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Molecular alterations in pancreatic precursor lesions: The path to invasive carcinoma but perhaps a chance for earlier detection?

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ABBREVIATIONS

BR: Borderline resectable
CA: Caliac artery
CAPS: International Cancer of the Pancreas Consortium
CEA: Carcinoembryonic antigen
CHA: Common hepatic artery
CI: Confidence interval
CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats
CT: Computed tomography
ctDNA: circulating tumor DNA
DFS: Disease free survival
ecDNA: extrachromosomal DNA
ECOG: Eastern Co-operative of Oncology Group
ED: Endoscopic drainage
EUS: Endoscopic ultrasound
FNA: Fine-needle aspiration
GEMM: Genetically engineered mouse models
HDR: Homology-directed repair
HPDE: Human pancreatic duct epithelial cell line
HRI: High-risk individuals
IPMN: Intraductal papillary mucinous neoplas
ITPN: Intraductal tubulo-papillary neoplasm
LA: Locally advanced
LCM: Laser capture microdissection
MCN: Mucinous cyst neoplasm
mFOLFIRINOX: modified-bolus fluorouracil, irinotecan, leucovorin, oxaliplatin
MRI: Magnet resonance imaging
NGS: Next Generation Sequencing
OS: Overall survival
PanIN: Pancreatic intraepithelial neoplasia
PARP: Poly-ADP ribose polymerase
PD: Percutanous drainage
PDAC: Pancreatic ductal adenocarcinoma
POPF: Postoperative pancreatic fistula
PPPD: Pylorus preserving pancreatoduodenectomy
PV: Portal vein
R: Resectable
RNAseq: Next Generation RNA Sequencing
SMA: Superior mesenteric artery
SMV: Superior mesenteric vein
SNV: Single nucleotide variant
TIME: Tumor immune microenvironment

1 INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) remains one of the most aggressive solid human cancers, while modest improvements in the outcome of patients in last decades must be acknowledged. With increasing incidence worldwide it is projected to become the second-leading cause of cancer-related death.[1,2] Across all stages, the median survival is a mere 12-14 months while in developed countries only around 10% of cases show 5-year survival.[3] This tumor type accounts for the majority (>95%) of malignant transformation occurring in the pancreas and current data locate its origin in the exocrine compartment. It derives from the epithelial-like ductal- or acinar cells forming the glandular structures.[4-6] Known risk factors for this carcinoma type are age, diabetes, smoking, alcohol, inflammation and family history.[reviewed in 7] A majority of these tumors develop in the head of the gland, possibly due to its dense ductal network and the sheer size of this region.[8] Early symptoms may include gastrointestinal discomfort, new-onset diabetes, weight loss and jaundice. However, because of its hidden location in the retroperitoneum as a metabolism-regulating gland, it remains often disconnected from the individuals' sensorium, resulting in late-stage diagnosis. Surgical treatment, including complete removal of the tumor burden, remains critical for enabling chances of cure. We generally assume that these tumors grow fast and disseminate early. However, limited data is available on the lag phase, before non-invasive pre-neoplastic lesions give rise to invasive carcinoma. Over the last two decades, the genomic, transcriptomic as well as proteomic landscape of PDACs has been deciphered. It characterizes distinctive subtypes and the overall complex molecular trajectory during pancreatic carcinogenesis.[9-13] Two important conceptual, promising research goals currently inspire clinical scientists to improve the outcomes of this devastating disease: Earlier detection and/or molecular driven, individualized therapies.

2.1. Multimodal treatment concepts for pancreatic duct adenocarcinoma (PDAC)

2.1.1 *Surgical treatment of PDAC and worrisome precursor lesions*

There is international consensus about the feasibility of surgical treatment as defined by multi-dimensional criteria, which take the anatomical-, biological- and conditional situation into account (ABC criteria).[14] Using multidetector CT-scans, the local

extension of the tumor is assessed in relation to the central arterial/venous vessel structures (SMA, CA, CHA, SMV, PV).[14,15] The main technical objective is complete removal also achieving negative resection margins (R0). All tumors confined to the pancreatic organ without affecting the central venous/arterial structures are considered primary resectable (R). Borderline resectable (BR) tumors that abut (<180°) the central venous/arterial vessel structures may be removed as well, using specific technical maneuvers (e.g. vessel reconstructions[16], vessel dissection[17]), but only if they do not exceed the inferior border of the duodenum. When central venous/arterial vessel structures are encased (>180°), the PDAC is considered locally advanced (LA), and therefore can no longer be safely removed. The same applies to patients at disseminated stages, who are generally allocated to palliative treatments. However neoadjuvant treatment concepts have become more popular and in selected cases with favorable tumor biology, LA- and BR tumors can be successfully transformed to resectable situations (see below).[18,19]

According to interdisciplinary consensus for resectable disease, patients will get surgery. The technique applied depends on the location and extent of the tumor and commonly includes partial pancreatoduodenectomy (Whipple procedure/PPPD), central pancreatectomy (Appleby procedure), and distal or total pancreatectomy. The technical innovations of minimally invasive surgery have preempted the safe exertion of laparoscopic and robotic surgery.[20,21] While head resections remain the domain for open and robotic procedures, left-sided pancreatectomies are also tenable for laparoscopic surgery.[22-24]

The role of pancreatic surgery is not only defined by radiographic indicators for malignant carcinoma. Patients with cystic precursor lesions (IPMN, MCN) that harbor low but defined risks for malignant transformation are screened at regular intervals for worrisome features or high-risk stigmata. These radiographic features are most accurately defined according to the revised *Fukuoka Criteria* and include cyst size, duct dilation, presence of mural nodules and irregularly shaped walls.[25,26] When these criteria are met, further diagnostic exploration or immediate pancreatic surgery is warranted in which case the aforementioned standard surgical procedures are applied. Similar to R0 resections in PDAC, resection margins need to be devoid of high-grade and invasive lesions to achieve recurrence-free survival.[27,28]

Surgical treatment for (pre-)malignant pancreatic disease is the prerequisite for curative concepts and its feasibility requires interdisciplinary consensus.

2.1.2 Systemic treatment of PDAC

For a long time, PDAC was considered untreatable with no perceivable positive effects testing various chemo reagents. Only in 2006, results from the CONKO-001 clinical trial informed about the clinical effects of Gemcitabine compared to untreated patients.[29] Since then, adjuvant therapies are considered a standard element in multimodal curative treatment concepts offered to PDAC individuals. Still, follow-up studies were only able to discriminate modest effects with this treatment and indicated average survival increments of around 2.8 months.[30] Novel agents and combinations have since been tested which ultimately initiated more successful clinical trials using paradigm-shifting multiagent cytotoxic regimens. Such treatments were initially investigated in metastasized patients in order to find more effective first-line alternatives in the palliative setting. Foremost the MPACT (8.5m vs 6.7m) and the PRODIGE (6.4m vs 3.3m) trials reported significant survival benefits when compared to single-agent treatment.[31,32] These landmark studies paved the way for subsequent adjuvant clinical trials. In the ESPAC-4 study, Gemcitabine plus Capecitabine showed moderate survival benefits over Gemcitabine alone (28m vs 25.5m). The study group around *Conroy T et al.* (PRODIGE-24 trial) demonstrated significantly improved OS (54.4m vs 35m) for adjuvant combination therapy mFOLFIRINOX (modified-bolus fluorouracil, irinotecan, leucovorin, oxaliplatin) compared to the single-agent gemcitabine in a highly selected cohort.[33] These excellent results have established the basis for current international recommendations with multi-agent adjuvant treatment in patients with good conditional status (ECOG 0-1).[34] Other, unsuitable patients for this treatment (ECOG ≥2) receive less toxic combinations with Gemcitabine and albumin-bound(nab-) Paclitaxel/Capecitabine or Gemcitabine/5-FU alone.

As has been established for other gastrointestinal carcinoma entities (esophageal-, gastric-, rectal cancer), pre-operative rounds of systemic therapy have also been incrementally tested among PDAC patients where up-front surgery appeared not to be the best choice. A multitude of observational data indicates efficient downstaging and improved margin-negative resection rates.[35,36] Recently, the ALLIANCE trial confirmed these observations, reporting 57% R0 resections when patients where pre-treated with 8 cycles of mFOLFIRINOX, thus identifying it as the most suitable pre-treatment modality at this point.[18] Other prospective trials (PREOPANC, Southwest Oncology Group) could produce similar effects meaning that neoadjuvant treatments might become the main recommendation for patients with BR- and LA- situations.[15,37,38]

Randomized data on neoadjuvant treatment versus up-front surgery in the resectable situation is currently not available but is underway (NCT04927780; NCT04340141) and will soon inform which, or if all, PDAC patients might potentially benefit from pre-treatment modalities.

Meanwhile, more profound insights into molecular alterations in PDAC have yielded many promising but few clinically effective targeted therapies useful for systemic treatment. Patients with gene alterations involved in the homologous recombination repair (*BRCA1/BRCA2)* have become typical candidates for maintenance treatment with Olaparib (PARP inhibitor) following first-line therapy.[39] Likewise, remarkable success has been achieved for patients with mismatch repair deficiencies (microsatellite instability), where PD-1 blockade showed objective responses in most cases (62%).[40] However, only a small selection of patients (<1%) are available for such a targeted approach. Other clinical trials (COMBAT, CITN11–01) focused on combination therapies with immunomodulatory agents and demonstrated clinical effects. [41,42] The most recently introduced molecular-guided therapy on the $KRAS^{G12C}$ mutant (Sotorasib, Adagrasib) will likely guide novel protocols for affected individuals in the near future.[43]

In line with adjuvant and neoadjuvant settings, multiagent cytotoxic regimens have greatly improved the outcomes of patients with advanced carcinoma. Based on the previously mentioned MPACT and PRODIGE trials, current first-line therapies include either gemcitabine and nab-paclitaxel or mFOLFIRINOX.[15] In addition, the NAPOLI-1 trial provided information about patients who have progressed under gemcitabine. It demonstrated improved survival rate under subsequent 5-FU/Irinotecan combination.[44] Ultimately, encouraging improvements in the palliative care (nutrition, pain management, management of thromboses, psychosocial needs) of PDAC patients have produced remarkable advances on overall survival and maintains a fundamental pillar in multimodal therapy.[45]

2.2. Clinical chances and hurdles for curative treatment of PDAC

Because most patients encountered and diagnosed in the clinics have locally advanced or disseminated pancreatic carcinoma, they are not amenable to surgery. For this reason, two-thirds of patients are directly allocated to palliative protocols which are responsible for the overall poor survival rates of PDAC cases. Recent developments using neoadjuvant treatments can induce pre-operative downstaging of

BR or LA tumors and have improved local control for a subset of eligible patients. Surgical treatment generally require radical measures as a prerequisite for removing all carcinoma cells. The technique applied depends on the location of the tumor and requires careful assessment of the anatomic situation and neighboring central vessels. These exacting abdominal operations are exceptionally demanding. Technical refinements over past decades have been able to significantly reduce morbidity and mortality after surgery.[46,47] Still, such abdominal surgeries should be performed in experienced and well-equipped high-volume pancreatic centers with adequate and interdisciplinary complication management.[48,49] Once the tumor is removed, adjuvant treatments are paramount to reduce risk of recurrence. Despite the more effective systemic treatment protocols of recent years, most patients eventually relapse with then limited treatment options and poor prognosis.[50] Studies on long-term survivors have identified perioperative and few molecular factors that could impact the chances of cure including small tumor sizes, negative nodal status, negative resection margin and presence of specific neoantigens.[51-53] At this point, such cases unfortunately remain anecdotal and a better understanding of the early carcinogenesis process and its molecular characteristics is key to discovering novel targets for earlier detection.

2.2.1. Precursor lesions giving rise to invasive carcinoma

From various histopathological and molecular association studies, we understand that pancreatic cancer must arise from noninvasive, precancerous lesions.[54] These lesions are mainly present in two distinct forms: microscopic pancreatic intraepithelial neoplasia (PanIN) and cystic precursors (IPMN, MCN). Classically, with increasing grades (low-grade; high-grade) of cytological and architectural atypia in the pancreatic ducts, invasive PanINs give rise to the majority of PDACs (>90%).[55] However, malignant transformation is not obligate and we know from autopsy studies of elder individuals or pancreatitis samples that PanINs regularly occur in the non-diseased pancreas.[56,57] The overall risk of malignant transformation from high-grade PanINs is not clearly defined. A much smaller proportion of PDACs derives from intraductal papillary mucinous neoplasia (IPMN, <10%). With increasing grades of dysplasia, IPMNs produce an abundance of mucus responsible for its macroscopic appearance (>0.5cm), a distinctive feature from PanINs. The risk of malignant transformation for incidental IPMNs is overall low (1.4-6.9% per year), but significantly increases once the main duct is involved (40-60% per year).[25,58-60] Nonetheless, due to their

macroscopic appearance and the overall low risk of malignant transformation, cysts are normally surveilled until worrisome features or high-risk stigmata in radiographic imaging are present. The least common precursors are mucinous cyst neoplasms (MCN, <2%), that are commonly found in the pancreatic body/tail of middle-aged females.[54] In contrast to IPMNs, the mucin-producing MCNs show an ovarian-type alignment of cells that do not communicate with the ductal system.[54] They harbor higher risks of malignant transformation and typically require immediate removal to prevent cancer.[25] The progression from non-invasive precursors to invasive carcinoma may be fast but they generate a conceptual opportunity for early detection.

2.2.2. Clinical follow-up of cystic precursor lesions

Late diagnosis of PDAC is a critical factor that imposes an overall poor prognosis for patients with this disease. Greater awareness of pre-cancerous lesions and a wider availability of sensitive diagnostic modalities (CT/MRI) has led to an enormous increase of incidental findings with macroscopic cystic lesions in the pancreas.[61] As previously mentioned, patients with identified uni-/multilocular cystic lesions without worrisome or high-risk stigmata are recommended to undergo annual surveillance with the use of appropriate imaging modalities.[25] Normally, around 1.4-6.9% per year will need surgery as a result of the follow-up.[25] After partial resection of their pancreas, patients need an intensified post-operative work-up as there is a low but maintained risk (4-10% per year) for development of lesions elsewhere in the gland.[28,62] More strikingly, many of those can transform into invasive lesions, so called concomitant carcinoma.[63,64] This is likely due to a molecular "*field defect*" and genomic heterogeneity of precursor lesions, which renders the entire gland at risk for developing carcinoma.[65-67] Some research suggests that surveillance can stop after five years of annual follow-up, due to the indolent growth of many of such lesions but this has ignited an intense debate across the community.[59,68,69] At any rate, we endorse close surveillance for patients with incidental finding of pancreatic cysts or post-surgery in cases of IPMNs long-term. The guidelines support the clinicians recommending adequate follow-up of affected individuals.

2.3 The cancer genome during pancreatic tumorigenesis

2.3.1. The molecular signatures of PDAC

Extensive genome sequencing studies demonstrated that PDACs accumulate around 60-200 somatic mutations (Single Nucleotide Variant) and other structural aberrations (copy number alterations, complex re-arrangements) in oncogenes and tumor suppressor genes.[9,70-72] The majority of these mutations occur in the driver genes *KRAS* (90-95%), *CDKN2A* (60-70%), *TP53* (50-80%), and *SMAD4* (40-60%), but are commonly associated with a wider range of low-abundance gene modifications, that target around 12 core signaling pathways.[71,73] While there appears to be homogeneity along the drivers, extensive heterogeneity in passenger gene alterations occur, which generally posits a challenge for finding actionable targets that can address a larger cohort of PDAC patients.[74-76] Cooperative research efforts has attempted to functionally and clinically categorize PDAC patient samples using molecular expression signatures (RNAseq). Two distinct transcriptional programs were identified, characterizing the basal (squamous) or classical subtypes with differential outcomes.[77] Meanwhile, other sub-classifications have been established, while their exact clinical relevance still needs to be determined.[11,12,71] In summary, the genomic underpinnings of the disease have been characterized in-depth. At the same time downstream molecular and cellular implications remain an underexplored field that needs to be better exploited for direct clinical applications.

2.3.2. Genomic alterations in pancreatic precursor lesions

Few studies have investigated the genomic characteristics of precancerous lesions. From histopathological association analyses of human tissue samples we characterize the increasing grades of dysplasia along changes in several driver genes.[78-80] These initial findings defined the pancreatic progression model in which *KRAS* is considered an initiating event while alterations in the tumor suppressors (*CDKN2A, TP53, SMAD*4) occur with sequential order from low- to high-grade or invasive lesions (**Figure 1**).[81] More recent NGS studies have confirmed these observations, while identifying some important distinctions between PanINs and cystic precursors (IPMNs).[82-85] The latter are driven to a lesser extent by *KRAS* alterations but more frequently contain *GNAS* and *RN43* mutations. In recent years, this strict molecular trajectory has been challenged by more complex signatures like that of “catastrophic shattering” (chromothripsis) which could release a definitive mechanism by which tumor

suppressor inactivation occurs simultaneously.[86] At any rate, there is a remarkable clonal heterogeneity in precancerous lesions with convergent evolution which appears to be present only at the later stages of the carcinogenic process.[67,87,88] Along the line, precursor lesions regularly occur as multifocal with distinct and/or parallel molecular characteristics. However, at this point, it is still impossible to predict the genomic composition ultimately responsible for downstream invasion.[64-66]

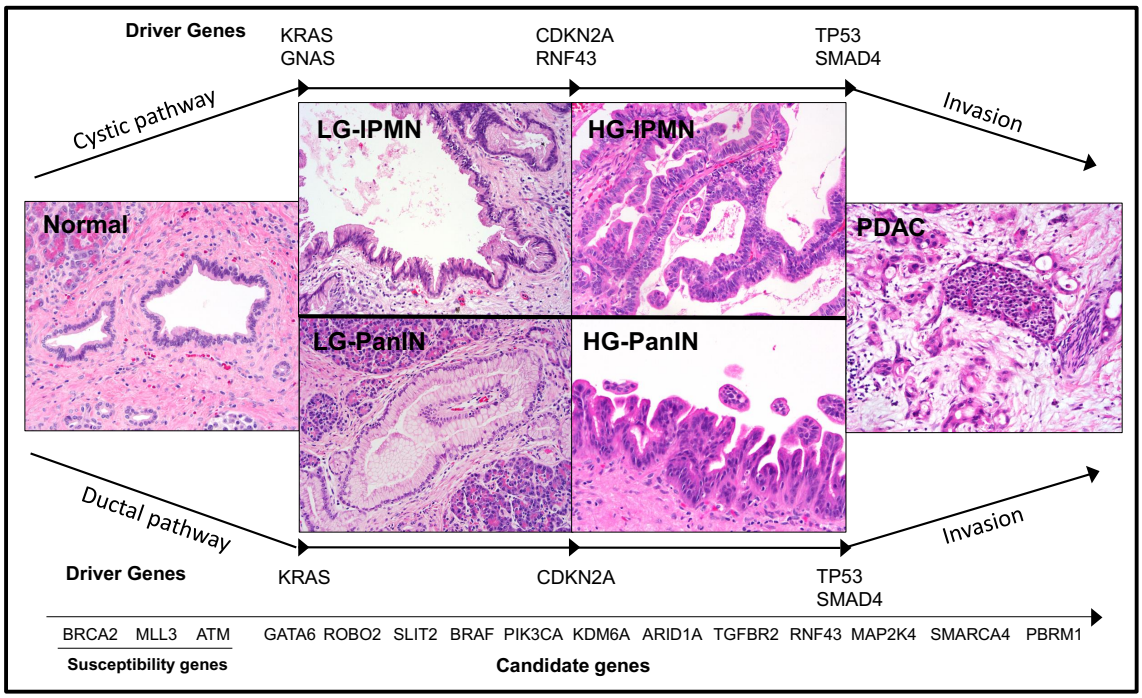

Figure 1. The accumulation of genomic alterations leads to dysplasia and ultimately to invasive PDAC. Two molecular pathways with specific driver gene alterations have been characterized, both driving pancreatic carcinogenesis. Figure extracted from own manuscript *Felsenstein M et al.* [89]

2.4. Experimental modeling for understanding of early pancreatic carcinogenesis

The molecular understanding of pancreatic carcinogenesis from human tissue samples has culminated in intense experimental investigation using animal- and *in vitro* culture models. Sophisticated, genetically engineered mouse models (GEMMs) generated de-novo pancreatic neoplasia (PanIN) with subsequent development of full-blown carcinoma and metastases, quite accurately aligned with the human disease condition.[90] The two essential mutated elements *KRAS* and *TP53* (KC; KPC mice) appear to drive the process of murine PDAC development, while other common drivers (*CDKN2A, SMAD4, BRCA2, PTEN*) modify specific biological and histological tumor characteristics.[91-95] Their implementation provides further evidence for the pancreatic

cancer progression model and allows for continuous and standardized experimental investigation precipitating accelerated carcinoma development. GEMMs were therefore used in the pre-clinical settings for drug testing, sometimes with predictive but often less indicative results.[96-98] A rather disappointing example resulted in differential responses of the Hedgehog-signaling inhibitor (IPI-926) exhibiting poor or even harmful responses in human patients.[99,100]

Compared to animal models, *in vitro* two-dimensional- (cell lines) and three-dimensional (organoid culture, spheroids) carcinoma models can be better standardized, are less expensive and time-consuming and enable the development of high throughput experiments on multi-omics and drug screening.[101-103] The various steps of pancreatic carcinogenesis have been similarly explored and resulted in an in-depth understanding of the required molecular signatures for tumorigenic transformation.[104-106] Recently, established transgenic models using the CRISPR/Cas9 technology, convincingly demonstrated *in vitro* carcinogenesis of three-dimensional culture systems.[107] The models successfully recapitulated the progression from (near-) normal pancreas cells to more invasive phenotypes by enriching cells with genetic perturbations dependent on environmental niche factors. Still, the two- and three-dimensional tissue culture represents an artificial *ex vivo* system and does not sufficiently take the patients' tumor micro-environment (stroma, immune cells) into account. Consequently, next-generation *ex vivo* models such as patient derived tissue slices or decellularized matrices are increasingly explored, in order to better recapitulate the human disease *ex vivo*.[108,109]

Despite recent valuable insights into the molecular mechanisms of pancreatic tumorigenesis, the derivation of effective treatment modalities from such studies remains disappointingly limited. A better understanding of the early phase of pancreatic carcinogenesis using genetically defined precursor cell models as well as next-generation *in vitro* cell cultures should significantly enhance the theoretical yield and adding translational knowledge to better address these endeavors.

2.5. Translational and clinical developments for early detection of PDAC

Based on mathematical models of genomic studies, the window of diagnostic opportunity for diagnosis of PDAC is estimated to be much longer than posited at this point.[74,87] Therefore, some research efforts focus on earlier detection. Despite remarkable progress in the sensitivity of diagnostic tools and molecular understanding

of the disease process, the population-wide screening of asymptomatic individuals is currently not justified.[110] It is widely acknowledged that we need to identify individuals with overall increased lifetime risk calculation, in order to establish cost-effective early detection while improving outcomes for the population overall.[111] Longitudinal studies conducted by the multidisciplinary International Cancer of the Pancreas Consortium (CAPS) identifes high-risk individuals (HRI) based on genomic alterations in “susceptibility” genes as well as documented disease in first and second degree blood relatives.[112] The observation of accumulated carcinoma development in blood relatives of affected individuals has led to the assumption that more than 10% of PDACs may underlie an inherited trajectory with deleterious mutations in susceptibility genes; many have possibly still not been identified at this point.[113,114] Indeed, individuals that fulfilled the HRI criteria (germline mutation carriers in susceptibility gene and/or affected first/second degree relative) and who received annual diagnostic work-up (CT/MRI, EUS) developed neoplastic progression at a rate of 2.8% during a median follow-up of 34 months, while 83% fulfilled criteria for surgical treatment (worrisome and/or high-risk stigmata) and were technically resectable.[115] Unfortunately, despite intensified screening, 17% of affected patients developed advanced carcinoma highlighting the diagnostic limitations of a disease with a particularly short diagnostic lead-time and aggressive nature.

Other exciting fields under ongoing investigation include molecular analyses of liquid biopsies from pancreatic juice/cyst fluid as well as plasma. The molecular (CEA, Amylase) and cytopathologic testing of larger or medium-sized pancreatic cysts via EUS-guided FNA is routinely indicated for further dignity assessment. More recently, sensitive high-throughput sequencing analyses allow for the detection of high-grade neoplasia via driver gene alterations (*TP53, SMAD4*).[116-118] This could prove interesting, not only in the presence of larger neoplastic cysts but also via collection of pancreatic juice after stimulation with secretin.[119,120] Further technical advances for improving the diagnostic sensitivity will most certainly yield powerful tools for early detection. The availability and ease of molecular testing in blood plasma offer an attractive vehicle for early detection and screening. Unfortunately current diagnostic sensitivities of NGS-based detections of mutated circulating tumor DNA (ctDNA) can only be warranted at disseminated stages which poses a problem for early discovery.[121,122] Technical optimization of clinical samples has allowed the detection of mutated DNA in the plasma patients with localized disease in 43% of the cases and

predicted recurrence around 6.5 months earlier than radiographic imaging.[123] Further advances in the detection of degradation-protected exosomal DNA may allow for more in-depth molecular information of ctDNA and so produce more accurate risk stratification.[124,125] Clearly, ctDNA enables efficient monitoring of disease recurrence and progression of patients undergoing (neo-)adjuvant treatment and has already been clinically employed in some institutions.[126-128]

2.6. Research aims

The core aim of my research plan was to address and provide novel insights on cancer genomics of PDACs and its precursors as well as exploring ways for improved and modern academic surgical treatment concepts. Working in an experimental laboratory with excellent expertise on the molecular and the histopathological trajectory of pancreatic carcinogenesis, I was seeking for genomic familiarities on pancreatic carcinoma precursor with its neighboring invasive lesions. Subsequently, I aimed at understanding and characterizing earlier stages of non-invasive precursors in order to better define the evolutionary consequences of specific driver gene alterations. Altogether, the data collected was paramount to providing additional evidence on the sequential concept of cancer invasion from cystic precursors, that could significantly aid in finding tools and biomarkers for earlier detection. Apart from analyses of clinical tissue samples, I also sought to investigate on *in vitro* models for further testing the molecular concept of disease progression. Starting my clinical training as an academic surgeon, great focus subsequently lied also on investigating modern concepts and tools to employ innovative robotic devices for complex pancreatic surgery. Another imminent field for patient survival after pancreatic surgery, remains adequate and optimized complication management. We sought to establish and characterize a novel interdisciplinary concept, for improving on clinical management of patients with pancreatic fistula. In the studies presented, I highlight the acquisition of in-depth experience on basic-, translational and clinical research that has provided a multidimensional research perspective on pancreatic diseases, which I deem essential for a sophisticated understanding of the underlying disease trajectory. Altogether, this has positioned my personal academic ambitions for advancing on improved molecular understanding of pancreatic carcinogenesis and uncovering novel paths for earlier detection.

2 PRESENTATION OF OWN WORK

3.1. IPMNs with co-occurring invasive cancers: neighbours but not always relatives

In the initial phase on my path towards clinician scientist on topics of pancreatic carcinoma development, we have explored the molecular trajectories of cystic precursor lesions. We were able to provide crucial evidence, that the co-occurrence of these pre-malignant lesions with PDAC, not always infer their origin, which only has been suspected for some time:

„Objective: Intraductal papillary mucinous neoplasms (IPMNs) are precursor lesions that can give rise to invasive pancreatic carcinoma. Although approximately 8% of patients with resected pancreatic ductal adenocarcinoma have a co-occurring IPMN, the precise genetic relationship between these two lesions has not been systematically investigated.

Design: We analysed all available patients with co-occurring IPMN and invasive intrapancreatic carcinoma over a 10-year period at a single institution. For each patient, we separately isolated DNA from the carcinoma, adjacent IPMN and distant IPMN and performed targeted next generation sequencing of a panel of pancreatic cancer driver genes. We then used the identified mutations to infer the relatedness of the IPMN and co-occurring invasive carcinoma in each patient.

Results: We analysed co-occurring IPMN and invasive carcinoma from 61 patients with IPMN/ductal adenocarcinoma as well as 13 patients with IPMN/colloid carcinoma and 7 patients with IPMN/carcinoma of the ampullary region. Of the patients with co-occurring IPMN and ductal adenocarcinoma, 51% were likely related. Surprisingly, 18% of co-occurring IPMN and ductal adenocarcinomas were likely independent, suggesting that the carcinoma arose from an independent precursor. By contrast, all colloid carcinomas were likely related to their associated IPMNs. In addition, these analyses showed striking genetic heterogeneity in IPMNs, even with respect to well-characterised driver genes.

Conclusion: This study demonstrates a higher prevalence of likely independent co-occurring IPMN and ductal adenocarcinoma than previously appreciated. These findings have important implications for molecular risk stratification of patients with IPMN.“

The abstract has been extracted from PMID: 29500184:

IPMNs with co-occurring invasive cancers: neighbours but not always relatives.
Matthäus Felsenstein, Michaël Noë, David L Masica, Waki Hosoda, Peter Chianchiano, Catherine G Fischer, Gemma Lionheart, Lodewijk A A Brosens, Antonio Pea, Jun Yu, Georgios Gemenetzis, Vincent P Groot, Martin A Makary, Jin He, Matthew J Weiss, John L Cameron, Christopher L Wolfgang, Ralph H Hruban, Nicholas J Roberts, Rachel Karchin, Michael G Goggins, Laura D Wood. **Gut. 2018** Sep;67(9):1652-1662.
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3.2. Multiregion whole-exome sequencing of intraductal papillary mucinous neoplasms reveals frequent somatic KLF4 mutations predominantly in low-grade regions

Subsequent efforts focused on in-depth und multi-regional characterization of cystic precursor lesions showing different histologic grades of dysplasia. We were able to identify a novel, unprecedented genomic marker that may play an important role for the early evolutionary process of disease progression:

„Objective: Intraductal papillary mucinous neoplasms (IPMNs) are non-invasive precursor lesions that can progress to invasive pancreatic cancer and are classified as low-grade or high-grade based on the morphology of the neoplastic epithelium. We aimed to compare genetic alterations in low-grade and high-grade regions of the same IPMN in order to identify molecular alterations underlying neoplastic progression.

