Diagnosis, differentiation and prevention in pancreatic diseases

Engels, M.M.L.

SECTION III

BIOMARKERS

ABSTRACT

Background and aims: In previous studies, methylated DNA markers (MDMs) have been identified in pancreatic juice (PJ) for detecting pancreatic ductal adenocarcinoma (PDAC). In this prospective multicenter study, the sensitivity and specificity characteristics of this panel of PJ-MDMs was evaluated standalone and in combination with plasma carbohydrate antigen 19-9 (CA 19-9).

Methods: Paired PJ and plasma were assayed from 88 biopsy-proven treatment-naïve PDAC cases and 134 controls (53 with normal pancreas, 23 with chronic pancreatitis [CP], 58 with intraductal papillary mucinous neoplasm). Bisulfite-converted DNA from buffered PJ was analyzed using long-probe quantitative amplified signal assay targeting 14 MDMs *(NDRG4, BMP3, TBX15, C13orf18, PRKCB, CLEC11A, CD1D, ELMO1, IGF2BP1, RYR2, ADCY1, FER1L4, EMX1, and LRRC4)* and a reference gene (methylated B3GALT6). Logistic regression was used to fit the previously identified 3-MDM PJ panel (*FER1L4, C13orf18, and BMP3*). Discrimination accuracy was summarized using area under the receiver-operating characteristic curve (AUROC) with corresponding 95% confidence interval (CI).

Results: Methylated *FER1L4* had the highest individual AUROC of 0.83 (95% CI, 0.78–0.89). The AUROC for the 3-MDM PJ D plasma CA 19-9 model (0.95; 95% CI, 0.92–0.98) was higher than both the 3-MDM PJ panel (0.87; 95% CI, 0.82–0.92)) and plasma CA 19-9 alone (0.91; 95% CI, 0.87–0.96) (P = .0002 and .0135, respectively). At a specificity of 88% (95% CI, 81%–93%), the sensitivity of this model was 89% (95% CI, 80%–94%) for all PDAC stages and 83% (95% CI, 64%–94%) for stage I/II PDAC.

Conclusion: A panel combining PJ-MDMs and plasma CA 19-9 discriminates PDAC from both healthy and disease control groups with high accuracy. This provides support for combining PJ and blood based biomarkers for enhancing diagnostic sensitivity and successful early PDAC detection.

John William Waterhouse - the Rescue (1890)

CHAPTER 5 - MULTIMODAL PANCREATIC CANCER DETECTION USING METHYLATED DNA BIOMARKERS IN PANCREATIC JUICE AND PLASMA CA 19-9: A PROSPECTIVE MULTICENTER STUDY.

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Megan M. L. Engels[1,2]*, Calise K. Berger[3]*, *Douglas W. Mahoney[5], Sanne A. Hoogenboom[1,2], Dhruv Sarwal[3], Derk C. F. Klatte[1,2], Jaime De La Fuente[3], Sonal Gandhi[4], William R. Taylor[3], Patrick H. Foote[3], Karen A. Doering[3], Adriana M. Delgado[3], Kelli N. Burger[5], Barham K. Abu Dayyeh[3], Aliana Bofill-Garcia[3], Bhaumik Brahmbhatt[1], Vinay Chandrasekhara[3], Ferga C. Gleeson[3], Victoria Gomez[1], Vivek Kumbhari[1], Ryan J. Law[3], Frank J. Lukens[1], Massimo Raimondo[1], Elizabeth Rajan[3], Andrew C. Storm[3], Eric J. Vargas Valls[3], Jeanin E. van Hooft[2], Michael B. Wallace[1,4], John B. Kisiel[3], and Shounak Majumder[3]*

**** Authors share co-first authorship***

AFFILIATIONS:

[1] Division of Gastroenterology and Hepatology, Mayo Clinic, Jacksonville, Florida
[2] Division of Gastroenterology and Hepatology, Leiden University Medical Center, Leiden, the Netherlands
[3] Division of Gastroenterology and Hepatology, Mayo Clinic, Rochester, Minnesota
[4] Division of Gastroenterology and Hepatology, Sheikh Shakhbout Medical City, Abu Dhabi, United Arab Emirates
[5] Division of Clinical Trials and Biostatistics, Mayo Clinic, Rochester, Minnesota

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the third leading cause of cancer-related death.[1] This is a result of global increase in incidence, and a high proportion of late-stage diagnoses with limited survival.[2] While pancreatic cancer screening is not recommended in average-risk populations, surveillance programs have been established for individuals at high risk, such as those with neoplastic pancreatic cysts [3-5] and familial or genetic predisposition.[6,7] Pancreatic cancer surveillance in high-risk individuals has demonstrated a shift toward a higher proportion of earlier stage PDAC diagnosis, and this stage shift has resulted in improved survival in screen-detected PDAC.[8-10]

Although plasma cancer antigen 19-9 (CA 19-9) is the most widely used tumor marker for treatment monitoring in patients with PDAC, it is ineffective as an early detection biomarker by itself and hence not currently utilized for surveillance.[6] Given 10% of the population cannot produce CA 19-9, its low sensitivity in early-stage PDAC, and false positive elevation in benign biliary and pancreatic diseases, there is a need for alternative and/or supplemental diagnostic biomarkers.[11] Diagnostic biomarker research in PDAC has burgeoned over the past decade with several genetic and epigenetic candidates,[12] yet there is a paucity of biomarkers that detect early-stage PDAC with high sensitivity and have reproducible results in rigorous independent and adequately powered prospective validation studies. Moreover, most studies to date have compared PDAC cases with normal healthy controls and have not reported biomarker distributions in inflammatory and low-grade neoplastic controls such as chronic pancreatitis and intraductal papillary mucinous neoplasms (IPMNs) with low-grade dysplasia.[12]

PDAC originates in the pancreatic ductal epithelium, and exfoliated neoplastic cells are shed in pancreatic juice (PJ). This offers biological rationale for developing molecular assays in PJ for detecting early-stage PDAC.[12] Moreover, an accurate diagnostic test for PDAC in PJ can serve as a complimentary test to blood-based PDAC biomarkers, blood-based multicancer cancer detection tests, and endoscopic ultrasound (EUS)–based pancreatic cancer screening in high-risk individuals. We have previously demonstrated that methylated DNA markers (MDMs) assayed from PJ can detect PDAC at meaningful sensitivity and specificity.[13,14] The PJ-MDMs were derived from candidate tissue MDMs discovered and validated by our group using unbiased reduced representation bisulfite sequencing aimed at distinguishing PDAC and high-grade precursors of PDAC from normal pancreas and low-grade precursor lesions.[15-17] These hypermethylated regions, individually, map to genes that operate in pathways known to be critical to tumorigenesis: transcriptional regulation and signal transduction. In a pilot study, we demonstrated that a 3-marker MDM panel assayed from PJ was able to detect all-stage PDAC with 83% sensitivity at a predefined 86% specificity cutoff.[13] This initial study included only a limited number of early-stage PDAC cases but established proof of concept.

