

Whole-exome sequencing of neoplastic cysts of the pancreas reveals recurrent mutations in components of ubiquitin-dependent pathways

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More than 2% of adults harbor a pancreatic cyst, a subset of which progresses to invasive lesions with lethal consequences. To assess the genomic landscapes of neoplastic cysts of the pancreas, we determined the exomic sequences of DNA from the neoplastic epithelium of eight surgically resected cysts of each of the major neoplastic cyst types: serous cystadenomas (SCAs), intraductal papillary mucinous neoplasms (IPMNs), mucinous cystic neoplasms (MCNs), and solid pseudopapillary neoplasms (SPNs). SPNs are low-grade malignancies, and IPMNs and MCNs, but not SCAs, have the capacity to progress to cancer. We found that SCAs, IPMNs, MCNs, and SPNs contained 10 ± 4.6 , 27 ± 12 , 16 ± 7.6 , and 2.9 ± 2.1 somatic mutations per tumor, respectively. Among the mutations identified, E3 ubiquitin ligase components were of particular note. Four of the eight SCAs contained mutations of the von Hippel-Lindau gene (*VHL*), a key component of the *VHL* ubiquitin ligase complex that has previously been associated with renal cell carcinomas, SCAs, and other neoplasms. Six of the eight IPMNs and three of the eight MCNs harbored mutations of *RNF43*, a gene coding for a protein with intrinsic E3 ubiquitin ligase activity that has not previously been found to be genetically altered in any human cancer. The preponderance of inactivating mutations in *RNF43* unequivocally establish it as a suppressor of both IPMNs and MCNs. SPNs contained remarkably few genetic alterations but always contained mutations of *CTNMB1*, previously demonstrated to inhibit degradation of the encoded protein (β -catenin) by E3 ubiquitin ligases. These results highlight the essential role of ubiquitin ligases in these neoplasms and have important implications for the diagnosis and treatment of patients with cystic tumors.

As the result of the increasing use of abdominal imaging in standard medical practice, pancreatic cysts are being identified with increasing frequency. Management of these cysts is concomitantly becoming a major clinical problem (1, 2). Cystic lesions occur in more than 20% of patients examined at autopsy (3), in as many as 19.6% of patients evaluated by MRI (4–6), and in as many as 2.6% of patients evaluated by computed tomography (7, 8). In the vast majority of cases, the cysts are identified as incidental findings in patients undergoing imaging for symptoms unrelated to pancreatic pathology. However, once a cyst is identified, it poses a challenging life-long management problem (1, 2, 9–13). Some cyst types are virtually always benign, some are low-grade malignant, and others are precursors to invasive pancreatic ductal adenocarcinomas (PDAs); PDAs are associated with a dismal prognosis (14–17). The distinction among cyst types is therefore critical for the effective management of patients with pancreatic cysts. Unfortunately, it is often difficult to determine the type of cyst from conventional clinical, radiographic, or cytologic findings (1, 2, 9–17).

Approximately 40% of cysts are nonneoplastic “pseudocysts” that develop as a complication of alcoholic, biliary, or traumatic acute pancreatitis (14–17). They are managed medically or by surgical drainage without resection. The neoplastic cysts (60% of the total cysts) are predominantly of four types: intraductal papillary mucinous neoplasms (IPMNs), mucinous cystic neoplasms (MCNs), serous cystadenomas (SCAs), and solid pseudopapillary neoplasms (SPNs) (18). SCAs, IPMNs, and MCNs are benign (i.e., noninvasive), but IPMNs and MCNs have the potential to progress to PDAs (i.e., become invasive lesions) if not surgically excised (17). Based on the age of patients undergoing surgical resection, some reports have suggested that there is a 5-y lag time from diagnosis of a large noninvasive IPMN (average age of 63.2 y) to diagnosis of an invasive cancer stemming from the IPMN (average age of 68.1 y). This provides a broad time window for curative resection if premalignant cysts are accurately identified (19). SPNs are regarded as low-grade malignant, but they can be cured by surgery if they are detected and removed before their widespread metastasis (20).

