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REVIEW

DNA methylation profile to aid in the diagnosis of pancreatic ductal adenocarcinoma and its role in disease progression

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ABSTRACT

There is no immunohistochemical or molecular marker to confirm the histologic diagnosis of pancreatic ductal adenocarcinoma (PDAC). This is particularly important in a scenario of unknown primary. Molecularly, PDAC is characterized by a limited set of driver mutations, and new predictive and prognostic markers are needed to guide novel therapies. Recent data show that DNA methylation profiles combined with complex machine learning algorithms are ideal tools to improve the diagnosis of PDAC. In addition, DNA methylation can be used to gain a deeper understanding of PDAC pathogenesis and further stratify this entity. Furthermore, exciting technologies have emerged, such as nanopore sequencing, which can be used to move these diagnostic tools from the postoperative to the intraoperative setting, or even as a liquid biopsy approach.

PLAIN LANGUAGE SUMMARY

Pancreatic cancer specimens are hard to diagnose with confidence because there aren't any specific tissue tests that can clearly confirm it. It's even harder to tell exactly where the cancer started in the body if the specimens are from metastases. While many cases of pancreatic cancer share similar traits at the molecular level, we still need better ways to predict how the disease will progress and how to treat it. Recent studies show that we can improve diagnosis by looking at chemical change patterns in DNA that affect how genes work. These chemical changes also help researchers understand how cancer develops and how to create more personalized treatments. New tools like nanopore sequencing could soon make it possible to detect pancreatic cancer more easily, even during surgery or through a simple blood test.

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1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer, with recent epidemiologic studies suggesting that by 2030, PDAC will be the second leading cause of cancer-related death [1]. Only a minority of PDAC patients, ca. 10–20%, can benefit from surgery [2], while the majority are diagnosed at a locally advanced or metastatic stage, where treatment typically involves a comprehensive approach, around systemic chemotherapy. A limited number of patients may benefit from targeted therapies, such as addressing *KRAS*^{G12C} mutations, homologous recombination deficiency, mismatch repair deficiency, or rare gene fusions, while the role of radiation remains controversial [3]. Hence, new predictive markers are needed to guide and expand therapeutic options.

The absence of specific molecular or immunohistochemical markers for definitive PDAC diagnosis is a significant hurdle for pathologists to overcome, particularly in metastatic settings or cancer of unknown primary (CUP) [4]. Moreover, PDAC has very few actionable targets, limiting clinical stratification and personalized therapy. The disease is primarily driven by alterations in four key genes: *KRAS*, *TP53*, *SMAD4*, and *CDKN2A* [5]. Recent advances in genome-wide epigenetic profiling, particularly DNA methylation analysis, have emerged as promising solutions to these challenges. DNA methylation patterns are highly tissue-specific [6,7]) and therefore have the potential to determine tissue of origin, improve diagnosis and subclassification of PDAC, and offer new insights into disease progression.

2. Current diagnostic challenges in PDAC

From a pathological perspective, PDAC poses two major diagnostic challenges:

The first challenge is the lack of specific diagnostic markers. There is no immunohistochemical (IHC) or molecular marker that definitively confirms PDAC. Most samples show positivity for CK7 (84.6–100%) and CK19 (~99.4%), while CK20 is variably positive (~20.1%), similar to other adenocarcinomas of the upper gastrointestinal tract [8,9]. ANXA10 is also expressed in ~ 78% of cases [10], and ~55% of samples are *SMAD4* mutated, showing loss of expression – thus making the IHC

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Article highlights

Genome-wide DNA methylation for the diagnosis of PDAC and other pancreatic tumors

- Several classifiers are available for the diagnosis of PDAC, and PDAC metastases, including in a cancer of unknown primary setting.
- Based on DNA methylation profiles, a landscape of pancreatic tumors was developed, offering a better understanding of the relationship between pancreatic tumors.
- Using DNA methylation profiles, a classifier was developed for the differential diagnosis of pancreatic tumors.

DNA methylation offers a better understanding of the pathogenesis of PDAC and its metastasis mechanism.

- Recent DNA methylation data reveal that PDAC liver metastases are different from PDAC peritoneal carcinomatosis and primary PDAC, while PDAC peritoneal carcinomatosis and primary PDAC are very similar at the epigenetic level.
- Acinar-to-ductal metaplasia preserves aberrant DNA methylation even after morphological recovery, leaving the cells "epigenetically primed" toward PDAC progression.
- PDAC precursors have different methylation profiles, with distinct patterns observed between pancreatic intraepithelial neoplasia, intraductal papillary mucinous neoplastic lesions of gastric and intestinal type, and mucinous cystic neoplasm.

DNA methylation offers new perspectives regarding PDAC liquid biopsy.