In this current multicenter study, we leveraged a larger independent sample set of prospectively collected PJ samples with the aim to assess the diagnostic performance of the previously reported panel of PJ-MDMs in distinguishing PDAC cases from both normal healthy controls and controls with pancreatic diseases including chronic pancreatitis (CP) and IPMNs and compare the diagnostic performance of the PJ-MDMs, stand-alone and in combination with paired plasma CA 19-9.

MATERIALS AND METHODS

STUDY DESIGN AND PARTICIPANT CHARACTERISTICS

Patients presenting to Mayo Clinic Rochester and Mayo Clinic Florida between January 2018 and August 2022 were prospectively screened for inclusion in this study. Patients were consented to Institutional Review Board protocols #17-005211, #18-011965, or #20- 012752 and phenotypical data were electronically entered into a secure database by trained study coordinators using a predefined case-report form. This study was conducted in accordance with the Declaration of Helsinki and STARD (Standards for Reporting Diagnostic Accuracy) reporting guidelines were followed.[18,19]

Cases included biopsy proven treatment-naïve primary PDAC or IPMN with high-grade dysplasia (HGD) or IPMN-associated pancreatic cancer. Patients with pancreatic biopsy within 3 days prior to PJ collection, prior pancreatectomy, recurrence of previously treated pancreatic cancer, treatment with chemotherapy class drugs

within 6 months prior to PJ collection, prior abdominal radiation, or other histologic variants of pancreatic cancer (neuroendocrine, adenosquamous, hepatoid, and acinar cell carcinoma) were excluded.

Controls included both individuals without any known pancreatic disease (normal controls) and those with benign pancreatic disease such as chronic pancreatitis or low-grade cystic neoplasm (cyst without worrisome or high-risk features, or benign/low-grade dysplasia on biopsy). Controls who received chemotherapy within the prior 6 months, or prior therapeutic abdominal radiation, or had undergone a pancreatic biopsy within 3 days before PJ collection were also excluded. Chronic pancreatitis was defined by the presence of radiological features including pancreatic calcifications and ductal dilation. For patients with pancreatic cysts, radiological features, biochemical cyst fluid markers when available (CEA, amylase, and *KRAS/GNAS*) and cytopathology were used to define the cyst as likely IPMN based on consensus between study team members (S.M., M.B.W., and M.M.L.E.); control status was further established by the absence of worrisome or high-risk features at the time of PJ collection as defined by the Fukuoka criteria.[4]

PJ COLLECTION AND STORAGE

Endoscopic procedures were performed after an overnight fast and under conscious sedation or anesthesia per institutional clinical standard of care. PJ collection was performed prior to fine needle aspiration/fine needle biopsy, brushing, or stent placements if performed during the same endoscopic procedure. Gastric fluid was first aspirated and discarded before intubation of the pylorus to minimize contamination. Synthetic human secretin (ChiRhoClin) at a dose of 0.2 mg/kg (or 16 mg for all patients 80 kg) was administered intravenously over 1 minute. With the endoscope positioned in the second portion of the duodenum, the dedicated aspiration catheter was passed through the instrument channel, and suction was only applied once the tip of the catheter was visualized in the vicinity of the papilla. From within the duodenum and without cannulation of the papilla of Vater, PJ was collected via suctioning. Fluid was continuously aspirated from the duodenum starting after secretin administration and continuing for 10 minutes or until 10 mL of fluid was aspirated. A proprietary DNA stabilization EDTA-based buffer solution (Exact Sciences) was added to the PJ immediately following collection in the endoscopy suite. Samples were subsequently aliquoted into 1 mL vials and stored at −80 C until use.

SAMPLE PREPARATION AND ASSAYS

DNA was purified from 850 mL of buffered PJ using the Maxwell RSC cfDNA plasma kit and RSC instrumentation (Promega). DNA was bisulfite converted with the EZ DNA Methylation-Gold kit (Zymo Research). A fixed weight of DNA (100 ng) per sample was assayed by Long-Probe Quantitative Amplified Signal (Exact Sciences) triplexes on the Roche 480 LightCyclers system. Seven triplexes were con-

figured, each with 2 unique PDAC MDMs, a common methylated reference gene (*B3GALT6*), and a specific FRET cassette assigned to each target for fluorescence detection and signal differentiation. The 14 candidate MDMs included *NDRG4, BMP3, TBX15, C13orf18, PRKCB, CLEC11A, CD1D, ELMO1, IGF2BP1, RYR2, ADCY1, FER1L4, EMX1*, and *LRRC4*. Absolute real-time quantitation of marker levels was determined using linear regression of plasmid derived standards for each of the MDMs. Using this fixed DNA input approach (n = 269) indicated suboptimal agreement of *B3GALT6* between the triplexes (**Supplemental Figure 1A**). MDM areas under the receiver-operating characteristic curve (AUROCs) for this fixed DNA input experiment are summarized in **Supplemental Figure 2**. The experiment was then repeated using all available residual DNA in cases and controls from the initial fixed volume of PJ using the same assay platform and candidate marker panel. The second experiment showed high fidelity of *B3GALT6* across the triplexes (correlation 0.999) (**Supplemental Figure 1B**). The data from this experiment coupled with availability of paired plasma samples for CA 19-9 testing constituted the final sample set for analysis (n = 222), as outlined in **Figure 1**. Laboratory personnel were blinded to patient data and case–control status.

