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New developments in the molecular mechanisms of pancreatic tumorigenesis

Matthäus Felsenstein, MD, Ralph H. Hruban, MD, and Laura D. Wood, MD PhD

Sol Goldman Pancreatic Cancer Research Center, Department of Pathology, Johns Hopkins University School of Medicine

Abstract

Pancreatic cancer is an aggressive disease with a dismal prognosis in dire need of novel diagnostic and therapeutic approaches. The past decade has witnessed an explosion of data on the genetic alterations that occur in pancreatic cancer, as comprehensive next generation sequencing analyses have been performed on samples from large cohorts of patients. These studies have defined the genomic landscape of this disease and identified novel candidates whose mutations contribute to pancreatic tumorigenesis. They have also clarified the genetic alterations that underlie multistep tumorigenesis in precursor lesions and provided insights into clonal evolution in pancreatic neoplasia. In addition to these important insights into pancreatic cancer biology, these large scale genomic studies have also provided a foundation for the development of novel early detection strategies and targeted therapies. In this review, we discuss the results of these comprehensive sequencing studies of pancreatic neoplasms, with a particular focus on how their results will impact the clinical care of patients with pancreatic cancer.

Keywords

pancreatic cancer; pancreatic ductal adenocarcinoma; cancer genome; next generation sequencing; somatic mutation

I. Introduction

Pancreatic ductal adenocarcinoma is one of the most aggressive human cancer types and is the third leading cause of cancer-related deaths in the United States¹. Despite intense research efforts in recent decades, only minor improvements to the generally dismal prognosis have been achieved: median survival under current chemotherapy regimens is still less than 12 months and overall 5-year survival rate only recently increased over 5%². The majority of patients are diagnosed at an advanced, disseminated stage when surgical cure is no longer attainable. Even patients eligible for surgical resection frequently suffer from local recurrence, metachronous metastasis, and chemotherapy resistance. A better understanding of the molecular alterations driving pancreatic carcinogenesis is critical to enable new strategies for earlier diagnosis and novel targeted therapies.

Pancreatic ductal adenocarcinoma is the most common malignant neoplasm of the pancreas and can be histologically diagnosed by its characteristic pathologic features³: neoplastic gland-forming epithelial cells invade into the surrounding stroma and induce an extensive inflammatory and desmoplastic reaction. The development and implementation of next-generation sequencing (NGS) and modern bioinformatic technologies has allowed the generation of an enormous amount of genomic data which has refined our understanding in pancreatic ductal adenocarcinoma development⁴⁻⁹. In this review, we focus on recent genomic insights into the molecular mechanisms driving pancreatic carcinogenesis, particularly focusing on tumor evolution and clinical applications.

II. Genetic landscape of pancreatic ductal adenocarcinoma

The malignant behavior of cancer cells is driven by somatic mutations in oncogenes and tumor suppressor genes^{10,11}. The accumulation of these genetic alterations significantly perturbs major signaling pathways, leading to the acquisition of a malignant phenotype. The most frequently altered driver genes in pancreatic ductal adenocarcinoma (the oncogene *KRAS* and the three tumor suppressor genes *TP53*, *CDKN2A*, *SMAD4*) have been thoroughly characterized in human tissue samples for some time (Table 1)¹²⁻¹⁵. However, recent comprehensive genomic analyses, including whole exome and whole genome sequencing studies, have provided a more detailed understanding of the complex genomic changes, orchestrated by a combination of chromosomal rearrangements, local copy number variations (CNV), and widespread single nucleotide variants (SNV)⁴⁻⁹.

The average number of non-synonymous somatic mutations in pancreatic ductal adenocarcinoma cohorts ranges from 26-101, a subset of which affect major signaling pathways and collectively contribute to the malignant behavior of pancreatic ductal adenocarcinoma cells. In addition to the four frequently altered driver genes, a wide range of genes are mutated in only a small fraction of pancreatic ductal adenocarcinomas, including *GATA6*, *ARID1A*, *RNF43*, *ATM*, *TGFBR2*, *MAP2K4*, *MLL3*, *PIK3CA*, *RBM10*, *ROBO2*, *SMARCA4*, *PBRM1*, *SLIT2*, *KDM6A*, *BRAF*, *BRCA2*, among others (Table 1)⁴⁻⁹. Some of these genes are also likely to be drivers of pancreatic tumorigenesis and reflect a degree of genetic heterogeneity among pancreatic cancers in different patients, superimposed on remarkable homogeneity demonstrated by the most frequently altered genes (*KRAS*, *CDKN2A*, *TP53*, and *SMAD4*). Mutations in some of these infrequently altered genes have been correlated with poor prognosis, providing evidence of their functional impact despite infrequent occurrence (Table 1)^{4,5,7}. Interestingly, in the event of *KRAS* wildtype pancreatic ductal adenocarcinoma, mutations in *BRAF* are a mutually exclusive hotspot, with similar oncogenic potential to drive pancreatic carcinogenesis⁵. The majority of analyses of the pancreatic cancer genome have focused on mutations in the coding region of well characterized genes. However, a recent study identified commonly mutated regulatory regions in pancreatic ductal adenocarcinomas, suggesting that mutations outside of the coding region are also likely to have functional significance¹⁶.