- Combined analysis of circulating DNA methylation biomarkers with protein markers achieves higher diagnostic accuracy in detecting early-stage PDAC.
- Successful differentiation between PDAC and chronic pancreatitis can be achieved with as little as six CpGs in cell-free DNA.
- Methylation profiles in cell-free DNA can distinguish PDAC from healthy samples with high accuracy.

profile minimally specific [11,12]. So far, ANXA10 has proven to be the only marker with promising sensitivity (90%) and specificity (66%), and notable precision (78% accuracy) in differentiating PDAC from cholangiocarcinoma [13]. However, its broad clinical adoption still requires validation in larger patient cohorts.

This lack of diagnostic markers is somewhat inconsequential in primary localized tumors but becomes particularly problematic in metastatic contexts. PDACs represent more than 20% of all unknown primary cancers, being the second most common cause of CUP [14]. Additionally, PDAC metastases to the liver are morphologically, immunohistochemically, and molecularly very similar to intrahepatic cholangiocarcinoma (iCCA). This distinction has direct therapeutic implications, as iCCA typically requires surgical resection while PDAC metastases are treated with chemotherapy. In these scenarios, a new tool is needed to confirm the diagnosis and guide appropriate therapy.

At the molecular level, approximately 60–90% of tumors show *KRAS* mutations, which is considered the most common driver in adenocarcinomas generally [15,16], hence not aiding the diagnostic process. While a broader spectrum of genomic alterations exists, these occur at low frequencies and have not, to date, led to wide targeted therapy implementation [17]. Even focusing on more peculiar and predominantly non-coding genomic regions, such as the phylogenetically perfectly conserved ultraconserved elements (UCE), does not bring additional information regarding the genomic profile of PDAC. Curiously, of all cancers, PDAC is the only one displaying a higher mutation rate in coding UCEs compared to non-coding UCEs, mainly because exon 2 of *KRAS* is an UCE [18].

The second challenge relates to the genetic uniformity stemming from the small number of driver mutations and limited stratification options. Moreover, most known prognostic markers are not strongly linked to disease progression. The molecular pathogenesis of PDAC is well-established – there are three potential precursors: pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasm (IPMN), and mucinous cystic neoplasm (MCN), all undergoing a stepwise malignant transformation from low-grade to high-grade and finally to invasive carcinoma. In parallel to these morphological transformations, the epithelial cells undergo *KRAS* mutations followed by *CDKN2A* inactivation and finally by *SMAD4* and *TP53* tumor suppressor loss [19]. Despite this understanding, it remains impossible to distinguish between PDACs arising from different precursors based on mutational profiles obtained by exome sequencing. At best, the CA19–9 antigen has shown some potential in PDAC screening; however, due to its well documented shortcomings, it has not been reliably implemented into routine diagnosis. Its limited utility as a standalone biomarker comes due to its lack of specificity as it is elevated in various malignancies (colorectal, gastric, lung, hepatobiliary) and benign conditions (e.g., cholangitis, pancreatitis, biliary obstruction, diabetes, black tea intake). It also yields false negatives in Lewis-negative individuals, and in jaundiced patients, CA19–9 levels are unreliable and cannot be corrected for hyperbilirubinemia [20]. Moreover, its sensitivity for detecting early-stage PDAC in asymptomatic individuals is too low (10–13%) [21]. Rather than functioning as a standalone, CA19–9 can, however, be approached as an additional criterion complementing other primary diagnostic methods.

Compared to other cancer types, none of the discovered molecular subtypes of PDAC have entered clinical practice. Based on unsupervised RNA-sequencing data, Bailey et al. proposed four prognostically relevant classes of PDAC: squamous, aberrantly differentiated endocrine exocrine, pancreatic progenitor, and immunogenic. Of these classes, the squamous subtype shows the worst survival. Although these data were proposed as potential opportunities for drug discovery, they did not enter clinical practice [22]. Similarly, the Cancer Genome Atlas Research Network used DNA-methylation data combined with gene expression, microRNA, and long non-coding RNA expression data using Similarity Network Fusion to propose two clusters that overlap with previously characterized basal-like and classical subtypes [16]. These classifications have not become practice-changing, largely because the complex multiomics approach is too labor-intensive and expensive for routine clinical use. Furthermore, the older Illumina Methylation array 450k was used for the DNA methylation in their study, and, since the new Illumina generation covers double the amount of CpGs, this new array could be *per se* sufficient to provide a clinically meaningful classification of PDAC, capable of also impacting the therapeutic approach.

3. Improving diagnosis – genome-wide DNA methylation classifiers for PDAC

DNA methylation-based classifiers have revolutionized tumor diagnostics. Their underlying principle is straightforward: they analyze genome-wide DNA methylation data in combination with machine learning algorithms to determine a histological diagnosis. Their success is best exemplified by the DNA methylation brain classifier [23], which has become a desirable WHO criterion for the diagnosis of several brain tumors. Such tools are increasingly under development, expected to become diagnostic aids for other types of neoplasms. For PDAC, developing similar classifiers presents unique challenges, especially for differentiating PDAC from other cancers originating from tissues of the endodermal foregut.

