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Epigenomics

Methylation of immune-regulatory cytokine genes and pancreatic cancer outcomes

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Aim: Given the immunosuppressive nature of pancreatic cancer, we investigated the relationship between epigenetic modification of immune-regulatory cytokine genes and pancreatic cancer outcomes. **Materials & methods:** We evaluated DNA methylation of 184 pancreatic tumor samples from The Cancer Genome Atlas for 111 CpG loci in seven cytokine genes: *IL10*, *IL6*, *IL8*, *TGFβ1*, *TGFβ2*, *TGFβ3* and *TNF*. We used Cox regression to evaluate the associations between methylation and overall survival, disease-specific survival and disease progression ($\alpha = 0.05$). **Results:** Poorer survival was associated with increased methylation in fifteen CpG probes in *TGFβ1*, *TGFβ2*, *TGFβ3* and *TNF*. We also detected improved outcomes for three loci in *IL10*, *IL8* and *IL6*. **Conclusion:** Epigenetic regulation of cytokine-related gene expression may be associated with pancreatic cancer outcomes.

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Keywords: cytokines • epigenetics • immune system • methylation • pancreatic cancer • progression • survival

Pancreatic cancer is a highly lethal cancer that is currently the third most common cancer-related death in the USA [1]. The 5-year survival rate is only 9% and over 90% of cases die from this aggressive malignancy [1,2]. One reason for these poor outcomes is the fact that pancreatic cancer cells are capable of modulating the immune response and establishing an immunosuppressive microenvironment that promotes tumor growth [3]. In this immune-inhibitory state, increased levels of T regulatory cells and myeloid derived suppressor cells impair the antitumor activity of effector cells, such as cytotoxic $CD8^+$ T cells, natural killer cells and dendritic cells [3]. In addition, helper $CD4^+$ T cells transition from a Th1 phenotype, which facilitates tumor-eliminating processes, to a Th2 phenotype, which supports more tumor-tolerating immune responses [3–5]. Current literature suggests that pancreatic cancer patients with higher levels of T regulatory cells [6], myeloid derived suppressor cells [7] and Th2-type T helper cells [8] have worse prognoses, while those with higher concentrations of immune effector cells have increased survival [3,9,10].

These changes in immune function are influenced in part by the aberrant expression and production of regulatory cytokines. Pancreatic tumor cells have been found to directly secrete the anti-inflammatory cytokines IL-10 and TGF-β, which skew T helper cell differentiation toward the Th2 phenotype and suppress tumor-killing Th1 responses [5,11]. Circulating levels of pro-inflammatory cytokines (e.g., IL-6, IL-8, TNF-α) have also been observed to be elevated in pancreatic cancer patients compared to normal controls [12–15]. Other studies have further detected increased genetic and protein expression of both pro-inflammatory (e.g., IL-1β, IL-6, IL-8, TNF-α) and anti-inflammatory (e.g., IL-10, TGF-β, IL-11) cytokines by pancreatic cancer cells [13,16–18]. Given the complex interplay between cytokines and immune function, the expression and serum levels of various cytokines have been found to be prognostic markers of pancreatic cancer progression in some small studies [13–15,17–23].

While serum and mRNA levels of cytokines have been evaluated in regards to survival, there is limited information as to whether prognosis is also affected by the epigenetic regulation of associated gene expression. DNA methylation is a form of epigenetic mark that involves the addition of a methyl group to cytosine bases at CpG dinucleotides. Aberrant DNA methylation patterns have been implicated in the development and progression of several cancers, perhaps by inducing genomic instability and altering gene expression to influence the tumor microenvironment [24–28]. Though current research on DNA methylation and pancreatic cancer has been fairly nascent, past studies have identified potential epigenetic biomarkers or methylation signatures of pancreatic cancer risk and progression [29–35]. However, these studies have been primarily epigenome-wide association studies or candidate gene studies that did not focus on genes related to the immune response. Hence, it is unknown whether the relationship between immune dysfunction and pancreatic cancer progression and mortality is attributed to the atypical methylation of cytokine-related genes. Furthermore, it has not been evaluated whether epigenetic regulation in these immune-regulatory genes impacts clinical outcomes through modifications in genetic expression. Discovering and understanding these relationships could potentially identify methylation biomarkers of survival and progression and allow for the development of novel methylation-targeted therapies.

Materials & methods

Data source

We performed a retrospective cohort study of pancreatic cancer patients from The Cancer Genome Atlas (TCGA). TCGA is a large-scale collaboration between the National Cancer Institute and the National Human Genome Research Institute that provides complete genomic sequencing and epigenetic data on 11,000 patients across 33 different tumor types. It involves the participation of twenty institutions across the USA and Canada and includes clinical data and comprehensive genomic profiling of both tumor and matched normal tissues [36]. For this study, we included all patients with a primary pancreatic cancer tumor sample (N = 184). All de-identified data was downloaded from TCGA using the *TCGAbiolinks* package in R [37].