Design: We performed multiregion whole exome sequencing on tissue samples from 17 IPMNs with both low-grade and high-grade dysplasia (76 IPMN regions, including 49 from low-grade dysplasia and 27 from high-grade dysplasia). We reconstructed the phylogeny for each case, and we assessed mutations in a novel driver gene in an independent cohort of 63 IPMN cyst fluid samples.

Results: Our multiregion whole exome sequencing identified KLF4, a previously unreported genetic driver of IPMN tumorigenesis, with hotspot mutations in one of two codons identified in >50% of the analyzed IPMNs. Mutations in KLF4 were significantly more prevalent in low-grade regions in our sequenced cases. Phylogenetic analyses of whole exome sequencing data demonstrated diverse patterns of IPMN initiation and progression. Hotspot mutations in KLF4 were also identified in an independent cohort of IPMN cyst fluid samples, again with a significantly higher prevalence in low-grade IPMNs.

Conclusion: Hotspot mutations in KLF4 occur at high prevalence in IPMNs. Unique among pancreatic driver genes, KLF4 mutations are enriched in low-grade IPMNs. These data highlight distinct molecular features of low-grade and high-grade dysplasia and suggest diverse pathways to high-grade dysplasia via the IPMN pathway.“

The abstract has been extracted from PMID: 33028669

Multiregion whole-exome sequencing of intraductal papillary mucinous neoplasms reveals frequent somatic KLF4 mutations predominantly in low-grade regions.

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Yip-Schneider, Rita T Lawlor, Roberto Salvia, Nicholas J Roberts, Elizabeth D Thompson, Rachel Karchin, Anne Marie Lennon, Yuchen Jiao , Laura D Wood.
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Multi-region Whole Exome Sequencing of Intraductal Papillary Mucinous Neoplasms Reveals Frequent Somatic *KLF4* Mutations Predominantly in Low-Grade Regions

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STATEMENT OF AUTHOR CONTRIBUTIONS

KF, WH, MF, QS, YJ, LDW designed the study. WH, MDM, CFD, MMK, CMS, MTY, RLT, RS, EDT, AML, LDW contributed to sample acquisition. WH, QS, JD, JC, PW acquired data. KF, MF, QS, JR, LZ, VBG, NR analyzed data. KF, WH, MF, QS, JR, LZ, VBG, CGF, AMB, MN, MJ, NJR, RK, YJ, LDW interpreted data. KF, LDW wrote the manuscript. All authors critically reviewed the manuscript. LDW provided study supervision.

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ETHICS APPROVAL

This study was approved by Institutional Review Board of The Johns Hopkins Hospital. Patients or the public were not involved in the design, conduct, reporting, or dissemination plans of our research.

Conflict of Interest

LDW receives research support from Applied Materials. The other authors declare no conflict of interest.

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Abstract

Objective: Intraductal papillary mucinous neoplasms (IPMNs) are non-invasive precursor lesions that can progress to invasive pancreatic cancer and are classified as low-grade or high-grade based on the morphology of the neoplastic epithelium. We aimed to compare genetic alterations in low-grade and high-grade regions of the same IPMN in order to identify molecular alterations underlying neoplastic progression.

Design: We performed multi-region whole exome sequencing on tissue samples from 17 IPMNs with both low-grade and high-grade dysplasia (76 IPMN regions, including 49 from low-grade dysplasia and 27 from high-grade dysplasia). We reconstructed the phylogeny for each case, and we assessed mutations in a novel driver gene in an independent cohort of 63 IPMN cyst fluid samples.

Results: Our multi-region whole exome sequencing identified *KLF4*, a previously unreported genetic driver of IPMN tumorigenesis, with hotspot mutations in one of two codons identified in >50% of the analyzed IPMNs. Mutations in *KLF4* were significantly more prevalent in low-grade regions in our sequenced cases. Phylogenetic analyses of whole exome sequencing data demonstrated diverse patterns of IPMN initiation and progression. Hotspot mutations in *KLF4* were also identified in an independent cohort of IPMN cyst fluid samples, again with a significantly higher prevalence in low-grade IPMNs.

Conclusion: Hotspot mutations in *KLF4* occur at high prevalence in IPMNs. Unique among pancreatic driver genes, *KLF4* mutations are enriched in low-grade IPMNs. These data highlight

distinct molecular features of low-grade and high-grade dysplasia and suggest diverse pathways to high-grade dysplasia via the IPMN pathway.

Keywords

intraductal papillary mucinous neoplasm; pancreatic precursor lesion; whole exome sequencing; KLF4; phylogenetic analysis; cyst fluid; somatic mutation

INTRODUCTION

Intraductal papillary mucinous neoplasms (IPMNs) are non-invasive cyst-forming pancreatic neoplasms that can progress to aggressive invasive pancreatic ductal adenocarcinoma (PDAC). IPMNs are classified based on the morphological dysplasia of their neoplastic epithelium – low-grade IPMNs have minimal atypia and low risk of malignant transformation, while high-grade IPMNs have severe atypia and are at higher risk for progression to invasive cancer.[1] IPMNs are frequently diagnosed incidentally on abdominal imaging, providing an important opportunity to prevent pancreatic cancer.[23] Guidelines for surveillance or surgical intervention in IPMN patients must balance the opportunity for cancer prevention with the morbidity and even mortality associated with overtreatment of low-risk lesions.[4–6] Current decision making relies largely on clinical and radiological features, but these approaches are not adequately sensitive nor are they specific for high-risk IPMNs.[56] Thus, there is a critical need to better understand the molecular alterations that drive the progression of low-risk IPMNs to those at high-risk for progression to invasive carcinoma, as these represent potential biomarkers for cysts requiring clinical intervention.

In contrast to the trove of genomic data describing invasive pancreatic cancers, the genomes of relatively few IPMNs have been analyzed. Previous comprehensive sequencing of small cohorts of IPMNs mostly focused on advanced lesions and revealed characteristic driver genes,[7–9] while targeted analyses in larger, more diverse cohorts have confirmed the prevalence of specific driver gene mutations that correlate with grade of dysplasia or histological subtype.[10] These initial studies relied on analysis of a single region from each IPMN, followed by comparison of clinical, pathological, and molecular characteristics across different patients. More recently, multi-region targeted next generation sequencing of IPMNs has revealed a surprising degree of intratumoral genetic heterogeneity, even with respect to well-characterized driver gene mutations in IPMNs, highlighting previously unappreciated genetic complexity in precancerous pancreatic lesions.[11–13] However, comprehensive multi-region sequencing has not yet been performed on IPMNs. Such analyses can define the genomic alterations associated with progression in individual lesions, uncover new driver genes, and define unique evolutionary patterns in precancerous lesions.

In this study, we report multi-region whole exome sequencing of distinct low-grade and high-grade regions of 17 human IPMNs without associated invasive carcinoma. The resulting data define evolutionary trajectories in IPMN progression and highlight genetic heterogeneity throughout these lesions. In addition, we identify a novel driver of early IPMN tumorigenesis with a unique evolutionary pattern and validate patterns of mutations in an

independent cohort of IPMN cyst fluid samples. Taken together, our results provide several key insights not possible through analysis of advanced cancers, highlighting the importance of direct analysis of precancerous lesions.

METHODS

Clinical Data

Electronic medical records were reviewed to document clinical information such as age, sex, family history, clinical presentation, imaging diagnosis, and outcome. These clinical data are summarized in online supplementary table 1.

Case selection and specimen acquisition

We retrospectively reviewed surgical pancreatectomy specimens from patients diagnosed with IPMN without associated invasive carcinoma between 2007 and 2016. Diagnostic hematoxylin-and-eosin stained slides were reviewed by pancreatic pathologists (WH, LDW) to identify IPMNs with distinct components of both low-grade and high-grade dysplasia and to select 1–3 blocks per case with regions of both grades of dysplasia. Because of recent previous reports of polyclonal origin in IPMNs,[12] we carefully selected IPMN cases in which morphological features suggested that the high-grade component arose in association with the co-existing low-grade component. We set the following histologic criteria for the case selection: IPMN with adequate quantities of both low-grade and high-grade components for genomic analysis in which (1) a high-grade component was in direct contact with a low-grade component, OR (2) if the low-grade component was close to but not directly attached to the high-grade component (in most instances growing in a different cystic space), then both components were located within the same formalin-fixed paraffin-embedded (FFPE) block. Of 118 resected specimens of high-grade IPMN retrieved from the database, we found 24 high-grade IPMN cases that met the aforementioned criteria, and morphologically distinct regions of each grade were identified in 32 blocks from these cases and selected for subsequent laser capture microdissection. The histological subtype of each sequenced region was determined by consensus of four pathologists (KF, EDT, RHH, and LDW).

Laser capture microdissection

Twenty to thirty 10um serial tissue sections from selected FFPE tissue blocks were cut onto membrane slides (Carl Zeiss MembranSlide 1.0 PEN; Carl Zeiss, Oberkochen, Germany). Deparaffinization and staining were performed as previously described.[14] Two to six morphologically distinct regions were microdissected from each case using laser capture microdissection (LMD7000, Leica, Wetzlar, Germany), resulting in DNA samples of adequate quantity and quality for whole exome sequencing from 76 IPMN regions from 17 cases, as well as a matched normal sample from each case. We did not obtain adequate DNA from the other 7 microdissected cases, which were excluded from further analyses.

Whole exome sequencing and data analysis

Genomic DNA libraries were prepared from the 93 FFPE DNA samples (76 IPMN samples, 17 normal samples) following Illumina's (Illumina, San Diego, CA) suggested protocol.

Human exome capture was performed following a protocol from Agilent SureSelect Human All Exon 50Mb Kit 5.0 (Agilent, Santa Clara, CA). The captured libraries were sequenced with XTEN sequencer (Illumina) with 150bp paired-end reads. Nonsynonymous mutations were called using a well-validated pipeline based on Mutect.[15] Details of the pipeline are presented in the online supplementary methods.

Phylogenetic analysis

To extract per-site features (mismatch frequency, insertion frequency, and deletion frequency), BAM alignment files were converted to tab delimited format using jvarkit (https://github.com/lindenb/jvarkit). We inferred phylogenies with Treeomics v1.7.12 based on all mutations that passed the filtering described above. Each phylogeny is rooted at the subject's normal sample and the leaves represent the distinct regions of the IPMN. Treeomics uses a Bayesian inference model to account for error-prone sequencing and varying neoplastic cell content to infer globally optimal trees using Mixed Integer Linear programming.[16] Gene names along lineages indicate an acquired non-synonymous mutation in the corresponding gene – genes are listed twice in the same phylogeny only if multiple distinct somatic mutations in that gene were identified. We display mutations in previously identified PDAC driver genes (significantly mutated genes in TCGA and ICGC PDAC studies),[1718] as well as mutations in genes that were mutated in at least three separate IPMNs and had a nonsynonymous mutation frequency >0.5 mutations per kb gene size in our cohort.

Analysis of IPMN cyst fluid

Pancreatic cyst fluid was collected from resected specimens in the surgical pathology laboratory (n=55) or at the time of endoscopic ultrasound (n=8). Genomic DNA was purified from pancreatic cyst fluid as described previously.[19] The hotspot loci in the *KLF4* gene were analyzed in IPMN cyst fluid using the Safe Sequencing System (Safe-SeqS),[20] which has been described in detail previously.[2122] Details are provided in the online supplementary methods.

RESULTS

Overall approach

In order to dissect the molecular events in low-grade and high-grade components of precancerous pancreatic lesions, we performed multi-region whole exome sequencing of 17 IPMNs containing regions of both low-grade and high-grade dysplasia (online supplementary table 2). We analyzed a total of 76 IPMN exomes, including 49 from low-grade regions and 27 from high-grade regions – the number of exomes analyzed per IPMN ranged from 2 to 6 (online supplementary table 2). In the majority of IPMNs in our cohort (14/17), the low-grade regions showed gastric differentiation, while the high-grade regions in the same IPMN were pancreatobiliary (online supplementary table 2). In addition, our cohort contained two mixed gastric-intestinal type IPMNs, as well as one that showed gastric differentiation in all regions analyzed (online supplementary table 2). In each case, matched normal samples were also analyzed by whole exome sequencing to exclude

germline variants and to identify somatic mutations. The average distinct coverage for our IPMN whole exome sequencing was 170x (range 38x–367x) (online supplementary table 3).

From the multi-region whole exome sequencing data, we identified a total of 3,090 nonsynonymous somatic mutations, with a mean of 41 nonsynonymous somatic mutations per analyzed IPMN region, corresponding to a mutation burden of 1.11 nonsynonymous mutations per megabase (Mb) (online supplementary table 4, online supplementary figure 1). Surprisingly, the mean number of somatic mutations did not differ between low-grade and high-grade regions – we identified a mean of 41 nonsynonymous somatic mutations (range 20–103) in low-grade regions compared to a mean of 41 (range 22–76) in high-grade regions (p=0.55, two-tailed Mann-Whitney U test) (figure 1A). These correspond to a mutation burden of 1.10 nonsynonymous mutations per Mb in low-grade regions and 1.12 non-synonymous mutations per Mb in high-grade regions. A mean of 10 nonsynonymous somatic mutations were shared among all samples analyzed from a given IPMN, while a mean of 26 were unique/private to low-grade regions and a mean of 24 were unique/private to high-grade regions. We then compared the neoplastic cell fraction (NCF) of mutations in low-grade and high-grade regions (figure 1B). There was no significant difference between low-grade and high-grade regions in the NCF of mutations shared in all samples of a given IPMN (p=0.51, two-tailed Mann-Whitney U test). In contrast, we identified a significant difference between low-grade and high-grade regions in the NCF of unshared mutations, with mutations in low-grade regions having a significantly lower NCF than those in high-grade regions (p<0.0001, two-tailed Mann-Whitney U test). Regions of high-grade dysplasia had a larger proportion of unshared mutations with a NCF approaching 1, suggestive of clonal mutations shared in all analyzed cells (figure 1B). Still, mutation signatures were similar among all samples analyzed, with an enrichment for C-to-T transitions (figure 1C).

Copy number analyses utilizing the whole exome sequencing data revealed scattered alterations without striking differences between low-grade and high-grade components in most IPMNs (online supplementary figure 2, online supplementary table 5). However, a minor subset of cases showed increased copy number alterations in high-grade regions (for example, IP2, IP9, IP29), suggesting accumulation of copy number alterations during neoplastic progression in some IPMNs (online supplementary figure 2).

Driver genes of IPMN tumorigenesis

Through multi-region whole exome sequencing, we confirmed the high prevalence of mutations in some previously identified pancreatic driver genes in IPMN cases, including *GNAS* (15/17 cases; 88%), *KRAS* (14/17 cases; 82%), *RNF43* (10/17 cases; 59%), and *CDKN2A* (5/17 cases; 29%) (figure 2A, online supplementary figure 3). Intriguingly, *TP53* mutations were uncommon in our cohort (2/17 cases; 12%), and we identified no mutations in *SMAD4* or *TGFBR2*. We also identified hotspot mutations in *CTNNB1* (3/17 cases; 18%) and inactivating mutations in *APC* (3/17 cases; 18%), demonstrating WNT signaling alterations in a subset of IPMNs as has been previously reported.[10] In addition, nonsynonymous mutations in *RBM10* were identified in 24% (4/17) of IPMNs in our study – mutations in this gene have been previously reported in both IPMNs and PDACs.[1323]

In addition to confirming the prevalence of mutations in previously characterized driver genes, our study also identified novel drivers of pancreatic tumorigenesis these IPMNs. The most striking of these new drivers was *KLF4*, which encodes a member of the Kruppel family of transcription factors. We identified somatic mutations in one of two hotspot codons (amino acids K409 and S411) of *KLF4* in 53% (9/17) of IPMNs in our study (figure 2A–2B). Both hotspots are located in the highly conserved C2H2 zinc finger domains in *KLF4* (figure 3A–3B). A total four different amino acid substitutions (K409Q, K409E, S411Y and S411F) were detected in these two hotspots, and two IPMNs in our cohort had two different *KLF4* mutations (K409E and S411F in IP5; K409Q and S411Y in IP7) (2/17 cases; 12%). We analyzed the four different *KLF4* hotspot mutations using Cancer-specific High-throughput Annotation of Somatic Mutations plus (CHASMplus).[24] All four different *KLF4* hotspot mutations had high CHASMplus PDAC scores (K409Q, p=0.042; K409E, p=0.041; S411Y, p=0.038; S411F, p=0.038), suggesting that these mutations are likely to be drivers of IPMN tumorigenesis. None of these amino acid substitutions were detected in the Genome Aggregation Database (gnomAD) of germline alterations (>250,000 alleles). Only *KLF4* K409Q has been previously reported in meningiomas and in three cases in previous IPMN analyses, but our study represents the first report of four different *KLF4* hotspot mutations at this high prevalence in epithelial neoplasms.[8132526]

In addition to these frequent mutations in *KLF4*, we report nonsynonymous mutations in *FBXW7* and *HUWE1*, each in 18% (3/17) of the IPMNs studied – these genes encode the components of an E3 ubiquitin ligase and are previously characterized tumor suppressor gene in other tumor types (figure 2A–2B, online supplementary figure 3).[2728] We also report mutations in *SETBP1* in 24% (4/17) of IPMNs; mutations in this gene have been reported in several other tumor types but have not been previously highlighted in pancreatic neoplasms.[2930] Other genes with prevalent somatic mutations in our cohort include *MUC16* (6/17 cases; 35%), *TTN* (5/17 cases; 29%), and *NBPF1* (5/17 cases; 29%). However, the large size of *MUC16* and *TTN* suggest that many (if not all) of these mutations may be passengers.

The comprehensive genomic analysis of multiple regions per IPMN, including regions of both low-grade and high-grade dysplasia, provides a unique opportunity to assess the timing of mutations in specific driver genes. In IPMNs with *KRAS* mutations, at least one mutation in this gene was shared in both low-grade and high-grade components (figure 2A–2B). Similarly, mutations in *GNAS* were almost always shared between low-grade and high-grade components. Most other mutated genes had a mixture of mutational patterns, including mutations limited to both low-grade and high-grade components. Of note, *TP53* mutations were consistently limited to high-grade components, as were mutations in the adherens junction protein *AJAP1* (figure 2A–2B).

Mutations in *KLF4* had a strikingly different pattern, with mutations frequently limited to the low-grade component of the IPMNs – *KLF4* hotspot mutations were limited to the low-grade component in 6 of 9 mutant cases and were shared between the low-grade and high-grade components of 3 of 9 (example in figure 4). In our cohort, the prevalence of *KLF4* mutations in low-grade and high-grade components was significantly different (figure 2B; 40% vs. 15%, p=0.049, two-tailed Mann-Whitney U test). There were no *KLF4* mutations that were limited to the high-grade components. When the previous three-tiered histologic

grading system for IPMN was applied, with low-grade IPMN divided into “low-grade” and “intermediate-grade” dysplasia, the prevalence of *KLF4* mutations was highest in “low-grade” (50%) compared to “intermediate-grade” (39%) or “high-grade” (15%; p=0.023, two-tailed Mann-Whitney U test vs. “low-grade”) (figure 3C). In addition, the prevalence of hotspot *KLF4* mutations in The Cancer Genome Atlas (TCGA) analysis of invasive PDAC (0.5%, 1/184 cases) was much lower than the prevalence in our cohort of precancerous lesions.[17]

Our multi-region sequencing data also allows examination of genetic heterogeneity in IPMNs, confirming previous observations about precancerous pancreatic neoplasia.[12] In IP24, two of the analyzed regions shared no somatic mutations with the other regions, suggesting that the analyzed tissue block contained multiple independent precancerous neoplasms. In this IPMN, the independent neoplasms demonstrably involved the same ductal space (figure 5). These observations provide further support for the hypothesis that at least a subset of IPMNs have polyclonal origin and are comprised of multiple independently arising clones that share no somatic mutations.[12] We also identified a distinct pattern of mutation in both *RNF43* and *KLF4* in which multiple mutations in the same driver gene were present in different regions of the same IPMN, suggesting convergent evolution with respect to mutations in some driver genes (figure 4). This pattern has been previously reported in *RNF43* but not *KLF4*, highlighting unique selective pressures on mutations in these two key driver genes.[1231]

Evolutionary Analyses of Non-invasive IPMNs

In order to analyze the evolutionary relationships of IPMN samples in more detail, we reconstructed lesional phylogenies using Treeomics, a computational approach specifically designed for noisy next generation sequencing data from tumor samples.[16] Treeomics calculates the probability that a mutation is present or absent in a particular sample based on the number of mutant and reference reads, with hyperparameters for the probability calculation depending on the estimated sample purity as well as other variables. Mutations are placed on the root of the tree by Treeomics only if that mutation was present with a high probability in all samples. Multiple important insights were evident from this analysis. First, our evolutionary analysis highlighted that in multiple samples (e.g. IP7, IP8, IP31), high-grade dysplasia arose without unique mutations in pancreatic driver genes, while in others (e.g. IP2, IP9) high-grade-specific mutations in driver genes such as *TP53* were identified (online supplementary figure 4). Second, in some samples with multiple distinct high-grade regions (e.g. IP7, IP20), we demonstrate that such regions are more closely related to low-grade regions than to each other, suggesting that the transition to high-grade dysplasia can occur multiple times in the same IPMN (figure 6, online supplementary figure 4). However, this pattern is not universal, as in other IPMNs (e.g. IP2) the high-grade components have a recent common ancestor (online supplementary figure 4). Our Treeomics analysis also confirmed independent genetic origin of different regions of IP24 and highlighted the parallel evolution of multiple distinct *RNF43* mutations in discrete subclones in IP20 (figure 6, online supplementary figure 4).

We also assessed quantitative features of the Treeomics phylogenies in detail. We observed varying lengths of the "trunk" of the phylogenetic tree, ranging from 0 mutations shared in all samples to 27 (online supplementary figure 4). The mean was ~12 truncal mutations, but there was a broad range - seven IPMNs had <10 truncal mutation while two IPMNs had >20. This highlights distinct evolutionary features in different IPMNs, underscoring that the selective forces governing neoplastic evolution may be variable between patients. Next, we compared the genetic relatedness of low-grade and high-grade regions using "genetic distance", defined as the total number of non-shared somatic mutations between two samples.[32] High-grade/high-grade sample comparisons had a lower mean genetic distance (36.1), when compared to low-grade/high-grade sample comparisons (44.2) and low-grade/low-grade sample comparisons (45.2). However, these results did not reach statistical significance (p=0.21 for LG/LG vs HG/HG, p=0.26 for LG/HG vs HG/HG, two-tailed Mann-Whitney U test).

Detection of KLF4 mutations in IPMN cyst fluid

In order to determine the prevalence of *KLF4* mutations in an independent cohort, we employed next generation sequencing analysis of human IPMN cyst fluid samples using the Safe Sequencing System (Safe-SeqS), a method designed to reduce sequencing errors and detect low-frequency mutations.[20] Clinical and pathological features of the analyzed cyst fluid samples are presented in the online supplementary table 6. Our Safe-SeqS assay was designed to assess the previously identified hotspots in *KLF4* at codons 409 and 411. The cyst fluid samples were from 63 IPMNs, including 26 low-grade and 37 high-grade IPMNs, with all diagnoses confirmed on pathological review of resected specimens. In this cohort of 63 cyst fluid samples, we identified *KLF4* mutations in 19 samples (30%), including 11 samples with 1 *KLF4* mutation, 7 samples with two distinct *KLF4* mutations, and 1 sample with three distinct *KLF4* mutations (table 1). The prevalence of multiple *KLF4* mutations in our cyst fluid samples (8 of 63, 13%) is similar to that identified in whole exome sequencing (2 of 17, 12%). Hotspot mutations in *KLF4* were identified in 12 of 26 (46%) low-grade IPMN cyst fluid samples, compared to 7 of 37 (19%) high-grade IPMN cyst fluid samples using Safe-SeqS (p=0.027, two-tailed Fisher's exact test). These results confirm the high prevalence of *KLF4* mutations in IPMNs as well as the enrichment of these in low-grade lesions. In addition, we identified significantly different *KLF4* mutation prevalence based on histological subtype, with mutations in 10 of 22 (45%) gastric-type IPMNs but only 3 of 22 (14%) intestinal-type IPMNs (p=0.045, two-tailed Fisher's exact test) (online supplementary table 7). The detectability of *KLF4* mutations in cyst fluid samples highlights their potential utility in preoperative risk stratification of IPMNs.

DISCUSSION

Using multi-region whole exome sequencing of IPMNs with both low-grade and high-grade components, we identified prevalent mutations in a previously unappreciated driver of pancreatic neoplasia: *KLF4*. Prevalent mutations at an oncogenic hotspot in *KLF4* have been previously reported in meningiomas and have been implicated as a universal genetic feature of the secretory subtype of meningioma.[25,26] In the pancreas, loss of heterozygosity at the *KLF4* locus has been reported in PDAC, but frequent somatic mutations in this gene have

not been previously reported.[33] Somatic mutations at hotspot positions in *KLF4* have been reported in three cases in previous whole exome sequencing studies of IPMNs, but mutations at the prevalence in our study (with somatic mutations in >50% of analyzed IPMNs) have not been previously reported.[8,13] This is likely due to the enrichment of *KLF4* mutations in regions of low-grade dysplasia, as previous comprehensive sequencing studies of IPMNs have focused on high-grade IPMNs or those with associated invasive carcinomas. We identified a total of 19 IPMNs previously analyzed by whole exome sequencing in the literature, the vast majority from high-grade IPMNs.[8,9,11–13] *KLF4* mutations were identified in three samples from two different studies, indicating a prevalence of 16% which is similar to the prevalence in high-grade IPMNs in our study. However, because *KLF4* mutations occurred in one or two samples in previous studies, they could not be separated from the much larger number of passenger mutations in these studies.

Several studies have assessed the expression levels of KLF4 in normal and neoplastic pancreas.[33–35] In the normal pancreas, KLF4 was found to be localized to the nuclei of pancreatic ductal epithelial cells.[33] Intriguingly, loss of KLF4 expression has been reported in a sizable proportion (>85%) of PDACs,[33] whereas increased expression was observed in human and mouse acinar-to-ductal metaplasia (ADM) and pancreatic intraepithelial neoplasia (PanIN) lesions.[34] We are aware of only one study to date describing KLF4 expression in IPMN.[35] This study demonstrated that KLF4 expression is restricted to a small proportion of highly mucinous cells in both human and mouse IPMN specimens, potentially representing regions of low-grade dysplasia.[35] Expanded analysis of KLF4 expression in larger IPMN cohorts and correlation with *KLF4* mutation status are warranted to clarify the relationship between *KLF4* mutation, expression, and role in tumorigenesis.

KLF4, also known as gut-enriched KLF (GKLF), is an important member of Kruppel-like transcription factor family with multiple putative functions.[36] *KLF4* was initially identified as a key regulator of cell fate decisions, such as cell proliferation, differentiation, and apoptosis. The identification of *KLF4* as one of the four “Yamanaka factors” that can reprogram differentiated somatic cells into pluripotent stem cells (*OCT3/4, SOX2, KLF4,* and *MYC*), as well as its essential role in the maintenance of genome stability, further substantiate its role in cell fate determination.[37] *KLF4* has been reported to have tumor suppressive functions in several tumor types, and both experimental and clinical evidence has shown that the loss of KLF4 protein expression can cause altered cell proliferation, differentiation, and precancerous changes in adult digestive organs.[36] These processes are mediated by multiple oncogenic pathways, including Wnt/β-catenin, TGF-β1, and $p21^{WAF1/Cip1}$ signaling.[36] Recent studies have also indicated that *KLF4* is a negative regulator of epithelial-to-mesenchymal transition (EMT), revealing several critical genes as a direct transcriptional targets of KLF4, including *CDH1, CDH2, VIM, CTNNB1, VEGF, and MAPK8*.[38]

In the pancreas, the functional role of *KLF4* varies at different points in tumorigenesis, with multiple studies suggesting pro-tumorigenic function in pancreatic tumor initiation and tumor-suppressive function in advanced PDAC.[34] Studies of *KLF4* in genetically engineered mouse models of pancreatic cancer have demonstrated overexpression of this gene in early pancreatic neoplasia, while experimental overexpression in pancreatic cancer cell lines led to

cell cycle arrest and growth inhibition.[3536] These genetically engineered mouse models suggested that dysregulation of the KLF4 signaling pathway promotes PDAC progression and metastasis, but paradoxically, *KLF4* ablation attenuates the formation of ADM and PanIN after pancreatic injury in the setting of mutant *KRAS*. Together with our data showing enrichment of *KLF4* mutations in low-grade regions, this raises the intriguing hypothesis that *KLF4* mutations are selected early in pancreatic tumorigenesis but are then selected against as lesions progress. Still, although the prevalence and pattern of *KLF4* mutations in our study provide strong evidence that *KLF4* is an oncogene in neoplastic pancreatic cysts, our sequencing data cannot provide mechanistic insights into the selective pressures in IPMNs. As such, we cannot further evaluate the hypothesis of temporal changes in selective forces with the current data. The functional impacts of hotspot *KLF4* mutations in pancreatic tumorigenesis remain to be determined and represent a critical direction of future investigation, and further experimental data in model systems will be required to support or refute this hypothesis.

The distinct mutation patterns of driver genes suggest their role in specific stages of pancreatic tumorigenesis. Mutations in the hotspots of the initiating oncogenes *KRAS* and *GNAS* were most often shared among all samples from a given IPMN, suggesting that these mutations occur early in tumorigenesis. In contrast to *KRAS* and *GNAS*, *TP53* mutations were not common, occurring in only 2 IPMNs and consistently limited to high-grade components. No mutations were identified in our IPMN cohort in *SMAD4*, despite frequent mutations in this gene in PDAC and IPMN-associated invasive carcinomas. These findings are consistent with previous studies suggesting that mutations in these genes occur very late in pancreatic tumorigenesis, and in particular that *SMAD4* mutations are typically limited to invasive carcinoma.[10143940]

As discussed above, in contrast to these later drivers, mutations in *KLF4* were uniquely enriched in regions of low-grade dysplasia, suggesting distinct selective forces on these mutations. The pattern of *KLF4* mutations suggests that, due to the complexities of clonal evolution, not all driver mutations in early pancreatic tumorigenesis can be detected by studying advanced cancers, highlighting the importance of direct analysis of precursor lesions. It is also important to note that many IPMNs lacked a high-grade specific driver gene, and copy number alterations were largely shared between matched low-grade and high-grade components, raising the possibility of a non-genetic driver of progression to high-grade dysplasia in a subset of cases. Overall, our data suggest that there is not a single universal genetic pathway to high-grade dysplasia.

