Comparison of Epigenetic and Genetic Alterations in Mucinous Cystic Neoplasm and Serous Microcystic Adenoma of Pancreas

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Mucinous cystic neoplasms and serous microcystic adenomas account for the majority of cystic tumors of pancreas. Mucinous cystic neoplasms and serous microcystic adenomas have different frequencies of progression to malignancy. The genetic and epigenetic alterations of these tumors have not been studied in detail. In this study, we compared methylation status of *p16*, *p14*, *VHL*, and *ppENK* genes by methylation-specific PCR (MSP), and genetic alterations including *K-ras* and *β-catenin* gene mutations, chromosome 3p loss, and microsatellite instability in 15 mucinous cystic neoplasms (10 benign and 5 borderline) and 16 serous microcystic adenomas. There were no significant differences between mucinous cystic neoplasms and serous microcystic adenomas in methylation of *p16* (14%, 2/14 and 12%, 2/16), *p14* (15%, 2/13 and 37%, 6/16), *VHL* (0/14 and 7%, 1/14), and *ppENK* (0/14 and 0/13), respectively. *K-ras* mutation was present only in mucinous cystic neoplasms but not in serous microcystic adenomas (33%, 5/15 *versus* 0/16; $P = .004$). In addition, LOH at 3p25, the chromosomal location of *VHL* gene, was present in 57% (8/14) of serous microcystic adenomas compared with in 17% (2/12) of mucinous cystic neoplasms ($P = .03$). No *β-catenin* mutation, microsatellite instability, or mutation of *transforming growth factor β type II receptor* was present in either type of tumors. In conclusion, *K-ras* mutations and allelic loss of VHL locus at 3p25, but not methylation, distinguished mucinous cystic neoplasms and serous microcystic adenomas. The differences in genetic alterations but not epigenetic alterations may explain the pathogenesis and progression to malignancy of these cystic tumors of pancreas.

KEY WORDS: Genetic alterations, Methylation, Mucinous cystic neoplasm, Pancreas, Serous microcystic adenoma.

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Cystic tumors of the pancreas are relatively uncommon, accounting for less than 5% of pancreas exocrine tumors (1–5). Benign and borderline mucinous cystic neoplasms (ICD-O 8470/0 and ICD-O 8470/1) and serous microcystic adenomas (ICD-O 8441/0) account for most of the cystic tumors (2–4). These two tumors have differences in progression to malignancy and clinicopathological features (6). Mucinous cystic neoplasms are classified as benign, borderline (uncertain malignant potential), carcinoma *in situ*, and invasive carcinoma based on the severity of dysplasia of the lining epithelium or presence of stromal invasion (2–4, 7). In contrast, almost all serous microcystic adenomas are benign and rarely progress to malignancy (4). Distinguishing between these tumor types is clinically challenging but important because mucinous cystic neoplasms are more prone to recurrence if incompletely excised and more likely to become malignant.

There are differences in the genetic alterations in these cystic tumors. Activating mutations of *K-ras* are reported in both benign and malignant mucinous cystic neoplasms (8–12), but mutations of *K-ras* proto-oncogene and alterations of *p53*, *p16* and *DPC4* tumor suppressor genes are more frequently present in invasive mucinous cystic neoplasms (9, 11–13), suggesting stepwise increase in

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genetic alterations in malignant tumors. In contrast, these genetic alterations are not present in serous microcystic adenomas (8–11, 14), which can occur as a manifestation of von-Hippel-Lindau disease due to germline mutations of the *VHL* gene (15–17). In addition, sporadic serous microcystic adenomas frequently have allelic loss of chromosome 3p25 and infrequently, somatic *VHL* gene mutations (15, 18).

Other molecular abnormalities have been reported in various pancreatic neoplasms. Aberrant methylation of CpG islands is a molecular mechanism that leads to silencing of tumor suppressor genes such as *p16* and *p14* in a variety of cancers and precursor lesions (19–22). Recently, methylation of the *ppENK* gene encoding met-enkephalin was found to be frequent in ductal adenocarcinoma (23) and intraductal papillary-mucinous neoplasm of pancreas (24). Mutations in exon 3 of the *β-catenin* gene, involving the sites phosphorylated by GSK-3 β for ubiquitination and degradation of *β-catenin,* stabilize *β-catenin* protein and in turn up-regulate the Tcf/Lef-1 family of transcription factors. *β-catenin* mutations have also been reported in diverse types of pancreatic carcinomas including pancreatoblastomas, pancreatic acinar cell carcinomas, and solid pseudopapillary carcinomas (25–28). Microsatellite instability due to alterations of mismatch repair genes such as *human Mut L homologue 1 (hMLH1)* and *human Mut S homologue 2 (hMSH2)* is present in medullary carcinomas of the pancreas (28, 29).

