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# Pancreatic intraductal tubulopapillary neoplasm is genetically distinct from intraductal papillary mucinous neoplasm and ductal adenocarcinoma

Olca Basturk[1], Michael F Berger[1], Hiroshi Yamaguchi[2], Volkan Adsay[3], Gokce Askan[1], Umesh K Bhanot[1], Ahmet Zehir[1], Fatima Carneiro[4], Seung-Mo Hong[5], Giuseppe Zamboni[6], Esra Dikoglu[7], Vaidehi Jobanputra[7,8], Kazimierz O Wrzeszczynski[7], Serdar Balci[3], Peter Allen[9], Naoki Ikari[10], Shoko Takeuchi[10], Hiroyuki Akagawa[10], Atsushi Kanno[11], Tooru Shimosegawa[11], Takanori Morikawa[12], Fuyuhiko Motoi[12], Michiaki Unno[12], Ryota Higuchi[13], Masakazu Yamamoto[13], Kyoko Shimizu[14], Toru Furukawa[15] and David S Klimstra[1]

*[1]Department of Pathology, Memorial Sloan Kettering Cancer Center, New York, NY, USA; [2]Department of Pathology, Tokyo Medical University, Tokyo, Japan; [3]Department of Pathology, Emory University, Atlanta, GA, USA; [4]Department of Pathology, Centro Hospitalar São João/Faculty of Medicine of Porto University and Institute for Research and Innovation in Health/Institute of Molecular Pathology and Immunology of the University of Porto (Ipatimup), Porto, Portugal; [5]Department of Pathology, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Korea; [6]Department of Pathology, University of Verona, Ospedale S.C.-Don Calabria-Negrar, Verona, Italy; [7]New York Genome Center, Molecular Diagnostics, New York, NY, USA; [8]Department of Pathology, Colombia University Medical Center, New York, NY, USA; [9]Department of Surgery, Memorial Sloan Kettering Cancer Center, New York, NY, USA; [10]Institute for Integrated Medical Sciences, Tokyo Women's Medical University, Tokyo, Japan; [11]Department of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai, Japan; [12]Department of Surgery, Tohoku University Graduate School of Medicine, Sendai, Japan; [13]Department of Surgery, Tokyo Women's Medical University, Tokyo, Japan; [14]Department of Gastroenterology, Tokyo Women's Medical University, Tokyo, Japan and [15]Department of Pathology, Tokyo Women's Medical University, Tokyo, Japan*

**Intraductal tubulopapillary neoplasm is a relatively recently described member of the pancreatic intraductal neoplasm family. The more common member of this family, intraductal papillary mucinous neoplasm, often carries genetic alterations typical of pancreatic infiltrating ductal adenocarcinoma (*KRAS*, *TP53*, and *CDKN2A*) but additionally has mutations in *GNAS* and *RNF43* genes. However, the genetic characteristics of intraductal tubulopapillary neoplasm have not been well characterized. Twenty-two intraductal tubulopapillary neoplasms were analyzed by either targeted next-generation sequencing, which enabled the identification of sequence mutations, copy number alterations, and selected structural rearrangements involving all targeted (≥300) genes, or whole-exome sequencing. Three of these intraductal tubulopapillary neoplasms were also subjected to whole-genome sequencing. All intraductal tubulopapillary neoplasms revealed the characteristic histologic (cellular intraductal nodules of back-to-back tubular glands lined by predominantly cuboidal cells with atypical nuclei and no obvious intracellular mucin) and immunohistochemical (immunolabeled with MUC1 and MUC6 but were negative for MUC2 and MUC5AC) features. By genomic analyses, there was loss of *CDKN2A* in 5/20 (25%) of these cases. However, the majority of the previously reported intraductal papillary mucinous neoplasm-related alterations were absent. Moreover, in contrast to most ductal neoplasms of the pancreas, MAP-kinase pathway was not involved. In fact, 2/22 (9%) of intraductal tubulopapillary neoplasms did not reveal any mutations in the tested genes. However, certain chromatin remodeling genes (*MLL1, MLL2, MLL3, BAP1, PBRM1*, *EED*, and *ATRX*)**

Correspondence: Prof Dr DS Klimstra, MD, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, NY 10065, USA.
E-mail: klimstrd@mskcc.org
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**were found to be mutated in 7/22 (32%) of intraductal tubulopapillary neoplasms and 27% harbored phosphatidylinositol 3-kinase (PI3K) pathway (*PIK3CA*, *PIK3CB*, *INPP4A,* and *PTEN*) mutations. In addition, 4/18 (18%) of intraductal tubulopapillary neoplasms had *FGFR2* fusions (*FGFR2-CEP55, FGFR2-SASS6, DISP1-FGFR2, FGFR2-TXLNA,* and *FGFR2-VCL*) and 1/18 (5.5%) had *STRN-ALK* fusion. Intraductal tubulopapillary neoplasm is a distinct clinicopathologic entity in the pancreas. Although its intraductal nature and some clinicopathologic features resemble those of intraductal papillary mucinous neoplasm, our results suggest that intraductal tubulopapillary neoplasm has distinguishing genetic characteristics. Some of these mutated genes are potentially targetable. Future functional studies will be needed to determine the consequences of these gene alterations.**
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Intraductal tubulopapillary neoplasm is a relatively recently described member of the pancreatic intraductal neoplasm family.[1–10] First reported by Tajiri *et al*.[2] under the heading of intraductal tubular carcinoma in 2004, the entity is now designated *intraductal tubulopapillary neoplasm*,[11] although limited papilla formation is seen only in a minority of cases.[1] The clinical findings of intraductal tubulopapillary neoplasm are often indistinguishable from those of intraductal papillary mucinous neoplasm of the pancreas, the prototype of the pancreatic intraductal neoplasm family. For example, both intraductal papillary mucinous neoplasms and intraductal tubulopapillary neoplasms present as a multinodular tumor with solid and cystic areas. Although the cystic areas may be less evident in intraductal tubulopapillary neoplasms, their intraductal nature may be appreciated radiographically in some cases. Also, preliminary studies suggest that, similar to intraductal papillary mucinous neoplasms, intraductal tubulopapillary neoplasms are less aggressive neoplasms than conventional pancreatic ductal adenocarcinomas, even if there is an associated invasive carcinoma.[1,10]

