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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.

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,