IPMNs are the most common type of neoplastic cyst, accounting for ~25–35% of the total cysts, whereas SCAs, MCNs, and SPNs account for ~20%, ~10%, and ~5% of pancreatic cysts, respectively. SCAs (Fig. 1A) are lined by cuboidal glycogen-rich epithelium with centrally placed round nuclei without atypia (17). The epithelium of SCAs is associated with a rich capillary network (21). IPMNs (Fig. 1B) arise within the normal ductal system and are lined by columnar mucin-producing cells that often form large papillary projections into ductal lumina (17). MCNs (Fig. 1C) are also lined by columnar mucin-producing cells, but in contrast to IPMNs, the neoplastic epithelium is associated with a characteristic ovarian-type stroma and the cysts do not communicate with the ductal system (17). MCNs nearly always occur in the body or tail of the pancreas in women, whereas IPMNs and SCAs can occur in any part of the pancreas and in both sexes. SPNs (Fig. 1E and F) are technically solid tumors, but the vast majority of them undergo cystic degeneration that clinically and radiographically

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mimics the other types of pancreatic cystic neoplasms (20). Like MCNs, they generally occur in women and do not communicate with the ductal system. Histologically, SPNs consist of uniform poorly cohesive cells supported by delicate small blood vessels. The neoplastic cells of SPNs do not have a normal counterpart in the normal pancreas.

To date, a definitive diagnosis of neoplastic cyst type can usually only be obtained following histopathological examination of surgically obtained specimens. The decision to resect pancreatic cysts surgically is based on the presumed type of cyst along with clinical parameters. Resection is performed on all cysts that are presumed to be SPNs. In contrast, cysts diagnosed as SCAs only require resection if they cause symptoms. Finally, patients with presumptive MCNs or IPMNs undergo surgery if they meet certain criteria, such as rapid growth or the presence of a mural nodule (1, 2, 9–13). The preoperative diagnosis of surgically excised cysts has been shown to be erroneous in one-third of cases and errors can lead to unnecessary surgical procedures (22). For example, there is no need to excise small asymptomatic SCAs, because they have essentially no malignant potential (17). However, SCAs are sometimes suspected to be IPMNs and therefore surgically excised (1, 2, 22). Major surgical procedures are often required for removal of these cystic lesions; thus, more accurate presurgical diagnosis has the potential to reduce the cost, morbidity, and occasional mortality associated with unnecessary surgery.

Cyst fluids can easily and safely be obtained from patients with pancreatic cysts by endoscopic aspiration (23–28). These fluids are often acellular, and therefore not typically useful for cytological diagnosis. However, such fluids can be analyzed for the presence of biochemical abnormalities, including those of DNA, and have the potential to inform diagnosis and improve the management of patients with these lesions (23–28). To set the stage for future molecular genetics-based diagnostic assays, we have here determined the sequences of the exomes, including all annotated coding genes, of representative cases of all four types of neoplastic cysts.

Results

Experimental Design. Neoplastic cysts are composed of a mixture of neoplastic epithelial cells and nonneoplastic cells [stromal, vas-