STATISTICAL METHODS AND SAMPLE SIZE JUSTIFICATION

The primary analysis aims were to (1) fit a 3-MDM panel (*FER1L4, C13orf18, BMP3*) identical to the 3- MDM PJ panel reported in our previous study,13 with and without the addition of plasma CA 19-9, to assess predictive accuracy and (2) assess the relative predictive performance of all 14 MDMs. Logistic regression of the endpoint PDAC (yes/no) was used to fit the 3-MDM panel comprising of the 3 most discriminant individual MDMs with/without the addition of CA 19-9. Bootstrap cross-validation was performed by randomly selecting an equivalent number of patients as the original data with replacement, refitting the models, and assessing the predictive accuracy in patients not selected to build the model. This was performed 500 times. Prediction accuracy was measured using the AUROC with corresponding 95% confidence interval (CI). Comparisons between paired AUROC from different submodels were based on the methods of DeLong.[20] Covariate effects of clinical characteristics on the predictive accuracy of models were based on a z-test between stratified AUROCs. To evaluate the relative predictive importance of individual MDMs, a random forest model utilizing all MDMs was trained and cross-validated for the prediction of PDAC (yes/no) status. Individual MDMs were ranked for their predictive importance by randomly permutating the MDM level of 1marker within the validation set while holding all other MDMs fixed. After permutation, the impact on model prediction accuracy was calculated as the mean decrease in accuracy. MDMs with slight change in prediction accuracy after permutation provide little predictive importance, whereas MDMs with larger changes have higher predictive importance. Random forest was selected for this assessment as it doesn't assume linear effects of the MDMs and is able to fit high-order interaction effects.

Hierarchical cluster analysis, using complete linkage, of MDMs was performed to assess similarities between MDMs levels across all patients. Sample size estimates were based on the methods developed by McKeigue.[21] We assumed that with an infinite sample size, the best performance a prediction model could attain would be a cross-validated AUROC of 0.9. Desiring to capture 90% of the information content of the infinitely trained model required a minimum of 77 cases and 77 controls for assessing a 14-MDM panel. Qualitative patient characteristics were summarized as a percent of group totals, whereas quantitative characteristics are summarized as a median (interquartile range). Throughout, a specificity threshold of 88% was selected to match the false positive rate of CA 19-9 35 U/mL in the control group of healthy controls, CP, and IPMN patients.

RESULTS

BASELINE CHARACTERISTICS

The case group comprised of 88 biopsy-proven treatment-naïve PDAC patients; 33% had early-stage disease (stage I/II) and 67% had late-stage (III/V) disease. The control group comprised of 134 participants including normal healthy controls (n = 53), CP patients (n = 23) and IPMN patients (n = 58). There were 2 patients who were hospitalized within 7 days after PJ collection with symptoms possibly related to endoscopic PJ collection. One patient was hospitalized with postprocedure acute exacerbation of chronic abdominal pain and discharged the following day with appropriate pain management. The other patient was also hospitalized with postprocedure abdominal pain after undergoing EUS fine needle aspiration of a pancreatic mass and biliary stent placement. This patient was managed conservatively and discharged 2 days later. Clinical and demographic characteristics of cases and controls are summarized in **Table 1**.

DIAGNOSTIC PERFORMANCE OF MDMS IN PJ

When considering individual marker performance, *FER1L4*, *C13orf18*, and *BMP3* had the highest AUROC levels, corroborating our previously published results (**Supplemental Table 1**). The distributions of these top 3 individual MDMs across the case and control groups and individual AUROCs are summarized in **Figures 2** and **3A**, respectively. Variable importance ranking indicated that *FER1L4* and *C13orf18* were the most informative PJ-MDMs for distinguishing cases from both normal and disease controls. When considering how individual PJ-MDMs cluster with one another, there were 2 subclasses observed. The subclass containing the top 6 most informative markers included *BMP3* (**Supplemental Figure 3**). Based on these results, we refit the previously published 3-MDM panel (*FER1L4, C13orf18,* and *BMP3*) to the data from the current sample set. The 3- MDM (*FER1L4*, *C13orf18*, and *BMP3*) PJ panel achieved an AUROC of 0.87 (95% CI, 0.82–0.92) and a crossvalidated AUROC of 0.86 (95% CI, 0.79–0.93). At an 88% specificity cutoff for the 3-MDM panel, the sensitivity for detecting any stage of PDAC was 73% (95%

CI, 62%–82%) and 55% (95% CI, 36%–74%) for stage I/II PDAC. False positives for normal controls, CP, and IPMN patients were 8% (95% CI, 2–18%), 9% (95% CI, 1%–28%), and 17% (95% CI, 9%–29%), respectively. When considering models combining *FER1L4* and *C13orf19* with *PRKCB*, *CD1D*, or *ADCY1*, which were the other MDMs in the top 6 subclass, we observed almost identical cross-validated AUROCs as the model with *BMP3* (**Supplemental Table 2**). The gene name, function, and known cancer associations are summarized in **Supplemental Table 3**.

DIAGNOSTIC PERFORMANCE OF PJ-MDM COMBINED WITH PLASMA CA 19-9

Plasma CA 19-9 (as a continuous variable) alone achieved an AUROC of 0.91 (95% CI, 0.87–0.96). For CA 19-9 35 U/mL, the sensitivity for any stage of PDAC was 86% (95% CI, 77%–93%) and 86% (95% CI, 68%– 96%) for stage I/II PDAC. Using this CA 19-9 threshold, the false positive rates for normal controls, CP patients, and IPMN patients were 6% (95% CI, 2%–18%), 35% (95% CI, 16%–57%), and 9% (95% CI, 3%–19%), respectively. There was no statistically significant difference in AUROCs between the 3-MDM PJ panel alone and plasma CA 19-9 alone (P = .1991). Combining plasma CA 19-9 and the 3-MDM PJ panel achieved an AUROC of 0.95 (95% CI, 0.92–0.98) (**Figure 3B**). The combination performed significantly better than both the 3-MDM PJ panel alone (P = .0002) and plasma CA 19-9 alone (P = .0135) (**Figure 3B**). At 88% specificity, sensitivity for the combination of 3-MDM PJ model + CA 19-9 was 89% (80%–94%) for all stages of PDAC and 83% (64-94%) for early-stage disease (stage I/II) (**Figure 4A**). The trade-off in positive tests thresholds for plasma CA 19-9 alone and the combination of 3-MDM PJ panel + plasma CA 19-9 is depicted in **Figure 4B**. From this figure, we can elicit the following: (1) at an equal number of false positives, the 3-MDM PJ panel + plasma CA 19-9 correctly identified more PDAC patients compared with plasma CA 19-9 alone (5 vs 3 patients); (2) there were fewer false positive normal patients using the 3-MDM PJ panel + plasma CA 19-9 compared with plasma CA 19-9 alone (1 vs 3 patients); and (3) the majority of false positive findings for the 3-MDM PJ panel + plasma CA 19-9 model consisted of IPMN patients, whereas when using CA 19-9 alone patients with CP were the majority of false positives. Furthermore, 22 patients (9 normal, 8 IPMN, 3 CP, and 2 PDAC) had CA 19-9 U/mL levels <2.1 U/mL. All 22 patients were considered negative by the 3-MDM PJ panel + CA 19-9 model.

