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Chromosome 9p21 Loss and p16 Inactivation in Primary Sclerosing Cholangitis-Associated Cholangiocarcinoma[1]

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Background. **Cholangiocarcinoma is a frequent complication of primary sclerosing cholangitis and is a leading cause of mortality in patients with this disease. The tumor suppressor gene p16 is commonly inactivated in many neoplasms; however, the role of p16 in the pathogenesis of cholangiocarcinoma is unclear. Therefore, we examined the role of p16 inactivation in the pathogenesis of cholangiocarcinoma associated with primary sclerosing cholangitis.**

Materials and methods. **Paraffin-embedded sections from 10 patients who developed cholangiocarcinoma in the setting of primary sclerosing cholangitis were examined. Chromosomal loss at 9p21 was determined using microsatellite analysis. Methylation of a CpG island in the promoter region of the p16 gene was determined using methylation-specific polymerase chain reaction. p16 inactivation was also determined using immunohistochemistry.**

Results. **Allelic loss at chromosome 9p21 was present in 9 of 10 tumors (90%). Methylation of the p16 promoter was present in 2 of the 8 tumors examined (25%). Four of seven tumors (57%) analyzed by immunohistochemistry demonstrated an absence of p16 nuclear staining.**

Conclusions. **Loss of chromosome 9p21 and inactivation of the p16 tumor suppressor gene are common events in primary sclerosing cholangitis-associated cholangiocarcinoma and may play a role in the high incidence of cholangiocarcinoma in patients with primary sclerosing cholangitis.** © 1999 Academic Press

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Cholangiocarcinoma develops frequently in patients with primary sclerosing cholangitis (PSC) and is currently a leading cause of death in patients with this disease [1–6]. However, the widespread biliary strictures associated with PSC make the diagnosis of cholangiocarcinoma particularly difficult. As a result, most patients are diagnosed with advanced, unresectable disease, and the median survival in patients with cholangiocarcinoma complicating PSC has been poor [1–3]. Liver transplantation has produced excellent long-term survival in patients with end-stage liver disease from PSC and has been advocated early in the course of the disease to avoid the mortality associated with cholangiocarcinoma [7–9]. However, only 20–30% of patients with both cholangiocarcinoma and PSC have cirrhosis and meet the usual criteria for liver transplantation [1, 2]. Recently, several studies have reported encouraging results using serum tumor markers [carcinoembryonic antigen (CEA) or carbohydrate antigen 19-9 (CA19-9)] or new radiological techniques to detect cholangiocarcinoma in patients with PSC [10–13]. However, further efforts are needed to develop screening strategies to identify patients with PSC at risk of developing cholangiocarcinoma.

Cytological examination of endoscopic brushings is commonly used to evaluate biliary strictures in patients with primary sclerosing cholangitis. This approach is limited by the low sensitivity of cytology in this setting [1]. Recently, genetic-based screening strategies have been used to identify cancer cells in clinical samples. K-ras mutations identical to those present in the primary tumor have been detected in the

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stool, blood, duodenal juice, bile, and pancreatic duct brushings of patients with pancreatic cancer [14–17]. We have recently demonstrated overexpression of p53 protein and mutation of the K-ras gene in 50 and 33% of patients with primary sclerosing cholangitis and cholangiocarcinoma, respectively [18]. Although mutations of the p53 and K-ras genes are two of the more common events in human cancer, their relatively low incidence in cholangiocarcinoma makes these markers less than ideal for a screening test. Inactivation of the tumor suppressor gene p16 is a frequent event in cancers of the pancreas and biliary tract and has been demonstrated in preneoplastic duct lesions associated with pancreatic cancer [19–21]. However, the role of p16 in the development of biliary tract cancers in patients with primary sclerosing cholangitis has not been examined. The aim of the present study was to determine whether allelic loss at the p16 locus on chromosome 9p21 or p16 inactivation as determined by immunohistochemistry occurs with sufficient frequency to be a useful marker of malignancy in patients with PSC.