However, intraductal tubulopapillary neoplasm has several distinguishing pathologic characteristics. Microscopically, the intraductal tumor nodules are typically cellular and punctuated by numerous tubules, which are prominent in most cases. The cells are cuboidal and relatively uniform but at the same time show substantial cytologic atypia. Although amorphous acidophilic secretions may be seen in rare cases, typically there is no visible intracytoplasmic mucin. In addition, the MUC expression profile of intraductal tubulopapillary neoplasm points to a gastro-pancreatic rather than intestinal lineage: MUC1 and MUC6 are expressed in most cases, while MUC2 and MUC4 are negative.[1,6,10] MUC5AC is reported to be only occasionally positive.[1,12]

With the introduction of routine genomic analyses, including next-generation sequencing,[13–15] various genetic alterations have been identified in intraductal papillary mucinous neoplasms. As in many ductal neoplasms including pancreatic ductal adenocarcinomas, activating *KRAS* mutations are the most common mutations and have been detected in the majority of intraductal papillary mucinous neoplasms.[14,16–22] *TP53* mutations occur in cases with high-grade dysplasia and *CDKN2A* may also be altered. More interestingly, activating *GNAS* mutations have been identified in approximately half of intraductal papillary mucinous neoplasms,[14,15,23–26] particularly in the intestinal subtype.[14,23,25] Inactivating mutations in the *RNF43* gene, a likely tumor suppressor and negative regulator of the Wnt signaling pathway,[27] are also seen frequently in intraductal papillary mucinous neoplasms.[14,24] Less common alterations involve *PIK3CA, SMAD4*, *BRAF*, *CTNNB1/β-catenin*, *IDH1*, *STK11*, *PTEN*, *ATM*, *CDH1*, *FGFR3,* and *SRC* genes.[16,24,28–30]

However, data on the genetic features of intraductal tubulopapillary neoplasm are fairly limited, partially due to the rarity of the neoplasm. In one of the first reported cases of intraductal tubulopapillary neoplasm, transcriptional profiling analysis and subsequent correspondence cluster analysis demonstrated that the transcriptional profile of intraductal tubulopapillary neoplasm differed distinctly from that of pancreatic ductal adenocarcinomas and other pancreatic cystic tumors.[31] Also, in contrast with intraductal papillary mucinous neoplasms, *KRAS, GNAS,* and *BRAF* mutations have been very rarely reported in intraductal tubulopapillary neoplasms.[6,7,9,12,32]

In the present study, we aimed to further define the genetic underpinnings of intraductal tubulopapillary neoplasm and analyzed 22 cases by targeted next-generation sequencing or whole-exome sequencing. Three of these cases were also subjected to whole-genome sequencing.

## Materials and methods

With approval of the Institutional Review Boards, the surgical pathology databases of Memorial Sloan Kettering Cancer Center, Emory University, Ipatimup, Tokyo Women's Medical University, Tokyo Medical University, and Tohoku University were searched for patients with diagnoses of pancreatic intraductal tubular carcinoma or intraductal tubulopapillary neoplasm. Twenty-two pancreatic neoplasms were identified for which the slides and

tissue blocks were available. The diagnosis was confirmed by the authors for each case. Medical records including pathology reports were reviewed to obtain clinical data. The choice of sequencing platforms (see below) was based on assays available at the institutions in the United States and Japan where the cases were collected. All methods included the major genes known to be altered in pancreatic ductal adenocarcinoma and intraductal papillary mucinous neoplasm, including *KRAS, TP53, CDKN2A, SMAD4, GNAS,* and *RNF43* (Supplementary Information Files 1a and 1b).

### Targeted Next-Generation Sequencing Using Illumina System (MSK-IMPACT)

Twenty 10-micron-thick sections of 18 cases (Cases #1–18) were cut from formalin-fixed, paraffin-embedded tissue blocks containing intraductal tubulopapillary neoplasms. From these sections, areas of interest were needle micro-dissected. For each patient, extraction of DNA was performed on dissected tissue, and where available on normal, non-pancreatic tissue (stomach, spleen, or duodenum). Deep coverage, targeted next-generation sequencing was then performed for a panel of 300 genes, listed in the Supplementary Information File 1a, known to undergo somatic genomic alterations in cancer, as previously described.[33,34] Briefly, massively parallel sequencing libraries (Kapa Biosystems, New England Biolabs) that contain barcoded universal primers were generated from 115 to 250 ng genomic DNA from the tumor material and matched normal tissue. After library amplification and DNA quantification, pools of barcoded libraries were subjected to solution-phase hybrid capture with synthetic biotinylated DNA probes (Nimblegen SeqCap) targeting all protein-coding exons from all 300 target genes as well as introns known to harbor recurrent translocation breakpoints. Each hybrid capture pool was sequenced to deep coverage in a single paired-end lane of an Illumina flow cell. Subsequently, the sequencing data were analyzed to identify multiple classes of genomic alterations (single-nucleotide sequence variants, small insertions/deletions, and DNA copy number alterations). For matched tumor/normal tissue pairs ($n=17$), somatic single-nucleotide variants and insertions and deletions were called using MuTect and the SomaticIndelDetector tools in Genome Analysis Toolkit, respectively.[35,36] For the only unmatched tumor ($n=1$), MuTect was run against a pool of unrelated DNAs from normal formalin-fixed, paraffin-embedded tissues, and variants were filtered out if they were present in the 1000 Genomes project at a population frequency of $>1\%$. All candidate mutations and insertions and deletions were reviewed manually using the Integrative Genomics Viewer.[37] The mean coverage was 400× for tumor DNA and 50× for normal DNA.