The complexity of DNA methylation data is characterized by extremely high dimensionality and highly correlated features; therefore, the first step in developing a classifier is to identify the subset of CpGs highly specific to PDAC and to reduce dimensionality while preserving discriminatory power. For this, the most common methods are using high standard deviation or differentially methylated probe analysis [24]. While the brain classifier uses 20,000 CpGs to distinguish among 91 classes, in a previously published intrahepatic pancreato-biliary tumor classifier [25], we succeeded in differentiating PDAC from intrahepatic cholangiocarcinoma using 2048 CpGs.

Next, the choice of machine learning algorithms significantly impacts classification performance. The brain classifier employs a Random Forest model [26] due to its ability to select the most important features and its explainability. However, for PDAC classification, Neural Networks have shown better performance compared to Random Forest or Support Vector Machine approaches [25].

Finally, a major challenge of classifier-based diagnostics is the closed-world assumption that classifiers can only select from a predetermined set of known classes. This limitation can lead to misclassification when samples belong to classes not represented in the training data. To address this issue, threshold-based methods are commonly employed, where samples with prediction confidence below a set threshold are classified as "unknown." Alternative approaches include implementing anomaly detection models to exclude samples different from known classes, measuring distance to class means, or using one-class classifiers.

4. Current status – DNA methylation profiles improve postoperative diagnosis and subclassification

Recent years have seen increased development of DNA methylation classifiers for PDAC diagnosis using various platforms and machine learning algorithms. These advances range from broad pan-cancer approaches to focused PDAC-specific classifiers aimed at improving PDAC diagnosis in both primary and metastatic settings (Table 1 and Supplementary Table S1).

Moran et al. developed a pan-cancer classifier ("EPICUP") for diagnosing CUP. Their classifier, built on a Random Forest algorithm, categorized tumors into 38 different types. It achieved 99.6% specificity, 97.7% sensitivity, and accurately predicted the primary cancer of origin in 87% of patients. Importantly, patients who received tumor-specific therapy based on the EPICUP classification had significantly improved survival compared to those who received empirical treatment [27]. While EPICUP's broad classification ability proved successful, the authors acknowledged inherent challenges, particularly in distinguishing between morphologically similar adenocarcinomas from different organs. The classifier's generalized approach, while useful in many settings, may lack the necessary specificity for more complicated tumor types such as PDAC. Notably, the classifier had a relatively limited representation of PDAC in its training ($n = 100$) and validation ($n = 57$) sets, with only nine metastatic cases in its validation cohort [27], potentially affecting classification accuracy for PDAC specifically.

In contrast to broad pan-cancer approaches, more focused classifiers have been developed specifically for PDAC diagnosis. To improve the diagnosis in a metastatic setting, we first addressed the differential diagnosis of iCCA versus PDAC liver metastases [25]. Using a reference cohort containing 205 PDAC, 144 iCCA and 50 normal bile duct samples, three different machine learning classifiers (Random Forest, Support Vector Machine and Neural Network) were developed that could differentiate between these three entities. To enhance classifier safety and increase accuracy, classification thresholds were added, and gastric adenocarcinoma and colorectal adenocarcinoma (common liver metastases) were used to develop an anomaly detection step. With these additional filters, the classifier achieved 100% accuracy with the Random Forest, 96.22% with the Support Vector Machine, and 99.07% with the Neural Network on the validation cohort ($n = 361$). The Neural Network classifier was subsequently tested on a bicentric cohort including PDAC metastases, reaching an accuracy of 95.45% with 8.33% unclassified samples [25]. Furthermore, when analyzing the correlation between CpG methylation and RNA expression, we found only 239/2048 differentially expressed CpGs between PDAC and iCCA mapping to known genes. Of these, 74% exhibited concordant changes – i.e., they were overexpressed when hypomethylated and under-expressed when hypermethylated – indicating a strong relationship between methylation and gene expression [25].

This classifier was further validated in a CUP-like scenario. To improve safety, eight additional carcinomas mimickers were added to the two existing ones, thus ensuring that any other cases other than PDAC, iCCA, and normal bile duct were excluded from diagnosis. This improved anomaly detection filter was validated on 3579 carcinomas, reaching an accuracy of 98.21%. In addition, the classifier was validated on the new array generation Infinium MethylationEPIC v2.0 930K, reaching perfect accuracy. In testing, the classifier achieved 93.75% accuracy on 16 PDAC metastases (15 non-liver) and 98.39% accuracy on the 124 non-PDAC metastases negative control [28]. Possible factors influencing the classifier results were found to be immune infiltrate [25], especially CD3+ T-cells induced lower classification scores [28] - an observation confirmed by false positives for mimicker carcinomas in lymph nodes [28].

