

Methylation Status of Tumor Suppressor Genes in Circulating DNA of PDAC Patients: Facts and Hopes

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Abstract

Pancreatic cancer is a very aggressive disease with high mortality rate and its high mortality rate makes its diagnosis difficult, especially when disease is at an advanced stage. Lack of efficient biomarkers until today makes its diagnosis difficult at early stages and during its progression. Methylation in the promoter of circulatory DNA, cirDNA of tumor suppressive genes is one of the important epigenetic phenomenon contributes to the pathogenesis of diseases and also opening a new venue for the discovery of non-invasive biomarkers. CirDNA methylation signatures represented an important paradigm shift in precision medicine and enabling early detection and monitoring the progression of pancreatic diseases. In this review we will provide an overview of differential methylation levels in PDAC with diagnostic and prognostic potential at the early stages of diseases.

Keywords: Pancreatic cancer, Circulatory DNA, Non-invasive biomarker, Epigenetic signature, Diagnosis, Prognosis

Introduction

Pancreatic cancer is one of the most lethal solid tumor malignancies and it is projected to become a leading cause of cancer related deaths in coming years [1]. It is often diagnosed at an advanced stage owing to the lack of specific symptoms and rapid invasion [2]. Overall survival rate in pancreatic cancer patients is around 10% (American Cancer Society) and only 15% patients are found to have respectable diseases [3]. Thus, aggressiveness of diseases may limit the early diagnosis, contributes to the high mortality rate. Until now, we are also lacking efficient noninvasive biomarkers to detect the early stage and the progression of diseases. Best-known FDA approved biomarker for prognostic surveillance is carbohydrate antigen CA19-9 [4]. However, sensitivity and specificity of this marker to detect the severity in PDAC patients is very low. When released by PDAC patients, CA19-9 monitors the progression and relapse of diseases [5]. While CA19-9 levels are also high in chronic pancreatitis patients CP along with PDAC [6]. Due to these limitations, the clinical use of CA19-9 is discouraged in PDAC patients unless use in combination of other circulating biomarkers. The collection and analysis of pancreatic juice is invasive and cumbersome, however more informative [6]. The lack of

robust noninvasive biomarker has encouraged scientists to explore more strategies.

The advancement in next generation sequencing (NGS) and high throughput omics profiling have provided a new paradigm in biomedical research to uncover an epigenetic basis of human diseases. This data has anticipated in exploring the sequence accumulation of genetic alterations in oncogene and tumor suppressor genes/methylation [7].

Aberrant methylation of gene promoter of certain tumor suppressor genes has been reported to lead the progression of cancer [8]. Methylation change is a common feature of different cancer types and occurs at early stages of cancer development [9]. DNA methylation may offer a more consistent and broadly applicable marker of tumor DNA in blood than mutations [10]. For e.g. GSTP1 is methylated in >90% of prostate cancer [11]. Stratifin is methylated in 96% of breast cancer [12] and HOX A9 and EN1 are methylated in 95% of ovarian tumors [13]. With regards to plasma methylated biomarker discovery, the pancreatic cancer field is still in the early stages, the number of studies carried out to date are small. The analysis of DNA methylation levels in cirDNA has proven the worthy approach for the early detection of pancreatic

diseases. Increasing evidences showed that this process is broadly more informative and sensitive than detecting DNA mutations. Moreover, sample collection is minimally invasive and allows proper follow up under negligible stress for the patient.

This review provided an overview and importance of the hyper-methylated genes in the early detection and progression of PDAC. We also compiled methylation levels of primary tissues biopsies and biological fluids from PDAC patients. Furthermore, we will describe the cirDNA methylation signature, differentially expressed in high-risk PDAC individuals and also current advancements made in developing diagnostic and prognostic biomarkers for PDAC patients.

Circulatory DNA and its Origin

The circulatory DNA comes from the nuclear as well as mitochondrial DNA liberated from the cell [14]. They are found in the physiological extracellular milieu, e.g., blood, lymph, bile, milk, urine, saliva, mucous suspension, spinal fluid, and amniotic fluid.

DNA is a very electrostatic molecule with an ability to make complex with other molecules or structures. It has been shown that cirDNA is present either in molecular or macromolecular complexes or internalized in vesicles (Such structures protect it from nucleases present in the circulatory system and reduce recognition as a danger signal by the immune system) [14]. CirDNA can equally be attached to the exterior of the cell membrane from which it can be detached and so can be freed in the circulatory system [15]. Different mechanisms are explored till date that permits the translocation of DNA from the intracellular to the extracellular compartment. The structural description of cirDNA is present in its origin like apoptosis, necrosis and phagocytosis. Studies which are done on different cell types, revealed that cirDNA was synthesized and spontaneously secreted through a regulated mechanism [14]. Abolhassani et al. have shown an active secretion of extracellular DNA from the HL60 cell line [16].

It has been demonstrated that the amount of cirDNA in the circulation compartment increases with the tumor cell number increases. It is reasonable to postulate that the total mutant cirDNA concentration accounts for the cirDNA deriving from malignant cells [17-19].

