



Original contribution

An assessment of chromosomal alterations detected by fluorescence in situ hybridization and p16 expression in sporadic and primary sclerosing cholangitis-associated cholangiocarcinomas

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Summary The objective of this study was to assess and compare the chromosome abnormalities present in sporadic and primary sclerosing cholangitis (PSC)-associated cholangiocarcinomas (CCAs) and biliary dysplasias. Histologic sections from 22 patients with CCA (16 sporadic and 6 PSC associated), 5 of whom had associated dysplasia, and 2 PSC patients with biliary dysplasia alone were assessed for chromosomal alterations with fluorescence in situ hybridization (FISH). FISH involved the use of a multiprobe set consisting of centromere-specific probes for chromosomes 3, 7, and 17 and a locus-specific probe for 9p21. The number of signals for each of these probes was enumerated in 50 nonoverlapping interphase nuclei, and the percentage of nuclei containing 0, 1, 2, and 3 or more signals was recorded for each probe. p16 expression was assessed using immunohistochemistry. Gain of at least 1 chromosome was identified in 19 of 22 (86%) invasive tumors and in 4 of 7 (57%) biliary dysplasias. Gain of 2 or more chromosomes (polysomy) was observed in 17 of 22 (77%) invasive tumors and in 3 of 7 (43%) biliary dysplasias. Homozygous loss of 9p21 was identified in 11 of 22 (50%) invasive tumors and in 3 of 7 (43%) biliary dysplasias. The patterns of chromosomal abnormalities detected by FISH in PSC-associated and sporadic CCAs were similar. Nine of 13 (69%) invasive tumors and 2 of 5 (40%) biliary dysplasias with complete loss of p16 expression by immunohistochemistry showed allelic loss of 9p21 by FISH. Polysomy and homozygous 9p21 deletion are common in both sporadic and PSC-associated CCAs and are frequently detectable in PSC-associated biliary dysplasia.

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1. Introduction

Malignant tumors of the intrahepatic and extrahepatic bile ducts, like many other solid tumors, exhibit numerical and structural chromosomal abnormalities by karyotypic

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analysis [1], DNA ploidy analysis [2,3], comparative genomic hybridization [4-6], and other molecular techniques [7,8]. In addition, alterations of the *P16* gene at chromosome band 9p21 are a commonly reported abnormality in cholangiocarcinoma (CCA) [1,3,5,8-11]. *P16* is a tumor suppressor gene that is commonly inactivated in a wide variety of malignant tumors [12] and in noninvasive precursor lesions [13,14]. The *P16* gene is inactivated via several mechanisms, including allelic loss, point mutation of the coding or promoter regions, and promoter hypermethylation [9-11,15-17].

CCA occurs both sporadically (ie, in patients without known risk factors) and in patients with known risk factors such as primary sclerosing cholangitis (PSC). PSC is a rare disorder in which chronic inflammation of the intrahepatic and/or extrahepatic biliary ducts leads to bile duct strictures. Approximately 10% to 20% of patients with PSC will develop CCA, and their lifetime risk of developing CCA is approximately 1500-fold greater than that of the general population [18].

Relatively little is known about the types of chromosomal alterations that occur in PSC-associated CCA and whether these genetic alterations are similar or different from those occurring in sporadic CCA. In addition, little is known about the spectrum of genetic abnormalities that occur in CCA precursors (ie, biliary ductal dysplasia).

Recently, we reported on the use of fluorescence in situ hybridization (FISH) as an adjunct to cytology for the diagnosis of malignant bile duct strictures [19]. Using a commercially available DNA probe set (UroVysion; Vysis Inc., Abbott Laboratories, Downers Grove, IL) containing centromeric probes for chromosomes 3, 7, and 17 and a locus-specific probe for 9p21, we identified malignant cells in cell preparations from bile duct brushings based on the finding of 5 or more cells with polysomy. Polysomic cells are defined as cells that show gains of 2 or more chromosomes. Used in this way, FISH was a sensitive and specific technique for diagnosing malignant bile duct strictures. In addition to being useful in detecting malignant cells in cytologic specimens, FISH can also be used to evaluate chromosomal abnormalities in paraffin-embedded tumors [20,21].

The goals of this study were to use FISH to determine the types of chromosomal alterations that occur in PSC-associated and sporadic CCAs and to determine whether these genetic alterations are similar or different. In addition, we sought to determine the types of chromosomal alterations that occur in precursors to CCA. Finally, we assessed p16 expression in these tumors to look for possible correlations between chromosomal alterations of the *P16* gene and p16 expression. The UroVysion probe set was used for these studies to correlate the results we obtained with paraffin-embedded CCA specimens to those we obtained with the same probe set on biliary duct brushing specimens.

2. Materials and methods

2.1. Patient population

As approved by the Mayo Clinic Institutional Review Board, we selected archival tissues from 24 patients who had undergone resection or liver transplantation for CCA or PSC for this study. Eight of these patients had PSC (6 with CCA and 2 with biliary dysplasia alone), and the remaining 16 had sporadic CCA. Seventeen patients were male and 7 were female. Patients' ages ranged from 25 to 77 years (median, 66 years; mean, 62 years). The ages of the patients with PSC-associated tumors ranged from 25 to 61 years (mean, 51 years), whereas those of the patients with sporadic tumors ranged from 51 to 77 years (mean, 67 years).

2.2. Histologic evaluation

Hematoxylin-eosin-stained sections from the 24 patients were reviewed by 3 investigators (T. C. S., R. D. D., and S. C. A.), and representative blocks containing invasive CCA and/or biliary dysplasia were selected for FISH analysis and p16 immunostaining. Patients with a diagnosis of PSC documented in their medical records were considered to have PSC-associated tumors. All other specimens were classified as sporadic tumors for this study.

