Int J Clin Exp Pathol (2009) 2, 310-326
www.ijcep.com/IJCEP810007

Review Article

Epigenetics and Epigenetic Alterations in Pancreatic Cancer

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Received 21 October 2008; Accepted 28 October 2008; Available online 15 November 2008

Abstract: Pancreatic cancer remains a major therapeutic challenge. In 2008, there will be approximately 37,680 new cases and 34,290 deaths attributable to pancreatic cancer in the United States (U.S.), making it the fourth leading cause of cancer-related death. Recent comprehensive pancreatic cancer genome project found that pancreatic adenocarcinomas harbored 63 intragenic mutations or amplifications/homozygous deletions and these alterations clustered in 12 signaling pathways. In addition to widespread genetic alterations, it is now apparent that epigenetic mechanisms are also central to the evolution and progression of human cancers. Since epigenetic silencing processes are mitotically heritable, they can drive neoplastic progression and undergo the same selective pressure as genetic alterations. This review will describe recent developments in cancer epigenetics and their importance in our understanding of pancreatic adenocarcinoma.
Keywords: Epigenetics, pancreatic cancer

Introduction and Background

Pancreatic cancer still remains a major therapeutic challenge. In 2008, there will be approximately 37,680 new cases and 34,290 deaths attributable to pancreatic cancer in the United States (U.S.), making it the fourth leading cause of cancer-related death [1]. For all stages, 1-year survival is 23% and 5-year overall survival from diagnosis is 4%. Median survival for patients with locally advanced disease is 9-12 months and, for patients with metastatic disease, the median survival is 3-6 months. The 5-year survival after curative resection is only 15-25%. Few agents have demonstrated significant benefit for patients with metastatic disease.

Numerous clinical and epidemiologic studies have demonstrated the importance of the early diagnosis of cancer and the early detection of neoplastic precursors as the most effective means of reducing cancer-related mortality. This has thus far been shown for colorectal [2], breast [3], cervical [4], and prostate cancer [5]. For example, as much as 50% of the recent decline in breast cancer mortality can be attributed to mammography and early treatment, particularly the treatment of pre-invasive lesions [6].

In the same fashion, a growing body of evidence supports the view that invasive pancreatic adenocarcinomas arise from histologically well-defined noninvasive lesions within the pancreatic ducts [7-9]. The early detection of these precursors could reduce the incidence and mortality from pancreatic adenocarcinoma. These precursor lesions include microscopic pancreatic intraepithelial neoplasias (PanINs), and macroscopic intraductal papillary mucinous neoplasms (IPMNs) [10] and mucinous cystic neoplasms (MCN) [11]. Initial efforts to identify these precursor lesions among individuals with a strong family history of pancreatic cancer have been successful in so far as these lesions can be identified with careful pancreatic imaging and surgically removed [12-15].

Most pancreatic ductal adenocarcinomas are thought to develop PanINs that progress from low-grade to high grade PanINs to invasive adenocarcinoma through a series of genetic and epigenetic alterations. The most common of these genetic abnormalities include extensive chromosomal losses and gains at selected loci [16] and mutations/deletions of oncogenes and tumor suppressor genes, including *KRAS*, *CDKN1A/p16*, *TP53*, *MADH4/SMAD4/DPC4*, and BRCA2 [17-22]. In

addition, telomere shortening is a common genetic abnormality observed in all stages of PanINs including the vast majority of earliest lesions (PanIN-1A) [23].

More recently, a comprehensive pancreatic cancer genome project was undertaken to profile the genetic abnormalities of pancreatic adenocarcinomas [24]. Twenty-four pancreatic ductal adenocarcinomas were analyzed for somatic mutations in 20,661 protein-coding genes by cycle sequencing with homozygous deletions and gene amplifications detected using Illumina 1Mb SNP arrays. Pancreatic adenocarcinomas harbored 63 intragenic mutations or amplifications/homozygous deletions and these alterations clustered in 12 signaling pathways [24].

