Ampullary Cancers Harbor ELF3 Tumor Suppressor Gene Mutations and Exhibit Frequent WNT Dysregulation

Highlights

- Three periampullary tumor types share a common molecular blueprint
- Frequent WNT pathway disruption can be a potential therapeutic target
- ELF3 is a frequently mutated tumor suppressor gene of periampullary tumors

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In Brief
Gingras et al. compare the genomic profiles of ampullary, distal bile duct, and duodenal adenocarcinomas. They find disruption of the WNT pathway, which could be used as a tumor subclassification for targeted therapy, a high frequency of ELF3 inactivating mutations, and microsatellite instability.

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Ampullary Cancers Harbor ELF3 Tumor Suppressor Gene Mutations and Exhibit Frequent WNT Dysregulation

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SUMMARY

The ampulla of Vater is a complex cellular environment from which adenocarcinomas arise to form a group of histopathologically heterogenous tumors. To evaluate the molecular features of these tumors, 98 ampullary adenocarcinomas were evaluated and compared to 44 distal bile duct and 18 duodenal adenocarcinomas. Genomic analyses revealed mutations in the WNT signaling pathway among half of the patients and in all three adenocarcinomas irrespective of their origin and histological morphology. These tumors were characterized by a high frequency of inactivating mutations of ELF3, a high rate of microsatellite instability, and common focal deletions and amplifications, suggesting common attributes in the molecular pathogenesis are at play in these tumors. The high frequency of WNT pathway activating mutation, coupled with small-molecule inhibitors of β-catenin in clinical trials, suggests future...
treatment decisions for these patients may be guided by genomic analysis.

INTRODUCTION

Though the pancreas, bile duct, and intestinal duodenum share common embryologic origins in the ventral endoderm, the adenocarcinomas arising in this region presumably originate from different epithelial cellular constituents present at the site (Zaret and Grompe, 2008). These tumors have been described in many different ways: intra-ampullary, periampullary, intra-ampullary papillary-tubular neoplasm, ampullary/ductal, periampullary-duodenal, and ampullary/not otherwise specified. The tumors clearly separated from the ampulla of Vater and localized in the bile duct, duodenum, or pancreatic duct have been identified as distal cholangiocarcinomas or distal bile duct (CAC), duodenal (DUOAC), or pancreatic ductal (PDAC) adenocarcinomas.

As recommended in the AJCC seventh edition 2009 staging system (Edge et al., 2009), the current subtype classification of ampullary adenocarcinoma (AMPAC) is based on the anatomical location from which the tumor is thought to arise (Edge et al., 2009), sometimes supplemented by histopathology and expression of differential markers (Adsay et al., 2012; Chang et al., 2013; Ehehalt et al., 2011; Morini et al., 2013). This classification is subjective and prone to inter-observer variability and can significantly impact treatment selection and therapeutic development (Ampoulach et al., 2011; Heinrich and Clavien, 2010; Romiti et al., 2012; Westgaard et al., 2013). Current treatment approaches do not distinguish patients based on subtypes, yet tumors may arise from at least the three epithelia that converge at that site, and some may arise from the ampulla itself, where little is known of the specialized epithelium that may be present. Malignancies that arise from different cellular origins often have vastly differing sensitivities to therapeutics. Post hoc analyses of clinical trials using histopathological criteria have not discerned such a difference and likely represent the inaccuracy of such a classifier. However, as most therapeutic development is focused on agents that target specific molecular mechanisms, a molecular characterization that would allow selection of patients for specific therapies would facilitate therapeutic development with the aim of improving outcomes and alleviate the impact of an inaccurate subjective classification.

For this study, we have assembled a large cohort of AMPAC with nearby DUOAC and CAC for comparison. We show that tumors from the duodenum, ampulla of Vater, and distal bile duct exhibit a common spectrum of features irrespective of their morphology, marker expression, and cellular origin. Here, we use the term “periampullary tumors” in this study to refer to the three tumor types of AMPAC, DUOAC, and CAC collectively, as defined by the AJCC seventh edition 2009 staging system (Edge et al., 2009), excluding cases that clearly arise from the pancreas (pancreatic adenocarcinoma [PDAC]).