Methylation exposures

DNA methylation profiles of TCGA pancreatic cancer samples were measured using the Illumina Infinium HumanMethylation450 (HM450) BeadChip methylation assay [38,39]. This assay measures the methylated and unmethylated signal intensities of over 450,000 CpG dinucleotide sites across the genome, which are enriched for regulatory elements and promoter regions [40]. Raw methylation intensities downloaded from TCGA were preprocessed using normal-exponential out-of-band (noob) background correction with dye-bias normalization and converted to β-values with the *minfi* package [38,41]. β-values were calculated from the corrected methylated (m_s) and unmethylated (m_u) signal intensities using the formula $\beta = m_s/(m_s+m_u)$ and range from 0 to 1, with larger values indicating higher levels of methylation [38].

We filtered CpG probes by dropping those that overlapped with single nucleotide polymorphisms (SNPs) with a minor allele frequency of ≤5% based on the CEU population [42]. These SNPs can interfere with probe binding, as well as influence methylation signal intensities since the HM450 assay can erroneously detect the common C to T polymorphism as a change in methylation [40,43]. We also dropped loci that had poor hybridization using the updated characterization file for the HM450 assay developed by Zhou *et al.* [44].

As a sensitivity analysis, we performed additional pre-processing and calculated β-values using the newly developed *SeSAMe* pipeline, which uses the P-value with out-of-band array hybridization (pOOBAH) method to further adjust for poorly performing probes with failed hybridization [45].

CpG probe selection

We conducted a candidate gene approach, focusing on CpG loci in proximity to genes coding for cytokines that have been associated with pancreatic cancer survival in previous studies: *IL10*, *IL6*, *IL8*, *TGFβ1*, *TGFβ2*, *TGFβ3* and *TNF*. We identified 113 total CpG loci across these seven genes from the annotation file of the HM450 assay [40]. One probe in *TGFβ2* was dropped due to failed hybridization and one probe in *TGFβ3* was dropped due to SNP overlap, leaving a total of 111 CpG loci (six for *IL10*, 11 for *IL6*, 2 for *IL8*, 25 for *TGFβ1*, 23 for *TGFβ2*, 16 for *TGFβ3* and 28 for *TNF*).

Assessment of genetic regulation & expression

Genetic regulatory elements associated with the selected CpG loci were determined using the core 15-state model of chromatin states for the pancreas tissue epigenome developed by the NIH Roadmap Epigenomics Mapping Consortium [46]. The core 15-state model was created with ChromHMM, which establishes chromatin state signatures using multivariate Hidden Markov Models [47] and indicates whether the genetic region is associated with regulatory elements, such as active transcription start sites (TSS), weak transcription, enhancers and heterochromatin [46]. Chromatin states were mapped to the CpG loci using the *GenomicRanges* package [48].

To evaluate whether the methylation sites were associated with changes in gene expression, we also downloaded Level 3 mRNA expression data from TCGA [49]. Raw expression reads were evaluated with RESM and standardized using the upper-quartile normalization approach [50].

Outcomes

Our outcomes of interest were overall survival, pancreatic cancer-specific survival and progression-free interval. For overall survival, patients were considered to have the event if they died from any cause. For pancreatic cancer-specific survival, events were defined as having died with the tumor or having pancreatic cancer listed as the cause of death. This outcome was missing for seven individuals due to missing data on tumor status. For progression-free interval, new tumor events were defined as progression of disease, local recurrence, distant metastasis, new primary tumors or disease-specific death. Data on these outcome end points were obtained from the standardized TCGA Pan-Cancer Clinical Data Resource (TCGA-CDR) [51].

Statistical analysis

Baseline characteristics were compared across alive and deceased patients using a Mann–Whitney U test for follow-up time, t-tests for age and tumor purity, and chi-square tests for all other variables. Four patients were missing ethnicity, which was predicted using the SNP and type I color channel switching probes on the HM450 array via the *SeSAMe* package [44,45]. All four individuals were predicted to be white.