The cellular pathways affected by somatic mutations in pancreatic ductal adenocarcinoma involve diverse cellular functions, including regulators of cell growth (G1/S-phase transition, *KRAS* signaling, hedgehog signaling, cell-cycle regulation), cell interaction with the

extracellular environment (proteins involved in invasion/angiogenesis, integrins, cell adhesion molecules, TGF β signaling, Wnt/Notch signaling, axon guidance pathways), and DNA repair mechanisms (DNA damage control, chromatin remodeling)⁴⁻⁹. Some of these pathways are targetable and thus have significant clinical application as discussed below.

In addition to the detected single nucleotide variants (SNV), comprehensive next-generation sequencing (in particular whole genome sequencing) has helped to identify more complex structural changes in the pancreatic ductal adenocarcinoma genome^{6,17,18}. Although the functional role of structural rearrangements remains to be determined, they can affect tumor suppressors such as *SMAD4* and *CDKN2A*, resulting in homozygous deletion or other inactivating alterations¹⁷. Based on these structural variants, pancreatic ductal adenocarcinoma subtypes have been defined as stable, locally rearranged, scattered, and unstable⁶. Crucially, deleterious germline and somatic mutations in *BRCA1*, *BRCA2*, *PALB2*, and other genes in the Fanconi anemia pathway are associated with the unstable subtype (>200 structural variation events), likely due to the resultant cancer's inability to repair double stranded DNA breaks¹⁹. In addition to these subtypes based specifically on chromosomal rearrangements, genome-wide analysis of somatic mutation types in over 100 pancreatic ductal adenocarcinomas identified four different mutational signatures²⁰. These signatures are related to age, APOBEC cytidine deaminases, *BRCA1/2* alterations, and mismatch repair deficiency (MMR). Intriguingly, the signatures related to age and APOBEC enzymes were present in all pancreatic ductal adenocarcinomas, while the *BRCA1/2* and mismatch repair deficiency signatures were only present in small subsets, presumably those with underlying alterations in these pathways²⁰.

In addition to categorization based on general patterns of genetic alterations or mutations in specific driver genes, comprehensive molecular analyses have also focused on classifying patterns of gene expression, resulting in novel classifications based on transcriptional signatures. These include the subtypes defined by Collisson *et al.* (classical, quasi-mesenchymal, exocrine) and those proposed by Bailey *et al.* (squamous, pancreas progenitor, ADEX, immunogenic) – these patterns were correlated with histologic features, clinical outcome, and chemo-sensitivity^{4,21}. However, some of the genes that define these subtypes are also expressed in non-neoplastic cells. The extent to which the gene expression patterns are intrinsic to the neoplastic cells (rather than a result of genes expressed in the desmoplastic stroma or infiltrating inflammatory cells) still needs to be defined.

Epigenetic changes contribute to altered gene expression in pancreatic ductal adenocarcinoma^{22,23}. The critical pancreatic ductal adenocarcinoma driver *CDKN2A* is frequently inactivated by promotor hypermethylation, demonstrating the impact of dysregulation conferred by epigenomic imprints^{24,25}. Methylation status may serve as a biomarker for the early detection of precursor lesions or pancreatic ductal adenocarcinomas²⁶⁻²⁹. However, the clinical significance of the numerous aberrantly methylated candidates still needs to be determined; a recently created methylation database may help in this regard³⁰. In addition to alterations in the expression of specific genes, large-scale epigenomic reprogramming has recently been suggested to play a role in metastatic progression of pancreatic ductal adenocarcinoma³¹.

In addition to the somatic mutations that play a critical role in driving pancreatic tumorigenesis in all pancreatic ductal adenocarcinoma patients, specific germline variants also contribute to the development of “familial” pancreatic ductal adenocarcinoma in ~10% of patients. Known genetic syndromes are associated with specific genetic alterations with an increased risk for pancreatic cancer, such as hereditary breast/ovarian syndrome (*BRCA 1/2*, *PALB2*), Li-Fraumeni syndrome (*TP53*), Lynch syndrome (*MLH1*, *MSH2*, *MSH6*, *PMS2*), familial atypical multiple mole melanoma syndrome (*CDKN2A*), and Peutz Jeghers syndrome (*STK11/LKB1*)^{32,33}. Heterozygous germline alterations in the *ATM* gene also lead to an increased risk of pancreatic cancer³⁴. Remarkably, whole genome sequencing of germline DNA from 638 patients with familial pancreatic cancer did not reveal a single gene that accounts for most of the familial aggregation of the disease³⁵. Instead, a large number of genes with rare deleterious germline variants were identified. These candidates require further characterization to determine if they are true familial pancreatic cancer genes or rare private germline changes of no clinical significance. As such, the genetic basis of the majority (>80%) of familial pancreatic cancer cases has not yet been elucidated.

Recent large scale sequencing efforts have provided a vast quantity of data, which has helped to define molecular subtypes that can facilitate an individualized approach to pancreatic cancer therapy. However, significant challenges remain in the implementation of personalized medicine for patients with pancreatic ductal adenocarcinoma. The first challenge is the rarity of targetable mutations in most pancreatic ductal adenocarcinomas; simply put, most of the genes mutated in pancreatic ductal adenocarcinoma are not targetable with currently available drugs³⁶. Second, clinical trials of molecular targets involved in downstream signaling pathways affected by driver gene mutations have, to date, been disappointing^{37,38}. Third, although most pancreatic cancers are relatively uniform compared to other tumor types, tumor heterogeneity can potentially challenge targeted therapy approaches since mutations in small subclones can rapidly lead to therapeutic resistance driven by newly selected variants³⁹. These challenges are discussed in greater detail below.

