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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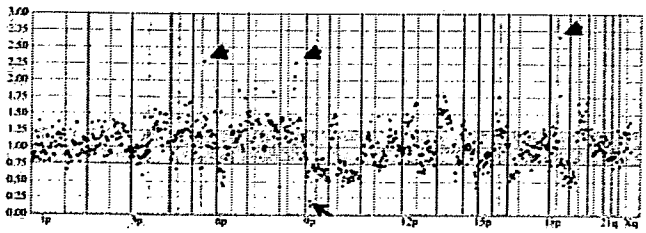

Fig. 1. Chromosomal copy number changes revealed by array-based comparative genomic hybridization. Representative array-based comparative genomic hybridization profile of a pancreatic adenocarcinoma. Copy number losses (ratio < 0.75) and gains (ratio > 1.25) were detected in both large fractions of the 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

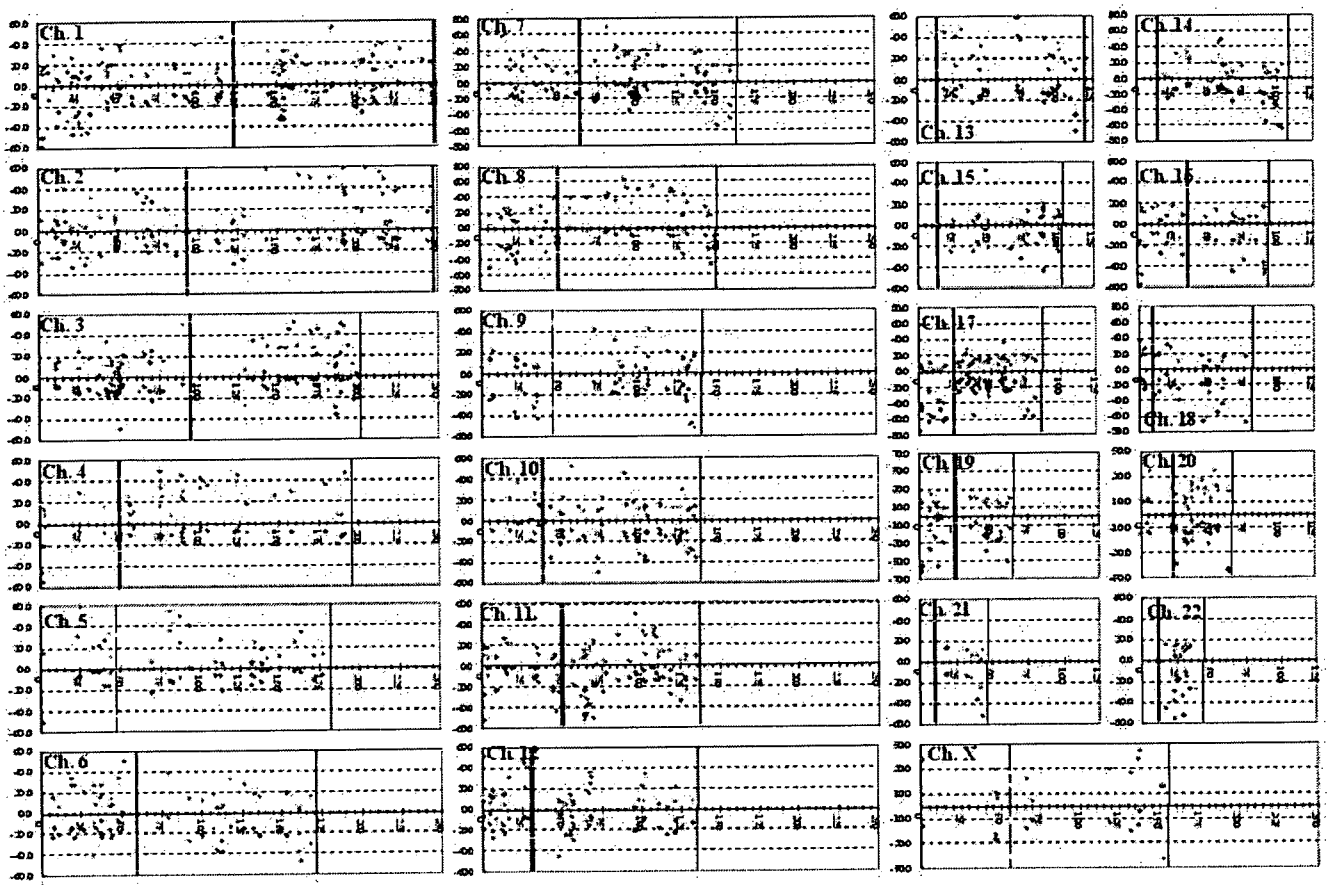

Fig. 2. Distribution of chromosomal copy number aberrations in pancreatic cancer. The horizontal axis indicates the physical distance (Mb) of the chromosomal loci from the telomere of the short arm. The vertical axis indicates the frequency (%) of tumors with chromosomal alterations (green, gain; red, 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