In addition to the widespread genetic alterations, it is now apparent that epigenetic mechanisms are also central to the evolution and progression of human cancers [25-29]. Since epigenetic silencing processes are mitotically heritable, they can drive neoplastic progression and undergo the same selective pressure as genetic alterations. Indeed, pancreatic cancers harbor numerous epigenetic alterations and these alterations can be observed in PanINs [30, 31] and IPMNs [31, 32] and their prevalence increases as lesions become of more advanced grade. This review will describe recent developments in cancer epigenetics and their importance in our understanding of pancreatic adenocarcinoma.

Epigenetics

Epigenetics is defined as heritable changes in gene expression that are not accompanied by changes in DNA sequence. There are two main categories of epigenetic mechanisms that affect mammalian gene expression at the chromatin level and fulfill the criterion of heritability: DNA methylation and histone modification.

DNA Methylation

DNA methylation in the mammalian genome arises due to covalent addition of a methyl group to the 5' position of cytosine in the context of the palindromic dinucleotide, CpG. This modification is established and maintained by a family of DNA methyltransferases (DNMTs), DNMT1, DNMT3a and DNMT3b [33-35]. DNMT1 binds and methylates the daughter strands of newly replicated DNA to preserve the parental methylation pattern [36]. The other two enzymes, DNMT3a and DNMT3b, function primarily as *de novo* methyltransferases and establish genome methylation during pre-implantation [37, 38]. DNA methylation is crucial for mammalian development; neither *DNMT1* nor *DNMT3b* homozygous knockout mice are viable, and *DNMT3a* knockout mice die 4 weeks after birth [38]. Patients with biallelic mutations in *DNMT3b* develop ICF syndrome (immunodeficiency, centromere instability and facial anomalies) [39]. Somatic knockout of *DNMT1* and *DNMT3b* results in complete DNA hypomethylation but single knockouts still retain DNA methylation indicating an ability of these enzymes to compensate for each other [40]. DNMT1 overexpression has been described in certain cancers [41-44] and Li *et al* have found that most pancreatic cancers harbor overexpression of DNMT1 (unpublished).

CpG Islands

There are two patterns of CpG methylation in normal human somatic cells: a majority of the genome (~98%) in which CpGs are relatively sparse (on average 1 per 100 base pairs (bp)) but highly methylated (approximately 80% of all CpG sites) [45], and a minor fraction (~2%) that comprises short stretches of DNA (~1,000 bp) with high CpG density (~1 per 10 bp) and largely methylation-free [46], known as CpG islands (CGIs). CpG islands were originally defined as regions of 200 bases or more with a (G+C)-content of at least 50% and a ratio of observed CpG frequency to expected CpG frequency of at least 0.6 [47]. There are fewer CpGs in the genome than predicted by its GC content and this pattern is thought to have arisen during evolution because methylated CpGs are liable to undergo spontaneous deamination and mutation to thymine. A more strict definition of CpG islands, the Takai-Jones criteria, provides a better association of CpG islands with 5' regions of genes and excludes most Alu repeats [48]. Unmethylated CpG islands often localize to the transcriptional start sites of genes.

Approximately 60% of human genes are associated with CpG islands, most of which were once thought to be unmethylated in all tissue types except in certain circumstances (e.g. genomic imprinting and X chromosome inactivation) [49]. However, it is clear that there are normal tissue-specific patterns of

CpG island methylation and some CpG island are prone to progressive methylation during aging and during the development of certain diseases such as cancer [50-52].

Recently, a comprehensive analysis of the methylation profile of CpG islands of normal tissues has been reported. Of 16,000 human promoters examined 3% of transcription start site (TSS)-associated CpG islands were normally methylated in somatic tissues [53]. Similarly, Illingworth *et al* reported that 6-8% of TSS-associated CpG islands were partially methylated [54]. In addition, Esteller *et al* highlighted the importance of environmental influences on DNA methylation by observing that twins can have significantly different lymphocyte methylation patterns [55]. Similarly, Matsubayashi found that normal duodenal mucosa from patients with pancreatic cancer was more likely to show CpG island methylation than patients with chronic pancreatitis even after adjusting for age [56].

In addition, many promoters that lack strictly defined CpG islands have nonetheless been shown to have tissue specific methylation patterns that strongly correlate with transcriptional activity [48, 57, 58]. For example, Sato *et al* demonstrated that many commonly overexpressed genes in pancreatic cancers undergo hypomethylation of their normally methylated CpG poor promoters [59]. Eckhardt *et al* using high-resolution methylation profiles of human chromosomes 6, 20, and 22 reported that genes without a CpG island in their 5'untranslated region showed an inverse correlation between mRNA expression and methylation [60]. The functional relevance of such CpG poor promoters and their methylation awaits further study [27, 61, 62].