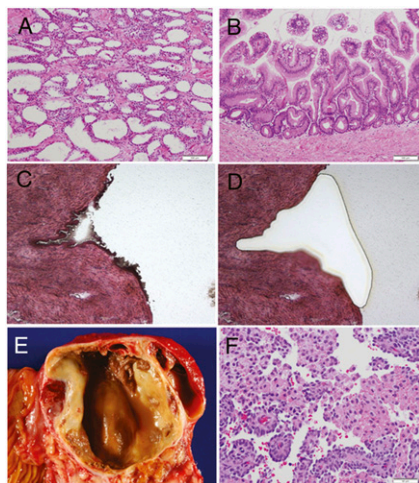


Fig. 1. Neoplastic cyst histopathology. (A) Typical SCA (SCA 38) shows centrally placed round nuclei without atypia. (Scale bar: 100 μ .) (B) Typical IPMN (IPMN 4) is lined by columnar mucin-producing cells that form large papillary projections into the ductal lumen. (Scale bar: 100 μ .) (C) Typical MCN is also lined by columnar mucin-producing cells, but the neoplastic epithelium is associated with a characteristic ovarian-type stroma. (Scale bar: 100 μ .) (D) Same MCN after laser capture microdissection of the columnar mucin-producing cells. (Scale bar: 100 μ .) (E) Gross appearance of a typical cyst-forming SPN (SPN 2). (Scale bar: 10 mm.) (F) Under the microscope, SPN 17 shows poorly cohesive cells supported by a delicate stroma. (Scale bar: 50 μ .)

cular, and inflammatory (17)] (Fig. 1). To maximize our ability to detect mutations, we carefully microdissected the neoplastic epithelial cells from the nonneoplastic cells. This was most difficult in the MCNs because of the cellular ovarian-type stroma present in these lesions (Fig. 1 C and D). Following microdissection, the neoplastic cell content of each of the cyst samples analyzed in this study was at least 33%.

DNA from 32 microdissected cysts, 8 of each of the four types, as well as matched DNA from normal tissues of the same patients was used in this study. The clinical and histopathological characteristics of the patients and their cystic lesions are detailed in **Dataset S1**. The DNA was ligated to adapters and amplified using standard Illumina protocols. The amplified DNA was then captured with a 50-Mb SureSelect Paired-End Target Enrichment System (Agilent). The captured DNA includes more than 20,000 coding genes (<http://www.sanger.ac.uk/gencode/>) plus all miRNAs in miRBase, v. 13 (<http://www.mirbase.org>). Sequencing was performed to relatively high depth on an Illumina GAI or HiSeq instrument [average unique coverage of 120-fold \pm 40 per base pair in the 64 libraries (32 from cyst DNA and 32 from matched normal DNA), **Dataset S2**]. This level of sequence coverage confers a >99% probability of detecting clonal heterozygous mutations present in DNA from cyst samples containing >33% neoplastic cells.

Analysis of SCAs. Using SNPs in the sequences captured by the SureSelect Paired-End Target Enrichment System, we were able to identify 15,190 \pm 428 heterozygous variants in the matched normal DNA samples of the eight patients with SCAs. Loss of heterozygosity (LOH) of at least one chromosomal region was identified in each of the eight SCAs studied (Fig. 2 and **Dataset S3**). The maximum degree of LOH (70% \pm 13%) confirmed the high fraction of neoplastic cell content achieved on microdissection. The only region that was lost in the majority of SCAs was on chromosome 3p (Fig. 2 and example in Fig. 3A). Seven of the eight SCAs lost chromosome 3p alleles, with the losses demarcated by bases 9,934,713–12,850,443. To determine whether this LOH was associated with reduplication of the remaining allele, we compared the copy number of all sequences lying within the regions of LOH with those of all other chromosomal regions in the same cysts. This was accomplished by comparing normalized tag counts through digital karyotyping (29), as explained in *Materials and Methods*. This analysis showed that in all seven SCAs exhibiting LOH of chromosome 3p, only one copy of chromosome 3p sequences remained in the tumor (assuming that the rest of the genome was, on average, diploid rather than polyploid).

