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Recurrent Rearrangements in *PRKACA* and *PRKACB* in Intraductal Oncocytic Papillary Neoplasms of the Pancreas and Bile Duct

Aatur D. Singhi, MD PhD[1,2], **Laura D. Wood, MD PhD**[3,4], **Emma Parks**[5], **Michael S. Torbenson, MD**[6], **Matthäus Felsenstein, MD**[3,7], **Ralph H. Hruban, MD**[3,4], **Marina N. Nikiforova, MD**[1], **Abigail I. Wald, PhD**[1], **Cihan Kaya, PhD**[1], **Yuri E. Nikiforov, MD PhD**[1], **Laura Favazza, DO**[1], **Jin He, MD PhD**[8], **Kevin McGrath, MD**[9], **Kenneth E. Fasanella, MD**[9], **Randall E. Brand, MD**[9], **Anne Marie Lennon, MD PhD**[10], **Alessandro Furlan, MD**[11], **Anil K. Dasyam, MD**[11], **Amer H. Zureikat, MD**[12], **Herbert J. Zeh, MD**[13], **Kenneth Lee, MD**[12], **David L. Bartlett, MD**[12], **Adam Slivka, MD PhD**[9]

[1]Department of Pathology, University of Pittsburgh Medical Center, Pittsburgh, PA, USA.

[2]Pittsburgh Liver Research Center, University of Pittsburgh Medical Center, Pittsburgh, PA, USA.

[3]Department of Pathology, Sol Goldman Pancreatic Cancer Research Center, Johns Hopkins University School of Medicine, Baltimore, MD, USA.

[4]Department of Oncology, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins School of Medicine, Baltimore, MA, USA.

[5]Carnegie Mellon University, Pittsburgh, PA, USA.

[6]Department of Laboratory Medicine and Pathology, Mayo Clinic Rochester, Rochester, MN, USA.

[7]Department of Surgery, Charité—Universitätsmedizin Berlin, Berlin, Germany.

[8]Department of Surgery, Johns Hopkins University School of Medicine, Baltimore, MD, USA.

[9]Department of Medicine, University of Pittsburgh Medical Center, Pittsburgh, PA, USA.

[10]Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Correspondence to: Aatur D. Singhi, MD PhD, UPMC Presbyterian Hospital, 200 Lothrop Street, Room A616.2, Pittsburgh, PA 15213, Phone: 412-864-1508, singhiad@upmc.edu.

Author Contributions

Study Concept and Design: ADS, LDW, RHH, MNN, AS

Acquisition of Data: ADS, LDW, EP, MST, MF, RHH, MNN, AIW, CK, YEN, LF, JH, KM, KEF, REB, AML, AF, AKD, AHZ, HJZ, DLB, AS

Analysis and Interpretation of Data: ADS, LDW, MST, RHH, MNN, AIW, CK, AS

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As added proof: The authors are aware of another study also demonstrating the DNAJB1-PRKACA fusion in oncocytic neoplasms of the pancreatobiliary tree, which provides further confirmation of our findings.

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[11]Department of Radiology, University of Pittsburgh Medical Center, Pittsburgh, PA, USA.

[12]Department of Surgery, University of Pittsburgh Medical Center, Pittsburgh, PA, USA.

[13]Department of Surgery, University of Texas Southwestern, Dallas, TX, USA.

Abstract

Background & Aims: Intraductal oncocytic papillary neoplasms (IOPNs) of the pancreas and bile duct contain epithelial cells with numerous, large mitochondria and are cystic precursors to pancreatic ductal adenocarcinoma (PDAC) and cholangiocarcinoma (CCA), respectively. However, IOPNs do not have the genomic alterations found in other pancreatobiliary neoplasms. In fact, no recurrent genomic alterations have been described in IOPNs. PDACs without activating mutations in *KRAS* contain gene rearrangements, so we investigated whether IOPNs have recurrent fusions in genes.

Methods: We analyzed 20 resected pancreatic IOPNs and 3 resected biliary IOPNs using a broad RNA-based targeted sequencing panel to detect cancer-related fusion genes. Four invasive PDACs and 2 intrahepatic cholangiocarcinomas from the same patients as the IOPNs, were also available for analysis. Samples of pancreatic cyst fluid (n=5, collected before surgery) and bile duct brushings (n=2) were analyzed for translocations. For comparison, we analyzed pancreatobiliary lesions from 126 patients without IOPN (controls).

Results: All IOPNs evaluated were found to have recurring fusions of *ATP1B1-PRKACB* (n = 13), *DNAJB1–PRKACA* (n = 6), or *ATP1B1–PRKACA* (n = 4). These fusions were also found in corresponding invasive PDACs and intrahepatic cholangiocarcinomas, as well as in matched pancreatic cyst fluid and bile duct brushings. These gene rearrangements were absent from all 126 control pancreatobiliary lesions.

Conclusions: We identified fusions in *PRKACA* and *PRKACB* genes in pancreatic and biliary IOPNs, as well as in PDACs and pancreatic cyst fluid and bile duct cells from the same patients. We did not identify these gene fusions in 126 control pancreatobiliary lesions. These fusions might be used to identify patients at risk for IOPNs and their associated invasive carcinomas.

Lay Summary:

Researchers identified a genetic alteration that is specific to intraductal oncocytic papillary neoplasms. This alteration might serve as a marker of patients at risk for these neoplasms.

