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Journal Title: Proceedings of the National Academy of Sciences
Volume: Volume 108, Number 52
Publisher: National Academy of Sciences | 2011-12-27, Pages 21188-21193
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1073/pnas.1118046108
Permanent URL: https://pid.emory.edu/ark:/25593/s5svz

Final published version: http://dx.doi.org/10.1073/pnas.1118046108

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Accessed April 30, 2020 4:41 AM EDT
Whole-exome sequencing of neoplastic cysts of the pancreas reveals recurrent mutations in components of ubiquitin-dependent pathways

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Contributed by Bert Vogelstein, November 4, 2011 (sent for review October 12, 2011)

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 CTNNB1, 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 cytotologic 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 5-9 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 pre-malignant 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. 1 D and F) are technically solid tumors, but the vast majority of them undergo cystic degeneration that clinically and radiographically


The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1118046108/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1118046108
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.

Cysts 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, vascular, 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/genoscope/) plus all miRNAs in miRBase, v. 13 (http://www.mirbase.org). Sequencing was performed to achieve high depth on an Illumina GAII or HiSeq instrument [average unique coverage of 120-fold ± 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 ± 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% ± 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. 3 A). 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-
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. 4A 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. 3. Representative LOH data based on the sequence evaluation of genenornes. 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.](image)

![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 only 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 cyt samples and mutations assessed are indicated.](image)
containing the \textit{VHL} gene were identified. The most commonly deleted region was on chromosome 17q, demarcated by nucleotides 53,790,884–53,939,507, which was observed in four of the eight samples (Fig. 2, Dataset S3, and example in Fig. 3B). Digital karyotyping showed that in these four cases, the LOH events were not associated with reduplication of the deleted chromosome 17q region.

Using the same criteria described above for SCAs to identify somatic mutations, a total of 211 nonsynonymous mutations within 191 genes were identified among the eight IPMNs (Dataset S4). There were 26 ± 12 nonsynonymous somatic mutations per tumor (Dataset S2), which is more than twice the number found in SCAs (P < 0.001) and approximately half as many as found in PDAs (P < 0.001).

There were six genes that were mutated in more than one of the eight IPMNs (Dataset S4). As expected, both \textit{KRAS} and \textit{GNAS} mutations were common, each identified in five tumors and always at codons 12 and 201, respectively (Dataset S4). Two mutations in Adenomatous polyposis coli (\textit{APC}) were observed. \textit{APC} is a well-known tumor suppressor gene whose mutations usually initiate colorectal tumorigenesis (44). One of the \textit{APC} mutations was a nonsense base substitution (R450X in IPMN 4), typical of the inactivating mutations that occur in the germline of patients with colorectal cancers. The second was a missense substitution (R99W in cyst 21) whose functional effects, if any, are unknown. Two different IPMNs had mutations in oligonucleotide/oligosaccharide-binding fold containing 1 (\textit{OBFC1}), and two others had mutations in calcium channel, voltage-dependent, T type, α-1 subunit (\textit{CACAN11}). There are no genetic or functional data implicating these two genes in neoplastic processes. Cancer-specific high-throughput annotation of somatic mutations (CHASM) analysis (45), which determines the probability that a given mutation alters the structural or biochemical properties of a protein, showed that the missense mutations in \textit{OBFC1} and \textit{CACAN11}, as well as the one in \textit{APC}, were not very likely to alter the function of the encoded proteins and may have been passengers. The CHASM scores of all missense mutations identified in this study are provided in Dataset S3.

Table 1. Recurrent genetic alterations in neoplastic cysts of the pancreas

<table>
<thead>
<tr>
<th>Cyst type</th>
<th>Sample ID</th>
<th>\textit{VHL}</th>
<th>\textit{RNF43}</th>
<th>\textit{KRAS}</th>
<th>\textit{GNAS}</th>
<th>\textit{CTNNB1}</th>
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<td>p.G12D</td>
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<td>None</td>
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<td>LOH, p.Y177X</td>
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<tr>
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</table>

The most commonly mutated gene in IPMNs was \textit{RNF43}, which is located on chromosome 17, nucleotides 53,786,037–53,849,930, within the small region of chromosome 17q experiencing LOH in IPMNs (Dataset S3). The protein encoded by \textit{RNF43} has been shown to have intrinsic E3 ubiquitin ligase activity (46). \textit{RNF43} was mutated in six of the eight tumors, including all four that had undergone chromosome 17q LOH. Five of the six mutations were base pair substitutions resulting in nonsense codons (Dataset S4). Based on the number and type of mutations in this gene, the probability that it functioned as a passenger was extremely low (<10^{-12}, binomial test). Each of the six \textit{RNF43} mutations was confirmed by an independent ligation-based assay (example in Fig. 4C). Although expression of \textit{RNF43} has been shown to be correlated with increased cell growth (47), the inactivating mutations of this gene in IPMNs leave little doubt that it suppresses neoplasia in pancreatic ductal epithelial cells.

**Analysis of MCNs.** Relatively few LOH events were identified in the MCNs compared with the IPMNs (Fig. 2 and Dataset S3). Only one region was lost in more than one tumor, and this region was on chromosome 17q and included the \textit{RNF43} gene (Fig. 3D). A total of 128 nonsynonymous somatic mutations distributed within 115 genes were identified among the eight MCNs (Dataset S4). There were 16.0 ± 7.6 nonsynonymous somatic mutations per tumor, more than in SCAs but less than in IPMNs (Dataset S2). Three MCNs harbored intragenic mutations in \textit{RNF43}, including
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 previously found in human cancers (https://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 SNPs. The most notable finding about SNPs 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 SNPs (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 (SNPs). 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 (HIFα) proteins leads to their binding to VHL. VHL then recruits an ubiquitin ligase complex that leads to the ubiquitination and subsequent degradation of HIFα. When VHL is inactivated, HIFα 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 HIFα. 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 SNPs 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 GAIIx/Hiseq Genome Analyzer, using one lane per sample, yielding 150 × 25 bp pairs (74). DNA was captured as described in ref. 41 using the primers listed in Dataset S6. Further details are provided in SI Materials and Methods.

ACKNOWLEDGMENTS. We thank J. Schaeffer, J. Ptak, N. Silliman, M. Whalen, and L. Dobony for expert technical assistance. This work was supported by the Lustgarten Foundation for Pancreatic Cancer Research; the Virginia and D. K. Ludwig Fund for Cancer Research; The Sol Goldman Pancreatic Cancer Research Center; the Joseph L. Rabinowitz Fund; the Michael Rolfe Foundation; the Stringer Foundation; the family of Ted and Julie Smith; and the Indiana University Comprehensive Cancer Center. Funding for this work was also provided by Institute Contracts N01-CN-43302, N01-CN-43309, and NCI grant P01CA134292.
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