### Targeted Cancer Gene Panel Sequencing Using Ion Ampliseq System (Ion AmpliSeq)

Twelve-micron-thick sections of two additional cases (Cases # 19 and #20) were cut from frozen tissue blocks containing intraductal tubulopapillary neoplasms. From these sections, areas of interest were needle micro-dissected. For each patient, extraction of DNA was performed on dissected tissue and on normal pancreatic tissue. Targeted next-generation sequencing was then performed on a panel of 409 genes using the Ion Ampliseq Comprehensive Cancer Panel (Thermo Fisher Scientific, Waltham, MA, USA) (Supplementary Information File 1b). Briefly, sequencing libraries that contain barcoded universal primers were generated from 10 ng genomic DNA from the tumor material and matched normal tissue using Ion AmpliSeq Library Kit 2.0 (Thermo Fisher Scientific) according to the manufacturer's instruction. After library DNA quantification, equimolar pools were generated consisting of up to 20 barcoded libraries. Library pools were sequenced by using Ion Proton System (Thermo Fisher Scientific). Subsequently, the sequencing data were deconvoluted to match all high-quality barcoded reads with the corresponding tumor samples, and genomic alterations (single-nucleotide sequence variants and small insertions/deletions) were identified. For matched tumor/normal tissue pairs, somatic single-nucleotide variants and insertions and deletions were called using Ion Reporter Software (Thermo Fisher Scientific). The mean coverage was 716× for tumor DNA and 585× for normal DNA.

Selected variations were validated by Sanger sequencing as follows: DNA was amplified by polymerase chain reaction and the AccuPrime polymerase chain reaction system (Thermo Fishers Scientific). The amplified products were treated with ExoSAP-IT (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) and sequenced using BigDye Terminator and a 3130xl Genetic Analyzer (Thermo Fishers Scientific) according to the manufacturers' instructions.

### Whole-Exome Sequencing Using SOLiD System

Tumor and normal tissues of another two cases (Cases #21 and #22) were dissected and collected separately from frozen sections under microscopic guidance. DNA was extracted using a ChargeSwitch gDNA Mini Tissue Kit (Thermo Fishers Scientific). The extracted DNA was constructed into a fragment library using the AB Library Builder System (Thermo Fisher Scientific). Libraries were quantified by using Bioanalyzer system (Agilent Technologies, Santa Clara, CA, USA). An appropriate amount of the constructed libraries were subjected to whole-exome enrichment using a TargetSeq Target Enrichment Kit (Thermo Fisher Scientific). The prepared exome libraries were sequenced using the massively parallel deep sequencer 5500xl SOLiD System (Thermo

Fisher Scientific) using the paired-end sequencing method. Data were analyzed using LifeScope software (Thermo Fisher Scientific) with mapping on the Human Genome Reference, GRCh37/hg19. All procedures were performed according to the manufacturers' instructions. Obtained data were annotated and stringently filtered to exclude false variation calls using our previously described programs developed in-house.[15] Copy number variations were calculated using the exome sequencing data as described previously.[38]

The mean coverage was 129 × for tumor DNA and 81.5 × for normal DNA.

### Whole-Genome Sequencing

Fresh frozen tumor material and matched normal tissues from three cases (Cases #7, #11, and #17) analyzed by MSK-IMPACT were also subjected to whole-genome sequencing at New York Genome Center, which was performed using Illumina paired-end chemistry on a HiSeqX sequencer at a coverage of at least 80 × for tumor DNA and 40 × for normal DNA.

For whole-genome sequencing and genotyping, DNA was extracted using Qiagen AllPrep DNA/RNA Mini Kit. DNA was quantified using the Qubit 2.0 Fluorometer, Invitrogen and quality was determined by using Agilent Bioanlyzer. DNA libraries were prepared using the KAPA Hyper Prep Kit (Kapa; Kapa Biosystems, Wilmington, MA, USA). For each sample library preparation, 100 ng of high molecular weight genomic DNA was fragmented using the Covaris LE220 system to an average size of 350 bp. Fragmented samples were end repaired and adenylated using Kapa's end-repair and a-tailing enzymes. The samples were then ligated with Bioscientific adapters and polymerase chain reaction amplified using KAPA Hifi HotStart Master Mix (Kapa; Kapa Biosystems). The DNA libraries were clustered onto flowcells using Illumina's cBot and HiSeq Paired End Cluster Generation kits as per the manufacturer's protocol (Illumina, San Diego, CA, USA). Sequencing was performed using 2 × 150 Illumina HiSeqX platform with v2.5 chemistry reagents. Genotyping was performed using HumanOmni2.5M BeadChips (Illumina).

Paired-end 2x150bp reads were aligned to the GRCh37 human reference using the Burrows-Wheeler Aligner (BWA aln v.0.7.8)[39] and processed using the best-practices pipeline that includes marking of duplicate reads by the use of Picard tools and realignment around insertions and deletions and base recalibration via Genome Analysis Toolkit ver. 2.7.4.[40] We employ the following variant callers: muTect v1.1.4,[35] LoFreq v2.0.0[41] (single-nucleotide sequence variants only), Strelka v1.0.13[42] (both single-nucleotide sequence variants and insertions and deletions), Pindel[43] and Scalpel[44] (insertions and deletions only) and return the union of calls, filtered using the default filtering criteria as implemented in each of the callers. Single-nucleotide sequence variants and insertions and deletions were annotated via snpEff, snpSift[45] and Genome Analysis Toolkit VariantAnnotator using annotation from ENSEMBL, COSMIC,[46] Gene Ontology and 1000 Genomes.

Structural variants, such as copy number variants as well as complex genomic rearrangements, were detected by the use of multiple tools: NBIC-seq[47] for copy number variant/structural variant calling, Delly,[48] Crest,[49] and BreakDancer[50] for structural variant calling. We prioritized structural variants in the intersection of callers and structural variants for which we can find additional split-read evidence using SplazerS.[51] Structural variants for which there was split-read support in the matched normal or that were annotated as known germline variants (1000 Genomes call set, DGV) were removed as likely remaining germline variants. The predicted sets of somatic structural variants were annotated with gene overlap (RefSeq, Cancer Gene Census) including prediction of potential effect on genes (eg, disruptive/exonic, intronic, intergenic, fusion candidate). In addition, copy number variants and loss of heterozygosity were also analyzed from the genotyping chip using Nexus (Biodiscovery) software.

## Results

### Clinicopathologic Features

The clinicopathologic features of the 22 cases (11 of which have been reported previously[1]) are summarized in Table 1. The mean patient age was 58 years, with a male to female ratio of 1.4. None of the patients received neoadjuvant chemotherapy or chemoradiation.