A high variation of the cirDNA amount in blood of patients depends upon the type of cancer like bladder, colorectal, ovarian, pancreas and breast cancer exhibiting larger amount of cirDNA as compared to thyroid and glioma cancer, respectively [18]. It is important to consider the cellular origins of the DNA found in the blood of cancer patients. The identification of genetic alterations in cirDNA specific to cancer cells has confirmed that the

source of cirDNA is tumor cell. A tumor is composed of malignant tumor cells and cells constituting the tumor microenvironment. Thus, stromal cells, endothelial cells, lymphocytes, and other immune cells also equally constitute a potential source of cirDNA release in relation to tumor progression [19]. Sources of cirDNA can be found in healthy cells, malignant cells and tumor microenvironment cells of cancer patients. Each of cellular compartments could lead to different processes to the release of DNA into the circulatory system.

Differential DNA Methylation Pattern in Pancreatic Tumor

Several gene promoters (CACNA1G, CDH1, CDKN2A, DAPK1, MGMT, MINT1-2- 31-32, MLH1, RARB, THBS1, and TIMP3) were analyzed for DNA methylation in pancreatic tumor PDACs xenografts [9]. All cancerous specimens showed aberrant DNA methylation at-least one locus except for MGMT, which was non-methylated in either neoplastic or normal control samples. CCNG1, PENK, ZBP genes were found to be differentially methylated in pancreatic-derived cell lines as compared with the healthy pancreas and further validated in pancreatic tumor [20,21]. Among abovementioned genes PENK, CGI (CpG islands) was found to be methylated in 91% of cases [22]. PENK and CDKN2A DNA methylation status was further assessed in different grades of pancreatic cancer: intraductal PDAC, extra-ductal PDAC (including one PNET) [23]. PENK methylation was found in 93% of invasive PDACs, of which 27% presented CDKN2A methylation. However, non-neoplastic specimens resected from matched healthy control tissues did not show any methylation of either gene. Few reports also showed the increase of DNA methylation pattern with inflammation of pancreatic tumor. BRCA1, APC, CDKN2A, and TIMP3 were methylated in 60%, 59%, 39%, and 31% of the PT cases, respectively [24]. NGS was also used to identify differential methylation levels in healthy and pancreatic cancer patients. Aberrantly methylated regions were more frequent in PDAC patients as compared to the healthy normal pancreas. Methylation of certain genes including C5orf38, DLX4, ELAVL2, EMX1, IRX1, NPR3, PITX2, SIM2, TBX5, TFAP2C, and VSTM2B were further validated by target-specific methods like MSP, direct bisulfite sequencing or methylation-sensitive restriction endo- nuclease PCR [25].

Epigenetic deregulation of non-coding genes, microRNAs (miRs) also plays an important role in tumor development. Besides tumor development, miRNA is involved in inflammatory processes playing a role in coordinating different processes of immune system, like immune cell differentiation, function, and recruitment. For eg. MiR124 and MiR10B showed aberrant methylation levels in PDAC patients as compared to healthy controls.

Hypermethylation of MiR124 was correlated with poor prognosis [26,27]. Additional evidence explored the role of MiR210 in epithelial to mesenchymal transition and also in poor prognosis of PDAC patients [28].

Methylation Levels of Circulatory DNA of Pancreatic Cancer Fluids

The methylation levels of the different genes have been studied to differentially diagnose pancreatic tumor from different biological fluids such as pancreatic juice and blood [22]. In pancreatic tumor, six methylated genes have been found as an indicative of advanced diseases while three (CLDN5, NPTX2 and SFRP1) amongst them were found to be methylated in pancreatic juice. In another study where, pancreatic juice was endoscopically or surgically collected from the patients with pancreatic disease like CP and pancreatic lesions [29] Quantitative analysis of 17 target genes revealed more methylation levels in pancreatic tumor samples (n= 56) as compared to the healthy control (n=11) also the number of methylated genes were highly profound in high-risk pancreatic tumor [29]. These results were very encouraging and were further conducted in pancreatic juice collected from a greater number of patients. DNA methylation levels of six methylated genes were also assessed in conjunction with mutant KRAS in pancreatic juice samples collected from 61 PT patients with 11 healthy controls. Mutant KRAS had a significant sensitivity in discriminating PDACs patients from healthy controls. The sensitivity in discriminating PT from healthy controls for gene CD1D was 79%, CLEC11A (7%), IKZF1 (62%), KCNK12 (79%), NDRG4 (72%), and 67% for PKRCB [30].

The collection of pancreatic juice is relatively complex process and may not considered as standard measure for early detection in asymptomatic individuals. To rule out this problem, cirDNA in plasma is gaining attention.

Melnikov et al. examined plasma specimens collected from PDAC patients with a panel of 56 frequently methylated genes [31]. By using microarray-based approach [32], five promoters CCND2, PLAU, SOCS1, THBS1, and VHL were used an aggregate bio-marker. This assay distinguished healthy pancreas from PDAC with 76% sensitivity and 59% specificity. Other studies have reported CCND2, SOCS1, and THBS1 hyper-methylation could be used as an important tool for cancer detection in pancreatic juice or tissues [33]. Another study done by Liggett et al identified promoters of 17 genes to identify methylation in PDAC and CP patients. Amongst them eight genes (BRCA1, CCND2, CDKN1C, MLH1, proximal and distal PGR promoter regions, SYK, and VHL) were useful to distinguish healthy pancreas from CP with sensitivity and a specificity of 82% and 78%, respectively.