Some genes do not display typical patterns of CpG methylation and gene expression. For example, *hTERT* encodes the catalytic subunit of telomerase, is expressed in most of telomerase positive tumors and its promoter CpG island is hypermethylated in many cancers and hypomethylated in telomerase negative normal tissues [62-64]. Recently, Renaud *et al* concluded that hTERT expression is induced when the *hTERT* CpG island is sufficiently hypermethylated to avoid binding of the CTCF repressor yet hypomethylated at certain CpGs thereby enabling the transcription complex to be formed [65]. Taken together, these studies highlight the fact that the functional significance of CpG islands cannot be reliably predicted by computational predictions of CpG density but require experimental evaluation.

Alterations in CpG Island Methylation in Pancreatic Cancer

Several of the classic tumor suppressor genes show aberrant promoter CpG island hypermethylation in a subset of pancreatic cancers. In addition, an increasing number of functionally important genes have been identified that undergo promoter methylation and transcriptional silencing in pancreatic and other cancers. The first classic tumor suppressor gene shown to undergo promoter methylation and gene silencing was the *p16/CDKN1A* gene where epigenetic silencing is limited to genes that are not targeted for genetic inactivation [66]. Other genetically inactivated tumor suppressor genes in pancreatic cancers, such as *p53*, *SMAD4* and *STK11* have not been found to undergo silencing by DNA methylation. Similarly, few of the classic tumor suppressor genes and DNA repair genes that are mutated in other cancers have been shown to undergo epigenetic silencing in pancreatic cancers. *hMLH1* has been shown to undergo methylation in a small percentage of pancreatic cancers [67-69] and is associated with microsatellite instability that is sometimes associated with a medullary histology [70, 71]. *E-cadherin* has been shown to be an infrequent target of DNA methylation [69] and gene silencing and *E-cadherin* silencing is associated with an undifferentiated phenotype and a poor prognosis [72]. But other classic tumor suppressors such as *VHL*, *Rb*, *PTEN* and *BRCA1* have not been found to be epigenetically silenced in pancreatic cancer. More recent studies have used a variety of gene discovery approaches to systematically identify genes that undergo promoter CpG island methylation.

Ueki *et al* used methylated CpG island amplification (MCA) coupled with representational difference analysis (RDA) and identified the gene preproenkephalin (*ppENK*), which encodes a native opioid peptide with growth-suppressor properties, and is methylated in most pancreatic cancers [73]. A similar approach was later used by Hagihara [74]. Sato *et al* compared global gene expression profiles of pancreatic cancer cell lines before and after treatment with the DNA methyltransferase inhibitor, 5-aza-2'-

deoxycytidine (5-Aza-dC) and identified 475 candidate genes induced in four pancreatic cancer cell lines, but not in a non-neoplastic pancreatic ductal epithelial cell line, and subsequent analyses confirmed aberrant hypermethylation of several genes in primary pancreatic cancers [75, 76]. Recently, we reported that an MCA approach applied to Agilent 44K promoter microarrays identified 606 genes differentially methylated in a pancreatic cancer cell line, compared to normal pancreas. This assay strategy also demonstrated high reproducibility and accuracy [77].

These approaches have led to the identification of several genes with tumor suppressor properties that are commonly inactivated in pancreatic cancers. For example, Sato *et al* compared the global gene expression profiles of IPMNs with that of normal pancreatic ductal epithelium samples and identified the cyclin-dependent kinase inhibitor, *CDKN1C/p57KIP2* as an underexpressed gene. CDKN1C is a potent inhibitor of several G1 cyclin complexes, and is a negative regulator of cell proliferation [78, 79]. *CDKN1C* is an imprinted gene located on chromosome 11p15.5, and a tumor suppressor whose inactivation leads to Wilm's tumor, and Beckwith-Wiedemann syndrome. Partial methylation of the *CDKN1C* promoter CpG island is commonly observed in pancreatic cancer cell lines and IPMNs with reduced CDKN1C expression. In addition to methylation of the *CDKN1C* promoter, complete hypomethylation of *LIT1*, an imprinting control region important for the regulation of CDKN1C expression, was detected in some pancreatic cancers as a result of deletion of the methylated *LIT1* allele at 11p15.5 rather than through loss of the epigenetic marks of imprinting [80].