Methylation, *β-catenin* gene mutations, or microsatellite instability have not been studied in either mucinous cystic neoplasms or serous microcystic adenomas, and their roles in the pathogenesis of the two distinct pancreatic cystic tumors are unclear. In this study, therefore, we compared aberrant methylation of the *p16*, *p14, ppENK,* and *VHL* gene and genetic alterations including *K-ras* and *β-catenin* mutations, loss of chromosome 3p25, and microsatellite instability in mucinous cystic neoplasms and sporadic serous microcystic adenomas.

MATERIALS AND METHODS

Patients and Specimens

Fifteen pancreatic mucinous cystic neoplasms (ICD-O 8470/0 or ICD-O 8470/1) and 16 sporadic serous microcystic adenomas (ICD-O 8441/0) were identified from the surgical pathology files of the University of Texas M. D. Anderson Cancer Center (11 mucinous cystic neoplasms and 14 serous microcystic adenomas) and Kyung Pook National University Hospital, South Korea (4 mucinous cystic neoplasms and 2 serous microcystic adenomas) from patients undergoing resection between 1991 and 2001. Formalin-fixed, paraffin-embedded tumor and non-neoplastic tissue specimens were collected. The histopathology of the tumors was reviewed (Fig. 1), and mucinous cystic neoplasms were further classified as benign (ICD-O 8470/0) and borderline (uncertain malignant potential, ICD-O 8470/1), as previously described (2–4). No mucinous cystic neoplasms with *in situ* carcinoma or invasive carcinoma were included in this study. Matching non-neoplastic pancreatic tissue was available from 13 patients with mucinous cystic neoplasms and 15 patients with serous microcystic adenomas. The study was approved by the Surveillance Committee (institutional review board) of the University of Texas M. D. Anderson Cancer Center.

DNA Extraction

Histologic sections were microdissected to obtain >70% neoplastic cellularity. DNA was extracted from microdissected tumors and non-neoplastic tissues as described previously (30).

Bisulfite Treatment of DNA and Methylation-Specific PCR

Bisulfite treatment of DNA for methylation assays was carried out as described previously (31, 32). Methylation of the *p16, p14, ppENK,* and *VHL* gene was determined by MSP as previously described (19, 21, 23, 33). Briefly, 2 μL of bisulfite-treated DNA was used as template for PCR reactions using primers specific for methylated and unmethylated alleles (Table 1). PCR products from methylated and unmethylated reactions were electrophoresed on 6% nondenaturing acrylamide gels. The gels were stained with ethidium bromide, and the proportion of methylated allele compared with unmethylated allele was determined by densitometry. The DNA of colon cancer cell line RKO or *Sss*I methylase-treated DNA (New England Biolabs, Beverly, MA) was used as positive control, and water, as negative control.

Sequencing of Exon 1 of *K-ras* and Exon 3 of *β-Catenin* Genes

Exon 1 of the *K-ras* proto-oncogene and exon 3 of the *β-catenin* gene were amplified by PCR and sequenced as described previously (34, 35). DNA sequencing was performed on the PCR product and amplification primers for each exon with a commercial DNA sequencing kit, according to the manufacturer's instructions (Applied Biosystems, Foster City, CA).

Loss of Heterozygosity of chromosome 3p25

Loss of heterozygosity (LOH) of chromosome 3p25 was determined by use of three dinucleotide