We evaluated methylation at three levels of analysis: at the level of the individual CpG locus, at the level of each chromatin state per gene, and at the level of the entire gene. For the chromatin state- and gene-level analyses, we used the average methylation across all CpG loci in the given region as the exposure. For all units of analysis, the relationship between methylation and expression of its respective gene was assessed by calculating the Spearman's correlation coefficient (ρ). We used Cox proportional hazards regression to assess the relationship between methylation and our three outcomes (overall survival, disease-specific survival and progression-free interval). Due to variation in methylation levels across CpG loci, we standardized methylation at each CpG site and evaluated each standard deviation increase in β-value as the unit of change. Patients were followed from time of diagnosis to the earliest of the given outcome event, last contact, or death. Tied event times were handled by calculating the exact likelihood.

We used independent surrogate variable analysis (ISVA) to identify potential batch effects and measurement error in confounders that may bias results [43,52]. In this step, the residual variation in DNA methylation across the array that was not associated with the outcome was first decomposed into independent components. Individuals with missing confounder information were dropped during the development of the independent components. Next, independent surrogate variables (ISVs) were constructed from the independent components that were significantly associated (using an alpha level of 0.05) with our confounders of interest: age (continuous), gender (male vs female), stage (I, II, III/IV), smoking (never, former, current), receipt of radiation therapy (yes vs no), receipt of chemotherapy (yes vs no) and sample plate. Since we planned to adjust for age, gender and stage in all fully adjusted models, we selected the ISVs that were significantly associated with any of the remaining potential confounders (smoking, receipt of radiation, receipt of chemotherapy or sample plate) as covariates in our models. ISVA identified eight total independent surrogate variables, six of which were included in our final models.

For each unit of analysis (CpG locus, chromatin state, or gene), we ran one model minimally adjusted for age and gender, and a fully adjusted model with age, gender, stage and the six ISVs as covariates. To evaluate the impact of probes with failed hybridization, we repeated all analyses using the β-values preprocessed by the *SeSAMe* pipeline. As there were no differences in the results for two β-value datasets, only the results from the noob-processed dataset are presented. To assess the influence of tumor purity, we performed a sensitivity analysis by re-running all models for overall survival with ABSOLUTE tumor purity score as an additional covariate. ABSOLUTE tumor purity scores for pancreatic cancer samples were obtained from the TCGA Research Network [53]. As a majority of the cohort

Table 1. Baseline characteristics of 184 patients with primary tumor samples in The Cancer Genome Atlas, by vital status.

Characteristic	Total (n = 184)		Alive (n = 85)		Deceased (n = 99)		p-value[†]
	n	%	n	%	n	%	
Follow-up time (mos), median (IQR)	15.6 (9.3, 22.6)		17.2 (11.3, 31.7)		13.1 (7.7, 19.9)		<0.01
Age, mean (SD)	64.8 (11.0)		63.2 (10.5)		66.1 (11.3)		0.07
Gender							0.31
– Female	82	44.6	34	40.0	48	48.5	
– Male	102	55.4	51	60.0	51	51.5	
Race							0.55
– White	166	90.2	77	90.6	89	90.0	
– Black	7	3.8	2	2.4	5	5.0	
– Asian	11	6.0	6	7.0	5	5.0	
Stage							0.21
– I	21	11.4	14	16.5	7	7.1	
– II	151	82.1	64	75.3	87	87.9	
– III/IV	9	4.9	4	4.7	5	5.0	
– Missing	3	1.6	3	3.5	0.0	0.0	
Smoking status							0.55
– Never	69	37.5	33	38.8	36	36.4	
– Former	60	32.6	29	34.1	31	31.3	
– Current	20	10.9	7	8.3	13	13.1	
– Missing	35	19.0	16	18.8	19	19.2	
Received radiation							0.09
– No	120	65.2	49	57.7	71	71.7	
– Yes	49	26.6	29	34.1	20	20.2	
– Missing	15	8.2	7	8.2	8	8.1	
Received chemotherapy							0.29
– No	41	22.3	15	17.6	26	26.3	
– Yes	133	72.3	64	75.3	69	69.7	
– Missing	10	5.4	6	7.1	4	4	
Tumor purity							0.11
– Mean (SD)	0.35 (0.18)		0.33 (0.20)		0.37 (0.17)		
– Missing	35	19.0	17	20.0	18	18.2	

[†]Mann-Whitney U test for follow-up time, t-test for age and tumor purity, χ^2 test for all other variables.

was white, we also conducted sensitivity analyses for overall survival among only white individuals to evaluate any potential differences by race/ethnicity.

For the chromatin state- and gene-level analyses, we performed additional subgroup analyses for overall survival stratified by gender and smoking (ever vs. never), as there is evidence of poorer pancreatic cancer survival among males [54] and smokers [55]. Heterogeneity was assessed using a separate model with a product term for methylation and the stratifying variable.