Keywords

kinase A; CCA; liver; diagnostic factor

Introduction

Intraductal oncocytic papillary neoplasms (IOPNs) of the pancreas and bile ducts are rare, cystic precursors to invasive carcinoma. IOPNs were originally described as a variant of intrahepatic cholangiocarcinoma (CCA), and only later were recognized to also occur in the duct system of the pancreas as a precursor to invasive pancreatic cancer (PDAC).[1, 2] Many of the clinical and pathologic features of pancreatic IOPNs overlap those with intraductal

papillary mucinous neoplasms (IPMNs) of the pancreas, and IOPNs were therefore classified as an IPMN subtype in 2010 by the World Health Organization (WHO).[3] Similarly, biliary IOPNs were subclassified as an intraductal papillary neoplasm of the bile duct (IPNB).[4]

Although IOPNs clinically and pathologically mimic other cystic lesions of the pancreas and bile ducts, IOPNs do have a number of distinctive features. In contrast to other IPMN and IPNB subtypes, IOPNs are composed of complex, arborizing papillae that are lined by multiple layers of cuboidal neoplastic epithelial cells with abundant intracytoplasmic mitochondria and uniform nuclei containing a single, prominent nucleolus. Of particular note, IOPNs do not harbor activating mutations in *KRAS* or *GNAS*, which are frequently found in both IPMNs and IPNBs.[5–7] In contrast, genomic alterations in *ARHGAP26*, *AXL1*, *EPHA8* and *ERBB4* have been identified in IOPNs, but for only two cases each.[5] Hence, in 2019, IOPNs of the pancreas were reclassified by the WHO as a separate neoplastic entity.[8]

While IOPNs are now recognized as a distinct neoplasm, at least within the pancreas, the underlying genomic alterations associated with IOPNs remain a mystery. Despite targeted next-generation sequencing, whole-exome sequencing and whole-genome sequencing studies, an entity-defining genomic alteration or group of genomic alterations within a common pathway have not been described for pancreatic or biliary IOPNs.[5] Recognizing that fusion genes can be missed using standard sequencing approaches, we hypothesized that the genetic drivers of IOPNs could be recurrent gene fusions. Moreover, considering *KRAS* wild-type PDACs often harbor recurrent kinase gene rearrangements, we further hypothesized that IOPN and IOPN-associated carcinomas may be characterized by recurrent gene fusions in genes coding for kinases.[9–11] Therefore, in order to elucidate whether gene fusions may be genetic drivers of IOPNs, we analyzed a series of pancreatic and biliary IOPNs, and, for a subset of cases, associated invasive carcinomas, using a broad RNA-based targeted sequencing panel of cancer-related fusion genes. We then analyzed corresponding pancreatic cyst fluid and bile duct brushings to determine if the fusion gene identified in the neoplastic cells could also be identified preoperatively by cyst fluid and bile duct brushing analysis.

Methods

Study samples

Study approval was obtained from the authors' respective institutional review boards. The anatomic pathology surgical archives from the Departments of Pathology at the University of Pittsburgh Medical Center (UPMC) and Johns Hopkins Hospital were queried for the diagnosis of intraductal oncocytic papillary neoplasm (IOPN). Cases with hematoxylin-and-eosin (H&E) stained slides and corresponding formalin-fixed paraffin-embedded (FFPE) tissue were retrieved and reviewed to confirm the diagnosis of an IOPN by a trained surgical pathologist with gastrointestinal tract and hepatopancreatobiliary subspecialty training (A.D.S., L.D.W. and/or R.H.H.). The diagnosis of an IOPN was based on the 2010/2019 WHO Classification of Tumours of the Digestive System.[3, 4, 8] In total, 23 IOPNs were identified, including 20 of the pancreas and three biliary IOPNs. For a subset of cases, an additional FFPE tissue block was obtained from an associated invasive carcinoma. Further,

patient pathology records were cross-referenced for the availability of a preoperative molecular testing specimen. Specifically, stored DNA and RNA from matched preoperative pancreatic cyst fluid submitted for PancreaSeqV2 testing and bile duct brushings submitted for BiliSeq testing were identified from the Molecular and Genomic Pathology (MGP) Laboratory at UPMC.

A separate non-IOPN control cohort was selected from the anatomic pathology surgical archives at UPMC to include diagnostic entities that may enter the differential diagnosis of both a pancreatic and a biliary IOPN. The diagnosis of each case was also confirmed (by A.D.S.). Finally, medical records including pathology reports were reviewed to obtain patient age, gender, and both size and location of the pancreatobilary lesions.

Nucleic acid extraction

Nucleic acid extraction, as well as subsequent DNA- and RNA-based targeted next-generation sequencing (NGS), was performed within the Clinical Laboratory Improvement Amendments-certified and College of American Pathologists-accredited MGP Laboratory at UPMC. Genomic DNA and mRNA was isolated from either FFPE tissue (surgical resection specimens), pancreatic cyst fluid obtained by endoscopic ultrasound-fine needle aspiration (EUS-FNA) or bile duct brushings obtained by endoscopic retrograde cholangiopancreatography (ERCP) using the DNeasy Blood and Tissue kit on automated QIAcube instrument (QIAGEN, Germantown, MD) or the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche, Indianapolis, IN) on Compact MagNA Pure (Roche, Indianapolis, IN). Extracted DNA and RNA was quantitated on the Glomax Discover using the QuantiFluor ONE dsDNA System and the QuantiFluor RNA system, respectively (Promega, Madison, WI).

DNA-based targeted next-generation sequencing

Amplification-based targeted DNA NGS for PancreaSeqV2 was performed with custom AmpliSeq primers for genomic regions of interest that included hotspots within *KRAS*, *GNAS*, *NRAS*, *HRAS*, *BRAF*, *RNF43*, *CTNNB1*, *VHL*, *PIK3CA*, *PTEN*, *TP53* and *SMAD4* with primer sequences and performance characteristics as previously described.[12] Similarly, BiliSeq DNA NGS-based testing was performed with custom AmpliSeq primers for targeted hotspots within the following genes: *AKT1*, *ALK*, *ATM*, *BRAF*, *CDKN2A*, *CTNNB1*, *EGFR*, *ERBB2*, *ERBB4*, *FGFR1*, *FGFR2*, *FGFR3*, *GNA11*, *GNAQ*, *GNAS*, *HRAS*, *IDH1*, *IDH2*, *KIT*, *KRAS*, *MET*, *NRAS*, *PDGFRA*, *PIK3CA*, *PTEN*, *SMAD4*, *TP53* and *VHL* as previously described.[13] Amplicons were barcoded, ligated with specific adapters and purified. DNA library quantity and quality checks were performed using the 4200 TapeStation (Agilent Technologies, Santa Clara, CA). The Ion Chef was used to prepare and enrich templates and enable testing via Ion Sphere Particles on a semiconductor chip. Massive parallel sequencing was carried out on an Ion S5 XL System according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA) and data was analyzed with the Torrent Suite Software v5.2.2 for point mutations, small insertions/deletions and copy number alterations. Each variant was prioritized according to the 2017 AMP/ASCO/CAP joint consensus guidelines for interpretation of sequence variants in cancer using a tier-based system.[14] Tier I, Tier II and Tier III variants were reported;