Park et al. and his coworkers investigated the DNA methylation levels of six genes (CDKN2A, NPTX2, PENK,

SFRP1, RASSF1A, and UCHL1) in the plasma collected from the individuals suffering with advanced and metastatic PDAC [34]. DNA methylation was detected in 81% of PDACs (13 of 16 cases) and 61% of CP (eight of 13 cases) with at least one gene affected in either condition. However, 4% of healthy pancreas (one of 29) showed DNA methylation. The most frequently methylated gene was NPTX2 (38% and 31% for PDAC and CP, respectively), However, the least methylated gene was RASSF1A (6% and 8% for PDAC and CP, respectively). High inter-individual variability was observed in this study due to small cohort size and other variations and significant differences were not made between PDAC and CP patients except for CDKN2A, which was specifically methylated in PDAC, but not in CP. NPTX2 hyper methylation levels was further confirmed in a larger cohort by the same authors [34]. Singh and colleagues recently described the methylation levels of these three genes [35]. Author compared methylation levels of NPTX2, PENK, SPARC and UCHL1 individual with PDAC and healthy controls by using QMSP. Quantitative DNA methylation levels were expressed as methylation indices (MIs) that is the fraction of the methylated copy number over total detectable cirDNA. This study detected higher MIs for all four genes and this MI was higher in PDAC patients as compared to the healthy individuals. Higher MIs of NPTX2 and SPARC were abundant in metastatic and advanced stage of PDAC patients, thus poor prognosis. While UCHL1 was only present in advanced stage of PDAC patients. This study claimed to use the combination of NPTX2, SPARC and UCHL1 genes as an efficient and valuable prognostic and diagnostic tool to differentiate the PDAC patients from CP and healthy controls. This study has been done in a smaller cohort, while provided efficient quantification method therefore, larger number of cohorts should be included to further validate this study.

To overcome an issue of scarcely abundant cirDNA, Yi et al., developed a single tube high-yield collection method termed methylation on beads (MOB) [36]. This study gained attention on the methylation levels of BNC1 and ADAMTS1 detected in PDAC samples from tumor stage I-IV, n=42 [36] while healthy controls did not. BNC1 methylation was profound in 33 patients out of 42 while 20 of 42 showed ADAMTS1 hyper-methylation. Combining these two genes increased the sensitivity (81%) but reduced the specificity (85%). Combination of these two genes could be used as promising diagnostic biomarker for the early detection of PDAC.

Utilization of cirDNA methylation detection tools for diagnostics is growing rapidly. Henriksen and his coworkers employed a panel of 28 genes to assess the methylation levels in plasma specimens of PDAC (95), CP (124) and healthy controls (59) [37]. They developed a diagnostic prediction model that optimizes the combinatory use of biomarkers to achieve the highest prediction power. This study resulted in a panel of eight genes (APC, BMP3,

BNC1, MESTv2, RASSF1A, TFPI2, and SFRP1-2) and demographic parameter patient's age (>65). This study was not quantitative but was able to differentiate malignant tumor from benign one with sensitivity (76%) and 83% of specificity irrespective of the stage of tumor. These studies were very encouraging however needs to be validated in independent cohorts to substantiate these results.

In another study Henriksen and coworkers used the previous set of genes and focused on the development of prognostic biomarker for PDAC [38]. They used multivariable Cox regression analysis and the stepwise backward elimination process [38] and developed survival prediction models. The final model included BNC1, GSTP1, SFRP1, SFRP2, and TFPI2 genes and an ASA physical status score of three (that is "severe systemic disease"). They concluded that methylation was associated with poor prognosis while SFRP2 methylation was correlated with longer survival. This study provided interesting hints, however needs to be done in larger independent cohorts to further confirm the findings.

The last and most recent finding by them employed the concept of using of methylation haplotype blocks (MHB) and machine learning algorithms. Adjacent CpG sites in mammalian genomes can be co-methylated due to the processivity of demethylases. CpG rich genomic regions exhibiting highly coordinated methylation are referred as MHBs [39].

A recent study by Lehmann-Werman et al. revealed how cirDNA methylation patterns could be employed to detect tissue-specific cell death in PDAC and CP patients [40]. cirDNA from plasma specimens of late stage PDAC individuals showed high levels of CUX2 and REG1A while healthy control showed minimum levels of methylation. Levels of these markers were lower as the grade of the lesion decreased which concluded that tissue-specific cirDNA levels in the plasma directly reflects cell death independent of the etiology of the disease. Additionally, PDAC patients showed higher levels of the ductal-specific marker CUX2 whereas CP affected individuals had higher levels of the acinar-ductal marker REG1A.

Finally, a large study done by Liu et al., and colleagues, where they have generated cirDNAmethylation profile of neo-plastic and matched non-neoplastic pancreatic tissue by using RRBS [41]. This study resulted in the identification of >350 PDAC specific DNA methylation signatures with the achievement of sensitivity (86%) and specificity (88%). This large database of PDAC specific methylation signature could harness for the identification of novel biomarkers and validation of existing ones.

