, with a median age of 64 years (range, 33 to 83 years). The patients were divided into two groups according to the chronological treatment period; i.e., patients who underwent surgery during the initial 20-year period (1980–1999; early group [EG]) and those who underwent surgery during the most recent 5-year period (2000–2004; late group [LG]).

Preoperative management and design of surgical procedures

Preoperative evaluation and strategies for management were performed as described previously.[25] Briefly, preoperative workup using image diagnosis included direct cholangiograms, ultrasonography, computed tomography (CT), and conventional abdominal angiography for the assessment of vascular involvement and for the mapping of the vascular anatomy. Concomitantly, patients with obstructive jaundice or obvious cholestatic liver damage principally underwent percutaneous transhepatic biliary drainage (PTBD). The type of hepatectomy was chosen based on the dominant location and extension of the tumor, vascular involvement, and hepatic functional reserve. In patients undergoing right hemihepatectomy or more extensive resection (resected liver volume, estimated using CT-volumetry, >50%), PVE for the resected liver segment was indicated to induce compensatory hypertrophy of the future remnant liver.[22–24]

The indocyanine green (ICG) retention rate at 15 min, after the serum total bilirubin level had decreased to less than 3 mg/dl, was the most reliable key datum for us to evaluate liver functional reserve in our strategy. Definitive surgery was planned 2 to 4 weeks after PVE and was typically performed when the serum total bilirubin level had decreased to less than 2 mg/dl.

Surgery

All the patients underwent hemihepatectomy, bisectionectomy, or more extensive resection with en-bloc resection of the caudate lobe and extrahepatic bile duct, as well as bilio-enterostomy using a Roux-en-Y jejunal limb. Systematic lymphadenectomy for the nodes at the hepatoduodenal ligament and upper part of the retropancreatic and celiac axis was also performed. All liver transections were performed employing the forceps clamp crushing method during hepatic artery and portal vein clamping for 15 min with 5-min intervals (Pringle's maneuver).

The surgical procedures are summarized in Table 1. Right-sided hepatectomy was performed in 63 patients, and left-sided hepatectomy was performed in 62 patients. Only 1 patient underwent central hepatic bisectionectomy. Combined portal vein resection and reconstruction was performed in 18 patients (14.3%), and 2 of these patients underwent concomitant resection and reconstruction of the hepatic artery. Hepatopancreatoduodenectomy (HPD)[26] was performed in 10 patients (7.9%) who had extensive distal bile duct cancer extension, in order to secure the distal bile duct margin. Reconstruction during HPD was conducted by end-to-side pancreaticojejunostomy, using a modified Child's method, in one stage. The amount of blood loss was measured from the volume of blood collected in the suction container plus the weight of gauze soaked with blood.

Blood transfusion strategy[27]

The liberal red blood cell (RBC) transfusion policy employed during the early time periods of this study was significantly restricted in the late 1980s. The most recent criteria consist of RBC transfusion for patients with a hemoglobin concentration of less than 7.5 g/dl during the operation or less than 7.0 g/dl postoperatively. Further, transfusions of fresh frozen plasma (FFP) have undergone a similar shift towards minimization over time. Regardless, FFP transfusion is indicated for patients undergoing extensive liver resection (i.e., >60% of the whole liver), those with poor hepatic functional reserve, or for postoperative hyperbilirubinemia of more than 3.0 to 5.0 mg/dl, at the discretion of the operating surgeon. The volumes of a unit of packed RBC and FFP are 130 ml and 80 ml, respectively.

Definitions of morbidity and mortality

Operative mortality included all in-hospital deaths. In regard to morbidity, all postoperative complications

Table 1. Surgical procedures and operative variables in 126 patients

	EG (1980–1999) ($n = 63$)	LG (2000–2004) ($n = 63$)	P value
Type of hepatectomy			
S1,4,5,6,7,8	0	2	
(ext) S1,5,6,7,8	25	36	
S1,2,3,4,5,8,	2	5	
(ext) S1,2,3,4,	36	19	
S1,4,5,8,	0	1	
Right-sided/left-sided	25/38	38/24[a]	0.016
Trisectionectomy	2 (3.2%)	7 (11.1%)	0.084
With PVE	22 (34.9%)	43 (68.3%)	<0.001
With PV	5 (7.9%)	13 (20.6%)	0.073
With PV + HA	2 (3.2%)	2 (3.2%)	1.000
With HA	0	0	1.000
With PD	3 (4.8%)	7 (11.1%)	0.164
Operation time (min)	639 ± 155	689 ± 161	0.212
Blood loss (g)	1480 ± 943	1912 ± 706	<0.001

Numerals indicate Couinaud's segment of the liver
PVE, preoperative portal vein embolization; PV, portal vein resection and reconstruction; HA, hepatic arterial resection and reconstruction; PD, pancreatoduodenectomy
[a] Excluding a patient with central hepatic bisectionectomy

that potentially influenced the outcome or lengthened the hospital stay were considered. Major complications were defined as those that resulted in organ failure or those that required a surgical or interventional radiological procedure. Those complications that could be managed and those that responded to conservative management without intervention or that resolved spontaneously were defined as minor complications.

Final staging of the disease

Histopathological diagnosis of the disease was assessed by pathologists, and the pathological staging of the disease was determined according to the criteria of the International Union Against Cancer (UICC) (sixth edition)[28] and the *General rules for surgical and pathological studies on cancer of the biliary tract* of the Japanese Society of Biliary Surgery (JSBS) (fifth edition).[29]

Statistics

Results are expressed as mean values with SD. Dichotomous variables were evaluated by χ^2 analysis or Fisher's test, as appropriate. The statistical significance of continuous variables was determined by Student's t-test or the Mann-Whitney test. The survival curve for the 126 study patients was generated by the Kaplan-Meier method, and the log-rank test was applied to compare survival between different groups. Results were considered significant when the P values were less than 0.05. All statistical analyses were performed using a statistical analysis software package (SPSS 11.5; SPSS, Chicago, IL, USA).

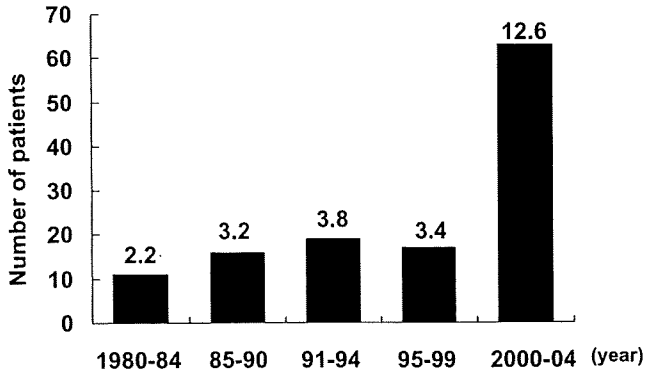

Fig. 1. Numbers of patients who underwent major hepatobiliary resection for hilar cholangiocarcinoma in the indicated 5-year periods. *Numbers above the columns* show the annual numbers of patients during the indicated period

Results

Morbidity, mortality, and survival

The annual number of patients who underwent major HBR for HCCa increased from 3.2 in the EG (1980–1999) to 12.6 in the LG (2000–2004; $P = 0.016$; Fig. 1). Although the overall in-hospital mortality rate was 3.9% (5/126), all the in-hospital mortalities occurred in the 1980s (Table 2), and the in-hospital mortality rate was 0% in 100 consecutive patients who were enrolled in the study since 1989. The mortality rate improved from 7.9% in the EG to 0% in the LG, but this difference did not reach the level of statistical significance ($P = 0.058$). The overall survival rates at 1, 3, 5, and 10 years were 82.4%, 43.9%, 35.2%, and 22.2%, respec-

Table 2. Clinical characteristics of patients who died in hospital

Case number	Age (years)	Sex	Type of hepatectomy[a]	POD	Preoperative complication	Main cause of death	Year
1	68	Male	Ext. S1,5,6,7,8	33		Hepatic failure	1981
2	71	Male	Ext. S1,5,6,7,8	95	Schistosomiasis	Hepatic failure	1984
3	66	Male	Ext. S1,5,6,7,8	30	Cholangitis	Sepsis, hepatic failure	1986
4	62	Male	Ext. S1,2,3,4	6		Intraabdominal bleeding, hepatic infarction	1987
5	72	Male	S1,2,3,4,5,8	25		Hepatic failure	1989