however, only Tier I and Tier II variants were used for subsequent analysis. The limit of detection of the assay for single nucleotide variants/indels was at 3% mutant allele frequency (AF). The minimum depth of coverage for testing was 1000x. For each mutation identified, an AF was calculated based on the number of reads of the mutant allele versus the wild-type allele and reported as a percentage.[12] Copy number variation analysis was performed as previously described.[15] The total depth of sequencing coverage of each sequenced region was normalized by the normal controls and calculated per sequenced case. A decrease in sequencing coverage below established cut-offs with simultaneous presence of sequence variant at high AF was considered a copy number loss. In contrast, an increase in sequencing coverage above established cut-offs were interpreted as a copy number gain. A gene amplification was defined by the presence of ≥6 copies of a variant as previously described and validated using fluorescence *in situ* hybridization analysis.[15, 16] Of note, corresponding germline DNA was not sequenced as part of this analysis.

RNA-based targeted next-generation sequencing

Targeted NGS-based testing from mRNA was also performed within the MGP lab at UPMC using the Oncomine Comprehensive Assay v3 (OCAv3) RNA primers (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol. The OCAv3 RNA panel (Supplementary Table 1) evaluates 760 fusion genes that comprises intergenic and intragenic regions among 51 cancer-associated kinase genes. Briefly, 50ng mRNA is reverse transcribed into cDNA and subject to multiplex PCR to amplify the regions of interest. Amplicons were barcoded, ligated with specific adapters and purified. RNA library quantity and quality check were performed using the 4200 TapeStation (Agilent Technologies, Santa Clara, CA). The Ion Chef was used to prepare and enrich templates and enable testing via Ion Sphere Particles on a semiconductor chip. Massive parallel sequencing was carried out on an Ion S5 XL System according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA) and data was analyzed with the Torrent Suite Software v5.2.2 and an in-house bioinformatics program, Variant Explorer (UPMC) for RNA fusions and RNA expression. The limit of detection of the RNA assay was 1–5% tumor cells. More than 50 fusion-specific reads that cross the fusion breakpoint are required to make a positive fusion call.

Whole transcriptome (RNA) sequencing and analysis

RNA sequencing libraries were prepared using the Illumina TruSeq RNA Exome Sample Preparation Kit v1, according to the manufacturer's protocol. Cluster generation and paired-end sequencing were performed on an Illumina HiSeq2500 using a HiSeq Paired-End (PE) Rapid Cluster Kit v2 and HiSeq Rapid SBS Lit v2 (Illumina). For detection of gene fusions, the sequencing reads were collected, and a quality filtering was applied based on Q-score.[17] The Chimerascan algorithm, which utilizes Tophat aligner, was used to identify fusion transcripts.[18, 19] The fusion transcripts detected with Chimerascan were filtered using custom filtering algorithm that utilizes the distance between breakpoints and various cancer databases, such as Atlas, CIS (RTCGD) and CHCG.[20–22] The junctions and the individual exon expression levels were used to visualize the fusion point.

For gene expression analysis, the filtered high-quality reads from HiSeq sequencing system were aligned to human genome (hg19-GRCh37) using TopHat aligner and the number of reads mapped to each gene was calculated using RSeM and featureCounts tools.[19, 23, 24] From the read counts, the differential expression analysis was performed using the edgeR package.[25] The expression levels for target genes implicated in the Protein Kinase A (PKA) and Epidermal Growth Factor Receptor (EGFR)/Extracellular signal-Regulated Kinase (ERK) signaling pathways were evaluated as previously published.[26] Genes with a fold change of >2 and a p-value of <0.05 were selected as differentially expressed genes and used in pathway analysis.

PRKACA and *PRKACB* fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization (FISH) was used to assess a break-apart probe to the *PRKACA* gene as previously described and the *PRKACB* gene.[27] For *PRKACA*, the telomeric clones (RP11–63F22, CTD-2003D17, and CTC-708A18) were labeled by nick translation with Spectrum Green and the centromeric clone (CTC-548K16) was labeled with Spectrum Orange (Abbott Molecular, Des Plains, IL). For *PRKACB*, break-apart telomeric and centromeric probes labeled with Spectrum Green and Spectrum Orange, respectively, were obtained commercially (Empire Genomics LLC, Williamsville, NY). Labeled clones were combined to create a dual-color, single fusion probe set. Formalin-fixed paraffin-embedded sections were cut at 4 μm thick, mounted on positively charged slides, baked for 15 min at 90°C, and then deparaffinized in xylene. Slides were dehydrated in 100% ethanol and allowed to air dry. Pre-treatment in 10 mM citric acid was followed by a NaCl protease treatment to remove proteins and non-DNA cellular components. The slide and probe were co-denatured and hybridized overnight. Slides were then washed and DAPI counterstain was applied as well as a glass coverslip. Visualization of the FISH signals was accomplished by using fluorescence microscope and pictures were captured by using a FISH imaging system (CytoVision, Leica Biosystems, Buffalo Grove, IL). Two technologists experienced in FISH independently scored 50 tumor nuclei for each case with positivity defined as >15% of tumor cells demonstrating split signals or isolated green signals. The expected FISH pattern for *DNAJB1-PRKACA* was the presence of isolated green signals (3’ end of *PRKACA*) due to an intrachromosomal deletion, while the *ATP1B1-PRKACA* and *ATP1B1-PRKACB* fusion genes should demonstrate split orange (5’ end of *PRKACA* and *PRKACB*) and green signals (3’ end of *PRKACA* and *PRKACB*).[27, 28] Overlapping cells (intact yellow signals) were excluded from analysis.

Results

The study cohort consisted of 23 patients who had a surgically resected IOPN (20 pancreatic and 3 biliary) (Figure 1 and Supplementary Figure 1). Their clinicopathologic features summarized in Table 1. The patients ranged in age from 25 to 75 years (mean, 57.4 years; median, 62 years) and the majority were male (16 of 23, 70%). The pancreatic IOPNs were relatively evenly distributed within the gland and ranged in size from 2.0 to 8.2 cm (mean, 4.7 cm; median, 4.6 cm). Data regarding the gross involvement of the pancreatic ducts were available for 14 cases. Two IOPNs involved the main pancreatic duct, five IOPNs a branch pancreatic duct and 7 IOPNs both the main and branch pancreatic ducts. Among the three

biliary IOPNs, two were centered within the left lobe of the liver, while the remaining biliary IOPN was located within the right lobe. The biliary IOPNs ranged in size from 2.8 to 4.3 cm and involved the intrahepatic bile duct with two cases also extending into the hilar bile duct. An associated invasive carcinoma was identified in association with 11 of 20 pancreatic IOPNs and in association with all three biliary IOPNs.