Challenges in Working with Methylated cirDNA

Methylated cirDNA presents a challenging substrate to work with, largely because cirDNA is diluted with

concentrations as low as < 10 ng per mL of plasma in healthy subjects [42]. The methylated component is an even smaller subfraction of this original amount. Thus, limited quantities of starting material are a common problem for researchers working with clinical samples. This problem can be overcome through the development of DNA purification methods, which accommodate larger volumes of plasma input [43,44]. Ongoing technical developments suggest that it is possible to analyze smaller amounts of DNA from even low volumes of plasma as starting material. The size of early stage tumors may limit the amount of DNA that is shed into the blood. However, there are many unknown parameters in estimating how much DNA is likely to be released into the blood stream starting from the number of cells per cubic centimeter of tumor tissue, which has been revealed to range from 1×10^8 to 1×10^9 per cm^3 depending on tumor size and the proportion of stromal components [45]. It is also possible to detect methylated DNA from the smaller tumors, which can be surgically removed. Another important thing is that the chosen targets are not methylated even at low levels in control blood samples therefore, the specificity of the test would be compromised. Another challenge in cirDNA studies is that cirDNA is fragmented. Most of the DNA present in plasma is fragmented. This corresponds to the smallest two bands of the apoptotic DNA ladder and reflects the apoptotic origin of the DNA [46]. This represents that DNA purification methods that perform for intact genomic DNA are not that efficient in extracting cirDNA [47] which further lead to further DNA losses. The collection and processing of clinical samples for the validation of plasma biomarkers also need to be taken in to the consideration. Blood in EDTA tubes can only be stored for a limited amount of time before processing DNA to the plasma DNA fraction [48]. Same day processing of blood samples is recommended so that the cirDNA does not get contaminated with leukocyte DNA. Problems can also arise from validation strategies where we compare samples from cancer patients and healthy controls. Insofar as in addition to cancer specific biomarkers, the patient samples are also likely to show changes due simply to the presence of inflammation. The resulting biomarker may not be able to differentiate between cancer and other less serious conditions with an inflammation component. These considerations are not specific to methylated DNA biomarkers and apply to biomarker discovery and validation generally. Developing a diagnostic test for rare cancer such as pancreatic cancer is technically more difficult than developing a test for common cancers such as breast and colorectal cancer. This is because the assay adopted for rare cancers must have an extremely low false positive rate if the test is to be clinically useful. Additionally, colorectal and breast cancer is at the local sites where a test can be done by using relatively non-invasive procedures of colonoscopy and fine needle biopsy, respectively, whereas pancreatic cancer can require surgery for the final diagnosis, thus the chances of getting false positive test results can be more serious for

patients. The problems associated with the requirement of high sensitivity can be overcome by targeting the screening test to at-risk populations with higher prevalence of the cancer, e.g., BRCA1,2 mutation carriers for ovarian cancer screening [48].

Discussion

Methylation of tumor suppressor genes is one of the most important epigenetic changes, which drive tumorigenesis. Circulating DNA has been known to reflect the molecular changes and tumor heterogeneity present in tumor tissue [38,49]. Previously published reports showed methylation signatures for pancreatic tumor tissue, which can easily predict the prognosis in patients [50], however cumbersome and invasive. Liquid biopsies are much easier and less invasive as compared to challenging process of tissue sampling and its usage is currently increasing in clinical setting. In this review we have focused on all the advancements made in the early diagnosis of PDAC based on methylation signature of liquid biopsies. Most of the studies we deciphered are focused on the genes, which are undergoing aberrant hypermethylation during the pathogenesis of diseases. Most of the studies showed the methylation levels at different stages of PDAC. For e.g. BNC1 levels were observed at all stages of PDAC however, ADAMTS1 gets methylated only at stage I and II of PDAC [49]. Therefore, this information provides us the information of using the combined DNA methylation of these genes to track the progression of diseases. Another worth mentioning aspect is that the target may also show the differences in its DNA methylation signature depending on the stage of the diseases. This may be relevant in diseases like PDAC, where inflammation plays an important role in aberrant DNA methylation [51,52]. For e.g. genes like CCND2, CDKN1C and MCH1 were able to differentiate PDAC patients from healthy individual with great sensitivity and specificity, however some genes were hyper methylated in CP individuals [53]. Moreover, ADAMTS1 showed higher frequency of methylation in stage I and II of PDAC patients but stage III and IV showed lower methylation frequency, hence supports methylation dynamics. Additional investigation needs to be performed on this to strongly correlate the DNA methylation levels with the diseases stage. Great efforts have been made in using highly predictive combination of DNA methylation patterns, quite obvious that a methylation level of individual gene is not seldom able to explore the complex biology of diseases. Therefore, the employment of multiple biomarkers enables the early detection and management of diseases during progression. Singh et al., described the usage of NPTX2, SPARC and UCHL1 which could efficiently differentiate PDAC patients from

the healthy controls and may be used as prognostic and diagnostic invasive biomarkers of diseases [35]. Moreover, prediction models are extensively used now a days and these can be integrated with DNA methylation status to develop robust predictive tools for PDAC patients. These predictive tools are also efficient in developing aggregate biomarkers that combined different diseases specific target [37]. These models were successfully able to distinguish PDAC patients from healthy controls and CP individuals with good specificity and sensitivity. However, detection and reproducibility of data in clinics still hinders. More number of patients needs to be involved to achieve the robust data. The different origins of cirDNA and genetic heterogeneity of tumor makes interpretation of results even more difficult. But the technological advancement made enables deconvolving the heterogeneity of cirDNA and pinpoint the tissue-specific and diseases specific molecules based on specific DNA methylation signatures [40]. This breakthrough will help to encourage the use of liquid biopsies and so the research in the biomarker discovery.