Table 1. Current studies proposing machine learning classifiers that use DNA methylation for PDAC and other pancreatic tumor prediction. The table includes studies focused on tissue of origin identification, differentiation between PDAC and other cancers or benign conditions, and early detection through liquid biopsy approaches.

Study	Objective	Description	Web page	Ref
Moran et al.	Identifying the tissue of origin in CUP cases	Random Forest classifier based on the DNA methylation profiles of 485,577 CpGs	N/A	[27]
Dragomir et al.	Differentiating between iCCA and PDAC liver metastases	Neural Network classifier based on 2048 most variable CpGs with anomaly detection and threshold filters	N/A	[25]
Calina et al.	Differentiating PDAC metastases from various anatomical regions	Updated Neural Network classifier based on 2048 most variable CpGs, with expanded anomaly detection for 10 different mimicker carcinomas and threshold filtering	https://classifier.tgc-research.de	[28]
Verschuur et al.	Classifying non-ductal primary pancreatic neoplasms and detecting non-pancreatic entities	Neural Network, Random Forest, and extreme gradient boosting machine learning classifiers based on the 5000 most variable CpGs, with a logistic regression model for non-pancreatic detection	https://github.com/averschuur/pancreas	[37]
Draškovič and Hauptman	Distinguishing between individual pan-adenocarcinoma entities	Classifier developed based on differentially methylated probes comparing one entity versus all others	N/A	[29]
Draškovič et al.	Differentiating between primary and metastatic liver adenocarcinoma and normal tissue	Classifier based on eight differentially methylated biomarkers using methylation-specific digital PCR	N/A	[30]
Wu et al.	Differentiating primary PDAC from chronic pancreatitis in tissue and plasma	Random Forest classifier using six CpG sites in the *PRKCB* gene	N/A	[31]
Ben-Ami et al.	Detecting PDAC early through liquid biopsy	Combined biomarker panel: CA19–9, TIMP1 and a 9-loci cfDNA panel covering 61 CpGs	N/A	[32]
Wu et al.	Detecting PDAC early through liquid biopsy	Support Vector Machine classifier trained on 56 CpGs selected through methylation haplotype block analysis	N/A	[33]

CUP – Cancer of Unknown Primary.
iCCA – Intrahepatic Cholangiocarcinoma.
PDAC – Pancreatic Ductal Adenocarcinoma.
cfDNA – Cell-Free DNA.

Targeted methylation studies have also shown promise. Draškovič and Hauptman investigated the potential of DNA methylation in developing diagnostic panels for adenocarcinomas, including PDAC. They used two complementary analytical methods: first, they analyzed all samples of each cancer type collectively (non-clustered approach); second, they first grouped samples within each cancer type into subgroups based on similar methylation patterns before analysis (clustered approach). Of all cancer PDAC and lung adenocarcinoma showed the fewest hypermethylated CpG sites. Using their first method, they identified two probes (cg01237565 and cg00955911) that were hypermethylated in PDAC, though these were also hypermethylated in normal pancreatic tissue. With their second method, they developed a four-probe panel (cg01237565, cg20178172, cg16788099, and cg19326153). They achieved diagnostic accuracies of 85.3% (non-clustered) and 94.5% (clustered). When applied to PDAC liver metastases, these panels maintained good accuracies: 86.8% (non-clustered) and 94.5% (clustered) [29]. These findings show that more complex approaches beyond simple differentially methylated CpG identification in one group versus all other groups are necessary.

In a subsequent study, the same research group validated previously identified DNA methylation biomarkers to differentiate between the most common primary liver tumors, hepatocellular carcinoma and iCCA, as well as between common liver metastases: PDAC and CRC. They used methylation-sensitive high-resolution melting for initial screening, followed by digital PCR for quantification. Using PROX1-AS1 promoter methylation as a biomarker for PDAC, they reached diagnostic accuracies of 93%-98% (dependent on threshold) for differentiating liver metastases from primary tumors and healthy tissue, and 86.7% sensitivity for primary PDAC detection. The limiting factor for clinical utility, however, constitutes the low detection sensitivity for metastatic lesions (80%). The comparable methylation patterns for selected genes observed between primary PDAC and PDAC liver metastases suggest preservation of methylation status across disease progression, though direct paired-sample comparisons were not performed [30]. Generally, such targeted methylation studies analyzing specific CpGs pave the way for liquid biopsy approaches for noninvasive PDAC diagnosis.