As part of routine clinical evaluation, preoperative specimens from 5 pancreatic IOPNs and 2 biliary IOPNs were also available. Among the 5 pancreatic IOPNs, pancreatic cyst fluid was obtained via endoscopic ultrasound-fine needle aspiration (EUS-FNA).[12] Similarly, endoscopic retrograde cholangiopancreatography (ERCP) was used to obtain bile duct brushings specimens from two patients with a biliary IOPN.[13]

Among the 20 surgically resected pancreatic IOPNs, DNA-based targeted next-generation sequencing was negative for genomic alterations in *KRAS*, *GNAS*, *NRAS*, *HRAS*, *BRAF*, *RNF43*, *CTNNB1*, *VHL*, *PIK3CA*, *PTEN*, *TP53* and *SMAD4*. Similarly, four IOPN-associated PDACs and five corresponding preoperative EUS-FNA obtained pancreatic cyst fluid specimens also tested negative. Molecular testing of three surgically resected biliary IOPNs for 28 genes commonly mutated in neoplasms involving the bile duct system did not identify pathologic genomic alterations. Further, two IOPN-associated intrahepatic CCAs and, as previously mentioned, two corresponding preoperative ERCP-obtained bile duct brushings were negative for genomic alterations.

Hypothesizing that the genetic drivers of IOPNs could be recurrent gene fusions not recognized by the previously performed clinical sequencing, we next performed RNA-based targeted sequencing of cancer-related fusion genes. All 20 pancreatic IOPNs harbored a gene fusion. These included ten with an *ATP1B1* (exon 1)-*PRKACB* (exon 2) fusion, six with a *DNAJB1* (exon 1)-*PRKACA* (exon 2) fusion, and four with an *ATP1B1* (exon 1)-*PRKACA* (exon 2) fusion (Figure 2). RNA-based targeted sequencing also identified an *ATP1B1-PRKACB* translocation in all three surgically resected biliary IOPNs. The aforementioned gene fusions were also present in corresponding IOPN-associated PDACs and intrahepatic CCAs. Further, the same fusion genes were detected in matched preoperative pancreatic cyst fluid and bile duct brushing specimens.

Fluorescence *in situ* hybridization (FISH) using a *PRKACA* and *PRKACB* break-apart probes (orange, 5’ end of PRKACA or PRKACB; green, 3’ end of PRKACA or PRKACB) were used to confirm the results of targeted RNA sequencing for Cases 1, 2, 5, 7, 10, 12, 13, 14, 15, 17, 21 and 23 (Supplementary Figure 2). For Cases 2, 7 and 12 that harbored a *DNAJB1-PRKACA* fusion gene, *PRKACA* break-apart FISH showed isolated green signals (3’ end of *PRKACA*) which is expected with an intrachromosomal deletion of the 5’ end of *PRKACA* and resulting fusion with *DNAJB1*. In comparison, the detection of an *ATP1B1-PRKACA* fusion gene for Cases 10, 13 and 15 was confirmed by the presence of split orange and split green signals. Among these IOPNs with a *PRKACA* rearrangement, *PRKACB* break-apart FISH demonstrated a wild-type copy number for *PRKACB* (yellow, overlapping orange and green signals). However, split orange and split green signals for *PRKACB* were present for Cases 1, 5, 14, 17, 21 and 23, which were found to have an

ATP1B1-PRKACB fusion gene by RNA-based targeted sequencing, but wild-type for *PRKACA*.

To determine if gene rearrangements in *PRKACA* and *PRKACB* were unique to IOPNs, RNA-based targeted sequencing was performed on a separate cohort of 126 non-IOPN pancreatobiliary lesions (Supplementary Table 2). *PRKACA* and *PRKACB* fusion genes were not identified in 68 PDACs with four arising from a classical IPMN, 13 extrahepatic and 10 intrahepatic CCAs with two arising from an IPNB, 3 gallbladder adenocarcinomas with one arising from an intracholecystic papillary-tubular neoplasm (ICPN) and 2 acinar cell carcinomas of the pancreas. Rearrangements in *PRKACA* and *PRKACB* were also absent in 30 pancreatic cystic neoplasms that commonly clinically mimic a pancreatic IOPN: two IPMNs with high-grade dysplasia, 15 IPMNs with low-grade dysplasia, four MCNs with low-grade dysplasia, three cystic pancreatic neuroendocrine tumors, three solid-pseudopapillary neoplasms, two serous cystadenomas, and a lymphoepithelial cyst.

The presence and predicted structure of the *DNAJB1-PRKACA*, *ATP1B1-PRKACA* and *ATP1B1-PRKACB* fusion transcripts were also confirmed by whole transcriptome sequencing of 4 pancreatic IOPNs. In comparison to 9 IPMNs (Supplementary Table 2, Cases 97–101, 105–106, 108 and 110), high levels of expression for exons 2 through 9 of *PRKACA* and *PRKACB* were identified for Cases 7 (*DNAJB1-PRKACA*) and 10 (*ATP1B1-PRKACA*), and Cases 8 and 14 (*ATP1B1-PRKACB*), respectively (Figure 3A through 3C). Both DNAJB1 and ATP1B1 had similar levels of expression in both IOPNs and IPMNs. Considering the *DNAJB1-PRKACA* fusion gene was previously described in fibrolamellar hepatocellular carcinomas (FL-HCCs) and whole transcriptome sequencing has implicated the Protein Kinase A (PKA) and Epidermal Growth Factor Receptor (EGFR)/Extracellular signal-Regulated Kinase (ERK) signaling pathways in their pathogenesis, comparative gene expression analysis was performed between IOPNs and IPMNs (Figure 3D).[26] The IPMNs had mutations in *KRAS* (n = 4) or in both *KRAS* and *GNAS* (n = 5); but, by RNA-based targeted sequencing and whole transcriptome sequencing, were negative for *PRKACA* and *PRKACB* fusion transcripts. Consistent with FL-HCCs, IOPNs harboring *PRKACA* fusion transcripts had high levels of expression for PKA and EGFR/ERK signaling target genes as compared to IPMNs. However, except for PRKACB, similar gene expression patterns for PKA and EGFR/ERK signaling target genes were seen between IOPNs with a *PRKACB* fusion gene and IPMNs.