2.3. Processing of paraffin sections for FISH

Five-micron paraffin sections were placed in a 90 °C oven for 15 minutes. The tissue sections were then placed in xylene for 30 minutes, followed by dehydration in 100% ethanol for 5 minutes. After air drying them for 3 minutes, we immersed the slides in 10 mmol/L of citric acid (80 °C, pH 6.0) for 35 minutes and 2× SSC at 37 °C for 5 minutes. The samples were then digested in a 0.2% pepsin solution (2500-3500 U/mg; Sigma, St Louis, MO) at 37 °C for 40 minutes. The specimens were subsequently dehydrated by immersion in 75%, 85%, and 100% alcohol solutions for 3 minutes each.

Ten microliters of UroVysion probe (Abbott Molecular Inc, Des Plaines, IL) was applied to the area of interest identified on the corresponding hematoxylin-eosin slide. Probe and target DNAs were codenatured and hybridized on a Vysis HYBrite instrument (Abbott Molecular Inc) set at a denaturation temperature of 73 °C for 5 minutes and a hybridization temperature of 37 °C for 10 to 16 hours. After hybridization, unbound probe was removed by washing in 2× SSC/0.1% NP-40 at 76 °C for 1 minute. Ten microliters of DAPI I (Abbott Molecular Inc) counterstain was applied, and the slides were coverslipped.

2.4. Enumeration of FISH signals in tumor and normal tissue specimens

Using standard accepted criteria for enumeration [20], the signal pattern for each of the 4 probes (CEP3, CEP7,

CEP17, and LSI 9p21) was enumerated in 50 consecutive nuclei from 10 specimens from healthy subjects, 7 specimens from patients with dysplasia, and 22 specimens from patients with CCA. The 10 normal specimens were areas of normal bile duct epithelium or hepatocytes in histologic sections that also contained tumors. The signal patterns in normal tissue were assessed to determine what the distribution of FISH signals is in normal tissue and to determine cutoffs for defining chromosomal abnormalities in tumor specimens. The percentage of cells demonstrating loss (<2 signals), gain (>2 signals), or polysomy (>2 signals in ≥ 2 probes) was calculated and recorded. CCA or dysplasia cases in which the percentages of cells showing a specific signal pattern (eg, gain of chromosome 3) that was 3 standard deviations higher than the mean observed in normal specimens were considered abnormal.

2.5. p16 immunohistochemistry

p16 expression was evaluated using a mouse monoclonal antibody to p16 (clone 16P07, NeoMarkers/Lab Vision, Fremont, CA) at a dilution of 1:80. Slides were immersed in 1 mmol/L of EDTA, pH 8.0, for 30 minutes in a preheated vegetable steamer (92 °C). The slides were then allowed to cool for 5 minutes and were rinsed with deionized water for 3 minutes. The slides were then placed on a Dako autostainer (DakoCytomation, Carpinteria, CA) and incubated with the p16 antibody for 30 minutes. Antigen-antibody reactions were visualized using a dextran polymer-based detection system, ENVISION+ (DakoCytomation) and DAB+ (3,3'-diaminobenzidine; DakoCytomation), as the substrate chromogen. Slides were counterstained with hematoxylin and coverslipped for microscopic examination.

The p16 stain for each case was reviewed independently by 2 investigators (T. C. S. and R. D. D.) who had no knowledge of the FISH findings and was interpreted as previously described [22]. Nuclear staining intensity higher than that of the cytoplasmic background staining was considered positive. Positive staining was further classified as diffuse (homogeneous throughout the tumor) or patchy (areas of positive and negative staining within the tumor). Cases that demonstrated no cytoplasmic or nuclear staining, or nuclear staining of the same or lesser intensity as with cytoplasmic staining, were considered negative. Stromal and/or inflammatory cells served as positive internal controls.

2.6. Statistical analysis

Statistical analysis was performed with SPSS for Windows software, version 11.5 (SPSS, Chicago, IL) using one-way analysis of variance and Pearson's χ^2 test, as appropriate. A *P* value lower than .05 was considered statistically significant.

3. Results

3.1. Histology

Histologic sections from the 24 patients were evaluated; the patients were categorized as non-PSC patients with CCA (*n* = 14), non-PSC patients with CCA and areas of dysplasia (*n* = 2), PSC patients with CCA (*n* = 3), PSC patients with CCA and dysplasia (*n* = 3), and PSC patients with dysplasia only (*n* = 2). Of the CCA cases, 9 were intrahepatic tumors (6 sporadic and 3 PSC associated), 8 were hilar tumors (5 sporadic and 3 PSC associated), and 5 were extrahepatic tumors (all sporadic). Of the dysplasia cases, 1 tumor was intrahepatic (PSC associated), 5 tumors were hilar (3 PSC associated and 2 sporadic), and 1 tumor was extrahepatic (PSC associated). Twenty-one of the 22 CCAs were moderately differentiated adenocarcinomas (grade 2-3 of 4). One sporadic CCA was poorly differentiated (grade 4 of 4). Of the 7 specimens with biliary dysplasia, 6 were classified to be of high grade and 1 was classified to be of low grade.

3.2. Normal value study and criteria for FISH abnormality

The percentages of nuclei in normal tissue with 0, 1, 2, and 3 or more signals for each probe are shown in Table 1. The signal distribution patterns were fairly similar for all 4 probes. The mean percentages of nuclei with 2 signals for chromosomes 3, 7, 17, and 9p21 varied from 52% to 63%. The mean percentages of nuclei with 0, 1, and 3 or more signals ranged from 9% to 12%, from 25% to 36%, and from 1% to 2%, respectively, for the 4 probes. The relatively high percentage of nuclei with no or only 1 signal in normal specimens has been variably observed in other normal value studies on paraffin-embedded tissue and is largely attributed to truncation of nuclei and, to a lesser extent, incomplete hybridization efficiency. The truncation rate can also be influenced by variability in nuclear size and degree of DNA condensation [20,23,24]. These values were used to establish cutoff values for the different types of chromosome signal patterns that were observed (eg, gain of chromosomes 3, 7, or 17; polysomy; loss of 9p21) based on the mean ± 3 standard deviations observed in normal tissue (Table 1). No normal tissue specimen included in the normal value study contained values that exceeded these cutoffs.