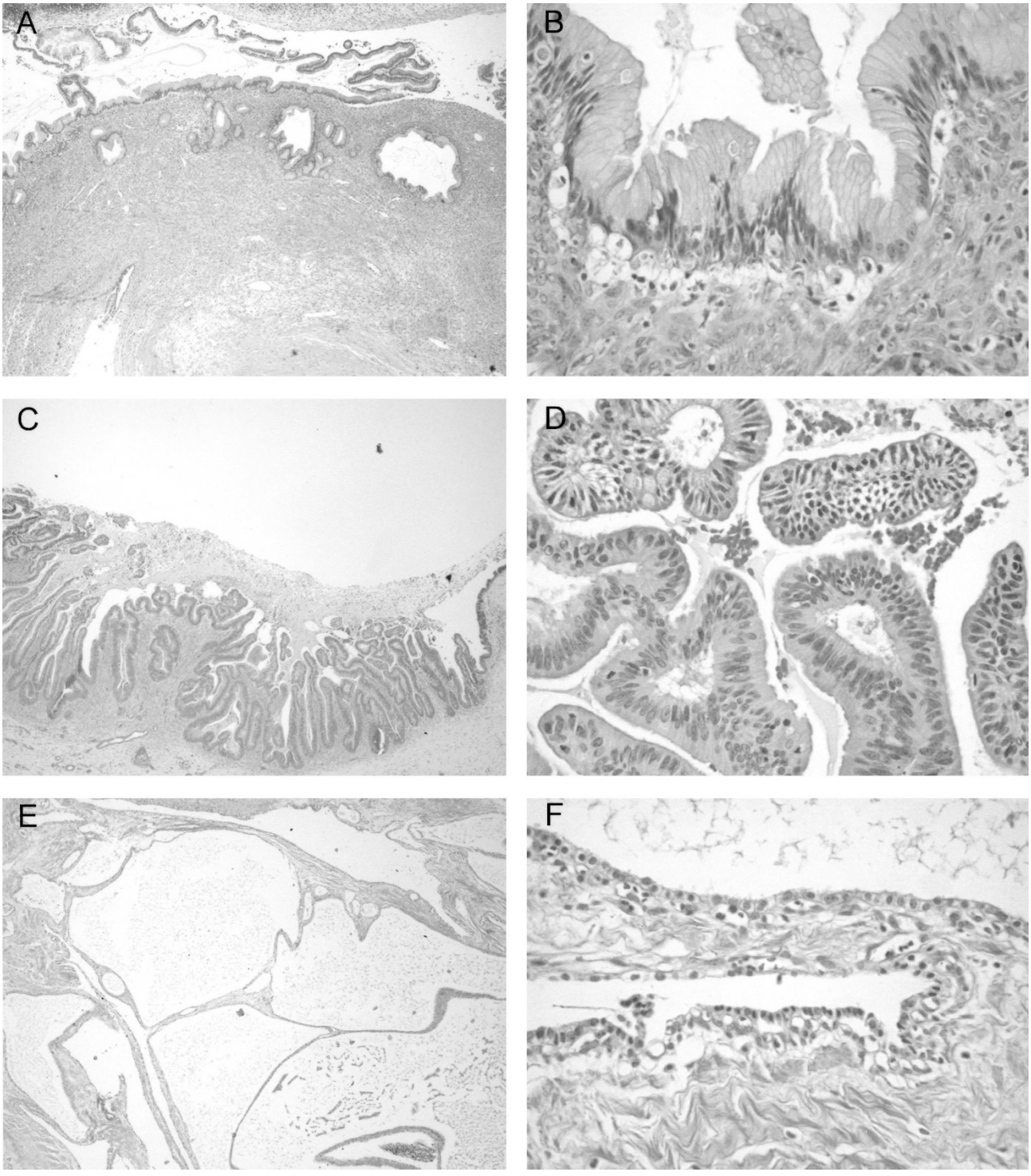

FIGURE 1. Histology of benign mucinous cystic neoplasm (**A–B**), borderline mucinous cystic neoplasm (**C–D**), and serous microcystic adenoma (**E–F**).

microsatellite markers near the *VHL* gene on chromosome 3 (D3S1110, D3S1038, and D3S1435) with PCR amplification using fluorescent dye–labeled primers (Invitrogen, Carlsbad, CA). The 5'oligonucleotide was end-labeled with 6-FAM fluorescent dye. PCR was performed in 15-μL reaction volumes containing 20 ng of DNA, 9 μL of ABI Prism True Allele PCR Premix (Applied Biosystems, Foster City, CA), and 5 pmol of each primer. PCR was performed using a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Foster City, CA) with the following conditions: 95° C for 7 minutes; 3 cycles of 94° C for 1 minute, 58° C for 30 seconds, and 72° C for 45 seconds; 42 cycles of 93° C for 45 seconds, 54° C for 30 seconds, 72° C for 40 seconds; and final extension at 72° C for 30 minutes. A

TABLE 1. Primers and PCR Condition for Methylation-Specific PCR for *p16, p14, ppENK,* and *VHL* Genes