RESULTS

In order to develop a molecular taxonomy for periampullary cancers and define subtypes with clinical relevance, we performed exome sequencing and copy-number analysis of 160 cancers arising in the periampullary region, 62 of these clearly arising from either the bile duct (n = 44) or the duodenum (n = 18) and 98 for which the epithelium of origin could not be clearly defined morphologically (AMPAC). Mutations were validated by deep and ultra-deep sequencing on a limited target region consisting of 71 recurrently mutated genes. RNA sequencing (RNA-seq) was performed on 30 patients: a 28-patient subset of the 98 ampullary tumors and a two-patient subset of the 18 duodenal tumors.

Clinical Characteristics and Subtyping

The clinical characteristics of our patient cohort are described in Table S1A. In this study, the anatomical primary site of origin of all tumors was defined using the AJCC seventh edition 2009 staging system (Edge et al., 2009). In addition, the tumors were also classified independently by cellular morphology and immunohistochemistry (IHC) staining (see Experimental Procedures) into intestinal, pancreatobiliary, or mixed subtypes (Table S1B). Since treatment may be determined based on subtypes defined by the combination of morphology and IHC even if these measures are somewhat subjective, it was an important objective of our study to assess the reliability and meaning of these subtypes. Subtyping according to IHC, the AMPAC tumors were 51% pancreatobiliary and 34% intestinal, with the remainder mixed. CAC was dominated by the pancreatobiliary subtype, 86% as expected; however, 11% of CAC exhibited an intestinal phenotype. In DUOAC, the intestinal subtype was 44%, with 22% pancreatobiliary and the remainder mixed.

By histological morphology, a smaller proportion of each tumor type was classified as pancreatobiliary (AMPAC, 37%; CAC, 77%; and DUOAC, 6%). The two methods of classification yielded concordant subtypes only 62% of the time for AMPAC tumors, 77% of the time in CAC, and 53% of the time in DUOAC. Although the two methods often disagreed, all three tumor types included in their numbers concordant cases of all three subtypes. Thus, tumors originating in each organ site in the periampullary region may be classified as any of the three subtypes, though this classification system is rarely applied to DUOAC or CAC tumors. These tumors were analyzed by genomic methods to further characterize their molecular properties.

Mutation Analysis

Exomes were sequenced to an average of 120-fold coverage resulting in 28,795 mutations across 152 patients. Eight additional patients were sequenced with targeted custom sequencing and were included in the study (see Supplemental Experimental Procedures, “Sequencing design and Mutation analysis,” and Tables S1C–S1E). Microsatellite instable (MSI) tumors were observed in 12 patients representing each organ cohort (Figure 1), accounting for 18,572 of the whole-exome sequencing (WES) discovery set. Using a method we developed based on the enzyme slippage of the homopolymer region (E.S., unpublished data), we identified two other patients among the targeted sequencing set (Figure S1A).

Excluding microsatellite instable (MSI) tumors and correcting for tumor purity, the median mutation rate did not vary
significant across the AMPAC, CAC, and DUOAC (3.8, 4.6, and 4.7 per Mb, respectively) but was clearly distinct from the MSI mutation rate (68, 127, and 108 per Mb, respectively) (Figures 1A, S1B, and S1C). Two-thirds of the hypermutated WES samples had germline mutation in genes associated with Lynch syndrome. Interestingly, PMS2, a gene that accounts for less than 5% of Lynch syndrome patients overall (Thompson et al., 2004) (OMIM #600259), was mutated in one half of our MSI patients (Figure 1B). Although MSI was more common in DUOAC than CAC patients, every morphologic category harbored at least one PMS2 germline mutation in this study. Leaving aside germline contribution, the overall frequency of MSI in AMPAC was 3%. MSI appeared to confer a survival advantage in AMPAC, as it does in other gastrointestinal (GI) cancers, as all 14 cases were alive ranging from 2 to 8 years after diagnosis (p = 0.04 with a lack of negative event) (Figure 1C).