We have previously described methods for the reliable identification of somatic mutations in next-generation sequencing data obtained from Illumina instruments (30, 31). Using stringent cri-

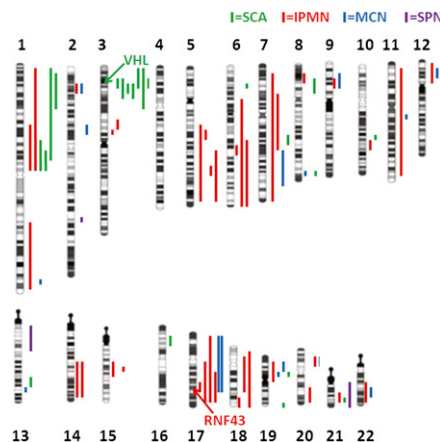


Fig. 2. LOH observed in neoplastic cysts of the pancreas. The lines indicate the observed regions of loss, with the different cyst types denoted by the indicated colors.

teria to avoid false-positive calls, we identified a total of 79 non-synonymous somatic mutations distributed within 71 genes among the eight SCA tumors (Dataset S4). There was an average of only 10 ± 4.6 nonsynonymous somatic mutations per tumor, far less than observed in PDAs [48 ± 23 per tumor (32); $P < 0.001$].

There were only two genes mutated in more than one SCA (Dataset S4). One of these was TBC1 domain family member 3F (*TBC1D3*), in which two missense mutations were observed (Dataset S4). This gene encodes a protein that stimulates the GTPase activity of RAB5A, which is important for early endosome trafficking (33, 34). Oncogenic properties of *TBC1D3* have been demonstrated previously *in vitro* and in mouse models, and the *TBC1D3* locus is amplified in 15% of primary prostate tumors (35) (36). However, whether the two *TBC1D3* mutations we identified are drivers or passengers (defined as mutations that did or did not directly contribute to oncogenesis, respectively) is not known. In contrast, the von Hippel–Lindau gene (*VHL*) located on chromosome 3p at position 10,158,319–10,168,746, is a bona fide tumor suppressor gene (37), and four of the SCAs contained mutations in this gene [N78S in SCA 23, W117L in SCA 35, C162W in SCA 38, and S80R in SCA 40 (Dataset S4)]. Three of these four (SCA 23, SCA 38, and SCA 40) showed evidence of LOH of the *VHL* chromosomal region (Dataset S3). Interestingly, the four tumors without detectable mutations of *VHL* (SCA 14, SCA 27, SCA 29, and SCA 37) had lost one allele of chromosome 3p within or adjacent to *VHL* (Fig. 2 and Dataset S3). We speculate that in these four cases, the *VHL* gene was inactivated by genetic alterations, such as deletions or translocations not detectable by sequencing, or by epigenetic mechanisms such as those responsible for *VHL* silencing in renal cell carcinomas (RCCs) (38).

SCAs have been identified in more than 15% of patients with *VHL* syndrome, a disease that predisposes to RCCs and other tumor types (39, 40). Of note, SCAs share several histomorphological