PATIENTS AND METHODS

Patient population.   One hundred thirty-nine patients with PSC were managed at The Johns Hopkins Hospital between January 1, 1984 and December 31, 1997. During this period, 26 patients with PSC had a biliary tract malignancy diagnosed either histologically or cytologically [1]. Pathological specimens from all 26 patients were reviewed, and sufficient material for genetic analysis was available from 10 patients [cholangiocarcinoma ($n = 9$) and small cell carcinoma of the gallbladder ($n = 1$)].

Mean age of the 10 patients was 43 ± 4 years, and 6 of the 10 patients were female. Seven of the 10 patients also had inflammatory bowel disease. A risk score was calculated for each patient at the time cholangiocarcinoma was diagnosed according to a multicenter survival model for PSC [22]. The mean risk score was 4.0 ± 0.4, and histological evidence of cirrhosis was present in four patients (40%). Two patients had unresectable tumors and had the diagnosis of malignancy made by percutaneous biopsy. Eight patients underwent laparotomy which included a biopsy ($n = 2$), resection of the extrahepatic biliary tree containing the tumor ($n = 3$), or orthotopic liver transplant (two unsuspected, one known cholangiocarcinoma).

Tissue samples.   Paraffin-embedded sections from 10 patients were used as a source of tumor and control DNA. Sections containing tumor were microdissected to enhance neoplastic cellularity to >50% of the sample as previously described [23]. Sections containing nonneoplastic tissue were used as a source of control DNA. Samples were digested in 1% SDS and proteinase K (0.5 mg/ml) overnight at 48°C followed by phenol/chloroform extraction and ethanol precipitation. Sections of each tumor mounted on lysine-coated glass slides were used for immunohistochemistry.

Microsatellite analysis.   Allelic loss at chromosome 9p21 was determined using seven highly polymorphic microsatellite markers. Oligonucleotides were obtained from Research Genetics (Huntsville, AL; D9S144, D9S157, IFN-α, D9S171, and D9S265) or synthesized by DNAgency (Malvern, PA) based on sequences in the Genome Database (D9S1748 and D9S1749). One marker from each primer pair was labeled with T4 polynucleotide kinase (New England Biolabs). Polymerase chain reaction (PCR) amplification was performed with 40 ng of DNA isolated from the tumor and nonneoplastic tissue as described previously. Products were separated in 8% denaturing urea–polyacrylamide–formamide gels and exposed to film. For infor-

mative cases, loss of heterozygosity (LOH) was scored if the intensity of one allele was decreased by 50% in the tumor when compared with the same allele in the control sample. Determination of homozygous deletion was based on the presence of one or markers demonstrating apparent retention flanked by markers showing clear LOH as previously described [24].

p16 methylation-specific PCR.   Methylation-specific PCR was used to determine the methylation status of a CpG island in the promoter region of the p16 gene in 8 of the 10 tumors [25]. Two hundred nanograms of tumor was modified with hydroquinone and sodium bisulfite at 50°C for 16 h. Modified DNA was then purified using the Wizard DNA purification system (Madison, WI), precipitated with ethanol, and resuspended in a Tris–EDTA buffer. The modified DNA was then amplified using both methylation- and unmethylation-specific primers as previously described. One primer pair recognizes a sequence in which CpG sites are unmethylated (bisulfite-modified to UpG) and the other pair recognizes a sequence in which CpG sites are methylated (unmodified by bisulfite). The primer sequences are localized to regions in and around the transcription start site of the p16 gene, a region shown to correlate with loss of gene expression. PCR products were directly loaded onto nondenaturing 6–8% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