Schoenfeld residuals were used to confirm no violation of the proportional hazards assumption. Martingale residuals, deviance residuals and δ-β values were reviewed to examine model fit. Wald tests with an alpha level of 0.05 were used to determine significance. All analysis was performed in R version 3.5.1 and all figures were created using the *Gviz* package [56].

Results

Our sample included 184 pancreatic cancer cases with primary tumor samples in The Cancer Genome Atlas database (Table 1). The median length of follow-up (censored at death or last contact) was 15.6 months (interquartile range: 9.3–22.6) and was longer for patients who were still alive (median 17.2 months) compared to those who were deceased (median: 13.1 months; $p < 0.01$). The mean age of pancreatic cancer diagnosis was 64.8 years (standard deviation [SD]: 11.0 years). The cohort was primarily white (90.2%) and consisted of 82 females (44.6%) and 102

males (55.4%). Over 90% of patients had tumors of early stage (Stage I/II). ABSOLUTE tumor purity data [53] was available for 149 samples (81%), with a mean score of 0.35 (SD 0.18). The pancreatic cancer tumor samples were spread across twelve different plates in the methylation assay. By the end of follow-up, there were 99 overall deaths (53.8%), 78 deaths due to pancreatic cancer (42.4%) and 109 disease progression events (59.2%).

CpG-level analyses

The 111 CpG probes of interest were associated with a variety of chromatin states in pancreas tissue. Flanking active TSS (28.0%) were the most common, followed by quiescent/low (26.1%), weak transcription (19.8%), active TSS (17.1%), enhancers (7.2%), strong transcription (0.9%) and weak repressed polycomb (0.9%).

Increased DNA methylation at several loci in *TGFβ1, TGFβ2* and *TGFβ3* was significantly associated with our three clinical end points. Each standard deviation increase in methylation at cg09926389 (hazard ratio [HR]: 1.34; 95% confidence interval [CI]: 1.03–1.73), located within the *TGFβ1* gene body, and cg04547554 (HR: 1.19; 95% CI: 1.00–1.43), cg23275502 (HR: 1.18; 95% CI: 1.01–1.37), and cg24767336 (HR: 1.30, 95% CI: 1.13–1.50), all located in proximity to the *TGFβ1* TSS, was associated with poorer overall survival (Table 2, Supplementary Figure 1). The HRs for these four CpG loci were similar for disease-specific survival (Table 2). In addition, each standard deviation increase in DNA methylation β-value at cg03313751, near the *TGFβ1* gene body, was associated with a 21–22% increased hazard for all three outcomes, which was borderline significant for overall (HR: 1.21; 95% CI: 0.99–1.47) and disease-specific mortality (HR: 1.22; 95% CI: 0.98–1.53) and statistically significant for disease progression (HR: 1.22; 95% CI: 1.02–1.47). Finally, increased methylation at cg03630756, near the *TGFβ1* gene body, was associated with a decreased hazard of pancreatic cancer-specific death (HR: 0.64; 95% CI: 0.42–0.96). Methylation at all of these sites had a weak to moderate correlation with *TGFβ1* gene expression (Table 2).

Within the *TGFβ2* gene body, each standard deviation increase in methylation at cg07810039 was associated with a 19–26% decreased hazard for all end points, which was borderline significant for overall mortality (HR: 0.81; 95% CI: 0.64–1.01) and statistically significant for disease-specific mortality (HR: 0.74; 95% CI: 0.57–0.97) and disease progression (HR: 0.79; 95% CI: 0.64–0.99). Methylation at this locus had a moderate negative correlation with *TGFβ2* gene expression (ρ = -0.436; $p < 0.001$, Supplementary Figure 2). Furthermore, increased methylation at cg16967578, which was not correlated with *TGFβ2* gene expression (ρ = 0.099; p = 0.190), was associated with a 45–52% increased hazard for all three outcomes (Table 2). Higher methylation at cg20698667 was associated with elevated hazards of overall (HR: 1.34, 95% 1.04–1.74) and pancreatic cancer-specific mortality (HR: 1.36; 95% CI: 1.03–1.81), and had a weak negative correlation with expression (ρ = -0.173; p = 0.020). Near the *TGFβ2* TSS, each standard deviation increase in methylation at cg16361301 was associated with a 23% higher hazard of disease progression (HR: 1.23; 95% CI: 1.01–1.51).