In total, 76 multi-region samples from 17 IPMN cases were analyzed by whole-exome sequencing in our study. In the majority of IPMNs (16/17 cases, 94%), low-grade and high-grade IPMNs shared common somatic mutations, suggesting evolution from a common ancestor. These observations raise the fundamental question of whether high-grade IPMN arises from low-grade IPMN. Because we analyzed a single resected IPMN specimen from each patient and thus observed each IPMN at a single point in time, we cannot directly observe this common ancestor of low-grade and high-grade IPMN. However, the molecular alterations predicted in the common ancestor by Treeomics are most consistent with those previously reported in low-grade IPMN.[10] Thus, our data suggest that the common ancestor

of the low-grade and high-grade IPMN regions we sequenced was low-grade IPMN, supporting the idea that high-grade IPMN typically arises from low-grade IPMN. Intriguingly, in addition to the shared mutations, each region we sequenced also independently accumulated a set of private mutations, even when the high-grade regions were in direct contact with low-grade regions within the same pancreatic duct (e.g., IP6, IP7). These results demonstrate independent evolution of both low-grade and high-grade regions after divergence from the common ancestor, suggesting that continued selection shapes the genetic alterations in both IPMN grades. However, the higher proportion of mutations in high-grade regions with a NCF near 1 suggests a clonal selection event that is unique to the development of high-grade dysplasia. We also identified one notable exception (IP24; 1/17 cases, 6%), in which the low-grade and high-grade components shared no somatic mutations. In this case, low-grade and high-grade regions arose as independent clones in the same duct.

Pancreatic cysts are frequently identified incidentally on abdominal imaging, creating the unique clinical problem of surveillance that balances cancer prevention with overtreatment.[41] To date, several different sets of guidelines have been released for the management of pancreatic cystic neoplasms, including those that can progress to invasive carcinoma, such as IPMN and MCN.[5642] In these guidelines, clinical decision-making relies largely on radiographic and clinical features, augmented by biochemical and cytologic analyses of cyst content. However, currently available diagnostic tools and algorithms are still imperfect. There is a discrepancy between the pre- and post-operative diagnosis in >30% of the pancreatic lesions, and 25% of patients who undergo surgery have pancreatic cysts without malignant potential, highlighting a need for improved approaches to preoperative diagnosis.[43–45] Recent studies have identified a combination of molecular and clinical features that classified cyst type with >90% sensitivity and >90% specificity.[1946] However, separating low-grade and high-grade precancerous cysts remains a challenging even with these advanced approaches. The results of our genomic analysis of IPMN progression may improve this discrimination and thus contribute to multidisciplinary assessment and management of IPMN.

More specifically, our results provide further insights into the use of molecular alterations in cyst fluid for IPMN risk stratification. *SMAD4* mutations were absent in our cohort of non-invasive IPMNs, and *TP53* mutations were uncommon and limited to areas of high-grade dysplasia, suggesting that alterations in these genes are specific markers for IPMNs at high risk of malignant progression or associated invasive carcinomas.[1947] In contrast, *KLF4* mutations were frequently limited to regions of low-grade dysplasia in our IPMNs analyzed by multi-region sequencing of tissue samples, and mutations in this gene were significantly more prevalent in cyst fluid samples from low-grade IPMNs. These data suggest that *KLF4* mutations may add to the discriminatory power of molecular cyst fluid analysis, though it is important to note that these mutations were also present in a smaller proportion of high-grade IPMNs. Thus, *KLF4* mutations are not an entirely specific marker of low-grade dysplasia and will likely need to be interpreted in combination with other clinical and molecular features to accurately assess the risk of IPMN progression. Furthermore, because a single IPMN can contain both low-grade and high-grade components, the finding of a mutation suggestive of low-grade dysplasia does not rule out a higher grade component

elsewhere in the IPMN. While the difference in prevalence of *KLF4* mutations in low-grade and high-grade IPMNs was statistically significant, these observations suggest significant challenges to their clinical utility, and thus the clinical impact of our study should be interpreted with caution. Assessment of *KLF4* mutations in larger cohorts will be required to clarify the value added by these mutations to existing risk stratification approaches. In addition, assessment of *KLF4* mutation prevalence in other pancreatic precancerous lesions and cyst types will be required to interpret the implications of these mutations in biospecimens.

Like all cancer genomics studies, our study has limitations. We analyzed a relatively small number of IPMNs. Still, this represents the largest cohort to date of IPMNs without associated cancer analyzed by whole exome sequencing, and we analyzed 76 IPMN exomes in total, a large increase in sample size from previous studies. Moreover, although we performed multi-region whole exome sequencing, we analyzed only a small proportion of the neoplastic epithelium in each case by microdissecting pathologically defined regions from 1–3 tissue blocks. The design of such multi-region sequencing studies represents a balance between the comprehensiveness of lesional sampling versus genomic analysis. In this study, we chose to comprehensively analyze the genome of the analyzed regions but not sample the whole IPMN. Of note, we employed the opposite balance in a recently published study in which we analyzed all available tissue from a cohort of IPMNs by targeted next generation sequencing of a small driver gene panel.[12] The latter approach allows comprehensive assessment of genetic heterogeneity with respect to known drivers but does not allow identification of new driver genes, which is a key finding in the current study. Taken together, the two approaches provide complementary insights into pancreatic tumorigenesis via the IPMN pathway.

It is also important to note that our cohort size did not permit us to perform computational methods of driver gene assessment such as MutSigCV.[48] As such, the importance of infrequently mutated genes in our study should be interpreted with caution, as the mutation prevalences have not been corrected for important confounders such as gene size, nucleotide context, and replication timing. Still, we identify *KLF4* mutations at oncogenic hotspots in >50% of analyzed IPMNs, and these specific mutations are predicted to be drivers based on CHASMplus analysis. Thus, methods such as MutSigCV are not required to confirm the driver gene status of *KLF4*. Another important caveat in our study is that we analyzed exomes from FFPE tissue, which has been documented to contain more artifacts than sequencing data from fresh or frozen tissue.[49] Although this is one possible explanation for the observation that we identified a similar number of mutations in IPMN samples to that previously identified in PDAC, an alternative explanation is also possible. Because we performed laser capture microdissection and analyzed multiple small, morphologically discrete regions of each IPMN, it is also likely that our experimental design allowed a higher sensitivity for subclonal mutations, particularly compared to bulk sequencing of paucicellular PDACs. A final caveat is that whole exome sequencing, as employed in our study, cannot identify all types of genomic alterations. Future studies using whole genome sequencing will be required to confidently place chromosomal rearrangements, chromothripsis, and whole genome doubling on the timeline of IPMN tumorigenesis.

In this study, we report comprehensive multi-region whole exome sequencing of pathologically well characterized IPMNs with both low-grade and high-grade components. This approach identified a new genetic driver of IPMN tumorigenesis and highlighted unique evolutionary processes not previously appreciated in precancerous pancreatic neoplasia. In addition, our results provide a novel biomarker that may refine risk stratification of IPMNs using cyst fluid analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ADM	acinar-to-ductal metaplasia
CHASMplus	Cancer-specific High-throughput Annotation of Somatic Mutations plus
FFPE	formalin-fixed paraffin-embedded
gnomAD	Genome Aggregation Database
HG	high-grade
IPMN	Intraductal papillary mucinous neoplasm
LG	low-grade
NCF	neoplastic cell fraction
PanIN	pancreatic intraepithelial neoplasia
PDAC	pancreatic ductal adenocarcinoma
Safe-SeqS	Safe Sequencing System
TCGA	The Cancer Genome Atlas

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Summary Box

What is already known about this subject?

- Intraductal papillary mucinous neoplasms (IPMNs) are the most common neoplastic cysts in the pancreas and can progress to invasive pancreatic adenocarcinoma.
- Comprehensive sequencing of small IPMN cohorts has identified driver genes in advanced lesions, and targeted multi-region sequencing has demonstrated genetic heterogeneity in IPMNs. However, comprehensive multi-region genomic analysis of IPMNs is required to elucidate the evolutionary features of neoplastic progression.

What are the new findings?

- Multi-region whole exome sequencing revealed that *KLF4* hotspot mutations (K409 and S411) were identified in >50% of the analyzed IPMNs, and these mutations were more frequently detected in regions with low-grade dysplasia than with high-grade dysplasia.
- *KLF4* mutations can be identified in IPMN cyst fluid samples using the Safe Sequencing System (Safe-SeqS), and these mutations are significantly more prevalent in cyst fluid from IPMNs with low-grade dysplasia.
- Phylogenetic analyses demonstrated diverse patterns of tumor initiation and progression, suggesting that there is not a single universal genetic pathway into high-grade dysplasia in IPMNs.

How might it impact on clinical practice in the foreseeable future?

- These results underscore the potential of *KLF4* hotspot mutations as a novel biomarker for pancreatic cyst risk stratification, as *KLF4* mutations are predominantly detected in low-grade IPMNs.
- Both inter-patient and inter-region genetic heterogeneity in IPMNs should be considered during the development of molecular approaches for pancreatic cyst assessment.

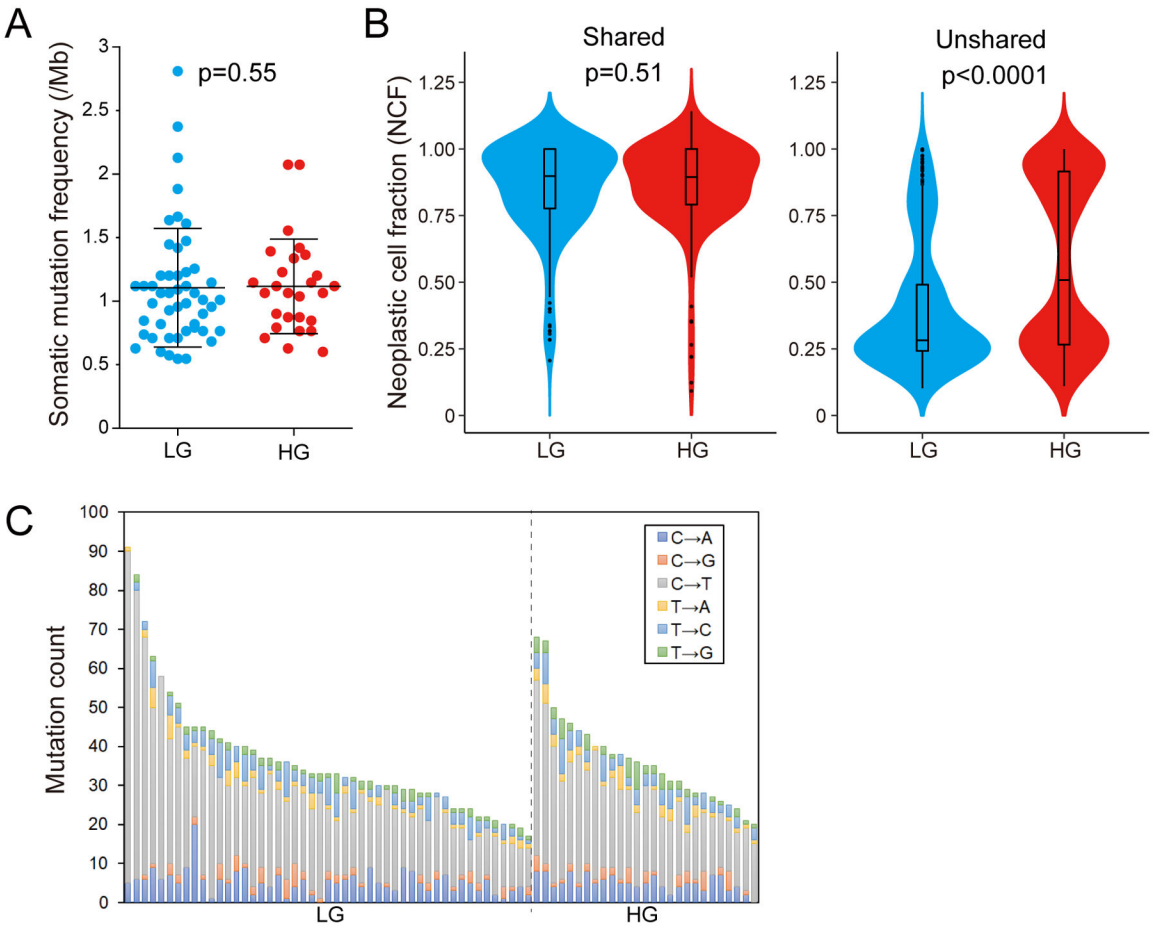

Figure 1: Whole exome sequencing of multi-region low-grade (LG) and high-grade (HG) IPMN samples.

(A) Comparison of the tumor mutation burden per megabase (TMB/Mb) between LG (n=49) and HG (n=27) IPMN regions. The lines and error bars indicate mean ± 1 standard deviation. (B) Violin plots showing the neoplastic cell fraction (NCF) of all mutations detected in LG (n=49) and HG (n=27) regions. The NCFs were calculated separately for shared mutations among all regions (left panel) and unshared mutations (right panel). (C) Proportion of base changes observed in each IPMN sample. Samples are organized by grade of dysplasia and descending total number of alterations.

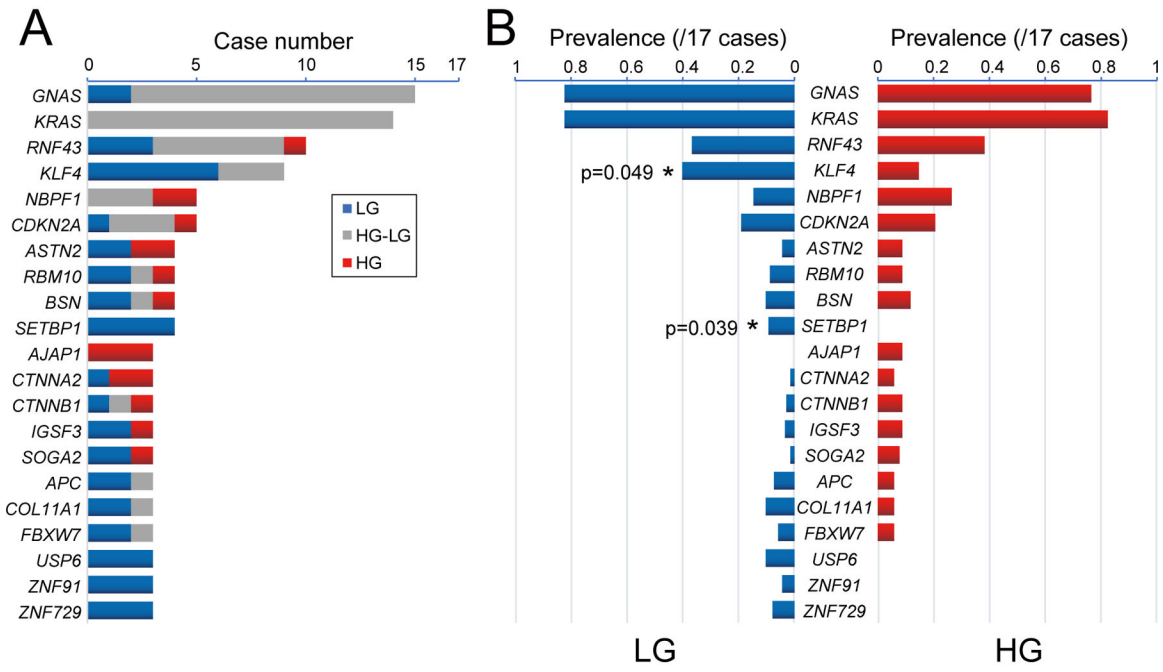

Figure 2: Driver mutations in low-grade (LG) and high-grade (HG) IPMN.

(A) Somatic nonsynonymous mutations in the most frequently mutated genes are categorized as shared between LG and HG IPMN (gray), limited to LG (blue), or limited to HG (red). Genes with mutations in >3 IPMNs and >0.5 mutations per kb gene size are included. (B) Comparison of prevalence nonsynonymous mutations between LG and HG samples. Genes showing significantly different mutation frequencies between LG and HG are indicated by asterisks (two-tailed Mann-Whitney U test).

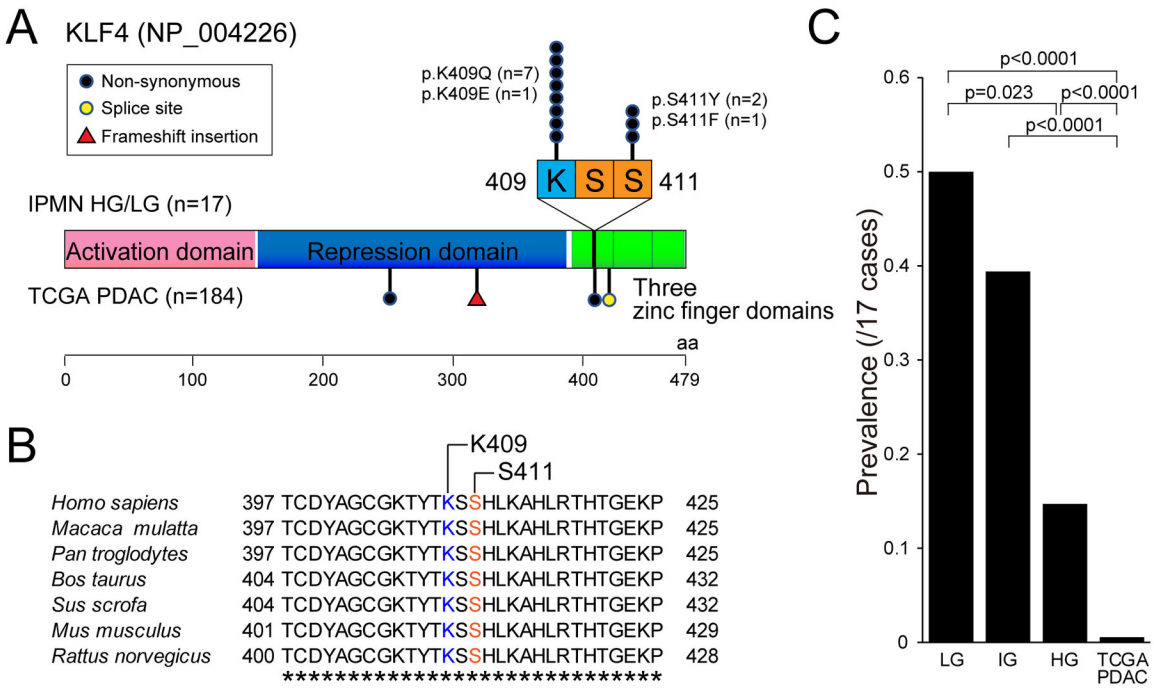

Figure 3: Characterization of recurrent hotspot mutations in *KLF4*.

(A) Gene schematic showing mutations identified in *KLF4* in our IPMN cohort (n=17 cases, 76 regions) and TCGA PDAC cohort (n=184 cases). All mutations in our IPMN cohort are located in two hotspots (K409 and S411) in first C2H2 zinc finger domain. (B) Amino acid sequences of different species around the hotspot mutations of *KLF4*. Completely conserved amino acids across all species are indicated by asterisks. (C) Comparison of *KLF4* mutation prevalence among “low-grade”, “intermediate-grade” and “high-grade” IPMN in our cohort and TCGA PDAC. The histologic grade in this panel is based on the previous three-tiered grading system for IPMN. p-values were calculated based on two-tailed Mann-Whitney U test.

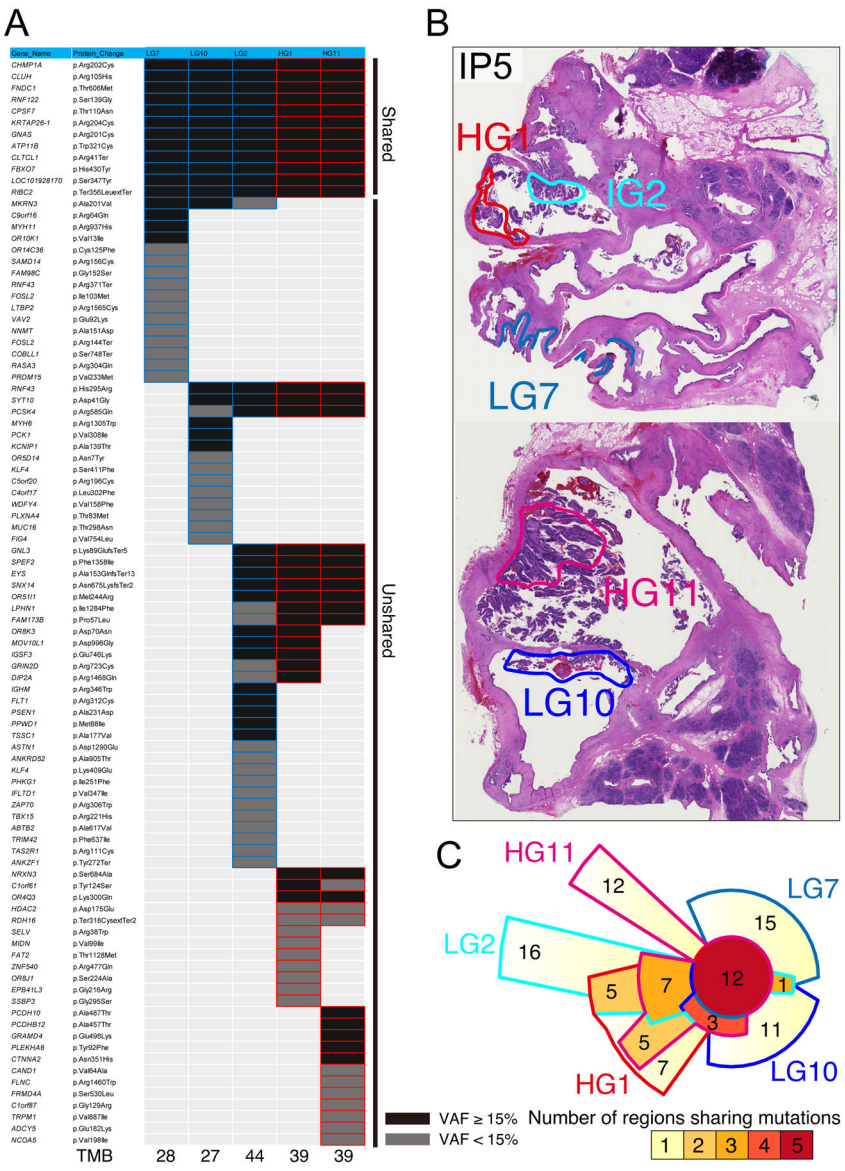

Figure 4: Distribution of somatic mutations in low-grade (LG) and high-grade (HG) regions of IP5.

(A) Heatmap depicting the distribution of nonsynonymous somatic mutations among five different regions of IP5 (three LG and two HG). Black boxes indicate mutations with VAF >15%, and grey boxes indicate VAF of 5–15%. (B) Representative images of neoplastic tissue of IP5 stained by hematoxylin and eosin. The colored circles indicate the microdissected regions for sequencing analysis. (C) Chow–Ruskey plot of shared and unique somatic mutations among five different regions of IP5.

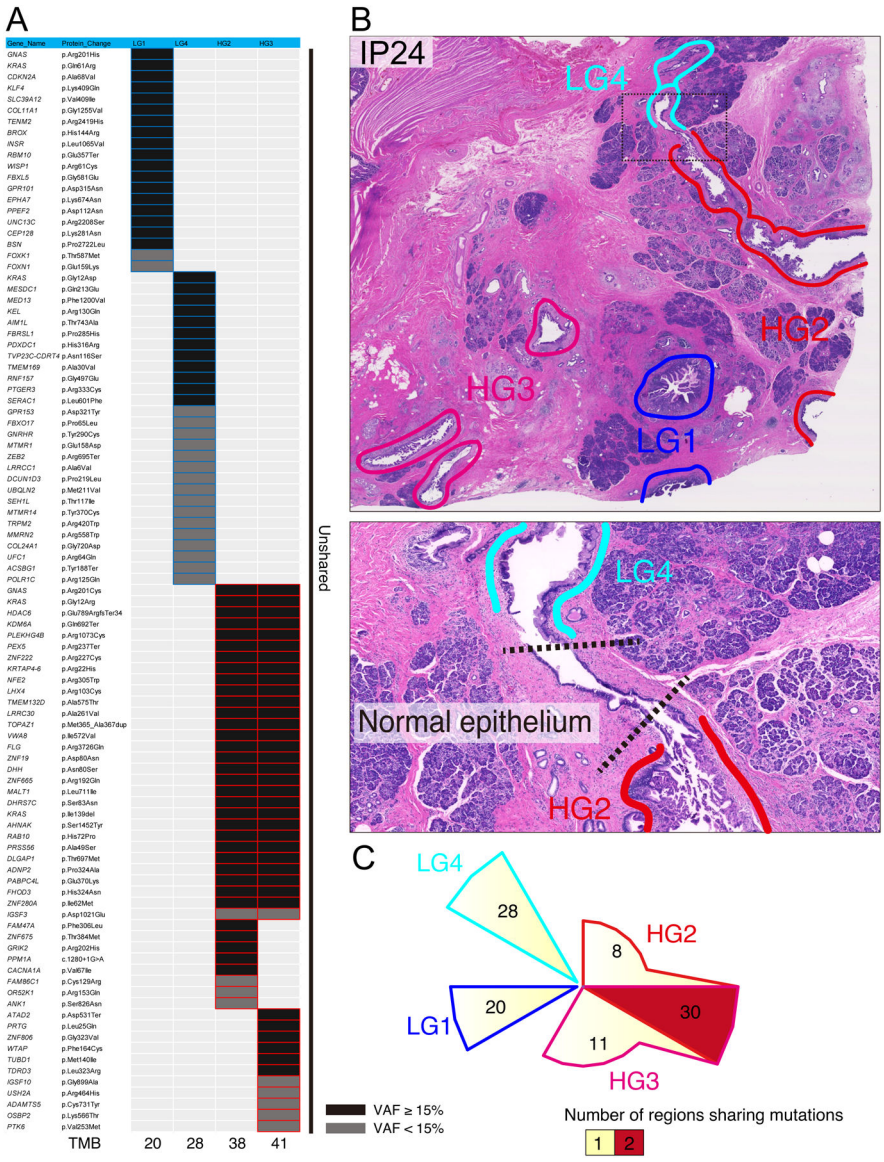

Figure 5: Distribution of somatic mutations in low-grade (LG) and high-grade (HG) regions of IP24.

(A) Heatmap depicting the distribution of nonsynonymous somatic mutations among four different regions of IP24 (two LG and two HG). Black boxes indicate mutations with VAF >15%, and grey boxes indicate VAF of 5–15%. (B) Representative images of neoplastic tissue of IP24 stained by hematoxylin and eosin. The colored circles indicate the microdissected regions for sequencing analysis. The bottom image shows the enlarged view of the black dotted circle in upper image. (C) Chow–Ruskey plot of shared and unique somatic mutations among four different regions of IP24.

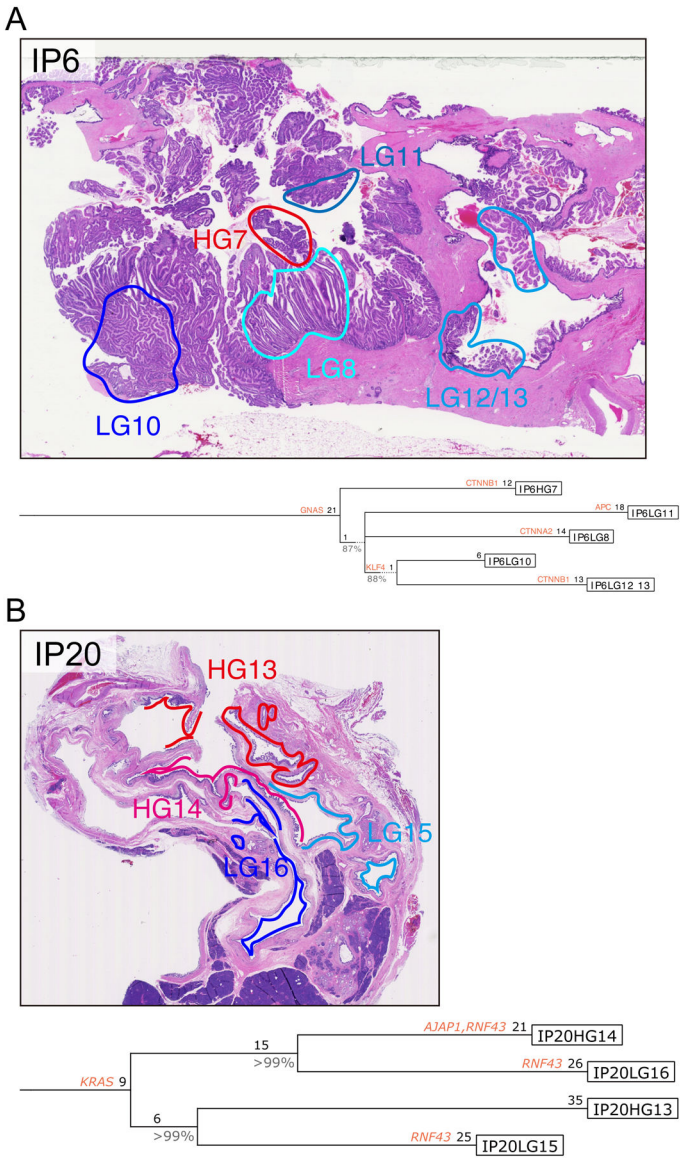

Figure 6: Representative IPMN phylogenies constructed using Treeomics.

Treeomics generated phylogenetic trees from all nonsynonymous mutations identified in each IPMN region (A, IP6; B, IP20). Potential driver gene mutations (including those identified in previous pancreatic cancer genomics studies, as well as those mutated in >3 IPMNs and >0.5 mutations per kb gene size in the current study) are indicated by their gene name on the lineage in which they occur. Numbers indicate the number of nonsynonymous somatic mutations occurring in each trunk or branch. Representative images of neoplastic tissue stained by hematoxylin and eosin are presented for both cases, with colored circles indicating the microdissected regions for sequencing analysis.

Table 1:

KLF4 Mutations Identified in Cyst Fluid Samples by Safe-SeqS.