Overall tumor size varied from 0.9 to 16 cm. All tumors exhibited typical entity-defining characteristics. Grossly, they were multinodular and solid to cystic. Microscopically, the tumors consisted of variably sized circumscribed nodules of back-to-back glands, resulting in large cribriform structures surrounded by fibrotic stroma (Figure 1). Within the tumor nodules, there were tightly packed small glands lined by cuboidal cells. In most cases there was no obvious intracellular mucin in the neoplastic cells. The nuclei were atypical and mitotic figures were readily identifiable (Figure 2). Of 21 resection cases, 17 (81%) had invasion.

Follow-up information was available for 18 patients. One patient (Case #7) died of perioperative complications 1 month after the surgery. One patient (Case #22) died of other disease(s) after 5 months. Three patients (19%) (Cases #17, #21, and #15) died of disease after 20, 25, and 125 months, respectively. The remaining 13 (81%) were alive after 9–173 months (median, 95). Of these, two patients (Cases #18 and #12) had a recurrence in the remainder of the pancreas after 9 and 12 months, respectively, and

**Table 1** Clinicopathologic features of the cases

| | |
|---|---|
| Mean age (range) (years) | 58 (21–75) |
| Male/female | 1.4 |
| *Type of specimen* | *n* (%) |
| Pancreaticoduodenectomy | 10 (48) |
| Distal pancreatectomy | 7 (33) |
| Total pancreatectomy | 3 (14) |
| Biopsy | 1 (5) |
| Unknown | 1 |
| *Tumor location* | *n* (%) |
| Head | 9 (47) |
| Body | 2 (11) |
| Tail | 5 (26) |
| Diffuse | 3 (16) |
| Unknown | 3 |
| Median overall tumor size (range) | 3.3 cm (0.9–16) |
| *Invasive component* | *n* (%) |
| Present | 17 (81) |
| Absent | 4 (19) |
| Unknown (biopsy case) | 1 |
| *Lymphovascular invasion* | *n* (%) |
| Yes | 7 (39) |
| No | 11 (61) |
| Unknown | 4 |
| *Resection margin* | *n* (%) |
| R0 | 15 (100) |
| R1 | 0 |
| Unknown | 7 |
| *Lymph node status* | *n* (%) |
| N0 | 16 (94) |
| N1 | 1 (6) |
| Unknown | 5 |
| Median follow-up (months) (range) | 48.5 (1–173) |
| *Status of known 18 cases* | |
| Died of perioperative complications | 1 |
| Died of other disease(s) | 1 |
| Died of disease | 3 |
| Alive WITH disease | 6 |
| Alive WITHOUT disease | 7 |

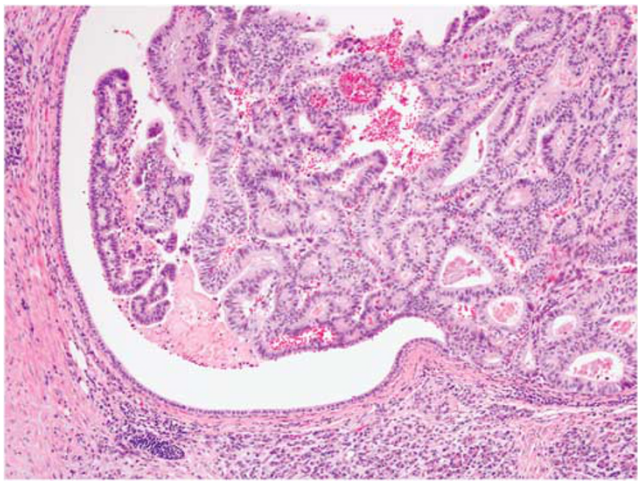

**Figure 1** Nodules typically filled the entire duct but continuity with the lining epithelium was identified in some.

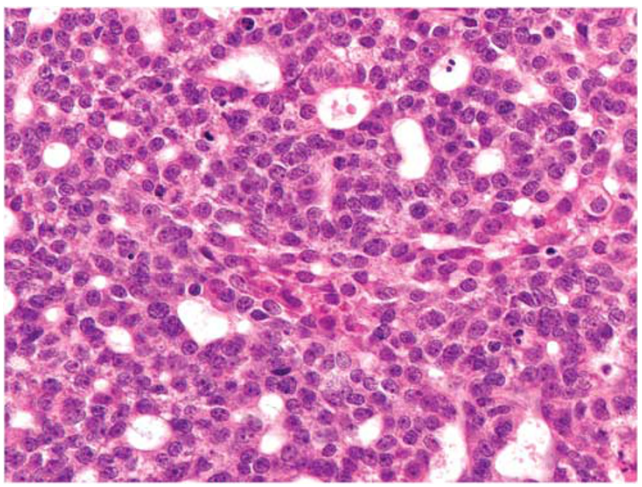

**Figure 2** Tumor cells contained modest amount of cytoplasm without obvious intracellular mucin. The nuclei were round to oval and atypical.

underwent completion pancreatectomy. Case #18 found to have lymph node metastases in the second surgery. Additional four patients (Cases #4, #15, #16, and #21) developed liver metastases after 78, 56, 10.5, and 17 months, respectively. Seven patients (44%) are alive without evidence of disease.

## Molecular Features

*Results of MSK-IMPACT, Ion AmpliSeq and Whole-Exome Sequencing.* Twenty tumor/normal tissue pairs and one unmatched tumor tissue sample from the 18 patients underwent MSK-IMPACT (intraductal and invasive tumor components of Case #16 and tumors from both Whipple and completion pancreatectomy as well as lymph node metastasis of Case #18 were analyzed separately). Four tumor/normal tissue pairs from four Japanese patients were analyzed using either Ion AmpliSeq (Cases #19 and #20) or whole-exome sequencing (Cases #21 and #22). The results of these genetic studies are summarized in Tables 2 and 3, and details regarding each of the individual tumors are described in the Supplementary Information Files 2–4.

By copy number analysis, 80% of intraductal tubulopapillary neoplasms (16/20, due to the lack of copy number alteration data from Ion AmpliSeq) were found to have gene amplifications and deletions, including loss of heterozygosity or copy-neutral loss of heterozygosity in whole or parts of chromosome arm 1p and recurrent gains/amplifications in chromosome arm 1q and 8 (Figure 3). There was also amplification of *MCL1* in 40% (8/20) and loss of *CDKN2A* in 25% (5/20). One case (Case #21) revealed amplification of *MCL3* as well as hemizygous loss of *MLL2* and *BAP1*.