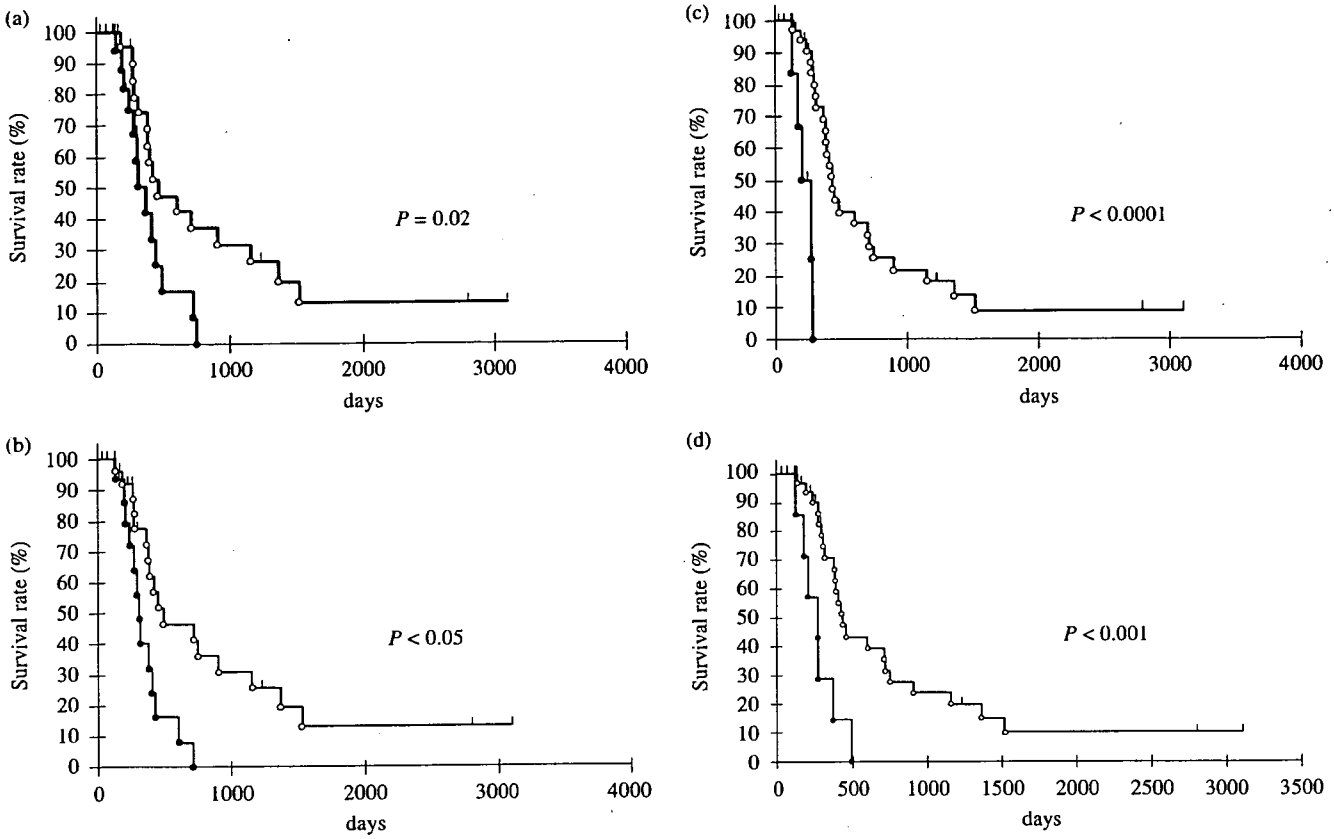

Fig. 3. Overall survival rate of pancreatic cancer patients according to the absence or presence of chromosomal abnormalities. (a) Overall survival rates of cases with chromosomal loss of the p73 locus on 1p36 (indicated as black dots) and cases without such loss (indicated as white dots). (b) Overall 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).

Table S1 Clinicopathological parameters of 43 pancreatic cancer cases analyzed by array-based comparative genomic hybridization.

Table S2 Numerical aberrations observed in 44 pancreatic adenocarcinoma cases examined by array-based comparative genomic hybridization.

Table S3 Loci lost frequently (>25% cases) in 44 pancreatic adenocarcinoma cases examined by array-based comparative genomic hybridization, arranged by region.

Table S4 Loci gained frequently (>25% cases) in 44 pancreatic adenocarcinoma cases examined by array-based comparative genomic hybridization, arranged by region.

Table S5 Association of sub-chromosomal numerical aberrations with selected clinicopathological parameters in pancreatic cancer.

Table S6 (A) Loci altered more frequently in moderately compared with well differentiated pancreatic adenocarcinomas. (B) Loci altered more frequently in poorly compared with moderately differentiated pancreatic adenocarcinomas.

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Surgical Outcomes of the Mass-Forming plus Periductal Infiltrating Types of Intrahepatic Cholangiocarcinoma: A Comparative Study with the Typical Mass-Forming Type of Intrahepatic Cholangiocarcinoma

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Abstract
Background The purpose of this study was to clarify the clinicopathologic characteristics and surgical outcomes of patients with the mass-forming (MF) plus periductal infiltrating (PI) type of intrahepatic cholangiocellular carcinoma (ICC).
Methods Between January 1, 1998, and December 31, 2004, a total of 94 patients with ICC underwent macroscopic curative resection, and the macroscopic type of the tumors was assessed prospectively. Among the 74 patients with the MF type ($n = 46$) and the MF plus PI type ($n = 28$) of ICC, multivariate analysis was conducted to identify the potential prognostic factors. The clinicopathologic data of the two groups were compared.
Results The results revealed two independent prognostic factors: presence/absence of intrahepatic metastasis and the macroscopic type of the tumor. ICCs categorized macroscopically as the MF plus PI type were significantly associated with jaundice ($p < 0.001$), bile duct invasion ($p < 0.001$), portal vein invasion ($p = 0.025$), lymph node involvement ($p = 0.017$), and positive surgical margin ($p = 0.038$).
Conclusion Identification of the macroscopic type of the tumor is useful for predicting survival after hepatectomy in patients with ICC. The MF plus PI type of ICC appears to have a more unfavorable prognosis, even after radical surgery, than the MF type of ICC.