Non-negative matrix factorization was used to evaluate the mutation signatures associated with periamplary tumors. We observed signature #1 at greater than 20% of the total signature in 9.6% of our entire tumor set (6% AMPAC and 21% CAC). Signature #1 is characterized by AC, AT > AN and is enriched in non-transcribed regions of the genome in samples from several cancer types (PDAC, medullloblastoma, breast tumor, AML, and CLL). However, signature #1 was also observed in the coding region of 18 out of 486 hepatocellular carcinoma (4%) and 31 out of 450 colorectal carcinoma (CRC) (7%) (Lawrence et al., 2013; Totoki et al., 2014; K.R.C., unpublished data). Whereas none of the known signatures have yet been associated with a difference in outcome, signature #1 was associated with poor outcomes in our study set (multivariate Cox proportional hazards p = 0.02) (Figure 2B).

The analysis of the periamplary tumors, excluding MSI patients, revealed 19 genes mutated significantly above background using MutSig-CV (Lawrence et al., 2013) (Figure 3A; Table S3A). Considering the ratio of inactivating to missense mutations, an additional three genes were brought in to the significantly mutated gene list (Table S3B) including PBRM1, RECQL4, and KDM6A. Gene expression data confirmed that the variants harboring missense mutation in the driver genes were expressed between 85% and 88% of the time (Table S3C).
Considering the 44 CAC alone, four genes were significantly mutated in this cancer: TP53, KRAS, SMAD4, and CDKN2A with the highest mutation incidence in TP53. Whereas intrahepatic CAC tumors frequently harbor BAP1, IDH1, and IDH2 (Nakamura et al., 2015), these were absent with the exception of a single IDH1 hotspot mutation in the periampullary CAC. This is in agreement with Nakamura et al. (2015), where no IDH1 mutations could be detected among 74 extrahepatic tumors (compared to a 5% mutation rate in intrahepatic tumor) and a less than 3% BAP1 mutation rate was found in extrahepatic tumors (compared to a 12.4% mutation rate in intrahepatic tumor).

**Alteration of Key Signaling Pathways**

The significantly mutated genes defined five pathways in periampullary tumors: TP53/cell division, RAS/PI3K, WNT, TGF-β, and chromatin remodeling pathways. We combined the point mutations and copy-number alterations (CNA) changes at the gene level within these five pathways to assess the impact of these pathways among the three anatomical sites (Figures 2A and 2B and Table S2).

**Figure 2. Mutation Signature in Periampullary Tumors**

(A) Heatmap of five dominant mutation signatures from NMF analysis of mutation spectrum for each subject. Intensity indicates the proportion of mutations for that subject attributed to the indicated signature. Subjects are sorted first by signature 1, then signature 6 from the highest to the lowest value. Only signatures with high penetrance are shown.

(B) Kaplan-Meier curve of survival in this cohort stratified by signature 1 levels (high, red line: signature 1 component >10% of all mutations; low, black line: otherwise, multivariate Cox proportional hazards p = 0.001). See also Figures S2A and S2B and Table S2.
The similarities and differences in gene mutations per tumor types and subtypes are illustrated in Figures S3A and S3B. The WNT pathway was mutated in 46% of patients overall but was clearly differentially mutated across the three tumor types, being more frequently mutated in DUOAC (72%) than in AMPAC (49%) or CAC (30%) (chi-square p < 0.05) (Tables S4A and S4B). This predominance of WNT pathway mutation in DUOAC was due mainly to more frequent mutations of APC and SOX9. Whereas the TP53, RAS, TGF-β signaling and chromatin remodeling pathways are deregulated in many tumor types, the WNT pathway deregulation is reported only in gastrointestinal tumors (Biankin et al., 2012; Cancer Genome Atlas, 2012). We reasoned that grouping the patients by our histological classification might enrich WNT mutation in the intestinal subtype relative to the pancreaticobiliary subtype. As expected, the intestinal subtype had 67% WNT pathway alterations compared to pancreaticobiliary with 30% WNT alterations, very close to the WNT frequency based on anatomical site (Figures 4B, S3A, and S3B; Tables S4A and S4B). Although we observe a gradient of WNT pathway disruption in tumors as their anatomical site moves away from the GI tract, WNT mutation is still frequent in CAC, or “pancreaticobiliary” subtype tumors.