POD, postoperative day
[a]Numbers indicate Couinaud's segment of the liver

Table 3 Postoperative course in 126 patients

	EG (1980–1999) (n = 63)	LG (2000–2004) (n = 63)	P value
Morbidity			
Major	15	6	0.031
Minor	23	28	0.364
Total	38 (60.3%)	34 (54.0%)	0.471
Mortality	5 (7.9%)	0 (0%)	0.058
Postoperative hospital stay (days)	74.4 ± 56.3	29.0 ± 11.8	<0.001
Median [range]	65 [15–429]	26 [23–63]	<0.001

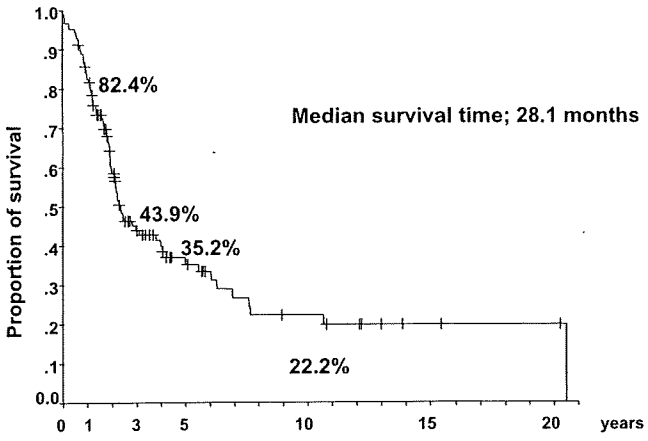

Fig. 2. Cumulative survival of patients with hilar cholangiocarcinoma over a 25-year period at our institution. The overall survival rates at 1, 3, 5, and 10 years were 82.4%, 43.9%, 35.2%, and 22.2%, respectively. The median survival was 28.1 months

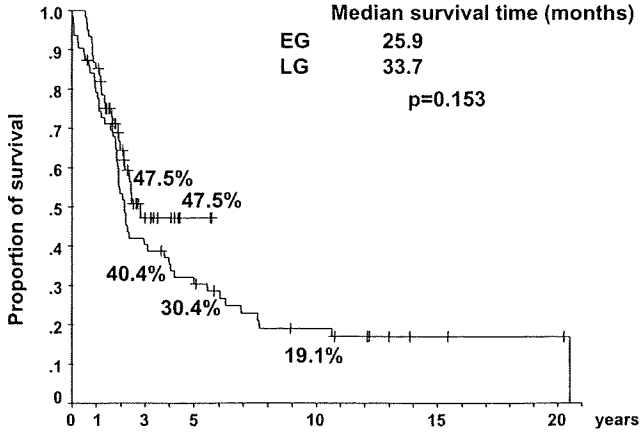

Fig. 3. Cumulative survival according to the chronological treatment period; i.e., the initial 20-year period (early group [*EG*]) and the most recent 5-year period (late group [*LG*]). In the EG, the overall survival rates at 3, 5, and 10 years were 40.4%, 30.4%, and 19.1%, respectively, and the median survival was 25.9 months. In the LG., the overall survival rates at 3 and 5 years were 47.5% and 47.5%, respectively, and the median survival was 33.7 months. The overall survival rate was similar in the two groups (P = 0.153)

tively (Fig. 2). The median survival in all patients was 28.1 months (range, 0.17 to 246.1 months), and the overall survival rate was similar in the two groups (P = 0.153; Fig. 3). Morbidity was documented in 57.1% of all patients (72/126), and the morbidity rates in the two groups were comparable (60.3% in the EG versus 54.0% in the LG; P = 0.471). However, major morbidity was significantly higher in the EG than in the LG (P = 0.031; Table 3).

Preoperative variables

Patient characteristics are summarized in Table 4, in which nine clinical variables are compared. The ICG retention value at 15 min was significantly better in the LG than in the EG (P < 0.001). Ninety-eight patients (77.8%) underwent preoperative biliary drainage in

Table 4. Characteristics of patients who underwent major hepatobiliary resection

Variables	EG (1980–1999) ($n = 63$)	LG (2000–2004) ($n = 63$)	P value
Sex (male/female)	46/17	44/19	0.693
Age (year)	61.7 ± 9.6	63.2 ± 7.9	0.610
[range]	[39–83]	[33–78]	
Preoperative biliary drainage performed	46 (73.0%)	52 (82.5%)	0.312
ICGR15 (%)	12.1 ± 5.9	8.7 ± 6.8	<0.001
Preoperative serum total bilirubin (mg/dl)	1.4 ± 0.9	1.1 ± 0.5	0.225
Total protein (mg/dl)	7.0 ± 0.6	6.9 ± 0.5	0.193
Albumin (mg/dl)	3.7 ± 0.4	3.7 ± 0.3	0.965
Hemoglobin (g/dl)	12.4 ± 1.4	12.4 ± 1.8	0.596
Platelet ($\times 10^4/\mu l$)	27.4 ± 11.5	24.6 ± 7.2	0.189

ICGR15, indocyanine green retention value at 15 min

Table 5. Blood transfusion data in patients who underwent major hepatobiliary resection

	EG (1980–1999) ($n = 63$)	LG (2000–2004) ($n = 63$)	P value
Blood transfusion			
Packed red blood cells			
Yes	31 (49.2%)	18 (28.6%)	0.019
No	32 (50.8%)	45 (71.4%)	
Mean volume of transfusion (U)	50 ± 8.8	1.3 ± 2.4	0.019
Median [range]	2 [0–56]	0 [0–12]	0.013
Fresh frozen plasma			
Yes	61 (96.8%)	52 (82.5%)	0.002
No	2 (3.2%)	11 (17.5%)	
Mean volume of transfusion (U)	96.6 ± 89.5	51.1 ± 47.2	0.001
Median [range]	70 [0–446]	40 [0–242]	<0.001

total, and there was no difference between the two groups (73.0% in the EG versus 82.5% in the LG; $P = 0.312$). For the other variables, there was no significant difference when comparing the EG and LG.

Operative variables

Surgical procedures are summarized in Table 1. The proportion of patients who underwent right-sided hepatectomy was significantly higher in the LG ($P = 0.016$), and the proportion of patients with preoperative PVE was also significantly higher in the LG than in the EG ($P < 0.001$). Trisectionectomy and portal vein resection and reconstruction were more frequently indicated in the LG than in the EG, but these differences did not reach the level of statistical significance ($P = 0.084$; and $P = 0.073$, respectively). Although the operation time was comparable in the two groups (689 versus 639 min, $P = 0.212$), intraoperative blood loss was significantly increased in the LG when compared with the EG (1912 versus 1480 ml; $P < 0.001$).

Blood transfusion

As shown in Table 5, RBC transfusion requirements were reduced, from 49.2% in the EG to 28.6% in the LG ($P = 0.019$), and the mean volume of transfused RBC decreased from 5.0 ± 8.8 U in the EG to 1.3 ± 2.4 U in the LG ($P = 0.019$). FFP transfusion requirements also decreased, from 96.8% in the EG to 82.5% in the LG, and the mean volume of transfused FFP decreased from 96.6 ± 89.5 U in the EG to 51.1 ± 47.2 U in the LG ($P = 0.001$).