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Intrahepatic cholangiocarcinoma (ICC) arising from the intrahepatic bile ducts has been reported to be a rare malignant tumor, accounting for approximately 5% to 10% of all primary liver cancers [1, 2]. However, an increase in the incidence and mortality of this cancer has been reported recently [3]. ICC is mainly recognized as a localized round tumor with a distinct border on the cut surface of the liver [2]. In 1997, the Liver Cancer Study Group of Japan (LCSGJ) proposed a new classification of ICC based on the macroscopic appearance of the tumors: the mass-forming (MF) type, periductal infiltrating (PI) type, intraductal growth type (IG), and mixed type containing more than one of the other three basic types [4].

The ICC has a tendency to spread diffusely along Glisson's sheath in a radial fashion from the original spherical tumor [5–7], and the MF plus PI type (MF-dominant type) is one of the most commonly encountered subtypes of ICC [8]. This tumor subtype occurs as a definitive mass in the liver, commonly causing infiltration along the portal pedicle and invasion of the wall of large vessels and bile ducts. It is usually detected only at an advanced stage [9]. There are only a few studies until now that have investigated the clinical significance of the macroscopic appearance of the tumor, with special reference to the MF plus PI subtype, as a prognostic indicator in patients with an ICC [10, 11].

We prospectively classified the 94 surgically resected cases at our hospital since 1998 on the basis of the gross appearance of the surgically resected tumors and examined the clinicopathologic characteristics and surgical outcomes of patients with the MF plus PI type of ICC compared with those of patients with the MF type of ICC.

Patients and methods

Between January 1, 1998 and December 31, 2004, a total of 94 patients with ICC underwent macroscopic curative resection at the Hepatobiliary and Pancreatic Surgery Division, National Cancer Center Hospital, Tokyo, Japan. ICC is defined as carcinoma arising from the second-order or more distal branches of the intrahepatic bile ducts. The criteria for resectability were the absence of (1) peritoneal dissemination, (2) bulky lymph node metastasis, (3) extensive invasion of the hepatoduodenal ligament, and (4) intrahepatic metastases in the remaining liver. Macroscopic curative resection was defined as the absence of apparent residual tumor in the operative field. The type of surgical procedure performed depended on both the tumor location and the mode of extension. In patients suspected to have lymph node involvement on the basis of preoperative imaging or intraoperative findings, lymph node dissection was performed around the hepatoduodenal ligament, posterior to the upper portion of the pancreatic head, and along the common hepatic artery.

The macroscopic typing of the resected tumor specimens was conducted prospectively and confirmed by microscopic examination according to the Classification of Primary Liver Cancer as: the mass-forming type (MF: characterized by the presence of a spherical mass with a distinct border in the liver parenchyma); the periductal-infiltrating type (PI: characterized by tumor infiltration along the bile duct, occasionally involving the surrounding blood vessels and/or hepatic parenchyma); or the intraductal growth type (IG: characterized by papillary and/or granular growth into the bile duct lumen, occasionally showing superficial extension). When more than one type of lesion was found, all of the types detected and the predominant type were recorded [4]. The distribution of the macroscopic tumor types in our cases was as follows: MF type, 47 (50%); MF plus PI type, 29 (31%); MF plus IG, 6 (6%); PI type, 8 (9%); IG type, 4 (4%). The 76 patients with the MF and MF plus PI type of ICC were the focus of the present study.

One hospital death (1%) was recorded in the group with the MF plus PI type of ICC and one patient with MF type of ICC was lost to follow-up. Patients were closely followed up every 3 months on an outpatient basis with measurement of the serum levels of carbohydrate antigen 19-9 (CA19-9) and carcinoembryonic antigen (CEA), chest radiography, and abdominal ultrasonography and/or computed tomography. The specific sites of the first tumor recurrence and the time until disease recurrence were recorded. Radiologic evidence of tumor recurrence was accepted as a criterion of recurrence even if the patient did not undergo a biopsy. When progression of the disease was confirmed by repeated imaging studies, the date of first detection of a suspicious radiologic finding was recorded as the date of the initial disease recurrence. The data of the hospital death case and the case lost to follow-up were excluded from the follow-up analysis. The median follow-up duration was 20 months (range 4–82 months).

Among the 74 patients, 14 potential prognostic factors, including the macroscopic type of the tumor, were investigated using the log-rank test. Pathologic factors associated with the tumor invasiveness, such as lymph node metastasis, portal vein (vp) or hepatic vein invasion (vv), bile duct invasion (b), and intrahepatic metastasis (im), were evaluated in relation to the tumor types. The tumors were further classified clinically into four groups as follows: (0, no tumor invasion of the portal vein, hepatic vein, or bile duct; 1, tumor invasion distal to the second branch of the portal vein or bile duct and/or invasion of a branch of the hepatic vein; 2, tumor invasion of the second branch of the portal vein or the bile duct, the major hepatic veins, and/or the short hepatic veins; 3, tumor invasion of the first branch of the portal vein or of the bile duct, tumor invasion of the inferior vena cava) according to the degree of "vp", "vv," and "b" [4]. A positive surgical margin was defined by histopathologic detection of tumor cells at the surgical margin.