Table 1 Centromeric and locus specific probe copy number of benign tissue

	0 signals	1 signal	2 signals	≥ 3 signals
CEP3	10 \pm 4.6%	25 \pm 9.2%	63 \pm 6.9%	2 \pm 2.6%
CEP7	9 \pm 4.3%	33 \pm 6.7%	56 \pm 7.4%	2 \pm 1.2%
CEP17	12 \pm 6.6%	34 \pm 6.4%	53 \pm 10.4%	1 \pm 1.4%
LSI 9p21	10 \pm 4.3%	36 \pm 5.9%	52 \pm 7.9%	2 \pm 3.2%

The results (mean ± 3 SD) are given as the percentage of 50 epithelial cells with indicated centromere or locus specific indicator number.

3.3. FISH abnormalities in CCA and dysplasia

Table 2 shows the percentages of nuclei containing 0, 1, and 3 or more signals for each FISH probe for each of the sporadic and PSC-associated CCA and dysplasia specimens. Thirteen of 16 (81%) sporadic CCA specimens contained polysomic cells. The percentages of tumor cells exhibiting polysomy in these 13 cases ranged from 6% to 100%. In the 3 sporadic CCA specimens that did not demonstrate polysomy, abnormally high percentages (26%-96%) of homozygous 9p21-deleted cells were observed. In addition, 1 of the 3 cases also exhibited gain of chromosome 7 and loss of chromosome 17. There was also evidence of 9p21

loss in most of the polysomic tumors, with 5 polysomic tumors showing evidence of homozygous 9p21 loss and 2 tumors showing hemizygous 9p21 loss. Similar results were found in the PSC-associated CCA cases in that 4 of 6 tumors demonstrated polysomy and the 2 that did not show polysomy had 9p21 loss. In total, 82% (18/22), 77% (17/22), and 64% (14/22) of CCAs showed gains of chromosomes 7, 3, and 17, respectively. Examples of the typical signal patterns observed in the CCA specimens are shown in Fig. 1.

The PSC-associated dysplasias were very similar to the PSC-associated carcinomas in that 60% (3/5) of the dysplasias demonstrated polysomy and the remaining

Table 2 FISH findings for sporadic and PSC-associated cholangiocarcinoma and dysplasias

Patient ID	CEP 3			CEP 7			CEP 17			9p21			% Polysomy
	0 sign.	1 sign.	≥3 sign.	0 sign.	1 sign.	≥3 sign.	0 sign.	1 sign.	≥3 sign.	0 sign.	1 sign.	≥3 sign.	
Cutoffs for abnormality	>24%	>53%	>10%	>22%	>53%	>6%	>32%	>53%	>5%	>23%	>54%	>12%	>5%
Sporadic cholangiocarcinoma ^a													
1 ^b	6	10	32	24	20	14	44	34	2	28	48	0	4
2	12	28	4	16	24	2	20	32	2	26	30	0	0
3	4	28	0	4	22	2	4	6	0	96	2	0	0
4	20	44	6	20	38	18	36	34	4	46	42	0	6
5 ^b	0	6	62	0	8	38	2	16	16	6	20	4	36
6	2	18	26	2	16	34	2	32	18	20	72	0	20
7	8	24	34	6	10	48	22	36	2	22	50	0	18
8	0	0	100	0	4	96	4	0	84	12	12	48	100
9	2	26	36	2	8	64	6	12	56	30	32	10	56
10	4	16	22	0	8	64	14	24	2	98	2	0	22
11	4	12	26	0	12	68	8	18	42	36	28	6	46
12	0	8	58	2	26	48	14	12	42	12	36	4	52
13	8	30	6	2	6	48	6	40	6	12	44	2	8
14	10	16	22	12	16	20	18	16	18	94	4	0	18
15	4	18	52	2	8	60	2	8	58	4	32	20	62
16	4	14	26	8	26	28	6	16	20	18	56	0	26
PSC cholangiocarcinoma ^c													
17 ^b	28	50	4	12	34	4	8	34	2	26	70	0	0
18 ^b	12	18	14	6	38	4	4	30	4	90	6	0	4
19	0	16	28	0	4	66	4	18	36	16	34	2	40
20 ^b	4	6	74	4	6	80	12	8	60	20	14	28	74
21	8	4	48	2	34	30	42	10	18	30	44	2	26
22	8	10	42	2	32	26	18	34	18	8	18	56	42
Dysplasia ^d													
1 ^b	0	12	4	2	30	4	6	24	0	8	18	2	2
5 ^b	2	8	0	34	14	4	48	22	0	20	12	0	0
PSC dysplasia ^e													
17 ^b	22	50	0	14	30	8	2	26	0	34	48	0	0
18 ^b	10	20	6	4	10	4	8	22	2	86	2	2	2
20 ^b	0	14	56	2	16	66	2	14	64	16	46	12	64
23	0	12	72	0	0	88	4	4	84	8	8	40	84
24	10	14	40	4	22	42	12	22	44	98	0	0	38

Note: Bolded numbers indicate signal patterns that exceed the normal value cut-offs.

^a Tissue from patients with cholangiocarcinoma and no history of PSC.

^b Represents patients with both a cancer and dysplasia specimen.

^c Tissue from patients with cholangiocarcinoma and a history of PSC.

^d Dysplastic tissue from patients without a history of PSC.

^e Dysplastic tissue from patients with a history of PSC.