For early diagnosis and screening applications, DNA methylation analysis of cell-free DNA (cfDNA) shows great promise. This can be done with or without prior amplification to reach the necessary DNA amounts. Several studies exploring such methodological approaches have recently started emerging. Wu et al. developed a Random Forest DNA methylation classifier that can differentiate PDAC from chronic pancreatitis. The authors subsequently refined their analysis to only 10 CpGs, achieving perfect accuracy on a small test set. When tested on cfDNA from plasma using whole-genome bisulfite sequencing, five of these 10 selected CpGs showed differential methylation between groups [31]. In a similar approach, Ben-Ami et al. performed PCR amplification and sequencing of 61 pancreas-specific CpGs from nine different loci on cfDNA, combining these with protein markers CA19–9 and TIMP1 to achieve high diagnostic power in discriminating early-stage PDAC from healthy controls (AUC 0.86) [32].

Taking a different approach, Wu et al. developed the "PDACatch" assay, a classifier focusing on methylation haplotype blocks (MHBs) rather than individual CpGs to detect early PDAC in blood samples [33]. MHBs are genomic regions with correlated methylation patterns across multiple CpG sites,

where the methylation status at one CpG site predicts that of nearby sites [34]. The researchers identified a 56-marker methylation signature from methylated and unmethylated haplotypes and trained a Support Vector Machine classifier that distinguished PDAC from healthy samples with high accuracy (AUC 0.91), which improved to AUC 0.94 when combined with CA19–9 [33]. While these studies provide a good proof of concept that DNA methylation can be used as a liquid biopsy screening tool, larger validation trials are needed to confirm performance in discriminating between different cancer types, chronic pancreatitis patients and early-stage PDAC, and to establish reproducibility in clinical practice.

In parallel, DNA methylation has been utilized to characterize the diverse spectrum of primary pancreatic tumors. Beyond PDAC, which is by far the most common pancreatic tumor, the pancreas gives rise to several types of solid and cystic tumors. Benhamida et al. compiled the first DNA methylation landscape of pancreatic tumors, showing that pancreatoblastoma and acinar cell carcinoma share similar DNA methylation profiles [35]. Taking a phylo-epigenetic approach, Simon et al. showed that pancreatic neuroendocrine carcinomas (PanNECs) are distinctly separate from pancreatic neuroendocrine tumors (PanNETs), as they arise from different cells of origin. Their methylation analysis revealed that PanNETs derive from endocrine cells of the Langerhans islands, whereas PanNECs originate from the exocrine (specifically acinar) compartment, sharing similarities only with the acinar cell profile of PDAC [36]. Further advancing DNA methylation for pancreatic tumor diagnosis, Verschuur et al. developed a classifier to distinguish between six primary solid pancreatic tumor types: PDAC, acinar cell carcinoma, pancreatoblastoma, PanNEC, PanNET, and solid pseudopapillary neoplasm. Using three different machine learning methods, their Random Forest classifier achieved 96.9% accuracy without thresholds and 100% accuracy with thresholds (using 13% of the samples). To address the rare occurrence of metastases to the pancreas, they also developed a logistic regression classifier to exclude non-pancreatic neoplasms, reaching nearly perfect accuracy [37]. Future versions of such classifiers will also need to cover pancreatic cystic lesions which can pose additional diagnostic challenges. For now, a panel of two methylated cfDNA markers (*TBX15, BMP3*) has been successfully identified by Majumder et al. to detect high-grade dysplasia in pancreatic cystic lesions (specifically IPMN and its intermediate precursor, MCN, serous cystadenoma, and inflammatory/nondysplastic cysts) [38], but not to specifically differentiate these cysts from PDAC as a separate entity.

DNA methylation has also proven valuable in characterizing new pancreatic tumor entities. Basturk et al. used DNA methylation to profile a newly described mesenchymal lesion – sclerosing epithelioid mesenchymal neoplasm of the pancreas. This neoplasm, characterized by epithelioid and spindle cells with CD99 and vimentin immunolabeling, lacked pathogenic mutations or fusions. DNA methylation analysis confirmed it as a unique entity with methylation patterns like angiomatoid fibrous histiocytoma [39]. Hence, the utility of methylation profiling in defining novel entities where other molecular approaches fall short.

Based on the review by Köelsche and von Deimling, in which the authors discuss brain and sarcoma classifier use in clinical practice [40] and also based on our observations regarding biological factors affecting classifier accuracy, a clinical implementation pipeline for the PDAC classifier has been proposed [28]. For a sample to be classified as PDAC, the DNA methylation classifier should identify it as PDAC (passing anomaly detection filters and reaching a calibrated score > 0.8), and at least one of four additional diagnostic criteria should support this diagnosis: history of PDAC, suggestive imaging, supportive mutational data, or compatible IHC findings. Samples that fail anomaly detection or have low calibrated scores without additional supporting criteria would be excluded from PDAC diagnosis. Two "gray zone" scenarios require additional approaches. When the classifier suggests PDAC, but no additional diagnostic criteria support this conclusion, we recommend performing additional imaging, DNA mutational analysis or even fusion analysis in cases with *KRAS* wild type [41], as well as expanding IHC analysis (incorporating ANXA10 to the panel to differentiate PDAC from iCCA) [13]. Conversely, if the classifier suggests "No match," but additional criteria suggest PDAC, repeating the DNA methylation analysis is recommended, potentially using samples from different metastatic sites or the primary tumor, as immune infiltration (especially in lymph node metastases) can affect classification scores. In such cases, other methylation classifiers such as the sarcoma classifier or brain classifier [23,42] can be used for additional information.