III. The mutational sequence driving pancreatic carcinogenesis

Because most patients with pancreatic ductal adenocarcinoma are diagnosed at an advanced stage of disease, novel approaches for early detection are greatly needed. A comprehensive understanding of the processes by which a normal pancreatic cell is transformed into a precursor lesion and then into an invasive carcinoma is critical to provide a rational foundation for early detection⁴⁰. Most pancreatic cancers are believed to develop from non-invasive precancerous lesions, histologically characterized either as pancreatic intraepithelial neoplasia (PanIN) when less than 5mm or intraductal papillary mucinous neoplasm (IPMN) when greater than 1cm⁴¹. Mucinous cystic neoplasms (MCN), a third type of precursor lesion, are less common and are characterized by the presence of ovarian-type stroma. These distinct precursor lesions represent three unique pathways in the development of pancreatic ductal adenocarcinoma, each with its own complement of somatic mutations^{41,42}. As precursor lesions progress, increasing grades of dysplasia are associated with accumulation of somatic mutations in key driver genes (Figure 1)^{24,43,44}. This conceptual model is based on data from multiple preceding studies that analyzed human precursor lesions with

immunohistochemical and molecular techniques, demonstrating that most driver gene alterations occur in pre-invasive lesions ⁴⁵⁻⁴⁸.

In PanIN lesions, somatic mutation in an oncogenic hotspot of *KRAS* is an early event that is almost universally found in PanINs (Figure 1). The activation of *KRAS* is subsequently followed by inactivation of tumor suppressors at higher grades of dysplasia, perhaps necessary to overcome oncogene-induced senescence ^{24,43,49-52}. The prevalence of *KRAS* mutations in PanIN lesions is greater than 90% (even in low-grade PanIN lesions) and mutations in *KRAS* almost always occur at specific hotspot positions (codons 12, 13, 61), leading to the activation of intracellular effector pathways ^{50,53}. Whole exome sequencing studies of PanIN lesions confirmed that *CDKN2A* may contribute in the transition to high-grade dysplasia, whereas aberrations in *TP53* and *SMAD4* occur late and predominantly drive invasiveness ^{24,43}.

The genetic alterations driving the formation of IPMNs overlap with those driving PanINs, but with some significant differences. Notably, in contrast to PanINs, IPMNs often harbor activating mutations in the oncogene *GNAS* and inactivating mutations in the tumor suppressor gene *RNF43* (Figure 1) ^{24,54}. More than 90% of IPMNs harbor one or both oncogenic events (*KRAS* and *GNAS*), highlighting their combined role in tumor initiation of cystic precursors ^{54,55}. As an oncogene, *GNAS* is usually targeted at a specific hotspot position (codon 201) ^{55,56}. Missense mutations in *GNAS* lead to constitutively active G-protein signaling with elevated cAMP levels and increased protein kinase A activity, but the exact oncogenic function of *GNAS* still needs to be further clarified ⁵⁶⁻⁵⁹. As is seen in PanINs, after the initiating oncogene mutation, loss of tumor suppressor gene function mediates the progression of IPMNs. Loss of *CDKN2A* expression occurs in 10-50% of IPMNs and is found in both low-grade and high-grade IPMNs ⁵⁴. *TP53* alterations are usually confined to high-grade IPMNs and their associated invasive cancers (20-40%), suggesting a role for *TP53* in malignant progression ^{54,60}. The inactivation of *SMAD4* is most commonly found in IPMN-associated invasive carcinoma ⁵⁴. Whole exome sequencing has further identified *RNF43* as an additional driver gene for IPMN, which is inactivated in 24% of cases analyzed ^{54,61}. The protein product of the *RNF43* gene functions as a tumor suppressor in the Wnt signaling pathway with intrinsic E3 ubiquitin activity and is commonly targeted by inactivating mutations combined with loss of heterozygosity (LOH) ^{62,63}.

Mucinous cystic neoplasms are the third and least prevalent preinvasive pancreatic lesion and are distinguished by unique clinical and pathological features, including their distinctive ovarian stroma ^{64,65}. Molecularly, MCNs resemble IPMNs, except that unlike IPMNs, MCNs do not typically harbor *GNAS* alterations. Whole exome sequencing of 8 MCNs identified *KRAS* (75%), *RNF43* (50%) and *TP53* (25%) as highly prevalent events in these lesions ⁶¹. Genetic changes accumulate with higher grade of dysplasia and invasive carcinomas associated with MCNs lesions can acquire all common driver gene alterations (*KRAS*, *TP53*, *CDKN2A*, *SMAD4*) ⁶¹.

The three most common precursor lesions that give rise to pancreatic ductal adenocarcinoma (PanIN, IPMN, and MCN) are each defined by a unique landscape of genetic alterations.