SPARC (secreted protein acidic and rich in cysteine, or osteonectin/BM40) is a gene that was first identified as aberrantly methylated in pancreatic cancer by treating pancreatic cancer cells with demethylating agents [81]. SPARC is a calcium-binding protein that interacts with extracellular matrix whose expression is often lost in pancreatic cancer cells through aberrant DNA methylation [81]. *SPARC* knockout mice grow cancers faster than mice expressing *SPARC* [82, 83], highlighting its growth inhibitory functions. SPARC influences cell migration, proliferation, angiogenesis (especially during wound healing), matrix cell adhesion, and tissue remodeling [81, 84]. Interestingly, juxta-tumoral fibroblasts often express SPARC and patients whose pancreatic cancers express SPARC in their peritumoral fibroblasts have a poorer prognosis [85].

Tissue factor pathway inhibitor 2 (TFPI-2) is a Kunitz-type serine proteinase inhibitor that acts against a wide range of proteases [86], and is thought to protect the matrix from degradation thereby counteracting tumor invasion and metastasis [87-89]. Sato *et al* identified aberrant methylation of *TFPI-2* in 73% (102/140) of pancreatic cancer xenografts and primary pancreatic adenocarcinomas. Restored expression of the *TFPI-2* gene in nonexpressing pancreatic cancer cells resulted in marked suppression in their proliferation, migration, and invasive potential in vitro [90].

Several genes of the GATA family have been investigated for epigenetic alterations in pancreatic cancer as other types of cancer. Fu *et al* demonstrated that *GATA-5* was frequently methylated in pancreatic cancers, whereas *GATA-4* was infrequently methylated and often overexpressed relative to normal ductal epithelium [91].

Another gene commonly silenced epigenetically in pancreatic cancer is *BNIP3*. Silencing of *BNIP3* was associated with CpG island methylation in the region of the transcription start site and treatment with the DNA methyltransferase inhibitor, 5-AZA-dC, restored hypoxic *BNIP3* expression and reversed resistance to hypoxia-induced death [92, 93]. Suppression of *BNIP3* expression in cell lines with RNAi also decreased sensitivity to 5-fluorouracil and gemcitabine, implicating *BNIP3* silencing as a potential drug resistance mechanism [94].

Some other targets of aberrant methylation in pancreatic cancer include *TSLC1* [95], *HHIP* [96], *MUC2* [97], *reprimo* [98], *CXCR4* [99] and *SOCS1* [100].

DNA Hypomethylation in Cancer

Although much of the focus of cancer epigenetics is on the inactivation of tumor suppressor genes by promoter methylation, the earliest observations of aberrant methylation in human cancer identified DNA hypomethylation. The global methylated

cytosine content is often reduced in cancer including pancreatic cancers [101] and this hypomethylation occurs both at normally methylated repeat sequences throughout the genome. Global DNA hypomethylation was first described in the early 1980s [102, 103], yet the cause of the global hypomethylation found in cancers is not well understood. One consequence of genome wide hypomethylation may be genomic instability, a characteristic of most pancreatic and other cancers [104, 105]. Interfering with DNA methylation functions such as by knocking out DNA methyltransferase leads to DNA hypomethylation and genetic instability [106]. Indeed, p53 deficient animals hypomorphic for *DNMT1* are more likely to get sarcomas [107]. However, in some experimental settings genomic DNA hypermethylation may be more important than hypomethylation; *Apc min* mice hypomorphic for *DNMT1* have a reduced risk of gastrointestinal neoplasia [108].