The continuous variables were classified into two groups according to the median value of each factor. All the variables were dichotomized for the analysis. Survival estimates were calculated by the Kaplan-Meier method. Multivariate regression analysis was performed using the Cox proportional hazards model, and factors associated with $p < 0.10$ were entered into the final model adopted. Comparison between patients with the MF type and MF plus PI type of ICC was performed by univariate analysis in terms of 12 clinicopathologic factors using the chi-squared test with Yates' correction. All statistical analyses were performed using the Software Package for Social Sciences, version 11.5J for Windows® (SPSS, Chicago, IL, USA). A two-sided $p < 0.05$ was considered to denote statistical significance.

Results

A total of 76 patients with the two types of the tumors (MF type or MF plus PI type) had undergone various kinds of

Table 1 Macroscopic types and operative procedures

Hepatectomy and other operative procedures	MF type (n = 47)	MF plus PI type (n = 29)	p
Left lobectomy	16 (34%)	16 (55%)	0.001
Left trisegmentectomy	6 (13%)	3 (10%)	
Central bisegmentectomy	1 (2%)	1 (3%)	
Right lobectomy	12 (26%)	9 (32%)	
Right trisegmentectomy	2 (4%)	0	
Segmentectomy/limited resection	10 (21%)	0	
Caudate lobe resection	21 (67%)	23 (79%)	0.006
IVC resection	5 (11%)	3 (10%)	1.000
Arterial resection and reconstruction	0	3 (10%)	0.052
Portal vein resection and reconstruction	4 (9%)	6 (21%)	0.167
Extrahepatic bile duct resection	12 (26%)	26 (90%)	< 0.001
Lymph node dissection[a]	26 (55%)	26 (90%)	0.002

MF: mass-forming; PI: periductal infiltrating; IVC: inferior vena cava

[a] Lymph node dissection in the hepatoduodenal ligament, retroperitoneal region along the hepatic artery, and behind the pancreas

hepatectomy and additional operative procedures (Table 1). The mean age of the patients was 64 ± 10 years (median 66 years; range 34–84 years). There were 28 women (37%) and 48 men (63%). Segmentectomy or limited resection was the most frequently performed procedure in the patients with the MF type of ICC (p = 0.02). The frequency of combined resection of the caudate lobe (p = 0.006), extrahepatic bile duct resection (p < 0.001), and/or lymph node dissection (p = 0.002) was significantly higher in the patients with the MF plus PI type of ICC. The incidence of concomitant resections of the inferior vena cava, hepatic artery, and portal vein was similar in the two groups. One patient with the MF plus PI type of ICC who underwent left lobectomy with combined resection of the caudate lobe, extrahepatic bile duct resection, and lymph node dissection died of liver failure caused by multiple liver abscesses 142 days after the operation.

The possible risk factors for survival in the patients with the MF type (n = 46) and MF plus PI type (n = 28) of ICC were examined using the log-rank test (Table 2). The poor prognostic factors according to the univariate analysis were the presence of intrahepatic metastases (p = 0.0002), portal vein involvement (p = 0.0538), lymph node involvement (p = 0.0061), a positive surgical margin (p = 0.0030), and the MF plus PI type of ICC (p = 0.0442). Fig. 1 shows the results of the survival analysis in the 74 patients with the MF or MF plus PI type of ICC. Among the possible prognostic factors identified by the univariate analysis, the following factors were found to be independently associated with a poorer prognosis: the presence of intrahepatic metastases (p < 0.001) and the MF plus PI type of ICC (p = 0.014), with hazard ratios [95% confidence intervals (CI)] of 3.560 (1.847–6.862) and 2.237 (1.175–4.259), respectively.

A comparison of the clinicopathologic factors in the patients with the MF and MF plus PI types of ICC is shown in Table 3. Significant differences were recognized between the two groups in terms of the presenting symptoms (p = 0.026) and the frequency of jaundice (p < 0.001), portal vein invasion (p = 0.025), bile duct invasion (p < 0.001), lymph node involvement (p = 0.017), and positive surgical margin (p = 0.038). Both groups had similar rates of intrahepatic metastasis and hepatic vein invasion. Patients with the MF plus PI type of ICC had a smaller mean tumor size than those with the MF type of ICC, but the difference was not statistically significant (p = 0.098).

The sites of initial recurrence were compared between the two groups (Table 4). The most frequent site of recurrence was the liver in patients with the MF type of ICC (16/35, 45.7%) and local recurrence (hepatic resection margin or bilioenteric anastomosis) was the most frequently encountered site of the initial recurrence in the patients with the MF plus PI type of ICC (9/26, 34.6%). Thus, local recurrence was more frequent in patients with the MF plus PI type of ICC (p = 0.0044).