Covariate analyses were performed to assess the effect of baseline characteristics on the predictive accuracy of the 3- MDM panel þ CA 19-9 (**Table 2**). There were no statistical differences based on patient age, sex, or diabetes status. There was a statistically significant difference based on family history of PDAC. Those without a family history had lower predictive accuracy compared with those with a family history. However, for the 85% of the patients without a family history, the AUROC was similar to the overall AUROC (ie, 0.95 [95% CI, 0.91–0.98] vs 0.95 [95% CI, 0.92–0.98]).

DISCUSSION

In this large prospective study, we demonstrate that a PJ-MDM panel combined with plasma CA 19-9 can distinguish PDAC from both normal healthy controls and pancreatic disease controls with high accuracy (AUROC = 0.95), and this panel demonstrates a high sensitivity of 83% for detecting early-stage PDAC. Leveraging this independent sample set, we also demonstrate that the candidate MDMs with the highest individual AUROCs for detecting PDAC in this study are identical to those reported previously, and the AUROC for the 3-MDM panel is similar to our previously reported results. The reproducibility of these findings provides further justification for clinical test development of a PJ-MDM assay for early detection of pancreatic cancer.

Advancements in biomarker discovery and validation for early PDAC detection have traditionally focused on next-generation sequencing using blood and pancreatic cyst fluid for genetic mutations.[22] However, there is a growing interest in developing PJ-based assays for PDAC detection and recent studies explore the use of DNA methylation biomarker candidates either alone or in conjunction with candidate genetic markers.[12] A study in pancreatic cyst fluid and PJ found mutant *KRAS/GNAS* not contributory in predicting HGD in IPMNs,[23] congruent with our past results for *KRAS*.[15,17] In that study, a higher prevalence and abundance of methylated *TBX15* was noted in IPMNs with HGD; formal sensitivity and specificity comparisons could not be performed due to their small number of surgically resected cases. Although *TBX15* was one of the MDMs assayed in this current study, it was not included in the 3-MDM panel based on lower individual AUROC and variable ranking importance compared with other MDMs assessed. However, in our previous work focused on cyst fluid MDMs, methylated *TBX15* assayed in pancreatic cyst fluid accurately identified pancreatic cystic lesions with HGD and cancer.[17] APC aberrant methylation has previously been described in PJ as a singular marker for PDAC with low false positive results (specificity 93%) for CP controls.[24] The 3-MDM PJ panel in our study had a comparable false positive rate of 9% in CP controls. Patients with CP are at a higher-than-average lifetime risk of PDAC, and the risk appears to be disease length dependent.[25] The low false positive rate of PJ-MDMs in CP patients noted in this study can be clinically impactful as a PDAC surveillance tool in this patient population in which blood CA 19-9 is often falsely elevated, and the distorted gland architecture and mass-forming inflammation presents challenges for detecting small cancers on both cross-sectional imaging and EUS.[26] While the 3-MDM PJ panel had a low false positive rate for patients with a normal pancreas or CP on imaging, there was a considerable false positive rate in the IPMN control group. As demonstrated in a recent systematic review, the specificity of candidate markers in literature is driven by the proportion of IPMN included in the control group, as false positive results occur most frequently in these patients.[12,13] These findings may reflect the multifocal and variable nature of dys-

plasia in IPMNs and the potential for undetected foci of HGD in the ductal epithelium of controls.[27,28] While we had previously reported elevated PJ-MDM levels in IPMN HGD cases in our previous publication, there were no IPMN-HGD cases in this current study.[15,17]

While there is consensus that blood CA 19-9 is a suboptimal standalone biomarker for PDAC detection, it has been observed in other recent studies that using blood CA 19-9 as an anchor biomarker may enhance diagnostic sensitivity for PDAC, even for early-stage cancer.[29] Thus, for a new class of biomarkers to be clinically impactful, it is important to demonstrate that it outperforms blood CA 19-9 alone or that the combination of CA 19-9 with the new biomarker panel enhances diagnostic accuracy compared with either alone. The role of paired blood CA 19-9 as an adjunct to a PJ biomarker panel was explored by Levink et al. in their recent study.[30] However, in their study combining CA 19-9 with a panel of PJ biomarkers did not enhance diagnostic performance compared with CA 19-9 alone. In our study, the combination of plasma CA 19-9 and PJ-MDM detected PDAC with significantly higher accuracy compared with either biomarker individually. Similarly, we have previously noted complementarity of CA 19-9 when combined with a blood-based MDM panel for PDAC detection.[14]

There is currently no standardized methodology for PJ collection for a diagnostic test.[31,32] In prior PJ biomarker studies, a collection duration between 5 and 10 minutes has been considered optimal for diagnostic yield without risking sample dilution and degradation.[15,33] Aspirating directly from the pancreatic duct poses the risk of local manipulation and endoscopic canulation of the pancreatic duct, which increases the risk of procedure-related pancreatitis.[32] Therefore, for clinical translation of a diagnostic test with potential application as a surveillance test, it would be critical to avoid the need for pancreatic duct canulation. Previous studies have shown secretin to be a safe drug with infrequent minor side effects.[34,35] Our approach of developing an assay using secretin-stimulated duodenal aspirates and minimizes the procedural risk while preserving diagnostic accuracy.

This study stands out as one of the few studies in the field of cancer biomarkers that has demonstrated diagnostic performance replication for a fixed set of candidate biomarkers in a large, adequately powered, independent, multicenter sample set. Other key strengths of the study are the inclusion of patients with nonmalignant pancreatic diseases within the control group and adequate representation of early-stage PDAC in the case group.