**Table 2** High confidence mutations and recurrent copy number variants identified by MSK-IMPACT, Ion AmpliSeq, or whole-exome sequencing

| *Case #* | *Analysis* | *Gene* | *Type of mutation* | *Protein* | *MCL amplification* | *CDKN2A loss* |
|---|---|---|---|---|---|---|
| 1 | MSK-IMPACT | JAK | Missense mutation | p.V1009D | Yes | No |
| | | DNMT3A | Missense mutation | p.R181H | | |
| | | TET2 | Missense mutation | p.E755K | | |
| | | ARHGAP26 | Missense mutation | p.R103Q | | |
| | | ROR2 | Missense mutation | p.R522Q | | |
| | | **MLL2** | Nonsense mutation | p.E3587* | | |
| 2 | MSK-IMPACT | **MLL2** | Nonsense mutation | p.R2471* | No | No |
| | | **MLL2** | Frame_Shift_Ins | p.L4518fs | | |
| 3 | MSK-IMPACT | NOTCH2 | Missense mutation | p.N632S | Yes | No |
| | | IRF4 | Missense mutation | p.E130G | | |
| | | DNMT3B | Missense mutation | p.G511C | | |
| | | BCOR | Frame_Shift_Ins | p.E1484fs | | |
| 4 | MSK-IMPACT | None | None | None | No | No |
| 5 | MSK-IMPACT | MAP2K1 | Missense mutation | p.E51G | Yes | No |
| | | FAM123B | Splice_Site | p.Q2048fs | | |
| | | **MLL3** | Frame_Shift_Del | | | |
| | | MAP2K1 | In_Frame_Ins | p.59_60insQK | | |
| 6 | MSK-IMPACT | EPHA2 | Missense mutation | p.A112T | Yes | No |
| | | BRCA2 | Missense mutation | p.G1771D | | |
| 7[a] | MSK-IMPACT | PTEN | In_Frame_Del | p.T319 | No | Yes |
| | | NPM1 | Missense mutation | p.S125L | | |
| | | **MLL3** | Missense mutation | p.K992M | | |
| 8 | MSK-IMPACT | None | None | None | Yes | No |
| 9 | MSK-IMPACT | **BAP1** | Splice_Site | p.E577_splice | No | No |
| 10 | MSK-IMPACT | INPP4A | Missense mutation | p.N308T | No | Yes |
| | | KDR | Missense mutation | p.M559I | | |
| 11[a] | MSK-IMPACT | AXIN1 | Missense mutation | p.E195* | No | No |
| | | **BAP1** | Missense mutation | p.R213H | | |
| | | FLT4 | Splice_Site | p.P1023R | | |
| | | PBRM1 | Missense mutation | | | |
| 12 | MSK-IMPACT | XPO1 | Missense mutation | p.L660F | No | No |
| 13 | MSK-IMPACT | **PIK3CA** | Missense mutation | p.G1049R | No | No |
| | | EED | Missense mutation | p.N194S | | |
| | | FGFR4 | Frame_Shift | p.R464Pfs*32 | | |
| 14 | MSK-IMPACT | **PIK3CA** | Missense mutation | p.H1047R | No | No |
| | | **BAP1** | Nonsense mutation | p.S319* | | |
| | | ATRX | Missense mutation | p.I47V | | |
| | | CRKL | Missense mutation | p.G136E | | |
| | | **MLL1** | Missense mutation | p.I962V | | |
| | | SPEN | Missense mutation | p.A3060V | | |
| 15 | MSK-IMPACT | SMARCA4 | Missense mutation | p.R885C | No | Yes |
| | | NTRK1 | Missense mutation | p.G181E | | |
| 16 intraductal & invasive tumor | MSK-IMPACT | ZFHX3 | Deletion | p.G3517_G3527del | No | No |
| 17[a] | MSK-IMPACT | None | None | None | Yes | No |
| 18 primary pancreatic tumor | MSK-IMPACT | CEBPA | Missense mutation | p.R86P | No | No |
| 18 recurrent pancreatic tumor | MSK-IMPACT | CDKN2A | Nonsense mutation | p.Y129* | No | No |
| | | RET | Missense mutation | p.L80R | | |
| 18 celiac LN metastasis | MSK-IMPACT | None | None | None | No | No |
| 19 | Ion AmpliSeq | TRIP11 | Missense mutation | p.L872H | Not applicable | Not applicable |
| 20 | Ion AmpliSeq | AXL | Missense mutation | p.R190H | Not applicable | Not applicable |
| | | PIK3CB | Missense mutation | p.L35V | | |
| 21 | Whole-exome seq | SYCP1 | Splice site mutation | — | Yes | Yes |
| | | USH2A | Missense mutation | p.R878C | | |
| | | SLC4A10 | Missense mutation | p.D208H | | |
| | | CTNNB1 | Missense mutation | p.S45F | | |
| | | CBLB | Missense mutation | p.G259V | | |
| | | **PIK3CA** | Missense mutation | p.E545K | | |
| | | EPHB3 | Missense mutation | p.Y855H | | |
| | | ETFDH | Missense mutation | p.G75D | | |
| | | FAT1 | Missense mutation | p.E2401K | | |
| | | FAM170A | Missense mutation | p.R65C | | |
| | | HIST1H4K | Nonsense Mutation | p.E64* | | |
| | | MYB | Missense mutation | p.R73Q | | |
| | | MUC12 | Missense mutation | p.N4428D | | |
| | | EHBP1L1 | Missense mutation | p.R1138H | | |
| | | UBASH3B | Missense mutation | p.E257K | | |
| | | KCNA5 | Missense mutation | p.A50V | | |