	Primer Sequence	Annealing Temperature, Cycles	Function of the Gene Product
p16	UF 5′-TTATTAGAGGGTGGGGTGGATTGT-3′ UR 5′-CAACCCCAAACCACAACCATAA-3′ MF 5′-TTATTAGAGGGTGGGGCGGATCGC-3′ MR 5′-GACCCCGAACCGCGACCGTAA-3′	60°C, 40 65 °C, 40	Cell cycle regulator, binds to CDK4 and CDK6, inhibiting phosphorylation of retinoblastoma protein
p14	UF 5′-TTTTTGGTGTTAAAGGGTGGTGTAGT-3′ UR 5′-CACAAAAACCCTCACTCACAACAA-3′ MF 5′-GTGTTAAAGGGCGGCGTAGC-3′ MR 5′-AAAACCCTCACTCGCGACGA-3′	60°C, 40	Cell cycle regulator, binds to human double minute2 (HDM2) gene product and prevents ubiquitin mediated degradation of p53
ppENK	UF 5′-TTGTGTGGGGAGTTATTGAGT-3′ UR 5′-CACCTTCACAAAAAAAATCAATC-3′ MF 5′-TGTGGGGAGTTATCGAGC-3′ MR 5′-GCCTTCGCGAAAAAAATCG-3′	62°C, 40	Encodes Met-enkephalin, which inhibits cell proliferation
VHL	UF 5′-GTTGGAGGATTTTTTTGTGTATGT-3′ UR 5′-GTTGGAGGATTTTTTTGTGTATGT-3′ MF 5′-GTTGGAGGATTTTTTTGTGTATGT-3′ MR 5′-GTTGGAGGATTTTTTTGTGTATGT-3′	60°C, 40	VHL protein ubiquitinates and degrades HIF-1 transcription factor, constitutively activated HIF-1 produces VHL disease associated tumors

Unmethylated forward, UF; unmethylated reverse, UR; methylated forward, MF; methylated reverse, MR.

0.25-μL aliquot of each fluorescent-labeled PCR product was combined with 12 μL of formamide and 0.5 μL of GENESCAN 400HD [ROX] size standard (Applied Biosystems). The samples were then subjected to capillary electrophoresis on an ABI 3700 DNA Analyzer using GENESCAN Analysis software (Applied Biosystems). Loss of a marker was considered to be present when the assay showed absence or decrease in intensity by >50% of one of two alleles from a tumor sample as compared with the paired control non-neoplastic tissue. Complete or partial loss of chromosome 3p25 was based on the pattern of loss of the three markers.

Microsatellite Instability and Alteration of the Transforming Growth Factor β Type II Receptor Gene

Microsatellite instability was evaluated by amplification of two mononucleotides repeats (BAT25 and BAT26) and three dinucleotide repeats (D2S123, D5S346, D17S250), as proposed by the NCI workshop (36), and alterations of the polyadenine tract of the *transforming growth factor β type II receptor* (*TGFβRII*) gene were evaluated using fluorescent dye-labeled primers as described previously (37). Allelic shift of a microsatellite marker was defined by the presence of at least one additional band in the tumor DNA as compared with in control DNA

Statistical Analysis

The differences in frequencies among clinicopathological factors and epigenetic and genetic alterations were analyzed by χ^2 or Fisher's exact test. All statistical analyses were performed using the SPSS statistical program (SPSS Inc., Chicago, IL).

RESULTS

Clinicopathological Characteristics of Patients and Tumors

The clinicopathological characteristics of the patients and their tumors are summarized in Table 2. Mucinous cystic neoplasms were located mainly in the pancreatic body (80%), whereas serous microcystic adenomas were evenly distributed among the head, body, and tail (44% in head, $P = .002$). Thirty-

TABLE 2. Clinicopathological Features of Mucinous Cystic Neoplasms and Serous Microcystic Adenomas

	Mucinous cystic neoplasms (n = 15) % (fraction)	Serous microcystic adenomas (n = 16) % (fraction)	*P* Value
Age (years ± SD)	51.1 ± 17.6	56.9 ± 11.8	NS
Sex (% female)	87 (13/15)	75 (12/16)	NS
Size (cm ± SD)	4.7 ± 3.4	5.6 ± 3.7	NS
Site			
Head	0 (0)	44 (7/16)	0.002
Body	80 (12/15)	31 (5/16)	
Tail	20 (3/15)	25 (4/16)	
Histology			
Benign	67 (10/15)	100 (16/16)	0.02
Borderline	33 (5/15)	0 (0)	

[a] NS, not significant.

three percent of mucinous cystic neoplasms were classified as borderline with uncertain malignant potential, but all serous microcystic adenomas were classified as benign ($P = .02$).