Discussion

Based on a study of cases collected from the member institutes of the committee of the LCSGJ, Yamasaki [12] reported a prevalence rate of the MF type (including the predominant MF type) of ICC of 78.6% among all cases of ICC (136/173). In the present study, the MF type (50%, 47/94) and the MF plus PI type (31%, 29/94) accounted for 81% (79/94) of all the patients with ICC. The IG type of ICC has been recognized as a distinct entity with a more

Table 2 Possible clinical and pathologic risk factors for survival

Factors	No. of patients	(%)	Survival rate (%)			Median survival	*p*
			1 Year	3 Years	5 Years	(months)	
Overall	74		69.5	35.5	31.1	24	
Age (median 66 years)							
>66	33	45	67.5	32.6	26.1	31	0.4105
≤66	41	55	72.0	37.4	37.4	22	
Sex							
Male	46	62	65.9	45.6	34.2	31	0.5067
Female	28	38	75.0	23.4	23.4	23	
Jaundice							
Absent	63	85	72.1	34.3	30.1	23	0.7171
Present	11	15	54.6	40.9	(–)	24	
Ca 19-9 (median 220 IU/ml)							
<220	37	51	75.7	46.9	37.5	25	0.1121
≥220	35	49	61.6	23.1	23.1	21	
CEA (median 3.0 mg/dl)							
<3.1	38	51	78.5	41.1	41.1	25	0.1121
≥3.1	36	49	60.0	26.9	–	22	
Size (median 5.0 cm)							
<5.0	36	49	79.0	46.2	30.8	25	0.2725
≥5.0	38	51	59.0	28.9	28.9	20	
Intrahepatic metastases							
Absent	48	65	88.3	44.1	44.1	31	0.0002
Present	26	35	41.9	12.6	–	10	
Portal vein involvement							
vp0-1	47	64	74.0	41.0	41.0	31	0.0538
vp2-3	27	36	61.1	25.1	12.6	17	
Hepatic vein involvement							
vv0-1	53	72	69.8	35.8	35.8	25	0.3629
vv2-3	21	28	69.1	32.0	–	22	
Bile duct invasion							
B0-1	37	50	77.8	44.2	38.7	31	0.0877
B2-3	37	50	61.0	21.9	–	22	
Histologic differentiation							
Well	16	22	81.3	42.9	34.3	24	0.6101
Mod./poor	58	78	66.0	32.3	32.3	22	
Lymph node involvement							
Absent	40	54	71.9	46.2	46.2	32	0.0061
Present	34	46	63.6	10.2	–	21	
Surgical margin							
Negative	51	67	71.9	47.0	47	0 32	0.0030
Positive	23	33	63.6	10.2	–	20	
Macroscopic type							
Mass-forming type	46	62	73.2	45.5	39.8	32	0.0442
Mass-forming plus periductal infiltration type	28	38	63.0	16.0	–	22	

favorable prognosis than that of the other types of ICC [13]. However, the clinical significance of the MF plus PI type of ICC as a prognostic indicator remains unclear. Yamamoto et al. [10] noted that patients with the MF plus PI type of ICC had a dismal prognosis owing to the high incidence of noncurative resection and lymph node

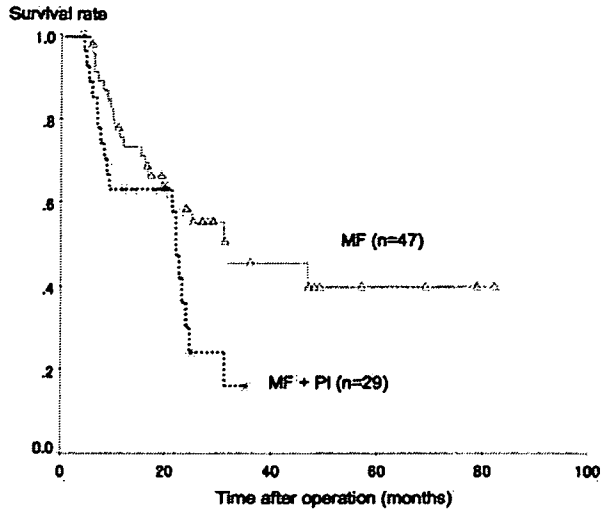

Fig. 1 Cumulative survival curve after surgery for cases of intrahepatic cholangiocarcinoma according to the gross appearance of the tumor. MF: mass-forming; PI: periductal infiltrating. $p < 0.0442$ (log-rank test)

metastases. On the other hand, Ohtsuka et al [11] reported that there was no significant difference in the survival rate between patients with this aforementioned type and other types of ICC. The results of the multivariate analysis in the present study suggested the MF plus PI type of ICC was associated with a poorer survival than the MF type of ICC. No definitive conclusion can be drawn from the limited number of cases from a single center, so cooperative multicenter trials that are powered adequately are necessary. However, the macroscopic classification might be useful for planning the surgical procedure and predicting the survival after hepatectomy in patients with the MF plus PI type of ICC because the recent advances in imaging techniques allow determination of the macroscopic type of ICC preoperatively [8].

One of the major speculations to explain the worse outcome of the MF plus PI type of ICC is that it represents a more advanced stage of the MF ICC [7]. The present study also showed that the MF plus PI type was more frequently associated with portal vein invasion and lymph node metastases, suggesting that it might represent a more advanced stage of tumor than the MF type of ICC. However, the tumor size and incidence of intrahepatic metastases, which are important components in defining the primary tumor stage, were not statistically different between the MF type and MF plus PI type of tumor. Two different modes of spread are generally recognized in patients with ICCs: lymph node metastasis spreading via lymphatics along Glisson's sheath and intrahepatic metastasis spreading via the portal venous system to the liver [9]. The stronger association of the MF plus PI type ICC with the former might be the primary reason for its more aggressive biologic nature.