Discussion

The combination of molecular analysis and careful histopathologic review has been useful in refining the classification of pancreatic and biliary neoplasms. IOPNs do not harbor the key genomic alterations typically found in other neoplasms of the pancreas and biliary tree. Instead, all IOPNs of the pancreas and biliary tree that we studied had recurrent *ATP1B1-PRKACB*, *DNAJB1-PRKACA* or *ATP1B1-PRKACA* fusion genes. These fusion genes were also present in corresponding IOPN-associated invasive carcinomas but absent in a diverse cohort of non-IOPN associated pancreatobiliary lesions.

The *DNAJB1-PRKACA* fusion gene is created by a 400 kb deletion in chromosome 19 that results in an inframe fusion of the first exon of *DNAJB1*, a member of the heat shock 40 protein family, to exons two through ten of the *PRKACA* gene.[29, 30] *PRKACA* encodes the cAMP-dependent protein kinase A (PKA) catalytic subunit alpha. Under the transcriptional control of the *DNAJB1* promoter, the fusion results in increased expression of the chimeric protein over native PRKACA and, consequently, increased protein kinase A (PKA) activity. To date, the *DNAJB1-PRKACA* fusion gene has only been described in fibrolamellar hepatocellular carcinoma (FL-HCC).[27, 31] Analogous to IOPNs, prior to the discovery of this fusion, the molecular basis for FL-HCC has been enigmatic and genomic alterations in common oncogenes and tumor suppressors, such as *KRAS* or *TP53*, have not been found in FL-HCC. However, the *DNAJB1-PRKACA* fusion gene has been identified in nearly 100% of FL-HCCs and, supported by findings in animal models, is a driver of liver tumorigenesis.[32, 33] Interestingly, the neoplastic cells of FL-HCC are histologically reminiscent of those seen in IOPNs as they are characterized by abundant, eosinophilic cytoplasm and a prominent nucleus. Moreover, by whole transcriptome sequencing, we showed IOPNs harboring the *PRKACA* fusion transcript had a similar gene expression pattern as reported for FL-HCCs.[26] Thus, the identification of recurrent *DNAJB1-PRKACA* fusions in IOPNs parallels FL-HCCs and establishes this fusion as a bona fide genetic driver for both FL-HCCs and IOPNs.

Recently, the spectrum PKA-associated fusion genes was expanded to include *ATP1B1-PRKACA* and *ATP1B1-PRKACB*.[34] Through transcriptome analysis of a large cohort of CCAs, Nakamura *et al.* identified *PRKACA* and *PRKACB* fusions with the *ATP1B1* gene in two extrahepatic CCAs.[34] The *ATP1B1* gene codes for the Na^+/K^+-transporting ATPase subunit beta 1, and, similar to whole transcriptome analysis described herein, both fusion genes robustly induce the expression of PRKACA and PRKACB by more than 14-fold over the mean expression levels observed in CCAs without a fusion gene. Cells with the *ATP1B1-PRKACA* and *ATP1B1-PRKACB* fusion genes demonstrated increased or comparable PKA activity relative to those with wild-type PKA. Moreover, these alterations were mutually exclusive from mutations in *KRAS* and *GNAS*. Although Nakamura *et al.* did not report the corresponding histopathology of their cases, it's reasonable to speculate that the two extrahepatic cholangiocarcinomas arose from biliary IOPNs.

The unique and ubiquitous nature of *PRKACA* and *PRKACB* fusion genes among pancreatic IOPNs and absence in non-IOPN associated pancreatic lesions supports the recent WHO reclassification of IOPNs as a separate diagnostic entity. In addition, our study would suggest that IOPNs of the bile duct represent a distinct category of biliary neoplasms and are closely related, by PKA fusion genes, to pancreatic IOPNs. However, the dysregulation of PKA signaling is not unique to IOPNs, as fusion unrelated dysregulation of PKA signaling has been observed in other pancreatobiliary neoplasms.[6, 7, 35, 36] For example, *GNAS* mutations are common in IPMNs.[6, 7, 12, 35, 37–3940] Gsα, which is encoded by *GNAS*, is the stimulatory alpha subunit of heterotrimeric G proteins. Dissociation of Gsα from the β and γ subunits due to activating *GNAS* mutations results in increased cAMP production. In turn, cAMP acts as a second messenger that interacts with and activates PKA. Thus, while IOPNs are genetically different from IPMNs and IPNBs, these neoplasms may share a unifying pathogenesis with other pancreatobiliary neoplasms. Further, this would suggest PKA

signaling is not responsible for the distinctive pathologic features of IOPNs, and, therefore, other signaling pathways/mechanisms may be involved. In fact, whole transcriptome sequencing of IOPNs with *PRKACA* and *PRKACB* fusion transcripts showed distinctly different gene expression patterns among PKA and EGFR/ERK signaling target genes.

Apart from advancing our understanding of the genetic basis of IOPNs, our study has important clinical implications as the fusions we identified could aid in the classification of cystic lesions in the pancreas. Some pancreatic cysts are entirely benign, while others have significant malignant potential. Fluid can be aspirated from pancreatic cysts and both the DNA and RNA from the cyst fluid can be sequenced. The patterns of genetic alterations identified in the cyst fluid can then be used to classify the cyst type, and guide management.[12, 41] Since IOPNs don't harbor mutations in the genes commonly sequenced in cyst fluid (*KRAS*, *GNAS*, *RNF43*, *CTNNB1*, *VHL*, *PIK3CA*, *TP53* and *SMAD4*), they could easily be misclassified as nonneoplastic cysts.[42] As *PRKACA* and *PRKACB* fusion genes are specific for IOPNs, and these fusions were detectable in preoperative pancreatic cyst fluid and bile duct brushings, a molecular screening assay could be designed to improve the preoperative diagnosis of pancreatobiliary IOPNs.