TGFB2 was also more frequently mutated in DUOAC than AMPAC and CAC, but this may have been secondary to MSI, which was in higher proportion in DUOAC. TGFB2 harbors an A homopolymer run of eight bases that is a frequent target of mutation in MSI patients, and 5 of the 12 TGFB2 mutations were at this site. Interestingly, SMAD4, a gene frequently mutated in PDAC, was the most commonly mutated gene of the TGF-β pathway in AMPAC and CAC, the tissue sites in closest proximity to the pancreas. Mutant KRAS was the major RAS signaling oncogene in all three tumor types. Overall, the RTK/RAS/PI3K pathway was activated in all periampullary patients at a statistically similar rate ranging from 84% to 94% among the three tumor types (Tables S4A and S4B).

Alterations in the SWI/SNF chromatin remodeling pathway were observed most frequently in ARID1A and ARID2. Overall, mutations in the SWI/SNF complex were equally frequent in the three tumor types.

Pathway Mutation Correlates with Disease Outcome
Multivariate analyses on the periampullary tumors as a group showed mutations in the TGF-β pathway are associated with better overall survival (multivariate Cox proportional hazard p = 0.0059, HR = 0.42) independent of stage, gender, subtype, and MSI status (multivariate Cox proportional hazard p = 0.029). Mutations in the PI3K pathway were also associated with better overall survival (multivariate Cox proportional hazards p = 0.036, HR = 0.43) (Figure S3C). Mutations in TP53, KRAS, WNT, and chromatin remodeling pathways showed no significant association with outcomes in multivariate modeling. Interestingly, TGF-β pathway mutations were also negatively associated with mutation signature 1 (multivariate ANOVA p = 0.02), possibly explaining the association with outcomes. However, the contribution of signature 1 to outcomes was still significant when considering TGF-β pathway mutations in the model, indicating that these two effects are not entirely redundant.

RNA Expression
RNA expression was analyzed in 28 AMPAC and 2 DUOAC. Due to the high frequency of mutation in WNT and the current development of therapeutic agents targeting β-catenin, we evaluated...
Figure 4. Major Altered Pathways in Periampullary Tumors

(A) Frequency of changes defined by somatic mutations or copy-number loss or gain is expressed as a percentage of cases for each gene. Inactivation (blue) or activation (red) is graded in intensity by percent of patients affected.

(B) Genetic alterations in the significantly mutated genes grouped by pathway are illustrated for each patient. Note WNT and PI3K signaling pathways could be found in the three tumor types and in each of their subtypes, including the pancreatobiliary subtype.

See also Figures S3A and S3B, wherein mutations in each gene are grouped by tumor type and subtype, and Figure S3C and Tables S4A and S4B.
the expression data using a previously developed WNT signature that included WNT antagonist, WNT agonist, and WNT target genes (Donehower et al., 2013). An increase in expression in these three gene groups as a result of the WNT pathway deregulation was noticed in colorectal cancer (Donehower et al., 2013). This could be explained by the fact that CTNNB1 activation resulted in an increased expression of targeted genes and the unrestricted WNT signaling set up a negative feedback loop of the WNT antagonist genes attempting to shut down signaling. In this study, we divided the patients into WNT mutated and those without (Figure 5, mutation panel). We then looked at the relative RNA expression in the two tumor groups for WNT antagonists, WNT agonists, and WNT targets (Figure 5, middle panel). The tumors with WNT mutations trend significantly toward higher overall WNT gene expression (p < 0.001) (Figure 5, lower panel). The WNT gene expression profile was also increased in some of the WNT non-mutated patients, indicating that some other mechanism affecting the WNT pathway might be at play.

Fusion analysis identified two noticeable non-recurrent fusions: SLC45A3-ELK4 used as a prognosis marker in prostate cancer, where its expression is elevated (Kumar-Sinha et al., 2012; Ren et al., 2014), and a LINE-MET fusion in a patient without any KRAS or TP53 driving mutations and a high MET expression (Figure S4; Table S5). LINE element insertions are found in PDAC, colon, hepatocellular, esophageal, and lung carcinoma (Paterson et al., 2015; Rodić et al., 2015).