Conclusion

This review has focused on all the advancements made in liquid biopsy of PDAC patients, exclusively aimed to elucidate the role and development of differential DNA methylation biomarkers to guide clinical management of PDAC. The usage of new technologies and array-based screening methods of DNA methylation detection could be more informative for diseases prognosis and developing personalized therapeutic strategies at early stage of PDAC. To achieve further benefits, more efforts need to be done in standardization of methods and validation of existing biomarkers in larger cohorts of pancreatic diseases. Table 1 is summarizing list of genes showing differential DNA methylation between healthy pancreas, pancreatic cancer and chronic pancreatitis in liquid biopsies.

Conflict of Interest

The authors declare no conflict of interest.

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DNA methylation in liquid biopsies of PDAC patients.								
Gene	Event	Characteristics of the study	Methods			Characteristics of the study	Event	References
ADAMTS1			qMSP			HC vs. PT	Hyper	Yi et al., 2013
APC			MSP			CP vs. PT	Hyper	Henriksen et al., 2016
BNC1			MSP			CP vs. PT	Hyper	Henriksen et al., 2016
			qMSP			HC vs. PT	Hyper	Yi et al., 2013
BRCA1			MSP			CP vs. PT	–	Henriksen et al., 2016
			MCAM			HC vs. CP	Hyper	Ligget et al., 2010
CCND2			qMSP			HC+CP vs. PT	Hyper	Matsubayashi et al., 2006
			MCAM	MSREP		HC vs. PT	Hypo	Melnikov et al., 2009
			MCAM			HC vs. CP CP vs. PT	Hyper Hypo	Ligget et al., 2010
CD1D			MSP	qMSP	RRBS	HC vs. CP HC vs. PT	– Hyper	Kisiel et al., 2015
CDKN2A			MSP			HC vs. CP CP vs. PT	– Hyper	Park et al. 2012
			MSP			CP vs. PT	–	Henriksen et al., 2016
			qMSP			HC+CP vs. PT	Hyper	Matsubayashi et al., 2006
CDKN2B			MSP			CP vs. PT	–	Henriksen et al., 2016
			MCAM			CP vs. PT	Hyper	Ligget et al., 2010
ESR1(A)			MSP			CP vs. PT	Hyper*	Henriksen et al., 2016
			MCAM			CP vs. PT	hypo	Ligget et al., 2010
FOXE1			qMSP			HC+CP vs. PT	Hyper	Matsubayashi et al., 2006
GSTP1			MSP					

Gene	Event	Characteristics of the study	Method			Characteristics of the study	Event	Reference
			MSP			CP vs. PT	Hyper*	Henriksen et al., 2016
HIC1			MSP			CP vs. PT	Hyper*	Henriksen et al., 2016
IKZF1			MSP	qMSP	RRBS	HC vs. CP HC vs. PT	– Hyper	Kisiel et al., 2015
KCNK12			MSP	qMSP	RRBS	HC vs. CP HC vs. PT	– Hyper	Kisiel et al., 2015
MGMT			MSP			CP vs. PT	–	Henriksen et al., 2016
			MCAM			CP vs. PT	Hyper	Ligget et al., 2010
MLH1			MSP			CP vs. PT	–	Henriksen et al., 2016
			MCAM			HC vs. CP CP vs. PT	Hyper hypo	Ligget et al., 2010
NDRG4			MSP	qMSP	RRBS	HC vs. CP HC vs. PT	– Hyper	Kisiel et al., 2015
NPTX2			MSP			HC vs. PT HC vs. PNET	Hyper –	Sato et al., 2003a
			MSP			HC vs. CP HC vs. PT	Hyper Hyper	Park et al., 2012
			MSP			CP vs. PT	Hyper	Henriksen et al., 2016
			qMSP			HC+CP vs. PT	Hyper	Matsubayashi et al., 2006
PENK			MSP			CP vs. PT	–	Henriksen et al., 2016
			MSP			HC vs. CP HC vs. PT	Hyper Hyper	Park et al., 2012
			qMSP			HC+CP vs. PT	Hyper	Matsubayashi et al., 2006
PRKCB			MSP	qMSP	RRBS	HC vs. CP HC vs. PT	– Hyper	Kisiel et al., 2015
RARB			MSP					
			MSP			CP vs. PT	Hyper	Henriksen et al., 2016
			MCAM			CP vs. PT	hypo	Ligget et al., 2010
RPRM			MSP			HC vs. CP vs. PT	Hyper	Matsubayashi et al., 2006