Table 3 Relation between the macroscopic classification and other clinicopathologic factors

Factors	MF type (n = 46)	MF plus PI type (n = 28)	p
Age (median 66 years)			
>66	22 (48%)	11 (39%)	0.630
≤66	24 (52%)	17 (61%)	
Sex			
Male	26 (57%)	20 (71%)	0.226
Female	20 (43%)	8 (29%)	
Symptoms			
Absent	35 (76%)	14 (50%)	0.026
Present	11 (24%)	14 (50%)	
Jaundice			
Absent	46 (100%)	17 (60%)	<0.001
Present	0	11	
CA 19-9 (median 220 IU/ml)			
>220	17 (39%)	18 (64%)	0.052
≤220	27 (61%)	10 (36%)	
Size (median 5.0 cm)			
>5.0	26 (57%)	10 (36%)	0.098
≤5.0	20 (43%)	18 (64%)	
Intrahepatic metastases			
Absent	29 (63%)	19 (68%)	0.803
Present	17 (37%)	9 (32%)	
Portal vein invasion			
vp0-1	34 (74%)	13 (46%)	0.025
vp2-3	12 (26%)	15 (54%)	
Hepatic vein invasion			
vv0-1	36 (78%)	17 (61%)	0.119
vv2-3	10 (22%)	11 (39%)	
Bile duct invasion			
B0-1	35 (76%)	2 (7%)	<0.001
B2-3	11 (24%)	26 (93%)	
Lymph node involvement			
Absent	30 (65%)	10 (36%)	0.017
Present	16	18	
Liver cirrhosis or hepatitis			
Absent	40 (87%)	27 (96%)	0.347
Present	6 (13%)	1 (4%)	
Surgical margin			
Negative	36 (78%)	15 (54%)	0.038
Positive	10 (22%)	13 (46%)	

The MF plus PI type of ICC usually behaves just like a perihilar bile duct carcinoma, requiring aggressive surgical management [9, 10, 14–16]. Lang et al. [16] emphasized the necessity of extended hepatectomy with complete tumor removal as the only treatment strategy that can yield

Table 4 Initial recurrence sites

Macroscopic type	MF type (n = 46)	MF plus PI type (n = 28)
Recurrence	35 (76.1%)	26 (92.9%)
Solitary recurrence	28 (80.0%)	20 (76.9%)
Local recurrence*	0	9 (34.6%)
Liver	16 (45.7%)	6 (23.1%)
Lymph nodes	8 (22.9%)	1 (3.8%)
Peritoneum	1 (2.9%)	2 (7.7%)
Pleural	0	1 (3.8%)
Lung	2 (5.7%)	0
Bone	1 (2.9%)	0
Skin	0	1 (3.8%)
Multiple recurrences	7 (20.0%)	6 (23.1%)
Liver and lymph nodes	3 (6.5%)	2 (7.7%)
Lung and lymph nodes	0	1 (3.8%)
Local recurrence and liver	1 (2.9%)	1 (3.8%)
Local recurrence and lymph nodes	0	1 (3.8%)
Skin, bone, and liver	1 (2.9%)	1 (3.8%)
Lung, liver, and lymph nodes	2 (5.7%)	0

*p = 0.0044

prolonged survival in these cases. However, patients with the MF plus PI type of ICC still had a higher incidence of a positive surgical margin compared with that in the patients with the MF type in the current study. Yamamoto et al. [10] also reported that microscopic curative resection was achieved in only 3 (17%) of 18 patients with the MF plus PI type of ICC. Thus, another major cause for the poor prognosis might be the difficulty of achieving microscopic curative resection even with extended surgical procedures in patients with the MF plus PI type of ICC.

The results of analysis of the recurrence patterns revealed that local recurrence was higher in patients with the MF plus PI type compared to those in patients with the MF type of ICC. Recently, several studies have demonstrated that the use of adjuvant radiotherapy improved the survival of patients with hilar cholangiocarcinoma with a positive microscopic surgical margin [17–19]. Additional local therapy as well as systemic chemotherapy might be indispensable in this patient group. However, the efficacy of intraoperative or adjuvant radiotherapy in patients with ICCs has been scarcely investigated. The accumulation of sufficient numbers of resected cases is extremely difficult owing to the rarity of the disease. Therefore, it is important for studies to be sponsored by large cooperative groups and for patients to be stratified and analyzed by stage and adequate power to show a difference.

Previous reports have suggested that the presence of lymph node metastases may be one of the most unfavorable prognostic factors in cases of ICC [14, 20, 21]. Lymphatic metastases may be more commonly encountered in patients with the MF plus PI type of ICC. The role of regional lymphadenectomy remains controversial [20, 22, 23], although it might be applied as a standard operative procedure in patients with the MF plus PI type of ICC because these patients usually have normal liver function without chronic hepatitis or cirrhosis [23]. The incidence of lymph node recurrence was found to be similar between patients with the MF type and the MF plus PI type of ICC in the current study.

Although extended hepatectomy, extrahepatic bile duct resection, and lymph node dissection were performed in patients with the MF plus PI type of ICC, these patients still had a poorer surgical outcome compared with that of patients with the MF type of ICC. Therefore, with the currently employed criteria for patient selection for surgery, it is still difficult to achieve complete tumor resection, and additional radiotherapy and/or systemic chemotherapy should be considered in the event of local or hepatic recurrence after surgical treatment in patients with the MF plus PI type of ICC.