For *TGFβ3*, each standard deviation increase in the methylation β-value at cg16292972, located near the *TGFβ3* TSS, was associated with a 26% elevated hazard of overall mortality (HR: 1.26; 95% CI: 1.01–1.57). However, methylation at this probe was not correlated with *TGFβ3* expression (ρ = 0.071; p = 0.350, Supplementary Figure 3). For cg24696715, increased methylation had a moderate negative correlation with *TGFβ3* gene expression (ρ = -0.479; $p < 0.001$) and was associated with a decreased hazard of overall (HR: 0.80; 95% CI: 0.63–1.01) and pancreatic cancer-specific mortality (HR: 0.76; 95% CI: 0.58–0.99). Higher methylation at cg06958766, which had a weak negative correlation with expression (ρ = -0.178; p = 0.020), was associated with a greater hazard of disease progression (HR: 1.32; 95% CI: 1.05–1.67).

In *TNF*, five CpG sites (cg17741993, cg20477259, cg09637172, cg05952498, cg02137984), all associated with either weak transcription or quiescent/low chromatin states, were associated with increased hazards of overall and disease-specific mortality (Table 2, Supplementary Figure 4). The HRs for these associations were stronger for disease-specific mortality (ranging from 1.35 to 1.55) compared to overall mortality (ranging from 1.26 to 1.38). For cg17741993 and cg05952498, the association for overall survival was borderline statistically significant (cg17741993: HR: 1.26; 95% CI: 0.98–1.62; cg05952498: HR: 1.34; 95% CI: 0.98–1.85), while the association for disease-specific survival was statistically significant (cg17741993: HR: 1.35; 95% CI: 1.02–1.78; cg05952498: HR: 1.55; 95% CI: 1.10–2.20). Methylation at all of these CpG loci had moderate inverse correlations with *TNF* expression (Table 2).

Elevated methylation in three CpG loci across *IL10*, *IL8* and *IL6* were inversely associated with our clinical outcomes. Higher methylation in cg14789529, located near the *IL10* three prime untranslated region (3′ UTR), had a weak negative correlation with expression (ρ = -0.229; $p < 0.001$) and was associated with reduced hazards

Table 2. Hazard ratios and 95% confidence intervals for the top associations between methylation in CpG loci and overall survival, disease-specific survival and progression-free interval.