Gene	Event	Characteristics of the study	Methods		Characteristics of the study	Event	References
SFRP1			MSP		HC vs. PT HC vs. PNET	Hyper -	Sato et al., 2003a
			MSP		HC vs. CP HC vs. PT	Hyper Hyper	Park et al., 2012
			MSP		HC vs. CP vs. PT	Hyper	Matsubayashi et al., 2006
			MSP		CP vs. PT	Hyper	Henriksen et al., 2016
			MCAM	MSP			
SOCS1			MSP				
			MSP				
			MCAM	MSREP	HC vs. PT	hypo	Melnikov et al., 2009
TFPI2			MSP		CP vs. PT	Hyper	Henriksen et al., 2016
			qMSP		HC+CP vs. PT	Hyper	Matsubayashi et al., 2006
THBS1			MSP				
			MCAM	MSREP	HC vs. PT	hypo	Melnikov et al., 2009
TJP2			MSP		HC vs. CP vs. PT	Hyper	Matsubayashi et al., 2006
UCHL1			MSP		HC vs. CP HC vs. PT	Hyper Hyper	Park et al., 2012
WNT5A			MI	MSP	HC vs PDAC, HC vs CP	Hyper	Singh et al., 2020
			MSP		CP vs. PT	Hyper*	Henriksen et al., 2016
SPARC			MI Q-MSP		HC vs PDAC, HC vs CP	Hyper*	Singh et al., 2020
UCHL1			MI Q- MSP		HC vs PDAC, HC vs CP	Hyper*	Singh et al., 2020
PENK			MI Q-MSP		HC vs PDAC, HC vs CP	Hyper*	Singh et al., 2020
NPTX2			MI Q-MSP		HC vs PDAC, HC vs CP	Hyper*	Singh et al., 2020

Table 1: List of genes representing differential DNA methylation between healthy controls (HC), pancreatic adenoductal carcinoma (PDAC) and chronic pancreatitis (CP).

References

1. Garg B, Giri B, Majumder K, Dudeja V, Banerjee S, Saluja A. Modulation of post-translational modifications in β -catenin and LRP6 inhibits Wnt signaling pathway in pancreatic cancer. *Cancer Letters.* 2017 Mar 1;388:64-72.
2. Wang L, Yang GH, Lu XH, Huang ZJ, Li H. Pancreatic cancer mortality in China (1991-2000). *World Journal of Gastroenterology: WJG.* 2003 Aug 15;9(8):1819.
3. Niederhuber JE, Brennan MF, Menck HR. The national cancer data base report on pancreatic cancer. *Cancer.* 1995 Nov 1;76(9):1671-7.
4. Ni XG, Bai XF, Mao YL, Shao YF, Wu JX, Shan Y, et al. The clinical value of serum CEA, CA19-9, and CA242 in the diagnosis and prognosis of pancreatic cancer. *European Journal of Surgical Oncology (EJSO).* 2005 Mar 1;31(2):164-9.
5. Locker GY, Hamilton S, Harris J, Jessup JM, Kemeny N, Macdonald JS, et al. ASCO 2006 update of recommendations for the use of tumor markers in gastrointestinal cancer. *Journal of Clinical Oncology.* 2006 Nov 20;24(33):5313-27.
6. Goonetilleke KS, Siriwardena AK. Systematic review of carbohydrate antigen (CA 19-9) as a biochemical marker in the diagnosis of pancreatic cancer. *European Journal of Surgical Oncology (EJSO).* 2007 Apr 1;33(3):266-70.
7. Sharma S, Kelly TK, Jones PA. Epigenetics in cancer. *Carcinogenesis.* 2010 Jan 1;31(1):27-36.
8. Warton K, Lin V, Navin T, Armstrong NJ, Kaplan W, Ying K, et al. Methylation-capture and next-generation sequencing of free circulating DNA from human plasma. *BMC Genomics.* 2014 Dec 1;15(1):476.
9. Baylin SB, Jones PA. A decade of exploring the cancer epigenome—biological and translational implications. *Nature Reviews Cancer.* 2011 Oct;11(10):726-34.
10. Warton K, Samimi G. Methylation of cell-free circulating DNA in the diagnosis of cancer. *Frontiers in Molecular Biosciences.* 2015 Apr 22;2:13.
11. Meiers I, Shanks JH, Bostwick DG. Glutathione S-transferase pi (GSTP1) hypermethylation in prostate cancer: review 2007. *Pathology.* 2007 Jun 1;39(3):299-304.
12. Umbricht CB, Evron E, Gabrielson E, Ferguson A, Marks J, Sukumar S. Hypermethylation of 14-3-3 σ (stratifin) is an early event in breast cancer. *Oncogene.* 2001 Jun;20(26):3348-53.
13. Montavon C, Gloss BS, Warton K, Barton CA, Statham AL, Scurry JP, et al. Prognostic and diagnostic significance of DNA methylation patterns in high grade serous ovarian cancer. *Gynecologic Oncology.* 2012 Mar 1;124(3):582-8.
14. Thierry AR, El Messaoudi S, Gahan PB, Anker P, Stroun M. Origins, structures, and functions of circulating DNA in oncology. *Cancer and Metastasis Reviews.* 2016 Sep 1;35(3):347-76.
15. Rykova EY, Morozkin ES, Ponomaryova AA, Loseva EM, Zaporozhchenko IA, Cherdyntseva NV, et al. Cell-free and cell-bound circulating nucleic acid complexes: mechanisms of generation, concentration and content. *Expert Opinion on Biological Therapy.* 2012 May 1;12(sup1):S141-53.
16. Abolhassani M, Tillotson JK, Chiao JW. Characterization of the release of DNA by a human leukemia-cell line hl-60. *International Journal of Oncology.* 1994 Feb 1;4(2):417-21.
17. El Messaoudi S, Thierry AR. Pre-analytical requirements for analyzing nucleic acids from blood. In *Circulating nucleic acids in early diagnosis, prognosis and treatment monitoring 2015* (pp. 45-69). Springer, Dordrecht.
18. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Science Translational Medicine.* 2014 Feb 19;6(224):224ra24-.
19. Mouliere F, Thierry AR. The importance of examining the proportion of circulating DNA originating from tumor, microenvironment and normal cells in colorectal cancer patients. *Expert Opinion on Biological Therapy.* 2012 May 1;12(sup1):S209-15.
20. Ueki T, Toyota M, Skinner H, Walter KM, Yeo CJ, Issa JP, et al. Identification and characterization of differentially methylated CpG islands in pancreatic carcinoma. *Cancer Research.* 2001 Dec 1;61(23):8540-6.
21. Ueki T, Walter KM, Skinner H, Jaffee E, Hruban RH, Goggins M. Aberrant CpG island methylation in cancer cell lines arises in the primary cancers from which they were derived. *Oncogene.* 2002 Mar;21(13):2114-7.
22. Natale F, Vivo M, Falco G, Angrisano T. Deciphering DNA methylation signatures of pancreatic cancer and pancreatitis. *Clinical Epigenetics.* 2019 Dec 1;11(1):132.