**Table 2 (Continued)**

| *Case #* | *Analysis* | *Gene* | *Type of mutation* | *Protein* | *MCL amplification* | *CDKN2A loss* |
|---|---|---|---|---|---|---|
| | | TM7SF3 | Frameshift Insertion | p.S246fs | | |
| | | CNTN1 | Missense mutation | p.P271L | | |
| | | OSBPL8 | Missense mutation | p.R318Q | | |
| | | CLK3 | Nonsense Mutation | p.Y36* | | |
| | | TP53 | Missense mutation | p.P113L | | |
| | | MYH13 | Missense mutation | p.G203R | | |
| | | MYH8 | Missense mutation | p.R1715H | | |
| | | KRT26 | Missense mutation | p.R93C | | |
| | | JMJD6 | Missense mutation | p.R95G | | |
| | | NOL4 | Missense mutation | p.T119M | | |
| | | LTBP4 | Missense mutation | p.G283D | | |
| | | ARHGAP35 | Nonsense mutation | p.S975* | | |
| | | FAM71E2 | Missense mutation | p.L329M | | |
| 22[b] | Whole-exome seq | CYR61 | Missense mutation | p.C39* | | |
| | | CHML | Missense mutation | p.D210Y | | |
| | | SCN9A | Missense mutation | p.S1594T | | |
| | | KALRN | Missense mutation | p.A364T | | |
| | | COL6A6 | Nonsense mutation | p.R1502H | | |
| | | PRR14L | Nonsense mutation | p.E658* | | |
| | | APC | Missense mutation | p.A2T | | |
| | | HIST1H3G | Missense mutation | p.M448K | | |
| | | WRN | Missense mutation | p.E226K | | |
| | | MUC2 | Missense mutation | p.A59E | | |
| | | KRAS | Missense mutation | p.S286T | | |
| | | DNASE1L2 | Missense mutation | p.S82F | | |
| | | NF1 | Missense mutation | p.K1111T | | |
| | | ZNF208 | Missense mutation | p.V30L | **YES** | **YES** |

[a]Cases 7, 11, and 17 were also subjected to whole-genome sequencing. [b]Patient has a history of neurofibromatosis type 1 (von-Recklinghausen's disease). Recurrent mutations are indicated in bold.

Except rare *PIK3CA* (Cases #13, #14, #21), *PIK3CB* (Case #20), *PTEN* (Case #7), and *BRCA2* (Case #6) mutations, the previously reported intraductal papillary mucinous neoplasm or pancreatic ductal adenocarcinomas-related mutations (eg, *KRAS, GNAS, RNF43, TP53, SMAD4*) were not identified in these intraductal tubulopapillary neoplasms. In fact, three tumors (Cases #4, #8, and #17) did not reveal any mutations by these methods. Others harbored mutations in genes involved in several additional signaling pathways pertinent to cancer biology such as chromatin remodeling pathway (*MLL1* in Case #14, *MLL2* in Cases #1 and #2, *MLL3* in Case #5, *BAP1* in Case #9, *PBRM1* in Case #11, *EED* in Case #13, and *ATRX* in Case #14), WNT-β catenin pathway (*CTNNB1* in Case #21, *APC* in Case #22, and *AXIN1* in Case #11), GAS6-AXL pathway (*AXL* in Case #20), Rho pathway (*ARHGAP26* in Case #1, *ARHGAP35* in Case #21, *ROR2* in Case #1, and *KALRN* in Case #22), tyrosine kinase pathway (*KDR* in Case #10, *FLT4* in Case #11, *NTRK* in Case #15, and *RET* in Case #18), and ephrin pathway (*EPHA2* in Case #6 and *EPHB3* in Case #21).

Interestingly, there was no difference between the separately analyzed intraductal and invasive tumor components of Case #16; both component revealed the same *ZFHX3* mutation (non-frameshift deletion). In contrast, separately analyzed primary and recurrent pancreatic tumors of Case# 18 found to have different mutations (Table 2). However, primary and recurrent pancreatic tumors as well as celiac lymph node metastasis of this case harbored the same *ALK* fusion (see below).

Of note, one of our patients (Case #22) had a history of neurofibromatosis type 1 (von-Recklinghausen's disease). Although we did not find any germline mutation in coding exons of *NF1* or *NF2*, we identified a somatic mutation in *NF1* (missense mutation at S82F) in this patient's tumor. The tumor also harbored a rare somatic mutation type in *KRAS* (missense mutation at A59E), which is different from common mutations found in pancreatic ductal adenocarcinomas that usually involve codon 12, 13, or 61.

In addition, 22% (4/18, due to the lack of fusion data from Ion AmpliSeq and whole-exome sequencing) of intraductal tubulopapillary neoplasms revealed *FGFR2* fusions (three in frame, one out of frame, and one mid-exon fusions) and 5.5% (1/18) revealed an *ALK* fusion (a deletion, which results in an in-frame fusion of *STRN* exon 3 and *ALK* exon 20) (Table 3) (*STRN-ALK* fusion was also confirmed by Archer FusionPlexTM Custom Solid Panel utilizing the Anchored Multiplex polymerase chain reaction (AMPTM) technology to detect gene fusions in tumor samples. The panel consists of 35 cancer-related genes previously reported to be involved in chromosomal rearrangements).

*Results of Whole-Genome Sequencing.* Fresh frozen tumor samples from three patients (Cases #7, #11, and #17) analyzed by MSK-IMPACT also under-

**Table 3** Rearrangements identified in our series

| *Case #* | *Gene 1* | *Gene 2* | *Site 1 description* | *Site 2 description* | *Fusion* |
|---|---|---|---|---|---|
| *FGFR2 fusions* | | | | | |
| #11 | FGFR2 | CEP55 | Exon 17 of FGFR2 | Exon 2 of CEP55 | Protein fusion: in frame (FGFR2-CEP55) |
| #12 | FGFR2 | SASS6 | Intron of FGFR2(−): 80 bp after exon 16 | Intron of SASS6(−): 586 bp before exon 7 | Protein fusion: in frame (FGFR2-SASS6) |
| #14 | FGFR2 | DISP1 | Intron of FGFR2(−): 276 bp before exon 17 | Intron of DISP1(+): 9Kb after exon 2 | Protein fusion: out of frame (DISP1-FGFR2) |
| #14 | FGFR2 | TXLNA | Intron of FGFR2(−): 687 bp before exon 17 | Intron of TXLNA(+): 1kb before exon 5 | Protein fusion: in frame (FGFR2-TXLNA) |
| #15 | VCL | FGFR2 | Exon 13 of VCL(+) | Intron of FGFR2(−): 535 bp before exon 17 | Protein fusion: mid-exon (FGFR2-VCL) |
| *ALK fusion*[a] | | | | | |
| #18 | STRN | ALK | Exon 3 of STRN (NM-003162) | Exon 20 of ALK (NM-004304) | Protein fusion: in frame (STRN-ALK) |

[a]Identified in the primary and recurrent pancreatic tumors as well as in the celiac lymph node metastasis

went whole-genome sequencing. Details of the individual tumors are described in Supplementary Information Files 5 and 6.