CpG site	Chr	Position	UCSC gene name	UCSC gene group	Chromatin state	β-value, mean (SD)	Expression		Overall survival	Disease-specific survival	Progression-free interval
							Spearman's ρ	p-value	HR (95% CI)[†]	HR (95% CI)[†]	HR (95% CI)[†]
IL10											
cg14789529	chr1	206941464	*IL10*	3′UTR	Weak transcription	0.75 (0.09)	-0.229	<0.001	0.70 (0.56, 0.89)	0.68 (0.52, 0.88)	0.80 (0.64, 1.00)
TGFβ2											
cg16361301	chr1	218519549	*TGFβ2*	1st Exon; 5′UTR	Active TSS	0.05 (0.02)	0.009	0.900	1.14 (0.93, 1.39)	1.15 (0.91, 1.45)	1.23 (1.01, 1.51)
cg20698667	chr1	218523325	*TGFβ2*	Body	Weak transcription	0.65 (0.14)	-0.173	0.020	1.34 (1.04, 1.74)	1.36 (1.03, 1.81)	1.17 (0.93, 1.46)
cg07810039	chr1	218524558	*TGFβ2*	Body	Weak transcription	0.46 (0.21)	-0.436	<0.001	0.81 (0.64, 1.01)	0.74 (0.57, 0.97)	0.79 (0.64, 0.99)
cg16967578	chr1	218575437	*TGFβ2*	Body	Weak transcription	0.80 (0.11)	0.099	0.190	1.45 (1.03, 2.02)	1.52 (1.05, 2.20)	1.50 (1.08, 2.09)
IL8											
cg04392234	chr4	74608458	*IL8*	3′UTR	Quiescent/low	0.82 (0.09)	-0.048	0.520	0.86 (0.61, 1.22)	0.70 (0.48, 1.02)	0.51 (0.36, 0.72)
TNF											
cg17741993	chr6	31544694	*TNF*	Body	Quiescent/low	0.78 (0.13)	-0.396	<0.001	1.26 (0.98, 1.62)	1.35 (1.02, 1.78)	1.12 (0.88, 1.42)
cg20477259	chr6	31544960	*TNF*	Body	Quiescent/low	0.55 (0.10)	-0.227	<0.001	1.35 (1.05, 1.74)	1.39 (1.06, 1.83)	1.11 (0.86, 1.44)
cg09637172	chr6	31545252	*TNF*	Body	Weak transcription	0.84 (0.13)	-0.389	<0.001	1.38 (1.01, 1.89)	1.48 (1.05, 2.08)	1.08 (0.81, 1.44)
cg05952498	chr6	31545257	*TNF*	Body	Weak transcription	0.74 (0.13)	-0.363	<0.001	1.34 (0.98, 1.85)	1.55 (1.10, 2.20)	1.11 (0.80, 1.55)
cg02137984	chr6	31545898	*TNF*	3′UTR	Weak transcription	0.87 (0.07)	-0.463	<0.001	1.33 (1.02, 1.74)	1.47 (1.09, 1.99)	1.10 (0.88, 1.37)
IL6											
cg26061582	chr7	22766209	*IL6*	TSS1500	Enhancers	0.07 (0.07)	-0.037	0.620	0.66 (0.44, 0.98)	0.72 (0.46, 1.12)	0.87 (0.63, 1.19)
TGFβ3											
cg06958766	chr14	76446087	*TGFβ3*	Body	Active TSS	0.66 (0.16)	-0.178	0.020	1.16 (0.91, 1.49)	1.17 (0.88, 1.53)	1.32 (1.05, 1.67)
cg24696715	chr14	76446681	*TGFβ3*	Body	Flanking Active TSS	0.57 (0.08)	-0.479	<0.001	0.80 (0.63, 1.01)	0.76 (0.58, 0.99)	0.89 (0.71, 1.11)
cg16292972	chr14	76448509	*TGFβ3*	TSS1500	Active TSS	0.04 (0.01)	0.071	0.350	1.26 (1.01, 1.57)	1.22 (0.95, 1.57)	1.06 (0.86, 1.31)
TGFβ1											
cg09926389	chr19	41837123	*TGFβ1*	Body	Weak transcription	0.73 (0.19)	0.378	<0.001	1.34 (1.03, 1.73)	1.42 (1.05, 1.92)	1.29 (0.99, 1.66)
cg03630756	chr19	41839506	*TGFβ1*	Body	Weak transcription	0.92 (0.02)	0.066	0.380	0.77 (0.53, 1.13)	0.64 (0.42, 0.96)	0.95 (0.67, 1.36)
cg03313751	chr19	41857626	*TGFβ1*	Body	Flanking active TSS	0.03 (0.01)	-0.316	<0.001	1.21 (0.99, 1.47)	1.22 (0.98, 1.53)	1.22 (1.02, 1.47)
cg04547554	chr19	41860013	*TGFβ1*	TSS200	Flanking Active TSS	0.02 (0.01)	-0.175	0.020	1.19 (1.00, 1.43)	1.25 (1.03, 1.52)	1.02 (0.79, 1.32)
cg23275502	chr19	41860019	*TGFβ1*	TSS200	Flanking active TSS	0.02 (0.02)	-0.161	0.030	1.18 (1.01, 1.37)	1.23 (1.04, 1.45)	0.85 (0.40, 1.80)
cg24767336	chr19	41860095	*TGFβ1*	TSS1500	Flanking active TSS	0.03 (0.02)	-0.160	0.030	1.30 (1.13, 1.50)	1.32 (1.12, 1.57)	1.04 (0.82, 1.33)

[†]Fully adjusted models adjusted for age, sex, race, stage and six surrogate variables.
Models were run using complete case analysis. Maximum sample sizes were 181 for overall survival, 174 for disease-specific survival and 181 for progression-free interval.
p-values and q-values for Cox proportional hazards models can be found in Supplementary Table 1.
TSS: Transcription start site.

of overall mortality (HR: 0.70; 95% CI: 0.56–0.89), disease-specific mortality (HR: 0.68; 95% CI: 0.52–0.88) and disease progression (HR: 0.80; 95% CI: 0.64–1.00, Supplementary Figure 5). For cg04392234, in the 3′ UTR region of *IL8*, each standard deviation increase in the methylation β-value was associated with a borderline 30% decreased hazard of disease-specific death (HR: 0.70; 95% CI: 0.48–1.02) and a significant 49% decreased hazard of disease progression (HR: 0.51; 95% CI: 0.36–0.72). Finally, increased methylation at cg26061582, near the *IL6* TSS, had a lower hazard of overall mortality (HR: 0.66; 95% CI: 0.44–0.98, Supplementary Figure 6). HRs for the remaining CpG loci are presented in Supplementary Table 1.

Chromatin & gene-level analyses

When evaluating the average methylation levels across chromatin states, we observed that higher methylation in weak transcription regions in *TNF* were associated with higher hazards of overall (HR: 1.43; 95% CI: 1.04–1.97) and pancreatic cancer-specific mortality (HR: 1.56; 95% CI: 1.10–2.21). We also found that increased methylation in weak transcription regions in *IL10* were associated with reduced hazards of overall mortality (HR: 0.70; 95% CI: 0.56–0.89), disease-specific mortality (HR: 0.68; 95% CI: 0.52–0.88) and disease progression (HR: 0.80; 95% CI: 0.64–1.00). For average methylation across entire genes, higher methylation in *IL8* was associated with a reduced hazard of disease progression (HR: 0.70; 95% CI: 0.54–0.91). There was no association for average methylation across any of the other genes (Table 3).