		1st *KLF4* mutation			2nd *KLF4* mutation			3rd *KLF4* mutation			Prevalence:any mut	p-value[+]	Prevalence: multiple mut	p-value[+]
		position	mut	MAF[*]	position	mut	MAF[*]	position	mut	MAF[*]				
LG IPMN (n=26)	Cyst 229	110249341	C>A	0.006	110249348	A>C	0.005				0.46	0.027	0.19	0.26
	Cyst 234	110249348	A>C	0.011										
	Cyst 244	110249348	A>C	0.346										
	Cyst 318	110249348	A>C	0.027										
	Cyst 792	110249348	A>C	0.002										
	Cyst 839	110249348	A>C	0.149	110249341	C>A	0.008							
	Cyst 840	110249348	A>C	0.014										
	Cyst 867	110249341	C>T	0.087	110249348	A>C	0.039	110249348	A>G	0.007				
	Cyst 903	110249348	A>C	0.013										
	Cyst 1307	110249348	A>C	0.213	110249341	C>T	0.079							
	Cyst 1308	110249341	C>A	0.375										
	Cyst 1315	110249341	C>A	0.158	110249348	A>C	0.064							
HG IPMN (n=37)	Cyst 358	110249348	A>C	0.288							0.19		0.08	
	Cyst 646	110249348	A>C	0.009										
	Cyst 674	110249348	A>C	0.180										
	Cyst 842	110249348	A>C	0.274	110249341	C>T	0.167							
	Cyst 843	110249348	A>C	0.018	110249348	A>C	0.003							
	Cyst 837	110249341	C>A	0.124										
	Cyst 1257	110249348	A>C	0.059	110249341	C>A	0.011							

LG, low-grade, HG, high-grade;

[*] MAF: Mutant allele frequency, reported as the mean of duplicate assays;

[+]: p-value for comparison of prevalence in LG and HG using two-tailed Fisher's exact test

3.3. Generation and characterization of a cell line from an intraductal tubulopapillary neoplasm of the pancreas

Besides the investigation of clinical samples, significant effort focused also on the understanding and modelling of the carcinogenic process in vitro. I have successfully established a relevant cell culture model for a rare cystic precursor lsion with subsequent molecular characterization. Such models will aid in the investigation of the biological and clinical consequences of specific molecular signatures:

„Intraductal tubulopapillary neoplasm (ITPN) is a distinct precancerous lesion in the pancreas with unique clinical and molecular features. Although in vitro studies in two-dimensional culture have led to numerous important insights in pancreatic cancer, such models are currently lacking for precancerous lesions. In this study, we report the generation and characterization of a cell line from a human pancreatic ITPN. Neoplastic cells were initially cultured in a three-dimensional organoid system, followed by transfer to two-dimensional culture. RNA sequencing revealed a gene expression profile consistent with pancreatic ductal origin, and whole genome sequencing identified many somatic mutations (including in genes involved in DNA repair and Wnt signaling) and structural rearrangements. In vitro characterization of the tumorigenic potential demonstrated a phenotype between that of normal pancreatic ductal cells and cancer cell lines. This cell line represents a valuable resource for interrogation of unique ITPN biology, as well as precancerous pancreatic lesions more generally.“

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Generation and characterization of a cell line from an intraductal tubulopapillary neoplasm of the pancreas

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Abstract

Intraductal tubulopapillary neoplasm (ITPN) is a distinct precancerous lesion in the pancreas with unique clinical and molecular features. Although *in vitro* studies in two-dimensional culture have led to numerous important insights in pancreatic cancer, such models are currently lacking for precancerous lesions. In this study, we report the generation and characterization of a cell line from a human pancreatic ITPN. Neoplastic cells were initially cultured in a three-dimensional organoid system, followed by transfer to two-dimensional culture. RNA sequencing revealed a gene expression profile consistent with pancreatic ductal origin, and whole genome sequencing identified many somatic mutations (including in genes involved in DNA repair and WNT signaling) and structural rearrangements. *In vitro* characterization of the tumorigenic potential demonstrated a phenotype between that of normal pancreatic ductal cells and cancer cell lines. This cell line represents a valuable resource for interrogation of unique ITPN biology, as well as precancerous pancreatic lesions more generally.

INTRODUCTION

Intraductal tubulopapillary neoplasms (ITPNs) are cystic pancreatic intraductal neoplasms characterized by distinct clinical, morphological, and molecular features (1, 2). This lesion was first described in 1992 as "tubular adenoma of the main pancreatic duct", and in 2010

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ITPN was recognized by the World Health Organization as a subtype of premalignant intraductal neoplasm of the pancreas distinct from the more common intraductal papillary mucinous neoplasm (IPMN) (3, 4). ITPNs are uncommon lesions, accounting for less than 3% of all intraductal pancreatic neoplasms. They are morphologically and immunohistochemically distinct from other intraductal neoplasms, with tubulopapillary growth pattern, high-grade cytologic atypia, scarce mucin production, and frequent necrosis (1, 3). The neoplastic cells in ITPNs express cytokeratin as well as MUC1 and MUC6 but typically lack expression of MUC2 and MUC5AC, again highlighting their distinct features compared to IPMNs. Invasive carcinomas co-occur in approximately 40% of ITPNs, and thus like IPMNs, ITPNs are regarded as pancreatic cancer precursor lesions (5). Although examined cohorts are not large, the outcome of carcinoma arising from ITPN seems distinct from that of PDAC, as ITPN-associated carcinomas infrequently metastasize and often show favorable outcomes (1, 6). Genomic analyses have revealed a unique pattern of driver genes in ITPNs, which typically lack somatic alterations in genes commonly associated with ductal pancreatic tumorigenesis, including *KRAS*, *GNAS*, *TP53*, and *SMAD4* (7, 8). Candidate drivers suggested in ITPNs include *CDKN2A*, genes involved in chromatin remodeling (*MLL*, *PBRM1*, *ATRX*), and genes of phosphatidylinositol 3-kinase (PI3K) pathway (*PIK3CA*, *PTEN*), among others (7). Recurrent fusions in *FGFR2* have also been reported in ITPNs (7). Taken together, these data highlight that ITPNs represent a distinct premalignant pancreatic neoplasm with unique clinical and molecular features.

The development of appropriate disease models is essential for investigating pancreatic tumorigenesis prior to malignant transformation. Unfortunately, there are few cell lines with which to model pancreatic precursor lesions. Human pancreatic duct epithelial (HPDE) cells have been reported as a near-normal pancreatic duct epithelial cell line, but immortalization using HPV E6/E7 proteins leads to perturbations in pathways associated with high-grade pancreatic precursor lesions (p53 and RB pathways) (9). Although HPDE represents an invaluable resource, these alterations call into question how faithfully it can recapitulate “normal” pancreatic duct biology. Previous propagation of human IPMNs has been achieved in murine xenografts, with one IPMN subsequently established as a cell line after *in vivo* propagation (10). In addition, multiple groups have reported derivation of cell lines from invasive carcinomas arising from IPMNs (11, 12). The scarcity of *in vitro* models of human premalignant pancreatic neoplasms highlights several difficulties in the establishment of such systems. First, pancreatic precursor lesions are often an incidental finding at time of pancreatic resection for invasive carcinoma and as such are often only identified in examination of fixed tissue, when harvesting of living cells is no longer possible. Second, human pancreatic neoplasms, even invasive carcinomas, are challenging to propagate *in vitro* in two-dimensional culture (13). Because of these difficulties with two-dimensional cell culture approaches, alternative strategies for propagation of pancreatic precursor lesions (such as murine and chicken egg xenografts as well as three-dimensional culture methods) are currently being explored (14–16). While such strategies may improve our ability to propagate human premalignant neoplasms, they lack the ease of culture and molecular manipulation of two-dimensional cell culture.

In this study, we derived a novel cell line (H58) from a surgically resected, pathologically confirmed pancreatic ITPN. Neoplastic cells were initially cultured in a three-dimensional

organoid system and subsequently propagated in two-dimensional culture over the course of >250 days and >30 passages. We report characterization of the new cell line with respect to its pancreatic ductal phenotype, *in vitro* correlates of malignancy, and molecular signatures. Our ITPN cell line represents a unique resource with which to interrogate this distinct premalignant neoplasm, as well as pancreatic cancer precursor lesions more generally.

MATERIALS AND METHODS

Institutional approval and informed consent

This study was reviewed and approved by the Johns Hopkins University Institutional Review Board. Written informed consent was obtained from the study participant.

Specimen collection and organoid derivation

Neoplastic tissue was collected from a patient with surgically resected ITPN, and organoids were isolated as previously described (17, 18). Briefly, tissue for culture was minced and incubated at 37°C for five hours in an Enviro Genie (Scientific Industries, Inc., NY) with 20 rpm rotation and 40 rpm rocking in advanced DMEM/F12 media (Invitrogen, catalog no. 11320033) containing collagenase type II 5 mg/mL (Life Technology, catalog no. 17101-015), dispase 1.25 mg/mL (Life Technology, catalog no. 17105-041), fetal bovine serum (FBS) (Gibco, catalog no. 26140079) 2.5%. After incubation, dissociated single cells were washed, mixed with Matrigel (BD Bioscience, catalog no. 356231), and seeded in Matrigel domes as described elsewhere (19). Matrigel domes were regularly supplemented with Human Feeding Media (HFM), which is based on AdvDMEM/F12 (Invitrogen, catalog no. 12491-015) supplemented with B27 (Invitrogen, catalog no. 17504044), 1.25 mM *N*-Acetylcysteine (Sigma, catalog no. A9165), 10 nM gastrin (Sigma, catalog no. G9020) and the growth factors: 50 ng/mL EGF (Peprotech, catalog no. 315-09), 10% RSPO1-conditioned media, 10% Noggin-conditioned media, 100 ng/mL FGF10 (Peprotech, catalog no. 100-26) and 10 mM Nicotinamide (Sigma, catalog no. 1094-61-7) (17, 18). When cell clusters reached confluence, Matrigel domes were broken down with ice-cold media, followed by organoid dissociation and transfer to fresh Matrigel. Passaging was performed in a 1:3–1:6 split ratio approximately once every two weeks.

Two-dimensional cell culture

After seven organoid passages, epithelial cell clusters were dissociated and split into both uncoated culture dishes and dishes coated with rat tail collagen type 1 (BD Bioscience, catalog no. 17100017) – both cultures were supplemented with 100% HFM. Over several passages, cells were gradually weaned from HFM and replaced with Advanced DMEM/F12 up to a ratio of 3:1 (AdvDMEM/F12:HFM). At higher ratios than 3:1, we observed increased vacuolization, cell death, and prolonged doubling times. H58 cells were maintained in two-dimensional culture over 30 passages and >250 days while supplementing AdvDMEM/F12:HFM (3:1) and regularly passaged after application of 0.25% Trypsin/EDTA at 70-90% confluence.

We kindly received HPDE cell line from the laboratory of Ming-Sound Tsao, MD, FRCPC, University Health Network, Canada. PANC-1 cells were purchased from ATCC

(CRL-1469). PANC-1 was cultured according to ATCC recommendations with reference to the original article (20). HPDE was cultured as previously described (9). All 2D cell lines were tested before experimental use to confirm identity by short tandem repeat (STR) analysis (GenePrint 10 System, Promega) and negative mycoplasma status by PCR (MycoDetect, Greiner Bio-One) by the Johns Hopkins University Genetics Resources Core Facility.

Mice Xenografts

Five, 6-8 week-old, female *nu/nu* mice were obtained from Jackson Laboratories (strain no. 002019). 3.5×10^6 H58 cells suspended in 100 μL of culture media were injected into the flank of each mouse. Tumor volume and weight for each mouse was determined twice weekly for 20 weeks.

DNA and RNA extraction

DNA was extracted from H58 cells in 3D culture and fresh-frozen non-tumor tissue (duodenum) using the PureLink Genomic DNA Mini Extraction Kit (Invitrogen, catalog no. K182000). RNA was extracted from H58 cells in 3D culture using a PureLink RNA mini extraction kit (Invitrogen, catalog no. 12183018A). DNA and RNA were quantified using the Qubit dsDNA HS Assay Kit (Invitrogen, catalog no. Q32851) and Qubit RNA BR Assay Kit (Invitrogen, catalog no. Q10210) respectively, according to manufacturer's protocols.

Analysis of KRAS and GNAS hotspots

DNA regions containing *KRAS* exon 2, *KRAS* exon 3, and *GNAS* exon 8 were amplified with OneTaq (NEB, catalog no. M0481) according to the manufacturer's protocol with the following modifications: 1) for *GNAS* exon 2, an annealing temperature of 62 °C and 30 cycles were used, and 2) for *KRAS* exon 2 and *KRAS* exon 3, an annealing temperature of 64 ºC and 40 cycles were. Primers for *KRAS* exon 2 were: 5'-CCCTGACATACTCCCAAGGA-3' and 5'-TGTAAAACGACGGCCAGTGAGGGTGTGCTACAGGGTGT-3'. Primers for *KRAS* exon 3 were: 5'-TGTAAAACGACGGCCAGTCCTAGGTTTCAATCCCAGCA-3' and 5'-CACCAGCAATGCACAAAGAT −3'. Primers for *GNAS* exon 8 were: 5'-TGTAAAACGACGGCCAGTCACCCCACGTGTCTTTCTTT-3' and 5'-AAAGAACCACCGCAATGAAC-3'. PCR products were purified with a Qiagen PCR Purification Kit (Qiagen, catalog no.) and Sanger sequenced by Genewiz, Inc. (Plainsfield, NJ).

Whole Genome Sequencing

Whole genome sequencing was performed by the Next Generation Sequencing Core of the Sidney Kimmel Comprehensive Cancer Center of Johns Hopkins University. Libraries for whole genome sequencing were prepared from H58 and matched normal DNA using the TruSeq Nano DNA Kit (Illumina), followed by 2 X 150 bp paired-end sequencing using a HiSeq2500 instrument (Illumina). Bcl2fastq v2.15.0 was used to generate FASTQ files from BCL files. FASTQ files were aligned to the human genome (G) using bwa v.0.7.7 (mem). Read groups were added and duplicate reads removed using Picard-tools v.1.119. Base call

recalibration was completed with GATK v.2.6.0 and BAM alignment files generated. Germline variants were called with HaplotypeCaller v.3.6.0. Somatic variants were called with MuTect2 v3.6.0. Structural variants (SVs) were called with lumpy v.0.2.11 and compared to normal using BEDtools. Germline variants and somatic mutations were annotated using ANNOVAR (v.2016-02-01) and FunSeq2 (v2.1.6). Somatic mutations in coding regions and non-coding regions with a FunSeq2 non-coding score > 1.5 were visually inspected in IGV (v2.4.8). SVs from Lumpy software were filtered to include only: 1) SVs not mapping to the mitochondrial genome, 2) SVs supported by at least 2 spanning paired-end reads (PE) and 2 split reads (SR), 3) SVs with an "Evidence Score" 0.0005 or less, and 4) SVs with PE/SR ratio between 1 and 3. Mutation signature and SVs profile were plotted by signeR (v1.4.0) and circlize (v0.4.6) packages, respectively. Default parameters were used unless otherwise specified.

Whole Exome Sequencing

DNA was extracted from archival formalin-fixed paraffin-embedded (FFPE) tissue samples from H58 ITPN (tumor) and duodenum (normal) using the QIAamp DNA FFPE Tissue Kit (Cat No: 56404). Exome capture, library preparation, and sequencing of the paired tumor and normal samples was conducted by the Next Generation Sequencing Core of the Sidney Kimmel Comprehensive Cancer Center of Johns Hopkins University. Briefly, exome capture and library preparation were performed with Agilent SureSelect Target Enrichment V5-post. DNA libraries were sequenced on a Illumina NovoSeq instrument to generate 2 X 150 bp paired-end sequence reads. Sequence reads were aligned to the human genome (hg38) using bwa mem (v0.7.15) with default parameters. Duplicate reads were removed using Picard tools (v1.119). GATK (v3.6) was used to call variants and Mutect2 (v3.6.0) was used to call somatic mutations in tumor compared to normal. Somatic mutations were annotated using ANNOVAR (v.2016-02-01). In order to make the results of the higher coverage FFPE tissue WES comparable to the organoid WGS, we set a threshold mutant allele frequency ≥10% in H58 FFPE WES, followed by visual inspection of each coding mutation identified in WGS or WES using IGV in both samples (v2.4.8).

RT-qPCR

500 ng of RNA from H58 cells in 2D culture was converted to cDNA using the High-Capacity RNA-to-cDNA Kit (Thermo Fisher, catalog no. 4387406) according to the manufacturer's protocol. Real time PCR for was conducted with the TaqMan Universal Master Mix II with UNG (Thermo Fisher, catalog no. 4440038) and the following gene specific primers according to the manufacturer's protocol: KRT19 (Thermo Fisher, catalog no. 4331182 Hs01051611_gH), SOX9 (Thermo Fisher, catalog no. 4331182 Hs00165814_m1), NEUROG3 (Thermo Fisher, catalog no 4331182 Hs01875204_s1), VIM (Thermo Fisher, catalog no. 4331182 Hs05024057_m1).

RNA Sequencing

RNA library preparation and sequencing were conducted by Genewiz, Inc. (Plainsfield, NJ). RNA libraries were prepared by mRNA enrichment by polyA selection. mRNA was then fragmented before random priming, cDNA synthesis, A-tailing, adapter ligation, and PCR amplification. RNA libraries were sequenced to generate a 2×150 bp paired-end reads using

a HiSeq instrument (Illumina, CA). Adapter sequences were removed from sequence reads using cutadapt (v.1.17). Reads were then mapped to the human genome (GRCh38) using HISAT2 (v2.1.0) before being assembled and gene transcripts per kilobase million (TPM) calculated using StringTie (v1.3.4). Fusion transcripts were detected by STAR-Fusion (v1.5.0). To reduce false positives, fusion events with fusion fragments per million total reads < 0.1 were removed (21). Three or more supporting paired-end reads were required for event detection (22). Default parameters were used unless otherwise specified.

Immunofluorescent staining

5×10^4 cells of HPDE, PANC-1, and H58 were seeded into chamber slides and cultured for 48 h. Culture media was removed, cells were washed twice with TBST (Tris Buffered Saline + 0.5% Tween20) and fixed with 4% PFA/PBS. For optimal penetration, fixed cells were permeabillized with 0.2% TritonX-100/TBST and subsequently blocked in blocking buffer (5% dry fat milk/TBST). Primary antibody diluted in blocking buffer (1:200 mouse-anti-panCK (Abcam, catalog no. ab7753); 1:500 Dylight594 conjugated rabbit-anti-Vim (Abcam, catalog no. ab154207) was applied and incubated for 2 h. Primary antibody solution was then removed, and cells were washed twice with TBST. Cells were then incubated with secondary antibody diluted in blocking buffer (1:500 Alexa fluor conjugated goat-anti-mouse (Abcam, catalog no. ab150117) for 1 h. Cells were washed with TBST and attachment wells removed. Stained cells were mounted in DAPI solution (Life technologies, catalog no. P36931) and each covered with a cover slip. Images were acquired after 24h incubation in dark at room temperature using a Nikon Eclipse Ti inverted microscope.

Karyotyping

Cytogenetic analysis was performed on cultured cells processed using standard techniques. Briefly, cells were treated with 0.06 μg/ml colcemid for 4 hours, incubated in hypotonic solution (0.075M KCl), and fixed in 3:1 methanol:glacial acetic acid. Slides were prepared and the metaphase chromosomes were treated with trypsin and stained with Leishman for G-banded karyotyping. Fifteen metaphases were analyzed. Chromosomal abnormalities were described based on the 2016 ISCN (International System for Human Cytogenomic Nomenclature).

Soft Agar Assay

2X culture media was prepared for each cell line assayed. PANC-1 received 2X DMEM with 20% FBS and 1% Penicillin/Streptomycin, HPDE received 2X AdvDMEM/F12 with 20% FBS and 1% Penicillin/Streptomycin, and H58 received 2X AdvDMEM/F12/HFM (2:1) with 20% FBS and 1% Penicillin/Streptomycin. We adjusted HPDE to grow in DMEM/F12 through gradual media replacement of SFM-Keratinocyte over several passages. Next, we prepared 0.5% and 1% agar solutions dissolved in cell culture grade water. 0.5% bottom layer agar was obtained by mixing 2X media with pre-warmed 1% Agar solution 1:1 and then 1 mL distributed along nine 12-well plates, considering triplicates for each cell line. 0.5% bottom agar layer solidified at room temperature after 30 min incubation. 0.25% top layer agar was prepared by mixing 2X media with pre-warmed 0.5% Agar solution 1:1. Top layer agar was kept at 42 ^{o}C while preparing cells. Cells were washed with PBS, trypsinized and re-diluted in media to calculate cell concentrations with automated cell counter.

Appropriate volumes of cell suspensions were added to each 0.25% top layer agar solution to obtain 1×10^4 cells/mL. 1 mL of warm top layer agar cell suspension was distributed along nine 12-well plates, considering triplicates for each cell line. Top agar layer cell suspension solidified at room temperature after 30 min incubation. 300 μL of respective 2X media was plated on top of agar layers and changed periodically. Cell colonies developed after 4 weeks incubation.

Cell Invasion Assay

Cell conditioned media from respective cell lines were collected and filtered through a 0.45μm membrane (Merck Milipore, catalog no. C3240). Conditioned media was supplemented with 20% FBS to act as a chemoattractant. Culture plates with Matrigel coated inserts from Cell Invasion Assay kit (Cell Biolabs, catalog no. CBA-110) were equilibrated at room temperature and inserts rehydrated with minimum essential media (MEM; Invitrogen, catalog no. 11095080) for 1 h at 37 °C. Cultured cells were washed with PBS, trypsinized, and washed again with PBS to remove any serum-containing media. Cells were diluted in serum-free MEM and cell concentrations calculated with automated cell counter (TC-20, Bio-Rad). 2.5×10^4 cells for each well were obtained, considering wells in duplicate for each cell line. MEM used for rehydrations was removed from wells and inserts. 500 μL of conditioned media with 20% FBS added to each well. Serum-free cell suspensions containing 2.5×10^4 cells were added to inserts and inserts placed into wells. Culture plates were incubated in 5% CO_2 at 37ºC for 48h to 96h. Media in both insert and bottom well were replaced daily to maintain the molecular gradient. After incubation, inserts were washed with PBS and cells from the interior of the insert removed with a Q-tip. Cells on the underside of the insert were washed with PBS and fixed in 4% PFA/PBS. Cells were stained in 0.2% Crystal violet/10%Ethanol/diH20 solution. Insert membranes were removed and mounted on plus microscopy slides with a cover slip and mounting solution. Membranes were imaged on an Olympus BX51 microscope and migrated cells in five distinct 10X high power fields quantified with ImageJ.

Clonogenic assay

Clonogenic assay was conducted as previously described (23). Briefly, cells were washed with PBS, trypsinized, and re-diluted in culture media. Cell concentrations were determined with an automated cell counter (TC-20, BioRad). 2000, 1000, 500, 200, 100 and 50 cells of each cell line were seeded in triplicate into 6-well plates. Cells were incubated for 4 h before receiving either 0 Gy, 1 Gy, 2 Gy, 4 Gy of X-ray radiation using a CIXD Biological Irradiator (Xstrahl Life Sciences). Cells were then incubated for 14 days, when colony formation was visible in all untreated wells. Cells were then washed with PBS and fixed in 4% PFA/PBS. Colonies were stained with 0.5% Crystal violet/10% Ethanol/diH20 solution. Isolated cell colonies were counted for each well at each seeding concentration.

MTT assay

MTT assays were performed to quantify the viability of the cells following treatment with G007-LK (MedChemExpress: Cat. No. HY-12438), a tankyrase inhibitor, and Olaparib (LC Laboratories, Cat. No. O-9201), a poly(ADP-ribose) polymerase inhibitor. H58 cells were seeded into 96-well plates at a concentration of 5000 cells per well and left to attach

overnight at 37˚C and 5% CO_2. G007-LK was then added to the culture medium to a final concentration of 0, 0.1, 0.5, 1, 2, 4 μmol/L. Olaparib was then added to the culture medium to a final concentration of 0, 1, 5, 10, 20, 40 μmol/L. After 96 hours, MTT (Thermo Fisher Scientific, Cat. No. M6494) was added to the culture media, and the cells were incubated for 4h at 37 ˚C. The culture media was then removed and the formazan crystals in the cells were solubilized using dimethyl sulfoxide (Sigma-Aldrich, Cat. No. 472301), with plate agitation for 10 min. Absorbance at 490 nm was the measure using a Bio-Rad Xmark Microplate Spectrophotometry (Bio-Rad, Hercules, California, USA).

RESULTS

Isolation and culture of neoplastic cells from human ITPN

Neoplastic tissue was harvested from a grossly identified cystic lesion in a pancreatoduodenectomy specimen from a male patient in his 60s. Grossly, the cyst was located in the pancreatic parenchyma and communicated with the duct system. Microscopic examination of the lesion revealed an intraductal neoplasm with minimal mucin and tubulopapillary growth of cuboidal neoplastic cells characteristic of ITPN (Figure 1A). While the lesion was entirely intraductal on frozen section examination at the time of tissue harvesting, a microscopic focus of invasive carcinoma was subsequently identified upon comprehensive review of formalin-fixed paraffin-embedded (FFPE) tissue sections. The harvested fresh tissue was processed to derive organoids embedded in Matrigel and supplemented with human feeding media (HFM). Analysis of oncogenic hotspots in pancreatic driver genes by Sanger sequencing of cultured organoids revealed no mutations in the oncogenic hotspots exons 2 and 3 of *KRAS* (codons 12, 13, and 61) and exon 8 of *GNAS* (codon 201). Three-dimensional cell clusters were passaged multiple times before transfer of the neoplastic cells to two-dimensional culture. When primary organoids were transitioned into a two-dimensional culture system, we were able to wean the cells from the growth factor enriched HFM. In two-dimensional culture, the ITPN cell line grew in Advanced DMEM F12/HFM (3:1) with a population doubling time of 36h (Figure 1B, Supplementary Table 1). This cell line, which we labeled H58, was passaged 30 times (including 6 passages in 3D culture and 24 passages in 2D culture) while maintaining cells >250 days in culture. Cells from H58 grow in monolayer without building clusters even at confluence. We observed anchorage dependent growth, as cells adhered solely in collagen type 1 coated flasks.

Thus, we report a new cell line derived from a primary pancreatic ITPN. We next sought to confirm the pancreatic ductal phenotype of H58 and characterize it on the molecular level.

Morphological and molecular characterization of ITPN cell line

Our ITPN cell line exhibited epithelial features both in morphology and protein expression. As is typical for epithelial cell lines, H58 cells adhered and grew in a cobblestone pattern in collagen-coated flasks (Figure 1C). H58 cells labeled strongly with antibodies directed against the epithelial marker cytokeratin (pKRT) by immunofluorescence, and there was no labeling with the mesenchymal marker vimentin (Vim) (Figure 1C). RT-qPCR confirmed ductal phenotype of the H58 cell line, with high expression of cytokeratin 19, modest

expression of Sox9, and no expression of neuroendocrine (Neurogenin 3) or mesenchymal (vimentin) markers (Figure 1D). In addition, we validated the origin of our cell line by short tandem repeat (STR) fingerprinting of the H58 cell line and normal tissue from the primary pancreatic resection specimen (Supplementary Figure 1).

RNA-Seq of RNA from H58 in 3D culture generated a total of 45,953,720 sequence reads, representing 13.8 Mb. 89.8% of bases had a quality score ≥ 30. 97.8% of sequence reads mapped to the reference genome (GRCh38), 92.7 % uniquely. Gene counts identified 12,096 expressed genes with tags per million (TPM) ≥ 1 (Supplementary Table 2). Genes associated with epithelial lineage were highly expressed, for example, KRT8, KRT18, and KRT19 (Supplementary Figure 2). Expression of SOX9 and PDX1 was also detected. Expression of genes associated with mesenchymal, neuroendocrine, acinar, and immune cell lineages were not detected or detected low levels (Supplementary Figure 2). Analysis of RNA-Seq data identified 11 fusion genes (Supplementary Table 3), with *ZC3H7A-BCAR4* fusion supported by the greatest number of spanning and junction reads.

Whole genome sequencing of DNA from H58 in 3D culture and fresh-frozen matched normal (N58) generated a total of 631,590,513 and 744,400,969 sequence reads with 99.73% and 99.71% mapping to the genome respectively. Across the genome, 13,337 somatic mutations consisting of 11,976 single base substitutions and 1,361 INDELs ≤ 95 nucleotides in length were identified in H58 (Supplementary Table 4). The mutation signature was predominantly C>T and similar to previously described signature 1A related to age (Figure 2) (24). Of these somatic mutations, 129 occurred in coding regions, including 36 synonymous mutations (27.9%), 83 nonsynonymous mutations (64.3%), 7 stop gain mutations (5.4%), 1 frame shift deletion (0.8%), 1 frame shift insertion (0.8%), and 1 in-frame deletion (0.8%) (Supplementary Figure 3; Supplementary Table 5). Of note, somatic mutations were not identified in *KRAS*, *CDKN2A*, or *SMAD4*, confirming the lack of most typical PDAC drivers this ITPN cell line. Somatic mutations were identified in known cancer driver genes, including genes involved in DNA damage response (single base substitutions in *BRCA2*, *MRE11A*, and *TP53*), as well as WNT pathway signaling (oncogenic hotspot mutation in *CTNNB1*, nonsense mutation in *APC*). To assess the role of the 13,208 non-coding somatic mutations we annotated mutations with FunSeq2 (25). 76 non-coding somatic mutations had a non-coding score (NCDS) > 1.5 and were predicted to be deleterious. These 76 non-coding somatic mutations were associated with 107 genes (Supplementary Table 6). Predicted deleterious noncoding variants were most frequently found in intronic regions (59.8%), promoter regions (17.8%), and untranslated regions (2.8%). Otherwise, predicted deleterious noncoding variants were found in regions distal to genes (≤10 kb) (Supplementary Figure 4).

To evaluate whether our H58 culture shared the genetic alterations found in bulk ITPN tissue, we compared the coding mutations identified in whole genome sequencing of H58 in culture to whole exome sequencing of H58 from FFPE tissue. Comfortingly, 90 of 129 (69.8%) coding mutations identified in H58 culture by whole genome sequencing were present in whole exome sequenced FFPE tumor tissue. Conversely, 90 of 115 (78.3%) coding mutations identified in whole exome sequenced FFPE tumor tissue were present in

whole genome sequenced H58 culture. Importantly, both samples shared somatic mutations in *TP53*, *ERBB2*, *APC*, *BRCA2*, *CTNNB1*, *MRE11* and *MUC4*.