By copy number analysis, similar to MSK-IMPACT, Cases #7 and #17 were found to have multiple copy number gains and losses in multiple chromosomes. No significant copy number alterations were identified in Case #11.

Mutation assessment revealed a total of 129 mutations within these three tumors, 67 mutations in Case #7, 28 in Case #11, and 34 in Case #17. Similar to MSK-IMPACT, whole-genome sequencing did not identify the vast majority of well-recognized intraductal papillary mucinous neoplasm-related (or pancreatic ductal adenocarcinoma-related) alterations. In fact, among the mutations identified by MSK-IMPACT, only *NPM1* and *PTEN* mutations were also detected by whole-genome sequencing, likely due to lower coverage of target regions with whole-genome sequencing. Of note, one case revealed a *MUC6* mutation (missense mutation, p.Pro1841Thr), which is not included in the panel of MSK-IMPACT.

## Discussion

Recent studies have helped to better characterize the prototype of the pancreatic intraductal neoplasm family, intraductal papillary mucinous neoplasm,[14,24,52–58] and have shown that the progression from intraductal papillary mucinous neoplasm with low-grade dysplasia to intraductal papillary mucinous neoplasm with associated invasive carcinoma is accompanied by a high number of molecular alterations (about 26 mutations per neoplasm), the most frequent ones being mutations in *KRAS*, *GNAS,* and *RNF43*.[14–16,20–27] These data have confirmed that intraductal papillary mucinous neoplasm has both similarities and differences in genetic progression patterns compared with conventional pancreatic ductal adenocarcinoma.[18,28,59–61]

However, the genetic characteristics of intraductal tubulopapillary neoplasm, the new member of the pancreatic intraductal neoplasm family,[1–10,62] have not been fully characterized yet as emerging studies have analyzed only selected gene mutations or a small number of cases.[6,7,9,24,32] For example, in a recent study based on targeted next-generation sequencing for a panel of 51 cancer-associated genes, no mutations were identified in three intraductal tubulopapillary neoplasms analyzed; although one case with coexisting intraductal papillary mucinous neoplasm and intraductal tubulopapillary neoplasm lesions revealed the same *GNAS* R201H mutation in both lesions and the intraductal tubulopapillary neoplasm also had an additional *NRAS* Q61L mutation.[24] With possible evidence to the contrary, in their investigation of somatic mutations in *KRAS, BRAF, PIK3CA, PTEN*, and *AKT1* in 11 intraductal tubulopapillary neoplasms and 50 intraductal papillary

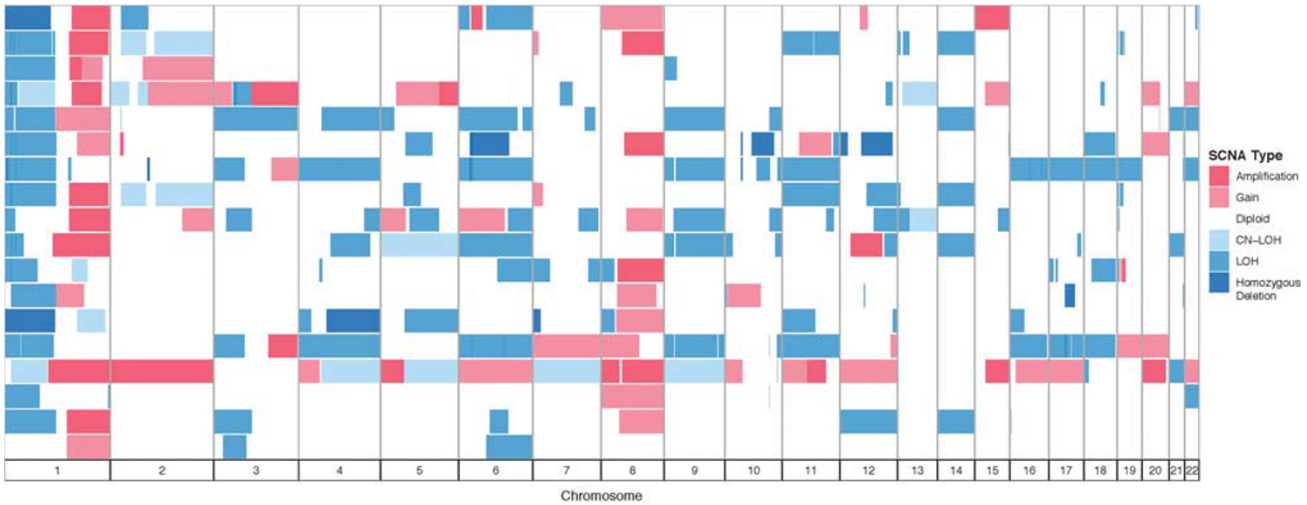


**Figure 3** Landscape of copy number alterations for intraductal tubulopapillary neoplasm. Allele-specific integer copy number analysis using FACETS revealed recurrent loss of heterozygosity in chromosome 1p and recurrent gains in chromosomes 1q and 8. CN-LOH, copy-neutral loss of heterozygosity; LOH, loss of heterozygosity.

