Peters *et al. J Cancer Metastasis Treat* 2021;7:32
DOI: 10.20517/2394-4722.2021.67

Journal of Cancer Metastasis and Treatment

Review

Open Access

Clinical implications of the molecular characterization of intraductal papillary mucinous neoplasms of the pancreas

Nicholas V. Peters, John W. Kunstman

Department of Surgery, Yale University School of Medicine, New Haven 06510, CT, USA.

Correspondence to: Prof. John W Kunstman, Department of Surgery, Division of Surgical Oncology, Yale University School of Medicine, 310 Cedar Street, FMB 130, New Haven 06510, CT, USA. E-mail: John.Kunstman@yale.edu

How to cite this article: Peters NV, Kunstman JW. Clinical implications of the molecular characterization of intraductal papillary mucinous neoplasms of the pancreas. *J Cancer Metastasis Treat* 2021;7:32. https://dx.doi.org/10.20517/2394-4722.2021.67

Received: 17 Mar 2021 **First Decision:** 21 Apr 2020 **Revised:** 5 May 2020 **Accepted:** 12 May 2021 **Published:** 11 Jun 2021

Academic Editor: Lucio Miele **Copy Editor:** Yue-Yue Zhang **Production Editor:** Yue-Yue Zhang

Abstract

Intraductal papillary mucinous neoplasm (IPMN) is a pre-malignant, mucin-producing epithelial lesion arising from pancreatic ducts. Observational reports define IPMN behavior as ranging from indolent, asymptomatic lesions to dysplasia that sometimes degenerate into pancreatic adenocarcinoma. The goal of IPMN management is risk-reducing surgery for high-risk cysts and observation of the remainder. Discriminating high- from low-risk IPMN disease still relies on imaging and clinical cyst characteristics. Here, we review the accepted classification of IPMN including the most common histological subtypes, their clinical features, and currently-accepted high-risk phenotypes. We then deeply examine the known molecular landscape of IPMN, which has largely been derived from post-resection analysis. This includes those gene variants unique to IPMN, chiefly GNAS and RNF43, but also examines the overlap between IPMN and conventional pancreatic adenocarcinoma. Utilizing molecular markers in the clinical setting relies on endoscopically-obtained cyst fluid and presumes that it accurately represents the molecular characteristics of the cystic epithelium. We synthesize existing data on mutational analysis from IPMN cyst fluid and consider the benefits and proper role of current commercially-available cyst fluid molecular analysis kits. We conclude that carefully interpreted molecular analysis of resected IPMN tissue reveals insights into its biology and natural history while cyst fluid analysis offers prognostication and data to guide treatment decisions. However, knowledge gaps remain, especially in characterizing IPMN molecular heterogeneity, time to progression, and correlating cyst fluid genotype data with surveillance strategies. As such, substantial additional research is required before the promise of true molecular guidance of IPMN management can be realized.

Keywords: Intraductal papillary mucinous neoplasm, pancreatic cancer, molecular analysis, GNAS, KRAS, RNF43

INTRODUCTION

Intraductal papillary mucinous neoplasm (IPMN) is defined as a grossly visible, mucin-producing epithelial neoplasm arising from pancreatic ducts[1,2]. Although long recognized as a pathologic entity under various terms such as mucinous duct ectasia, mucin hypersecreting tumor, or intraductal papillary mucinous tumors, IPMN was first codified as a unique entity by the World Health Organization in 1996[3]. Following adoption of this standard definition, knowledge of IPMN including its characteristics and natural history has greatly expanded. Initial efforts focused on describing the histopathologic features of IPMN and the prevalence of associated invasive pancreatic adenocarcinoma. Determining the proper timing for surveillance of IPMNs and surgical intervention remain ongoing challenges. Recently, improving tools for genomic analysis has allowed a much deeper understanding of the underlying biology of these pre-malignant lesions. However, the molecular origin of IPMN and its implication for IPMN behavior remain poorly understood.

Postmortem analysis demonstrates that cystic masses in the pancreas are exceedingly common and strongly correlate with age[4]. Due to the increased use, availability, and quality of cross-sectional diagnostic imaging, more pancreatic cystic lesions are being diagnosed in patients with unclear clinical relevance[5-7]. In patients without a history of pancreatitis, IPMN is by far the most common incidentally-diagnosed cystic lesion of the pancreas. IPMN is, by definition, a premalignant lesion, in which pancreatic adenocarcinoma can arise within these cysts. However, the majority of IPMNs do not progress to invasive disease or even high-grade dysplasia; thus, there are no screening recommendations for detection of IPMN in standard risk individuals[8,9]. As most IPMNs are incidentally discovered, the vast majority are asymptomatic at the time of diagnosis. In such patients, recommendations are individualized and can involve several modalities, including imaging, endoscopy, and rarely, immediate surgery. Molecular analysis of IPMN can occur following surgical resection or via endoscopic sampling. Surgical sampling is advantageous as cyst tissue is readily available and can be directly examined; however, this requires resection. In the non-operative setting, endoscopic ultrasound (EUS) can be used to locate the pancreatic lesion and fine needle aspiration (FNA) can obtain cyst endothelial tissue in select cases. However, EUS-guided aspiration is most commonly used to obtain cyst fluid. Biochemical analysis of aspirated pancreatic cyst fluid has long been utilized to discriminate mucinous *vs.* serous lesions[10] but modern tools have allowed molecular assays as well. While cyst fluid of many IPMNs is aspirated, as discussed below, the resulting personalized molecular data infrequently alter treatment plans in the current era. This is in part because of an incomplete understanding of IPMN biology and malignant potential[5,11,12], as well as limited molecular analysis tools and their associated costs. The role of molecular analysis in IPMN management is an area of ongoing investigation and continues to rapidly evolve. This manuscript will review the progress in defining IPMN as a clinical entity, the current molecular understanding of the disease, and available means to assess these findings and their clinical utility in patients with IPMN.