One cause of DNA hypomethylation is nutritional deficiency. Low folate status or defective folate or vitamin B12 metabolism causes methyl group deficiency which is thought to cause genetic instability by lowering S-adenosyl-methionine concentration, reducing global DNA methylation and the synthesis of thymidine from uracil [109]. Uracil misincorporation in place of thymidine leads to an imbalanced nucleotide pool and increased occurrence of DNA strand breaks [110], which increases genomic instability [111] and is thought to contribute to cancer development. Low vitamin B12 levels or low folic acid intake is a risk factor for pancreatic cancer [112]. In addition, Matsubayashi found that cancer with defective *MTHFR* genotypes are associated with higher levels of chromosomal losses and reduced levels of *LINE1* element methylation [101]. Such observations raise the possibility that nutritional causes of methyl group deficiency could accelerate tumorigenesis in neoplasms that are genetically unstable and have already lost or inactivated genes such as *MTHFR* involved in methyl group metabolism.

Gene Hypomethylation and Overexpression

DNA hypomethylation also occurs at the 5′ regions of certain genes in pancreatic and other cancers and is associated with gene overexpression. For example, Rosty *et al* identified overexpression of *S100A4* was associated with hypomethylation at specific CpG sites within the first intron [113]. In an attempt to identify additional hypomethylation targets in pancreatic cancer, Sato *et al* examined microarray profiles to identify genes that were overexpressed in pancreatic cancer but not normal pancreata and normal pancreatic ducts as a screen for hypomethylated genes. Among the identified transcripts, about 200 genes were found to be re-induced after combined treatment with 5Aza-dC and TSA. DNA hypomethylation of promoter CpGs were identified in over half of the genes with this expression pattern including *claudin4*, *lipocalin2*, *14-3-3sigma/ stratifin*, trefoil factor 2, *S100A4*, *mesothelin*, *PSCA*, *S100P* and *maspin* [114].

Methyl-CpG Binding Proteins (MBDs)

The information stored by hypermethylated CpG islands is in part interpreted by methyl-CpG binding proteins (MBDs). MBDs help establish a transcriptionally inactive chromatin environment. This family of proteins consists of five well-characterized members (MeCP2, MBD1, MBD2, MBD3 and MBD4) [115]. MeCP2 was first characterized as a 'translator' which represses transcription of methylated DNA through the recruitment of a histone deacetylase (HDAC) complex [116, 117]. Most hypermethylated promoters are occupied by MBD proteins, whereas unmethylated promoters generally lack MBDs. Treatment of cancer cells with a demethylating agent causes CpG island hypomethylation, MBD release and gene re-expression, reinforcing the notion that association of MBDs with methylated promoters is methylation-dependent [118, 119]. It is currently thought that the specific profile of MBD occupancy for hypermethylated CpG islands of tumor-suppressor genes might be gene- and tumor- specific.

MBDs like DNMTs also recruit histone methyltransferases (HMTs) [118]. A limited number of studies have examined the significance of MBDs in pancreatic cancer [120].

Histone Modifications in Cancer

Histones are globular proteins with protruding N-terminal tails that are THE main site of biochemical modifications including acetylation, methylation, phosphorylation, and ubiquitination [118, 121]. The specificity of certain histone modifications to influence transcription led to the 'histone code

hypothesis' which predicts that histone modifications act sequentially or in combination to alter chromatin structure and form a code that may be read by nuclear factors [122-127]. Lysine acetylation neutralizes the charge between DNA and histone tails and correlates with chromatin accessibility and transcriptional activity. Lysine methylation can have different effects depending on which residue is modified.

Hypermethylated CpG islands of silenced tumor-suppressor genes are known to display a particular histone code characterized by deacetylation of histones H3 and H4, methylation of H3 lysine 9 (H3K9), H3 lysine 27 (H3K27), and H4 lysine 20 (H4K20), and demethylation of H3 lysine 4 [128-131]. On the other hand, methylation of histone H3 lysine 4 (H3K4) and H3 lysine 36 is associated with transcribed chromatin [125, 130]. Histone modifications also function to recruit other effector proteins [132]. Acetylated lysines are recognized by bromodomains within nucleosome remodeling complexes. An interaction between methylated H3K4 and the chromodomain of the helicase Chd1 recruits activating complexes to chromatin. In contrast, methylated H3K9 and H3K27 are bound by heterochromatin protein 1 (HP 1) and Polycomb-group (PcG) proteins, respectively, which mediate chromatin compaction. A given lysine can have up to three methyl groups, and this ''methyl state'' can influence chromodomain binding. PcG proteins preferentially interact with trimethylated H3K27, while HP1 shows preference for both di- and trimethylated H3K9 [133].