p16 immunohistochemistry.   p16 immunohistochemistry was performed as previously described [24]. Seven-micrometer paraffin sections from each tumor were deparaffinized with xylene and rehydrated. Antigen retrieval was performed by microwaving deparaffinized sections for 10 min in 10 mM Citra buffer (BioGenex, San Ramon, CA). Sections were blocked with 3% hydrogen peroxide in methanol followed by 1.5% normal horse serum in 0.1% bovine serum albumin (BSA)/0.1 M phosphate-buffered saline (PBS). Slides were then incubated overnight at 4°C with the monoclonal p16 antibody, Ab-1 (Oncogene Research Products, Cambridge, MA), diluted 1:40 (2.5 μg/ml) in 0.1% BSA/0.1 M PBS. Immunolocalization was performed by reacting the sections with biotin-labeled anti-mouse IgG and then preformed avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA). After zinc-enhanced 3,3′-diaminobenizidine tetrahydrochloride substrate (Sigma, St. Louis, MO) was added to localize the p16 monoclonal antibody binding, slides were counterstained with hematoxylin. Sections incubated with normal horse serum were used as negative controls. Normal human oral mucosa served as positive controls. In addition, inflammatory and reactive stromal cells were used as internal positive controls. All sections were reviewed by a single pathologist (A.R.). Biliary tract tumors were considered negative for antibody detection only when none of the malignant cells expressed p16.

RESULTS

Pathological analysis.   Pathological characteristics of all 10 tumors are listed in Table 1. Tumor site was classified according to Nakeeb *et al.* [26]. Tumors were most common in the perihilar region ($n = 7$), but were also located in the gallbladder ($n = 2$) or intrahepatically ($n = 1$). Five of the ten tumors were localized to the wall of the bile duct (Stages I and II). Six of the eight operatively explored patients underwent conventional resection of their tumor or liver transplantation. Resection margins were microscopically free of tumor in three of these six patients.

Microsatellite analysis.   Allelic loss at chromosome 9p21 was determined using closely spaced microsatellite markers at the p16 locus (Table 1). Nine of the ten tumors (90%) demonstrated LOH with at least one

TABLE 1

Tumor Characteristics[a]

Patient	Age	Histological type	Site	TNM stage	9p21 allelic status	p16 methylation status	p16 IHC staining
CC1	44	CCA	Perihilar	I	LOH	Methylated	−
CC2	54	CCA	Perihilar	I	LOH	Unmethylated	ND
CC3	23	CCA	Perihilar	I	LOH	Methylated	−
CC4	26	CCA	Perihilar	II	LOH	Unmethylated	+
CC5	49	CCA	Perihilar	II	LOH	Unmethylated	−
CC6	36	CCA	Perihilar	III	LOH	Unmethylated	+
CC7	47	CCA	Perihilar	IVa	Retention	ND	ND
CC8	61	CCA	Intrahepatic	IVb	LOH	ND	−
CC9	49	CCA	Gallbladder	IVa	LOH	Unmethylated	ND
CC10	41	Small cell	Gallbladder	IVb	LOH	Unmethylated	+

[a] IHC, immunohistochemistry; CCA, cholangiocarcinoma; LOH, loss of heterozygosity; ND, not done.

marker at chromosome 9p21 (Fig. 1). LOH was present with all informative markers tested in 8 of 10 tumors (80%). LOH involving the p16 locus and more distal regions of chromosome 9p was observed in one tumor. Retention of both alleles at chromosome 9p21 was observed in only one tumor. No homozygous deletions were detected.

p16 methylation status. Methylation-specific PCR was used to determine the methylation status of the p16 promoter region in 8 of the 10 tumors (Table 1). Two of the eight tumors (25%) demonstrated methylation of the CpG promoter region. Methylation has been associated with transcriptional block of p16 in cell lines and correlates with absent p16 immunohistochemical staining in head and neck cancer [24, 25, 27].

p16 immunohistochemistry. Loss of p16 expression was determined in 7 of the 10 tumors using immunohistochemistry. Four of the seven tumors (57%) demonstrated complete absence of p16 nuclear staining with staining of the surrounding nonneoplastic tissue. All four of these tumors demonstrated LOH at chromosome 9p21. Two of these cases also demonstrated methylation of the p16 promoter region. Nuclear staining was present in the remaining three cases (each with LOH at chromosome 9p21). The remaining three tumors did not contain an adequate positive control and were not scored.