OVERVIEW OF IPMN MANAGEMENT

Current guidelines for management are based on three decades of accrued data on the correlation of clinical and radiographic features with IPMN natural history. The goal of IPMN management is to prevent progression to overt pancreatic adenocarcinoma while avoiding unnecessary surgery or overly burdensome surveillance. Recent technological advances allowing rapid genetic characterization of cyst fluid have been utilized for research purposes and are beginning to be used in the clinical setting as well. Commercial assays for cyst fluid genetic analysis are now in widespread use[13-17] although evidence supporting routine

employment is lacking. Despite these advances, management principles remain focused on cross-sectional imaging and endoscopic cyst characteristics. While many groups have offered guidelines, the most widely accepted international consensus recommendations can be summarized [Figure 1][18-20]. In addition, areas where input from molecular analysis may be particularly useful have been indicated.

These strategies have greatly clarified and improved the management of IPMN, but some patients still develop late-stage pancreatic adenocarcinoma despite careful surveillance. Moreover, 60%-80% of patients submitted for resection lack high-grade dysplasia or early invasive disease[21], implying surgery may not have been necessary at that time. Finally, most IPMNs seem to convey a field effect on the pancreas with multifocal disease and recurrence being the norm. Thus, even patients undergoing successful surgery still require ongoing observation. These features greatly complicate IPMN treatment and surveillance. It is hoped that better molecular understanding of IPMN will clarify these questions and guide improved management strategies in the future. This work reviews the molecular characterization of IPMN, mutational information from cyst fluid and resection specimen analysis, and the clinical implications of IPMN in the context of histologic subtype. Furthermore, it discusses commercially available genetic analysis kits and their utility and niche in clinical decision-making.

CONVENTIONAL CLASSIFICATION OF IPMN

Several classification schemes have been used to describe IPMN: anatomic examination, histology, spectrum of dysplasia and others. Together these classification systems are used in clinical guidelines and are commonly reported in the IPMN literature but can be incongruent. As a result, correlating these classifications with natural history, prognosis, and malignant potential of IPMN is challenging and inconsistent[15,22-24]. Molecular designations are increasingly being recognized but are yet to be comprehensively integrated into classifying IPMN. Importantly, recent studies have consistently found IPMN to be a heterogeneous lesion, with multiple geographically and genetically distinct regions residing within a single cyst or group of cysts. It seems likely that this contributes to the inconsistencies earlier studies observed between IPMN classification and behavior[25,26].

Anatomic classification - main duct, branch duct, or mixed

Macroscopic examination is the basis for classifying IPMN as a main duct (MD), branch duct (BD), or mixed lesion[5]. This has been consistently recognized as an important clinical factor from the earliest guidelines[27] to the present day[19,20]. In resected IPMN specimens, early studies reported a higher risk of malignancy among main duct lesions (31%-70%) as opposed to branch-duct lesions (3%-25%)[5,11,12,28]. Risks in mixed IPMN are generally considered analogous to main duct IPMN[29,30]. Sub-group analysis of BD-IPMN has greatly informed current management strategies[31-33] with significantly increased rates of invasive adenocarcinoma found in BD-IPMNs with (1) cyst size greater than 3 cm; (2) presence of a mural nodule; and (3) associated dilation of the main pancreatic duct (i.e., mixed IPMN)[31].

As noted above, resection is advocated for MD-IPMN in acceptable surgical candidates[18,19,34], which sometimes entails total pancreatectomy. Management of BD-IPMN remains more nuanced. The previously described "high risk stigmata" and "worrisome features" based on imaging and endoscopic findings drive decision-making in these patients[20]. The Pancreatic Surgery Consortium clarified the relative risks of these possible IPMN features in 2018, with the presence of jaundice most predictive of high-risk (i.e., high-grade dysplasia or invasive) lesions (57/58), while cyst size > 3.0 cm, mural nodule, pain symptoms, and weight loss were also associated with high-risk lesions to a lesser degree[35]. Another study reported the presence of a radiographic mural nodule was the most predictive feature of invasive disease[36].