Histopathological variables (Table 6)

Histologically, 55 patients (43.7%) showed positive surgical margins, including proximal and/or distal bile ducts and excisional margins. Sixty-one patients (48.4%) had lymph node metastasis, and direct tumor invasion to the liver parenchyma was confirmed in 32 patients (25.4%). There were no differences between the two groups in terms of surgical margins, histological grade, lymph

Table 6. Histopathological variables in 126 patients

Variables	EG (1980–1999) (n = 63)	LG (2000–2004) (n = 63)	P value
Surgical margins			
Positive/negative	27/36	28/35	1.000
Histological grade			
Well, pap/others	29/34	19/44	0.067
Lymph node metastasis			
Present/absent	29/34	32/31	0.593
Invasion to the liver parenchyma			
Present/absent	14/49	18/45	0.414
Staging (JSBS)			
I, II/III, IV	32/31	25/38	0.367
Staging (UICC)			
I, II/III, IV	49/14	52/11	0.656

JSBS, Japanese Society of Biliary Surgery; UICC, International Union Against Cancer

node metastasis, or invasio n into the liver parenchyma. Sixty-nine patients (54.8%) had stage III or IV disease according to the JSBS system, and there was no difference in the histological staging, using the JSBS or the UICC system, when comparing the two groups.

Postoperative hospital stay

The overall mean length of postoperative hospital stay was 50.2 ± 46.2 days (median, 38 days; range, 5–429 days) in all patients, with the mean length being 74.4 ± 56.3 days in the EG, and 29.0 ± 11.8 days in the LG ($P < 0.001$). The mean length of postoperative hospital stay in patients with minor morbidity was 65.4 ± 19.3 days (median, 68 days; range, 30–105 days) in the EG, and 34.1 ± 11.1 days (median, 33 days; range, 19–63 days) in the LG ($P < 0.001$).

Discussion

While the management of liver tumors with hepatectomy is relatively safe, with a mortality rate of less than 5% at most institutions,[30–33] major HBR for HCCa is a technically demanding procedure. The present study demonstrated that the mortality rate following major HBR for HCCa tended to decrease over the past several decades, although this difference did not reach the level of statistical significance. Of note, the mortality rate for major HBR for HCCa was 0% for the most recent 100 patients who had been enrolled since 1989. Because the present treatment strategy of preoperative biliary drainage, preoperative PVE, and major HBR with caudate lobectomy was performed by more than ten attending surgeons throughout the course of this study, it is likely that this is a practical approach for the general population of hepatobiliary surgeons.

The reason for the increase in the number of patients undergoing resection of HCCa at our center since 2000 is unclear, but may be related to the increasing information available to patients and their families regarding disease and hospital capacity, surgical volume, and outcomes. Indeed, as the incidence rate of hepatobiliary malignancies is thought to be lower than that of gastrointestinal malignancies, it seems that the number of patients with hepatobiliary malignancies suitable for resection was likely to be concentrated at a few major centers. Thus, the low rate of mortality in our LG is consistent with the notion that hospital surgical volume is inversely correlated with mortality rates.[18–21]

In recent years, patients with obstructive jaundice have tended to undergo biliary drainage at a low range of serum total bilirubin; consequently, the recovery from cholestatic liver damage may be quite rapid, and this may be the reason that the ICG retention value was better in the LG than in the EG. In tumors that are resectable by either right-sided or left-sided hepatectomy, right-sided resection is the preferred method for patients with a satisfactory functional reserve of the future remnant liver; therefore, right-sided hepatectomy and PVE were more frequently indicated in the LG than in the EG.

Although the operation time was comparable in the two groups in our study, the intraoperative blood loss was approximately 400 ml more in the LG that in the EG. The absolute blood volume loss of 1912 ml in the LG was comparable to that seen in another recent large series of hilar malignancies treated with HBR.[34] The definitive reason for the larger blood loss in the LG remains unclear, but it may be related to extended surgical procedures, including portal vein resection and reconstruction, trisectionectomy, and HPD, which tended to be more frequently indicated in the LG than in the EG.

While the blood transfusion requirement was significantly reduced in the LG when compared with the EG, 28.6% of patients in the LG still underwent RBC transfusion, and 82.5% underwent FFP transfusion; figures that are higher than the transfusion requirement reported in other studies.[34,35] The main purpose of FFP administration is to induce volume expansion without homologous RBC transfusion,[27] which would otherwise be associated with an increased risk of postoperative hyperbilirubinemia[27,34] or immunosuppression.[36] However, the liberal use of FFP results in a higher perioperative cost and a higher risk of transmission of infectious diseases, making minimization of FFP transfusions desirable. Although the proportion of patients receiving FFP decreased, from 96.8% in the EG to 82.5% in the LG, the mean volume of transfused products decreased from 96.6 U in the EG to 51.1 U in the LG. Further minimization of transfusion may help to improve outcomes.

In the present study, the proportion of patients with cancer-positive surgical margins exceeded 40%, which is higher than that reported in another large series, which achieved more than 70% of patients with cancer-negative surgical margins.[4–6,11–13,15–17] Regardless of this finding, the 5-year survival of the patients with positive surgical margins was not necessarily low. Sixty-one patients (48.4%) had lymph node metastasis and more than half of the patients were diagnosed as having stage III or IV disease according to the JSBS system. These data suggest that the patients in this series were not necessarily shifted to an early stage. Thus, the relatively high rate of positive surgical margins in the present study compared with rates in other studies may be attributed either to the strict diagnostic criterion of bile duct margins that we used or to differences in institutional diagnostic criteria.[37] Another possibility is that a positive surgical margin is not a significant prognostic factor.[38] Indeed, several patients in our series have survived for more than 5 years without tumor recurrence, despite having positive surgical margins. Further study to clarify the diagnostic accuracy of bile duct margins and the impact of positive bile duct margins on survival would be of benefit.

In our series, 40% of patients with stage II disease, according to the JSBS system, had positive bile duct margins. Tumor extension in the bile duct consisted of perpendicular and horizontal extensions along the bile duct. While aggressive surgical strategies to combat horizontal extension, such as trisectionectomy or HPD, may be selected, the safety, efficacy, and indications for HPD in patients with biliary cancer remain controversial. In fact, trisectionectomy to minimize future remnant liver volume may be a burden for patients with cholestatic liver damage and may result in serious complications, such as liver failure. When considering patients for trisectionectomy or HPD, we must pay special attention to the safety of such invasive procedures, considering the reported high morbidity and mortality rates associated with HPD for biliary cancer.[26,39,40]

Although overall postoperative morbidity in HBR for HCCa is still high,[2] we found that the rate of major complications and length of hospitalization in patients with minor morbidity was significantly reduced in our LG, which likely accounts for the reduced total length of hospitalization in the LG when compared with the EG.

In summary, improvements were documented in major morbidity rates, length of hospital stay, and the mortality rate in the LG when compared with the EG. The overall survival rate was similar in the two groups. Blood transfusion requirements were significantly reduced in the LG when compared with the EG. However, the high proportion of patients with positive surgical margins remains a significant problem.

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Genome-wide array-based comparative genomic hybridization analysis of pancreatic adenocarcinoma: Identification of genetic indicators that predict patient outcome

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We analyzed the subchromosomal numerical aberrations of 44 surgically resected pancreatic adenocarcinomas by array-based comparative genomic hybridization. The aberration profile ranged widely between cases, suggesting the presence of multiple or complementary mechanisms of evolution in pancreatic cancer, and was associated with lymph node metastasis and venous or serosal invasion. A large number of small loci, previously uncharacterized in pancreatic cancer, showed non-random loss or gain. Frequent losses at 1p36, 4p16, 7q36, 9q34, 11p15, 11q13, 14q32-33, 16p13, 17p11-13, 17q11-25, 18q21-tel, 19p13, 21q22 and 22q11-12, and gains at 1q25, 2p16, 2q21-37, 3q25, 5p14, 5q11-13, 7q21, 7p22, 8p22, 8q21-23, 10q21, 12p13, 13q22, 15q13-22 and 18q11 were identified. Sixteen loci were amplified recurrently. We identified novel chromosomal alterations that were significantly associated with a range of malignant phenotypes. Gain of LUNX, HCK, E2F1 and DNMT3b at 20q11, loss of p73 at 1p36 and gain of PPM1D at 17q23 independently predicted patient outcome. Expression profiling of amplified genes identified Smurf1 and TRRAP at 7q22.1, BCAS1 at 20q13.2-3, and VCL at 10q22.1 as potential novel oncogenes. Our results contribute to a complete description of genomic structural aberrations and the identification of potential therapeutic targets and genetic indicators that predict patient outcome in pancreatic adenocarcinoma. (*Cancer Sci* 2007; 98: 392–400)