It is worth noting that there are few limitations to our study. Although it represents one of the largest series of pancreatic and biliary IOPNs to be molecularly analyzed, the number of cases evaluated is relatively small and concluding gene rearrangements in *PRKACA* and *PRKACB* are a universal feature of IOPNs would be premature. Using FL-HCCs as an analogy, the majority of these tumors are characterized by the *DNAJB1-PRKACA* fusion gene, but a small proportion instead have inactivating mutations in *PRKAR1A*, a negative regulatory subunit of PKA.[28] These *PRKAR1A*-mutated FL-HCCs are reported to occur in the setting of the Carney complex syndrome, a rare autosomal disorder associated with germline alterations in *PRKAR1A*. In contrast to FL-HCCs, IOPNs are not considered to be among the neoplasms included within the Carney complex syndrome. Further, whole exome sequencing of IOPNs has not identified recurrent mutations in genes implicated in PKA signaling.[5] Another issue with this study is the lack of orthogonal confirmation of all cases for a *PRKACA* or *PRKACB* fusion gene. Orthogonal confirmatory testing of detected genomic alterations assures technical validity. We did validate the fusions in a subset cases using break-apart FISH for *PRKACA* and *PRKACB* and whole transcriptome sequencing, and the RNA-based targeted fusion gene panel we employed has been both internally and externally validated and is currently used within our clinical lab for patient care.[43] Lastly, we acknowledge our pancreatobiliary cohort of non-IOPN associated lesions may not be exhaustive to truly gauge whether *PRKACA* and *PRKACB* fusion genes never occur in non-IOPN pancreatobiliary neoplasms. Nevertheless, the cases chosen for analysis include those that are within the clinicopathologic differential diagnosis for both pancreatic and biliary IOPNs.

In summary, we report IOPNs and IOPN-associated carcinomas of the pancreas and bile duct harbor recurrent *ATP1B1-PRKACB*, *DNAJB1-PRKACA* or *ATP1B1-PRKACA* fusion genes. The recurrent nature of these fusion genes helps establish them as genetic drivers of IOPNs, and because these gene rearrangements do not occur in other tumors of the pancreatobiliary system, our findings support the WHO classification of IOPNs as a distinct

neoplastic entity from IPMNs and IPNBs. Finally, the identification of *PRKACA* and *PRKACB* fusion genes in preoperative pancreatic cyst fluid and bile duct brushings suggests that these gene fusions could potentially improve the clinical detection of pancreatobiliary IOPNs and associated carcinomas in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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What you need to know:

BACKGROUND AND CONTEXT:

No genetic alterations have been identified in intraductal oncocytic papillary neoplasms.

NEW FINDINGS:

Fusions were found in *PRKACA* and *PRKACB* genes in pancreatic and biliary IOPNs, as well as in PDACs and pancreatic cyst fluid and bile duct cells from the same patients. These gene fusions were not found in 126 control pancreatobiliary lesions.

LIMITATIONS:

This was a retrospective study of 23 resected IOPNs. Studies are needed to determine how these gene fusion might contribute to development of these neoplasms.

IMPACT:

These gene fusions might be used to identify patients at risk for IOPNs and their associated carcinomas.

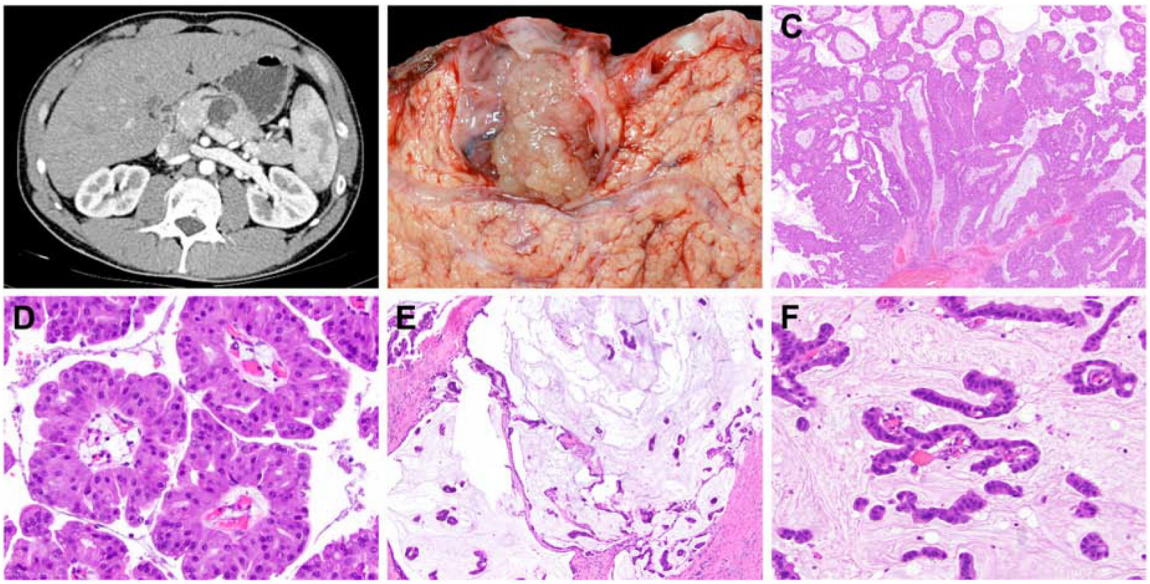

Figure 1.

Imaging and pathologic features of an intraductal oncocytic papillary neoplasm (IOPN) of the pancreas. Abdominal computed tomography (CT) of pancreatic IOPNs demonstrates involvement of either the main pancreatic duct, a branch duct (A) or both. Macroscopically, a typical IOPN is characterized by tan and friable, papillary excrescences within a cystic structure (B). By microscopic examination, these neoplasms are composed of complex, arborizing papillae (C) that are lined by multiple layers of cuboidal neoplastic epithelial cells with abundant eosinophilic, granular cytoplasm and uniform nuclei containing a single, prominent nucleolus (D). Pancreatic ductal adenocarcinoma can be found arising in association with an IOPN and often exhibits extracellular mucin accumulation (E) in which neoplastic cells are suspended, resembling a colloid carcinoma (F).