Our study does have certain limitations. The majority of IPMN controls in our study did not have a histologically confirmed dysplasia grade, and we did not have any IPMN-HGD in the case group. Hence, the results of this study should not be

applied in the context of cyst surveillance. We also did not have germline mutation status on the vast majority of cases and controls, limiting the ability to assess the performance of PJ-MDMs specifically in the context of underlying germline variants. However, the majority of high-risk individuals in a pancreas cancer surveillance program have a structurally normal pancreas,[36,37] and the MDM panel in this study had a low false positive rate in patients with a normal pancreas. Moreover, our model's performance appeared better in patients with a family history of PDAC. While studies specifically targeting high-risk individuals are warranted, these results support the further exploration of this biomarker panel as a screening tool in high-risk individuals undergoing pancreas cancer surveillance. The CA 19-9 assay used in this study was not adjusted for the FUT genotype. The incorporation of a personalized genotype-adjusted blood CA 19-9 assay may potentially further enhance the diagnostic accuracy of this multimodal panel combining PJ MDMs and blood CA 19-9. Although we aimed to expand early-stage PDAC cases in the current study, only 33% of the cases were stages 1 and 2. While this is higher than the proportion of PDAC clinically diagnosed at a localized stage, it reflects a major challenge in advancing early detection research, namely access to early-stage PDAC biosamples. Last, the use of plasma CA 19-9 as a continuous variable in the model is not aligned to how CA 19-9 values are interpreted in current clinical practice. In future diagnostic algorithm setting studies, we anticipate defining a threshold at which CA 19-9 is most informative while minimizing the false positive rate in nonmalignant pancreatic diseases. The relatively low event rate of pancreatic cancer, even among people at high familial and genetic risk, poses a significant challenge for prospective validation of novel early detection assays. This will need to be addressed in appropriately designed future studies along with a cost-effectiveness analysis to define the true impact of PJ biomarkers in pancreatic cancer screening.

In conclusion, a multimodal biomarker panel combining DNA methylation in PJ and blood CA 19-9 is highly accurate for detecting PDAC and distinguishing it not only from normal healthy controls, but also from patients with nonmalignant inflammatory and dysplastic pancreatic diseases. In this study, using an independent PJ sample set, the 3 PJ-MDMs with highest individual AUROCs were identical to our previous study, reinforcing prior discovery and validation efforts. Future work will focus on developing clinical-grade assays for the most informative MDMs, setting a diagnostic algorithm in a large multicenter validation study for the intended-use population of high-risk individuals undergoing pancreas cancer surveillance. It is anticipated that a molecular PJ test that detects HGD/p/pDAC with high accuracy will enhance the diagnostic accuracy of endoscopic evaluation for screen-detected pancreatic lesions and potentially individualize pancreatic cancer screening intervals in high-risk individuals. Our eventual goal is to translate these findings into a clinical tool for detecting PDAC at an early stage and improving patient outcomes.

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TABLES

Table 1. Clinical and Demographic Characteristics of the Study Population

	Normal (n = 53)	CP (n = 23)	IPMN (n = 58)	PDAC (n = 88)
Age, y	58.1 (45.9–65.5)	59.4 (54.9–69.9)	70.1 (64.4–76.8)	70.8 (64.0–77.7)
Female	36 (67.9)	11 (47.8)	32 (55.2)	48 (54.5)
Tobacco				
Missing	0	0	0	2
Current	9 (17.0)	9 (39.1)	5 (8.6)	8 (9.3)
Former	17 (32.1)	5 (21.7)	22 (37.9)	39 (45.3)
Never	27 (50.9)	9 (39.1)	31 (53.4)	39 (45.3)
Alcohol				
Missing	5	0	3	6
Former	5 (10.4)	6 (26.1)	10 (18.2)	19 (23.2)
<3 times/wk	16 (33.3)	5 (21.7)	20 (36.4)	27 (32.9)
>3 times/wk	4 (8.3)	4 (17.4)	10 (18.2)	10 (12.2)
Never	23 (47.9)	8 (34.8)	15 (27.3)	26 (31.7)
Diabetes				
No	46 (86.8)	15 (65.2)	44 (75.9)	64 (72.7)
Yes: type II	7 (13.2)	6 (26.1)	14 (24.1)	22 (25.0)
Yes: unknown	0 (0.0)	2 (8.7)	0 (0.0)	2 (2.3)
Fam history of PDAC				
Missing	1	1	2	1
No	44 (84.6)	20 (90.9)	42 (75.0)	78 (89.7)
Yes	8 (15.4)	2 (9.1)	14 (25.0)	9 (10.3)
Stent				
No	50 (94.3)	18 (78.3)	57 (98.3)	73 (83.0)
Yes	3 (5.7)	5 (21.7)	1 (1.7)	15 (17.0)
Stage				
I				10 (11.4)
II				19 (21.6)
III				23 (26.1)
IV				36 (40.9)

Values are median (interquartile range) or n (%).
CP, chronic pancreatitis; IPMN, intraductal papillary mucinous neoplasm; PDAC, pancreatic ductal adenocarcinoma.

Table 2. Covariate Analyses of Baseline Characteristics

	No	Yes	*P* Value
Age ≥65 y	0.98 (0.95–1)	0.93 (0.88–0.98)	.0844
Male	0.97 (0.93–1)	0.94 (0.88–0.99)	.2946
Diabetes	0.91 (0.83–0.99)	0.99 (0.96–1)	.0701
Family history	0.95 (0.91–0.98)	1 (1–1)	.0033

Values are area under the receiver-operating characteristic curve (95% CI).