In the subgroup analyses, higher methylation in loci located on the enhancer region of *IL6* was associated with an increased hazard of overall death among never smokers (HR: 1.58; 95% CI: 1.02–2.46), and a decreased hazard of overall death among ever smokers (HR: 0.56; 95% CI: 0.37–0.84; p-interaction < 0.01). For loci in the active TSS of *TGFβ2*, higher methylation was associated with increased overall mortality among ever smokers (HR: 1.40; 95% CI: 1.00–1.97), but not among never smokers (HR: 0.86; 95% CI: 0.51–1.27; p-interaction = 0.04). Furthermore, higher average methylation in *IL8* was associated with a decreased hazard of overall mortality among females (HR: 0.63; 95% CI: 0.41–0.95), but not among males (HR: 1.19; 95% CI: 0.83–1.72; p-interaction = 0.03). There was no other significant interaction across smoking or sex for the other genomic regions (data not shown).

Sensitivity analyses

We observed comparable results for overall survival when further adjusting for tumor purity or performing analyses among only whites (Supplementary Table 2). The HRs for methylation in individual CpG loci, chromatin states and entire genes were generally consistent across the different analytical methods. The only exception was for cg23275502, near the TSS in *TGFβ1*, which had an unstable HR in the tumor purity sensitivity analyses due to small cell bias and a tight range of methylation among nonwhites. When we re-ran the models for this CpG locus among only whites, we did not observe any major differences in the HRs between the main and tumor purity sensitivity analyses (Supplementary Table 2). Likewise, there were no major changes in the HRs for the models assessing average methylation across chromatin states and entire genes (Supplementary Table 3).

Since this was a candidate gene approach where we treated each gene as a unique hypothesis, we did not perform correction for multiple comparisons for the main CpG-level analyses. However, if we were to apply the Benjamini and Hochberg procedure to control the false discovery rate (FDR) at a q-level of 0.05 in each gene [57], only cg14789529 in *IL10* and cg24767336 in *TGFβ1* would still be considered significant for overall and disease-specific survival, and cg04392234 in *IL8* would still be significant for progression-free interval (Supplementary Table 1). On the other hand, several loci across *IL10*, *TGFβ1*, *TGFβ2*, *TGFβ3* and *TNF* in the minimally adjusted models for overall survival would still meet the FDR-corrected threshold for significance (Supplementary Table 2).

Discussion

In this study, we examined the associations between DNA methylation in proximity to cytokine-related genes and overall survival, disease-specific survival and progression-free interval among pancreatic cancer patients from TCGA. We observed significant associations for all three outcomes for methylation in several CpG probes across *TGFβ1*, *TGFβ2* and *TGFβ3*. In addition, elevated methylation in five CpG loci in *TNF* was associated with increased overall and disease-specific mortality, while higher methylation in CpG loci across *IL10*, *IL6* and *IL8* appeared to have a protective association. These results may improve our understanding of cytokine-related mechanisms and can contribute toward the development of survival models and targeted therapy for pancreatic cancer.

TGF-β is an immunosuppressive cytokine that has been found to be directly secreted by pancreatic cancer tumor cells [5]. Past literature has shown that higher serum concentrations of TGF-β are associated with lower pancreatic

Table 3. Hazard ratios and 95% confidence intervals for the associations between average methylation across CpG loci in specific genomic regions and overall survival, disease-specific survival and progression-free interval.