Cytogenetic analysis revealed an aneuploid karyotype with multiple clonal structural aberrations (Supplementary Figure 5). Structural variant analysis using whole genome sequencing data identified 173 structural variants including 30 deletions (17.3%), 73 duplications (42.2%), 32 interchromosomal translocations (18.5%), and 38 inversions (30.0%) (Figure 2) (Supplementary Table 7). Interestingly, the structural variant analysis detected a fusion between genes *ZC3H7A* and *BCAR4* that was also detected by RNA-Seq (Supplementary Figure 6; Supplementary Tables 3 and 7). The *ZC3H7A-BCAR4* fusion was also validated by PCR amplification and Sanger sequencing.

Together, these results indicate that H58 is a cell line with a pancreatic ductal phenotype, with genomic and transcriptomic alterations characteristic of ITPNs.

Analysis of transformed phenotypes of ITPN cell line

Cancer cell lines frequently exhibit transformed phenotypes with respect to both tumor formation and invasion. As H58 was uniquely derived from a precancerous lesion, we sought to comprehensively assess its phenotype with respect to these features of transformation. H58 cells were implanted bilaterally into flanks of immunodeficient nu/nu mice. No tumors were identified after 6 months of observation, indicating limited tumorigenic potential in this assay. In a soft agar assay, no H58 colonies grew after 6 weeks of culture, confirming the anchorage dependency observed in culture in collagen-coated flasks (Figure 3A). This phenotype was similar to that observed for HPDE but strikingly different from the pancreatic cancer cell line PANC-1, which readily formed colonies in the soft agar assay.

The invasive properties of the H58 cell line were assessed by standardized Cell Invasion Assay 48h and 96h after plating (Figure 3B) (26). Although invasion of the ITPN cell line was observed after 48h, significantly fewer ITPN cells invaded compared to the pancreatic cancer cell line PANC-1 (mean 236 vs 469 cells/10 HPF; $p=0.0036$; student's t-test). The difference between the invasive ability of H58 and PANC-1 was even more striking at 96h (mean 406 vs 1202 cells/10 HPF; $p<0.001$; student's t-test). By comparison, the normal duct cell line (HPDE) invaded minimally in this assay, with significant differences from H58 at both 48h (236 vs 56 cells/10 HPF; $p<0.001$; student's t-test) and 96h (406 vs 58 cells/10 HPF; $p<0.001$; student's t-test) (Supplementary Table 8). The results of the Cell Invasion Assay demonstrate that H58 shows an invasive capability between that HPDE and PANC-1, consistent with its origin from a high-grade precancerous lesion.

In order to assess colony formation in cell lines and their replicative activity after radiation we performed a clonogenic assay. We applied either no or low dosages of radiation (1Gy, 2Gy, 4Gy) directly after seeding various cell concentrations (Figure 3C; Supplementary Table 9). In this assay, the H58 cell line was relatively radioresistant after low-dose emittance (surviving fraction 2.28 at 1Gy; 3.74 at 2Gy; 0.47 at 4Gy). This represents greater survival compared to HPDE cells (surviving fraction 0.15 at 1Gy; 0.53 at 2Gy; 0.19 at 4Gy). In contrast, the pancreatic cancer cell line PANC-1 was unable to form colonies after radiation treatment, with no surviving cells after any radiation dose.

The generation of a patient derived cell line with matched genomic data provides a unique opportunity to test the sensitivity of targeted agents based on the identified somatic alterations. As we identified a nonsense somatic mutation in *APC* and a nonsynonymous somatic mutation in *BRCA2* in H58, we tested sensitivity of the H58 cell line to a tankyrase 1/2 specific inhibitor (G007-LK) and a PARP1 inhibitor (olaparib) (27–31). The H58 cell line was not sensitive to tankyrase inhibition, as IC50 was not reached even with a G007-LK concentration of 4 μmol/L (Supplementary Figure 7). This result is in keeping with previous reports that cancer cell lines with *APC* mutations outside of the mutation cluster region are not sensitive to tankyrase inhibition, as this is the case with the *APC* somatic mutation identified in H58 (27). Conversely, the H58 cell line was sensitive to PARP1 inhibition as the IC50 for olaparib was 9.23 μmol/L, which is between the IC50 for olaparib in MDA-MB-436 cells (3 μmol/L) and Capan-1 cells (>10 μmol/L), both of which harbor mutations in *BRCA1* (Supplementary Figure 7) (29).

DISCUSSION

In this study, we report a novel cell line (H58) derived from a human pancreatic ITPN, a cystic precancerous lesion. Previous reports of derivation of cell lines from precancerous pancreatic lesions have relied on murine xenografts or other growth promoting systems to support early passages in culture (10, 11). In this study, we utilized culture in a three-dimensional organoid system for multiple passages, followed by transfer into a two-dimensional culture system. While in organoid culture, we utilized the previously reported growth factor enriched HFM, which contains factors that promote ductal epithelial growth and stem cell differentiation (EGF, FGF-10, Gastrin I, PGE2), as well as TGFβ inhibition (A83-01, mNoggin), Wnt pathway activation (Wnt3a, R-spondin), and essential vitamin supplementation (Nicotinamide, B27) (17, 18). We cannot determine whether the three-dimensional culture, enriched media, or both were the critical factors that supported the initial propagation of this unique neoplasm. Still, this approach may be useful to establish additional cell lines of precancerous lesions from the pancreas or other organs.

One important caveat of our study is that, although frozen section at the time of initial tissue processing revealed only intraductal neoplasia, a focal invasive carcinoma was identified on comprehensive review of formalin-fixed paraffin-embedded (FFPE) tissue sections. Our method of tissue harvesting, in which small superficial tissue fragments from the cyst wall were selected, should minimize the potential for harvesting carcinoma, which invaded into the underlying stroma. Still, we cannot exclude the possibility that our tissue was harvested from a region with carcinoma. As discussed below, the phenotype of our cell line in culture suggests that it represents premalignant rather than malignant cells. Of note, similar caveats were presented in a previous study of in vitro propagation of IPMNs (10).

Intriguingly, the H58 cell line showed a phenotype intermediate between the pancreatic duct epithelium cell line HPDE and pancreatic cancer cell line PANC-1. H58 demonstrated classic features of primary pancreatic duct epithelial cells in culture: cobblestone appearance, expression of epithelial cytokeratins and other ductal markers, and anchorage dependence (9). H58 continued to show growth factor dependency in two-dimensional culture and required supplementation with HFM even after many passages. The cell line did

not produce tumors in *nu/nu* mice, as is often found with precursor or normal cells (9, 32). However, in the invasion assay, the invasive properties of H58 were between those of HPDE and PANC-1.

In contrast to HPDE, H58 displayed some features typically associated with cancer cell lines, including increased plating efficiency, reduced population doubling time, unlimited population doubling, and aneuploidy – some of these features likely facilitated the cells' transition to two-dimensional culture. The ITPN cell line displayed resistance to low dose irradiation (1 and 2 Gy) *in vitro*, in stark contrast to the pancreatic carcinoma cell line PANC-1 which displayed radiosensitivity at all dosages. Of note, PANC-1 has a mutation in *TP53*, which is known to impact response to radiation, though such response is likely complex and multifactorial (33). The intact and even enhanced colony formation of the H58 cell line after radiation treatment suggests that it has intact DNA repair mechanisms in response to ionizing radiation (33, 34). Thus, the somatic mutations in DNA repair genes in H58, including p.Y88C in *TP53*, do not affect the cell line's response to ionizing radiation, at least at the doses used in this assay.

Genomic characterization of ITPNs has previously demonstrated a paucity of somatic alterations in commonly mutated pancreatic driver genes (7). Instead, ITPNs harbor a heterogeneous range of somatic alterations, including mutations in chromatin remodeling genes and components of the PI3K pathway (7). WGS sequencing of the organoids used to derive H58 revealed missense mutations in DNA repair genes (*TP53*, *BRCA2*, *MRE11*), oncogenic hotspot mutation in *CTNNB1*, nonsense mutation in *APC,* and multiple putative chromosomal rearrangements, including one in *ZC3H7A-BCAR4* that was supported by both WGS and RNA-Seq data. Importantly, we show that the majority of coding somatic mutations identified in H58 in culture were also present in the primary ITPN tumor tissue, confirming the shared clonal origin. There are multiple possible explanations for the discrepant mutations, including genetic heterogeneity in the primary ITPN, differences in sensitivity due to coverage differences in the whole genome and whole exome sequencing approaches, and acquisition of mutations during in vitro culture. Mutations in *TP53, BRCA2,* and *CTNNB1* have been previously reported in ITPNs (7). Intriguingly, the *ZC3H7A-BCAR4* fusion has been previously reported in cervical cancer, but to our knowledge this is the first report in pancreatic neoplasia (35).

The somatic mutations in multiple DNA repair genes raise the possibility that our cell line has a homologous recombination deficiency (HDR) phenotype. Though we identified many structural rearrangements, the number (173) is less than the reported threshold for an "unstable" genome (36). Still, the sensitivity of this cell line to the PARP inhibitor olaparib, which is specifically effective in cells with DNA repair defects, suggests that the nonsynonymous *BRCA2* mutation has a functional impact on DNA repair (30, 31). Thus, this cell line represents a unique model in which to study DNA repair defects in precancerous lesions.

This is the first report of a cell line derived from a patient with ITPN, an uncommon pancreatic cancer precursor lesion with distinct molecular features. This cell line is a unique resource to study the genetics and biology of pancreatic tumorigenesis prior to the

development of invasive carcinoma, offering a disease model for pre-clinical investigation of ITPNs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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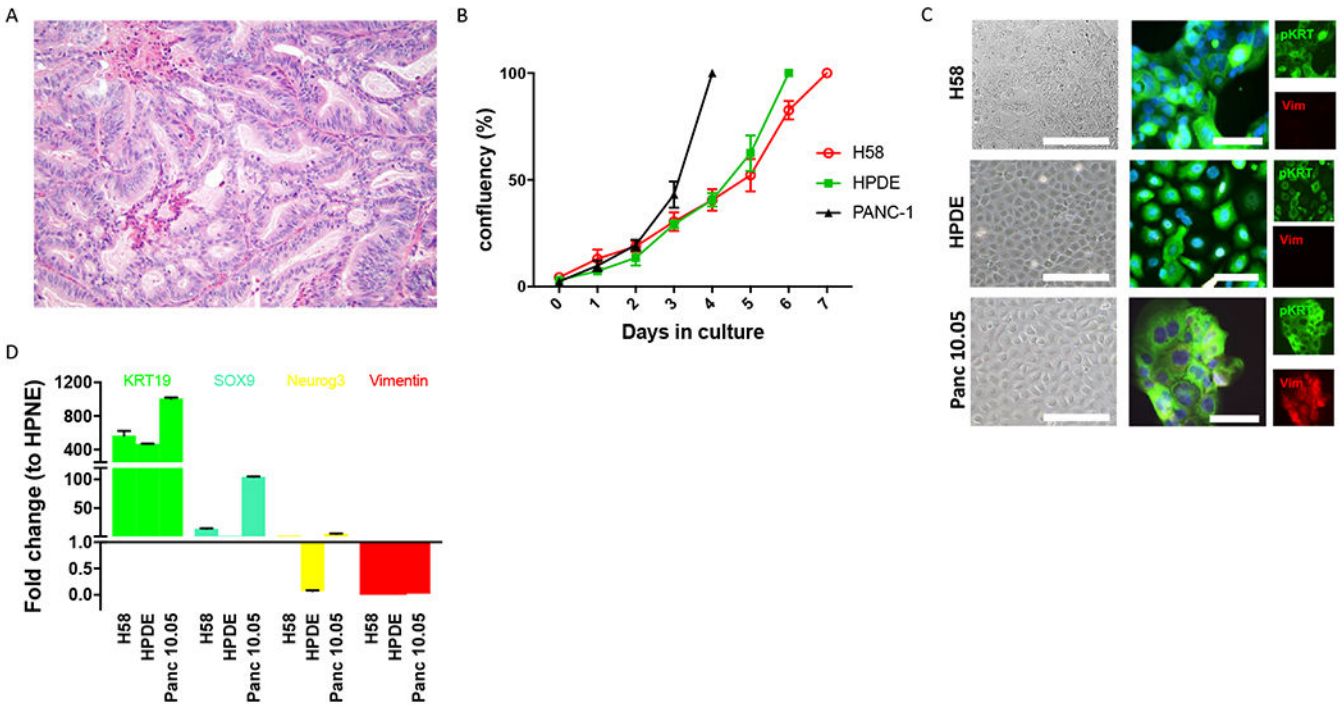

Figure 1.

Morphological and molecular features of H58 cell line. A. H&E section (20X) of human pancreatic ITPN from which H58 was derived. The neoplastic cells are organized in closely packed tubules with minimal intracellular mucin. B. The *in vitro* growth rate of H58 is similar to that of pancreatic ductal cell line HPDE and slower than that of the pancreatic cancer cell line PANC-1. C. Immunofluorescence analysis for pan-cytokeratin (green) and vimentin (red) demonstrates an epithelial phenotype of all three analyzed cell lines. D. RT-PCR analysis confirms the ductal epithelial phenotype of H58, with expression of KRT19 and SOX9.

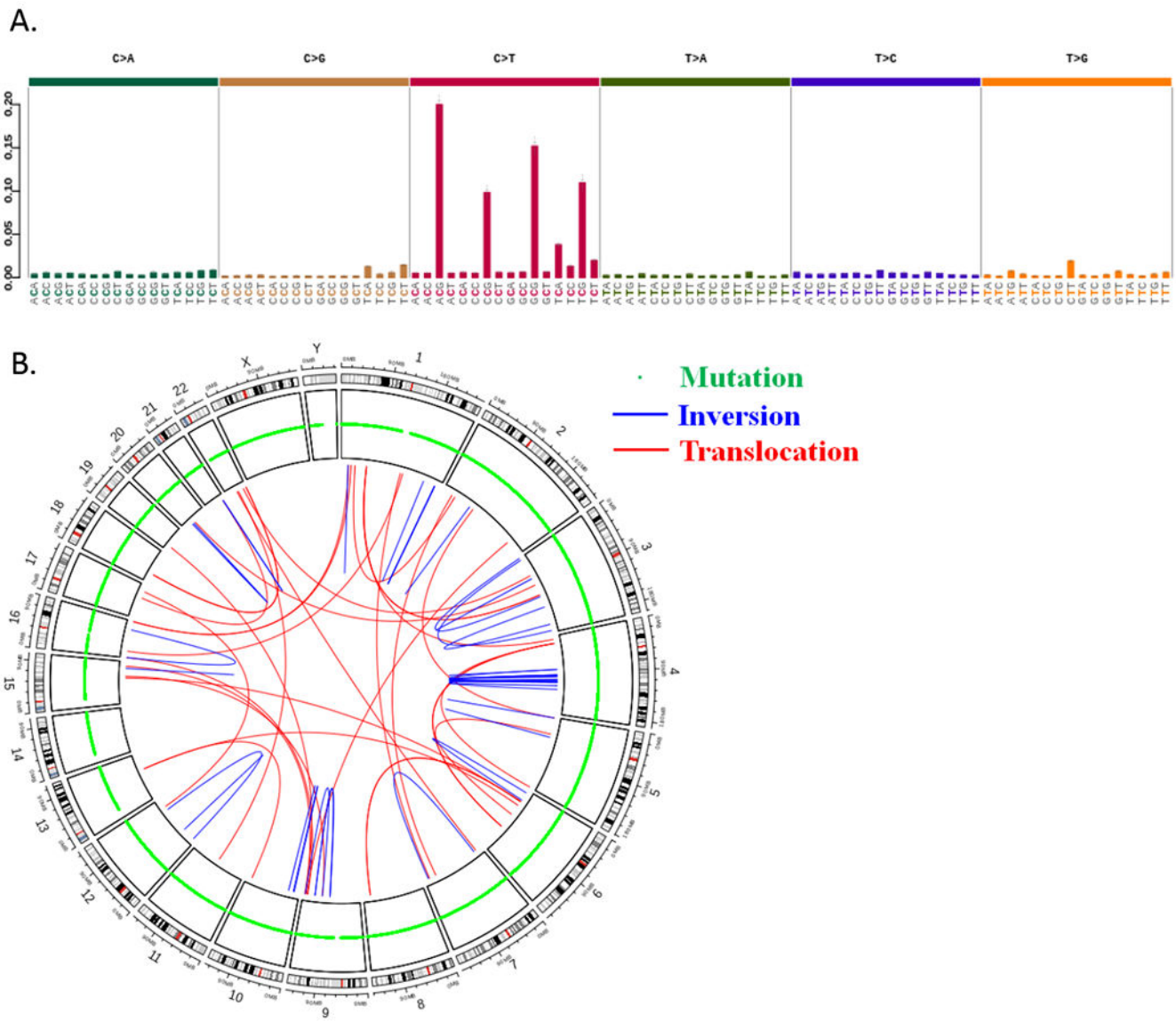

Figure 2.

Results of whole genome sequencing of H58 cell line. A. Mutational signature of single base substitutions shows a preponderance of C>T changes. B. CIRCOS plot shows somatic mutations and structural alterations throughout the genome of H58.

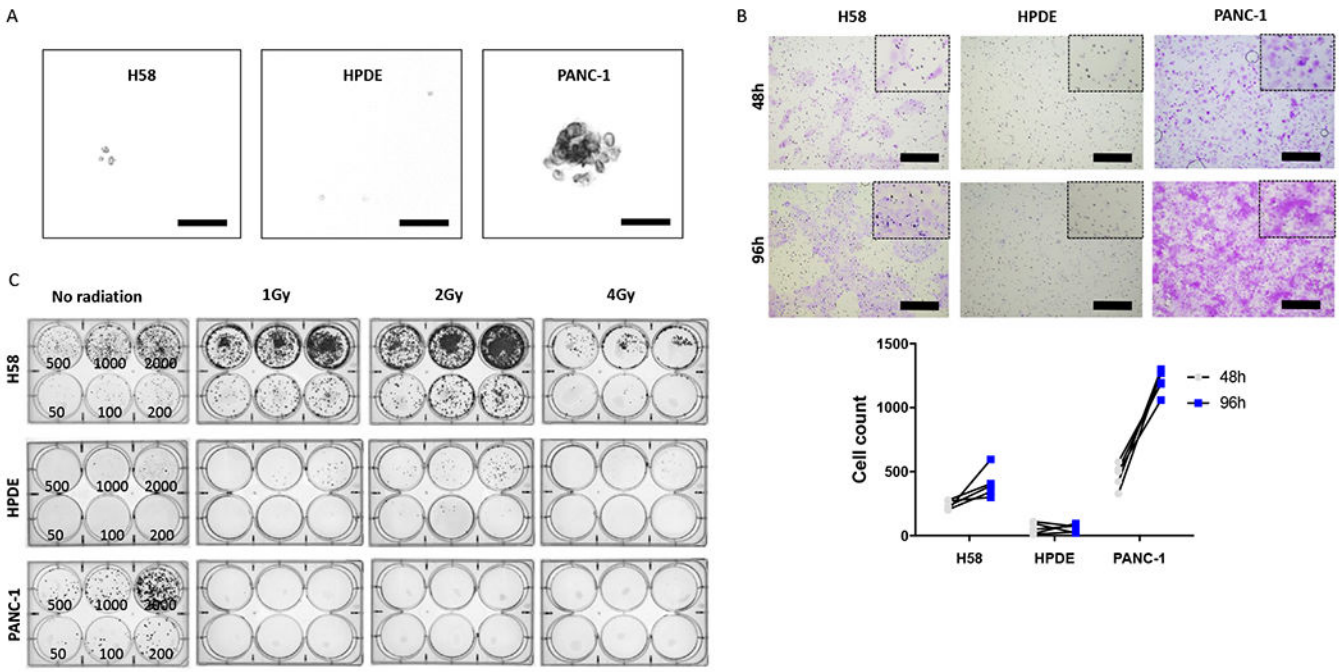

Figure 3.

Tumorigenic characteristics of H58 cell line. A. Soft agar assay shows minimal colony formation by H58 cells, in contrast to the pancreatic cancer cell line PANC-1. B. H58 has invasive capability between that of HPDE and PANC-1 in cell invasion assay. C. Clonogenic assay demonstrates enhance colony formation after ionizing radiation in the H58 cell line.

3.4 Robot-assisted pancreatic surgery-optimized operating procedures: set-up, port placement, surgical steps

As a clinician scientist in the field of modern academic pancreatic surgery, technical aspects still need to be improved in order to reduce morbidity and mortality in our patients. We have optimized and established standardized operating procedures for robotic pancreatectomies. This way we were able to promote a standardized concept for other academic centers who seek to establish innovative robotic programs for pancreatic surgery:

„Even in most complex surgical settings, recent advances in minimal-invasive technologies have made the application of robotic-assisted devices more viable. Due to ever increasing experience and expertise, many large international centers now offer robotic-assisted pancreatic surgery as a preferred alternative. In general however, pancreatic operations are still associated with high morbidity and mortality, while robotic-assisted techniques still require significant learning curves. As a prospective post-marketing trial, we have established optimized operating procedures at our clinic. This manuscript intends to publicize our standardized methodology, including pre-operative preparation, surgical set-up as well as the surgeons' step-by-step actions when using pancreatic-assisted robotic surgery. This manuscript is based on our institutional experience as a high-volume pancreas operating center. We introduce novel concepts that should standardize, facilitate and economize the surgical steps in all types of robotic-assisted pancreatic surgery. The "One Fits All" principle enables single port placement irrespective of the pancreatic procedure, while the "Reversed 6-to-6 Approach" offers an optimized manual for pancreatic surgeons using the robotic console. Novel and standardized surgical concepts could guide new centers to establish a robust, efficient and safe robotic-assisted pancreatic surgery program.“

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ORIGINAL ARTICLE

Robot-assisted pancreatic surgery—optimized operating procedures: set-up, port placement, surgical steps

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Abstract
Even in most complex surgical settings, recent advances in minimal-invasive technologies have made the application of robotic-assisted devices more viable. Due to ever increasing experience and expertise, many large international centers now offer robotic-assisted pancreatic surgery as a preferred alternative. In general however, pancreatic operations are still associated with high morbidity and mortality, while robotic-assisted techniques still require significant learning curves. As a prospective post-marketing trial, we have established optimized operating procedures at our clinic. This manuscript intends to publicize our standardized methodology, including pre-operative preparation, surgical set-up as well as the surgeons' step-by-step actions when using pancreatic-assisted robotic surgery. This manuscript is based on our institutional experience as a high-volume pancreas operating center. We introduce novel concepts that should standardize, facilitate and economize the surgical steps in all types of robotic-assisted pancreatic surgery. The "One Fits All" principle enables single port placement irrespective of the pancreatic procedure, while the "Reversed 6-to-6 Approach" offers an optimized manual for pancreatic surgeons using the robotic console. Novel and standardized surgical concepts could guide new centers to establish a robust, efficient and safe robotic-assisted pancreatic surgery program.

Keywords Robotic-assisted pancreatic surgery · Pancreatic surgery · Standard operating procedures · One fits all · Reversed 6-to-6 approach

Background

Even in most challenging surgical interventions of the retroperitoneum, minimally invasive techniques are increasingly evaluated for their feasibility and efficacy. Indications for laparoscopic interventions have already demonstrated advantages over open pancreatic surgeries in some instances [1, 2]. Results of robotic-assisted interventions from high-volume centers suggest even broader application [3–5]. Due to high costs, the application of these technologies is limited to a few large international centers, so that universally applied Standard Operating Procedures have not yet been established. Some studies also indicate that significant center-specific and time-dependent differences prevail, thus any benefits of robotic-assisted pancreatic surgery may develop only after extended learning curves [6–9]. However, company marketing and the general popularity of such technologies increase daily. To minimize the rate of any serious complications, which may occur during the learning curve, standardization in experienced centers is extremely important. From our high-volume center-specific experiences, we present the following Optimized Operating Procedures, for the setting of robotic-assisted pancreatic surgery. This protocol should enable other centers to establish a robust-, time efficient- and safe robotic-assisted pancreatic surgery program.

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Logistics and informed consent

Robot-assisted technology is novel, so that it is necessary to explain to the patient both the risks and advantages compared to conventional operating methods. We generally inform patients about current international opinions and studies to provide individual, evidence-based recommendations when considering this surgical technology's pros and cons.

This prospective post-marketing study (CARE study; E/A4/084/17) was conducted with IRB approval, which was necessary to collect data of a unique cohort receiving surgical treatment with the da Vinci Xi (Sunnyvale, USA) Surgical System (DRKS00017229).

Hospitalization

Initial surgical assessment and indication for surgery is obtained at our Charité Pancreatic Outpatient Center. Specialized and experienced pancreatic surgeons inform and consent selected patients for robotic-assisted surgery. Anesthesiologists are consulted to thoroughly examine the patient before scheduling the operation. In the context of a professionalized ERAS (enhanced recovery after surgery) program, patients are surveilled before and after surgery by expertly skilled personnel. Patient admission to the hospital occurs one day prior to the operation for final clinical evaluation.

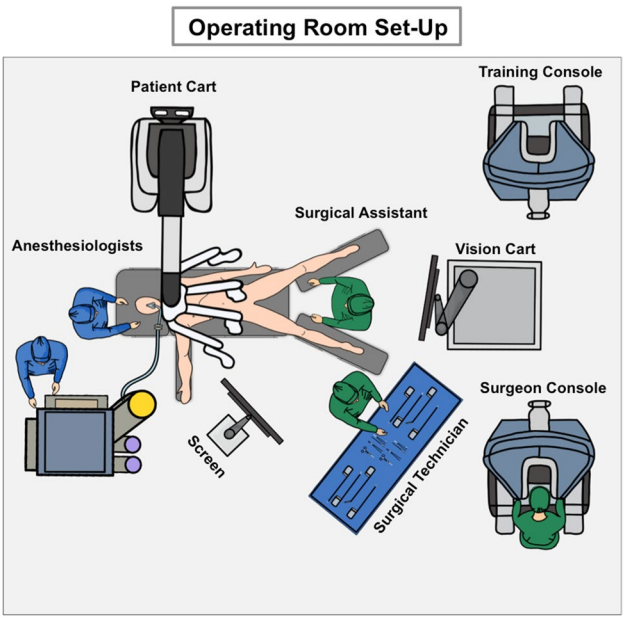

Fig. 1 The operating room set-up: The set-up of the operating room is essential for process optimization and facilitated communication during the robotic-assisted procedure. We were able to limit the number of team members to adjust to any spatial constraints when working with robotic devices. This can be easily re-organized to address center-specific demands

The positioning of the patient

To avoid positioning and collision injuries, patients are placed on a soft vacuum mattress, and metal protectors are attached for safety and shield the surgical area. The left arm is positioned at the patient's side, while the right arm is stretched out to give anesthesiologists easy access (Fig. 1). The patient's legs are placed in French Position while their body remains in 12° reverse Trendelenburg inclination.

The positioning of the ports

Irrespective of the pancreatic procedure, we set out all the robotic and assistant trocars prior to attachment of the robotic arms. Our center has established a "One Fits All" principle. When performing pancreatoduodenectomies (PD), total pancreatectomies (TP) and Appleby procedures (AP), four robotic trocars (8 mm) and two assistant trocars (15 mm/5 mm) are needed. For Distal Pancreatectomies (DP) however, we introduce a single assistant trocar (15 mm). Figure 2 indicates the exact port placement.

First, we place 8 mm trocar (R3) umbilical. After a pneumoperitoneum has been established, a diagnostic laparoscopy is then performed. If there is a good overview, other robotic trocars (R1, R2, R4) are placed in an imaginary horizontal line using standardized distances to avoid robotic arm collisions (see Fig. 2). During this process, any intra-abdominal adhesions are removed using laparoscopic instruments, before introducing the remaining assistant trocars (A1, A2).

Alignment of the da Vinci patient cart

The da Vinci Xi (Sunnyvale, USA) Patient Cart is aligned with the operating table on the left side (Fig. 1). The camera is now being introduced (R3). After the focal point has been adjusted intra-abdominally, the robotic system and arms are set up fully automatic. On demand, the required instruments are introduced into the patient and connected with the robotic arms (Fig. 3) (Table 1).