FIGURES

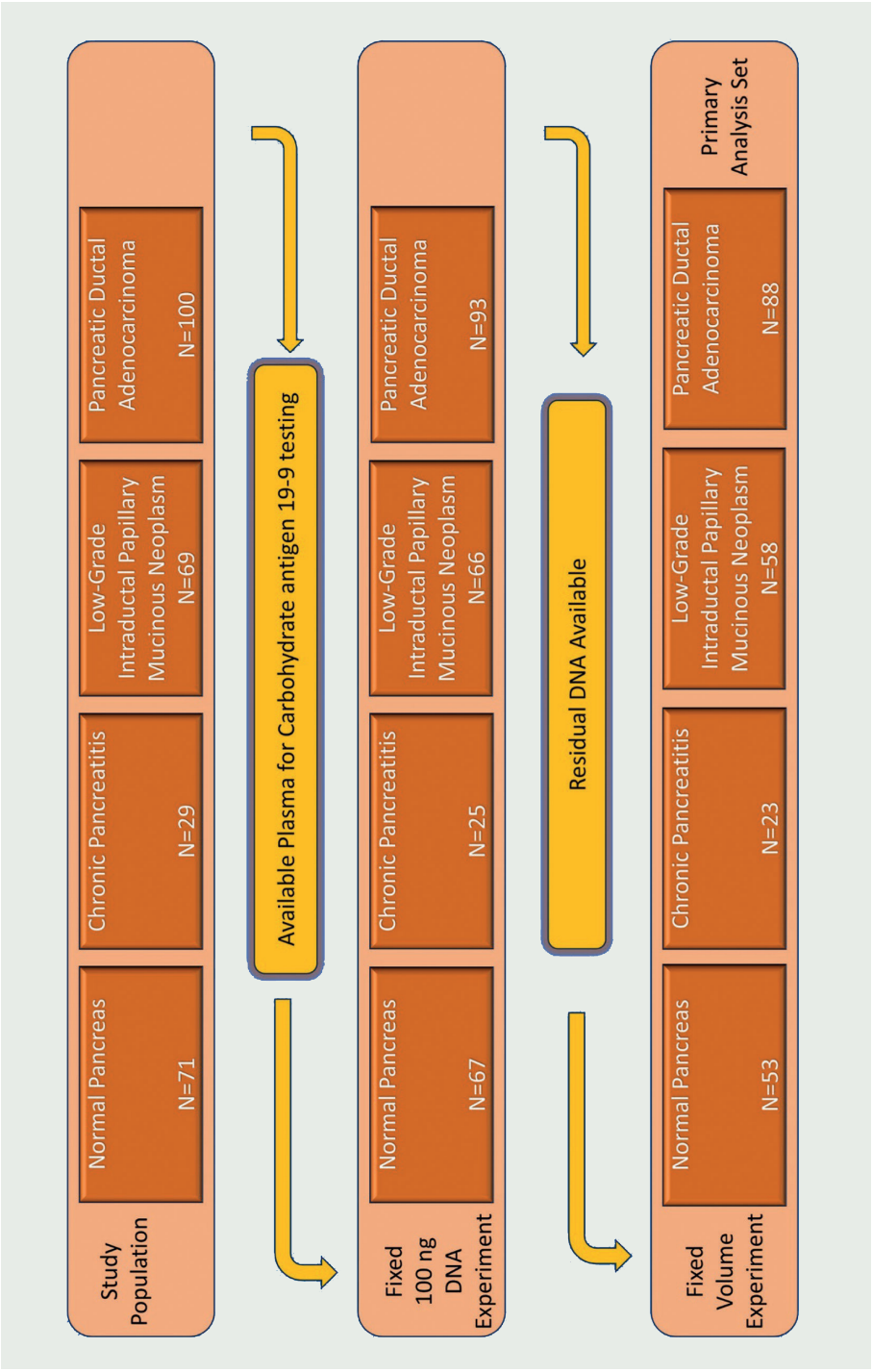


Figure 1. *Study flow diagram*

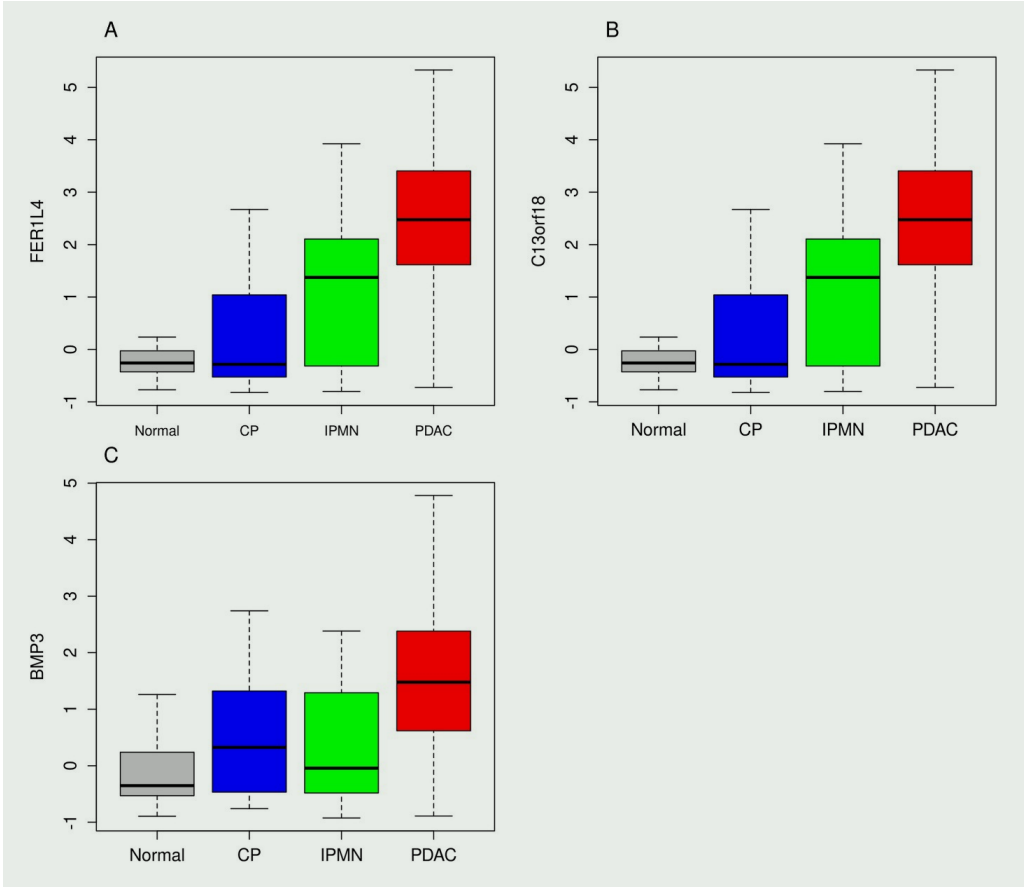


Figure 2. *Boxplots showing marker distributions in case and control groups for the top 3 individual MDMs.*