Finally, PcG proteins function as transcriptional repressors that silence genes through chromatin modification [134]. The precise molecular mechanisms of Polycomb repressive complex (PRC)-mediated repression are still poorly understood. The PcG complex can inhibit transcription by preventing ATP-dependent nucleosome remodeling by the SWI/SNF complex as well as by directly blocking the transcription initiation machinery (See review [134]). PcG and MBD proteins collaborate in long-term gene silencing events such as X-chromosome inactivation and imprinting [135]. PcG proteins are thought to serve as recruitment platforms for DNMTs involved in the hypermethylation of tumor suppressor genes [136]. The ability of chromatin modifications to facilitate aberrant DNA methylation highlights uncertainty as to the primacy of DNA methylation or histone modifications as initiators of aberrant epigenetic gene silencing during cancer development.

Histone Modifications in Pancreatic Cancer

A global gene expression analysis of pancreatic cancers before and after treatment of histone deacetylase inhibitors [75, 76] has been described, but overall few studies have examined genes that are regulated by histone modifications in pancreatic cancers.

Genes of the mucin family have been shown to undergo histone alterations in pancreatic cancers in association with gene overexpression. Mucins are high molecular weight glycoproteins several of which are produced by pancreatic cancers. Yamada *et al* revealed that the 5' region of *MUC1* transcriptional start site (TSS) were enriched in tri/dimethylated H3K9 marks as well as methylated DNA in non-expressing cells [97, 137]. The TSS of *MUC2* is highly enriched in di- and tri-methylated H3K4, acetylated H3K9, and acetylated H3K27 in *MUC2* expressing pancreatic cancer cells. Vincent *et al* demonstrated that *MUC4* transcription activity was directly affected by DNMT3A, DNMT3B, HDAC1 and HDAC3, DNA methylation and repressive histone code of one region in 5'UTR of *MUC4*, regarding one Sp1 binding site was significantly associated with MUC1 silencing [138].

Antisense and Epigenetic Regulation

Several microRNAs (miRNAs) have been identified that are regulated by DNA methylation in pancreatic cancer [77] and other cancers [139-141]. Non-coding RNA such as miRNAs and natural antisense transcripts (NATs) are known to be involved in post-transcriptional gene silencing (PTGS). For example, certain miRNAs regulate DNA methylation. *miR-29* inhibits DNMT3 activity and transfection of *miR-29* into *miR-29* negative cell lines inhibits DNA methylation [142].

Transcriptional gene silencing (TGS) caused by small interfering RNAs (siRNA) was first observed in transformed tobacco plants and gene suppression was associated with DNA methylation [143]. Until recently, it was not known if a similar TGS mechanism existed in mammals. However in 2004, Morris *et al*

demonstrated that siRNA targeting the *EF1alpha* promoter could induce transcriptional silencing and *de novo* methylation [144]. Recently, Tufarelli *et al* investigated a rare case of alpha-thalassaemia caused by a deletion that did not affect the *HBA2* gene but relocated a *LUC7L* gene. Its expression gave rise to NATs that overlapped both coding and promoter region of *HBA2* and led to aberrant methylation of the promoter CpG island [145].

Although several studies have demonstrated that RNA-dependent transcriptional silencing can occur in mammals, the mechanism for silencing identified is generally been chromatin mediated. Whether or not such silencing alters DNA methylation is controversial. For example, Ting *et al* has observed that small double stranded RNA targeted to *CDH1* in colon cancer cells induced TGS by H3K9 methylation without DNA methylation [146]. Feinberg and Cui *et al* examined whether cancer cells have aberrant expression of antisense transcripts and if such transcripts cause tumor suppressor gene silencing. They found that some AML cases contained *p15* NATs that were associated with chromatin silencing and demonstrated that expression of antisense RNAs from transgenes could silence tumor suppressor gene expression and lead to secondary DNA methylation and that such silencing represented a hit and run effect because it persisted after removal of the antisense RNA [147]. The mechanisms by which noncoding RNAs induce gene silencing are just beginning to be understood. The elucidation of these mechanisms may ultimately lead to therapeutic strategies to epigenetically silence oncogenes.