Aberrant Methylation of p16, p14, ppENK, and VHL Genes

Examples of methylation are shown in Figure 2. One control pancreatic tissue adjoining a serous microcystic adenoma was methylated at *p14*, but no other control pancreatic tissues had methylation of the *p14*, *p16*, *ppENK*, or *VHL* genes.

Methylation of *p16* and *p14* was present in 14% (2/14) and 15% (2/13), respectively, of mucinous cystic neoplasms, and in 12% (2/16) and 37% (6/16) of serous microcystic adenomas (P = not significant, Table 3). Methylation of *VHL* was present in 7% (1/14) of serous microcystic adenomas but was not found in mucinous cystic neoplasms (0/14). No methylation of *ppENK* was present in either 14 mucinous cystic neoplasms or 13 serous microcystic adenomas. The frequencies of methylation of *p16*, *p14*, and *VHL* were not different between mucinous cystic neoplasms and serous microcystic adenomas or between benign and borderline mucinous cystic neoplasms. The methylation of *p16* and *p14* was not concordant in either mucinous cystic neoplasms or serous microcystic adenomas.

Genetic Alterations

Mutation in exon 1 of the *K-ras* gene was present in 33% (5/15) of mucinous cystic neoplasms, but no mutations were found in serous microcystic adenomas (0/16, $P = .004$, Fig. 3A, Table 3). Four *K-ras* mutations were present in codon 12 and one in codon 13. There were no significant differences in mutation frequency between benign and borderline mucinous cystic neoplasms.

LOH of chromosome 3p25 was present in 57% (8/14) of serous microcystic adenomas compared with only 17% (2/12) of mucinous cystic neoplasms ($P = .03$, Fig. 3B, Table 3). Allelic loss of chromosome 3p25 was more common in mucinous cystic neoplasms with *K-ras* mutations than tumors without *K-ras* mutations (40%, 2/5 *versus* 0/7, $P = .04$) but was not associated with other clinicopathological characteristics in either type of cystic tumors.