Copy-Number Alteration

The majority of CNAs involved entire chromosomes or chromosome arms as opposed to focal events, which are common in gastrointestinal tumors. Arm-level deletions outnumbered amplifications across all tumors (Figure 6A). The three tumor types shared four arm-level amplifications and nine arm-level deletions. AMPAC shared amplification of 1q and deletion of 1p and 8p CAC (Table S6A). AMPAC shared no events specifically with DUOAC, making AMPAC marginally more similar to CAC in its CNA pattern. AMPAC also had two unique amplifications on 5p and 6p, whereas 3q amplification was unique to CAC, and 6p was unique to DUOAC.

A combined GISTIC analysis revealed as expected a focal deletion of 9p23.1, involving CDKN2A (Table S6B). A focal deletion in chromosome 9 removed the promotor and 5′ end of KDM4C (Figure 6B). Although present in every tumor type, it was only statistically significant in AMPAC (Table S6B). This deletion resulted in a significant decrease in expression of KDM4C as well as the upstream UHRF2 (Figure 6B, inserts). Interestingly, overexpression of both genes has been associated with a pro-growth effect on colon cancer cells (KDM4C) and a much lower disease-free survival and overall survival in patients with colon cancer (UHRF2) (Lu et al., 2014; Kim et al., 2014). KDM4C forms also complexes with β-catenin (Kim et al., 2014; Yamamoto et al., 2013).

DISCUSSION

This study compares the genetic constitution of ampullary cancer with two nearby tumor types with pathologic classification. Historically, ampullary cancers have been classified as intestinal
Figure 6. Copy-Number Alteration
(A) Nexus GISTIC analysis of copy-number alteration by anatomical site. Upper blue panel shows copy-number gains and the lower red panel shows copy-number losses for each tumor type. Blue arrows demark changes characteristic of a given anatomical site.

(legend continued on next page)
or pancreaticobiliary subtypes based on immunohistochemistry and/or cellular morphology. The genomic analysis mirrored these results by the finding that some ampullary tumors exhibit properties of intestinal tumors such as microsatellite instability, ELF3 mutation, and disruption of WNT signaling.

We found that the classification approaches of the three periampullary tumors are often discordant with one another. No unique molecular characteristics were specifically associated with one tumor subtype or one tumor type. Interestingly patients from each tumor type and subtype exhibited alterations in WNT pathway genes, including nearly one-fourth of the CAC tumors and one-fourth of the pancreaticobiliary tumors. Other studies using subtype classification different from ours report WNT pathway mutations in AMPAC pancreaticobiliary (PB) subtype (Achille et al., 1996; Hechtman et al., 2015). Transcriptional changes in AMPAC tumors in WNT signaling genes was increased, as expected, in tumors with WNT mutation, reinforcing a molecular dichotomy. With half of the patients across the three tumor types harboring WNT mutation, this could impact greatly the choice of treatment since several WNT pathway targeted therapies are in development. Ampullary, duodenal and distal bile duct adenocarcinoma could be regarded as a WNT ± entity from the perspective of treatment. Thus, the molecular data suggest that clinical testing for WNT signaling status might be beneficial to patients in the near future, making this a stepping stone to personalized medicine.

The identification of ELF3 as a significantly mutated gene with an inactivating mutation pattern is also of interest. It was reported at lower frequency in bladder and biliary tract cancers, but not in any other cancer so far. ELF3 encodes an ETS-domain transcription factor. By interacting with promoter regions, ELF3 is implicated in the regulation of several genes during epithelial cell differentiation (Oliver et al., 2012). One of the genes transactivated by ELF3 is TGFBR2, a prime initiator of TGF-β signaling, a pathway with a dual role in tumorogenesis, suppressing tumor progression at early stages but enhancing invasion and metastasis at later stages (Roberts and Wakefield, 2003). The tumor-suppressor antiproliferative function of ELF3 was previously noted in studies on colorectal, prostate, and oral squamous cancer cells (Iwai et al., 2008; Lee et al., 2003, 2008; Shatnawi et al., 2014) and more recently in biliary tract cancer cell line (Nakamura et al., 2015). Such studies showed that ELF3 directly binds to the promoter region of EGR1 (Lee et al., 2003) and TGFBR2 (Lee et al., 2003), increasing the transcription of these two tumor suppressor genes in CRC, whereas ELF3 binding to androgen receptor (AR) (Shatnawi et al., 2014) and matrix metalloproteinase-9 (MMP9) (Iwai et al., 2008) promoters suppressed the transcriptional activity of these tumor growth- and invasive- ness-promoting genes in prostate and squamous cancers, respectively. However, recent observations also suggested an oncogenic functional role in CRC development when ELF3 is amplified, and its upregulated expression correlated with cancer progression and decreased patient survival (Wang et al., 2014).