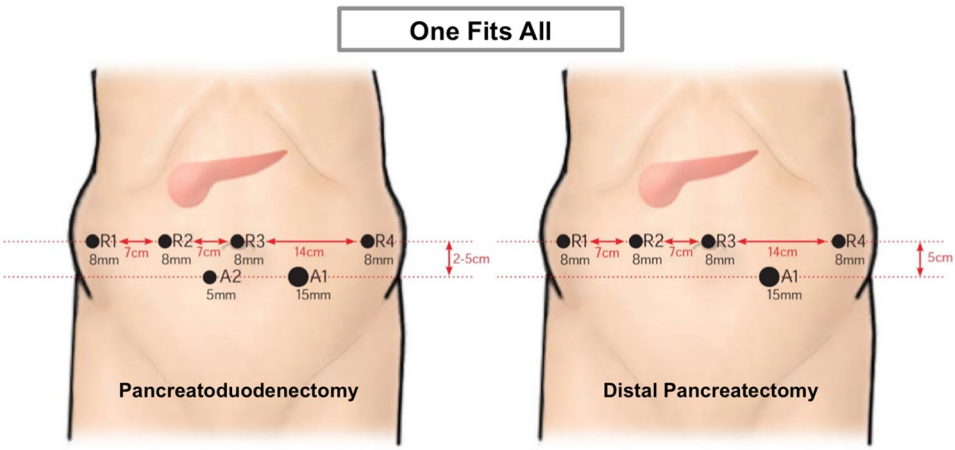

Fig. 2 Port placement: Following the principle "One Fits All", all trocars are positioned the same way irrespective of the pancreatic procedure. We start with the umbilical R3 position for diagnostic laparoscopy. We sequentially place trocars R1, R2 and R4 along a horizontal trajectory. Distances R1–R3 measure 7 cm to one another, while R4 is laid out at the left hemi-abdominal side in double distance (14 cm). Ultimately, assistant trocars (A1, A2) are positioned 3–5 cm below the umbilical horizontal line. The set-up for DP only differs in the lack of a second assistant trocar (A2)

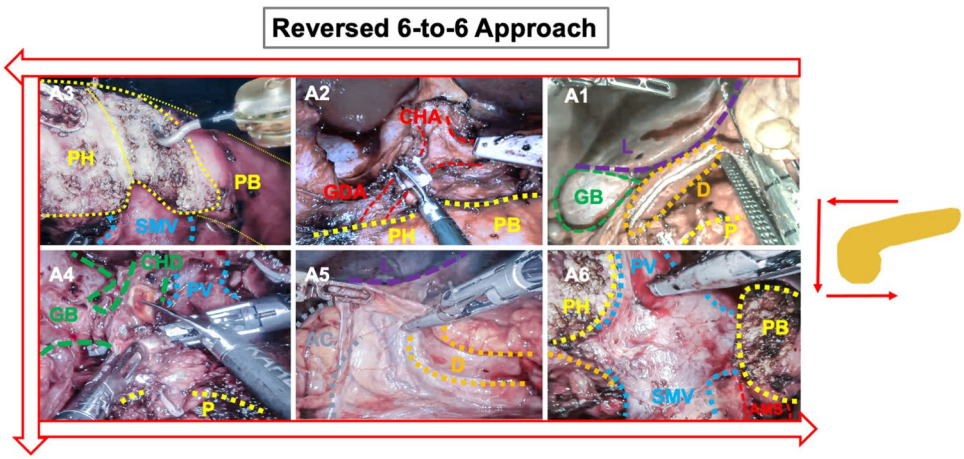

Fig. 3 The "Reversed 6-to-6 Approach": following the "Reversed 6-to-6 Approach", we optimized the surgical steps best suited for robotic-assisted pancreatoduodenectomy. We start dissecting directly at the pancreas, before releasing the specimen from surrounding structures in anti-clockwise orientation. This systematic approach allowed us to economize operating time. *L* liver, *D* duodenum, *GB* gallbladder, *CHA* common hepatic artery, *GDA* gastroduodenal artery, *P* pancreas; *PH* pancreatic head, *PB* pancreatic body, *PV* portal vein, *CHD* common hepatic duct, *AC* ascending colon, *SMA* superior mesenteric artery

Table 1 Robotic ports and instruments: robotic arms are connected with specialized instruments for robotic-assisted surgery

Trocar	Port	Size (mm)	Instrument
Robotic trocar	R1	8	Tip-up Fenestrated Grasper
Robotic trocar	R2	8	Fenestrated Bipolar Forceps
Robotic trocar	R3	8	Endoscope
Robotic trocar	R4	8	Vessel Sealer Extend, Permanent Cautery Hook
Assistant trocar	A1	15	Forceps, Scissor, Covidien EndoGia
Assistant trocar	A2	5	Forceps

Most instruments are used in defined port positions but may be adjusted and customized during each phase of the operation. According to the "One Fits All" principle, we list the distinct trocar/port positions coupled with common robotic instruments used in that position

Steps for structured robotic-assisted pancreatoduodenectomy (PD)

Operating procedures have now been significantly optimized and economized based on our center-specific experience. During the period of 2017–2021, we were able to conduct > 125 robotic-assisted pancreatic surgeries and > 70 PDs. We believe that this process optimization is particularly important in the robotic setting due to its limited spatial overview when compared to the open situs operations. For this reason, our team established a novel concept, named the "Reversed 6-to-6 Approach":

A: Resection

A1: Entering the bursa omentalis to expose the pancreatic organ

When dissecting the greater omentum at the gastrocolic ligament, which enables the access to the bursa omentalis, the Tip-Up Fenestrated Grasper (R1) is utilized. The stomach is mobilized using the Fenestrated Bipolar Forceps (R2) as well as the Vessel Sealer Extend (R4) for dissection of the ligament. The stomach is then dissected directly at the post-pyloric plane using a Covidien (Dublin, Ireland) EndoGia (A1) with a purple cartridge (60 mm). The result is that the stomach can be mobilized into the upper-left quadrant providing an optimal view early on.

A2: Exposing the pancreas at the resection level

The next step includes dissection of the pancreas at the caudal edge to expose the mesenterico-portal axis. While retracting the liver using the robotic arm 1, Fenestrated Bipolar Forceps (R2) and the Vessel Sealer Extend (R4) instruments are needed for careful dissection. Additional support is provided using instruments via assistant trocars (A1, A2). Subsequently, cranial structures are exposed by conducting complete lymphadenectomy with the dissection of all-important vessel structures within the hepatoduodenal ligament (Common hepatic artery, CHA/Gastroduodenal artery, GDA/Right gastric artery, RGA). Identification of the GDA branching-point enables a safe ligation of the artery via Hem-o-lock clips (A1). The portal vein structure is then fully exposed at the cranial edge. At this point, the pancreatic body can be mobilized along the mesenterico-portal axis.

A3: Cutting the pancreas at the resection level

Following the mobilization from cranial and caudal edges, the pancreas is then carefully dissected along the mesenterico-portal axis using the Permanent Cautery Hook (R4).

A4: Preparation of hilar structures

Along the "Reversed 6-to-6 Approach", the hilar structures can subsequently be dissected and visualized. Identification of the hepatocholedochus duct enables dissection right behind the main cystic branch using a scissor (A1). Subsequently, we perform anterograde cholecystectomy using the Permanent Cautery Hook (R4).

A5: Kocher Maneuver

The Kocher Maneuver is realized using the Fenestrated Bipolar Forceps (R1) and Vessel Sealer Extend (R4) instrument. These are progressed along the duodenum in a craniocaudal direction until reaching the ligament of Treitz. The release of these latter segments allows the flection of the jejunal loop into the right upper quadrant. The jejunal loop is subsequently parted using the Covidien (Dublin, Ireland) EndoGia with purple cartridge (45 mm) (A1) to establish the alimentary loop.

A6: Completion at the mesenterico-portal axis

Finally, the dissection of the pancreatic head and the uncinate process is completed along the portal vein and superior mesenteric artery (AMS) using the Fenestrated Bipolar Forceps (R1) and Vessel Sealer Extend (R4). Small branches are clipped (A1), and the resection specimen is removed using a retrieval bag.

B: Reconstruction

B1: Reconstruction of the hepaticojejunostomy

The alimentary loop is commonly opened at counter mesenteric position using the Permanent Cautery Hook (R4), followed by anastomosis of the hepaticojejunostomy in back-to-front direction using a continuous PDS suture (5–0), realized by Large Needle Driver (R4) und Tip-Up Fenestrated Grasper (R2). Prior to closure of the hepaticojejunostomy, a trans-anastomotic stent (2mm × 3cm) is positioned (PDS 5/0) to ensure biliary drainage.

B2: Reconstruction of the pancreato-gastrostomy

A suitable position for anastomosis is marked at the back wall of the stomach using the Permanent Cautery Hook (R4).

A Prolene 5/0 suture along the incision line in purse-string technique is applied using the Large Needle Driver (R4) and Tip-Up Fenestrated Grasper (R2). The stomach is now incised using the Permanent Cautery Hook (R4), and when a clear view of the back wall is achieved, a robotic-adjusted pancreatogastrostomy is executed using our recently developed mattress-seam technique (Vicryl 3/0) [10]. The procedure is conducted with the Tip-Up Fenestrated Grasper (R2), and Large Needle Driver (R4) and a trans-anastomotic splint (2mm × 3cm) is positioned into the pancreatic duct. By pulling the mattress-seam sutures, the pancreatic tail is directly drawn into the stomach, fully covered by gastric mucosa. Finally, the outer pancreatogastrostomy is tightly sealed by purse-string sutures.

B3: Reconstruction of the gastroenteric anastomosis

Antecolic gastroenterostomy finalizes the reconstruction using continuous V-Loc 4-0 sutures. Again, the surgeon proceeds in a back-to-front direction using the Tip-Up Fenestrated Grasper (R2) and Large Needle Driver (R4) instruments.

B4: Disconnection of da Vinci robotic system

Before finalizing the procedure, the abdominal situs is inspected for minor bleeding, cauterized and rinsed to remove any remaining intraabdominal debris (R2). The instruments are removed under careful observation, and all robotic arms are disconnected before the entire patient cart is pulled back from the patients' site. Subsequently, the bag containing the resection specimen is removed through a 5 cm mid-line retrieval incision. We also use this incision to conduct a haptic re-evaluation of all anastomoses, or, in rare circumstances, even to perform the reconstruction of the gastroenteric anastomosis entirely via this incision (hybrid approach). This allows for more flexibility during the initial stages of the learning curve.

B6: Drains and closure

As our institutional standard, we place drains through trocar incisions, which scan the regions around the pancreatogastrostomy and the hepaticojejunostomy (R4 position). The integral planes of mid-line retrieval incision and trocar wounds are closed with sutures.

Steps for structured distal pancreatectomies (DP)

The new standard for non-oncologic distal pancreatectomies is the minimally invasive resection of the pancreatic tail [11]. With this in view, we sought to establish standard, robotic-assisted distal pancreatectomies at our center. With respect to the individual surgical indication (benign versus malign), this procedure can be conducted with spleen preservation (Kimura Maneovre) or via complete oncologic clear-up [12]. As spleen-preserving procedures are rarely performed, we describe the individual steps of an oncologic distal pancreatectomy.

A: Resection

A1: Entering the bursa omentalis to expose the pancreatic organ

When dissecting the greater omentum at the gastrocolic ligament, which enables good access to the bursa omentalis, we use the Tip-Up Fenestrated Grasper (R1) instrument to mobilize the stomach and the Vessel Sealer Extend (R4) for dissection of the ligament. This is performed progressing from the right-medial peri-gastric plane to the left colic flexure using the Fenestrated Bipolar Forceps (R2) und Vessel Sealer Extend (R4) instruments, while the short gastric arteries are regularly dissected. Instruments maneuvering through assistant trocars (A1, A2) may provide additional support and a better overview. After full mobilization of the stomach, we recommend fixing the stomach via a single-armed suture with a straight needle, which we introduce sub-xiphoidal. It is then transfixed at the greater curvature and tied from the outside after the needle is re-released sub-xiphoidally. This enables an excellent overview of the bursa omentalis and the entire pancreatic organ.

A2: Exposing the splenic vessels

For malignant indications, an exact localization and planning of the resection plane is necessary and can be realized intra-abdominally via an ultrasound device (A2). At the resection level, the splenic artery is exposed at the cranial pancreatic edge, using the Fenestrated Bipolar Forceps (R2) and Vessel Sealer Extend (R4) instruments. Next, Hem-o-lock clips are introduced for safe ligation of the artery (A1). The splenic vein is commonly found at the pancreatic body's caudal edge, which is equally ligated via Hem-o-lock clips (A1). The pancreas can now be inflected from the Gerota fascia beneath.

A3: Cutting the pancreas at the resection level

Subsequently, the Covidien (Dublin, Ireland) EndoGia (A1) with a black cartridge (60 mm), reinforced by Seamguard Mesh, is introduced and tunneled at the resection site and dissects the pancreas at the required position.

A4: Retrieval of the resection specimen

The specimen can be released after thorough preparation of the pancreatic tail. It includes lymphadenectomy and mobilization of the spleen, using the Fenestrated Bipolar Forceps (R2) and Vessel Sealer Extend (R4) instruments. The specimen is transferred into a retrieval bag and recovered using an extended incision at position A1.

B6: Drains and closure

As an institutional standard, we place drains through trocar incisions, which scan the regions around the resection margin and the Koller's Pouch (R4 position). Integral planes of retrieval incision and trocar wounds are sutured to close them.

Outcome parameters using optimized operating procedures

In the recent series of robotic-assisted pancreatectomies at our center, we were able to report good outcome parameters, after having implemented novel concepts, such as the "One Fits All" principle and "Reversed 6-to-6 Approach" [4]. Of particular relevance in the evaluation of this novel approach are the parameters, such as mean procedure time, in-hospital stay, complication rates and oncologic outcomes (Table 2). Like other large international centers, we present similar positive outcome parameters, while offering a standardized approach, feasible for majority of patients. There is also a measurable reduction in operating time compared to other centers, likely due to our steep learning curve (Fig. 4). Altogether, this indicates successful process optimization through standardization.

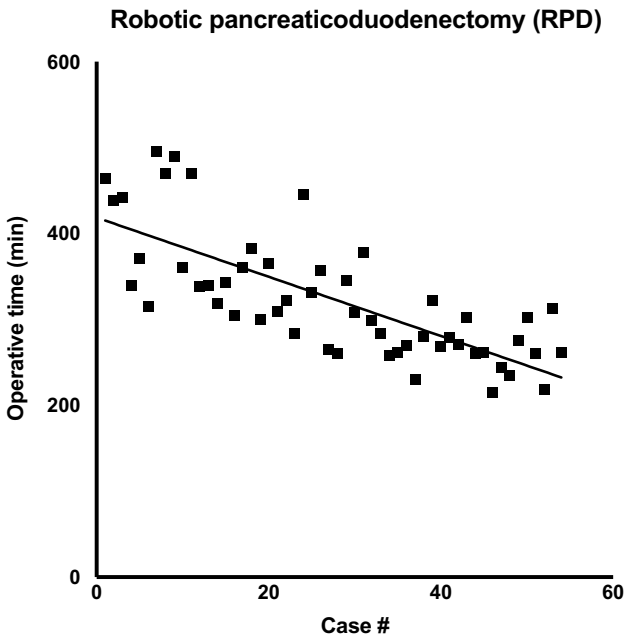

Fig. 4 Institutional learning curve: regression analysis of operating time over the course of 54 RPDs as a result of process optimization. Overall, we were able to halve procedure time over the course of our first series, reflecting a particularly steep learning curve

Table 2 Clinical outcome parameters: main outcome parameters after robotic pancreaticoduodenectomy from various international centers were derived from published reports

	Charité Universitätsmedizin Berlin, Timmermann et al. 2021 [10]	University of Pittsburgh Medical Center, Zureikat et al. 2020 [9]	University of Hongkong, Zhang et al. 2019 [7]	Johns Hopkins University, Van Oosten et al. 2020 [8]	Shanghai Jiaotong University SOM, Shi et al. 2020 [6]
Case number	54	500	100	96	200
Procedure time (min)	325	415	358	474	279
In-hospital stay (days) 15	8	18	8	21.8	
Morbitdity (%)	63	69	58	62.5	36
POPF (%)	18.6	20.2	24	13.5	7.4
PPH (%)	20.2	NA	22	6.2	10
30d Mortality (%)	5.3	1.8	3	2.1	2.5
R0 Resection (%)	83.9	85	100	NA	95
Lymph node harvest	16.5	28	7	NA	16.3

Not surprisingly, most experienced centers show improved outcomes likely due to advanced learning curves. However, even within our early stage of the learning curve, we were able to cut down operating time, after having implemented optimized operating procedures

Conclusion

The rising popularity in the health care setting as well as intense marketing of robotic-assisted techniques warrants its broader application and distribution, particularly expected in pancreatic surgery. While the implementation of novel techniques is indispensable for innovation in the surgical field, it sometimes involves unforeseeable risks. Important studies from centers that have already established large programs for robotic-assisted pancreatic surgery have indicated that good performance and major benefits are only achieved after a long learning curve [6, 7]. At this point, professional training centers have been implemented to train international surgeons in conducting safe robotic-assisted pancreatic procedures [13]. They facilitate the acquisition of proficient skills at the robotic console, prior to their application on patients. However, they are not able to provide immediate surgical guidance on step-by-step operating procedures, necessary to acquire consistent outcome parameters in complex surgical settings. In this manuscript, we emphasize and were able to show that institutional process optimization and standardization, may shorten such learning curves. For this reason, we strongly propose to broadly implement standard operating procedures, which may facilitate intra- and inter-institutional process optimization as well as providing guidance for novel centers to establish a robust, time efficient- and safe robotic-assisted pancreatic surgical program.

Considering the continuous development of this technology, such recommendations need to be regularly discussed and re-evaluated to establish national and international consensus.

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Availability of data and material Not applicable.

Code availability Not applicable.

Declarations

Conflicts of interest Matthäus Felsenstein, Karl Hillebrandt, Mathilde Feist, Lea Timmermann and Christian Benzing have no conflict of interest or financial ties to disclose; Moritz Schmelzle: Merck Serono GmbH, Bayer AG, ERBE Elektromedizin GmbH, Amgen Inc., Johnson & Johnson Medical GmbH, Takeda Pharmaceutical Limited, Olympus K.K., Medtronic GmbH, Intuitive; Thomas Malinka: Intuitive; Johann Pratschke: Intuitive, Johnson & Johnson Medical GmbH.

Ethics approval CARE study obtained IRB approval under E/A4/084/17.

Consent to participate Informed consent was obtained from all individual participants included in the study.

Consent for publication The authors affirm that human research participants provided informed consent for publication of the images in Fig. 3.

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3.5 Internal drainage for interdisciplinary management of anastomotic leakage after pancreaticogastrostomy.

Pancreatic surgery remain one of the most dreaded interventions for visceral surgeons due to considerably high morbidity and mortality. Pancreatic fistula following pancreatoduodenectomy regularly results in hazardous complications including hemorrhages and sepsis. We have employed and optimized an interdisciplinary algorithm, that allowed us to efficiently address anastomotic leakage and fistula though an internal drainage, resulting in earlier resolution and discharge of our patients. This innovative approach will guide less invasive efforts to address complications after pancreatic surgery.

"*Background: Anastomotic leakage and postoperative pancreatic fistula (POPF) may occur after pancreatic head resection, also in the setting of pancreato-gastric reconstruction. For adequate complication management, a variety of non-standardized treatments are available. Still, data on clinical evaluation of endoscopic methods remain scarce. Based on our interdisciplinary experience on endoscopic treatment of retro-gastric fluid collections after left-sided pancreatectomies, we developed an innovative endoscopic concept with internal peri-anastomotic stent placement for patients with anastomotic leakage and/or peri-anastomotic fluid collection.*
Methods: Over the period of 6 years (2015 - 2020) we retrospectively evaluated 531 patients after pancreatic head resections at the Department of Surgery, Charité – Unversitätsmedizin Berlin. Of these, 403 received reconstruction via pancreatogastrostomy. We identified 110 patients (27.3%) with anastomotic leakage and/or peri-anastomotic fluid collection and could define four treatment groups which received either conservative treatment (C), percutaneous drainage (PD), endoscopic drainage (ED), and/or re-operation (OP). Patients were grouped in a step-up approach for descriptive analyses and in a stratified, decision-based algorithm for comparative analyses. The study's primary endpoints were hospitalization (length of hospital stay) and clinical success (treatment success rate, primary/secondary resolution).
Results: We characterized an institutional, post-operative cohort with heterogenous complication management following pancreato-gastric reconstruction. The majority of patients needed interventional treatments (n=92, 83.6%). Of these, close to one-third (n=32, 29.1%) were treated with endoscopy-guided, peri-anastomotic pigtail stents for internal drainage as either primary, secondary and/or tertiary treatment modality.

Following a decision-based algorithm, we could discriminate superior primary- (77,8% vs 53.7%) and secondary success rates (85.7% vs 68.4%) as well as earlier primary resolutions (11.4 days, 95%CI [5.75-17.13] vs 37.4 days, 95%CI [27.2-47.5]) in patients receiving an endoscopic compared to percutaneous management. Conclusion: This study underscores the importance of endoscopy-guided approaches for adequate treatment of anastomotic leakage and/or peri-anastomotic fluid collections after pancreatoduodenectomy. We herein report a novel, interdisciplinary concept for internal drainage in the setting of pancreato-gastric reconstruction."

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Internal drainage for interdisciplinary management of anastomotic leakage after pancreaticogastrostomy. **Matthäus Felsenstein**, Ann-Christin Amini, Sophie Dorfer, Mengwen Hu, Ruonan Wang, Lea Timmermann, Karl Herbert Hillebrandt, Christian Benzing, Uli Fehrenbach, Uwe Pelzer, Igor Maximillian Sauer, Johann Pratschke, Christian Jürgensen, Thomas Malinka. **Surg Endosc. 2023** Mar 6. doi: 10.1007/s00464-023-09964-1.

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ORIGINAL ARTICLE

Internal drainage for interdisciplinary management of anastomotic leakage after pancreaticogastrostomy

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Abstract

Background Anastomotic leakage and postoperative pancreatic fistula (POPF) may occur after pancreatic head resection, also in the setting of pancreato-gastric reconstruction. For adequate complication management, a variety of non-standardized treatments are available. Still, data on clinical evaluation of endoscopic methods remain scarce. Based on our interdisciplinary experience on endoscopic treatment of retro-gastric fluid collections after left-sided pancreatectomies, we developed an innovative endoscopic concept with internal peri-anastomotic stent placement for patients with anastomotic leakage and/or peri-anastomotic fluid collection.

Methods Over the period of 6 years (2015–2020) we retrospectively evaluated 531 patients after pancreatic head resections at the Department of Surgery, Charité–Unversitätsmedizin Berlin. Of these, 403 received reconstruction via pancreatogastrostomy. We identified 110 patients (27.3%) with anastomotic leakage and/or peri-anastomotic fluid collection and could define four treatment groups which received either conservative treatment (C), percutaneous drainage (PD), endoscopic drainage (ED), and/or re-operation (OP). Patients were grouped in a step-up approach for descriptive analyses and in a stratified, decision-based algorithm for comparative analyses. The study's primary endpoints were hospitalization (length of hospital stay) and clinical success (treatment success rate, primary/secondary resolution).

Results We characterized an institutional, post-operative cohort with heterogenous complication management following pancreato-gastric reconstruction. The majority of patients needed interventional treatments ($n=92$, 83.6%). Of these, close to one-third ($n=32$, 29.1%) were treated with endoscopy-guided, peri-anastomotic pigtail stents for internal drainage as either primary, secondary and/or tertiary treatment modality. Following a decision-based algorithm, we could discriminate superior primary—(77,8% vs 53.7%) and secondary success rates (85.7% vs 68.4%) as well as earlier primary resolutions (11.4 days, 95%CI (5.75–17.13) vs 37.4 days, 95%CI (27.2–47.5)] in patients receiving an endoscopic compared to percutaneous management.

Conclusion This study underscores the importance of endoscopy-guided approaches for adequate treatment of anastomotic leakage and/or peri-anastomotic fluid collections after pancreatoduodenectomy. We herein report a novel, interdisciplinary concept for internal drainage in the setting of pancreato-gastric reconstruction.

Keywords Endoscopy-guided drainage · Peri-anastomotic stent · Intramural drainage · Anastomotic leakage · Pancreatic fistula

Anastomotic leakage after pancreatoduodenectomy remains a technical bottleneck resulting in increased morbidity and mortality [1]. Various treatment modalities are widely used to address intraabdominal fluid collections and fistulas after pancreatic surgery [2]. Following pancreato-enteric reconstruction, anastomotic leakage can be a significant source of pancreatic fistulas and peri-anastomotic fluid collection. This regularly demands immediate intervention due to

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potential hazardous complications such as hemorrhages and sepsis [3]. For complication management commonly considers conservative (antibiotics, octreotide, parental nutrition, irrigation of intraoperative drains), interventional (percutaneous drain, endoscopic drain), and/or surgical treatments in step-up strategies [2, 4, 5].

However, a combination of treatment modalities is regularly needed for complete resolution culminating in prolonged hospitalization and delayed re-convalescence [6]. Previous studies demonstrated the advantages of endoscopic management for peri-gastric fluid collections after distal pancreatectomy [7, 8]. This has been further translated to other indications while few studies included patients after pancreatoduodenectomy [9–12]. Initially, Tilara et al. described clinical success with trans-gastric stents for treatment of peri-gastric fluid collections early after pancreaticoduodenectomy [9]. A subsequent study by Al-Efishat M et al. were able to demonstrate comparable technical and clinical success rates of endoscopy-guided, transmural stent placement over percutaneous drainage in a matched-pair analyses [11]. Still, studies on pancreatic fistula with fluid collections after pancreato-enteric reconstruction remain scarce. Therefore, many pancreatic centers primarily rely on percutaneous and operative interventions which challenges the implementation of modern enhanced recovery programs (ERAS) for pancreatic procedures [13, 14].

Our institutional experience treating peri-gastric fluid collections and pancreatic fistula after distal pancreatectomies has helped us to further advance on interventional techniques [15]. For reconstruction after pancreatoduodenectomy, we regularly perform a refined technique of pancreatogastrostomy in both, open and robotic procedures [16, 17]. Still, anastomotic leakage occurs during the postoperative course but can now be directly addressed by endoscopic intervention. In cases of confirmed anastomotic leakage, we regularly perform endoscopy-guided, peri-anastomotic stent placement for internal drainage of peri-anastomotic fluid collection. Positive experiences with early endoscopic intervention on selected patients created some optimism about effectively decreasing hospitalization and morbidity. We sought to retrospectively analyze the safety and efficacy of our novel treatment modality following pancreatoduodenectomy with pancreato-gastric reconstruction.

Methods

Patients

The study enrolled all patients that underwent open or minimal-invasive (robotic) pancreatoduodenectomy with pancreato-gastric reconstruction at Charité–Universitätsmedizin Berlin from January 2015 to December 2020 ($n = 403$). We selected patients fulfilling radiographic (CT-scan) criteria for peri-anastomotic fluid collection or endoscopic assessment of anastomotic leakage ($n = 110$). At our institution, treatments normally started only after one of these diagnostic modalities was conducted. Therefore, patients showing biochemical leakage (POPF A) without detection of fluid collection in CT-scan or dehiscence during endoscopy were not included in the study. We retrospectively analyzed the clinical course and complication management in a prospectively collected database. Demographics and baseline characteristics, including age, sex, surgery technique, pre-/peri-operative information, as well as histopathologic diagnoses were documented. All patients gave their informed consent for statistical evaluation of their clinical course after pancreatic surgery (Approval Institutional ethics board EA1/341/20).

Definition of treatment groups

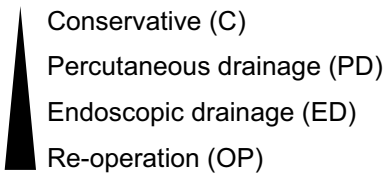
The complication management during the study period after diagnosis of peri-anastomotic fluid collection and/or anastomotic leakage included four main treatment modalities:

(1) Conservative (C)–Treatment with only antibiotics, somatostatin analogues and/or irrigation of intraoperative drainages
(2) Percutaneous drainage (PD)–Treatment with CT-guided drainage placement
(3) Endoscopic drainage (ED)–Placement of endoscopy-guided internal pigtail stents
(4) Re-operation (OP)–Treatment with re-operation

For statistical evaluation, the treatment groups were generated based on two distinct concepts:

Cohort characterization (results "Cohort characterization and Treatment course of patients with anastomotic leakage after pancreatogastrostomy")

Patients were grouped in a step-up approach for descriptive analyses, meaning that patients were allocated according to their most invasive procedure for treatment of anastomotic leakage:



Comparative analyses (results "Clinical resolution of anastomotic leakage after treatment")

Patients were grouped in a stratified, decision-based algorithm, in which primary-, secondary- and tertiary interventions were defined. The algorithm allowed us to directly compare the technical modalities at given phases in sometimes alternating sequences of treatment modalities during the post-operative clinical course:

(i) Primary intervention–First intervention after diagnosis of peri-anastomotic fluid collection and/or anastomotic leakage
(ii) Secondary intervention–Post-procedural intervention after primary intervention
(iii) Tertiary intervention–Post-procedural intervention after secondary intervention

The primary endpoints of the study were hospitalization (length of hospital stay) and clinical success (treatment success rate, primary/secondary resolution) of interventional treatment modalities. We defined clinical resolution as a sub-clinical, ambulatory situation without further need for interventions, and antibiotics, while maintaining normal mobility and food intake (= day of discharge). Therefore, clinical resolution directly correlated with in-hospital stay. However, due to often alternating treatment modalities (range: 0–4) for diagnosed anastomotic leakage, we created the decision-based algorithm (*comparative analyses*). Secondary endpoints considered post-interventional infectious and drainage parameters, morbidity/mortality and disease-specific cumulative survival.

Peri-anastomotic drainage technique

In cases of clinical and radiographic suspicion of a peri-anastomotic leakage, we inspected the anastomosis through direct endoscopy. Anastomotic leakage could typically be identified by pus secretion or direct visualization, without the need of endoscopy-guided ultrasound (EUS). Following contrast medium injection and simultaneous fluoroscopy, we used a guidewire to place double-pigtail stents peri-anastomotic. If direct endoscopy did not visualize a dehiscence or size did not match with pre-interventional radiologic findings, the use of additional EUS confirmed (or excluded) any perianastomotic fistula with fluid collection, which was subsequently drained by the internalized drainage in the technique described previously [15]. In cases of larger dehiscence and necrotic material, we performed additional endoscopic necrosectomy. The size of dehiscence determined the number of stents used (range: 1–5). In cases of drained fluid collections, aspirates underwent microbiologic analyses. Once internal drainages were in place, external drainages were removed, generally at the same day (internalization). We scheduled endoscopic removal of internal stents around 6 weeks after discharge.

Statistics

For the data analyses, we used the statistical software package SPSS (IBM Corp. Released 2021. IBM SPSS Statistic, Version 28.0. Armonk) and visualized using Prism (GraphPad Software, La Jolla California USA). For descriptive analyses of patients' demographics, we performed one-way Anova for continuous and Fischer's exact for categorical variables. We applied ordinal logistic regression to determine independent predictors of complication management. For comparative outcome analyses and follow-up of treatment groups, Cox-regression was used, controlling for underlying disease and tumor stage. Kaplan-Mayer regression was used to compare clinical resolution.

Results

Cohort characterization

During the study period from 2015 to 2020 a total of 795 pancreatic resections were performed. Of those, 531 patients (66.8%) underwent pancreatoduodenectomies (PPPD/Whipple), 216 left-sided pancreatectomies (27.2%) and 48 patients (6.0%) had other surgical procedures (total pancreatectomy, central pancreatectomy, enucleation etc.). Following PPPD/Whipple, 403 patients (73%) received pancreatogastric reconstruction of which 110 patients (27.3%) fulfilled inclusion criteria for comparative evaluation of treatment approaches for either anastomotic leakage and/or pancreatic fistula. Most patients received a PD ($n = 50$; 45.5%) as a single interventional treatment modality while around 16.4% ($n = 18$) were sufficiently treated through conservative concepts. Close to one-third (n = 32; 29.1%) of our patients were treated with peri-anastomotic pigtail stents (ED) for internal drainage of peri-anastomotic fluid collections as primary-, secondary-, and/or tertiary treatment modality. Ultimately, 12 patients (10.7%) needed re-operation (OP). Patient characteristics of weighted treatment groups are listed in Table 1.