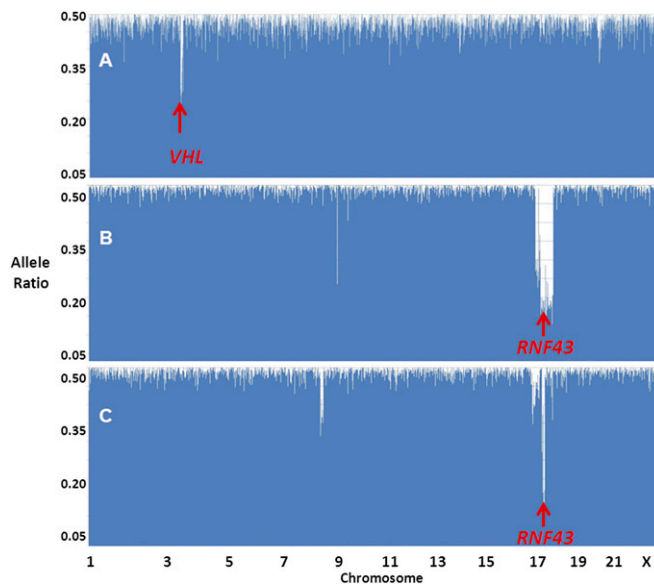


Fig. 3. Representative LOH data based on the sequence evaluation of genome-wide SNPs. The positions of each chromosome are indicated on the x axis. The y axis indicates the ratio of the two alleles of each SNP. In normal cells, this fraction is 0.5; in a pure neoplastic cell population in which every cell loses one allele of a locus, the allele ratio is 0.0. At a locus at which at least some neoplastic cells had undergone LOH, allele ratios greater than 0.0 reflect nonneoplastic cells that “contaminated” the microdissected cell population plus the fraction of neoplastic cells that had not undergone LOH at that locus. (A) SCA 40, exhibiting LOH of the region on chromosome 3p containing the *VHL* gene. (B) IPMN 12, exhibiting LOH of the region on chromosome 17q containing the *RNF43* gene. (C) MCN 169, exhibiting LOH of the region on chromosome 17q containing the *RNF43* gene.

characteristics with RCCs, including excessive glycogen production and a distinctive recruitment of vasculature (angiogenesis). All four mutations in *VHL* identified in SCAs were identical to ones previously identified in RCCs (<http://www.sanger.ac.uk/genetics/CGP/cosmic/>); three of the four were identical to ones previously described in the germline of patients with *VHL* syndrome, and the fourth (W117L) was at the same amino acid position found to be mutated in the germline of patients with *VHL* syndrome. This provides conclusive evidence of the inactivating nature of these mutations in human cells. In each of these four cases, we additionally confirmed that the *VHL* mutations in the cysts were somatic using independent ligation-based assays (Fig. 4 A and B).

To our knowledge, no mutations of *VHL*, or any other gene, have been identified in the fluids obtained from SCA cysts. Demonstration of such mutations in samples obtained by fine needle aspiration could be useful from a diagnostic standpoint, as discussed above. To determine whether *VHL* mutations could be identified in the fluids from SCAs, we designed a customized chip containing *VHL* gene sequences and used it to capture libraries from the DNA of 18 SCA cyst fluids. In nine (50%) of these cases, we identified point mutations in *VHL* (Dataset S5). Four of the mutations were predicted to inactivate the encoded protein’s function, because two produced nonsense codons, one was a 1-bp deletion producing a frameshift, and one altered the splice donor site. Of the five missense mutations, four were identical to those observed in the germline of patients with *VHL* syndrome. Similar capture of *VHL* genes from 28 IPMNs and three MCNs revealed no *VHL* mutations.

Analysis of IPMNs. In a previous study, in which we analyzed 169 well-annotated cancer genes, we identified recurrent mutations of Guanine nucleotide binding protein (G protein), alpha stimulating activity polypeptide 1 (*GNAS*) and v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) in IPMN samples and showed that these mutations were present in 66% and 81% of IPMNs, respectively (41). *KRAS* mutations had previously been identified in IPMNs as well as in PDAs (42, 43). To extend these analyses, we performed whole-exome sequencing on DNA from eight microdissected high-grade IPMNs and matched normal tissues.

LOH of at least one chromosomal region was identified in seven of the eight IPMNs (Fig. 2 and Dataset S3). No losses of the region

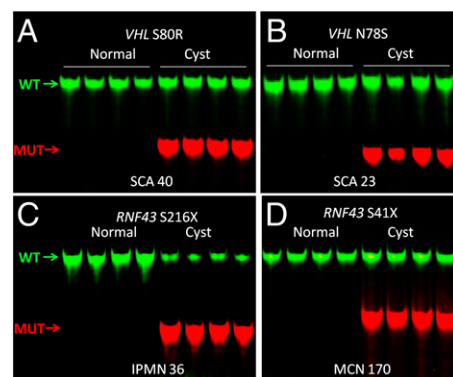


Fig. 4. Ligation assays used to confirm mutations in *VHL* and *RNF43*. Each lane represents the results of ligation of one of four independent PCR products, with each containing 100 template molecules. The ligation products were then size-separated on a denaturing acrylamide gel. The green bands are 6-carboxyfluorescein-labeled oligonucleotide probes that ligate to an unlabeled oligonucleotide when WT alleles are present. The red bands are hexachlorofluorescein-labeled oligonucleotide probes that ligate to the same unlabeled oligonucleotide when mutant (MUT) alleles are present. The WT- and MUT-specific oligonucleotide probes were of different lengths (~32 and ~12 bases, respectively); thus, they migrated at different positions in the acrylamide gel. The cyst samples and mutations assessed are indicated.