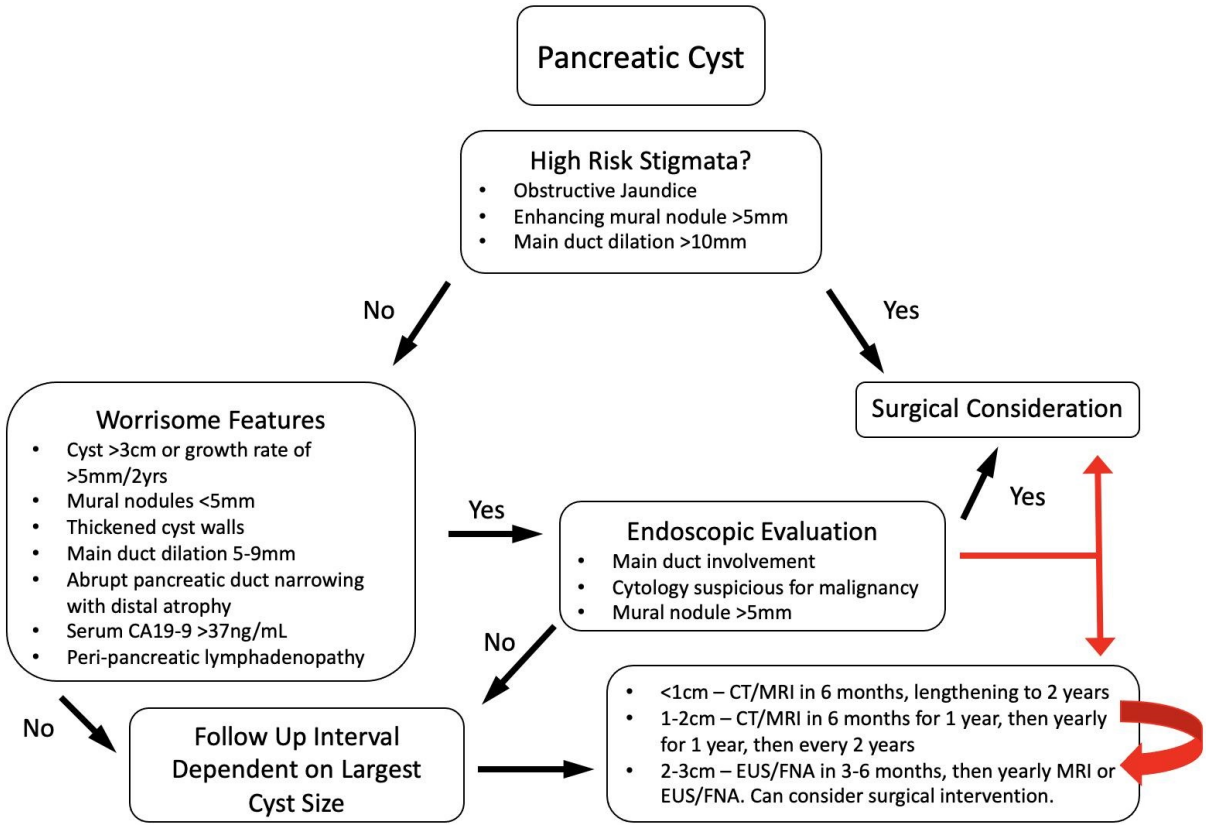

Figure 1. Summary of clinical management of pancreatic cysts. Adapted from Tanaka *et al.*[20] *Pancreatology* 2017. Current clinical management guidelines for pancreatic cystic lesions summarized in a flow chart. Red arrows denote specific areas in the flow chart where novel molecular data may be able to assist in clinical decision making.

Histology and dysplasia distinction

Histological analysis of resected IPMN has defined four primary distinct subtypes: gastric, intestinal, pancreatobiliary, and oncocytic[37-42]. The appearance of the epithelial cyst lining on microscopy and differential mucin protein expression profiles form the basis of histologic subtype differentiation[22,37,43] [Table 1]. Although conventional pathology reporting often conveys a ubiquitous histologic subtype to a particular IPMN, multiregional examination has revealed that multiple histologic subtypes can exist within a single lesion[25,38]. As described below, this can have molecular implications as well. Additionally, some have suggested the oncocytic subtype is a distinct entity from conventional IPMN[44,45].

A substantial number of studies have attempted to risk-stratify IPMN lesions by their histological subtype[46-51]. A systematic review by Koh *et al.*[52] reviewed 14 studies with 1617 unique patients with IPMN that consisted of 900 IPMNs with noninvasive features and 717 with invasive disease. They found that pancreatobiliary subtype IPMN has the highest likelihood of (1) an invasive component (67.9%); (2) presence of a mural nodule (56.6%); and (3) tumor recurrence (46.3%). Conversely, gastric-subtype IPMN had the lowest likelihood of an invasive component (10.2%) or tumor recurrence (9.4%).

The most important driver of patient survivorship with regards to IPMN is the degree of dysplasia[53]. Conventionally, IPMN is understood as a premalignant lesion that progresses from low-grade to high-grade dysplasia to invasive pancreatic carcinoma[25,54]. Historically, dysplasia was classified as low-, moderate-, or high-grade. However, international consensus has now simplified this to low- or high-grade dysplasia

Table 1. Immunohistochemical staining profiles of IPMN subtypes

IPMN subtype	(+) Staining	(-) Staining	Ref.
Gastric	MUC5AC MUC6 PDX1	MUC1 MUC2	[37,43,46,134,144]
Intestinal	MUC2 MUC5AC CDX2 PDX1	MUC1	[22,37,42,43,46,134,144]
Pancreatobiliary	MUC1 MUC5A/C MUC6	MUC2 CDX2	[22,37,42,43,46,134]

The staining patterns of IPMN stubtypes are shown here. The classically defined oncocytic IPMN is excluded from this table because of its rarity and recent sequencing and outcome data suggests that it may be distinct from IPMN. IPMN: Intraductal papillary mucinous neoplasm.

only[55,56], with most lesions previously characterized as moderate relegated to the low-grade dysplasia group. Those patients with invasive IPMN experience a pronounced decrement in overall survival[53]. While the overall 5-year survival of resected IPMN-associated adenocarcinoma (42%) is superior to that of pancreatic adenocarcinoma not associated with IPMN (19%)[57], this is misleading as survival is closely correlated with the invasive histologic subtype seen arising from the parent IPMN. The vast majority are of two subtypes: tubular or colloid carcinoma. A third variant, anaplastic carcinoma is rarely observed. The 5-year survival of IPMN-derived colloid carcinoma is markedly improved compared to tubular carcinoma, 57%-83% *vs.* 24%-37%, respectively[5,57]. The survival for patients with IPMN-derived tubular adenocarcinoma parallels those with conventional pancreatic adenocarcinoma not associated with IPMN[58]. Tubular carcinoma is associated with the pancreatobiliary and gastric IPMN subtypes, whereas colloid carcinoma is associated with the intestinal subtype[50,59]. The pancreatobiliary subtype has the highest incidence of harboring an invasive component and is strongly associated with the more aggressive tubular carcinoma. To investigate these differences in biological behavior several studies have investigated the mutational landscape of IPMN[24,60-63].