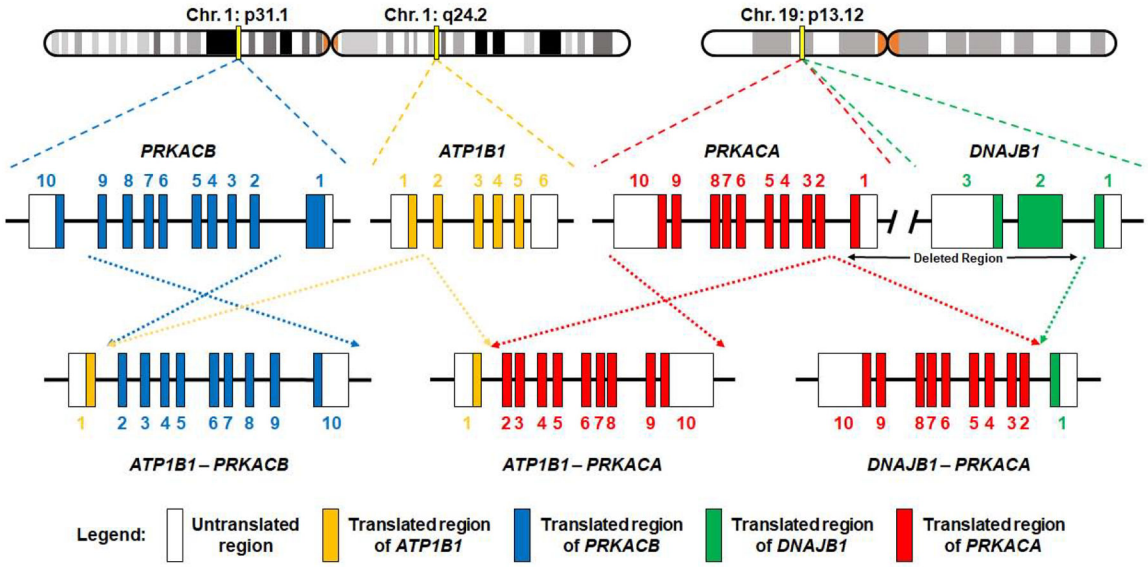

Figure 2.

Schematic representation of inframe fusion genes between *ATP1B1* (exon 1)-*PRKACB* (exon 2), *ATP1B1* (exon 1)-*PRKACA* (exon 2) and *DNAJB1* (exon 1)-*PRKACA* (exon 2). A translocation between the long and short arms of chromosome 1 results in an *ATP1B1-PRKACB* fusion gene, while the *ATP1B1-PRKACA* fusion gene is due to a translocation between the long arm of chromosome 1 and the short arm of chromosome 19. In contrast, a single 400 kb deletion in the short arm of chromosome 19 leads to the *DNAJB1-PRKACA* fusion gene.

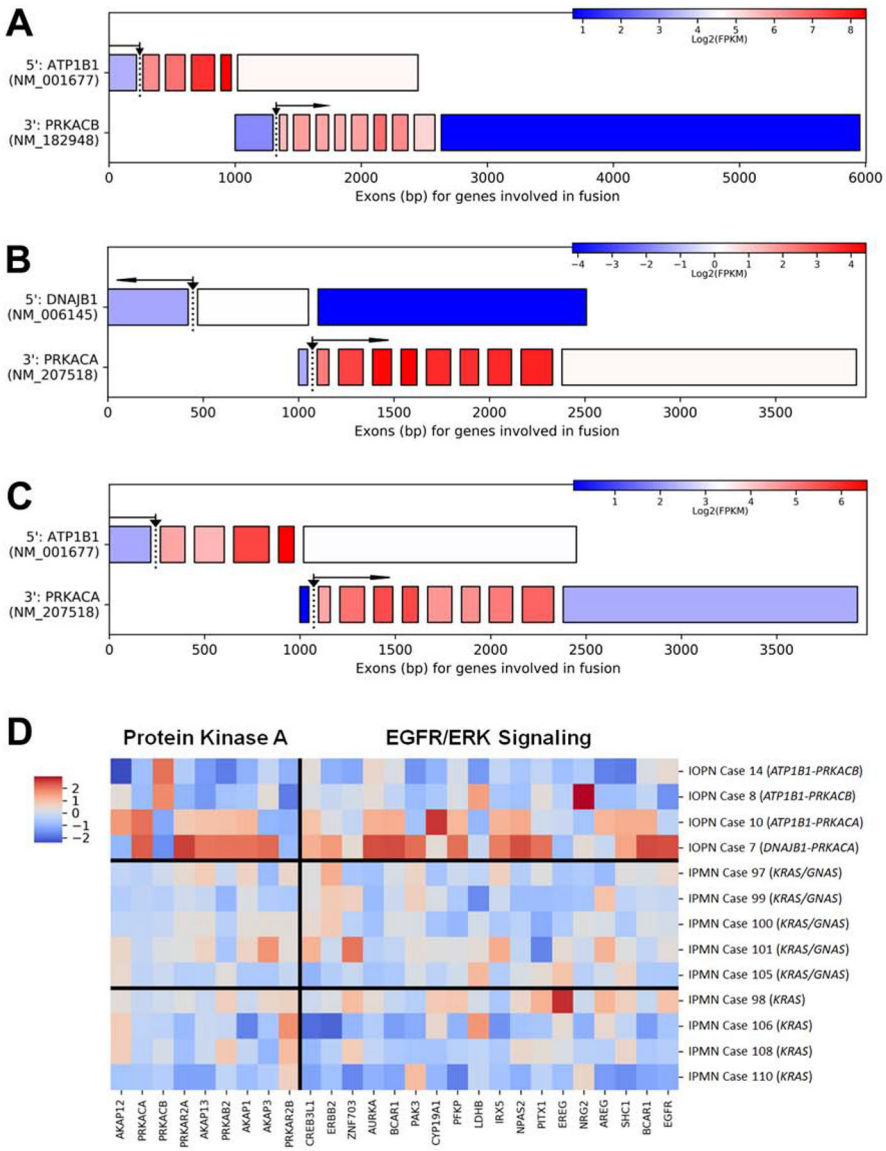

Figure 3.