one of the two tumors with LOH of the *RNF43* locus. Two of the alterations were nonsense mutations (S41X and R371X), and the third was a missense mutation (R127P) (Dataset S4). This type of mutational pattern, with an overrepresentation of inactivating mutations, is characteristic of tumor suppressor genes and is similar to that observed for *RNF43* in IPMNs (Table 1). One of the MCNs with a truncating mutation was a low-grade MCN (MCN 166), whereas the other two tumors with *RNF43* mutations were high-grade MCNs.

In addition to these *RNF43* mutations, *KRAS* mutations were found in six MCNs and *TP53* mutations were found in two. The *KRAS* mutations were all at codon 12, in accord with previous studies of MCNs (41, 48). The *TP53* alterations were identical to those observed previously in other cancer types (<http://www.sanger.ac.uk/genetics/CGP/cosmic/>). Because *TP53* mutations are often associated with aggressiveness, it is possible that the MCNs with mutations in this gene are the ones most likely to progress to PDAs. Finally, there were two other genes (*MUC4* and *POTEJ*) that were mutated in more than one MCN, but analysis of the mutations by CHASM did not suggest that they would have substantial effects on protein function (Dataset S4).

Analysis of SPNs. The most notable finding about SPNs was the paucity of genetic alterations identified. Only one of the eight tumors studied exhibited any LOH whatsoever (Figs. 2 and 3 and Dataset S3). The absence of LOH was not attributable to contamination of the microdissected samples with nonneoplastic cells; this possibility could be excluded both by histopathological analysis and by the high fraction of *CTNNB1* mutant alleles, as discussed below (39–59%, Dataset S4).

As with LOH, the number of point mutations was also very low (2.9 ± 1.8 mutations per tumor). This number is lower than any of the other cyst types ($P < 0.001$) and, indeed, is less than any tumor type yet evaluated by genome-wide sequencing (49, 50), including pediatric tumors (51). Five of the eight tumors contained only one or two somatic mutations (Dataset S4). Nevertheless, every tumor had a missense mutation of *CTNNB1*, and all these mutations were at codon 32, 33, 34, or 37. This region of the encoded protein (β -catenin) is critical for regulation of the protein (52–54). When phosphorylated at serine or threonine residues lying between codons 32 and 37, β -catenin is degraded by E3 ubiquitin ligases. Mutations within this region inhibit phosphorylation and the consequent degradation of the protein (55, 56). Previous studies have shown that *CTNNB1* is nearly always mutated in SPNs (57, 58). Our study shows that the *CTNNB1* mutations are the only mutations that are evident in these tumors in many cases, even when all coding genes are assessed by sequencing. An evaluation of these tumors for translocations or epigenetic events may lead to the identification of additional alterations. If not, this tumor type would represent an exception to the widely held view that several sequential mutations are required for the formation of solid tumors in adults (59).

Discussion

The results described above have implications for both basic and applied research. It is fascinating that all four types of cysts are associated with defects in genes that are either components of ubiquitin ligase complexes (SCAs, IPMNs, and MCNs) or render them resistant to degradation by such complexes (SPNs). *RNF43*, genetically inactivated in both types of mucinous cysts (IPMNs and MCNs), has intrinsic E3 ubiquitin ligase activity (46). There has been relatively little research on this gene's product, although it has been shown to be present in a complex that regulates p53-mediated apoptosis (60). Based on these past studies, it is likely that the tumor suppressor effects of the WT *RNF43* gene product are a result of its ubiquitin ligase activity. To confirm this hypothesis, it will be important to determine the proteins that *RNF43* ubiquitinates in vivo as well as to demonstrate that such ubiquitination is essential for its tumor-suppressive role.