MOLECULAR CLASSIFICATION OF INTRADUCTAL PAPILLARY MUCINOUS NEOPLASMS

Molecular analysis is a broad term for evaluating human tissues via analysis of DNA or RNA transcripts, or indirect analysis of their downstream products including proteins and other effector molecules. With regards to IPMN, there is general agreement that molecular analysis potentially holds key insights into the natural course of IPMN including defining disparate subtype behavior and crucial factors leading to malignant degeneration. Conventional pathologic analysis as described above has been critically important in defining IPMN and generating the current clinical guidelines for management and there is substantial enthusiasm that molecular techniques will serve to further these efforts.

There are two major categories of genetic material for molecular analysis: pancreatic cyst fluid and direct tissue analysis. Cyst fluid, which can be obtained via endoscopic modalities, is unique as it can risk-stratify lesions thereby informing surveillance strategies and aiding selection of patients for surgical resection. Direct tissue analysis is more robust, but is often limited to those patients who have undergone surgery. This can inform future risk and clinical decision-making; however, the greatest value of this analysis is better elucidation of the underlying biology of IPMN. Much of our current understanding of IPMN molecular biology comes from direct analysis of resected IPMN.

The landscape of somatic mutations in pancreatic ductal adenocarcinoma (PDAC) has been carefully defined by numerous investigators over the past three decades. It is therefore unsurprising that the typical

variants present in conventional PDAC are regularly observed at varying frequencies in IPMN, given its status as a precursor to PDAC [Table 2]. The hallmark genes most commonly mutated in PDAC, Kirsten rat sarcoma virus (KRAS), tumor protein 53 (TP53), cyclin-dependent kinase inhibitor 2A (CDKN2A), and deleted in pancreatic cancer-4 (DPC4, more commonly SMAD-4), can be all observed in IPMN as well. Initial investigations into the mutational landscape of IPMN sought to characterize these mutations and assess for the presence of recurrent variants private to IPMN to define the substantial differences in behavior between IPMN, IPMN-associated PDAC, and conventional PDAC (not associated with IPMN).

Somatic variants unique to intraductal papillary mucinous neoplasms

The current molecular understanding of IPMN as a unique genetic entity emerged in 2011 with two seminal manuscripts that performed whole exome analysis of multiple cystic lesions of the pancreas, including IPMN[64] and targeted analysis of IPMN cyst fluid[61]. These complementary studies identified the PDAC-related mutations noted above, but also defined variants unique to IPMN. Specifically, frequent recurrent mutations were found in GNAS complex locus (GNAS) and ring finger protein 43 (RNF43) in IPMN with other unique IPMN-specific variants occurring with much less consistency. Subsequent studies have confirmed that mutations in GNAS are the second-most common variant in IPMN after KRAS, being observed in 41%-75% of all IPMN[61,65,66]. This is histology-specific, with > 90%-100% of intestinal-subtype IPMN but only 40%-70% of other histologic subtypes demonstrating mutations in GNAS[61,65]. GNAS mutations are uncommonly reported in conventional PDAC[67] but are present in 33% of resected PDAC cases associated with IPMN[68]. GNAS encodes a subunit of guanine nucleotide-binding protein that, when activated, leads to cell growth and proliferation[69-71]. The dominant recurring mutation found in IPMN is at R201 with R201C/H accounting for most reported variants. Additional R201 or Q227 variants have been reported, but common to all these variants is constitutive activation of GNAS by reducing the rate of GTP hydrolysis[24,67]. While these mutations have been observed in various neoplastic lesions ranging from osseous fibrous dysplasia to growth hormone-secreting adenomas of the pituitary, within the digestive tract they cluster in mucinous and pre-malignant neoplasms, such as pyloric gland adenoma, appendiceal mucinous neoplasms, and IPMN of the pancreas and biliary tree[72]. As such, it appears that GNAS is an early mutation in IPMN tumorigenesis and may synergize with other molecular changes to promote transformation but is unlikely to lead to invasive PDAC in isolation[72]. No GNAS-directed therapies are currently clinically relevant with regards to IPMN or PDAC.

The second-most commonly mutated gene specific to non-invasive IPMN is RNF43, with studies of resected IPMN reporting 14%-75% carrying an RNF43 variant[24,26,61,64,66,73]. RNF43 is a ubiquitin E3 ligase that targets cytosolic frizzled receptors (FZD) for ubiquitination and degradation. FZD is the upstream regulator of both canonical and non-canonical Wnt pathways[74]. Similar to colon cancer, RNF43 inactivating mutations and subsequent loss of heterozygosity reduces FZD ubiquitination and upregulates Wnt signaling thereby promoting tumorigenesis[74]. Unlike GNAS, hotspot mutations are not observed in RNF43 and inactivation can occur via a combination of frameshift indels, missense, and nonsense mutations[66,73]. Moreover, intronic, epigenetic, and post-translational changes also play a role in reducing the RNF43 regulation of the Wnt pathway. This finding was highlighted in one study of 57 resected IPMN lesions where a somatic RNF43 mutation was found in only 14% of cases but decreased RNF43 protein expression was observed in 29.5%[73]. Antibodies for FZD receptors are currently under investigational development and could be potential therapeutic targets in cancers with *RNF43* mutations[75].

Numerous additional somatic mutations at a lower frequency have been reported in sequencing studies of resected IPMN tissue. While their prevalence is too low to be regarded as critical for generic IPMN development, these infrequent mutations may hold insight into sub-populations of IPMN that could become clinically relevant and thus warrant reporting and further investigation. Mutations in *ATM* and

Table 2. Comparison of common somatic mutations in IPMN and conventional PDAC

Gene	IPMN, no/low-grade dysplasia	IPMN, high-grade dysplasia	IPMN-associated PDAC	Conventional PDAC
KRAS	43%-89%[13,66]	32%-71%[13,66]	61%[13,66,76]	92%-100%[67,145,146]
GNAS	40%-90%[13,61,65,66]	42%-72%[13,61,65,66]	33%-61%[13,61,65,66,76]	< 5%[67]
RNF43	10%-11%[66]	25%-75%[66]	18%[66,76]	< 5%[67]
CDKN2A/p16	< 5%[13,66]	0%-16%[13,66]	5%[13,66,76]	82%-98%[147,148]
TP53	< 5%[13,66]	18%-21%[13,66]	21%[13,66,76]	50%-75%[149]
SMAD-4/DPC-4	< 5%[13,66]	< 5%[13,66]	15%[13,66,76]	90%[150,151]

The frequencies of somatic mutations in *IPMN* with progressive levels of dsyplasia, compared with conventional PDAC. IPMN: Intraductal papillary mucinous neoplasm; PDAC: pancreatic ductal adenocarcinoma.