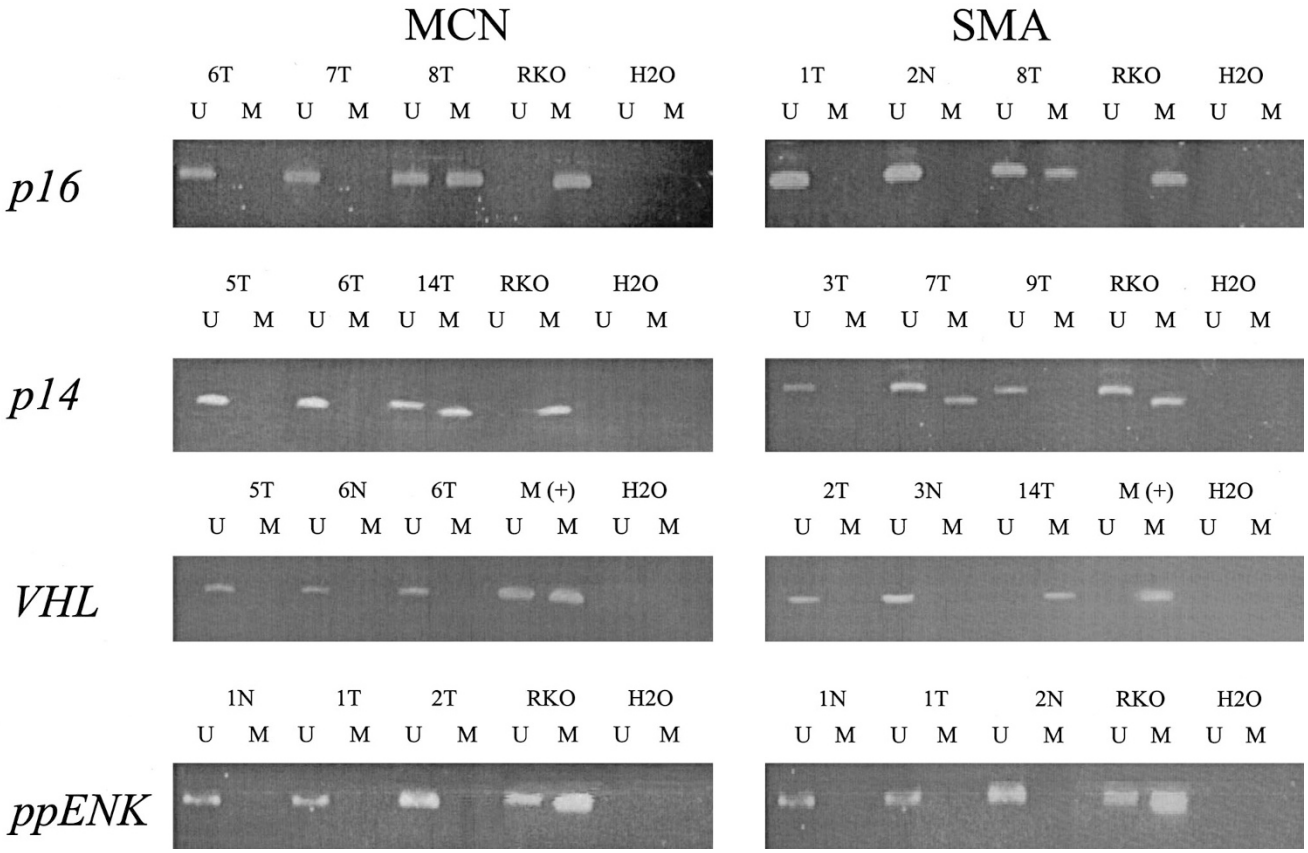

FIGURE 2. Methylation analysis of *p16*, *p14*, *ppENK*, and *VHL* genes by methylation-specific PCR. PCR products using primers for unmethylated (U) and methylated (M) alleles of bisulfite-treated DNA from tumor (T) and positive and negative control samples. MCN, mucinous cystic neoplasm; SMA, serous microcystic adenoma; RKO, positive control from colon cancer cell line; M(+), positive control DNA treated with Sss1 methylase; H_2O, lanes without DNA used as negative control. MCN 8T and SMA 8T are methylated at *p16*, MCN 14T and SMA 7T at *p14*, and SMA 14T at *VHL* genes. All other samples shown in the gels are unmethylated.