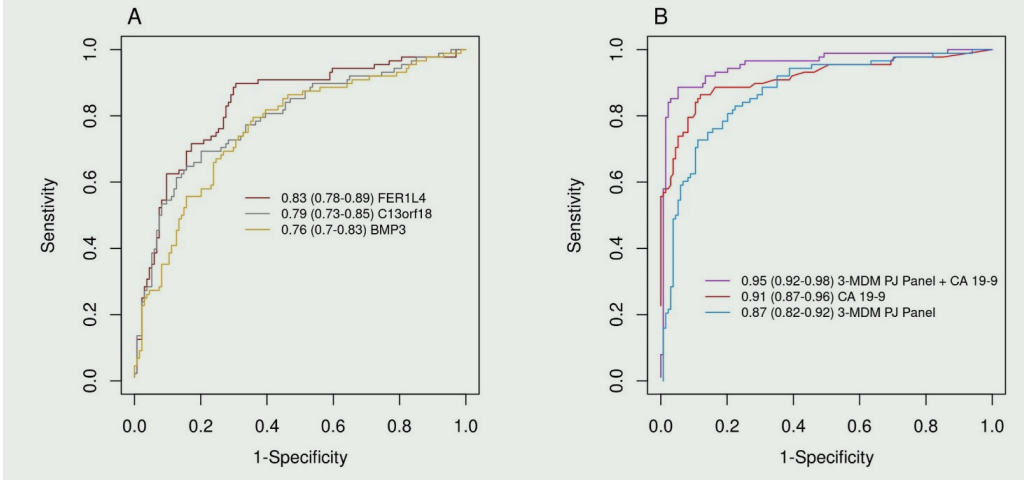


Figure 3. *Receiver operating characteristic curves demonstrating the AUROC: (A) the 3 MDMs with highest individual AUROC; (B) comparing the 3-MDM PJ panel alone, plasma CA 19-9 alone, and the combination of both.*

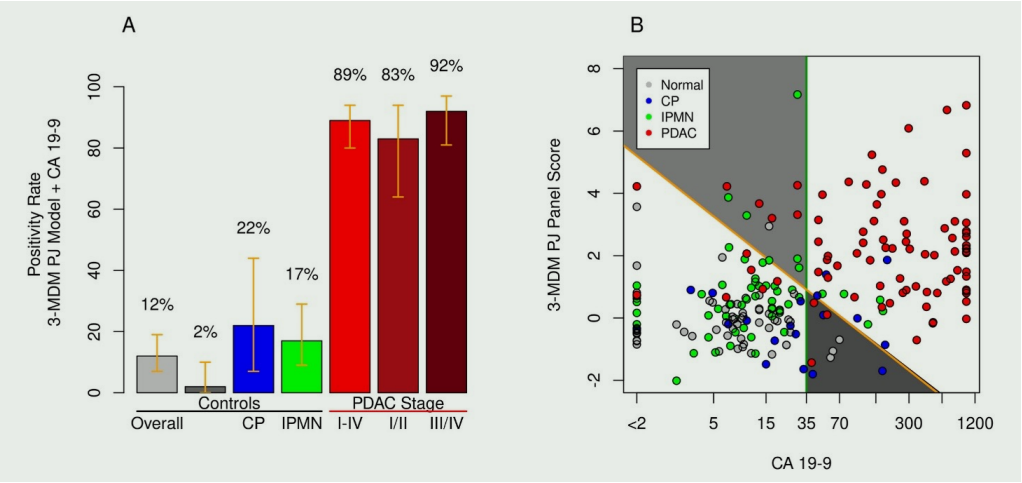
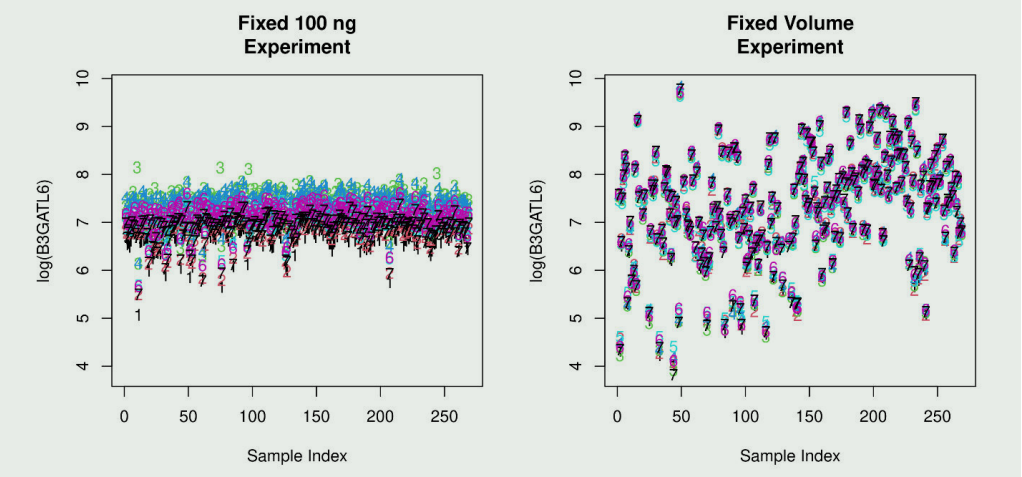
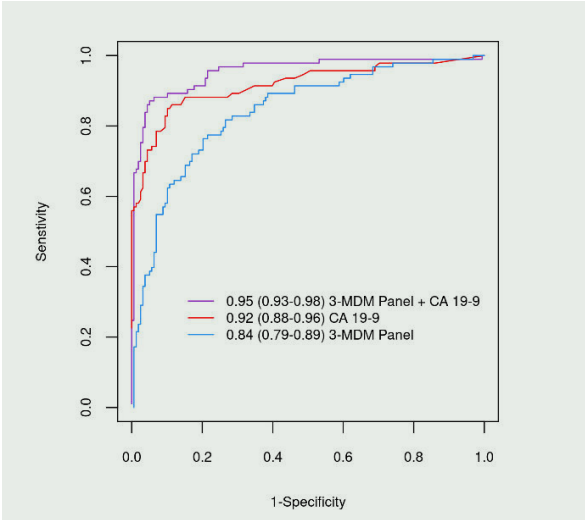


Figure 4. A) *PJ-MDMs + CA 19-9 positivity rates for case and control groups. (B) Depiction of sensitivity and specificity trade-offs of PJ-MDMs + CA 19-9 relative to CA 19-9 and MDMs alone. The green vertical line represents the clinical decision point of CA 19-9 and the diagonal orange line represents the decision curve for 3-MDM PJ + plasma CA 19-9. The dark gray area of the figure identifies cases and controls that were positive by plasma CA 19-9 alone but negative based on the 3-MDM PJ panel + plasma CA 19-9 model. The light gray area of the figure identifies cases and controls that were negative by plasma CA 19-9 alone but considered positive based on the 3-MDM PJ panel + plasma CA 19-9. Individual patients are colored as gray (healthy controls), blue (CP), green (IPMN), and red (PDAC).*