Table 1 Postoperative Management after PPPD with clinically relevant perianastomotic fluid collection

	Treatment groups*								
	Conservative (C)	%	Percutanous drain (PD)	%	Endoscopic drain (ED)	%	Re-operation (OP)	%	Statistics (p)**
Total cases	18	16.3	50	45.5	30	27.3	12	10.9	
Age	64.1 (±10.1)		63.9 (±12.4)		67.0 (±9.2)		62.3 (±13.1)		*0.76*
Sex	M 14 W 4	77.8 22.2	M 29 W 21	58 42	M 26 W 4	86.7 13.3	M 7 W 5	58.3 41.7	*0.05*
Body mass index (BMI)	25,7 (±4.9)		25.2 (±4.4)		26,2 (±6.6)		27.1 (±6.2)		*0.89*
ASA	ASA1: 1 ASA2: 9 ASA3: 8	5.6 50.0 44.4	ASA1 2 ASA2 30 ASA3 17	4.1 61.2 34.7	ASA1 0 ASA2 16 ASA3 13	0 55.2 44.8	ASA1 1 ASA2 7 ASA3 4	8.3 58.3 33.3	*0.69*
Surgery technique	Open: 14 Robotic: 4	77.8 22.2	Open: 45 Robotic: 5	90.0 10.0	Open: 22 Robotic: 8	73.3 26.7	Open: 7 Robotic: 5	58.3 41.7	*0.12*
Previous abdominal surgery	No: 12 Yes: 6	66.7 33.3	No: 33 Yes: 17	66.0 34.0	No: 7 Yes: 23	19.4 80.6	No: 5 Yes: 7	41.7 58.3	*0.13*
Operation time (min)	347.2 (±68.2)		353.8 (±84.3)		289.1 (±59.0)		331.7 (±59.5)		*0.06*
Surgery indication	Benign: 6 Malign: 12	33.3 66.7	Benign: 13 Malign: 37	26.0 74.0	Benign: 7 Malign: 24	23.3 76.7	Benign: 4 Malign: 8	33.3 66.7	*0.39*
Tumor entity	PDAC: 7	38.9	PDAC: 17	34.0	PDAC: 11	36.7	PDAC: 5	41.7	
	Duodenal/Papillary: 2	11.1	Duodenal/Papillary: 3	6.0	Duodenal/Papillary: 3	10.0	Duodenal/Papillary: 1	8.3	
	Ampullary: 1 Distal bile duct: 2 Other: 6	5.6 11.1 33.3	Ampullary: 5 Distal bile duct: 8 Other: 17	10.0 16.0 34.0	Ampullary: 1 Distal bile duct: 6 Other: 9	3.3 20.0 30.0	Ampullary: 1 Distal bile duct: 1 Other: 4	8.3 8.3 33.3	*0.62*

*Group assignment depending on most invasive treatment during course of hospitalization (step-up approach)

**ANOVA for continous variables or Fisher-Exact test for categorical variables

Statistical comparison on patient demographics were primarily conducted on interventional (PD, ED, OP) groups. The latter identified sex as single significant parameter, likely due to uneven distribution already present in the baseline characteristics of our cohort (76 male vs 34 female). In addition, pre-treatment conditions (BMI, previous abdominal surgery, ASA) and peri-operative variables (operative time, surgical technique) were not predictive of treatment allocation in ordinal logistic regression analyses (Table 2).

Treatment course of patients with anastomotic leakage after pancreatogastrostomy

Following the pancreatoduodenectomy, all patients were admitted to the ICU (100%). Most patients were transferred to regular wards at postoperative day 1 (42.7%; median 2.0). On average, the timing of complication (anastomotic leak-age) occurred at day 7.5 (± 4.6 days; range: 1–25 days) dis-covered either by CT-scan or endoscopic validation. Postop-erative parameters as surrogates for systemic inflammation (CRP/Leukocytes) averaged 155.4 mg/l (± 96.2 mg/l; range 10–467.8 mg/l) and 14.5/nl (±5.9/nl; range 3.8–38.9/nl) at day of diagnosis. Drainage lipase values were 18,052.4 U/l (±48,217.2 U/l; range 3–327,687 U/l) at time of diagnosis. Treatment started at postoperative day 9.5 (±5.9 days) for PD, while ED treatment was performed at postoperative day 14.5 (±6.4 days; $p<0.01$). ICU re-admission was needed in 11 cases (22%) for PD- vs. 12 cases (38.7%) for ED group ($p=0.15$, Fisher´s Exact). Total duration (days) on ICU of respective treatment groups is shown in Fig. 1A. Length of in-hospital stay was 19.4 days for C (±10.9 days), 30.5 days (±16.1 days) for PD, 25.0 days (±11.4 days) for ED and 47.3 for OP (±26.9 days) (Fig. 1B , $p<0.001$, one-sided ANOVA). Overall complications in treatment groups were documented according to classifications of Clavien-Dindo (Fig. 1C) and the ISGPS society (Table 3). In-hospital mortality of primary interventions was significantly reduced in the interventional groups (PD, ED) compared to re-operation (Fig. 1D). Comparing the total drainage placement of interventional groups, drainage remained significantly longer in ED compared to PD group (Fig. 1E), due to varying treatment standards. Cox-Proportional-Regression analyses on patients with malignant diagnosis (PDAC, ampullary cancer, papillary cancer, distal bile duct cancer) revealed different

Table 2 Ordinal logistic regression analysis of pre-operative and peri-operative variables

Variable	Odds ratio	Estimate	95% Confidence interval		*p*-value
			Lower	Upper	
Sex (female vs male)	0.794	−0.231	0.321	1.963	0.617
Body mass index (BMI)	1.145	0.135	0.405	3.236	0.798
Age	0.997	−0.001	0.963	1.038	0.977
ASA (ASA 1/2 vs 3)	1.191	0.175	0.544	2.608	0.661
Diabetes mellitus II	0.955	−0.046	0.367	2.487	0.925
Smoking	0.955	−0.157	0.443	1.647	0.639
Alcohol	1.091	0.087	0.525	2.269	0.815
Past abdominal surgery	1.173	0.159	0.468	2.940	0.734
Tumor entity (malign vs benign)	1.297	0.260	0.281	5.983	0.739
T stage (T3/4 vs T1/2)	0.931	−0.072	0.560	1.548	0.782
Operation technique (robotic vs open)	1.513	0.414	0.895	2.557	0.122
Operation time	0.996	−0.004	0.992	1.000	0.066

Dependent variable: Ascending treatment groups in terms of invasiveness

cumulative survival in weighted treatment groups, showing reduced survival for patients who needed re-operation (Fig. 1F; Table 4).

Clinical resolution of anastomotic leakage after treatment

Primary success rate, meaning that the first intervention led to resolution, varied between groups (Fig. 2A). On average, we achieved primary success in the conservative group in 69.2%, in the PD group in 53.7%, in the ED group in 77.8% and in the OP group in 50.0% ($p = 0.58$, Fischer´s Exact). A direct comparison of the interventional groups PD and ED indicated no significant difference ($p = 0.16$, Fischer´s Exact). Following the primary intervention, inflammatory parameters (CRP/Leukocytes) at post-intervention day 3 immediately decreased in the interventional groups (PD, ED) while parameters in the OP group remained high (Fig. 2B). Comparing inflammatory parameters at post-intervention day 3 in both, primary- and secondary intervention settings, showed a tendency for decreased infectious parameter in the ED as compared to the PD group (Table 5). Analyzing the clinical resolution of primary interventions, we observed earlier resolution in the ED group, compared to the PD- and OP group which reaches statistical significance ($p = 0.02$, Log-rank test; Fig. 2C). A secondary modality for anastomotic leakage was required in eight cases (30.8%) for the conservative-, 31 cases (46.3%) for the PD-, two cases (22.2%) for the ED- and in four cases (50%) for the OP group (Fig. 1). Following secondary treatment, success rates with interventional groups were 68.4% in the secondary PD group, 85.7% in the secondary ED group and 100% in the secondary OP group, respectively. Kaplan-Maier analyses on resolution after secondary intervention only characterized a tendency towards reduction in the ED group compared to the PD group ($p = 0.11$, Log-rank test**,** Fig. 2D). In summary, treatment stratification into decision steps, discriminated advantages of ED over PD treatment. Thus, in some observations (inflammatory parameters, resolution after primary intervention) endoscopic treatment outperformed percutaneous intervention.

Endoscopic drainage

In 29 patients ED was performed either as a primary or secondary treatment modality (three cases as tertiary intervention). Based on positive experiences with the treatment of fluid collections after distal pancreatectomy, we expanded and increasingly applied the technique to patients after pancreatoduodenectomy with pancreato-gastrostomy (PG) (Fig. 3A). Facilitated monitoring after surgery by endoscopic inspection reflects the inherent advantage of applying the PG reconstruction. In cases of confirmed anastomotic leakage with subsequent endoscopic treatment, we placed at least one plastic pigtail stent (median 1, range: 1–5) right across the dehiscence (Fig. 3B). On average a single (median 1, range: 1–2) intervention sufficed (technical success rate of 93.3%). EUS was only needed in 31.3% (10 out of 32 cases) for assessment of peri-anastomotic fluid collection. The percutaneous drainage was sometimes used as a landmark for correct stent placement but was generally removed after the procedure (internalization) (Fig. 3C). In a few cases with larger dehiscence and abscess formation with mature walls, we additionally performed necrosectomy prior to stent placement (6 out of 32 cases). Adverse events directly linked to endoscopic intervention were not documented during the observational period (complication rate:

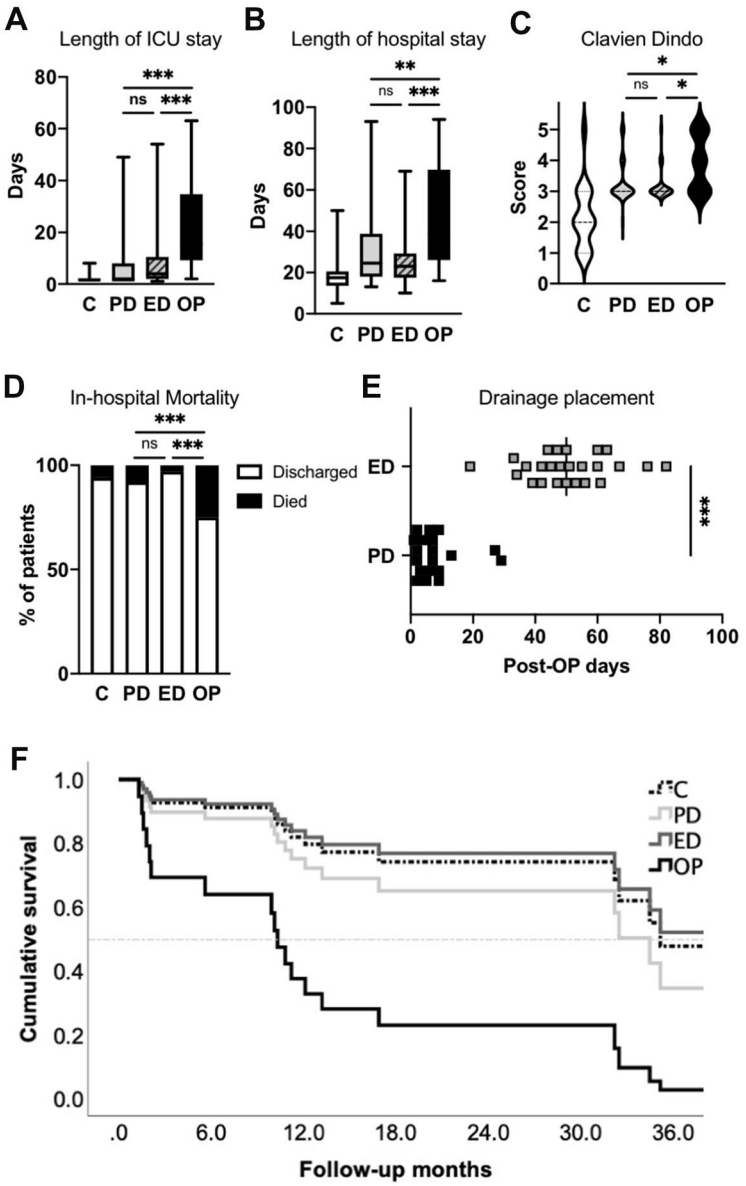


Fig. 1 **A**–**D** Comparative analyses of weighted treatment groups for length of ICU stay, length of hospital stay, Clavien-Dindo classification and in-hospital mortality (one-way ANOVA with Bonferroni multiple comparison). **E** Comparative analyses of ED- and PD treatment groups for total drainage placement in-situ (Welch's unpaired *t*-test). **F** Analyses of cumulative survival (Cox-Proportional-Regression) of treatment groups, controlling for underlying disease

0%). Endoscopic re-interventions for stent correction were needed in two cases (6.3%). On average, drainages were electively removed around 6 weeks after discharge (54.1 postoperative days; ± 28.2 days). Variances occurred due to the different clinical courses of included individuals. Incidental stent migration, detected in routine X-rays prior to second intervention, occurred in seven patients (21.8%) and were not associated with any clinical deterioration.

Discussion

Across pancreatic cancer surgery centers post-operative fistula and anastomotic leakage are widely regarded the Achilles' heel [18]. We have learned that complication management is key for decreasing morbidity and mortality after pancreatic resection [3, 19]. Through institutional experience as a high-volume center, we to further advanced on interdisciplinary concepts for treating pancreatic fistulas and anastomotic leakage. This study demonstrates the feasibility, efficiency, and safe application of an endoscopic technique with the placement of peri-anastomotic stents for internal drainage of peri-anastomotic fluid collection after pancreatoduodenectomy. Consequently, we demonstrate earlier resolution and reduced hospitalization of patients with anastomotic leakage, better aligning with modern ERAS concepts that ultimately allow for earlier admission of oncologic patients to adjuvant treatments.

EUS-guided concepts for the drainage of peri-luminal fluid collections are not new and have been tested for some time. The main indications remain in the treatment of digestive fluids after pancreatic surgery for preventing severe

Table 3 Post-operative complications after pancreatic surgery (ISGPS definition of 2016)

	Treatment groups									
	Conservative (C)	%	Percutanous drain (PD)	%	Endoscopic drain (ED)	%	Re-operation (OP)	%	Statistics*	Statistics**
Total cases	18	16.4	50	45.5	30	27.3	12	10.9		
Pancreatic fistula (POPF)	A: 18 B: 0 C: 0	100 0 0	A: 0 B: 50 C: 0	2 98 0	A: 0 B: 30 C: 0	0 100 0	A: 0 B: 0 C: 12	0 0 100	*<0.001*	*NA*
Hemorrhage (PPH)	None: 16 A: 0 B: 1 C: 1	88.9 0 5.6 5.6	None: 43 A: 1 B: 3 C: 3	86.0 2 6.0 6.0	None: 23 A: 0 B: 6 C: 1	76.7 0 20 3.3	None: 3 A: 0 B: 1 C: 8	25 0 8.3 66.7	*<0.001*	*0.24*
Surgical site infections (SSI)	None: 16 1/2: 2 3: 0	88.9 11.1 0	None: 0 1/2: 0 3: 50	0 0 100	None: 0 1/2: 0 3: 30	0 0 100	None: 1 1/2: 0 3: 11	8.3 0 91.7	*<0.001*	*NA*
Deleayed gastric emptying (DGE)	None: 15 Yes: 3	83.3 16.7	None: 36 Yes: 14	72.0 28.0	None: 28 Yes: 2	93.3 6.7	None: 8 Yes: 4	66.7 33.3	*0.09*	*0.16*

NA Not applicable

*Chi^2 test for categorical variables (all groups)

**Fisher's exact test for categorical variables (PD vs ED)

Table 4 Cox-regression overall survival analysis of patients diagnosed with PDAC

Variable	Hazard ratio*	Standard error	Estimate	95% Confidence Interval		*p*-value
				lower	upper	
Conservative (C)			*6.738*			0.081
Percutanous Drain (PD)	1.435	0.615	0.346	0.430	4.788	0.557
Endoscopic Drain (ED)	0.882	0.795	0.025	0.186	4.195	0.875
Re-Operation (OP)	4.896	0.739	4.617	1.150	20.847	0.032

*Hazard calculated with conservative group as indicator

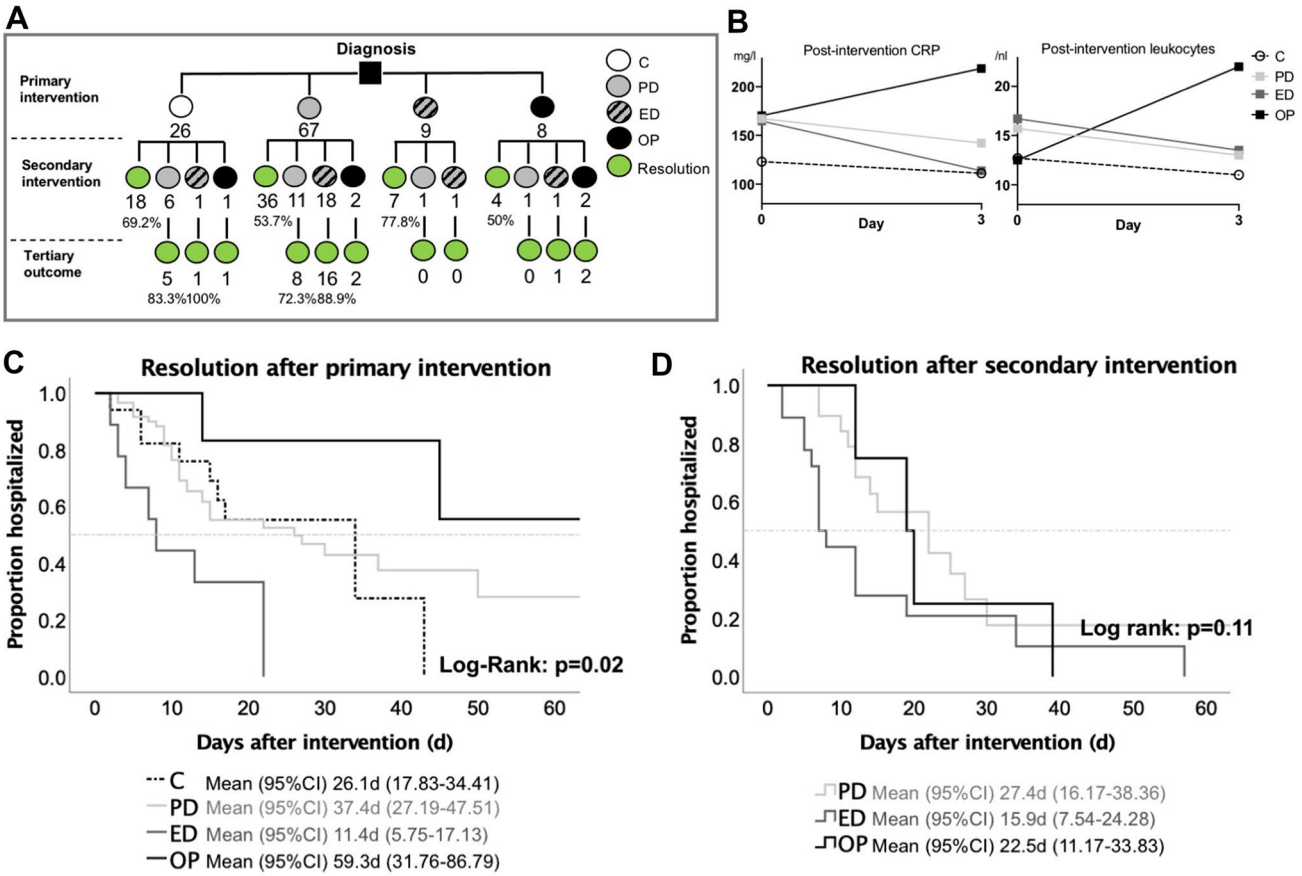


Fig. 2 **A** Decision tree of patients, regularly receiving combinatory treatments (primary-/secondary-/tertiary treatment) for anastomotic leakage and/or peri-anastomotic fluid collection. Below annotated, the number of patients and success rates of respective sub-groups. **B** Post-procedural development of infectious parameters. **C**–**D**. Kaplan-Maier analyses of clinical resolution after the primary- and secondary intervention

complications. Such techniques have been initially conducted in pseudocysts or peri-gastric fluid collections after distal pancreatectomies, however focusing on late abscess formation with mature walls [7, 8, 20]. We and others have shown that the trans-gastric route allows for uncomplicated and safe internal drainage even in the absence of abscess formation for early post-operative treatment of clinically relevant pancreatic fistulas [15, 21]. Compared to percutaneous interventions, success rates revealed advantages of transmural drainage systems in some settings [7, 22]. Positive experiences have also been documented for other indications, including bariatric surgery and liver resections [10, 23].

In our series, we routinely performed reconstruction with pancreato-gastric anastomosis after pancreatoduodenectomy (Whipple procedure/PPPD) [16, 17]. This surgical technique facilitates early monitoring of pancreatic fistulas and anastomotic leakage, using contrast agents through

Table 5 Infectious parameters after interventions

Intervention group	Post-intervention (d3)* Primary intervention											Post-intervention (d3)* Secondary intervention										
	n	CRP (mg/l)	STD	Range	Statistics**	Post-hoc***	Leukocytes (/nl)	STD	Range	Statistics**	Post-hoc***	n	CRP (mg/l)	STD	Range	Statistics**	Post-hoc***	Leukocytes (/nl)	STD	Range	Statistics**	Post-hoc***
Conservative (C)	18	111.2	±71.9	20.3–315.3	0.018		11.1	±4.0	5.0–18.7	< 0.001		–	–	–	–	0.015		–	–	–	< 0.001	
Percutanous Drain (PD)	67	141.9	±81.5	18.5–444.4		ns	13.1	±5.6	4.0–33.7		ns	19	108.2	±36.1	36.2–87.3		ns	12.4	±6.3	9.3–15.4		ns
Endoscopic Drain (ED)	9	113.7	±69.9	39–0-234.9			13.7	±5.5	6.8–25.8			20	91.4	±93.3	7.5–323.6			10.7	±5.7	7.9–13.4		
Re-Operation (OP)	8	218.8	±114.4	60.3–345.6			21.9	±13.3	6.0–39.5			5	215.3	±123.1	42.9–271.5			25.4	±13.8	8.2–42.5		

* Laboratory results at day of intervention (± 1d)

** One-sided ANOVA

*** Bonferroni multiple comparisons

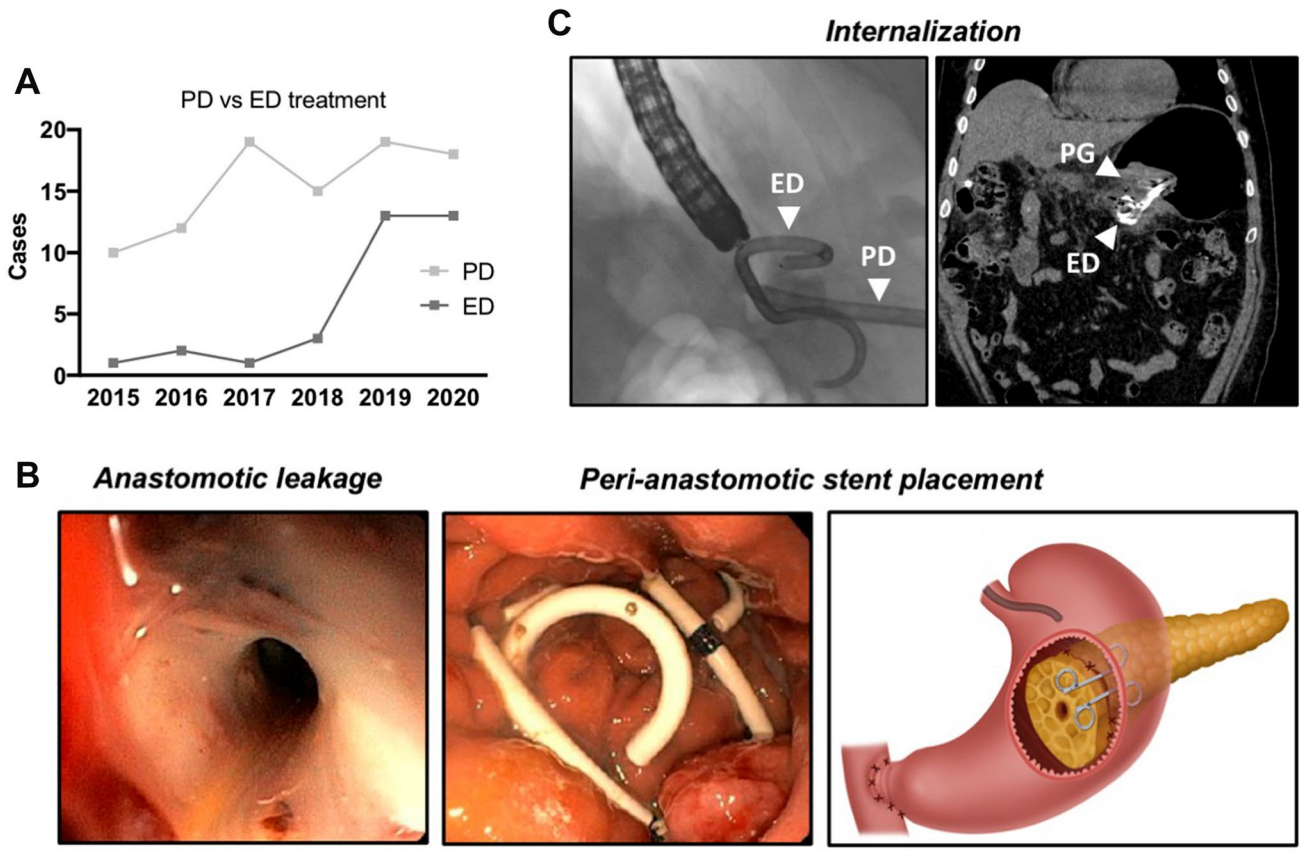


Fig. 3 **A** The number of endoscopic treatments over the years 2015–2020. **B** Representative pictures during endoscopic intervention showing large dehiscence of the PG with subsequent placement of two plastic pigtail stents. **C** A guidewire and the tip of the percutaneous drainage (PD) helped to correctly place the peri-anastomotic pigtail stents (ED). Representative post-interventional CT-scan shows correct stent placement and internal drainage of peri-anastomotic fluid collection at the pancreato-gastrostomy (PG)

located nasogastric tubes and/or endoscopic intervention. In cases with detectable dehiscence, early stent placement was conducted (on average at POD 14.5) and demonstrated primary success rates of 77.8% (7/9 patients) while this was documented in only 53.7% (36/67 patients) of cases with percutaneous drainage. Also in the setting of secondary intervention, endoscopic drainage showed advantages over percutaneous management (85.7% in ED groups vs. 68.4% in PD group). Despite limited sample sizes, our study adds to accumulating evidence of using transmural over percutaneous interventions as preferred treatment for peri-anastomotic fluid collections and pancreatic fistula. At a minimum, such approaches should be widely established as a versatile and safe adjunct to conventional percutaneous techniques. Future studies with larger patient cohorts may define algorithms and characteristics of which patients most likely benefit from either of these treatment modalities.

The rationale for internalizing external drainages lies in the aim of earlier recovery, reduced hospitalization and an improved quality of life. Although it remains difficult to accurately define resolution, studies could show similar or even reduced hospital stay in cases with endoscopic stent placement when compared to percutaneous techniques [7, 24]. This was also a conclusion of a recent meta-analysis even when certainty of evidence remains limited overall [25]. In our study, we examine advantages of earlier resolution via ED compared to PD in the setting of primary intervention. Similar tendencies were observed in the setting of secondary interventions, not reaching statistical significance however, likely due to the patients' overall more complicated clinical courses. Also, laboratory surrogates suggested more effective fistula treatment of ED over PD in both, primary and secondary interventions. Despite limited sample sizes, we are confident that our preliminary observation of reduced hospitalization through internalization can be confirmed when using a stratified, decision-based algorithm for com-parative analyses.

Several studies reported adverse events or complications after endoscopic stent placement [21, 25]. However, we were not able to directly assign complications to the endoscopic treatment, which may be routed in our technique which

uses a pre-existent dehiscence, not puncturing the gastrointestinal wall.

Data on experiences of transmural drainage after pancreatoduodenectomy are scarce, possibly due to the often distant location of fluid collections in the peri-hepatic cavity after pancreato-jejunostomy. Tilara A et al. reported nine patients after pancreatoduodenectomy, while using a trans-gastric route and not documenting specific success rates for this cohort [9]. Al-Efishat M et al. demonstrated similar success rates and outcomes between ED and PD in a matched-pair analyses, ultimately including 20 patients after pancreatoduodenectomy, again using procedures puncturing the intestinal wall [11]. Our study is unique, in that we include a large cohort of patients treated with an innovative approach using peri-anastomotic stents in the setting of pancreato-gastric reconstruction after pancreatoduodenectomy. This combination enabled a standardized interdisciplinary work-flow between surgeons, radiologists and endoscopists with measurable benefits for our patients suffering from complications such as anastomotic leakage and pancreatic fistula.

As a retrospective study, we cannot overcome the inherent selection bias. During the study period 2015–2020, there was an increase of patients selected for our adopted technique due to positive experiences, while the percutaneous technique were more prevalent at earlier time points. Overall, sample sizes of conceptional groups were limited, such that statistical analyses is challenged and therefore need to be interpreted with caution. We seek to collect prospective data in order to obtained more robust analyses on these treatment modalities. In addition, this study was performed at a single institution, due to the project-related demands of a specific surgical technique (pancreatogastrostomy) in combination with excellent endoscopic expertise for ultrasonography-guided intervention. Challenges also lay in the often heterogenous and individual approaches of complication management, which we attempted to address by analyzing cohorts with a stratified, decision-based algorithm (primary, secondary, tertiary interventions) while being aware of its limitation to control for other more complicated confounders. Lastly, decisions were sometimes based on individual clinical preferences not always allowing for standardized, clinical algorithms.