A WNT-independent CTNNB1 transactivation facilitating tumor development was also reported (Wang et al., 2014). Such dual function has also been observed in breast and prostate cancer (Longoni et al., 2013; Oliver et al., 2012; Shatnawi et al., 2014). It could be argued that when ELF3 inactivation occurs early in tumor development, it provides a moderate growth advantage by suppressing TGF-β signaling. The fact that we found ELF3 mutation in a duodenal adenoma with intraepithelial neoplasia and dysplasia components (DUOAC 707) and that 75% of the tumors with ELF3 mutation were lower-grade tumors (stage I or II) could support this hypothesis. The ELF3 functional switch might depend on tumor stage and expression of other factors and/or be associated with its expression level, some genes being transactivated only when ELF3 is overexpressed. In any case, ELF3 is implicated in the development of periampullary tumors, and its exact functional role during periampullary tumor development will need to be investigated further.

**EXPERIMENTAL PROCEDURES**

**Clinical Data**

A total of 160 tumors (98 AMPAC, 44 CAC, and 18 DUOAC) were collected by the different groups participating in this study: Australian Pancreatic Cancer Genome Initiative (APGI), Baylor College of Medicine Elkins Pancreatic Center (BCM) as a member of The Cancer Research Banking, MD Anderson Cancer Center (MDA), and Technical University of Dresden (TUD). Ethical approval was obtained from each of these institution’s research ethic boards. All patients underwent surgical pancreaticoduodenectomy with curative intent without known residual disease. Clinical data variables including race, sex, age, familial history, operative procedure, pathological findings, and survival from the date of initial surgery to the date of death or last follow up are presented in Table S1A.

**Tumor Classification**

A section of the tumor was fixed in formaldehyde and embedded in paraffin (FFPE). H&E-stained and immunohistochemistry slides from the FFPE tissue were examined by the pathologists from the original site of collection to confirm diagnosis of the specific tumor section and to grade expression of subtype markers. All slides were then centrally reviewed by a single pathologist (A.G.) who was blinded to all clinical, molecular, and pathological data at the time of review and scoring. The distinction between pancreatic, biliary, ampullary or intestinal carcinoma was based on the anatomical site from which the carcinoma was thought to arise using the guidelines recommended in the AJCC seventh edition 2009 staging manual (Edge et al., 2009).

**Histology and Morphology**

Tumors were classified as pancreaticobiliary, intestinal, or mixed morphological subtype based on the cellular morphology. A morphology similar to colorectal adenocarcinoma (tall often pseudostratified columnar epithelium with oval nuclei forming elongated glands) was defined as intestinal type. Morphology similar to pancreaticobiliary carcinoma (small solid nest of cells with rounded nuclei surrounded by desmoplastic stroma and forming simple or branching rounded glands) was defined as pancreaticobiliary type. Mixed histological types contained a mixture of both intestinal and pancreaticobiliary types with 80% or less of the cells with either morphology. Grade of differentiation was also noted as well as presence of adenoma, signet-ring cells, or mucinous cells (Table S1B).
Immunohistochemistry Staining
FFPE sections were stained with antibodies against MUC1 and CDX2 (see Supplemental Experimental Procedures). This two-antibody panel has previously been validated by our group to predict prognosis in ampullary carcinoma (Chang et al., 2013), and the methods we used were the same as employed in that study. Briefly, expression was evaluated by estimating the percentage of positively stained carcinoma cells and the intensity of the staining (0 absent, 1+ weak, 2+ intermediate, and 3+ strong). H scores were calculated for both markers by multiplying the percentage of stained cells by the intensity of the staining. The ratio of the CDX2/MUC1 H score defined the subtypes: a ratio of 2 and above and smaller than 0.5 were considered intestinal and pancreatobiliary, respectively. Inter-observer variability was associated to a mixed subtype (Table S1B).