Still, common themes among all three lesions include early oncogene mutations that initiate tumorigenesis and the later loss of tumor suppressor genes that drives tumor progression. This deep understanding of the genomic alterations in pancreatic cancer precursor lesions has major clinical implications, since early detection has the potential to drastically improve the clinical course of patients with pancreatic neoplasms. In particular, mutations in *TP53* and *SMAD4* are limited to high-risk lesions, including high-grade dysplasia and invasive cancer, and thus are optimal targets for novel strategies to detect pancreatic neoplasms at high risk for transformation to invasive carcinoma. In addition, further investigation in effector pathways of critical driver genes may clarify the individual roles of each alteration in the selective growth advantage and recalcitrant behavior of neoplastic cells, potentially identifying new methods to target these common aberrations early on⁶⁶.

IV. Clonal evolution leading to disease progression

The prevalence and distribution of genetic alterations in all types of cancer can be metaphorically represented as mountains and hills on the cancer genome landscape⁶⁷. The pancreatic cancer landscape is dominated by four mountains, all of which are altered in the majority of pancreatic ductal adenocarcinomas and are regarded as unequivocal drivers in pancreatic cancer initiation and progression. However, the majority of somatic mutations in pancreatic ductal adenocarcinoma occur in genes that are altered at low prevalence, only some of which are likely to be true driver genes^{5,7,9}. These less prevalent mutations accumulate during tumor growth and result in superimposed genetic heterogeneity, which may contribute to poor therapy response and resistance in individual cancers⁶⁸⁻⁷⁰. Comprehensive analyses of multiple lesions from the same patient are currently underway in many tumor types, which will provide new insights into this heterogeneity in cancer evolution⁷¹. These studies include multi-region and even single cell sequencing, as well as deep next generation sequencing and *in situ* mutation detection approaches, which will enable a more complete understanding of the evolutionary dynamics and heterogeneity in pancreatic cancer progression⁷²⁻⁷⁶.

Nowell elegantly described the evolutionary process of tumor growth as an adaptive Darwinian process driven by somatic mutations with sequential subclonal selection⁷⁷. Due to the normal error rate of DNA replication, each cell division naturally results in approximately 3 somatic mutations, most of which affect intergenic regions without functional consequences^{78,79}. Increased mutation rate, induced by mutagenic exposure (exogenous) and/or genetic instability (endogenous), accelerates this system of Darwinian evolution by providing additional genetic variation on which selection can act^{11,80,81}. Remarkably, mathematical models incorporating stem cell division rates suggest that normal DNA replication errors (without additional genetic instability) can account for the somatic mutations identified in many tumor types⁸².

At the initiation of neoplasia, a somatic mutation occurs to confer a selective growth advantage followed by clonal expansion. Then, throughout tumorigenesis, additional somatic mutations become clonally fixed because they provide survival advantage, such as increased proliferation, resistance to apoptosis, or evasion of the immune system^{79,83}. The

timing of selectively neutral passenger alterations is less clear – some likely occur before the initiating mutation while others accumulate during the process of clonal evolution^{78,84,85}.

The role of a mutation can be context dependent. A previously neutral mutation can become a driver, based on its micro-environmental influences, interdependence with other mutations, or location within the bulk tissue; similarly, the phenotype conferred by an early driver mutation may not be selected later in tumorigenesis^{79,86,87}. On the other hand, clonal expansion of a newly acquired beneficial mutation may be restrained by competition (clonal interference) with other clones for resources in the tumor microenvironment⁸⁸.

In the case of pancreatic cancer development, the initiating event is likely a somatic mutation at a key oncogenic hotspot. As previously described, *KRAS* mutation occurs early in pancreatic tumorigenesis, as they are present in all grades of PanIN lesions. However, the proportion of cells with a *KRAS* mutation (also known as the variant allele frequency or VAF) increases gradually with increasing grade of dysplasia, possibly reflecting clonal expansion over time⁵⁰. This is also true for the other driver genes *CDKN2A*, *TP53*, and *SMAD4*, in which VAF increases from HG-PanIN to invasive carcinoma lesions^{43,45,60}. However, these allele frequencies should be interpreted with some caution, as the VAF is dependent on the neoplastic cellularity of the analyzed samples, which is exceedingly difficult to control for in such experiments.

Although the critical role of driver genes in pancreatic tumorigenesis is well established, the exact order and pace at which these alterations occur is intensely debated. Notta *et al.* recently challenged the stepwise progression model for pancreatic tumorigenesis, and inferred that a single catastrophic chromotrypsis event can lead to a simultaneous loss of multiple tumor suppressor genes¹⁸. These studies highlighted the importance of genomic instability, structural rearrangements, and polyploidization in pancreatic tumorigenesis¹⁸. Such a punctuated evolution pattern may at least partially explain the rapid progression of disease observed in some patients with pancreatic cancer⁸⁹. However, although such catastrophic chromosomal events may drive tumor progression in a subset of patients, it does not explain the typical stepwise accumulation of driver mutations typically found within most precursor lesions⁹⁰. In addition, recent whole exome studies with a cohort of 456 pancreatic ductal adenocarcinomas observed that such catastrophic patterns are uncommon, and when they do occur only rarely target known oncogenes or tumor suppressor genes^{4,18}. Ultimately, the pattern of evolution appreciated in a particular study is likely dependent on the type of alteration analyzed in the study, with point mutations accumulating sequentially and copy number alterations in a more punctuated manner⁹¹.