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資料は準備いたします。

Modulator: Minoru ESAKI, Hepatobiliary Pancreatic Surgery Division

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Prognosis of Perihilar Cholangiocarcinoma: Hilar Bile Duct Cancer versus Intrahepatic Cholangiocarcinoma Involving the Hepatic Hilus

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Background: Clinically hepatobiliary resection is indicated for both hilar bile duct cancer (BDC) and intrahepatic cholangiocarcinoma involving the hepatic hilus (CCC). The aim of this study was to compare the long-term outcome of BDC and CCC.

Methods: Between 1990 and 2004, we surgically treated 158 consecutive patients with perihilar cholangiocarcinoma. The clinicopathological data on all of the patients were analyzed retrospectively.

Results: The overall 3-year survival rate, 5-year survival rate, and median survival time for BDC patients were 48.4%, 38.4 %, and 33.7 months, respectively, and 35.8%, 24.5 %, and 22.7 months, respectively, in CCC patients (P = .033).

On multivariate analysis, three independent factors were related to longer survival in BDC patients: achieved in curative resection with cancer free margin (R0) (P = .024, odds ratio 1.862), well differentiated or papillary adenocarcinoma (P = .011, odds ratio 2.135), and absence of lymph node metastasis (P < .001, odds ratio 3.314). Five factors were related to longer survival in CCC patients: absence of intrahepatic daughter nodules (P < .001, odds ratio 2.318), CEA level ≤2.9 ng/mL (P = .005, odds ratio 2.606), no red blood cell transfusion requirement (P = .016, odds ratio 2.614), absence or slight degree of lymphatic system invasion (P < .001, odds ratio 4.577), and negative margin of the proximal bile duct (P = .003, odds ratio 7.398).

Conclusions: BDC and CCC appear to have different prognoses after hepatobiliary resection. Therefore, differentiating between these two categories must impact the prediction of postoperative survival in patients with perihilar cholangiocarcinoma.

Key Words: Hilar bile duct cancer—Intrahepatic cholangiocarcinoma—Hepatobiliary resection.

Hilar cholangiocarcinoma remains a challenging disease, and the prognosis is often dismal, even after aggressive surgery including hepatobiliary resection with caudate lobectomy.[1] Previous reports have included a limited number of resected cases, and reports of large, single-center studies are not common.[2–13]

Based on the anatomical origin of the tumor, hilar cholangiocarcinoma and perihilar cholangiocarcinoma are potentially divisible into two categories: hilar bile duct cancer (BDC) and intrahepatic cholangiocarcinoma involving the hepatic hilus (CCC). BDC originates in the epithelium of the common hepatic, right or left hepatic duct, whereas CCC

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originates in the intrahepatic bile duct or bile ductules.

In the clinical setting, curative resection for both BDC and CCC involves hepatobiliary resection with regional lymphadenectomy. Many previous studies have treated BDC and CCC as the same entity; thus, the clinicopathological differences between BDC and CCC remain unclear, and the clinical usefulness of differentiating between these two groups has not been elucidated.

We have distinguished between BDC and CCC based on pathology and have collected the clinicopathological data since the 1980s. Thus, a review of these patients' data is crucial for determining future strategies.

The aims of this study were to review the long-term outcome of major hepatobiliary resections done over the last 15 years for BDC and CCC using a similar treatment strategy in a single center and to characterize the prognostic factors affecting the long-term outcome for each group to clarify the differences between groups.

PATIENTS AND METHODS

Between January 1, 1990 and December 31, 2004, 225 patients were admitted to our department with a tentative diagnosis of perihilar cholangiocarcinoma. The following patients were excluded: 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 had a hilar bile duct resection or a minor hepatectomy, and patients with gallbladder cancer or benign biliary stricture based on postoperative pathology. Thus 158 patients, consisting of 99 patients (62.7%) with hilar bile duct cancer (BDC) and 59 patients (37.3%) with intrahepatic cholangiocarcinoma involving the hepatic hilus (CCC), treated with major hepatobiliary resection were enrolled in this study (Table 1). The patient population consisted of 52 women and 106 men, with a median age of 65 years (range, 33–83 years).

The medical records that had been collected, including the hospital charts, operation records, and pathology reports, were analyzed retrospectively.

Our standard management strategies and surgical procedures have been described previously.[14] Briefly, after a preoperative imaging diagnosis of tumor extension was made, biliary drainage was done to ensure that the patient recovered from cholestatic liver damage, if necessary. To induce compensatory hypertrophy of the future remnant liver, preoperative portal vein embolization (PVE)[15,16] for the liver segment to be resected was done if the estimated resection volume exceeded 50–55% of the whole liver; 71 patients (44.9%) underwent PVE.

All patients underwent major hepatobiliary resection with a hepatectomy involving two or more sectors and systematic lymphadenectomy of the nodes located at the hepatoduodenal ligament, the upper part of the retropancreatic nodes, and the celiac nodes, as well as skeletonization of the hepatic hilus. Operative mortality included both death within 30 days of surgery and all in-hospital death. Morbidity included all postoperative complications that affected the outcome or lengthened the hospital stay. The surgical procedures are summarized in Table 2.

Histopathological Evaluation, Pathological Diagnosis, and Staging

First, the extrahepatic bile duct was incised longitudinally from the distal to the proximal margin. The anatomical orientation of the individual vessels and the surgical margins of the resected specimen were assessed macroscopically. The en bloc dissected lymph nodes were classified according to the anatomical location. Both the proximal and distal margins of the bile duct were routinely evaluated using intraoperative frozen section.