SF3B1 have been reported in 5%-17% in resected IPMN[66,76]. ATM is a cell cycle regulator through modulation of DNA damage repair pathways, while SF3B1 is involved in RNA splicing. RNA splicing has recently been implicated in a crucial mechanism of progression certain cases of PDAC[77] and presents an interesting target for investigation in IPMN. Additional rare mutations occurring in < 4% of IPMN include variants in known oncogenic genes such as *CTNNB1*, *STK11*, and *CDH1*. Interestingly, *CTNNB1* mutation is also highly prevalent in another cystic pancreatic lesion, solid-pseudopapillary neoplasm[64], but this entity is distinct from IPMN. As the unifying molecular mechanisms of IPMN progression are elucidated, further investigation into these rarer aberrations will be critical in incrementally improving clinical management by detecting new therapeutic targets.

Somatic variants common to intraductal papillary mucinous neoplasms and invasive pancreatic cancer

Most commonly observed mutations in IPMN mirror those seen in conventional PDAC. The frequency of these variants in IPMN appears to correlate with their prevalence in PDAC and the degree of dysplasia within an IPMN lesion [Table 2][24]. As such, KRAS variants are the most common somatic mutations found in IPMN, occurring in 50%-80% of lesions[61-63,78]. This correlates to the near-ubiquitous presence of mutated KRAS in invasive PDAC[67,79,80]. Overall, landscape studies of all PDAC cases demonstrate that GNAS is mutated or overexpressed in 6%-11%; presumably, this represents a substantial portion of those cases of PDAC derived from IPMN[67,81,82].

RAS is a monomeric, G-family proto-oncogene involved in regulation of cell proliferation, differentiation, and survival. Of its three isoforms, KRAS is the most frequently mutated gene in human cancer[83,84]. In IPMN, KRAS is also commonly mutated with reports varying from 40%-89%[13,66,76]. Early literature suggested a positive correlation between the frequency of *KRAS* mutation and grade of dysplasia[63,80]. However, larger and more recent sequencing studies examining IPMN heterogeneity demonstrate the rate of *KRAS* mutation may not correlate closely with grade of dysplasia. Intracystic heterogeneity has been a long-recognized but understudied phenomenon in IPMN in that a particular cyst may have multiple regions with varying degrees of dysplasia or histology. This heterogeneity extends to the genetic level and the prevalence of *KRAS* mutations in IPMN makes it the ideal marker to study this phenomenon. Recently, in situ hybridization has been utilized to demonstrate disparate KRAS variants from spatially distinct areas within a single IPMN lesion[25,60,85]. The most common loci for *KRAS* mutations in IPMN are in exon 1 (G12x, G13x, Q61x)[86] and are identical to those reported in conventional PDAC and many other cancers. All of these are activating mutations with *Q61x* mutations conveying a favorable prognosis[67]. Circulating tumor DNA with $KRAS^{G12D}$ mutation is associated with early distant metastasis and poor outcomes in resected PDAC[87]. Despite considerable research, no approved KRAS-targeting therapies exist[88,89] although novel approaches have been recently reported. This includes exosomes impregnated with KRAS targeting

siRNA[90], small molecules specifically targeting $KRAS^{G12C}$ mutations[91], and $KRAS^{G12D}$ knockouts using the CRISPR/Cas-9 system, but none of these have progressed beyond the investigational stage[92].

The tumor suppressor TP53 has also be implicated as a driver mutation in *PDAC*[67,76,93,94]. TP53 is an essential regulator involved in cell growth, protection against mutation accumulation, and suppression of oncogene activation[95]. Sequencing of resected IPMN reveals that *TP53* mutations are more common in high grade dysplasia and invasive IPMN (15%-20%) as compared with low grade IPMN (0%-5%)[13,66]. An immunohistochemistry (IHC) study of 206 resected pancreas lesions including precursor lesions (IPMN and PanIN), corroborates this finding by reporting abnormal TP53 staining in 0% low grade dysplastic lesions, 42% high grade dysplastic lesions, and 68% invasive ductal carcinomas[37]. It has recently been shown that TP53 function can be moderated via alternate mechanisms such aberrations in transcript splicing[96]. Whether this occurs in IPMN and IPMN-associated PDAC has not yet been investigated. Like KRAS, no TP53-directed therapies are currently in clinical use.

CDKN2A (also known as p16) is a tumor suppressor gene that is recurrently mutated in PDAC[67,94,97] and has also been reported in IPMN[13,66]. CDKN2A is a cell cycle regulator involved in the transition from G1 to S phase[98]. Sequencing data from resected IPMN specimens reveal *CDKN2A* mutation rate of 0%-18%, with an increasing frequency in lesions with high grade dysplasia, mirroring *TP53* and KRAS variants[13,66]. Interestingly, IHC studies have reported a much more frequent loss of *CDKN2A* expression: 50%-100% of high-grade dysplasia and invasive IPMN, and 10%-51% in low-grade dysplasia IPMN[24,99-101]. The mechanism of this expression loss is by epigenetic silencing through hypermethylation of CDKN2A promoters in 80% of IPMN lesions, again with an increasing prevalence with higher grades of dysplasia[102,103]. Multiple pharmaceutical agents targeting methylation are in clinical use, but thus far have been largely limited to hematologic malignancies. Some clinical trials with epigenetic drugs as potentiators of cytotoxic chemotherapy have been performed in PDAC, but the results have been modest.