Another evolutionary step in the fate of a cancer cell is dissemination and clonal expansion at distant sites. The degree of heterogeneity and the clonal composition of metastatic lesions can provide insights into the timing of metastatic progression^{70,72,92,93}. Such inter- and intra-metastatic genetic heterogeneity is of crucial clinical importance, as it underlies the development of therapeutic resistance, particularly to targeted therapies^{68,69,94-96}. Resistance to therapy occurs when treatment selects for an already-present resistant clone, which can expand once sensitive cells are killed.

Two complementary models of the time point of metastasis have been proposed: the “linear progression” model describes a stepwise accumulation of genetic changes until a cell acquires autonomous fitness to disconnect from the primary tumor at a late stage, and the “parallel progression” model infers early dissemination and independent accumulation of genetic changes in both the primary and metastatic site^{68,93}. Recently, multiple whole exome and whole genome sequencing studies in disseminated pancreatic cancer have analyzed genetic heterogeneity at metastatic sites, in comparison to the other metastases as well as to the primary tumor^{72,92}. In pancreatic ductal adenocarcinoma, mutations in well-established driver genes appear to be widely uniform within the primary tumor and among the distinct metastases. Further mathematical modeling based on these sequencing data suggests a broad window of time between tumor initiation and the acquisition of metastatic ability⁹². These findings provide some support for the linear progression model predominating in pancreatic ductal adenocarcinomas, which infers late dissemination of specific metastatic subclones from the primary tumor. However, there is also marked genetic heterogeneity among metastases, conferred almost entirely by passenger mutations, likely due to neutral evolution after the acquisition of the driver mutations that confer malignancy^{78,84}.

Clonal evolution in cancer is a highly complex process which has only recently begun to be studied in detail. The resulting intratumoral heterogeneity may contribute to the emergence of therapeutic resistance. Future studies, perhaps even down to the single cell level, will more comprehensively characterize this heterogeneity, phylogenetic relationships, and clonal evolution, providing critical insights into tumor initiation and progression as well as a rational foundation for early detection and therapy in pancreatic cancer.

V. Exploiting molecular alterations in the clinical setting

Great efforts are being exerted to translate the many insights provided by recent next generation sequencing studies into clinical practice⁹⁷. Genetic alterations may be used to develop diagnostic markers for early detection, biomarkers for disease progression or therapeutic responsiveness, and novel targeted therapies. Molecular alterations are already used to select subsets of patients most likely to respond to targeted therapy in other tumor types. For example, only colorectal cancers without mutations in *KRAS* or *BRAF* respond to therapies targeting the epidermal growth factor receptor⁹⁸⁻¹⁰². As such, assays for these mutations are routinely performed before instituting these particular therapies. Molecular knowledge of pancreatic cancer progression generated by recent sequencing studies has significantly improved our understanding of the evolutionary processes that drive pancreatic ductal adenocarcinoma progression. However, genetic knowledge about pancreatic cancer has not had as much impact on clinical practice as it has for other tumor types³⁶. Here, we highlight a few specific examples of the clinical applicability of molecular alterations – these will almost certainly be expanded with further advances in sequencing technology and therapeutic targeting.

Perhaps one of the first clinical applications of molecular genetics will be in early detection. One major challenge in improving the overall-survival of pancreatic ductal adenocarcinoma patients lies in the fact that patients almost always present with advanced stage disease; at

this stage, surgical cure is no longer possible, and patients literally have billions of cancer cells in their bodies. However, knowledge of precancerous lesions that precede the development of pancreatic ductal adenocarcinoma can provide a strategy for early detection and complete eradication of all neoplastic cells. Although precursor lesions likely take years to develop, the most common precursor lesion (PanIN) does not produce symptoms, and virtually all are undetectable by currently available imaging techniques¹⁰³⁻¹⁰⁵.

Mathematical models analyzing evolutionary patterns in metastatic lesions offers an estimated broad time-window (10 years) before an initiating mutation in the pancreas progresses to the parental founder cell of a pancreatic ductal adenocarcinoma⁹². This suggests a broad period of time for early detection. As one considers early detection of precursor lesions, it is important to remember that most PanIN lesions as well as cystic precursors (IPMNs) carry a low risk for progression to invasive carcinoma. The benefits of early detection must be balanced against the risks of overtreatment¹⁰⁶⁻¹⁰⁹.

Because of the low-incidence of pancreatic ductal adenocarcinomas in the general population (life-time risk 1.3%) and the high-cost of the most sensitive imaging techniques, population-wide screening has not been recommended thus far^{110,111}. Instead, most efforts have focused on identifying high-risk individuals (HRI) in whom there is an increased likelihood of detecting high-grade dysplasia or small pancreatic ductal adenocarcinomas by surveillance compared to the general population^{103,112,113}. The inherited germline alterations that predispose to pancreatic cancer include mutations in *BRCA1*, *BRCA2*, *PALB2*, *CDKN2A*, *STK11/LKB1* (Peutz-Jeghers), *ATM*, *TP53*, *PRSS1*, and genes associated with mismatch repair defects (Lynch-Syndrome)¹¹⁴. As previously discussed, it is anticipated that there are additional familial susceptibility genes for pancreatic ductal adenocarcinoma that have not yet been defined³⁵. Therefore, HRI have also been identified based on their cancer family history.