The resected specimen was fixed in 10% formalin, after that multiple, 5-mm thick, thin slice sections of the resected specimen were prepared in alignment with the computed tomography (CT) plane. In every section, the biliary anatomy was identified in relation to the vasculature. Then, sagittal, thin slice sections every 3 to 5 mm were added to precisely determine tumor extension around the hepatic hilum. Distal tumor extension was clarified based on the serial perpendicular sections to the longitudinal axis of the distal bile duct. In every case, histological tumor extension was investigated in 20 to 40 sections, and lymph node involvement was independently evaluated.

The criteria used to discriminate between BDC and CCC depended primarily on the location of the main tumor, as is schematically shown in Fig. 1. BDC was defined as a tumor originating in the upper common, right or left hepatic duct. Representative cases of BDC and CCC on the slice section of resected specimen are illustrated in Fig. 2. To evaluate the origin or the dominant spatial location of the tumor, hematoxylin-eosin staining was routinely used; elastica stain was additionally used to delineate the elastic

TABLE 1. *Patient characteristics and preoperative variables*

Variable	BDC (n = 99)	CCC (n = 59)	P value
Age (years)	64, [33–83]	66 [34–82]	
Gender (men / women)	69/30	37/22	
Preoperative biliary drainage (performed)	77 (78%)	16 (27%)	<.001
ICGR15 (%)	8.4 [0.8–48.1]	7.1 [0.2–63.2]	
CEA (ng/mL)	2.5 [0.7–22.1]	2.9 [0.8–560]	
CA19-9 (U/mL)	101 [1–14,750]	306 [1–256,800]	.006

[range].
ICGR15 indicates indocyanine green retention value at 15 minutes; CEA, carcinoembrionic antigen; CA19-9, carbohydrate antigen 19-9.

TABLE 2. *Surgical procedures and operative variables*

	BDC (n = 99)	CCC (n = 59)	P value
Type of hepatectomy			
Left hepatectomy	42 (42%)	29 (49%)	
Left trisectionectomy	5 (5)	9 (15)	
Central bisectionectomy	1 (1)	1 (2)	
Right hepatectomy	49 (49)	18 (31)	
Right trisectionectomy	2 (2)	2 (3)	
with PD	10 (10)	0	.012
with PV	18 (18)	14 (24)	
with PVE	53 (54)	18 (31)	.005
Right-sided hepatectomy	51 (52)	20 (34)	.033
Operation time (minutes)	655 [302–1125]	616 [372–950]	.051
Intraoperative blood loss (g)	1670 [446–5087]	1574 [445–7530]	
Red blood cell transfusion performed	34 (34)	26 (44)	
Postoperative morbidity	51 (52)	32 (54)	
In-hospital mortality	0 (0)	2 (3)	.066

[range]
PD, pancreatoduodenectomy; PV, resection and reconstruction of the portal vein; PVE, portal vein embolization prior to the resectional surgery.
Percentages are described in parentheses.

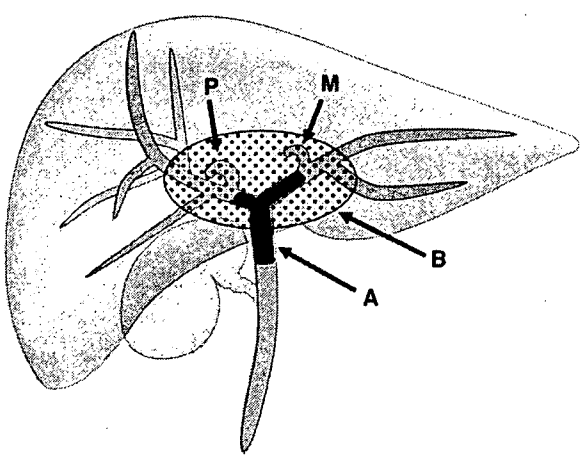

FIG. 1. Tumor origin for differentiating hilar bile duct cancer (BDC) and intrahepatic cholangiocarcinoma involving the hepatic hilus (CCC). Histopathologically, tumors thought to be originated in the black pasted area (**A**) were defined as BDC, and also tumors thought to be originated in the area with dot spots (**B**) were defined as CCC. P, right posterior sectional branch of the bile duct; M, left medial sectional branch of the bile duct.

fibers of the hepatic hilum and the intrahepatic Glisson's capsule in difficult cases of differentiating BDC from CCC. Namely, we can estimate special tumor domination whether inside or outside of hilar plate by the aid of elastica stain.

Macroscopically, the BDC tumors were classified as being polypoid and nodular or infiltrating. The CCC tumors were classified by the pathologists as being mass forming or non mass forming (periductal infiltrating or intraductal growth) type[17] on the plane of the thin slice section.

Pathological TNM classification was determined according to the criteria of the International Union Against Cancer (UICC) (sixth edition),[18] using the chapter dealing with extrahepatic bile duct cancer for BDC and that dealing with liver cancer for CCC. During the study period, the histopathological diagnoses were recorded and accumulated, then reviewed by the pathologists for this paper.

Follow-Up

All patients were followed at our outpatient clinic, where chest x-rays, abdominal ultrasound, CT, and the measurement of CEA and CA19-9 levels was done every 3–6 months after surgery. In principle, postoperative