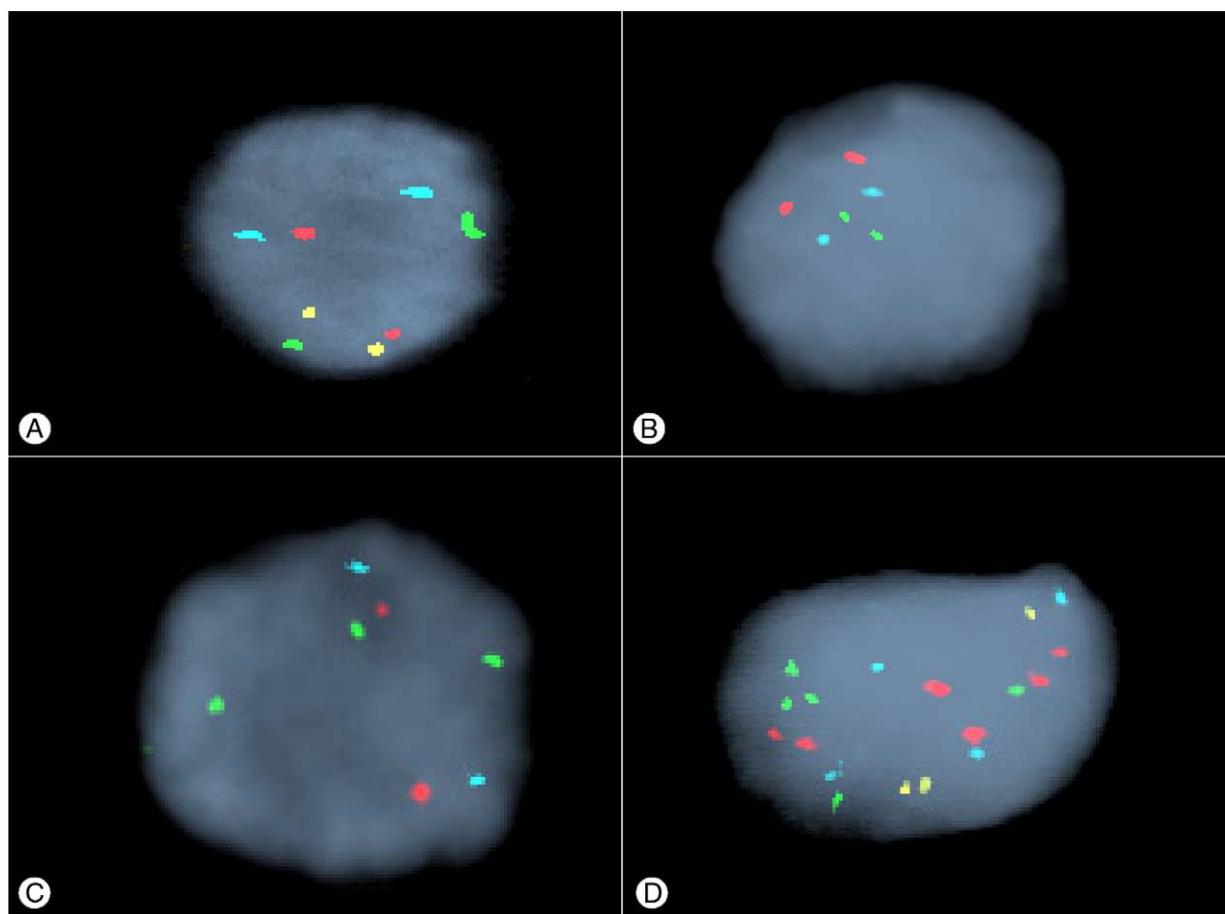


Fig. 1 Representative examples of FISH results. A, Normal cell with 2 signals for each of the 4 probes. B, Homozygous 9p21 deletion with loss of both copies of the 9p21 probe. C, Trisomy 7 and homozygous 9p21 deletion with 3 copies of the CEP7 probe and no copy of the 9p21 probe. D, Polysomy with gain of all 4 probes. CEP3 is illustrated in red; CEP7, in green; CEP17, in aqua; and LSI 9p21, in gold.

2 specimens had homozygous 9p21 loss. Gains of chromosomes 7, 3, and 17 were seen in 80% (4/5), 60% (3/5), and 60% (3/5) of PSC-associated dysplasias, respectively. In contrast, the 2 non-PSC-associated dysplasias had neither

increased polysomic cells nor cells with homozygous 9p21 deletion. Of note, 3 of the 4 dysplasias without polysomy were associated with carcinomas not showing polysomy.

Comparisons of the mean number of probe signals for each chromosome based on tissue diagnosis (benign, dysplasia, carcinoma) are summarized in Table 3. There

Table 3 Comparison of chromosome and LSI signal copy number in cells from carcinoma, dysplasia and benign tissue

Probe	Tissue	# Cells	Mean	Std. deviation	P-Value*
CEP 3	Benign	500	1.56	0.69	$P < .001$
	Dysplasia	350	2.21	1.45	
	Carcinoma	1100	2.45	1.91	
CEP 7	Benign	500	1.54	0.69	$P < .001$
	Dysplasia	350	2.27	1.47	
	Carcinoma	1100	2.60	1.94	
CEP 17	Benign	500	1.43	0.71	$P < .001$
	Dysplasia	350	2.03	1.30	
	Carcinoma	1100	1.97	1.53	
LSI 9p21	Benign	500	1.46	0.70	$P < .001$
	Dysplasia	350	1.18	1.18	
	Carcinoma	1100	1.12	1.06	

* One way ANOVA; significant difference ($P < .05$) in the mean number of signals by diagnosis.