New screening methods incorporating molecular alterations are being investigated. These include molecular analysis of fine needle aspiration (FNA) samples of pancreatic cysts, as well as duodenal fluid collected endoscopically and circulating tumor DNA (ctDNA) (Table 2). Molecular analysis of cyst fluid can help to distinguish neoplastic precursors from benign cystic lesions¹¹⁵. Mutations in *KRAS* or *GNAS* are present in >96% of patients with a mucin-producing premalignant neoplastic cyst (IPMN or MCN), and these mutations can be detected in cyst fluid with excellent sensitivity and specificity^{55,115}. Detection of such mutations can distinguish potentially harmful precursor lesions from benign cysts (such as serous cystadenomas), but at least to date, they do not reliably distinguish IPMNs with low-grade dysplasia from IPMNs with high-grade dysplasia. The assessment of ploidy by DNA cytometry may provide a simple method to suggest grade of dysplasia in IPMN cyst fluid¹¹⁶. However, more accurate markers (such as mutations in late driver genes like *TP53* and *SMAD4*) will be required to distinguish cysts requiring immediate clinical intervention from those that can be safely monitored.

Genetic alterations from cystic and non-cystic precursor lesions can also be detected in duodenal fluid collected after secretin stimulation, albeit with less sensitivity and specificity than in analysis of directed FNA samples^{117,118}. As *KRAS* and *GNAS* occur at the lowest grades of dysplasia, they are unlikely to be markers for high-risk lesions. However, detection

of *TP53* mutations in duodenal fluid samples can be an indication of worrisome high-risk lesions such as high-grade PanINs, high-grade IPMNs, and small invasive pancreatic ductal adenocarcinomas, which could have otherwise escaped diagnostic imaging criteria ⁶⁰. More recently, a novel digital NGS technique was reported that detected even low-abundance mutations down to 0.1-1% mutation prevalence ¹¹⁹. In this study, mutations in *TP53* and *SMAD4* were frequently identified in duodenal fluids from patients with pancreatic ductal adenocarcinoma, distinguishing them from a control population and even from patients with IPMNs with high specificity. Interestingly, high-risk mutations could be detected months prior a definitive clinical diagnosis of pancreatic ductal adenocarcinoma, highlighting the promise of this technique to facilitate earlier diagnosis. Still, such techniques need to be validated in large cohorts before broad implementation, even in high-risk individuals.

In addition to analysis of cyst and duodenal fluid, another form of so-called “liquid biopsy” can be collected from peripheral blood. Circulating tumor DNA (ctDNA) is readily detectable in patients with metastatic pancreatic cancer but also in almost half of the patients with localized disease ^{120,121}. However, the sensitivity of ctDNA in the detection of non-invasive lesions is limited. Still, this approach can be used to detect invasive cancers earlier and to follow patients with known cancer ^{120,121}. Mutations detected in ctDNA prior to intervention predicted mutations found in the resected tumor, demonstrating that ctDNA represents a surrogate for genetic alterations in the tumor ¹²¹. Thus, sequencing of ctDNA can be used to non-invasively detect mutations that confer therapeutic resistance, and it can also predict recurrence earlier than CT scan and is correlated with survival among metastatic and resected patients ^{122,123}.

Despite the low prevalence of targetable mutations identified in pancreatic ductal adenocarcinoma, it is worthwhile to consider some of the therapeutic targets that have been identified. To date, few agents have obtained approval from the United States Food and Drug Administration (FDA): the use of the EGFR inhibitor erlotinib demonstrated a survival benefit in pancreatic ductal adenocarcinoma patients in combination with Gemcitabine versus Gemcitabine alone ^{124,125}. However, targeting major genomic events in pancreatic cancer, such as oncogenic *KRAS* signaling, have been disappointing until now ^{126,127,128}.

Still, deeper understanding of altered signaling pathways has suggested other targeted treatment strategies. For example, promising pre-clinical results demonstrated treatment response to porcupine inhibitors (Wnt/Notch signaling) in pancreatic ductal adenocarcinomas with loss-of-function mutations in *RNF43*; this approach is currently being investigated in a clinical trial (NCT01351103) ⁶². In addition, patients with alterations in BRCA-mediated repair of DNA double strand breaks (*BRCA2*, *PALB*, *ATM*) are sensitive to DNA-crosslinking agents (MitomycinC, Cisplatin, Carboplatin) and poly ADP-ribose polymerase (PARP) inhibitors, which induce additional DNA damage that affect cancer cells selectively ^{129,130}. Pre-clinical success was also reported by CDK4/6 inhibitors in p16^{INK4A} deficient cell lines and may represent actionability for at least a subset of *CDKN2A* deficient pancreatic ductal adenocarcinomas ¹³¹. In addition, promising results of *BRAF*-inhibitors in metastatic melanoma, which obtained FDA approval, may be transitioned into pancreatic ductal adenocarcinoma trials of *BRAF*-mutated tumors ¹³². Finally, although uncommon (occurring only in about 1% of pancreatic ductal adenocarcinomas), defects in DNA

mismatch repair may indicate a subset of pancreatic ductal adenocarcinomas that are specifically responsive to immunotherapy. A potential mechanism suggested in other tumor types is that increased immune cell infiltration occurs due to neo-antigens as a result of a higher mutational load¹³³⁻¹³⁵. Clinical trials for immune checkpoint inhibitor therapies in pancreatic ductal adenocarcinoma are currently underway (NCT02907099; NCT02305186).