DISCUSSION

Cholangiocarcinoma occurs commonly in patients with PSC and is difficult to diagnose at an early, resectable stage [1–4, 28]. Biliary tract malignancies from 10 patients were analyzed for allelic loss at chromosome 9p21, methylation of the p16 promoter, and p16 inactivation by immunohistochemistry. Allelic loss at the p16 locus on chromosome 9p21 was present in 90% of the tumors. Loss of p16 expression was identified in 57% of the evaluable tumors.

Cholangiocarcinoma was initially reported as a frequent complication of PSC by Rosen *et al.* [2]. Although the reported incidence of cholangiocarcinoma in PSC varies considerably from 7 to 42% [1–6], a recent population-based study has reported an incidence of 11.2% during a 10-year period following the diagnosis of PSC [6]. The risk of cholangiocarcinoma does not correlate with the length or severity of PSC [1]. In fact, most patients diagnosed with cancer do not have cirrhosis and often have recently been diagnosed with PSC, suggesting that the earliest neoplastic changes often precede the clinical symptoms of PSC [1, 6]. Survival following the diagnosis of cholangiocarcinoma in patients with PSC has been dismal with the exception of those small cancers detected incidentally at the time of liver transplantation [1, 2, 7, 8].

The molecular events underlying the pathogenesis of cholangiocarcinoma are not well defined. Genetic alterations of K-ras, p16, and DPC-4 as well as p53 overexpression, have all been reported in cholangiocarcinoma [18, 20, 29–31]. Yoshida *et al.* recently sequenced the p16 gene in 25 biliary tract cancers including 13 cases of cholangiocarcinoma [20]. Missense mutations were identified in 6 of these 13 patients (46%). The frequency of p16 mutations was greater than the percentage of tumors containing a K-ras (8%), p53 (9%), or p15 (0%) mutation. Interestingly, allelic loss at chromosome 9p21 was identified in only one of these 13 tumors using microsatellite analysis, albeit with only two markers outside of the p16 locus. Genetic events occurring in precursor lesions to cholangiocarcinoma are even less well understood. K-ras mutations have been detected in atypical biliary epithelium associated with anomalous pancreatobiliary duct junction and congenital biliary dilation [32, 33]. Moreover, Hruban *et al.* have identified K-ras mutations in 7% of benign bile duct proliferations identified on liver biopsy, suggesting that K-ras mutations may be an early event
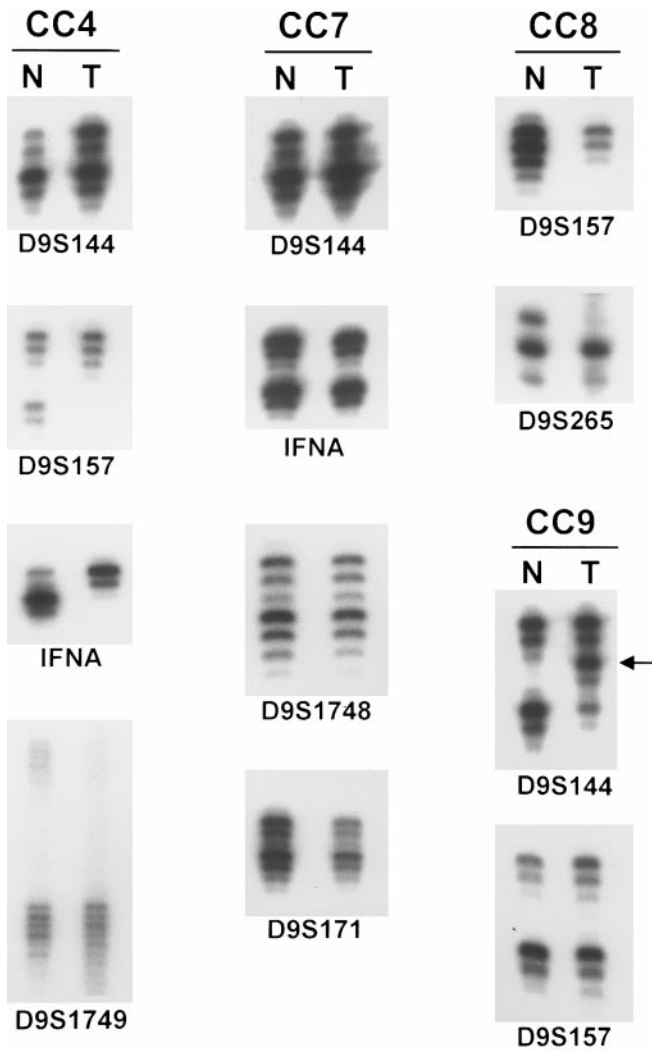
FIG. 1. Microsatellite analysis at chromosome 9p21 in cholangiocarcinoma. Sample numbers are indicated at the top, and microsatellite loci are indicated at the bottom. Allelic loss of the entire region analyzed was present in samples CC4, CC8, and CC9. A novel allele was present in sample CC9 with D9S144 (arrow). Retention of chromosome 9p21 was present in sample CC7. N, normal; T, tumor.