SMAD-4 is a highly conserved signal transduction protein in the transforming growth factor Beta (TGF-β) pathway where it functions as a tumor suppressor by inhibiting epithelial cell growth[104]. Homozygous deletions have been reported in 30% of PDAC[105], while only 3% of IPMNs were reported to have mutations in SMAD-4[13]. Notably, SMAD-4 loss in PDAC is associated with a poor prognosis[106]. IHC of resected IPMN tissue revealed conserved expression in non-neoplastic and low-grade dysplastic tissue with loss of expression in high-grade dysplasia and IPMN-associated invasive PDAC[37,100]. Although one study reported SMAD-4 loss of heterozygosity of 80%-90% of PDAC and 22% in IPMN, IHC may again be more informative as only ~50% of PDAC and very few IPMNs actually demonstrate loss of *SMAD-4* expression[107]. In total, it appears that SMAD-4 inactivation is rare in IPMN but more common in invasive disease and usually occurs via deletion, rather than a silencing mutation or other mechanisms.

IMPLICATIONS OF THE SOMATIC MUTATIONAL LANDSCAPE OF IPMN

As in other cancers, the accumulation of driver mutations is implicated in the development of IPMN-associated invasive PDAC. However, the reality is much more complex than the archetypal progression from normal epithelium to adenoma and carcinoma as classically described in colorectal cancers[108,109]. Sequencing analysis of spatially distinct regions within resected IPMN specimens has identified considerable heterogeneity at the molecular level. Tan *et al.*[66] demonstrated that KRAS-wild type high-grade dysplasia can exist in an IPMN with KRAS-mutant low-grade dysplasia. Despite this, *KRAS* mutations often appear to be one of the earliest *IPMN* driver mutations during progression to invasive PDAC, as phylogenetic and whole-exome sequencing data do not reveal any shared mutations in surrounding tissue that precedes KRAS[25,76]. In addition, using an in-situ hybridization approach, heterogeneity in individual

KRAS clones can be demonstrated in a single patient[25,76]. In metastatic untreated PDAC, the metastatic lesions contained identical driver mutations as compared with the primary lesion with heterogeneity in subsequent passenger variants[110]. Synthesizing these data is challenging but some conclusions can be derived. The diverse mutational landscape present in non-dysplastic or low-grade IPMN with increasing uniformity in advanced PDAC suggests clonal selection is a hallmark of IPMN malignant transformation [Figure 2]. The recognition of *TP53* and *SMAD-4* as late-occurring mutations often associated with high-grade or invasive disease, has broadened our understanding of worrisome features. Using resected tissue specimens to characterize molecular differences of low-grade *vs.* high-grade and invasive IPMN informs the interpretation of cyst fluid analysis and cyst wall biopsy. Appreciation of IPMN as a heterogeneous lesion through multi-focal analysis offers context in interpreting earlier single biopsy studies and has important implications for the natural history of low-grade IPMN. It also implies that variants of KRAS or GNAS alone are likely not sufficient to drive the development of invasive disease in IPMN. Interestingly, polyclonality observed in precancerous lesions of other organ systems has been attributed to environmental carcinogen exposure[111-113] resulting in a field defect to the affected tissue. This has not been demonstrated experimentally in the pancreas but may be an area for future inquiry. Lastly, the oncocytic histology subtype has recently been scrutinized based on recent molecular data. Sequencing studies of oncocytic IPMN reveal that *KRAS* and *GNAS* mutations are not present and *RNF43* mutations are rare, suggesting that the mutational landscape and biological behavior of these lesions is dissimilar to other IPMN sub-types[44,45]. It is possible that oncocytic-subtype IPMN may be reclassified in the future as a distinct pathologic entity separate from IPMN.

HERITABLE CAUSES OF PANCREATIC CANCER & IPMN

While accounting for 2%-10% of pancreatic cancer[114-117], three heritable pathways for developing pancreatic cancer have been discussed: (1) Hereditary tumor predisposition syndromes; (2) tumor syndromes stemming from chronic inflammation; and (3) familial pancreatic cancer (FPC). FPC is defined as at least two first-degree relatives with PDAC, which are not at increased risk from other syndromes[117]. There is a body of literature establishing a link between numerous specific germline mutations and pancreatic cancer: ATM, BRCA1/2, CDKN2A, MLH1, MSH2, PALB2, PMS2, PRSS1, and STK11[118-125].

The literature base regarding heritable IPMN is more limited; however, in a 2019 study of resected IPMN tissue Skaro *et al.*[125] found that 7.3% of patients carried germline mutations in one of the 94 genes captured in the TruSight Cancer probe, and 2.9% of patients carried a germline mutation specifically associated with pancreatic cancer. They also found that patients with IPMN and a concurrent invasive cancer were more likely to have pancreatic-specific germline mutations, than patients with IPMN alone[125]. This study suggests that there may be a link between FPC and IPMN development; however, currently there is a paucity of data on the topic.

CLINICAL IMPLICATIONS OF IPMN MOLECULAR ANALYSIS

Despite years of study, the management of IPMN remains a major challenge for clinicians. As the vast majority IPMNs fall into a low or intermediate risk category, determining optimal need and timing for surgical intervention is difficult. Once reaching intermediate risk, usually due to questionable worrisome features or increased size on imaging, the next evaluation typically includes endoscopy with ultrasound and fine needle biopsy. Thoughtful interpretation of pancreatic cyst fluid analysis, in the context of the genetic and pathologic studies discussed above, offers a relevant diagnostic tool with the potential for personalized risk-stratification and informing clinical decisions.