Currently, several centers have implemented a new approach to clinical trials of targeted therapy. In large scale randomized “basket” trials (such as IMPaCT), the efficacy of genotype-guided therapies with existing targeting agents are currently being investigated in pancreatic cancer (along with many other tumor types) based on individual tumor genotype^{36,136,137}. Such studies of genotype-guided therapy in pancreatic ductal adenocarcinoma will be required to implement personalized medicine for patients with pancreatic cancer.

In summary, to date, signaling pathways of the four main pancreatic ductal adenocarcinoma driver genes are not druggable. Thus, current strategies for molecularly targeted therapies in pancreatic cancer focus on mutations that occur at lower prevalence. In order to define which of the low prevalence mutations are best targeted, a deeper pathological and cellular understanding of specific gene mutations is crucial. In addition, mutations can improve survival by providing biomarkers for early detection, as survival of pancreatic ductal adenocarcinoma patients is highly correlated with their stage at diagnosis. Although genetic alterations can be detected in multiple types of bio-specimens, including blood and duodenal fluid, the precise clinical applications of such assays have not yet been standardized.

VI. Future directions

The cellular and biological implications of the vast majority of genetic changes found in individual pancreatic ductal adenocarcinomas are still not well understood. Our molecular knowledge is predominantly based on sequencing studies at a single time point, complicating our ability to perform in-depth analysis of the evolutionary trajectory of disease progression. This is particularly challenging to investigate in human specimens because in the follow-up of operatively resected patients, no additional tumor samples are typically harvested for routine clinical care. Serial biopsies for research studies can overcome this hurdle, but such analyses can be augmented by reproducible experimental models to assess the molecular alterations and downstream consequences in a longitudinal fashion. As our knowledge grows, the following questions need to be addressed: what process is driven by each driver gene mutation: invasion, proliferation, or dissemination? Which of the infrequently altered genes are truly drivers? To answer these pressing questions, the functional impact of specific somatic mutations on cellular signaling and other phenotypes also needs to be more completely characterized.

Studies of genetically engineered mouse models (GEMMs) have largely supported the insights from human samples. For example, pancreas-specific expression of mutant *KRAS* alone leads to the development of PanIN-like structures and with some latency to subsequent pancreatic ductal adenocarcinoma development¹³⁸. When targeting additional drivers (*CDKN2A*, *TP53*, *SMAD4*), pancreatic ductal adenocarcinoma development with rapid

progression can be reproduced, with a higher prevalence of invasion and metastasis that mimics the clinical course of human disease¹³⁹⁻¹⁴¹. Future models could support the functional role of low-prevalence mutations found in recent large-scale sequencing attempts, such as *ATM*, *MLL3*, Axon-guidance genes (*ROBO1/2*, *SLIT*), *KDM6A*, *GLI3* and others.

GEMMs also provide a tool for clinically important questions, as they can be used as a model for drug testing prior to clinical trials¹⁴². EGFR- and VEGF-inhibitors were successfully tested in GEMMs that recapitulated therapeutic responses found in patients^{143,144}. However, results in GEMMs are not always indicative of clinical success in human pancreatic cancer patients. For example, the Hedgehog-signaling inhibitor IPI-926 showed an exceptional response with improved drug delivery and survival in a GEMM system, but results in human clinical trials were disappointing^{145,146}.

A promising new development is the use of three-dimensional (organoid) culture systems, which enable the analysis of local growth and invasiveness of human cancer cells over time¹⁴⁷. There are major advantages of this system over two-dimensional culture and animal models, as organoids enable time-lapse imaging and spatial characterization during pancreatic cancer cell invasion¹⁴⁸. The flexibility offered by the growth of human organoids directly from dissociated primary tissue or even from biopsies offers an extremely valuable methodology for examining the biologic behavior of individual patient samples, with a much higher success rate than establishment of traditional two-dimensional cell cultures¹⁴⁹. For example, such three-dimensional cultures can be used as a surrogate for treatment response.

Genetic changes can be induced in *in vitro* cell models, including two-dimensional cell culture as well as organoids. Attempts to model the pancreatic cancer progression sequence in cultured human cells have confirmed the malignant potential of specific drivers^{148,150,151}. Recent developments in genome engineering using CRISPR/Cas9 (Clustered regularly interspaced palindromic regions/CRISPR-associated proteins) gene editing system have facilitated improved genomic modification of cultured cells by avoiding the use of unnatural exogenous or leaky transgene expression systems¹⁵². In this case, the functional impact on pancreatic carcinogenesis can be accurately analyzed following each genetic hit. The CRISPR/Cas9 technology may also improve the process of developing new animal models^{153,154}.

Modern experimental developments in modeling pancreatic carcinogenesis enable us to understand the biological consequences of genetic alterations found in recent large-scale sequencing studies. In order to augment data from *in silico* analyses of the functional impact of mutations, a combination of studies in *in vitro* and *in vivo* models will highlight the most promising genetic alterations to be further explored for the development of novel clinically relevant approaches, including new strategies for early diagnosis and targeted therapy of pancreatic cancer.