nearby, p15 and p16β, which is an alternative transcript to p16 [27]. The third mechanism of p16 inactivation involves methylation of 5′ regulatory regions, or discrete regions of CG dinucleotides called CpG islands [25]. Evidence is mounting that methylation of critical regulatory genes may play an important role in tumorigenesis.

Allelic loss at chromosome 9p21 and inactivation of the p16 gene occur frequently in many human cancers including non-small cell lung cancer, head and neck squamous cell cancer, and pancreatic cancer and are also present in precursor lesions associated with these tumors [19, 21, 24, 36–38]. Reed *et al.* have demonstrated inactivation of the p16 gene in 83% of patients with squamous cell cancer of the head and neck using immunohistochemistry and genetic analysis [24]. Inactivation of the p16 gene determined by immunohistochemistry has correlated strongly with genetic analysis of p16, supporting the use of this technique [24]. Immunohistochemistry is particularly useful when limited tissue is available for genetic analysis as in the case with percutaneous fine-needle aspirates or endoscopic brushings.

The p16 gene is also inactivated in more than 95% of invasive pancreatic cancers [19]. Wilentz *et al.* recently demonstrated an increasing incidence of p16 inactivation with the histological progression of precursor pancreatic ductal lesions [21]. p16 was inactivated in 30% of 30 flat duct lesions, 27% of 15 papillary lesions without atypia, 55% of 67 papillary lesions with atypia, and 71% of the 14 lesions classified as carcinoma *in situ.* The high frequency of allelic loss at 9p21 and p16 inactivation (90 and 71%, respectively) in patients with primary sclerosing cholangitis-associated cholangiocarcinoma suggests that these genetic changes might also be useful early markers of neoplasia in patients with primary sclerosing cholangitis.

SUMMARY

Allelic loss at chromosome 9p21 and p16 inactivation are the most frequent genetic events identified to date in primary sclerosing cholangitis-associated cholangiocarcinoma. Identification of 9p21 loss or p16 inactivation in patients with primary sclerosing cholangitis may identify patients at high risk of developing cholangiocarcinoma. Further studies characterizing the genetic changes present in premalignant biliary epithelium from patients with primary sclerosing cholangitis are warranted.

in the progression of cholangiocarcinoma [34]. However, the role of p16 in the early neoplastic changes associated with cholangiocarcinoma is unknown.

The tumor suppressor gene p16 is frequently inactivated in a wide variety of human cancers [19, 24, 27]. Unlike other tumor suppressor genes that are predominantly inactivated by point mutations, p16 is inactivated by at least three distinct mechanisms [27]. p16 is inactivated by point mutation or deletion in many familial atypical multiple mole/melanoma kindreds. However, this mechanism is fairly infrequent in most tumor types with the exception of cancers of the pancreas (40%) and biliary tract (46%) [19, 20]. A second mechanism of inactivation involves small (<200-kb) deletions of both p16 alleles [35]. Deletions at this locus often include at least two other potential targets

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