DNA Methylation Alterations in Pancreatic Cancer Precursors

The timing of epigenetic alterations during cancer development vs cancer progression provides evidence as to their likely significance. Support for the contribution of DNA methylation alterations to pancreatic cancer susceptibility is shown by the fact that many epigenetically silenced genes are silenced in pancreatic cancer precursors. For example, DNA methylation can be detected in PanINs and IPMNs and the prevalence of methylation increases with neoplastic grade [7, 32]. Some of the genes that have been identified as silenced include *p16*, *RELN*, *TFPI-2* and *ppENK* [30, 148]. Methylation abnormalities have also been identified in mucinous cystic adenomas of the pancreas [149].

Epigenetic Alterations and Cancer Stem Cells

Recent reviews have emphasized that epigenetic abnormalities might play a seminal role in the earliest steps in cancer initiation [25, 150, 151]. In addition, markers of cancer stem cells are likely to be under epigenetic regulation [152]. Some studies have implicated a stem cell hierarchy to pancreatic cancer cells and to other solid cancers [153], but conclusive evidence that pancreatic cancer stem cells exist has yet to be demonstrated [154], nor have epigenetic influences on cancer stem cells been conclusively demonstrated. Normal stem cells are regulated in part by polycomb proteins that repress gene expression until cellular differentiation. For example, the PcG proteins suppressor of zeste 12 (SUZ12) and embryonic ectoderm development (EED), which form the Polycomb repressive complex 2 (PRC2) are associated with nucleosomes that are trimethylated at H3K27 [155]. Many PcG targets are methylated in adult cancer cells including pancreatic cancer cells [77, 150, 156]. There is also considerable overlap between genes that are aberrantly hypermethylated in cancer are also frequently hypermethylated in stem cells [157]. Ohm *et al* demonstrated that many polycomb target genes are modified by a 'bivalent' promoter chromatin pattern consisting of the repressive mark, methylated H3K27, and an active mark, methylated H3K4 in embryonic stem cells so that they are held in a 'transcriptional ready state [150]. Several groups hypothesized that reversible repression of these regulatory genes by histone modification in stem or progenitor cells might be replaced by permanent silencing by aberrant DNA methylation, locking the cell into a perpetual state of self renewal and thereby predisposing to tumor initiation and subsequent progression [158, 159]. It will be interesting to determine if environmental signals could drive neoplastic development by acting on epigenetic signals in stem cells.

Mechanisms of Aberrant DNA Hypermethylation in Cancer

The causes of aberrant hypermethylation in pancreatic and other cancers remain poorly understood. As indicated above, aging is associated with an increase in DNA

methylation in normal tissues that is likely to predispose to the epigenetic silencing of certain tumor suppressor genes [56, 160, 161], and some studies indicate that cancers of older patients have more aberrant methylation than those in younger patients [73]. Precancerous lesions also undergo aberrant methylation that is often present in early stages of neoplasia and increases with neoplastic stage and studies indicate that epigenetic influences can alter the genetic profile of a cancer, supporting the notion that epigenetic influences can occur prior to early genetic events [162, 163]. Some studies have indicated that chronically inflamed tissues may have more aberrant methylation, though interestingly a recent report indicated that ulcerative colitis-associated cancers had low levels of aberrant methylation [164]. One interesting report found that sites of DNA repair could become foci of aberrant methylation as the DNA repair apparatus also recruits DNMTs and SIRT1 [165]. A methylator phenotype (CIMP or CpG island methylator phenotype) has been proposed as a mechanism by which cancers acquire aberrant methylation at hundreds of sites [166]. A small subset of colorectal cancers have been shown to have aberrant methylation of a unique set of genes that do not undergo frequent methylation in normal tissues or other colon cancers and these cancers have a very high prevalence of *BRAF* mutation, suggesting that there are mechanisms that can lead to a CIMP phenotype [167]. It is not known if dietary or other environmental factors can influence the propensity to hypermethylated CpG islands. In the same fashion that dietary deficiency of methyl groups can lead to hypomethylation, it is possible that dietary excess of methyl groups can cause hypermethylation. For example, the phenotype of the agouti mouse can be modified by its dietary level of methyl groups [168].