This study confirms the importance of endoscopy-guided approaches as a primary treatment or adjunct procedure to address complications after pancreatoduodenectomy. We herein report a novel, innovative technique for internal drainage of peri-anastomotic fluid collections in the setting of pancreato-gastric reconstructions.

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Declarations

Disclosure The authors Matthäus Felsenstein, Ann-Christin Amini, Sophie Dorfer, Mengwen Hu, Ruonan Wang, Lea Timmermann, Karl Herbert Hillebrandt, Christian Benzing, Uli Fehrenbach, Uwe Pelzer, Igor Maximillian Sauer, Johann Pratschke, Christian Jürgensen, Thomas Malinka declare no conflict of interest or financial ties related to the study.

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3 DISCUSSION

With the continuous aging of the population and overall demographic changes in Western countries, there is a perceivable and quite evident increase of patients diagnosed with PDAC.[2] Further improvements on diagnostic modalities and optimized surgical processes have enabled safe and curative concepts for many patients at early stage diagnosis.[129-131] Recent developments on neoadjuvant treatments further pushed the boundaries pro resectability such that survival benefits likely range even higher than currently estimated.[3] Still, in the setting of margin negative resection with complete micro- and macroscopic tumor removal, the majority of patients eventually relapse, likely due to not identified microscopic carcinoma cells, micro-metastases and ineffective adjuvant therapies.[18,19] In addition, the majority of patients are diagnosed at disseminated stages, so curative concepts are no longer possible. Although more effective systemic therapies are available, the individuals' survival benefit in the palliative setting remains limited unless rare molecular, actionable targets can be detected.[40,44,132] Realistic short-/mid-term chances for survival improvements of affected PDAC patients with local disease involve, (i) effective pre-operative treatment with down-staging of the primary tumor site and preventing micro-metastasis, (ii) peri-operative advances through technical refinements on pancreatic surgical procedures could limit hazardous complications which regularly impact the individuals amenability to adjuvant treatments as well as (iii) finding novel post-operative combination or targeted therapies that can reduce drug resistance and treatment failure. These clinical demands for optimized multimodal oncologic therapy require a professional interdisciplinary infrastructure with centralization at high-volume academic pancreatic cancer centers.[49] As such, our department has greatly focused on improving the peri-operative measures (ii) that falls to our hands as academic surgeons. We offer modern minimal-invasive including robotic surgery as well as effective interdisciplinary concepts for complication management and within this framework we usually enable early adjuvant treatment for the majority of our patients.[133,134] In addition, we have explored peri-operative factors that impacted and define treatment success and failure at our institution, as we continuously seek for solutions to improve the clinical outcome of our patients.[48,135,136]

Pancreatic surgical procedures remain one of the most complex and technically demanding abdominal interventions. Therefore, clinical research in the surgical field greatly focuses on minimal-invasive procedures that could reduce complications and

the overall treatment burden. Indeed, advantages lie in the faster recuperation with a reduced length of hospital stay, reduced wound infections and margin positivity.[137] Laparoscopic as well as robotic procedures, are innovative and effective minimal-invasive modalities for pancreatic intervention and tumor resection.[138,139] Even in the setting of complex pancreatoduodenectomy with multi-step gastrointestinal reconstruction, robotic procedures are increasingly adopted, proving a safe and cost-effective alternative (**Figure 3**).[140,141]

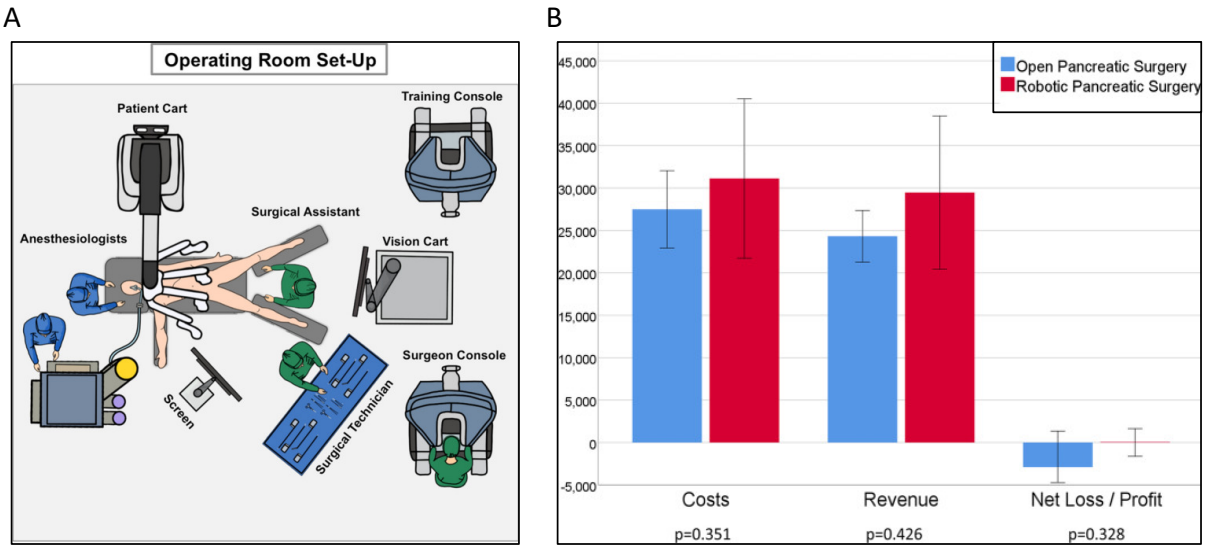

Figure 3. A. Set-up of the robotic device and surgical team during a pancreatic procedure in the operating room. Extracted from own manuscript under 3.4. B. Similar to other studies, we could demonstrate cost-effectiveness during the implementation phase of our procedure. Figure modified from *Benzing C et al.*[141]

However, a bottleneck remains the sometimes extensive learning curves needed for the surgeon's training until equilibrated outcome measures can be achieved.[142] Some studies reported increased mortality and complication rates during the implementation phase, an unacceptable side-effect from the individuals perspective.[143] Therefore, significant care and rigorous measures remain paramount, for implementation of novel technologies into the surgical field. In the conducted study **under 3.4.**, we were able to present our optimized and structured operating procedures from extensive institutional experience in both robotic pancreaticoduodenectomy as well as distal pancreatectomy.[133,144] The study defines a structured step-by-step manual based on evidence of rapidly improving learning curves during the adoption phase at our institution. The surgeons spatial perspective within the abdominal cavity changes drastically and therefore required various technical adoptions for safe performance of pancreatoduodenectomies or distal pancreatectomies. Such practice guidelines may support other pancreatic cancer programs in conducting safe and efficient robotic

pancreatic surgery during their learning curves. This will become ever more important, as industrial marketing and the general popularity of such technologies increases the widespread and clinical demands worldwide.

Another major technical obstacle that significantly impacted the outcome of treated PDAC patients is imposed by surgical complications.[48] Abdominal fluid collections and postoperative pancreatic fistula (POPF) are frequent side effects after complex pancreatic surgery in both, open or minimal-invasive procedures.[145] Such problems regularly occur due to anastomotic leakage (**Figure 4**). It has been identified the Achilles' heel after complex pancreato-enteric reconstructions, but is required for complete removal of pancreatic head tumors. As such, they regularly result in increased morbidity and mortality with prolonged hospitalization and delayed recovalescence.[146] Based on vast interdisciplinary experience for complication management at our institution, we have developed efficient interventional tools for drainage of abdominal fluid collections.[147] In **3.5.**, we have implemented and investigated a novel modality for endoscopic treatment (ED) of peri-anastomotic fluid collection after pancreatogastrostomy. In such setting, the anastomotic dehiscence can be endoscopically visualized and pigtail stents can be placed through guidewires for internal drainage. The conceptional idea for such modalities is to achieve earlier recovery, reduced hospitalization and improved quality of life, as prolonged irrigation therapy using conventional percutaneous drainages (PD) requires professional handling and in-patient observation. Previous work has shown such tendencies after distal pancreatectomies with mean difference of -3.84 days (95% CI − 6.12 to − 1.55; P < 0.01) across studies in a larger meta-analysis.[148] However, only few studies reported experiences on transmural approaches following pancreatoduodenectomies. We have identified two meaningful studies, that included a limited number of patients (<20) and demonstrated similar clinical success rates of ED compared to PD after pancreatoduodenectomy.[149,150]

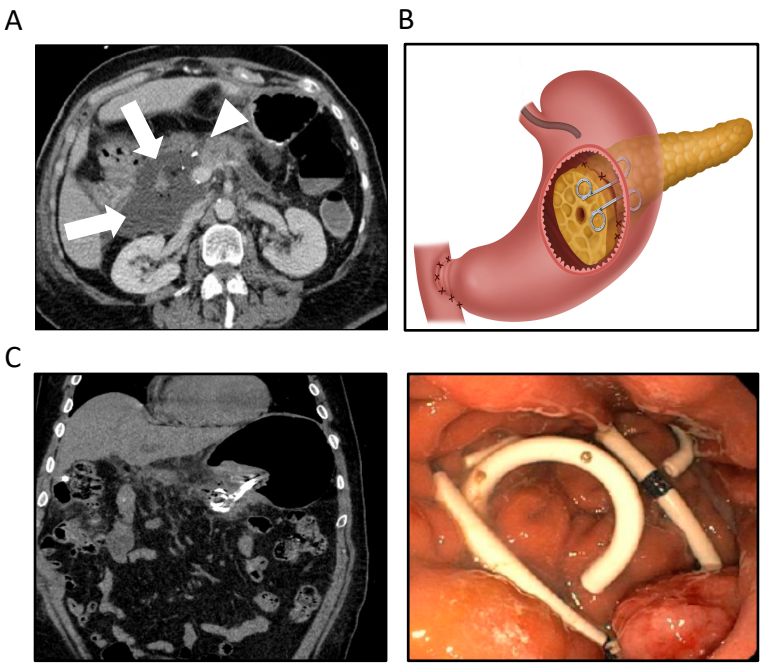

Figure 4. A. Computed tomography (CT) following pancreatoduodenectomy. Larger fluid collection (arrow) at pancreato-enteric anastomosis (arrow head) strongly suggests anastomotic leakage. Figure modified from *Planz V et al.*[151] B. Illustration depicts endoscopic placement of two peri-anastomotic pigtail stents. C. Peri-anastomotic pigtail stents in-situ during radiographic (left) and endoscopic (right) follow-up. Figures B&C extracted from own manuscript under 3.5..

In our study, we were able to demonstrate efficient treatment of one-third of our patients suffering from anastomotic leakage as either primary, secondary and/or tertiary treatment modality. We could discriminate superior primary- (77.8% in ED vs 53.7% in PD) and secondary success rates (85.7% in ED vs 68.4% in PD) and overall earlier resolution of peri-anastomotic fluid collection when compared to percutaneous management. This work has majorly contributed to expanding the interdisciplinary toolkit for this treatment-specific complication and now offers a versatile and safe adjunct to conventional percutaneous techniques, ultimately aiming at earlier admission of oncologic patients to adjuvant treatments.

As a clinician scientist, current challenges in the peri-operative management (ii) of PDAC patients were academically addressed, which culminated in evidence-based conclusions and technical advancements that will likely have a positive impact on the outcome of our patients.

Technical developments in high-throughput analyses of recent decade(s) greatly illuminated the molecular trajectories of malignant diseases, including PDAC. This has become ever more important in the discovery of novel paths for earlier detection and the development of effective targeted therapies. For instance, the tremendous success of a biomarker-driven therapy in treating malignant melanoma and leukemia has driven

and initiated the current progress in precision medicine across many cancer entities.[152,153] However, when compared to other gastrointestinal counterparts (gastric, colorectal, esophageal cancers), PDAC lags significantly behind in this ongoing endeavor.[154] The reasons are manifold but partly lie in undruggable driver genes along with extensive heterogeneity of low-abundance gene alterations, the complex interplay of carcinoma cells with the stroma and the "cold" immunosuppressive tumor immune microenvironment (TIME).[155] Despite extensive efforts and progress in the molecular trajectory of the disease and apart from few examples, the majority of targeted therapies fail to improve the survival of PDAC patients at this point in time.[155] In addition, it must be acknowledged that major breakthroughs for improved survival of patients with gastrointestinal malignancies, such as colorectal or gastric cancers, were primarily due to the development of cost-effective diagnostic modalities for early detection (endoscopy). On the other hand, without the molecular and histopathologic understanding of the carcinogenic process undergoing precursor metaplasia (adenoma, ulcers), concepts for earlier detection would not have arisen.[156] For some time now, a similar process has been extrapolated for pancreatic carcinoma.[81] Different grades of pancreatic precursor lesions (PanIN, IPMN, MCN) align with molecular alterations in driver genes characterizing a modified "adenoma-to-carcinoma" sequence (**Figure 5**).

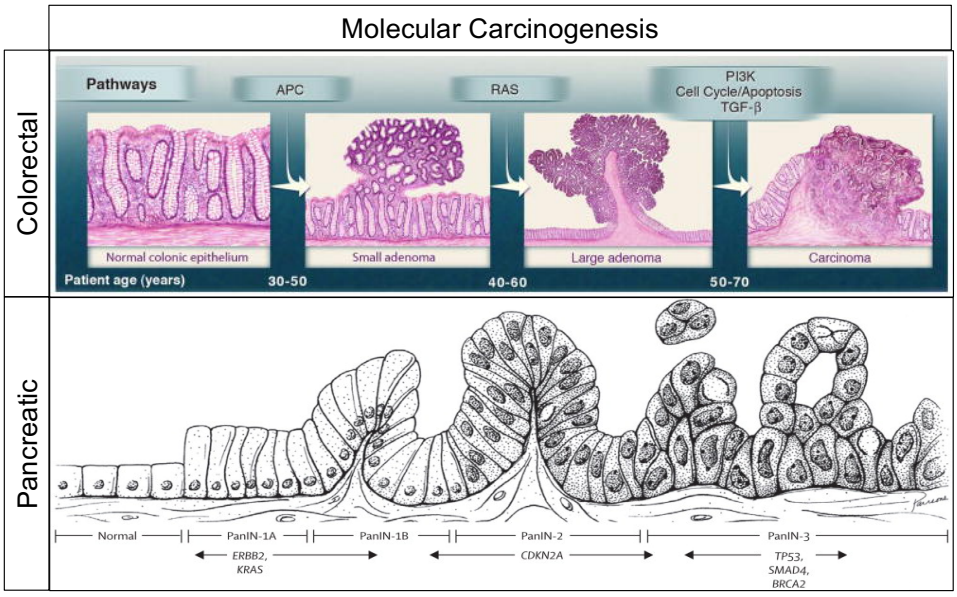

Figure 5. Based on molecular and histopathological information of pancreatic cancer precursor lesions, a sequential "*adenoma-to-carcinoma*" model has been characterized, which closely resembles the "*Vogelgram*" described for colorectal carcinogenesis. Figures modified from *Vogelstein B et al.* and *Hruban RH et al.* [81,157]

Unfortunately, to a large extent, this process remains microscopic. Removal of pancreatic precursors also entails technically demanding and complicated pancreatic surgery. We currently monitor macroscopic lesions until they have already started to invade (worrisome features; high-risk stigmata).[25] In view of the dismal prognosis once invasive, this represents an overall dissatisfying situation. Only recently, translational and clinical attempts began to include the molecular information of DNA shed into secreted fluids of the pancreas or blood stream and harbor the potential to discern high-grade from low-grade neoplasia.[116,119,123,124] Other larger scale screening approaches include the genomic germline information to detect high-risk individuals for developing pancreatic malignoma.[112] Either of these diagnostic concepts will most likely yield effective modalities for earlier detection soon.

In conjunction with this ongoing progress, our studies provided significant insight into the molecular trajectory from precursor to invasive carcinoma. For instance, in **3.1.** we were able to demonstrate that the co-occurrence of cystic IPMNs with invasive carcinoma does not always infer that the carcinoma is derived from its neighboring precursor. Following Laser Capture Microdissection (LCM), where we separated the cyst epithelium from the invasive areas, we subsequently performed Next Generation Sequencing (NGS) and molecularly analyzed their relationship based on strategic bioinformatical likelihood calculations. At least one-sixth of PDACs did not share any driver gene alterations present in the IPMN, which defined them as (molecularly) unrelated. Multifocal neoplasia in the pancreas has been observed previously and a so called molecular "*field defect*" of the pre-conditioned pancreatic duct system has helped to explain such phenomena.[64,158] However, it has long been assumed that all PDACs co-occurring with IPMNs, must directly derive from their neighboring cystic precursors. But distinct kinds of precursors seem to co-occur (PanIN, IPMN, MCN), which all harbor the potential for incremental dysplasia and invasion. These results have also significant clinical implications, as this situation likely renders the endoscopic sampling of cyst fluid for diagnostic molecular purposes much more complex than precedented. Nonetheless, such insights may help us to disentangle the underpinnings of clonal heterogeneity during pancreatic carcinogenesis.

Follow-up studies from the research group further characterized the remarkable heterogeneity in cystic precursor lesions.[67,87] These results were also confirmed at single-cell level.[88] To elaborate on the neoplastic progression of earlier-stage cystic precursors and their relationship among neighboring precursors, we performed multi-

regional exome sequencing on 76 Low- and high-grade lesions from total of 17 patients (in **3.4.)**. We have uniquely discovered a novel driver gene (*KLF4*) with high abundance (>50%) and enrichment in low-grade lesions. The data provided further evidence of diverse phylogenetic patterns in the progression from low-to-high grade lesions. Using a computational analytic tool (Treeomics), we were unable to identify a universal molecular pattern for progression from precursor to carcinoma across patients. Apart of in-depth tissue sampling, molecular analysis of cyst fluid has helped us to assess its potential power for risk stratification, in order to discriminate low- from high-grade lesions. The results of our study need to be interpreted with some caution, as only a limited number (17 patients) as well as a small proportion of the patients‘ IPMN were analyzed. However, the strategy of multi-regional sampling of large number of well-characterized cystic precursors enables us to uniquely define stratified genomic signatures in low- versus high grade IPMN lesions.

Together with other recently published data, it becomes increasingly evident, that PDAC evolves from heterogenic molecular clusters in precursor lesions, with subsequent convergent clonal evolution of cells undergoing additional dysplasia which develops into invasive carcinoma (**Figure 6**).[159] The molecular and cellular characteristics that confer improved clonal fitness, towards invasion in specific, remain poorly understood and appear to be rooted beyond recurrent driver gene alterations.

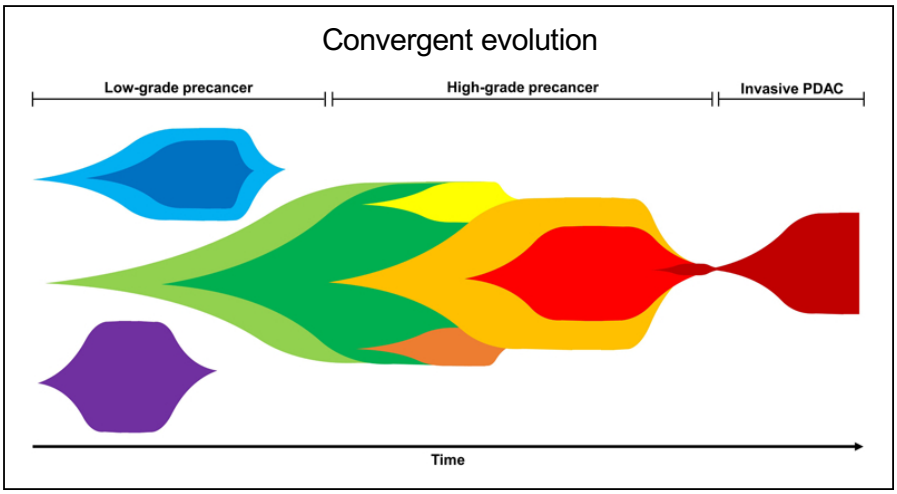

Figure 6. Remarkable heterogeneity of subclones in co-occurring low-grade precursors may lead to convergent evolution at later stages with heterogeneity in driver genes. A dominant clone ultimately leads to development of full-blown carcinoma. Figure modified from *Fischer CG et al*.[67]

For better dynamic understanding of evolutionary concepts and investigation of molecular phenotypes, versatile models for standardized downstream analyses would be helpful. Hitherto, *ex vivo* animal or cell culture models were engineered to effectively

mimic the disease process.[93,107] However, the ultimate translational value in terms of testing and developing effective agents for clinical practice, have remained scarce. Focusing on the precursor cell biology, we have explored concepts to harness modern genome engineering technologies (CRISPR/Cas9) in order to investigate pancreatic carcinogenesis (FE1747/1-1). To date, only a single normal pancreatic ductal cell line ($HPDE^{E6E7}$) and a compendium of carcinoma cell lines are available. Despite extensive technical efforts, the development of monoclonal precursor cell lines with defined genomic alterations in driver genes are still under investigation by our group. In turn, we successfully derived a cystic precursor cell line from primary tissue employing three-dimensional organoid culture (in **3.5.**). During initial stages, a living biobank for cystic precursor lesions has been composed that was subsequently characterized on a genetic and transcriptional level.[160] We then transferred the precursor cells (H58_ITPN) derived from an ITPN (Intraductal tubulo-papillary neoplasm) patient into two-dimensional tissue culture and thoroughly characterized its tumorigenic features. The technical novelty lies in successful derivation and survival of non-carcinogenic precursor cells using protocols for three-dimensional organoid culture and their careful transfer into two-dimensional tissue culture (**Figure 7**).

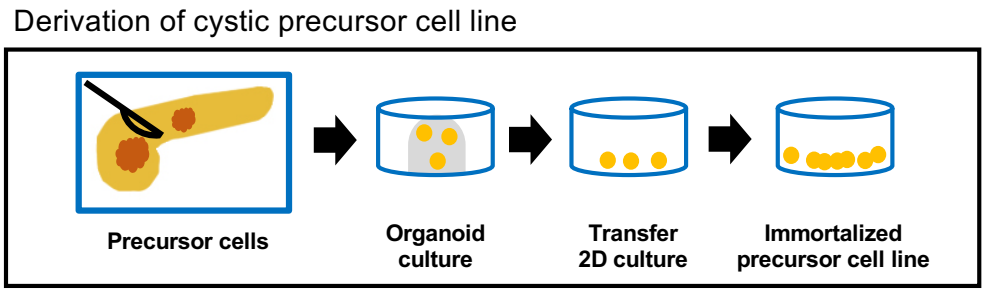

Figure 7. Primary tissue from cystic precursors (IPMN, MCN) can be derived through stepwise, gradual transfer from three- to two-dimensional tissue culture, enabling long-term culture of non-invasive precursor cells.

Characterization of the novel cell line revealed phenotypic properties of normal- or other precursor cell lines, with no tumor formation *in vivo* as well as low tumorigenicity in standardized *in vitro* assays. Genomic and transcriptomic analyses elucidated unique signatures devoid of somatic alterations in commonly mutated pancreatic driver genes. However, we were able to report a range of somatic mutations in other genes involved in PI3K-, DNA damage- and Wnt signaling pathways, as a distinctive feature of ITPN pathobiology. Based on genomic alteration in genes involved in DNA damage response (HDR phenotype), we were also able to provide functional data on treatment response with PARP1 (Olaparib) and a tankyrase inhibitor (G007-LK). We identified

sensitivities towards these agents indicating its usefulness to study DNA repair defects in a translational approach. Despite the overall rarity of this precursor entity, the cell line could provide a broader insight on evolutional mechanisms of cystic precursor biology and carcinogenesis.

Pancreatic carcinoma is a deadly disease with complex genomic alterations accumulating in variant phylogenic arrangements along the entire disease process. This is initiated by clonal ancestors towards invasive cell colonies with aggressive or even dislocating behavior towards metastatic resettlements. The discoveries generated by in-depth multi-omic data (genomics, transcriptomics, proteomics) dismantled the complex perturbation of interwoven intracellular signaling pathways, that are likely responsible for iterative resistance towards single agent therapy. However, similar larger scale molecular characterization of patients with precursor lesions, may ensure some chances for the development of effective clinical tools for early detection. My work has significantly contributed to the compile of data on genomic signatures of precursor lesions. Subsequent work, on clinical datasets of patients undergoing pancreatic surgery has helped to standardize innovative technologies and improving on postoperative complication management that could more immediately improve the clinical outcome of our patients. The enormous anthology of basic research-, translational- and clinical efforts demonstrate the unfaltering commitment of researchers to steer many more patients into curative stages. This triad together with multi-disciplinary initiatives can make such endeavors conceivable in near future.

4 FUTURE DIRECTIONS

Future work will ensure progress on the investigation of the molecular principles of pancreatic carcinogenesis as well as optimization of peri-operative variables, all directed towards curative PDAC therapy, which remains the bottleneck of mid-/long-term disease morbidity and mortality. The triad experiences with basic, translational and clinical research remain the building blocks upon which novel interdisciplinary principles and innovative paths for earlier detection will be explored. This I believe, remains the cornerstone for improving the clinical outcomes of PDAC patients. Multiple studies are underway to further our understanding of molecular pancreatic carcinogenesis. Together with my research group (*Molecular Carcinogenesis Lab, https://www.experimental-surgery.de/Projects/molecular_carcinogenesis/*), we have

established two-dimensional and three-dimensional tissue cultures of primary duct and carcinoma cells, and followed-up on gene editing techniques for gene modification in pancreatic driver genes and subsequent derivation of novel culture models. Our group has also optimized ways on derivation of single-cell (-nuclei) data from fresh human pancreatic precursor tissue, which should uncover transcriptomic signatures that may drive pancreatic precursor cells into invasive transformation. Collaboratively we also screen for more complex chromosomal aberrations and extrachromosomal DNA (ecDNA) in PDAC samples with enriched neoplastic content (organoids) using short- and long-read sequencing techniques. Such studies are aimed at exploring putative mechanisms of therapy resistance. In clinical studies, we are investigating technical modalities to improve interdisciplinary complication management after pancreatic surgery. Altogether, ongoing efforts focus on contributing to the corpus of knowledge and experience that can support diagnostic tools for early detection and improve the therapy of patients suffering from pancreatic neoplasia.

5 SUMMARY

With rising incidence and prevalence PDAC are soon expected to become the second leading cause of cancer related deaths. At time of diagnosis, few curative therapeutic options are available due to systemic dissemination. Surgery in combination with (radio-)chemotherapy for patients with primary resectable or borderline-resectable tumors offer a minimal chance of cure. Recurrence rates are high and overall survival rates are poor. For this reason, research efforts must focus on early detection of precancerous lesions or early stage carcinoma. PDAC develops through a complement of genetic alterations already present in pancreatic carcinoma precursor lesions. These precursors are histologically characterized by the following types of dysplasia: Pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasm (IPMN) and mucinous cystic neoplasm (MCN). As these precursor lesions progress, increasing grades of dysplasia are associated with the accumulation of somatic mutations in the key driver genes *KRAS*, *CDKN2A*, *TP53*, *SMAD4*, *GNAS* and *RNF43*. Complex molecular disruption of core signaling pathways ultimately leads to cancer progression and metastasis. Apart from single nucleotide variants (SNVs), other complex chromosomal aberrations during pancreatic carcinogenesis occur and are likely responsible for the aggressive nature and rapid development of PDAC.

Cooperative efforts to categorize PDAC patient samples, both functionally and clinically, based on expression signatures, uncovered the fact that PDAC samples essentially follow two distinct transcriptional programs, characterized as basal (squamous) or classical. Other sub-classifications have been applied, while their exact clinical relevance still needs to be evaluated. However, we still lack profound understanding of the evolutionary concepts which ultimately drive dysplastic precursor cells towards invasive carcinoma development. There is a real need to identify and define cells with pre-invasive hallmarks and eventually be able to detect them in patient samples which could guide us towards earlier detection as well as adopting paths for modern precision medicine for a molecularly complex disease.

In my research, we delineated the molecular distinction and phylogenetic relationships of cystic precursor lesions that co-occur with invasive carcinoma. The results have fueled the concept of a pre-existent "*field defects*", possibly found in a subset of affected individuals. Building up on published data, subsequent studies were able to characterize the extensive heterogeneity present in IPMN precursors, defining molecular signatures that drive low-grade IPMNs into higher-grade lesions and ultimately establishing molecular proof that PDACs directly originate from high-grade IPMNs.[87] A process that likely takes around 3-4 years. Technical progress in analyzing liquid biopsies, such advances will aid in the multi-disciplinary venture to develop tools for early detection. However, we have to acknowledge that only a minor fraction of PDACs derive from macroscopic cysts. At least similar effort needs to focus on the precursor biology of PanINs. These lesions are barely detectable in patient samples which renders any progress difficult. Molecularly engineered *ex vivo* models will assist in their understanding and aid in a thorough investigation of pancreatic carcinogenesis. In view of this, we have provided a valuable *in vitro* model for ITPN pathogenesis and we are currently working on models to develop ductal precursor cell lines that will harbor main pancreatic driver genes. Downstream single-cell analyses in novel three dimensional models will aid in the complex interplay between the tumor cells, stromal and immune cells.

Apart from such visionary endeavors, we should focus on the optimization of patient care. Academic centers specifically underlie the predicament of evidence-based decision making while offering individualized therapy concepts to oncologic patients. Close monitoring and documentation of peri-operative variables therefore remains a fundamental task for clinician scientists to facilitate developing, adopting and

optimizing therapeutic modalities. In the multi-disciplinary context and as a surgeon in a high-volume pancreatic cancer center, primarily the peri-operative variables (ii) are directly modifiable factors to be addressed. The work presented here has focused on technical refinements via optimization of innovative robotic surgery as well as improving on interdisciplinary concepts for efficient complication management. We are aiming at offering the best individual treatment decisions while sharing our experiences with the entire clinical research community and reporting on our evidence-based results on a regular basis.

This work highlights the ongoing route in offering many more PDAC patients curatively intended therapies while improving the safety and efficiency of such treatments. In order to improve survival from this aggressive disease, we highlight the necessity to cooperate and think in multi-disciplinary concepts as only joint inter-disciplinary efforts may culminate in effective treatments for patients with pancreatic neoplasia. On the basis of this work, I mean to motivate clinicians including academic and oncologic surgeons, to overall deepening the biologic understanding of PDAC in the clinical routine as many translational solutions are inevitably rooted at the molecular level.

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8 ERKLÄRUNG

Hiermit erkläre ich, dass

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Berlin, 05.05.2023

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