5. Molecular subtyping of PDAC based on DNA methylation and its role in disease progression

While the mutational pathogenesis of PDAC and its precursors (PanIN, IPMN, and MCN) is well-characterized [43–45], their epigenetic aspects have not been fully explored. Genome-wide studies have demonstrated that DNA methylation plays a significant role in PDAC initiation and progression. Using methylation-specific PCR, Sato et al. found that at least one of seven genes aberrantly methylated in PDAC was also methylated in approximately 80% of IPMN cases. They observed increasing numbers of methylated loci with disease progression, implicating *de novo* CpG island methylation in IPMN malignant evolution [46]. These findings were extended to PanIN by a subsequent study showing that eight genes methylated in PDAC also exhibited abnormal methylation in over 70% of early PanIN lesions, with methylation frequency increasing with PanIN grade. This pattern supports the hypothesis that epigenetic alterations accumulate during neoplastic transformation [47]. In an effort to investigate the timing of the aberrant epigenetic accumulation events, Hong et al. identified several genes (*BNIP3, PTCHD2, SOX17, NXPH1* and *EBF3*) more likely to be differentially methylated in high-grade than in low-grade IPMN, highlighting progression-associated epigenetic changes [48].

Liffers et al. characterized distinct DNA methylation profiles to differentiate between PanIN, gastric IPMN (gIPMN), and intestinal IPMN (iIPMN). PanIN and gIPMN displayed remarkably similar methylation and gene expression signatures traceable to the ductal cell compartment, while iIPMN exhibited

a distinctly divergent profile, suggesting a different cell of origin. The study not only identified specific molecular markers corresponding to phenotypic variations (*TFF3* expression in iIPMN and differential *MUCL3* expression between gIPMN and PanIN) but also showed no significant differences in methylation patterns between low-grade and high-grade lesions. While the classification of gIPMNs and iIPMNs as distinct biological entities is a relevant differentiation, the study further underscores the molecular and epigenetic heterogeneity among PDAC precursor lesions [49].

Leoni et al. explored the characterization of pancreatic MCN by analyzing their epigenetic profiles within a pancreatic, hepatic, and ovarian landscape. Notably, rather than clustering with PDAC, both high-grade, low-grade, and invasive MCNs showed greater epigenetic similarity to mucinous ovarian tumors, suggesting a potential common origin [50]. Emerging methylation profiling hints toward biologically distinct PDAC precursors. In the future, meaningful stratification could inform prognosis and refine the development of novel therapies – particularly since precursor lesions are often histologically unidentifiable – while also enabling the identification of specific methylated genes that may contribute to PDAC development or serve as clinically relevant biomarkers.

An investigation into the cell-of-origin sets up an interesting framework. Lo et al. compared methylation profiles across acinar cells, ductal cells, PanIN, and PDAC, concluding that PanIN likely arises from acinar cells [51]. This supports the known murine model of acinar-to-ductal metaplasia (ADM), a reprogramming event triggered by injury or inflammation that facilitates neoplastic transformation [52]. Although it is disputed whether the process similarly translates in humans [53], their model sees PDAC development following an acinar-to-ADM-to-PanIN-to-PDAC progression pathway rather than originating directly from ductal cells. The epigenetic analysis revealed that PanIN lesions exhibit an intermediate methylation state between normal cells and PDAC in 97.6% of PanIN-specific differentially methylated regions, suggesting they are "epigenetically primed" for progression to PDAC, once again, supporting a shared epigenetic lineage [51].

In a subsequent murine study, the same researchers demonstrated that acinar cells which recovered after ADM retained aberrant DNA methylation in PI3K pathway genes even after returning to their normal phenotype. This "methylation memory" suggests a potential molecular mechanism for increased cancer susceptibility following recurrent pancreatitis [54]. Complementing these findings, Espinet et al. identified two distinct PDAC subtypes based on methylation patterns of repeat elements in human epithelial tumor cells. The MC1 (methylation cluster 1) subtype showed higher methylation and low interferon signaling, while MC2 (methylation cluster 2) exhibited lower methylation with high interferon signaling and worse patient outcomes. Their lineage analysis suggested different cellular origins for these subtypes, with MC1 appearing to originate from acinar cells and MC2 from ductal cells [55]. This dual-origin hypothesis is compatible with the acinar-origin model proposed by Lo et al., while suggesting a parallel ductal-origin pathway for more aggressive PDAC subtypes.