Pancreatic adenocarcinoma is a leading cause of cancer-related death worldwide; the 5-year survival rate for patients that underwent surgery remains below 5%.[1] Pancreatic adenocarcinoma appears to successively acquire genetic aberrations in genes involved in the regulation of cell proliferation, the central ones being early activating mutations of the K-ras oncogene, followed by inactivation of the p53, p16 and DPC4 TSG.[2] The application of chromosome CGH,[3] karyotype and allelotype studies in pancreatic cancer has also revealed a large number of complex structural and numerical aberrations at the subchromosomal level.[4–11] Recurrent aberrations reported concern copy number gain on 3q, 5p, 7p, 8q, 11q, 12p, 17q, 19q and 20q and loss on 1p, 3p, 4q, 6q, 8p, 9p, 10q, 12q, 13q, 15q, 17p, 18q, 19p, 21q and 22q.[8,12] aCGH methods have recently been developed and used in studies of various malignancies, including pancreatic cancer. The latter used cell lines,[13–18] and a small number of primary cases[14,15] or xenografts,[19] to confirm previously described regional alterations and identify novel ones. Although some of these loci are known to contain oncogenes or TSG,[2] the role that copy number alterations of most of the above loci play in pancreatic cancer genesis or progression, if any, is far from being fully evaluated. From these and previous studies, it is also evident that there exists substantial variation in the reported aberrations between studies as well as between individual cases.

The aim of the present study was to examine the SNAP of pancreatic cancer to identify novel loci that contain genes for which copy number status is likely to be relevant to pancreatic carcinogenesis or associated with clinically relevant parameters. For this, we used aCGH to examine a comparatively large number of well-characterized primary cases and LCM to allow more accurate analysis. In addition, mRNA expression analysis of loci exhibiting amplifications was carried out to identify genes that are amplified recurrently and overexpressed in pancreatic cancer.

Materials and Methods

Tumor samples. Forty-four methanol-fixed pancreatic ductal adenocarcinomas from 43 patients were examined (Suppl. Table 1). These included 33 specimens from patients who had undergone surgery at the National Cancer Center Hospital between 1994 and 2003, and 11 xenografts that were produced following the orthotopic implantation of tumors in severe combined immunodeficient mice, as described previously.[20] Forty-two samples were of primary tumors, one of a liver metastasis and one of a pancreatic xenograft of a liver metastasis, the corresponding primary of which was also examined. Tumor classification was carried out according to the Japan Pancreas Society guidelines.[21] The study was approved by the institutional review board of the National Cancer Center.

LCM and whole-genome amplification. LCM was carried out with a PixCell II (Arcturus Engineering, Mountain View, CA, USA). At least 5000 tumor cells per sample were recovered. Genomic (test) DNA was extracted by standard procedures. Sex-matched high molecular weight human genomic DNA (Promega, Madison, WI, USA) was sheared randomly (HydroShear; Gene Machines, San Carlos, CA, USA) and used as reference DNA. Both test and reference DNA were amplified

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Abbreviations: aCGH, array-based comparative genomic hybridization; BAC, bacterial artificial chromosome; CGH, comparative genomic hybridization; HD, homozygous deletion; LCM, laser-capture microdissection; PCR, polymerase chain reaction; SNAP, subchromosomal numerical aberration profile; TSG, tumor suppressor gene.

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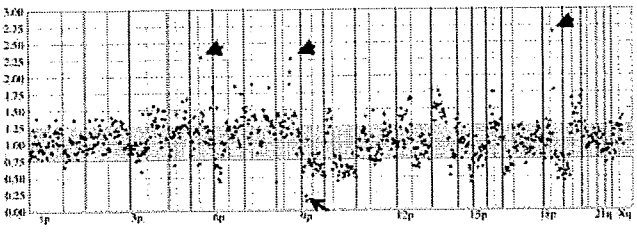

Fig. 1. Chromosomal copy number changes revealed by array-based comparative genomic hybridization. Representative array-based comparative genomic hybridization profile of a pancreatic adenocarcinoma. Copy number losses (ratio < 0.75) and gains (ratio > 1.25) were detected in both large fractions of the chromosome arms and small chromosomal regions. Amplifications (ratio > 2.00, arrowheads) and homozygous deletions (ratio < 0.25, arrow) were also identified in this tumor. The average signal ratios (test:reference) of two normalized signals from duplicated spots are given from chromosome 1p telomere (left) to Xq telomere (right). The vertical dotted and continuous lines indicate the position of the centromere and telomere of each chromosome, respectively.

using an adaptor ligation-mediated whole-genome PCR, as described previously.[22]

Array-based CGH. A custom-made CGH array ('MCG Cancer Array-800 ver. 2') was used, consisting of 800 duplicated target BAC clones that correspond to chromosomal loci of potential importance in various cancers (listed at http://www.cghtmd.jp/CGHDatabase/microarray/mcg800_array_e.htm). Labeling of the DNA probes, hybridization, data acquisition and data normalization were carried out as described previously.[23–25] Based on control experiments,[26] we considered a signal ratio <0.75 or >1.25 to indicate loss or gain, respectively, and a ratio of <0.25 or >2.00 to indicate HD or amplification, respectively.

The validity of our aCGH data was confirmed by fluorescence *in situ* hybridization, PCR (Suppl. Fig. 1), loss of heterozygosity analysis and immunohistochemistry for selected genes.[26]

Expression profiling of primary xenografts. We used xenografts for gene expression analysis due to their abundance in tumors cells compared with primary tumors. We focused on the relationship between amplification and overexpression; additional gene expression profiling results will be submitted in a subsequent publication.

Total RNA was extracted from frozen xenograft samples, biotin-labeled cRNA synthesized and hybridized to a probe array (HG-U95Av2, Affymetrix) and data acquired as described.[27] A probe set signal log ratio (SLR) of the gene expression level in the tumor relative to the control (normal pancreas) >1.5 was defined as indicating overexpression.

Statistical analysis. The χ^2 test was used to assess the statistical significance, set at 0.05, of intergroup differences in the frequency of aberrations of individual loci. The relationship between clinicopathological parameters and the number of aberrations per case was evaluated using Student's unpaired *t*-test. Survival curves were calculated using the Kaplan–Meier method, and differences in survival periods were analyzed with the log-rank test.

Results

Range of numerical aberrations. We constructed and analyzed the genomic profile of 44 pancreatic adenocarcinomas using aCGH. Subchromosomal numerical aberrations were revealed in all but two (42/44) of the tumors examined (Fig. 1). The number of aberrations differed widely between cases (Suppl. Table 2; Suppl. Fig. 2). Apart from the two cases in which no copy number changes were observed, a third case showed changes in only 11 loci (all gains), whereas nine cases (20%) had alterations in more than 50% of loci. In most cases (34/44), the number of gains was higher than the number of losses ($P < 10^{-7}$). Overall, however, the loss rate was similar to the gain rate (19% of loci altered on average per case for both). Similarly, amplifications were observed more frequently, in terms of number of cases and number of aberrations per case, than HD. Most loci showed aberrations in at least one case, the majority showing loss or gain in 2–25% and 0–20% of cases, respectively.

Loss. The most frequently lost loci were 17p13.3 (ABR, in 75% of cases), 18qtel (CTDP1, SHGC-145820, 68%) and 18q21 (SMAD7, 66%). The loci containing the p16 (9p21), p53 (17p13.1), SMAD4 and DCC (both at 18q21) genes were lost in 41, 55, 61 and 30% of cases, respectively. In total, 33 loci with frequent (>50%) losses were identified at 1p36, 4p16.3, 7q36, 9q34.3, 11p15, 11q13, 14q32-33, 16p13.3, 17p11.2, 17p13.1-3, 17q11-qter, 17q21.2, 17q25, 18q21, 18qtel, 19p13.2-3, 21q22.3, 22q11.23 and 22q12.1-2 (Fig. 2). The chromosome arms with the highest number of loci lost, taking into account only loci that were lost in >25% of cases, included, in descending order of frequency, 1p, 11q, 17p, 10q, 8p, 18q, 22q, 6q, 9p, 14q and 17q (Suppl. Table 3).