Aberrant DNA Methylation as a Diagnostic Marker

Methylation-specific PCR (MSP)-based assays to detect aberrant DNA hypermethylation have been evaluated in patients with pancreatic cancer. Fukushima *et al* first reported the utility of detecting methylation biomarker in pancreatic juice by MSP. Pancreatic juice samples were collected either intraoperatively, from 92 patients undergoing pancreaticoduodenectomy for benign (n = 20) and malignant periampullary disease (n = 72) or endoscopically (by duodenal aspiration after secretin infusion), and from 13 patients undergoing investigation for pancreatic disease. Methylated *ppENK* was detected in the pancreatic juice of 30 (66.7%) of 45 patients with pancreatic ductal adenocarcinoma, in 4 (44.4%) of 9 patients with intraductal papillary-mucinous adenocarcinoma, and in 7 (41.2%) of 17 patients with other periampullary carcinomas. Methylated *p16* was detected in a lower percentage of these patients (11.1%, 11.1% and 23.5%, respectively). In contrast, methylated *ppENK* and *p16* were not detected in 32 patients with non-malignant periampullary disease. However they found that methylated *ppENK* and *p16* was present in the duodenum of 90.5% and 28.6% and furthermore 88.9% and 11.1%, respectively, of pancreatic juice samples obtained by duodenal aspiration from patients without cancer. They concluded that detection of methylated *ppENK* and *p16* in pure pancreatic juice obtained by direct cannulation of the pancreatic duct to avoid duodenal secretions which normally have methylated DNA [169].

Matsubayashi *et al* quantified methylated DNA levels in the pancreatic juice of patients with benign and malignant pancreatic disease using quantitative MSP (QMSP) and demonstrated that a concentrations of >1% methylated DNA in 2 or more of 5 QMSP markers (*TFPI-2*, *p16*, *ppENK*, *SPARC*, *NPTX2*) was highly accurate in distinguishing patients with pancreatic cancer from those with pancreatitis or a normal pancreas [170]. Yan *et al* analyzed pancreatic juice for *p53* mutations, *KRAS* mutations and *p16* methylation [171]. The *p53* mutations were detected with a functional yeast assay in which yeast with transfected mutant *p53* PCR products are identified by a red color [171]. Other studies have used MSP to detect methylation alterations of *p16*, *p14*, *ppENK*, *NPTX2* and *TFPI-2* in pancreatic juice in preliminary studies [172-175]. Parsi *et al* demonstrated the diagnostic utility of quantifying aberrantly methylated DNA concentration in endoscopic brush samples of biliary strictures. Methylation analysis with QMSP was performed on endoscopically obtained brush samples from the biliary and pancreatic duct from 130 individuals with biliary tract strictures. Using with 3-gene (*NPTX-2*, *Cyclin D2*, and *TFPI-2*) panel, 73.2% of patients with pancreatic adenocarcinoma has positive methylation in 1 or more genes,

compared to only 13.6% of individuals with non-neoplastic conditions. They defined a cut-off of at least one gene positive for methylation as >1% methylated *TFPI2* and >3% methylated *NPTX2* and *Cyclin D2* [176].

Although plasma or serum is a very attractive sample for clinical use, and the detection of cancer-associated changes in DNA (mutations, methylation, and rearrangements) in serum, has been reported [177, 178] (for review, see [179]), results to date have been variable [180-182]. Studies evaluating mutant *KRAS* detection in plasma suggest that such mutant DNA is not readily detected in patients with pancreatic cancer until the disease is more advanced [183], although this may depend on the detection method. It is suspected that circulating tumor DNA is usually released into the bloodstream via necrotic or apoptotic pathways [184, 185], although in some instances it reflects the presence of circulating tumor cells. Interestingly, recent estimations of the concentrations of tumor-derived DNA in plasma, based on the detection of a confirmed tumor-specific mutations, demonstrate that concentrations of circulating mutant DNA is very low (<0.2%) in patients with early stage colon cancer and increases in concentration with tumor stage [186]. Since individual point mutations are not common to every tumor type, aberrant DNA methylation patterns may be a preferred method for identifying DNA alterations derived from cancer. Before such a goal can be realized, more information is needed regarding the methylation landscape of circulating DNA in healthy individuals.

Acknowledgements

This work was supported by the National Cancer Institute grant (CA120432, CA62924), the V foundation and the Michael Rolfe Foundation.

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