mucinous neoplasms, Yamaguchi *et al.* identified *PIK3CA* mutations in 3 (27%) intraductal tubulopapillary neoplasms, but in none of the intraductal papillary mucinous neoplasms. *PIK3CA* mutations were also significantly associated with strong immunoexpression of phosphorylated AKT in their cases. In contrast, mutations in *KRAS* were found in none of the intraductal tubulopapillary neoplasms but were found in 26 (52%) intraductal papillary mucinous neoplasms.[7] In a subsequent study, the same group investigated somatic mutations in *KRAS, BRAF, PIK3CA,* and *GNAS* in 14 intraductal tubulopapillary neoplasms and 15 gastric-subtype intraductal papillary mucinous neoplasms.[9] Similar to their first study, they identified *PIK3CA* mutations in three (21%) intraductal tubulopapillary neoplasms, but in none of the intraductal papillary mucinous neoplasms. Also, *KRAS* mutations were found in only 1 (7%) intraductal tubulopapillary neoplasm and 12 (80%) intraductal papillary mucinous neoplasms, *BRAF* mutation in only 1 (7%) intraductal tubulopapillary neoplasm, and *GNAS* mutations only in 9 (60%) intraductal papillary mucinous neoplasms.[9] A recent molecular analysis of the biliary counterpart of these neoplasms also revealed the very low prevalence of alterations in common oncogenic signaling pathways in 20 biliary intraductal tubulopapillary neoplasms.[63]

In this study, we explored the genetic characteristics of 22 intraductal tubulopapillary neoplasms, the largest series analyzed to date, by high depth targeted next-generation sequencing for large panels of key cancer-associated genes (MSK-IMPACT or Ion AmpliSeq) or by whole-exome sequencing. Selected intraductal tubulopapillary neoplasms were also subjected to whole-genome sequencing. Our results show that, although loss of *CDKN2A* appears to be present at least in a subset (%25), intraductal tubulopapillary neoplasms do not commonly harbor the previously reported intraductal papillary mucinous neoplasm-related (or pancreatic ductal adenocarcinoma-related) alterations. However, consistent with the findings of Yamaguchi *et al.*,[7,9] six (27%) intraductal tubulopapillary neoplasms harbored phosphatidylinositol pathway mutations (three *PIK3CA*, one *PIK3CB*, one *INPP4A,* and one *PTEN* mutation). In addition, certain chromatin remodeling genes (*MLL1, MLL2, MLL3, BAP1, PBRM1*, *EED,* and *ATRX*) were found to be mutated in seven (32%) intraductal tubulopapillary neoplasms. Eight (40%) intraductal tubulopapillary neoplasms also had *MCL1* amplification.

*MLL1 (KMT2A)*, *MLL2 (KMT2D),* and *MLL3 (KMT2C)* are members of the myeloid/lymphoid or mixed-lineage leukemia (MLL) family. Each gene encodes a different component of a histone H3 lysine 4 methyltransferase complex and is responsible for the transcriptional regulation of developmental genes including the homeobox gene family.[64] These genes have also been implicated as tumor suppressors due to their frequent mutations in multiple types of human tumors. For example, mutations of *MLL2* are common in various types of B-cell lymphoma[65] and are reported in epithelial tumors such as non-small cell lung carcinoma[66] and renal cell carcinoma.[67] Mutations and LOH of *MLL3* are reported in various types of lymphoma, as well as in gastrointestinal carcinomas, including cholangiocarcinoma,[68–71] and urothelial carcinoma.[72] Similarly, *BAP1*, belongs to the family of deubiquitinating enzymes, encodes an enzyme that binds to the breast cancer type 1 susceptibility protein (BRCA1), and acts as a tumor suppressor.[73,74] Mutations of *BAP1* are one of the frequent inactivating mutations in intrahepatic cholangiocarcinomas[75,76] and mesotheliomas.[77,78] Finally, *MCL1* is involved in the regulation of apoptosis *versus* cell survival, and in

the maintenance of viability. It mediates its effects by interactions with a number of other regulators of apoptosis.[79,80] *MCL1* is frequently amplified in neural, lung, breast, and gastrointestinal cancers.[81]

In a recent study, potential involvement of somatic mutations in chromatin remodeling genes including *MLL2* and *MLL3* has also been reported in 20% of pancreatic ductal adenocarcinoma patients who have prolonged overall and progression-free survival, compared with wild-type tumors, and these observations appear to be independent of other clinical variables.[82] Similarly, mutation of other epigenetic regulators has been described in other pancreatic neoplasms and has been associated with clinical outcome. For instance, improved outcome was reported in patients with *DAXX/ATRX* alterations in metastatic pancreatic neuroendocrine tumors,[83] although primary tumors with *DAXX/ATRX* mutations appear to have a poorer outcome.[84] Intraductal tubulopapillary neoplasms, even if they have an associated invasive carcinoma, are also less aggressive compared with conventional pancreatic ductal adenocarcinomas.[10] Therefore, whether tumors significantly driven by mutations in epigenetic regulators may be inherently less aggressive is an intriguing speculation, and genetic alterations identified in this study are likely to be more than mere epiphenomena for their potential role in the tumorigenesis of intraductal tubulopapillary neoplasms.

More importantly, some of these genetic alterations might be potentially targetable. For example, *PIK3CA* mutation is of special interest as it is a potential therapeutic target for inhibition of the PI3K pathway.[85,86] Similarly, *BAP1* mutation may also confer sensitivity to drugs targeting chromatin remodeling, such as histone deacetylase inhibitors.[87] Multiple *FGFR2* (fibroblast growth factor receptor 2) fusions, including a previously described *FGFR2-TXLNA* fusion,[88] detected in four (22%) intraductal tubulopapillary neoplasms are also of importance. *FGFR2* fusions, an important class of genetic driver, have been detected in a diverse array of cancer types including cholangiocarcinoma, breast carcinoma, and lung squamous cell carcinoma among others.[89–92] Moreover, several independent studies reported that cells harboring *FGFR* fusions show enhanced sensitivity to the FGFR inhibitors, suggesting that the presence of *FGFR* fusions may be a useful biomarker of tumor response to FGFR inhibitors.[89,92]

In summary, the present comprehensive analysis helps validate the morphologic distinction of intraductal tubulopapillary neoplasm from other types of pancreatic neoplasms. More importantly, it demonstrates potentially targetable genetic alterations in intraductal tubulopapillary neoplasms, such as kinases (*PIK3CA* and *FGFR2*) and tumor-suppressor genes (*BAP1* and *BRCA2*). Further analysis of these genetic alterations in biologically distinct pathway will likely shed new light on the mechanisms of intraductal tumor formation in the pancreas and reveal new therapeutic targets for patients with these neoplasms.

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## Disclosure/conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (http://www.nature.com/modpathol)