Homozygous deletions. Twenty-six loci with HD were detected, nine of which were in more than one case (Table 1). HD were detected in 11 cases (25%), seven of which in only one locus. The 1p35-36.33 region contained the highest number of loci deleted (six). The most frequently deleted locus was 9p21 spanning the p16 gene, whereas the locus containing SMAD4 (18q21) was deleted in one case.

Gains. Loci with frequent (>50% cases) gains were identified at 1q25.2-q25.3, 2p16, 2q21.2, 2q23-q37, 2q31, 2q33, 2q34, 3q25.1, 5p14.2, 5q11.2-q13.2, 7q21.1, 7p22, 8p22, 8q21, 8q22-q23, 10q21.1, 12p13.33, 13q22, 15q13-q22 and 18q11.2 (Fig. 2; Suppl. Table 4). The most frequently gained locus was 7q21.1 (71%) containing the HGF gene. The loci spanning the KRAS2 (12p12.1) and KRAG (12p11.2) genes were gained in 45 and 20% of cases, respectively. The NRAS (1p13), MYC (8q24), MDM2 (12q14.3) and AKT1 (14q32.2)[28] loci were gained in 45, 43, 36 and 18% of cases, respectively.

Amplifications. Amplifications were observed in 37 tumors. The seven cases in which no amplification was observed included six with few aberrations, and, interestingly, one case with 419 aberrations. Nineteen cases had amplifications in more than 1%, and three cases in more than 5% of loci examined.

Sixteen loci were amplified in five cases or more (>10%) (Table 2). The most frequently amplified locus was 18q11.2 containing RBBP8. 7q34 (BRAF) was amplified in four cases, whereas 12p12.1 (KRAS2), 1p13 (NRAS) and 8q24 (MYC) were amplified in two cases each.

Association of SNAP with clinicopathological parameters. A number of clinicopathological parameters were associated with the degree and type of aberration (Suppl. Table 5). Overall, cases with a phenotype indicating increased malignant potential had a higher degree of aberrations. Smaller tumors and tumors with higher venous or perineural invasion histological scores had a higher total number of aberrations than tumors that were larger or with lower invasion scores. No other clinicopathological parameters examined, such as the sex, primary tumor location, macroscopic type (infiltrative or nodular), degree of differentiation (Suppl. Table 6), infiltration or otherwise of certain neighboring tissues, pattern of such infiltration (INF α, β, or γ), or spread within the main pancreatic duct had significant correlation with SNAP (data not shown).

Association with venous invasion. Venous invasion-negative tumors had markedly different SNAP than venous invasion-positive tumors, although it should be noted that only a small number of negative tumors was examined. The loci lost or gained more frequently in the venous invasion-positive tumors are shown in

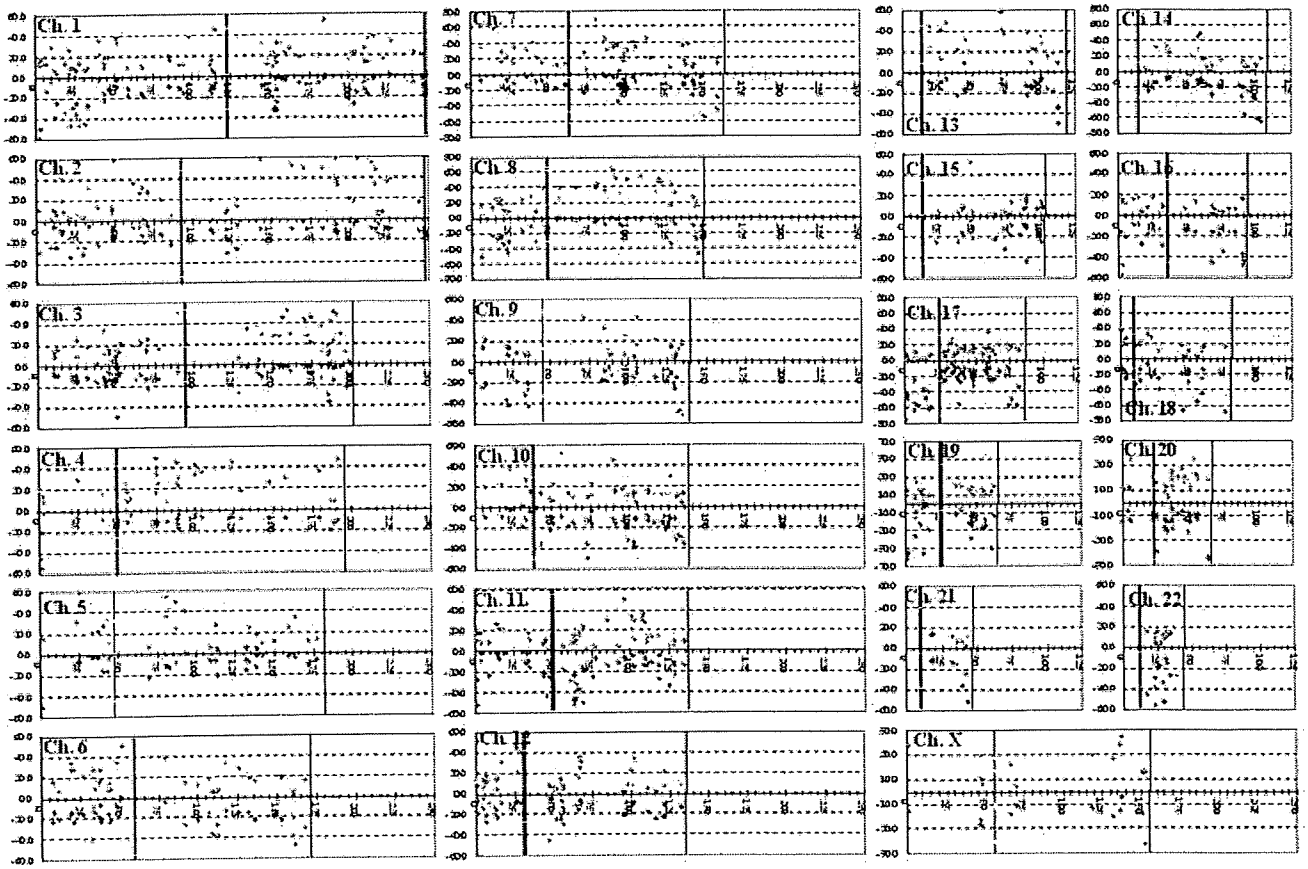

Fig. 2. Distribution of chromosomal copy number aberrations in pancreatic cancer. The horizontal axis indicates the physical distance (Mb) of the chromosomal loci from the telomere of the short arm. The vertical axis indicates the frequency (%) of tumors with chromosomal alterations (green, gain; red, loss). The vertical dotted and continuous lines indicate the positions of the centromere and telomere of each chromosome, respectively.

Table 1. Loci deleted homozygously in more than one case

Locus†	No. cases	Percentage of cases
9p21 (p16)	5	11
16p13.3 (ABCA3)	3	7
1p36.1 (p73)	2	5
5p15 (TERT)	2	5
11p15 (HRAS)	2	5
17q25 (MAFG)	2	5
18q21 (SMAD7)	2	5
18qtel (CTDP1,SHGC-145820)	2	5
19p13.3 (ABCA7)	2	5

†Known cancer-related genes contained in the respective clones are shown in parentheses.

Table 2. Loci amplified in more than 10% of cases

Locus†	No. cases	Percentage of cases
18q11.2 (RBBP8)	10	23
7q21.1 (HGF)	9	20
2q31 (PMS1)	7	16
11q13.3 (BCL1,FGF4)	7	16
2q34 (ERBB4)	6	14
11q13 (CCND1)	6	14
7q22.1 (Smurf1)	6	14
8q21 (NBS1)	5	11
2p16 (GTBP)	5	11
7p22 (ETV1)	5	11
2q21.2 (LRP1B)	5	11
2q35 (HUP2)	5	11
6q22 (ROS1)	5	11
8p11.2-p11.1 (FGFR1)	5	11
7q22.1 (CYP3A4)	5	11
7q22.1 (TRRAP)	5	11

†Known cancer-related genes contained in the respective clones are shown in parentheses.