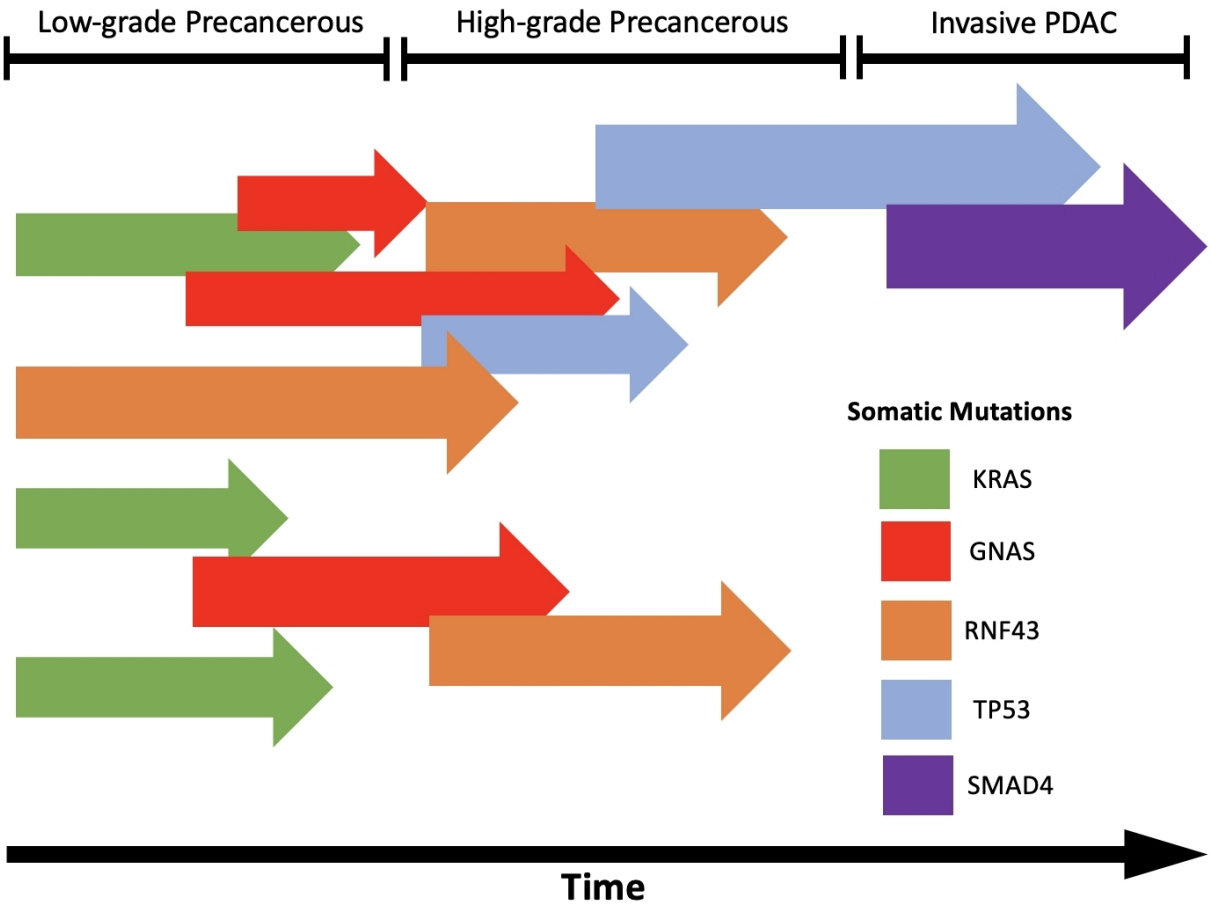

Figure 2. Schematic of clonal expansion of IPMN to invasive PDAC. Adapted from Fischer *et al.*[25] Intraductal Papillary Mucinous Neoplasms Arise from Multiple Independent Clones, Each with Distinct Mutations, *Gastroenterology* 2019. This schematic illustrates multiple precancerous cells undergoing clonal expansion with in an IPMN. Clones with distinct mutations in known driver genes (*KRAS* & *GNAS*), may acquire further mutations and vie for dominance while displaying increasing levels of dysplasia. Eventually enough key mutations accumulate, and a single clonal becomes dominate and displays invasive behavior.

Cyst fluid molecular analysis

Clinical cytology studies from EUS-FNA samples of IPMN lesions have historically been heavily factored into clinical decision-making when available. However, low cellularity, low interobserver reliability, and indeterminate results often obscure the clinical picture[126,127]. For instance, when cytology reveals high-grade dysplasia, it is strongly supportive of proceeding with resection. However, such clarity is rare. The more typical result is a paucicellular specimen or visualization of "atypical" cells, which should not be interpreted as dysplastic. Aspiration via fiberoptic endoscopic instruments invariably results in atypical cellular morphology and these findings more often complicate rather than simplify management. Instead, studies have looked to other markers to assist in risk-stratifying pancreatic cystic lesions. A number of studies have proven that molecular analysis of IPMN cyst fluid is technically feasible, allowing for genetic and epigenetic analysis[128-133]. These have investigated cytopathology, proteomics with particular focus on mucin expression[134], biochemical analysis including levels of CEA[10,135] and Das-1[136], telomere status[137,138], miRNA[130,132], and mutations in *GNAS*, *KRAS*, *RNF43*, *SMAD4*, and *TP53*[139,140].

In a study investigating EUS fine needle aspiration of pancreatic cyst fluid, *KRAS*/*GNAS* mutations were detected via genetic analysis of cell-free DNA (cfDNA) in 56/56 IPMN. Consistent with sequencing data discussed above, this study found that the combination of *KRAS*/*GNAS* mutation and an alteration in

TP53/PIK3CA/PTEN had an 89% sensitivity and 100% specificity for high risk IPMN[140]. A major criticism regarding genetic analysis of cyst fluid aspiration involves the volume of cyst fluid required to obtain enough DNA for sequencing, sometimes 1 mL or more[126,128]. A novel "through the needle biopsy" technique was developed to capture cyst wall and allow for a richer histological evaluation and provide more material for genetic analysis[141].