23. Fukushima N, Sato N, Ueki T, Rosty C, Walter KM, Wilentz RE, et al. Aberrant methylation of preproenkephalin and p16 genes in pancreatic intraepithelial neoplasia and pancreatic ductal adenocarcinoma. *The American Journal of Pathology.* 2002 May 1;160(5):1573-81.
24. Peng DF, Kanai Y, Sawada M, Ushijima S, Hiraoka N, Kitazawa S, et al. DNA methylation of multiple tumor-related genes in association with overexpression of DNA methyltransferase 1 (DNMT1) during multistage carcinogenesis of the pancreas. *Carcinogenesis.* 2006 Jun 1;27(6):1160-8.
25. Zhao Y, Sun J, Zhang H, Guo S, Gu J, Wang W, et al. High-frequency aberrantly methylated targets in pancreatic adenocarcinoma identified via global DNA methylation analysis using methylCap-seq. *Clinical Epigenetics.* 2014 Dec 1;6(1):18.
26. Nakata K, Ohuchida K, Mizumoto K, Kayashima T, Ikenaga N, Sakai H, et al. MicroRNA-10b is overexpressed in pancreatic cancer, promotes its invasiveness, and correlates with a poor prognosis. *Surgery.* 2011 Nov 1;150(5):916-22.
27. Ma L, Reinhardt F, Pan E, Soutschek J, Bhat B, Marcusson EG, et al. Therapeutic silencing of miR-10b inhibits metastasis in a mouse mammary tumor model. *Nature Biotechnology.* 2010 Apr;28(4):341-7.
28. Takikawa T, Masamune A, Hamada S, Nakano E, Yoshida N, Shimosegawa T. miR-210 regulates the interaction between pancreatic cancer cells and stellate cells. *Biochemical and Biophysical Research Communications.* 2013 Aug 2;437(3):433-9.
29. Matsubayashi H, Canto M, Sato N, Klein A, Abe T, Yamashita K, et al. DNA methylation alterations in the pancreatic juice of patients with suspected pancreatic disease. *Cancer Research.* 2006 Jan 15;66(2):1208-17.
30. Kisiel JB, Raimondo M, Taylor WR, Yab TC, Mahoney DW, Sun Z, et al. New DNA methylation markers for pancreatic cancer: discovery, tissue validation, and pilot testing in pancreatic juice. *Clinical Cancer Research.* 2015 Oct 1;21(19):4473-81.
31. Melnikov AA, Scholtens D, Talamonti MS, Bentrem DJ, Levenson VV. Methylation profile of circulating plasma DNA in patients with pancreatic cancer. *Journal of Surgical Oncology.* 2009 Feb 1;99(2):119-22.
32. Melnikov AA, Scholtens DM, Wiley EL, Khan SA, Levenson VV. Array-based multiplex analysis of DNA methylation in breast cancer tissues. *The Journal of Molecular Diagnostics.* 2008 Jan 1;10(1):93-101.
33. Matsubayashi H, Sato N, Fukushima N, Yeo CJ, Walter KM, Brune K, et al. Methylation of cyclin D2 is observed frequently in pancreatic cancer but is also an age-related phenomenon in gastrointestinal tissues. *Clinical Cancer Research.* 2003 Apr 1;9(4):1446-52.
34. Park JK, Ryu JK, Yoon WJ, Lee SH, Lee GY, Jeong KS, et al. The role of quantitative NPTX2 hypermethylation as a novel serum diagnostic marker in pancreatic cancer. *Pancreas.* 2012 Jan 1;41(1):95-101.
35. Singh N, Rashid S, Rashid S, Dash NR, Gupta S, Saraya A. Clinical significance of promoter methylation status of tumor suppressor genes in circulating DNA of pancreatic cancer patients. *Journal of Cancer Research and Clinical Oncology.* 2020 Mar 7:1-1.
36. Tan AC, Jimeno A, Lin SH, Wheelhouse J, Chan F, Solomon A, et al. Characterizing DNA methylation patterns in pancreatic cancer genome. *Molecular Oncology.* 2009 Dec 1;3(5-6):425-38.
37. Henriksen SD, Madsen PH, Larsen AC, Johansen MB, Drewes AM, Pedersen IS, et al. Cell-free DNA promoter hypermethylation in plasma as a diagnostic marker for pancreatic adenocarcinoma. *Clinical Epigenetics.* 2016 Dec 1;8(1):117.
38. Henriksen SD, Madsen PH, Larsen AC, Johansen MB, Pedersen IS, Krarup H, et al. Cell-free DNA promoter hypermethylation in plasma as a predictive marker for survival of patients with pancreatic adenocarcinoma. *Oncotarget.* 2017 Nov 7;8(55):93942.
39. Guo S, Diep D, Plongthongkum N, Fung HL, Zhang K, Zhang K. Identification of methylation haplotype blocks aids in deconvolution of heterogeneous tissue samples and tumor tissue-of-origin mapping from plasma DNA. *Nature Genetics.* 2017 Apr;49(4):635-42.
40. Lehmann-Werman R, Neiman D, Zemmour H, Moss J, Magenheimer J, Vaknin-Dembinsky A, et al. Identification of tissue-specific cell death using methylation patterns of circulating DNA. *Proceedings of the National Academy of Sciences.* 2016 Mar 29;113(13):E1826-34.
41. Liu XD, Wu H, Li Y, Liu X, Zhang Z, Yu L, et al. 686P Early detection of pancreatic ductal adenocarcinoma using methylation signatures in circulating tumour DNA. *Annals of Oncology.* 2019 Oct 1;30(Supplement 5):mdz247-013.
42. El Messaoudi S, Rolet F, Mouliere F, Thierry AR. Circulating cell free DNA: preanalytical considerations.