Table 3. HD were not observed in the venous invasion-negative tumors (0/5 vs 19/37, $P = 0.03$). In the venous invasion-negative tumors, 178 and 84 loci, respectively, were lost or gained more frequently than in the positive tumors; these included frequently amplified loci (3/5) such as the ones containing FGF7 (15q13-q22), BRAF (7q34) ($P \leq 4.1 \times 10^{-5}$), ROS1 (6q22), GTBP (2p16) ($P = 0.0004$) and HGF (7q21.1) ($P = 0.02$).

Association with lymph node metastasis. Unlike venous invasion, few differences were observed when the genomic profiles of lymph node metastasis-positive and lymph node metastasis-negative tumors were compared, although only six negative tumors were examined. Only three loci showed significant differences in their signal ratios, 9p13 (SCYA21), 11q22 (ATM) and 17q12 (RAD51L3). Xq28 (MAGEA2) was lost more frequently in the lymph node metastasis-negative group (3/6 vs

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Table 3. Loci altered frequently in the venous invasion-positive pancreatic adenocarcinomas

Chromosomal locus	Contained cancer-related gene	Sub-chromosomal loss detected				P value†
		Venous invasion-positive cases		Venous invasion-negative cases		
		n	%	n	%	
19p13.3	ABCA7	26	70	0	0	0.002
9q34.3	ABCA2	25	68	1	20	0.040
1p36.33	TP73	22	59	0	0	0.012
11q13	FGF3	22	59	0	0	0.012
4p16	GAK	20	54	0	0	0.023
11q12	LTBP3	20	54	0	0	0.023
20q13	Livin	20	54	0	0	0.023
18q22	BCL2	19	51	0	0	0.030
5p14.2	CDH10	25	68	0	0	0.004
8q24	OPG	21	57	0	0	0.017
3q27-q29	TP63	20	54	0	0	0.023
8q24.1	NOV	20	54	0	0	0.023
14q22.3	RBBP1	19	51	0	0	0.030

†χ^2 test.

Table 4. Loci altered frequently in pancreatic adenocarcinoma cases with short-survival (<1 year) compared with long-survival periods

Chromosomal locus	Contained cancer-related gene	Sub-chromosomal loss detected				P value†
		Venous invasion-positive cases		Venous invasion-negative cases		
		n	%	n	%	
1p36.33	TP73	9	69	4	21	0.006
8q24.3	GLI4	5	38	1	5	0.018
Xq12	AR	5	38	1	5	0.018
11q13	STIP1, FOLR1	8	62	5	26	0.046
20q11.2	LUNX, TOP1	5	38	0	0	0.003
18p11.3	TGIF	6	46	1	5	0.006
4q13-q21	AREG	4	31	0	0	0.010
6q21	CCNC	4	31	0	0	0.010
10q21.1	PCDH15	10	77	6	32	0.012
1p32	RLF	5	38	1	5	0.018
2q36	Cul3	8	62	4	21	0.020
17q23	PPM1D	8	62	4	21	0.020
4q21	GRO1	6	46	2	11	0.022
1p36.2	KIAA0591(KIF1B)	7	54	3	16	0.023
4q21	GRO2	7	54	3	16	0.023
13q32	GPC5	7	54	3	16	0.023
8q22-q23	EIF3S6	9	69	6	32	0.036

†χ^2 test.

5/36, $P = 0.037$). Four loci, all on 7q21-22 (containing the HGF, DMTF1, MLL5 and CDK6 genes), were gained more frequently in the lymph node metastasis-positive group (all $P < 0.05$).

Association with survival. Thirty-two cases had survival data amenable to analysis. The genomic profiles of cases with a survival period shorter ($n = 13$) or longer than ($n = 19$) 1 year were compared. Four and 13 loci, respectively, were lost or gained more frequently in the short-compared with the long-survival group (Table 4). In contrast, only two loci were lost (6q25/ESR1 and 22q11.23/ADRBK2) and none gained more frequently in the long-compared with the short-survival group. Loss of 1p36 (p73) and 11q121-3 was associated with both short-term survival and evidence of venous invasion, whereas gain of 7q21-22 was associated with both short-term (<3 years) survival and the presence of lymph node metastases.

Kaplan–Meier analysis showed that loss of 1p36 (p73) ($P = 0.02$; Fig. 3a), gain of 17q23 (PPM1D) ($P < 0.05$; Fig. 3b) and particularly gain of the LUNX locus at 20q111-12 ($P < 0.0001$; Fig. 3c) were significantly associated with prognosis, whereas loss of the STIP1 or FOLR1 locus (11q13), gain of the TOP1 (20q11-12) and gain of MUC3 or Smurf1 loci (7q21-22) were not. Loci adjacent to LUNX on 20q11 were further analyzed; gain of the HCK ($P < 0.001$; Fig. 3d), E2F1 ($P < 0.005$; data not shown) and DNMT3b loci ($P < 0.05$; data not shown), but not TGIF2, were also associated with prognosis, albeit not as closely as LUNX.

Potential oncogenes revealed by expression profiling analysis. Eighty-one loci were amplified in at least one case in the group examined; these loci contained 15 genes that were overexpressed in at least one case (Table 5). Of the individual amplifications observed, 14.7% (20/136) resulted in overexpression. Only four genes were amplified and overexpressed in more than one case: Smurf1 (7q22.1), BCAS1 (20q13.2-3), which was the most frequently overexpressed, VCL (10q22.1) and TRRAP