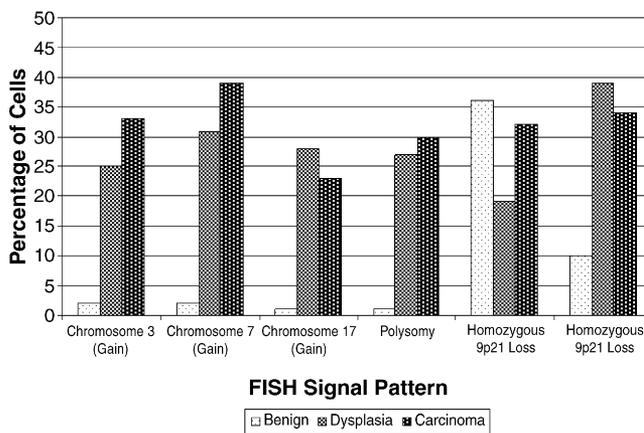


Fig. 2 Percentage of cells demonstrating gains or losses in benign, dysplastic, and carcinoma specimens.

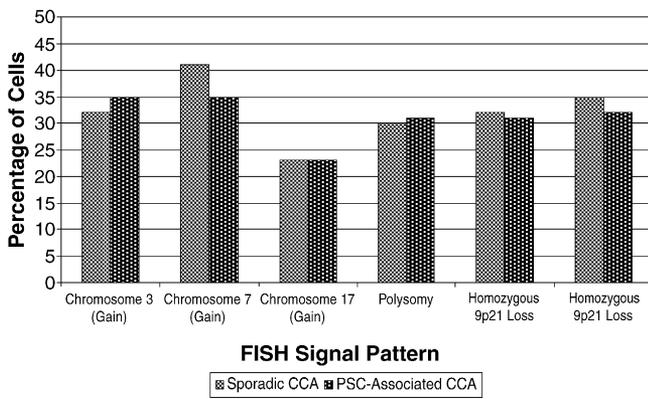


Fig. 3 Comparison of genetic alterations observed in sporadic and PSC-associated CCAs.

was a significant ($P < .001$) increase in the mean number of CEP signals (CEPs 3, 7, and 17) observed in dysplasia and CCA specimens as compared with normal tissue. In contrast, the mean number of probe signals for 9p21 was significantly ($P < .001$) decreased in both dysplasia and CCA specimens as compared with normal tissue.

Fig. 2 shows that the percentage of cells demonstrating the CEP probe gains and that of cells demonstrating loss of one or both of the 9p21 probes significantly ($P < .001$) increased when comparing normal with dysplasia and CCA results, respectively. There was no significant difference ($P > .05$) between PSC-associated CCA signal patterns and sporadic CCA signal patterns for any of the 4 probes analyzed (Fig. 3).

3.4. p16 immunohistochemistry

A summary of the FISH and p16 immunohistochemistry results for all patients is shown in Table 4. Among the 22 CCAs that were examined, 6 (27%) showed normal nuclear p16 expression, 3 (14%) showed patchy loss of p16 expression, and 13 (59%) showed complete loss of p16 expression in the tumor. Representative examples of tumors showing normal p16 expression and complete loss of p16 expression are shown in Fig. 4. Five of the 7 (71%) dysplasias showed complete loss of p16 expression, 1 dysplasia showed patchy p16 loss, and another had normal p16 expression. There appeared to be a correlation between the mean percentage of cells demonstrating homozygous 9p21 loss and attenuated p16 expression by immunohistochemistry. In specimens with normal p16 expression, the mean percentage of cells demonstrating homozygous loss by FISH was 20% as compared with 24% and 44% in specimens with patchy or no p16 expression, respectively. However, this increase was not significant ($P = .197$).

4. Discussion

We have previously reported on the use of the UroVysion probe set to detect CCA in bile duct brushings [19,25]. In

the current study, FISH of paraffin-embedded CCAs and biliary dysplasias was successfully used to document consistent patterns of chromosomal alteration, including single chromosome gain, polysomy (gains of ≥ 2 chromosomes), and homozygous 9p21 deletion. Polysomy was identified in 77% (17/22) of the CCA specimens. Gain of chromosome 7 was the most common chromosomal gain, which is consistent with cytology-based FISH studies at our institution that found gains of chromosome 7 in a large percentage of CCA cells [26]. The epidermal growth factor receptor gene is on chromosome 7 (7p12) and may be involved in the development of CCA [27,28].

Many of the chromosomal alterations present in the invasive CCA of PSC patients are also present in coexistent dysplastic foci. The mean signal number for chromosomes 3, 7, and 17 was significantly higher in cells from dysplasia and

Table 4 Summary of FISH and immunohistochemistry results

Specimen #	FISH result		p16 Immunohistochemistry
	Gains	Losses	
Cholangiocarcinoma from patients with no history of PSC			
1 ^a	Polysomy 7, 9 ^b , 17		Patchy loss
2	None	9 ^b	Patchy loss
3	None	9 ^b	Loss
4	7	9 ^b , 17	Normal
5 ^a	Polysomy	None	Patchy loss
6	Polysomy	9 ^c	Loss
7	Polysomy	None	Normal
8	Polysomy	None	Normal
9	Polysomy	9 ^b	Loss
10	Polysomy	9 ^b	Loss
11	Polysomy	9 ^b	Loss
12	Polysomy	None	Normal
13	Polysomy	None	Loss
14	Polysomy	9 ^b	Loss
15	Polysomy	None	Loss
16	Polysomy	9 ^c	Loss
Cholangiocarcinoma from patients with history of PSC			
17 ^a	None	3, 9 ^d	Normal
18 ^a	3	9 ^b	Loss
19	Polysomy	None	Normal
20 ^a	Polysomy	None	Loss
21	Polysomy	9 ^b , 17	Loss
22	Polysomy	None	Loss
Dysplasia specimens from patients with no history of PSC			
1 ^a	None	None	Loss
5 ^a	None	7, 17	Loss
Dysplasia specimens from patients with history of PSC			
17 ^a	7	9 ^b	Patchy loss
18 ^a	None	9 ^b	Loss
20 ^a	Polysomy	None	Loss
23	Polysomy	None	Normal
24	Polysomy	9 ^b	Loss

^a Represents patients with both a cancer and dysplasia result.