Nucleic Acid Isolation
Samples were retrieved and had full face sectioning performed in OCT embedding media to verify the presence of carcinoma in the sample to be sequenced and to estimate the percentage of malignant epithelial nuclei in the sample relative to stromal nuclei. Macrodissection was performed if possible to excise areas of non-malignant tissue. DNA and RNA extraction was performed at the center of collection following their own protocol with all samples being tracked using unique identifiers though out the process (see Supplemental Methods). DNA was shipped and quantified at BCM-Human Genome Sequencing Center (HGSC) using the PicoGreen DNA Assay.

SNP Array Assays
SNP arrays were processed at the HGSC for each sample using the Illumina Infinium LCG Assay according to the manufacturer’s guidelines. Specifically, assays were performed with Human Omni2.5-8 BeadChips (illumina, catalog no. WG-311-2513), interrogating 2.5 million SNP loci with a MAF detection limit of 1% (see Supplemental Experimental Procedures). SNP calls were collected using Illumina’s GenomeStudio software (version 2011.1) in which standard SNP clustering and genotyping were performed with the default settings recommended by the manufacturer. Data from samples that met a minimum SNP call rate of 0.9 were considered passing and were included in subsequent analyses. Results were analyzed on Nexus (BioDiscovery).

Sequencing
Library preparation, whole (Rainbridge et al., 2011) and targeted exome capture, and regular and ultra-deep sequencing on HiSeq 2000 platform are detailed in Supplemental Experimental Procedures. In brief, 152 samples were whole-exome sequenced and their mutations validated with a custom design targeted exome capture. The targeted capture consisted of a panel of 71 genes covering 0.25 Mb, and the probes were designed by Nimblegen (genes are listed in Supplemental Experimental Procedures). These genes were selected on the basis they were significantly mutated and/or had high impact in the development of MAPPC, PDAC, DUOAC, CAC, and other pancreatic tumor types. The selective targeted capture was also used in discovery on eight samples received at a later date (six samples) or of low purity (two samples with <10% tumor). The mutations identified with the targeted capture were validated with ultra-deep (single-molecule reconstruction) sequencing.

Data Analysis
Primary Data Analysis
Initial sequence analysis was performed using the HGSC Mercury analysis pipeline (https://www.hgsc.bcm.edu/software/mercury). First, the primary analysis software on the instrument produces .bcl files that are transferred off-instrument into the HGSC analysis infrastructure by the HiSeq Real-time Analysis module. Once the run is complete and all .bcl files are transferred, Mercury runs the vendor’s primary analysis software (CASAVA), which demultiplexes pooled samples and generates sequencing reads and base-call confidence values (qualities). The next step is the mapping of reads to the GRCh37 Human reference genome (http://www.ncbi.nlm.nih.gov/projects/ genome/assembly/grc/human/) using the Burrows-Wheeler aligner (Li and Durbin, 2009) (BWA; http://bio-bwa.sourceforge.net/) and producing a BAM (Li et al., 2009) (binary alignment/map) file. The third step involves quality recalibration (using GATK; DePristo et al., 2011; https://www.broadinstitute.org/gatk/) and, where necessary, the merging of separate sequence-event BAMs into a single sample-level BAM. BAM sorting, duplicate-read marking, and realignment to improve in/del discovery all occur at this step.

Cancer Data Analysis
Primary BAM files were separately run through Atlas-SNP (Shen et al., 2010), Atlas-Indel, and Pindel (Ye et al., 2009). Data were aggregated for each tumor/normal pair, and variants were cross-checked for each tissue pair. Variant annotation was performed using Annovar (Wang et al., 2010), COSMIC (Forbes et al., 2011), and dbSNP (Sherry et al., 2001). Variant filtering was performed to remove low-quality variants. Cohort-level data processing was performed to remove additional false somatic calls by filtering against a cohort of normal tissues.