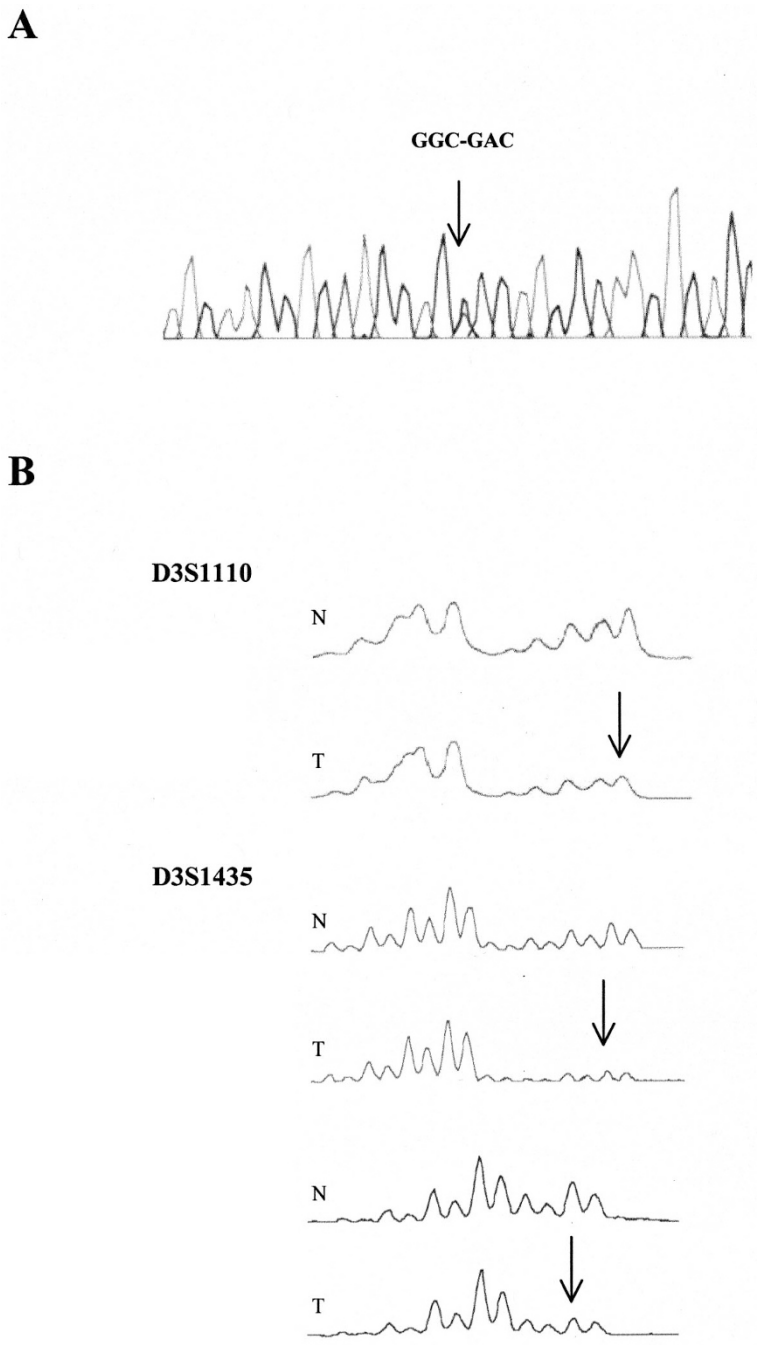

FIGURE 3. A, nucleotide sequencing of *K-ras* gene in mucinous cystic neoplasm. The mutation is indicated by an **arrow**. The wild-type and mutated nucleotide sequences are shown above the **arrow. B**, allelic loss of chromosome 3p in mucinous cystic neoplasm and serous microcystic adenomas using dinucleotide microsatellite markers (D31110 and D31435). The lanes from normal DNA (N) have two alleles and the lanes from tumor DNA (T) show loss of one allele, indicated by arrows.

No mutations in exon 3 of *β-catenin*, microsatellite instability, or alterations of *TGFβRII* were present in mucinous cystic neoplasms or serous microcystic adenomas (Table 3).

DISCUSSION

Epigenetic alterations have not been studied previously in mucinous cystic neoplasms (benign and borderline) or sporadic serous microcystic adenomas. In our study, we found infrequent methylation of *p16* and *p14* in mucinous cystic neoplasms and serous microcystic adenomas, rare methylation of *VHL* gene in serous microcystic adenomas, and no methylation of *ppENK* gene in either type of cystic tumor. These findings suggest that silencing of these genes by methylation may play a role in the tumorigenesis of some mucinous cystic neoplasms

TABLE 3. The Frequencies of Epigenetic and Genetic Alterations in Mucinous Cystic Neoplasms and Serous Microcystic Adenomas of Pancreas

		Mucinous Cystic Neoplasms			Serous Microcystic Adenomas (n = 16) % (fraction)	*P* Value
		Benign (n = 10) % (fraction)	Borderline (n = 5) % (fraction)	Total (n = 15) % (fraction)		
Methylation	p16	10 (1/10)	25 (1/4)	14 (2/14)	12 (2/16)	NS
	p14	22 (2/9)	0 (0/4)	15 (2/13)	37 (6/16)	NS
	VHL	0 (0/9)	0 (0/5)	0 (0/14)	7 (1/14)	NS
	PpENK	0 (0/9)	0 (0/5)	0 (0/14)	0 (0/13)	NS
Genetic alterations	K-ras	30 (3/10)	40 (2/5)	33 (5/15)[a]	0 (0/16)[a]	0.004[a]
	3p25 allelic loss	20 (2/10)	0 (0/2)	17 (2/12)[a]	57 (8/14)[a]	0.03[a]
	β-catenin	0 (0/10)	0 (0/5)	0 (0/15)	0 (0/16)	NS
	Microsatellite instability	0 (0/10)	0 (0/5)	0 (0/15)	0 (0/16)	NS
	TGFβRII	0 (0/10)	0 (0/5)	0 (0/15)	0 (0/16)	

NS, not significant.
[a] Mucinous cystic neoplasms versus serous microcystic adenomas.

and serous microcystic adenomas. However, there is no distinct methylation profile that can distinguish these two cystic tumor types.