^b Homozygous 9p21 loss (loss of both FISH signals).

^c Hemizygous 9p21 loss (loss of 1 FISH signal).

^d Hemi- and Homozygous 9p21 loss.

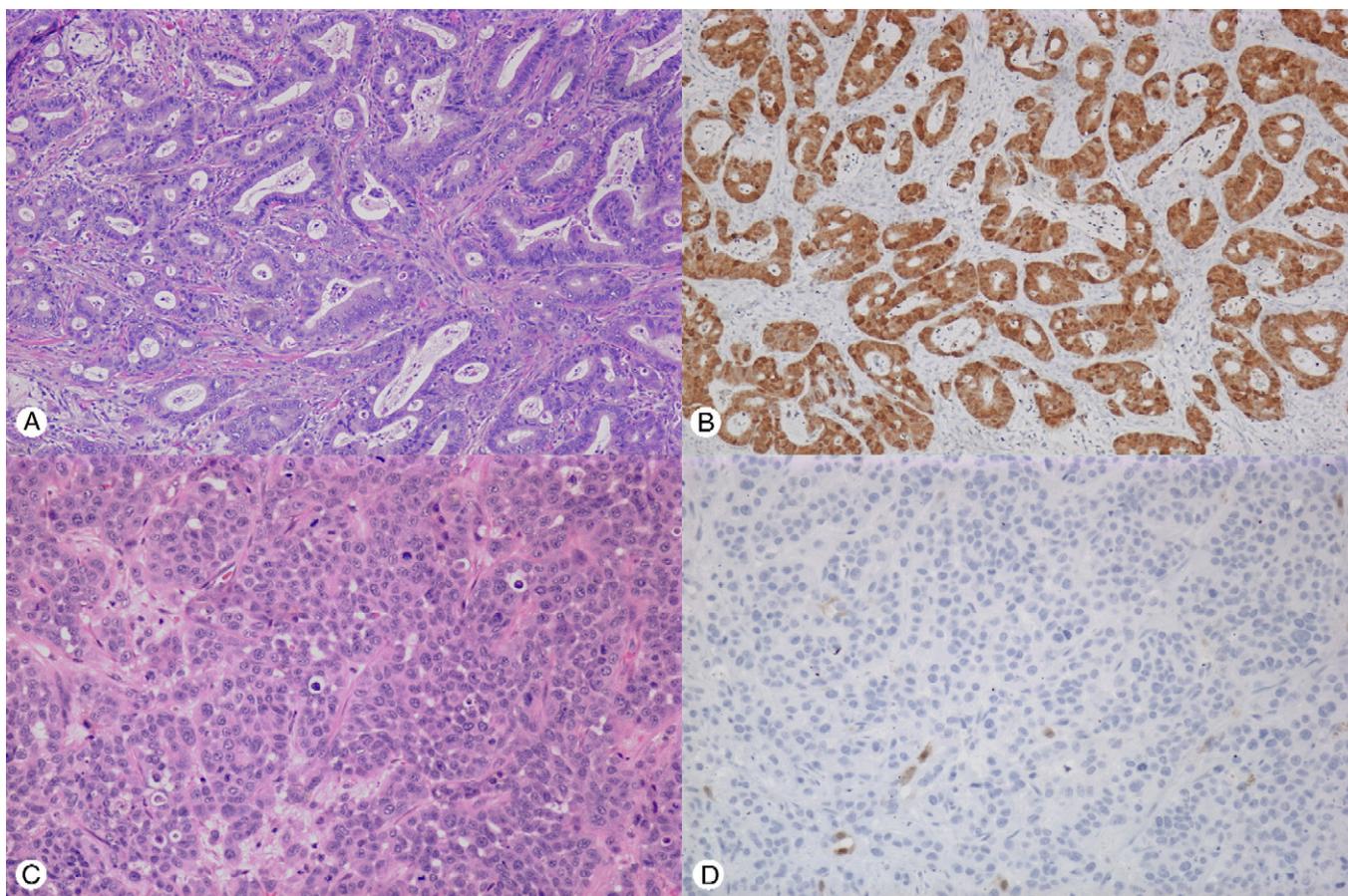


Fig. 4 Representative examples of p16 immunohistochemistry. A and B, Case with no 9p21 deletion and preserved p16 expression: hematoxylin-eosin staining in panel A and immunohistochemical staining for p16 in panel B. Almost all tumor cell nuclei are strongly positive for p16 expression. C and D, Case with homozygous 9p21 deletion and loss of p16 expression: hematoxylin-eosin staining in panel C and immunohistochemical staining for p16 in panel D. Almost all nuclei of tumor cells demonstrate loss of p16 expression. Stromal cells serve as internal positive controls.

carcinoma as compared with benign tissue but not significantly greater in CCA as compared with dysplasia. The percentage of cells with gains of chromosomes 3, 7, and 17 and the percentage of polysomic cells were also significantly increased in dysplasia and carcinoma as compared with benign tissue. These data are consistent with those of previous studies from other body sites, showing that FISH is capable of detecting abnormalities in dysplastic or preneoplastic cells [29]. These findings suggest that we should be able to use FISH to detect alterations in endoscopic brushings. PSC patients who develop CCA typically have a poor prognosis, but early diagnosis with prompt liver transplantation markedly improves outcomes.