Genetic alterations in PDAC pathogenesis are well characterized, and analyses of primary and metastatic lesions have revealed surprisingly few differences in driver gene mutations [56,57]. This either suggests that the mutations in primary tumors are sufficient to induce distant metastases or that other molecular alterations drive PDAC metastases.

To investigate this question, we took advantage of a comprehensive dataset of PDAC liver metastases and PDAC peritoneal carcinomatosis to obtain a deeper understanding of the epigenetic changes of PDAC during cancer cell spread. We observed that PDAC peritoneal carcinomatosis samples tend to group with primary PDAC, while PDAC liver metastases grouped separately. More specifically, PDAC liver metastases show a hypomethylation of enhancers and promoters compared to PDAC peritoneal carcinomatosis and primary PDAC, particularly at loci associated with epithelial-mesenchymal transition (EMT) processes. Pathway analysis revealed no differentially methylated pathways between primary PDAC and peritoneal metastases; however, liver metastases showed significant EMT-related methylation differences when compared to both groups [28]. Altogether, these observations led us to speculate that PDAC peritoneal carcinomatosis is not a true metastasis, but only a large primary reaching the peritoneal borders and spreading into the abdominal cavity. Of note, this study did not analyze ideally metachronous matched primary-metastasis samples, which would have offered us a unique evolutionary perspective of the tumor. Beyond DNA methylation, other epigenetic modifications, such as chromatin modifications, were proposed as potential mechanisms explaining the metastatic process. For example, McDonald et al. observed that a reprogramming of chromatin domains during PDAC evolution increased glucose metabolism via the pentose phosphate pathway in metastatic sites [58].

6. Conclusion

In conclusion, DNA methylation profiling shows promise in addressing diagnostic challenges in PDAC where conventional approaches lack specificity. Array-based methods are already improving postoperative diagnosis, while emerging nanopore sequencing technologies could potentially reduce processing time from days to hours, enabling intraoperative use. Machine learning improves classification accuracy while managing tumor heterogeneity, and advances in targeted amplification techniques have reduced input DNA requirements, opening the door toward early diagnosis through minimally invasive liquid biopsies. As research progresses, methylation analysis may evolve to become complementary to PDAC diagnostics alongside current molecular and histopathological methods, though the path from promising research tools to clinical standards is still in need of large-scale validation.

7. Future perspectives – DNA methylation profiles improve preoperative and intraoperative diagnosis

As emerging data indicate that methylation patterns could reflect divergent biological origins, DNA methylation profiling may also contribute to a better understanding of PDAC pathogenesis and help uncover key regulatory pathways in tumor progression, offering opportunities for risk stratification, early

detection and prevention. Building upon recent progress, current DNA methylation classifiers show potential for diagnosing PDAC across various anatomical regions and distinguishing between the most common solid tumors within the pancreas. While these advances significantly improve diagnostic pathology, the methodology remains too slow for intraoperative application, and the required DNA quantity is too high for liquid biopsy approaches using blood, pancreatic cyst fluid, or brush cytology material. Emerging technologies promise to overcome these limitations and deliver much more efficient sequencing results.

Nanopore sequencing represents such a significant methodological advance for DNA methylation analysis, capable of reducing the diagnostic timeline from several days to a few hours [59]. Presenting as an improvement to current applications, the nanopore technology could enable intraoperative DNA methylation diagnosis, essentially functioning as a molecular frozen section diagnostic tool [60,61], capable of detecting tumor infiltration in surgical resection margins (Figure 1). Of note, nanopore limitations include requiring high-quality, high molecular weight DNA that may restrict certain tissue analyses, potential failure to detect weak methylation signals, and, although it offers direct detection of various modifications, the algorithms for signal interpretation have largely been trained using data from bisulfite sequencing, potentially inducing the same biases of the original method [62–64]. Moreover, while rapid for sequencing-based methods, its real-time detection speed is not comparable to the near-instantaneous capabilities of techniques such as Surface-enhanced Raman spectroscopy [65].

Recent advances in machine learning algorithms will make such classifiers more precise and significantly more interpretable, making it possible to combine laboratory data, imaging, morphology, and molecular data in a multimodal fashion. Autoencoder-based models [66,67], such as MethylNet [68], address the high dimensionality issues and help with both denoising and imputation, and feature interpretability [69]. Transformer models have been adapted from large language models and applied to DNA methylation data. By treating methylation patterns or genomic regions analogous to sequences, using self-attention to capture relationships across CpG sites or between DNA sequence context and methylation state, they are applied in read-level methylation data analysis [70], methylome imputation [71], and methylation site prediction [72,73]. Graph Neural Networks (GNNs) can model the relational structure of DNA methylation data, representing methylation profiles as graphs where nodes can be CpG sites or genomic regions, and edges encode biological relationships (genomic proximity, co-methylation correlations, or regulatory interactions). For example, MSI-XGNN uses a GNN on a gene-methylation graph to classify microsatellite instability with high accuracy while identifying key immune-related biomarkers [74].