In contrast, *VHL* has been the subject of intense research since its identification as the gene responsible for *VHL* syndrome (61). The most well-studied function of *VHL* involves its role in

angiogenesis (62, 63). When cells are well-oxygenated, prolyl hydroxylation of hypoxia inducible factor 1, alpha subunit (*HIF α*) proteins leads to their binding to *VHL*. *VHL* then recruits an ubiquitin ligase complex that leads to the ubiquitination and subsequent degradation of *HIF α* . When *VHL* is inactivated, *HIF α* proteins are stabilized, resulting in the expression of numerous genes that stimulate angiogenesis even when cells are well-oxygenated.

There are at least two ways in which *VHL* mutations might stimulate SCA formation. First, it has been noted that there is a rich capillary network within SCAs, presumably a result of the abnormal activation of *HIF α* . This network could disturb local hemodynamics, facilitating the production of cyst fluid, and the increased local concentration of growth factors could stimulate epithelial cell proliferation (21). A second possibility involves the stabilization of microtubules, which is a well-documented but less extensively studied function of the *VHL* protein (64, 65). In the absence of *VHL*, primary cilia are absent or defective (66). In view of abundant evidence linking certain types of cysts to defects in primary cilia, it has been suggested that the microtubule stabilization function of *VHL* is key to its role in suppressing cysts (66). Several mouse models in which the *VHL* gene has been inactivated develop various cysts (although not SCAs) (67–70), suggesting fertile avenues for future research on this topic.

Our results also have potentially important diagnostic implications. As mentioned previously in this report, the distinction among various types of cysts before surgical intervention is critically important for patient management (1, 2, 9–17). Cyst fluid can be readily obtained from such patients and subjected to analytical assays (22, 23, 26, 27, 71–73). Our results show that the analysis of only five genes (*VHL*, *RNF43*, *CTNNB1*, *GNAS*, and *KRAS*) can usually distinguish among cyst types (Table 1): All eight SCAs had intragenic mutations of *VHL* or LOH in or adjacent to *VHL* and did not contain mutations of the other four genes; all eight IPMNs had alterations of *RNF43*, *GNAS*, or *KRAS* and never had *VHL* or *CTNNB1* mutations; MCNs always harbored *KRAS* or *RNF43* mutations but never contained *GNAS*, *CTNNB1*, or *VHL* mutations; and SPNs always contained *CTNNB1* mutations and never contained mutations of the other four genes. When combined with clinical and radiological data, the molecular genetic analysis of cyst fluid could thereby lead to more accurate diagnosis. For IPMNs and MCNs, it will be important to determine whether the number and type of genetic alterations in *GNAS*, *RNF43*, and other genes can be used to help gauge the risk for progression to invasive cancers. The examination of a large number of cyst fluid samples from patients with all four cyst types will be required to determine the added value of molecular genetic analyses for these and other diagnostic purposes.

Materials and Methods

Patients and Specimens. The present study was approved by the Institutional Review Boards of the Johns Hopkins Medical Institutions, Memorial Sloan-Kettering Cancer Center, Wayne State University, Emory University, and the University of Indiana. DNA was purified and libraries were prepared as described (74). Captured DNA libraries were sequenced with the Illumina GAllx/HiSeq Genome Analyzer, using one lane per sample, yielding 150 (2 × 75) base pairs from the final library fragments. Sequencing reads were analyzed and aligned to human genome hg18 with the Eland algorithm in CASAVA 1.7 software (Illumina). Mutations were confirmed as described in ref. 41 using the primers listed in Dataset S6. Further details are provided in *SI Materials and Methods*.

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