Comparative transcriptome analysis of pancreatic IOPNs and intraductal papillary mucinous neoplasms (IPMNs). Fusion transcripts for *ATP1B1-PRKACB* (Case 8, A and Case 14 [data not shown]), *DNAJB1-PRKACA* (B, Case 10) and *ATP1B1-PRKACA* (C, Case 7) were identified in 4 pancreatic IOPNs by whole transcriptome (RNA) sequencing. In addition, high expression levels of exons 2 through 9 of *PRKACB* and *PRKACA* were detected in IOPNs as compared to IPMNs, while similar levels of expression were seen with ATP1B1 and DNAJB1. (D) Previous studies using whole transcriptome sequencing of fibrolamellar hepatocellular carcinomas (FL-HCCs) harboring the *DNAJB1-PRKACA* fusion gene have identified dysregulated expression for Protein Kinase A (PKA) and Epidermal Growth Factor Receptor (EGFR)/Extracellular signal-Regulated Kinase (ERK) signaling target genes.[26] Similar to FLHCCs, IOPNs with a *PRKACA* fusion gene showed altered expression for PKA and EGFR/ERK signaling target genes as compared to IPMNs and

IOPNs with a *PRKACB* fusion gene. Except for PRKACB, the expression profile among PKA and MAPK/ERK signaling target genes was essentially the same between IPMNs and IOPNs with a *PRKACB* fusion gene.

Table 1.

Clinical and pathologic findings of 23 patients with an intraductal oncocytic papillary neoplasm of the pancreatobiliary duct.

Case	Age (years)	Sex	Origin	Type	Location	Size (cm)	Associated Carcinoma	Gene Fusion
1*	43	F	Pancreas	Branch Pancreatic Duct	Pancreatic Body	3.4	No	*ATP1B1* (exon 1) - *PRKACB* (exons 2 to 10)
2*	25	M	Pancreas	Branch Pancreatic Duct	Pancreatic Body	5.1	Yes***	*DNAJB1* (exon 1) - *PRKACA* (exons 2 to 10)
3*	51	F	Pancreas	Branch Pancreatic Duct	Pancreatic Body	4.7	No	*ATP1B1* (exon 1) - *PRKACB* (exons 2 to 10)
4	67	M	Pancreas	Branch Pancreatic Duct	Pancreatic Head	4.5	No	*ATP1B1* (exon 1) - *PRKACB* (exons 2 to 10)
5	54	M	Pancreas	Mixed Main and Branch Pancreatic Duct	Pancreatic Head	3.7	Yes***	*ATP1B1* (exon 1) - *PRKACB* (exons 2 to 10)
6	48	F	Pancreas	Branch Pancreatic Duct	Pancreatic Body	4.5	Yes***	*ATP1B1* (exon 1) - *PRKACB* (exons 2 to 10)
7*	57	M	Pancreas	Mixed Main and Branch Pancreatic Duct	Pancreatic Tail	5.3	Yes	*DNAJB1* (exon 1) - *PRKACA* (exons 2 to 10)
8	64	M	Pancreas	Mixed Main and Branch Pancreatic Duct	Pancreatic Head	7.0	Yes***	*ATP1B1* (exon 1) - *PRKACB* (exons 2 to 10)
9*	27	M	Pancreas	Mixed Main and Branch Pancreatic Duct	Pancreatic Body	4.5	No	*DNAJB1* (exon 1) - *PRKACA* (exons 2 to 10)
10	72	M	Pancreas	Pancreatic Duct**	Pancreatic Head	6.0	Yes	*ATP1B1* (exon 1) - *PRKACA* (exons 2 to 10)
11	47	M	Pancreas	Main Pancreatic Duct	Pancreatic Head	2.5	Yes	*ATP1B1* (exon 1) - *PRKACA* (exons 2 to 10)
12	74	M	Pancreas	Pancreatic Duct**	Pancreatic Head and Body	6.0	Yes	*DNAJB1* (exon 1) - *PRKACA* (exons 2 to 10)
13	52	F	Pancreas	Pancreatic Duct**	Pancreatic Tail	2.5	No	*ATP1B1* (exon 1) - *PRKACA* (exons 2 to 10)
14	65	M	Pancreas	Mixed Main and Branch Pancreatic Duct	Pancreatic Tail	8.2	No	*ATP1B1* (exon 1) - *PRKACB* (exons 2 to 10)
15	63	M	Pancreas	Mixed Main and Branch Pancreatic Duct	Pancreatic Head	2.0	No	*ATP1B1* (exon 1) - *PRKACA* (exons 2 to 10)
16	64	M	Pancreas	Pancreatic Duct**	Pancreatic Head	6.0	Yes	*DNAJB1* (exon 1) - *PRKACA* (exons 2 to 10)
17	57	M	Pancreas	Pancreatic Duct**	Pancreatic Head	3.5	No	*ATP1B1* (exon 1) - *PRKACB* (exons 2 to 10)
18	67	M	Pancreas	Main Pancreatic Duct	Pancreatic Tail	2.5	Yes	*ATP1B1* (exon 1) - *PRKACB* (exons 2 to 10)
19	50	F	Pancreas	Mixed Main and Branch Pancreatic Duct	Pancreatic Body	6.5	Yes	*DNAJB1* (exon 1) - *PRKACA* (exons 2 to 10)
20	75	F	Pancreas	Pancreatic Duct**	Pancreatic Head	5.1	No	*ATP1B1* (exon 1) - *PRKACB* (exons 2 to 10)
21*	65	M	Bile Duct	Intrahepatic and Hilar Bile Ducts	Left Liver Lobe	3.5	Yes***	*ATP1B1* (exon 1) - *PRKACB* (exons 2 to 10)
22*	62	M	Bile Duct	Intrahepatic Bile Duct	Right Liver Lobe	2.8	Yes	*ATP1B1* (exon 1) - *PRKACB* (exons 2 to 10)
23	71	F	Bile Duct	Intrahepatic and Hilar Bile Ducts	Left Liver Lobe	4.3	Yes***	*ATP1B1* (exon 1) - *PRKACB* (exons 2 to 10)

Abbreviations: cm, centimeters; F, female; M, male.

*In addition to the patient's surgical specimen, the corresponding preoperative specimen (pancreatic cyst fluid or bile duct brushing/biopsy) was molecularly evaluated.

** The level of involvement (e.g. main pancreatic duct, branch pancreatic duct or mixed main and branch pancreatic duct) is unknown.

*** The associated carcinoma was also molecular evaluated for the presence of the indicated gene fusion.