Clinica Chimica Acta. 2013 Sep 23;424:222-30.

43. Keeley B, Stark A, Pisanic II TR, Kwak R, Zhang Y, Wrangle J, et al. Extraction and processing of circulating DNA from large sample volumes using methylation on beads for the detection of rare epigenetic events. *Clinica Chimica Acta.* 2013 Oct 21;425:169-75.

44. Warton K, Lin V, Navin T, Armstrong NJ, Kaplan W, Ying K, et al. Methylation-capture and next-generation sequencing of free circulating DNA from human plasma. *BMC Genomics.* 2014 Dec 1;15(1):476.

45. Del Monte U. Does the cell number 10⁹ still really fit one gram of tumor tissue?. *Cell cycle.* 2009 Feb 1;8(3):505-6.

46. Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch RD, et al. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. *Cancer Research.* 2001 Feb 2;61(4):1659-65.

47. Devonshire AS, Whale AS, Gutteridge A, Jones G, Cowen S, Foy CA, et al. Towards standardisation of cell-free DNA measurement in plasma: controls for extraction efficiency, fragment size bias and quantification. *Analytical and Bioanalytical Chemistry.* 2014 Oct 1;406(26):6499-512.

48. Warton K, Samimi G. Methylation of cell-free circulating DNA in the diagnosis of cancer. *Frontiers in Molecular Biosciences.* 2015 Apr 22;2:13.

49. Yi JM, Guzzetta AA, Bailey VJ, Downing SR, Van Neste L, Chiappinelli KB, et al. Novel methylation biomarker panel for the early detection of pancreatic cancer. *Clinical Cancer Research.* 2013 Dec 1;19(23):6544-55.

50. Thompson MJ, Rubbi L, Dawson DW, Donahue TR, Pellegrini M. Pancreatic cancer patient survival correlates with DNA methylation of pancreas development genes. *PLoS One.* 2015 Jun 3;10(6):e0128814.

51. Angrisano T, Pero R, Brancaccio M, Coretti L, Florio E, Pezone A, et al. Cyclical DNA methylation and histone changes are induced by Ips to activate cox-2 in human intestinal epithelial cells. *PLOS One.* 2016 Jun 2;11(6):e0156671.

52. Pero R, Angrisano T, Brancaccio M, Falanga A, Lombardi L, Natale F, et al. Beta-defensins and analogs in *Helicobacter pylori* infections: mRNA expression levels, DNA methylation, and antibacterial activity. *PLOS One.* 2019 Sep 19;14(9):e0222295.

53. Liggett T, Melnikov A, Yi QL, Replogle C, Brand R, Kaul K, et al. Differential methylation of cell-free circulating DNA among patients with pancreatic cancer versus chronic pancreatitis. *Cancer: Interdisciplinary International Journal of the American Cancer Society.* 2010 Apr 1;116(7):1674-80.