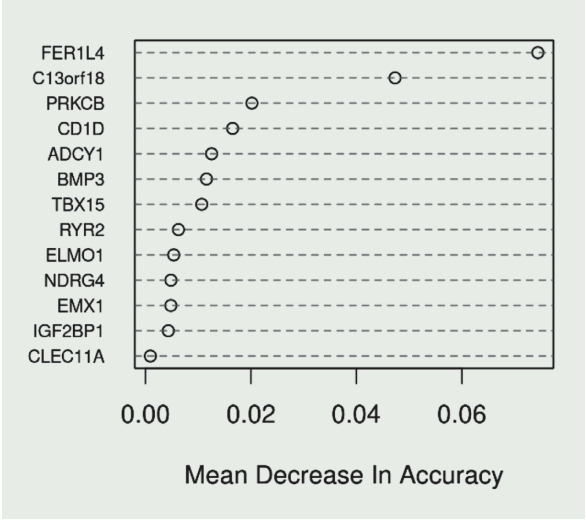
SUPPLEMENTAL FIGURES



Supplemental Figure 1A and B. *Quality control sample index plot of B3GALT6 across the 7 triplexes represented by a different color and number for the fixed DNA input and fixed volume experiments. The fixed DNA input experiment (A) showed a drift in B3GALT6 values whereas the fixed volume experiment (B) showed consistent B3GALT6 values across the triplexes*



Supplemental Figure 2. *Receiver operating characteristic (ROC) curves demonstrating the area under receiver operating characteristic curve (AUROC) for the 3-MDM PJ panel alone, plasma CA 19-9 alone and the combination of both when using a fixed DNA input.*



Supplemental Figure 3. *Relative importance of individual MDMs when ordered by mean decrease in prediction accuracy after permutating marker levels within the test sets.*

Supplemental Table 1.

AUROC (95% CI) summaries of individual PJ-MDMs

	All controls vs PDAC	Normal controls vs PDAC	Diseased controls vs pdac
FER1L4	0.83 (0.78-0.89)	0.90 (0.84-0.95)	0.79 (0.72-0.86)
C13orf18	0.79 (0.73-0.85)	0.80 (0.72-0.87)	0.79 (0.72-0.86)
BMP3	0.76 (0.70-0.83)	0.83 (0.76-0.9)	0.72 (0.64-0.79)
PRKCB	0.76 (0.69-0.82)	0.81 (0.73-0.88)	0.72 (0.65-0.80)
CD1D	0.75 (0.68-0.81)	0.82 (0.75-0.89)	0.70 (0.62-0.78)
ADCY1	0.71 (0.64-0.79)	0.78 (0.7-0.86)	0.67 (0.59-0.75)
TBX15	0.66 (0.59-0.74)	0.72 (0.64-0.81)	0.62 (0.54-0.71)
IGF2BP1	0.66 (0.58-0.73)	0.74 (0.65-0.82)	0.60 (0.52-0.69)
EMX1	0.65 (0.58-0.72)	0.71 (0.63-0.8)	0.61 (0.52-0.69)
RYR2	0.61 (0.54-0.69)	0.71 (0.62-0.80)	0.55 (0.46-0.64)
ELMO1	0.61 (0.53-0.68)	0.67 (0.57-0.76)	0.56 (0.48-0.65)
NDRG4	0.60 (0.52-0.67)	0.64 (0.55-0.74)	0.56 (0.48-0.65)
CLEC11A	0.60 (0.52-0.67)	0.68 (0.59-0.77)	0.54 (0.45-0.63)
LRRC4	0.52 (0.44-0.59)	0.60 (0.50-0.70)	0.46 (0.38-0.55)

Supplemental Table 2.

Cross-validated AUROC metrics for adding MDMs to the model with *FER1L4* and *C13orf18*

MDM	Without CA 19-9	With CA 19-9
BMP3	0.863 (0.793-0.928)	0.945 (0.896-0.984)
PRKCB	0.867 (0.800-0.930)	0.946 (0.896-0.987)
CD1D	0.865 (0.797-0.929)	0.946 (0.900-0.985)
ADCY1	0.862 (0.794-0.925)	0.945 (0.897-0.985)

Supplemental Table 3.

Marker	Gene Name	Molecular Function	Location of Hyper-methylation	Cancer Associations	PMID
ADCY1	adenylate cyclase 1 (brain)	Signal transduction	Promoter	Oncogenic and tumor suppressor activity in cancers	35832555
BMP3	bone morphogenetic protein 3	Growth factor/cell proliferation	5' UTR	Hypermethylated in multiple cancers	31665064
c13orf18 (RUBCNL)	rubicon like autophagy enhancer	Endocytic trafficking and autophagy	Gene body	Hypermethylated in cervical cancer	23522960
CD1D	Cluster of differentiation 1D	MHC protein/antigen presentation	5' UTR	Tumor suppressor activity PDAC	28419443
CLEC11A	C-type lectin domain containing 11A	Growth factor/cell proliferation	Gene body	AML progression	38466706
ELMO1	engulfment and cell motility 1	Immune response/ phagocytosis	Gene body	Hypermethylated in multiple cancers	35668246
EMX1	empty spiracles homeobox 1	Transcriptional regulation	Gene body	Hypermethylated in PDAC	25276247
FER1L4	Fer-1 like family member 4 (pseudogene)	lncRNA/gene expression regulation	Gene body	Tumor suppressor activity CRC, HCC, GC	35198876
IGF2BP1	insulin-like growth factor 2 mRNA binding protein 1	Growth factor/cell proliferation	5' regulatory	Oncogenic and tumor suppressor activity in cancers	29954406
LRRC4	Leucine-rich repeat-containing protein 4	Synaptic adhesion/cell signaling	Promoter	Tumor suppressor activity glioma	16723503
NDRG4	N-myc downstream regulated gene 4	Signal transduction/ cell cycle	Promoter	Tumor suppressor activity multiple cancers	25749388
PRKCB	Protein kinase C beta type	Cell proliferation, differentiation, apoptosis	Promoter	Hypermethylated in multiple cancers	35174160
RYR2	Ryanodine receptor 2	BMP pathway signaling	Promoter	Hypermutated in multiple cancers	34079583
TBX15	T-box 15	Transcriptional regulation	Gene body	Up regulated in multiple cancers	37328486