Ultra-deep Sequencing Analysis
Duplicate reads were aggregated and consensus variants were defined as the variant being present in 90% of the reads contributing to both halves of the duplex molecule. Subsequent filtering was employed to remove variants in which there was either mapping error (tested using BLAST) or sequence error non-consensus block rate of 50% (Altschul et al., 1990). Variants detected in this dataset were annotated using ANNOVAR, COSMIC, and dbSNP annotations (Forbes et al., 2011; Sherry et al., 2001; Wang et al., 2010).

Mutational Signature
Mutations signatures were generated from a set of over 6,000 somatic mutations across a range of cancer types using non-smooth non-negative matrix factorization (nnSNMF) (K.R.C., unpublished data; Pascual-Montano et al., 2006). The solution resulted in 21 distinct mutational signatures, similar to those previously reported (Alexandrov et al., 2013; Gaudjoux and Seoighe, 2010), many of which could be correlated with previously published mutational modes, including APOBEC, UV radiation exposure, POLE hypermutation (Lawrence et al., 2013), and CpG island mutation. Mutations for this cohort were compared against the solved NMF to generate a mutational decomposition for each of the tumor samples. Samples were aggregated and compared using hierarchical clustering and other correlative statistics to clinical covariates.

Tumor Purity and Normalization Mutation Rate
Tumor purity was estimated using ASCAT and the tumor variant allelic fraction of driver genes. The average of both analyses was plotted against the number of mutations in each tumor, and the slope value was used to approximate the number of mutations that would have been identified in 100% tumor cellularity (Figure S1A).

Significantly Altered Genes
Several approaches were taken to dissect genes and pathways which were mutated more often than by chance in this dataset. We used the final MAF file (Tables S6A and S6B) to calculate significantly mutated genes using MutSig-CV and an inactivation bias test (Lawrence et al., 2013; Totoki et al., 2014).

Microsatellite Instability Coefficient
For details, see Supplemental Experimental Procedures.

Multivariable Cox Analysis
Cox proportional hazards analysis was performed using the survival (Therneau, 2000) package in R (R Development Core Team, 2008). We included age at diagnosis, gender, stage, grade, tumor type, histologic subtype (HIC), and mutation status (WNT, TGFB, TP53, KRAS, PI3K, and chromatin remodeling) in the multivariate Cox analysis. Country of origin and ethnicity were not included as covariates since they had no associated effect with survival.

RNA-Seq
Total RNA was prepared using the AllPrep RNA/DNA isolation kit (QiAGEN). RNA integrity was confirmed (RIN > 7.0) on a Bioanalyzer (Agilent). RNA-seq libraries were prepared using the TruSeq Stranded total RNA LT library kit (Illumina) following the manufacturer’s instructions. 100-base-pair-end sequencing was then performed to a minimum depth of 50 million reads of each sample on an Illumina HiSeq2000 sequencer.

Transcript Expression Analysis
Gene Expression of the WNT Pathway
The profiles in the RNA-seq dataset were quantile normalized, and log-transformed expression values were then centered to SDs from the median across
sample profiles. Tumors were split into two groups (those with WNT canonical pathway mutations and those without such mutations) and were scored for relative activity in the WNT pathway. The gene signature score within each tumor profile was defined as the average of the centered values for the WNT signature genes.

**Fusion Analysis**

The deFuse software version 0.6.1 (McPherson et al., 2011) with default settings was used to detect fusion genes. The deFuse results were further filtered by removing identified read through fusions, selecting coding regions, selecting in-frame (open reading frame) genes and selecting samples with a deFuse confidence score of >80%. This filtering resulted in a list of candidate fusion genes. To characterize these candidate fusion genes, we took each spanning junction read and using the BLAT tool in UCSC genome browser examined where the reads mapped. The fusions that mapped with 100% identity to each part of the identified fusion (gene1 or gene2) were selected for further analysis. This filter removed genes that mapped to multiple locations. Next, each RNA BAM from candidate fusion genes was examined in IGV, looking for stacked soft clipped reads, changes in coverage, at the identified fusion breakpoints. The sequence of each soft clipped read was brought into the UCSC genome browser and mapped using BLAT. Only fusions that had reads that matched (100%) the identified fusion genes were considered further.

**ACCESSION NUMBERS**

The accession number for the sequence data reported in this paper is dbGAP: PRJNA280134.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, four figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.12.005.

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