To date miR-216, Das-1, and combination panel of tumor suppressor and proto-oncogenes mutations have shown promise in differentiating benign tumors from IPMN with high-grade dysplasia or invasive components[130,136,138,140]. However, the majority of these studies are retrospective, limiting their applicability in the prospective setting. At present, no clinical trials have been opened utilizing molecular markers in comparing cyst management strategies [a current ongoing multi-institutional trial does compare high- *vs.* low-intensity surveillance regimens, but this utilizes clinical features only (ECOG-ACRIN EA2185)]. Also, and perhaps more importantly, none of these studies have final tissue pathology from surgical resection to correlate with the cyst fluid marker. Thus, it is impossible to truly ascribe a high- or low-risk designation to a particular marker. The first publication from the prospective ZYSTEUS trial[85] sought to overcome that concern and reported that the 12 patients who underwent fine needle aspiration of their IPMN all yielded either *KRAS* or *GNAS* mutations. The aspirate was fractionated into a cellular component and a liquid component, but the liquid component did not yield cfDNA of sufficient quality for analysis in 25% of patients, often due to viscosity. As this prospective trial continues to mature and accrue more patients it may offer important insights into the utility and practicality of using genetic analysis of cyst fluid for risk stratification and clinical decision making in the management of patients with IPMN.

To unify existing molecular knowledge and assist practicing clinicians in risk management of cystic pancreatic lesions, commercial molecular diagnostic kits have recently become widely available. These kits generally require aspirated cyst fluid (~600 μL) which, in combination with clinical factors, is subjected to a targeted molecular analysis. A panel of oncogenes and tumor suppressor gene variants associated with high-risk lesions is assessed as well as DNA quantity/quality. These kits signify the first attempt at personalized management of cystic lesions of the pancreas. This approach has revolutionized other tumor types, with OncotypeDx in breast cancer staging and treatment being the archetypal example[142,143]. Clearly, much work remains, but several clinical scenarios regarding IPMN management could benefit from molecular analysis. For example, asymptomatic patients with low-risk imaging but a concerning mutation profile may benefit from more aggressive intervention. However, a frail patient with a clinically-concerning IPMN but found to have a reassuring molecular profile may be best served by close surveillance.

Two commercially-available kits are in widespread use in the United States: PancraGEN[16] (Interpace Diagnostics) and PancreaSeq[17] (University of Pittsburgh Medical Center). Both tests screen for > 20 mutations associated with pancreatic cystic lesions. PancreaSeq utilizes next generation sequencing, while PancraGEN uses Sanger sequencing. The mutations captured by both kits also include variants common to non-mucinous lesions, such as VHL variants in serous cystadenoma. Reports provide the relevant genomic analysis and, in combination with clinical risk factors, stratify lesions as low-, moderate-, or high-risk. As always, patient factors such as candidacy for surgery or willingness to undergo surveillance remain critical components to the shared decision-making process.

In practice, data are sparse but seem to support selective employment of these assays. In general, patients with conflicting clinical, personal, or imaging risk profiles benefit the most from the currently available molecular assays. Patients with worrisome imaging features but cyst fluid analysis questioning the diagnosis of IPMN (i.e., low CEA) are ideal candidates for molecular profiling. In such patients, the presence or

absence of a GNAS or KRAS variant can serve to conclusively direct management. Routine employment of molecular techniques to patients with small, reassuring IPMNs or those with clear high-risk stigmata (such as jaundice) is not supported by evidence at this time. For example, in a patient with IPMN-associated jaundice, a reassuring molecular profile would not alter recommendations for intervention. Judicious use of these tests is also indicated as they are costly and sometimes result in a substantial out-of-pocket expense to the patient in the United States. Guidelines for appropriate usage of molecular testing will be reliant on the accrual of prospective data in the future.

CONCLUSION

IPMN is the most common cystic pre-malignant pancreatic lesion. However, the natural history and molecular underpinnings of its malignant transformation have not been fully characterized and it therefore remains a challenging entity to manage. Decades of observational studies have laid the groundwork for its histopathologic classification and the current consensus towards management based on clinical and imaging risk factors. With the application of modern molecular investigative techniques to both resected surgical specimens and endoscopically-obtained cyst fluid aspirates, it is hoped that a deeper molecular understanding of IPMN can allow informed design of improved care strategies. At present, our growing knowledge of IPMN biology has begun to create new opportunities for personalized management but has also uncovered previously-unappreciated molecular complexity.

Mutations private to IPMN, defined by examination of resected IPMN tissue, hold potential in defining novel therapeutic targets to reverse, halt, or slow the process of malignant progression. These studies can also inform the post-resection risk of recurrence or synchronous pathology, and therefore help improve surveillance paradigms. In addition, cyst fluid analysis holds tremendous clinical potential to risk-stratify lesions prior to resection. Ultimately, this could assist in determining optimal treatment or surveillance regimens. First-generation commercially available genetic analysis kits are already in practice, and their optimal role in routine practice is being explored. A prospective study aimed to specifically investigate the clinical utility of molecular analysis tools in guiding clinical decision making is the next logical step. While individualized patient-specific management remains paramount, even with cyst fluid genetic analysis, the rapidly evolving field of IPMN molecular analysis promises continued future improvement and augmentation of IPMN management strategies.

DECLARATIONS

Authors' contributions

Performed literature review, made significant contributions to concept and design of figure/tables, and wrote the manuscript: Peters NV, Kunstman JW

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

Both authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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