Homozygous deletion of 9p21, the site of the *P16* tumor suppressor gene, was identified in most cases (Table 2). This abnormality was identified in 50% (11/22) of cancer specimens and 60% (3/5) of PSC-associated dysplasia specimens. This finding is consistent with findings of previous reports, showing that the *P16* gene is one of the most commonly inactivated tumor suppressor genes in human cancers and is frequently altered in CCA [12,30]. p16 has been shown to act

through the retinoblastoma-cyclin-dependent kinase and p53 pathways as a critical cell cycle regulator. Inactivation of the *P16* gene leads to an unchecked progression through the cell cycle that is characteristic of malignant tumors. FISH is able to detect allelic deletion of the *P16* gene. However, other mechanisms that can lead to p16 inactivation, which FISH cannot detect, include promoter methylation and point mutation [9-11,15,17]. The contributions of homozygous allelic 9p21 deletion to p16 inactivation in CCA are variable. Using microsatellite analysis, Ahrendt et al [17] reported heterozygous allelic loss of 9p21 in 9 of 10 tumors but no homozygous deletion. In a study on 51 CCAs, homozygous 9p21 deletion was detected in 4% of cases by molecular DNA analysis [9,11]. Our data suggest that homozygous 9p21 loss is more common than as suggested by previous studies. One advantage that FISH has over techniques such as microsatellite analysis is that it can detect genetic alterations, such as homozygous loss, that are present in only a small subset of the tumor cells, whereas homozygous loss is difficult to detect by microsatellite analysis owing to contamination by normal cells.

The relationship between allelic loss of 9p21 and expression of p16 protein in CCA is shown in Table 4. The 9p21 probe used in this study spans the *P16* gene; thus, the finding of homozygous 9p21 loss by FISH would be expected to result in an absence of p16 expression. Thirteen of 22 (59%) invasive tumors and 5 of 7 (71%) dysplasias showed complete loss of p16 expression (Table 4). There were 4 CCAs and 2 dysplasias with homozygous 9p21 loss in 85% or more of the nuclei, and each of these showed complete loss of p16 expression. For the remaining 18 CCA cases in which less than 85% of the nuclei showed homozygous loss by FISH, 9 showed loss of p16 expression, 3 showed patchy loss, and 6 showed normal p16 expression. The complete loss of p16 expression in cases without evidence of 9p21 loss by FISH can be explained by the fact that there are other mechanisms, such as promoter methylation and point mutation, that can lead to *P16* gene inactivation. For example, a study on 21 sporadic CCAs by Caca et al [10] showed that 69% (9/13) of tumors with allelic loss of 9p21 also had coexistent *P16* promoter methylation and that all 9 of these cases had lost p16 expression.

Although there was an overall good correlation between allelic loss of 9p21 and loss of p16 expression in our study, the correlation was questionable in 4 cases. Two cases with allelic loss of 9p21 showed patchy staining of the tumor cells in some areas and complete loss of p16 expression in other areas (patients 1 and 2; Tables 2 and 4). This pattern of staining could be related to heterogeneity within the tumor cell population with regard to allelic loss of 9p21 and/or *P16* gene inactivation. In fact, the percentage of nuclei with 9p21 loss in each of these cases was quite low (Table 2) and thus may account for the heterogeneity of p16 staining. A slightly more puzzling finding was that of 2 cases with allelic loss of 9p21 and diffuse p16 expression (patients 4 and 17; Tables 2 and 4). This could be a result of a false-positive FISH result caused by inadequate hybridization and/or fading of 9p21 FISH signals causing an appearance of 9p21 deletion.

In summary, by evaluating paraffin sections of CCA and biliary dysplasia with FISH, we have provided further support for the validity of using FISH as a diagnostic tool in biliary brushing specimens. We have also shown that FISH can detect chromosomal abnormalities in preinvasive dysplastic lesions of the biliary tract, which may have implications for improving earlier detection of biliary tract malignancies. In addition, we have directly compared the chromosomal alterations detectable by FISH in PSC-associated and sporadic CCAs. The advantage of FISH over molecular techniques is its ability to specifically target tumor cells and detect heterogeneity of genetic changes within tumor cell populations. Polysomy and inactivation of the *P16* tumor suppressor gene via homozygous deletion of 9p21 are common abnormalities in both sporadic and PSC-associated CCAs, and these abnormalities are frequently detectable in high-grade preinvasive biliary lesions in PSC patients. Further characterization of the genetic altera-

tions in preinvasive and invasive biliary lesions will help in our understanding of the pathogenesis of these tumors and may aid in the development of methods for early diagnosis and screening.