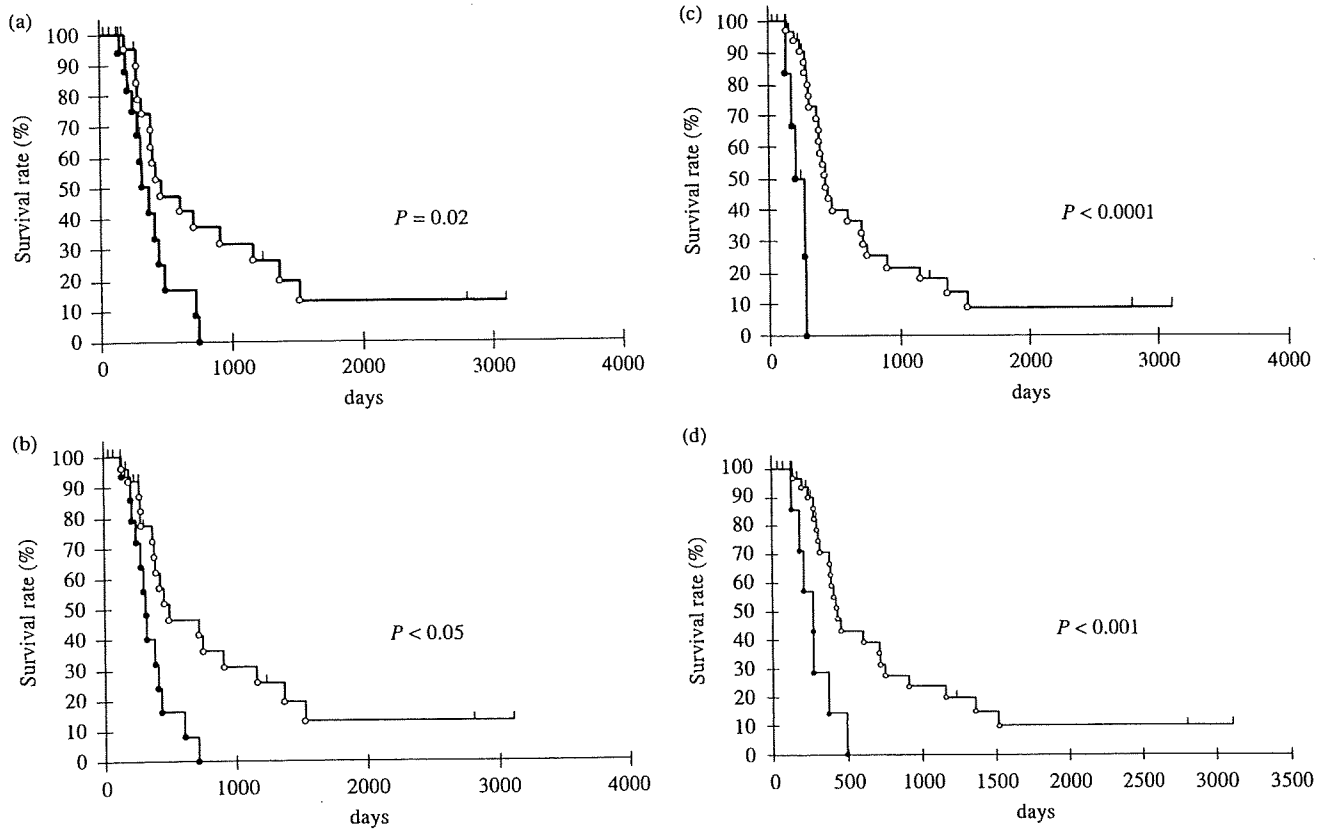

Fig. 3. Overall survival rate of pancreatic cancer patients according to the absence or presence of chromosomal abnormalities. (a) Overall survival rates of cases with chromosomal loss of the p73 locus on 1p36 (indicated as black dots) and cases without such loss (indicated as white dots). (b) Overall survival rates of cases with chromosomal gain of the PPM1D locus on 17q23 (indicated as black dots) and cases without such gain (indicated as white dots). Overall survival rates of cases with chromosomal gain of the (c) LUNX and (d) HCK loci on 20q11 (indicated as black dots) and cases without such gain (indicated as white dots). Survival curves were calculated by the Kaplan–Meier method.

(7q22.1) (Table 5). Genes that were contained in loci frequently amplified but not overexpressed included RBBP8 (18q11.2), LRP1B (2q21.2) and HGF (7q21.1). It should be noted that Smurf1 protein overexpression was also detected in pancreatic cancer clinical samples, as part of a separate study (F. Suzuki, T. Shibata, S. Hirohashi, J. Inazawa, I. Imoto, unpublished data).

The expression levels of genes on 20q11 were examined in more detail, because of the close association of four loci on 20q11 with survival. Eleven genes on 20q11 (BLCAP, RALY, GSS, ID1, NCOA6, TPX2, COX4I2, EPB41L1, BCL2L1, DNCL2A, CTNNBL1) were overexpressed. BCL2L1 (or BCL-x1) expression was also associated with lymph node metastasis of the xenografted tumors in mice (data not shown). It should be noted that two adjacent loci, TNFRSF6B and ZNF217 (20q13), were amplified in three cases each.

Discussion

This study represents the first genome-wide analysis of the subchromosomal numerical aberration profile (here designated SNAP) of a substantial number of pancreatic cancer cases by aCGH and is the first to establish its relationship with particular clinicopathological parameters of known prognostic value. It examined a number of primary tumors large enough to exclude randomly observed alterations from being considered as likely candidates, as would be the case in smaller-scale studies. In all previous studies except one,[29] case selection was based on the exclusion of samples that did not possess a high degree of neoplastic cellularity, which translates to a high copy number ratio error probability. In the present study, tumors were subjected to LCM so as to exclude non-tumor DNA from the analysis and thus increase both the number of available cases and the accuracy of the derived copy number ratio.

One of the striking findings of the study was the wide range in the number and pattern of aberrations observed between cases. Whereas many cases showed few aberrations and two had none whatsoever, 20% of cases showed alterations in more than 50% of loci examined. Importantly, the loss rate and range reported here (17%, 0–46%) is in very close agreement with the one reported in a comprehensive genome-wide allelic loss study of pancreatic cancer (15%, 1.5–32%).[30] It should be noted that it was not possible to know whether alterations of adjacent loci represented single amplicons or losses or whether they were independent events. Our results indicate that in the majority of pancreatic adenocarcinomas genomic instability occurs at the subchromosomal level, affecting a varying but large number of genes, and suggests the presence of multiple or complementary patterns of tumor evolution. Based on the association of SNAP with clinicopathological parameters revealed here, it is fair to assume that some of these aberrations contribute to tumor progression whereas others are the result of it. For the remaining cases showing a low SNAP or absence of aberrations, alternative mechanisms leading to tumor progression may be in place, such as DNA methylation or mismatch repair system aberrations,

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Table 5. Correlation of amplification with overexpression in pancreatic cancer genes both amplified and overexpressed in at least one xenograft

Gene	Locus	Amplified cases (%)	Overexpressed cases (%)	Amplified and overexpressed in the same case (%)	Amplified or gained and overexpressed in the same case (%)
Smurf1	7q22.1	36	25	25	25
BCAS1	20q13.2-q13.3	18	83	17	33
VCL	10q22.1	18	33	17	17
TRRAP	7q22.1	36	17	17	17
SRI	7q21.1	9	83	8	33
Cul3	2q36	27	42	8	33
TPD52	8q21	9	42	8	33
EFNB2	13q33	9	83	8	17
PDAP1	7q22	27	17	8	17
ZNF217	20q13	9	25	8	17
PLAU	10q24	18	17	8	8
WHSC1	4p16.3	9	17	8	8
CDK4	12q14	9	17	8	8
CYP3A4	7q22.1	36	8	8	8
CCNE1	19q11	18	8	8	8
OPG	8q24	9	33	0	33
BARD1	2q34	9	25	0	25
ELE1,MSMB	10q11.2	9	42	0	17
RAP1B	12q14	9	25	0	17
KRAS2	12p12.1	18	17	0	17
DHFR,MSH3	5q11.2-q13.2	9	17	0	17
TPR	1q25	9	17	0	8
MLL5	7q22.3	27	8	0	8
SSXT	18q11.2	27	8	0	8
PEG10	7q21.3	9	8	0	8
NBS1	8q21	9	8	0	8

mutations or small deletions, or chromosomal translocations and rearrangements not accompanied by numerical aberrations. These mechanisms may act in a way complementary to that of numerical aberrations in pancreatic carcinogenesis, so that in cases with high or low SNAP the above-mentioned mechanisms would be expected to play a minor role whereas other alternative mechanisms would be expected to play a minor or major role respectively.

A number of clinicopathological parameters was associated with SNAP, including, importantly, survival probability. Overall, cases with a phenotype indicating increased malignant potential had a higher SNAP. Specific loci, the loss or gain of which is associated with particular clinicopathological characteristics, were identified and are delineated in detail in the results section. Although only a small number of negative tumors was examined, it is noteworthy that loci associated with venous invasion were different from those associated with lymph node metastasis. Our results therefore appear to indicate that invasiveness and metastatic ability result from diverse and distinct molecular mechanisms in pancreatic cancer.

Despite the aforementioned genomic complexity, we identified genes the copy number status of which is associated with survival and may therefore be of prognostic value. Gains of the LUNX (20q11.2), AREG (4q13-q21) and CCNC (6q21) loci were detected exclusively in the short-survival group. Loss of 1p36 (p73) and 11q12-13 was associated with both short-term survival and evidence of venous invasion, whereas gain of 7q21-22 was associated with both short-term survival and the presence of lymph node metastases. Combining the above observations, we identified candidates most likely to yield clinically relevant results. A strong association was revealed between the copy number status of a number of loci at 20q11 and prognosis, mainly concerning the LUNX (PLUNC) locus ($P < 0.0001$) but also including adjacent loci containing HCK, E2F1 and DNMT3b. LUNX is upregulated and has been proposed as a marker for detection of micrometastases in non-small-cell lung cancer.[31] E2F1 activates the transcription of genes that encode proteins necessary for DNA replication, and is deregulated in most tumors.[32] DNMT3b may contribute to tumorigenesis by improper *de novo* methylation and silencing of the promoters of growth-regulatory genes, and its expression may be of clinical significance in breast cancer.[33] Although our data refer to loci rather than individual genes, the significance of the copy aberrations of the above loci has not been described previously in pancreatic or other cancers. Two loci on 20q13 were amplified in three cases each, whereas a further 12 genes on 20q11, including BCL2L1, were overexpressed. BCL2L1 is a BCL2-independent apoptosis regulator located in close proximity to LUNX. Its overexpression has already been linked to short survival times in pancreatic cancer[34,35] and other malignancies, and was also found to be associated with lymph node metastasis in the present study. Amplification and overexpression of BCL10 and BCL6 were also recently described in pancreatic carcinoma.[15] The 11q13.3 locus, containing another BCL family member, BCL1, was found to be amplified frequently in our study, which together with our findings on BCL2L1 described above may indicate a role for the BCL family in pancreatic carcinogenesis. The BCL2L1 overexpression and association with the metastatic phenotype may partially explain the effect the 20q11 region copy number status has on survival. However, we tend to think, in agreement with a similar proposal,[36] that our findings are more indicative of the fact that many (but not all) genes collectively confer selective advantage, in varying degrees of involvement, within the 20q11 region.