Integration of DNA methylation with other omics data, such as transcriptomics and proteomics, provides a more comprehensive disease perspective. DeepAutoGlioma [75] fuses transcriptome and methylome data to support glioma subtype diagnosis. Meanwhile, Multi-omics Attention Deep Learning Network (MOADLN) uses attention-based fusion to balance the contribution of different omics data to avoid bias toward one data type, thus outperforming other baseline methods in cancer classification [76]. Yassi et al. offer a comprehensive overview of machine learning in cancer epigenetics [77]. Particularly for PDAC, integrative multi-omics approaches may uncover previously undetected molecular patterns, creating more efficient personalized treatment strategies.

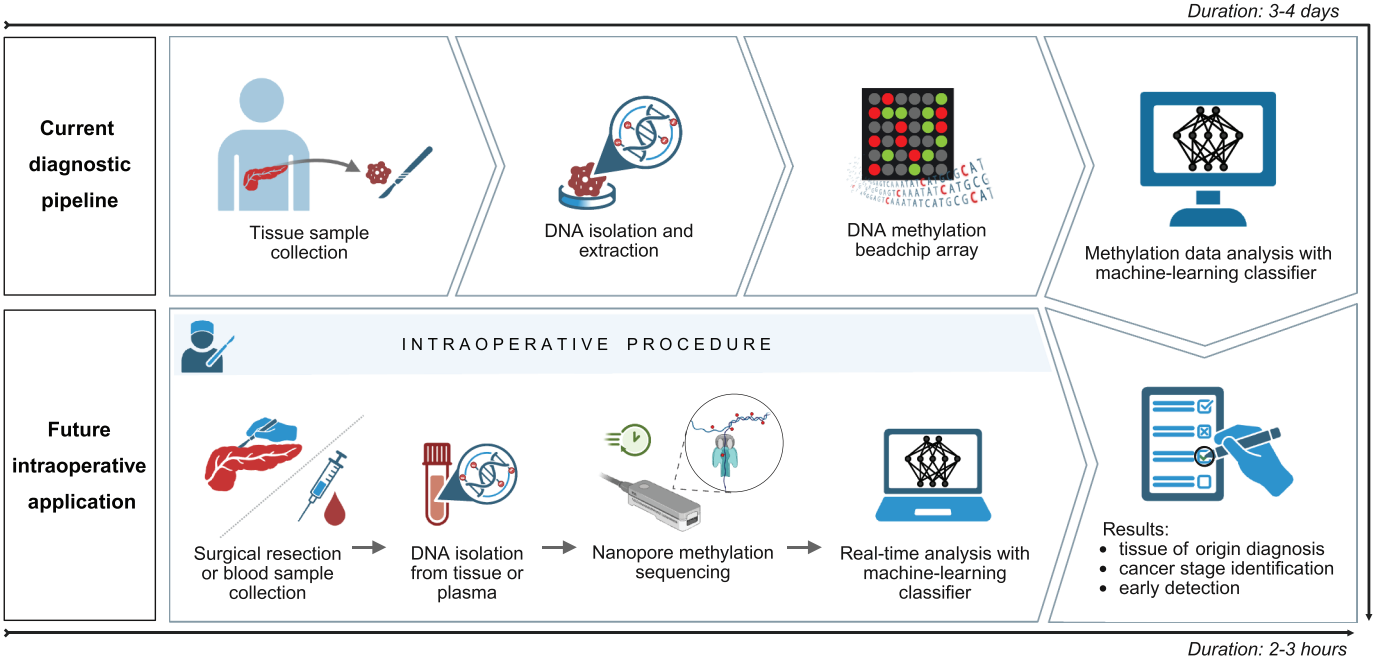

Figure 1. Current diagnostic pipeline using DNA methylation for PDAC and proposed future diagnostic application in an intraoperative/liquid biopsy setting. Created with BioRender.com/i53d5sj.

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The authors used ChatGPT (OpenAI, GPT-4) and DeepL (www.deepl.com) for language and grammar proofreading only. No content was generated using these tools.

Author contributions

Elena Grafenhorst and Mihnea P. Dragomir designed the framework. Elena Grafenhorst prepared the figure and table. Elena Grafenhorst, Teodor G. Calina, and Mihnea P. Dragomir contributed to writing the manuscript. Mihnea P. Dragomir supervised the work. Elena Grafenhorst, Teodor G. Calina, and Mihnea P. Dragomir reviewed and edited the draft. All authors read and approved the final manuscript.

Disclosure statement

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