The Rb/p16 pathway is frequently inactivated in pancreatic carcinogenesis by homozygous/heterozygous deletions, mutations, and/or methylation (38). In our study, the frequency of methylation of the *p16* gene in mucinous cystic neoplasms and serous microcystic adenomas is similar to the reported frequencies of its methylation in pancreatic ductal adenocarcinomas, intraductal papillary-mucinous neoplasm with carcinoma *in situ* or invasive carcinoma, and high-grade pancreatic intraepithelial neoplasia (23, 24, 39). In contrast, previous studies of benign and borderline intraductal papillary-mucinous neoplasms and serous microcystic adenomas reported no methylation of *p16* gene but more frequent methylation of *p16* gene in mucinous cystic neoplasms with invasive carcinoma (11, 23). $p14^{ARF}$ binds to human double minute 2 (HDM2) gene product to prevent HDM2 ubiquitin–mediated degradation of p53 (40). Methylation of *p14* has been reported in various cancers and in colitis-associated dysplasia and neoplasia in ulcerative colitis patients (21, 22), but we report for the first time *p14* methylation in pancreatic neoplasms.

Recently, frequent tumor-specific methylation of *ppENK,* which encodes met-enkephalin, has been reported in pancreatic ductal adenocarcinoma, intraductal papillary-mucinous neoplasm, and pancreatic ductal intraepithelial neoplasia (23, 24). Met-enkephalin is a growth inhibitory factor and decreases DNA synthesis, mitosis, and growth of cells (41). However, in the present study, no methylation of *ppENK* was present in either type of pancreatic cystic tumors. Thus, methylation of *ppENK* appear to be a selective event in pancreatic adenocarcinomas and intraductal papillary-mucinous neoplasms and may not play any role in the pathogenesis of MCNs and serous microcystic adenomas.

The complex of VHL protein, elongin C, elongin B, and CLU2 enzymatically adds ubiquitin to HIF-1 transcription factor, which is degraded by proteosomal complex. Impaired binding of VHL protein to elongin C and HIF-1 due to mutation, or lack of VHL protein due to deletion or methylation, fails to ubiquitinate HIF-1. This alteration constitutively activates HIF-1 that promotes VHL disease-associated tumors (42). Syndromic serous microcystic adenomas are due to germline mutations of the *VHL* gene, and sporadic serous microcystic adenomas have somatic alterations of the *VHL* gene including deletion and mutations (17, 18). In our study, we found more frequent loss of chromosome 3p25 in serous microcystic adenomas compared with mucinous cystic neoplasms. Methylation of the *VHL* gene was reported in sporadic and VHL disease-associated tumors such as renal cell carcinoma and hemangioblastoma (43). In the present study, methylation of the *VHL* gene was detected in only one serous microcystic adenoma, indicating that silencing of the *VHL* gene by methylation has only a minor role in sporadic serous microcystic adenomas.

In our study, *K-ras* mutations were present in mucinous cystic neoplasms but not serous microcystic adenomas, and the frequency of *K-ras* mutations was similar between benign and borderline subtypes of mucinous cystic neoplasms. Our finding corroborates results of previous studies that have shown less frequent *K-ras* mutations in benign and borderline subtypes of mucinous cystic neoplasms (9, 10, 12). In contrast, the frequency of K-ras mutations is more common in mucinous cystic neoplasms with *in situ* carcinoma or invasive carcinoma (9, 10–13). Our study and the previous studies (9, 11–13) support the concept that there is a progressive increase in the frequencies of the genetic alterations, including *K-ras* mutation, and *p53*, *p16*, and *Dpc4* gene alterations in mucinous cystic tumors with histological progression: the ge-

netic alterations are more common in mucinous cystic tumors with *in situ* carcinoma or invasive carcinoma than in benign and borderline subtypes.

We found that mucinous cystic neoplasms and serous microcystic adenomas lacked *β-catenin* mutation or microsatellite instability, in contrast to pancreatoblastomas, pancreatic acinar cell carcinomas, and solid-pseudopapillary carcinomas that have *β-catenin* mutation (25–27) and medullary subtypes of pancreatic carcinomas that have microsatellite instability (28).

It is important to distinguish mucinous cystic neoplasms from serous microcystic adenomas preoperatively because the former are more prone to recurrence if incompletely resected and can transform into invasive carcinoma. There is no distinctive methylation profile based on the limited number of CpG islands analyzed in the current study, but these two cystic tumor types have distinct histology and can be easily distinguished. Evaluation of *K-ras* gene mutation and chromosome 3p loss in aspiration biopsy specimens may be of help to distinguish mucinous cystic neoplasms from serous microcystic adenomas because of different frequencies in the tumor types: *K-ras* mutation favors mucinous cystic neoplasms, and 3p25 allelic loss without *K-ras* mutation favors serous cystic adenoma.

In conclusion, distinct genetic alterations rather than epigenetic alterations may account for the different pathogenesis of these two types of pancreatic cystic tumors. These findings may have diagnostic implications.

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