References

- [1] Rijken AM, Hu J, Perlman EJ, et al. Genomic alterations in distal bile duct carcinoma by comparative genomic hybridization and karyotype analysis. *Genes Chromosomes Cancer* 1999;26:185-91.
- [2] Baron TH, Harewood GC, Rumalla A, et al. A prospective comparison of digital image analysis and routine cytology for the identification of malignancy in biliary tract strictures. *Clin Gastroenterol Hepatol* 2004;2:214-9.
- [3] Abou-Rebyeh H, Al-Abadi H, Jonas S, Rotter I, Bechstein WO, Neuhaus P. DNA analysis of cholangiocarcinoma cells: prognostic and clinical importance. *Cancer Detect Prev* 2002;26:313-9.
- [4] Lee JY, Park YN, Uhm KO, Park SY, Park SH. Genetic alterations in intrahepatic cholangiocarcinoma as revealed by degenerate oligonucleotide primed PCR-comparative genomic hybridization. *J Korean Med Sci* 2004;19:682-7.
- [5] Koo SH, Ihm CH, Kwon KC, Park JW, Kim JM, Kong G. Genetic alterations in hepatocellular carcinoma and intrahepatic cholangiocarcinoma. *Cancer Genet Cytogenet* 2001;130:22-8.
- [6] Wong N, Li L, Tsang K, Lai PB, To KF, Johnson PJ. Frequent loss of chromosome 3p and hypermethylation of RASSF1A in cholangiocarcinoma. *J Hepatol* 2002;37:633-9.
- [7] Chariyalertsak S, Khuhaprema T, Bhudisawasdi V, Sripa B, Wongkham S, Petmitr S. Novel DNA amplification on chromosomes 2p25.3 and 7q11.23 in cholangiocarcinoma identified by arbitrarily primed polymerase chain reaction. *J Cancer Res Clin Oncol* 2005;131:821-8.
- [8] Kim DG, Park SY, You KR, et al. Establishment and characterization of chromosomal aberrations in human cholangiocarcinoma cell lines by cross-species color banding. *Genes Chromosomes Cancer* 2001;30:48-56.
- [9] Tannapfel A, Sommerer F, Benicke M, et al. Genetic and epigenetic alterations of the INK4a-ARF pathway in cholangiocarcinoma. *J Pathol* 2002;197:624-31.
- [10] Caca K, Feisthammel J, Klee K, et al. Inactivation of the INK4a/ARF locus and p53 in sporadic extrahepatic bile duct cancers and bile tract cancer cell lines. *Int J Cancer* 2002;97:481-8.
- [11] Tannapfel A, Benicke M, Katalinic A, et al. Frequency of p16(INK4A) alterations and *K-ras* mutations in intrahepatic cholangiocarcinoma of the liver. *Gut* 2000;47:721-7.
- [12] Liggett W, Sidransky D. Role of the p16 tumor suppressor gene in cancer. *J Clin Oncol* 1998;16:1197-206.
- [13] Hustinx SR, Leoni LM, Yeo CJ, et al. Concordant loss of MTAP and p16/CDKN2A expression in pancreatic intraepithelial neoplasia: evidence of homozygous deletion in a noninvasive precursor lesion. *Mod Pathol* 2005;18:959-63.
- [14] Fahmy M, Skacel M, Gramlich TL, et al. Chromosomal gains and genomic loss of p53 and p16 genes in Barrett's esophagus detected by fluorescence in situ hybridization of cytology specimens. *Mod Pathol* 2004;17:588-96.
- [15] Taniai M, Higuchi H, Burgart LJ, Gores GJ. p16INK4a promoter mutations are frequent in primary sclerosing cholangitis (PSC) and PSC-associated cholangiocarcinoma. *Gastroenterology* 2002;123:1090-8.
- [16] Yoshida S, Todoroki T, Ichikawa Y, et al. Mutations of p16Ink4/CDKN2 and p15Ink4B/MTS2 genes in biliary tract cancers. *Cancer Res* 1995;55:2756-60.
- [17] Ahrendt SA, Eisenberger CF, Yip L, et al. Chromosome 9p21 loss and p16 inactivation in primary sclerosing cholangitis-associated cholangiocarcinoma. *J Surg Res* 1999;84:88-93.

- [18] Burak K, Angulo P, Pasha TM, Egan K, Petz J, Lindor KD. Incidence and risk factors for cholangiocarcinoma in primary sclerosing cholangitis. *Am J Gastroenterol* 2004;99:523-6.
- [19] Kipp BR, Stadheim LM, Halling SA, et al. A comparison of routine cytology and fluorescence in situ hybridization for the detection of malignant bile duct strictures. *Am J Gastroenterol* 2004;99:1675-81.
- [20] Qian J, Bostwick DG, Takahashi S, et al. Comparison of fluorescence in situ hybridization analysis of isolated nuclei and routine histological sections from paraffin-embedded prostatic adenocarcinoma specimens. *Am J Pathol* 1996;149:1193-9.
- [21] Brunelli M, Eble JN, Zhang S, Martignoni G, Delahunt B, Cheng L. Eosinophilic and classic chromophobe renal cell carcinomas have similar frequent losses of multiple chromosomes from among chromosomes 1, 2, 6, 10, and 17, and this pattern of genetic abnormality is not present in renal oncocytoma. *Mod Pathol* 2005; 18:161-9.
- [22] Geradts J, Hruban RH, Schutte M, Kern SE, Maynard R. Immunohistochemical p16INK4a analysis of archival tumors with deletion, hypermethylation, or mutation of the CDKN2/MTS1 gene. A comparison of four commercial antibodies. *Appl Immunohistochem Mol Morphol* 2000;8:71-9.
- [23] Ventura RA, Martin-Subero JI, Jones M, et al. FISH analysis for the detection of lymphoma-associated chromosomal abnormalities in routine paraffin-embedded tissue. *J Mol Diagn* 2006;8:141-51.
- [24] Wilkens L, Gerr H, Gadzicki D, Kreipe H, Schlegelberger B. Standardised fluorescence in situ hybridisation in cytological and histological specimens. *Virchows Arch* 2005;447:586-92.
- [25] Klump B, Hsieh CJ, Dette S, Holzmann K, Kiebetalich R, Jung M, et al. Promoter methylation of INK4a/ARF as detected in bile-significance for the differential diagnosis in biliary disease. *Clin Cancer Res* 2003;9:1773-8.
- [26] Moreno Luna LE, Kipp B, Halling KC, Sebo TJ, Kremers WK, Roberts LR, et al. Advanced cytologic techniques for the detection of malignant pancreatobiliary strictures. *Gastroenterology* 2006; 131:1064-72.
- [27] Gwak GY, Yoon JH, Shin CM, Ahn YJ, Chung JK, Kim YA, et al. Detection of response-predicting mutations in the kinase domain of the epidermal growth factor receptor gene in cholangiocarcinomas. *J Cancer Res Clin Oncol* 2005;131:649-52.
- [28] Yoon JH, Gwak GY, Lee HS, Bronk SF, Werneburg NW, Gores GJ. Enhanced epidermal growth factor receptor activation in human cholangiocarcinoma cells. *J Hepatol* 2004;41:808-14.
- [29] Fahmy M, Skacel M, Gramlich TL, Brainard JA, Rice TW, Goldblum JR, et al. Chromosomal gains and genomic loss of p53 and p16 genes in Barrett's esophagus detected by fluorescence in situ hybridization of cytology specimens. *Mod Pathol* 2004;17:588-96.
- [30] Rashid A. Cellular and molecular biology of biliary tract cancers. *Surg Oncol Clin N Am* 2002;11:995-1009.