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Figure 1. Genetic alterations that drive pancreatic tumorigenesis. Pancreatic ductal adenocarcinoma arises from two different types of precursor lesions: pancreatic intraepithelial neoplasia (PanIN) and intraductal papillary mucinous neoplasm (IPMN). Both pathways are defined by common low-risk precursors (LG-PanIN and LG-IPMN), which can give rise to more advanced precursors (HG-PanIN and HG-IPMN) at high risk for transformation to invasive cancer. In both pathways, activating mutations in oncogenes are early events, with *KRAS* mutation occurring in LG-PanIN and both *KRAS* and *GNAS* mutations occurring in LG-IPMN. Tumor suppressor gene loss (including *CDKN2A*, *TP53*, and *SMAD4* in both pathways as well as *RNF43* specifically in IPMNs) occurs in more advanced precursor lesions, often limited to HG-PanIN/HG-IPMN or associated invasive cancer. The timing of mutations in genes altered at low prevalence (“candidate genes”) in these pathways is less clear due to the infrequent occurrence of these mutations.

Table 1:

Mutated genes in recent comprehensive sequencing analyses of PDACs

Driver gene	Signaling pathway	Mutation prevalence (%) [*]	Targeted therapy	Prognostic impact ^{***}
<i>KRAS</i>	KRAS signaling	91		
<i>TP53</i>	Cell cycle	61		
<i>CDKN2A</i>	Cell cycle	44 ^{**}	CDK4/6 inhibitor	poor prognosis Oshima M 2013 <i>Ann Surg</i> ¹⁵⁵
<i>SMAD4</i>	TGFβ signaling	40 ^{**}		poor prognosis Blackford A 2009 <i>Clin Cancer Res</i> ¹⁵⁶ Oshima M 2013 <i>Ann Surg</i> ¹⁵⁵
Candidate driver gene				
<i>GATA6</i>	Wnt/Notch signaling	17 ^{**}	Gamma secretase inhibitor	improved prognosis Zhong L 2011 <i>PLoS one</i> ¹⁵⁷
<i>ARID1A</i>	Chromatin remodeling	8		
<i>RNF43</i>	Wnt/Notch signaling	8 ^{**}	Porcupine Inhibitor	improved prognosis Dal Molin M 2015 <i>Clin Cancer Res</i> ¹⁵⁸
<i>TGFBR2</i>	TGFβ signaling	6		
<i>MAP2K4</i>	KRAS signaling	5		
<i>MLL3</i>	Chromatin remodeling	5		
<i>PIK3CA</i>	KRAS signaling	5 ^{**}	mTOR Inhibitor	poor prognosis
<i>RBM10</i>	RNA processing	4 ^{**}		improved prognosis Witkiewitz AK 2015 <i>Nature</i> ⁵
<i>ATM</i>	DNA Repair	4		poor prognosis Russell R 2015 <i>Nat Commun</i> ¹⁵⁹ Kamphues C 2015 <i>Pancreas</i> ¹⁶⁰
<i>ROBO2</i>	Axon Guidance	3		poor prognosis Bailey P 2012 <i>Nature</i> ⁵
<i>SMARCA4</i>	Chromatin remodeling	3		
<i>PBRM1</i>	Chromatin remodeling	3		
<i>SLIT2</i>	Axon Guidance	3		
<i>KDM6A</i>	Chromatin remodeling	3		poor prognosis Bailey P 2012 <i>Nature</i> ⁵
<i>BRAF</i>	KRAS signaling	2	BRAF inhibitor	Schultz NA 2012 <i>Pancreas</i> ¹⁶¹
<i>BRCA2</i>	DNA Repair	2	PARP inhibitor	Golan T 2017 <i>Br J Cancer</i> ¹⁶²

^{*} Whole exome sequencing datasets (740 PDACs): Biankin AV 2012; Waddell N 2015; Witkiewitz AK 2015; Bailey P 2016

^{**} Excluded datasets of Biankin et al. and Bailey et al., which did not consider copy number variants (CNV)

^{***} Reported in expression/genomic databases (onclink.com; TCGA) and selected publications

Table 2:
Potential applications of next generation sequencing to diagnosis of pancreatic neoplasms

Specimen	Procedure	Evidence	Sensitivity*	Specificity*
Tumor biopsy	EUS-FNA	Kameta E 2016 <i>Onco Lett</i> ⁶³ Young G 2013 <i>Cancer Cytopathol</i> ⁶⁴ Bournet B 2015 <i>J Clin Gastroenterol</i> ⁶⁵	80-95%	95-100%
Cyst fluid (IPMN)	EUS-FNA	Springer S 2015 <i>Gastroenterology</i> ¹¹⁵ Wu J 2011 <i>Sci Trans Med</i> ⁵	75-96%	92-98%
Pancreatic juice	Endoscopic juice collection	Yu J 2016 <i>Gut</i> ⁸⁹ Sadakari Y 2014 <i>Clin Trans Gastro</i> ¹¹⁷ Kanda M 2013 <i>Clin Gastroent Hepat</i> ⁶⁰	60-85%	80-100%
Blood	Circulating tumor DNA	Bettegowda C 2014 <i>Sci Trans Med</i> ¹²¹ Sausen M 2015 <i>Nature</i> ²⁰ Pietrasz D 2016 <i>Clin Cancer Res</i> ⁶⁶	80-90%	90-100%

EUS, Endoscopic ultrasound; FNA, Fine needle aspiration

* Sensitivity/specificity to detect oncogenic hotspot mutations (*KRAS*, *GNAS*)