Loss of 1p36 (p73) and gain of 17q23 (PPM1D) were also significantly associated with prognosis. As mentioned earlier, 1p36/p73 loss was also associated with evidence of venous invasion in our study. p73, like its homolog p53, is able to induce apoptosis and has been reported to predict clinical outcome

in bladder cancer.[37] PPM1D amplification abrogates p53 tumor-suppressor activity. PPM1D is located within one of the most commonly amplified regions in breast cancer.[38] Gain of 17q21-q24 has also been associated with poor prognosis in ovarian clear cell adenocarcinomas, in which both PPM1D and APPBP2 were identified as likely amplification targets,[39] but, like p73, the PPM1D locus has not been previously reported to be of prognostic significance in pancreatic cancer.

Examination of the association between SNAP and expression provided a satisfactory filter for candidate genes. Only 15 of the 81 loci amplified and 14.7% (20/136) of individual amplifications observed contained genes that were overexpressed concurrently. This concordance level lies between those observed in breast cancer[40,41] and colon cancer,[42] in which 44–62% and 4%, respectively, of genes showing amplifications were overexpressed. It is, however, significantly lower than the one recently reported for pancreatic cancer cell lines, in which 60% of the genes within highly amplified genomic regions displayed associated overexpression,[14] a discrepancy that may partially be explained by the different source used (primary tumors vs cell lines) and the fact that we examined loci rather than genes. More than one target gene was overexpressed in some amplicons in our study, a finding not in disagreement with the above study.[14] We identified four genes contained in loci that were amplified and that were overexpressed recurrently: Smurf1 and TRRAP, both at 7q22.1, BCAS1 (20q13.2-3), and VCL (10q22.1). Smurf1 acts as a negative regulator of transfroming growth factor β signaling.[43] It was amplified in six cases overall and overexpressed concurrently in four. Although, as mentioned, gain of the Smurf1 locus was not associated with poor prognosis, 7q21-22 gain was associated with the presence of lymph node metastasis and was detected significantly more frequently in the short-term (<3 years) survival group. TRRAP is an essential cofactor for both the c-Myc and E1A/E2F oncogenic transcription factor pathways and interacts specifically with the E2F-1 transactivation domain. Its inclusion among the four genes both amplified and overexpressed lends further support to the association between E2F1 gain and poor survival revealed here. The fact that Smurf1 and TRRAP are amplified in pancreatic cancer was reported recently, albeit only in cell lines.[16] We show that amplifications of these genes also occurs in primary tumors and that they are recurrently accompanied by overexpression, therefore presenting as very likely novel oncogenes in pancreatic cancer. BCAS1 (20q13), reported to be amplified and overexpressed in breast cancer,[44] was the most frequently overexpressed gene among the ones contained in loci recurrently amplified, and may therefore have a similar role in pancreatic cancer; 20q13 was also one of the most frequently amplified loci in a recent aCGH study on pancreatic cancer.[14,15] Finally, 10q22-24 contained another novel candidate, vinculin, an intracellular protein with a crucial role in the maintenance and regulation of cell adhesion and migration.[45] KRAS2 and 20 other genes have recently been identified as potential target genes on 12p.[36] This finding is in partial agreement with our study, in which five loci on 12p were amplified and KRAS2 was amplified in two cases.

Numerous recurrent, non-random, patterns of subchromosomal aberrations have emerged through our analysis. Thirty-three loci with frequent losses (>50% cases) were identified. The chromosome arms found to contain the highest number of loci lost are in agreement with allelic loss[30] and chromosome CGH studies, with the additional detection of losses at 5p, 8q, 9q, 11p, 16p and 20q. The most frequently lost loci were: 17p13.3 (in 75% of cases), containing ABR, a multifunctional cellular signaling regulator and a putative TSG in medulloblastoma, 18qtel (CTDP1, SHGC-145820, 68%) and 18q21 (66%), containing SMAD7, a member of the SMAD family, although all three are close to either the p53 or the DPC4 locus. HD were detected in 25% of cases, affecting 26 loci. The 1p353-6.33 region contained the highest number of loci deleted (six) or lost (16). The most frequently deleted locus was 9p21 spanning the p16 gene, the inactivation of which is known to play an established role in pancreatic carcinogenesis. The above regions (17p, 18q and 1p353-6) have been reported previously to show frequent loss.[19] The most frequently gained locus was 7q21.1 (71%) containing the HGF gene, which encodes a cytokine involved in initiating cell migration. Some regions in which gains were observed frequently, at 6p21 (2 loci), 11q22 (7 loci) 12p12 (three loci) and 17q12 (Suppl. Table 4), have been previously proposed as novel amplicons.[16] Sixteen loci were amplified frequently (>10%), although, again, the possibility of another gene being amplified within these loci cannot be excluded. Two of the most frequently amplified loci were on 11q13, in agreement with a report by Holzmann *et al.* on 13 pancreatic cancer cell lines and six primary tumors.[15]

Novel loci likely to play important roles in pancreatic carcinogenesis and in the acquisition of certain malignant phenotypes were identified. Genes associated with prognosis or established histopathological indicators of malignancy, or showing both numerical aberrations and overexpression, may represent novel oncogenes. The copy number alterations of the p73 and PPM1D loci, the 20q11 region, including LUNX, and the loci amplified that contained genes concurrently overexpressed, particularly Smurf1, shown here may be of great importance for predicting clinical outcomes and setting new therapeutic targets in pancreatic cancer but will require prospective studies in order to be firmly established.

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Supplementary material

This material is available as part of the online article from:

The following supplementary material is available for this article:

Fig. S1. Homozygous deletions detected by array-based comparative genomic hybridization were validated by polymerase chain reaction and gel electrophoresis for selected cases and genes. Genes contained in the two most frequently deleted loci, p16 (left panel gel, cases 12, 18 and 41) at 9p21 and ABCA3 (right panel gel, cases 44 and 47) at 16p13.3 were examined (exons 2 and 28, respectively). Homozygous deletions were confirmed in all five cases examined, whereas wild-type products were detected in all control tissues used. The control tissue for p16 consisted of the corresponding normal tissue in one of three cases examined; corresponding normal tissue was not available for the other two cases (cases 12 and 18) as they derived from xenografts. The control tissues for the cases examined for homozygous deletions of the ABCA3 gene consisted of: (a) the corresponding normal tissues of both cases and (b) a third case (case no. 40), in which the array-based comparative genomic hybridization signal ratio indicated loss of heterozygosity of the ABCA3 gene, but not homozygous deletion, and its corresponding normal tissue.

Fig. S2. Range of numerical aberrations observed between cases. The total number of (a) numerical aberrations, (b) losses and (c) gains observed ranged widely between cases. In two cases no copy number changes were observed (a–c), whereas nine cases (20%) had alterations in more than 50% of loci (>400 loci) (a). Although the loss range was wider than the gain range (b,c), in most cases the number of gains was higher than the number of losses. Overall, however, the loss rate was similar to the gain rate (19% of loci altered on average per case for both).