Genomic region	N CpG loci	β-value, mean (SD)	Expression		Overall survival		Disease-specific survival		Progression-free interval	
			Spearman's ρ	p-value	HR (95% CI)†	p-value	HR (95% CI)†	p-value	HR (95% CI)†	p-value
IL10										
Entire gene	6	0.79 (0.07)	-0.479	<0.001	0.94 (0.76, 1.17)	0.577	0.88 (0.70, 1.12)	0.298	0.91 (0.75, 1.12)	0.368
Weak transcription	1	0.75 (0.09)	-0.254	0.001	0.70 (0.56, 0.89)	0.003	0.68 (0.52, 0.88)	0.003	0.80 (0.64, 1.00)	0.050
Quiescent/low	5	0.79 (0.07)	-0.499	<0.001	1.01 (0.82, 1.26)	0.911	0.95 (0.75, 1.19)	0.641	0.95 (0.77, 1.16)	0.584
TGFβ2										
Entire gene	23	0.22 (0.03)	-0.321	<0.001	0.99 (0.77, 1.28)	0.960	1.06 (0.81, 1.39)	0.660	1.18 (0.93, 1.50)	0.180
Active TSS	8	0.05 (0.02)	-0.277	<0.001	1.00 (0.80, 1.26)	0.972	1.02 (0.79, 1.30)	0.893	1.16 (0.94, 1.42)	0.167
Flanking active TSS	8	0.07 (0.05)	-0.350	<0.001	0.96 (0.74, 1.25)	0.776	1.01 (0.77, 1.34)	0.936	1.24 (0.99, 1.57)	0.067
Weak transcription	5	0.61 (0.08)	-0.377	<0.001	0.97 (0.78, 1.20)	0.768	1.02 (0.81, 1.30)	0.849	1.00 (0.82, 1.22)	0.996
Enhancers	1	0.35 (0.13)	0.132	0.094	1.11 (0.88, 1.39)	0.383	1.14 (0.89, 1.47)	0.307	1.15 (0.93, 1.43)	0.197
Quiescent/low	1	0.72 (0.13)	0.311	<0.001	1.07 (0.84, 1.36)	0.584	1.04 (0.80, 1.36)	0.753	0.97 (0.77, 1.22)	0.793
IL8‡										
Entire gene	2	0.48 (0.04)	0.009	0.915	0.89 (0.69, 1.14)	0.358	0.76 (0.57, 1.00)	0.052	0.70 (0.54, 0.91)	0.008
TNF										
Entire gene	28	0.60 (0.07)	-0.443	<0.001	1.14 (0.88, 1.48)	0.318	1.19 (0.90, 1.59)	0.225	1.04 (0.82, 1.32)	0.756
Weak transcription	9	0.78 (0.07)	-0.390	<0.001	1.43 (1.04, 1.97)	0.026	1.56 (1.10, 2.21)	0.013	1.08 (0.82, 1.43)	0.580
Quiescent/low	19	0.52 (0.07)	-0.417	<0.001	1.05 (0.82, 1.33)	0.714	1.08 (0.82, 1.41)	0.592	1.02 (0.81, 1.28)	0.857
IL6										
Entire gene	11	0.43 (0.06)	-0.032	0.689	0.96 (0.77, 1.21)	0.733	0.95 (0.74, 1.23)	0.717	0.99 (0.80, 1.22)	0.898
Flanking active TSS	4	0.39 (0.09)	-0.328	<0.001	1.05 (0.82, 1.33)	0.701	0.99 (0.75, 1.30)	0.941	1.03 (0.82, 1.28)	0.805
Enhancers	4	0.27 (0.05)	0.191	0.015	0.91 (0.76, 1.10)	0.339	0.93 (0.75, 1.16)	0.537	0.96 (0.79, 1.15)	0.644
Weak repressed PolyComb	1	0.50 (0.17)	0.274	<0.001	0.88 (0.72, 1.08)	0.215	0.88 (0.70, 1.11)	0.294	0.91 (0.75, 1.10)	0.325
Quiescent/low	2	0.77 (0.08)	0.049	0.540	1.14 (0.85, 1.53)	0.387	1.21 (0.85, 1.70)	0.290	1.14 (0.86, 1.52)	0.372
TGFβ3										
Entire gene	16	0.39 (0.05)	-0.211	0.007	0.91 (0.72, 1.15)	0.441	0.93 (0.72, 1.20)	0.585	1.12 (0.90, 1.38)	0.317
Active TSS	10	0.31 (0.04)	-0.160	0.043	0.98 (0.78, 1.24)	0.879	1.00 (0.77, 1.28)	0.970	1.17 (0.94, 1.46)	0.169
Flanking active TSS	5	0.47 (0.08)	-0.221	0.005	0.86 (0.69, 1.09)	0.220	0.88 (0.67, 1.15)	0.342	1.05 (0.84, 1.30)	0.676
Strong transcription	1	0.81 (0.03)	-0.264	0.001	0.88 (0.72, 1.09)	0.244	0.87 (0.69, 1.09)	0.211	1.01 (0.80, 1.26)	0.955
TGFβ1										
Entire gene	25	0.32 (0.02)	0.336	<0.001	1.20 (0.94, 1.52)	0.142	1.27 (0.96, 1.67)	0.090	1.09 (0.86, 1.38)	0.478
Active TSS	1	0.08 (0.04)	0.105	0.184	0.98 (0.71, 1.36)	0.915	0.94 (0.67, 1.33)	0.741	0.83 (0.60, 1.15)	0.260
Flanking active TSS	14	0.04 (0.01)	-0.384	<0.001	1.11 (0.86, 1.45)	0.418	1.15 (0.86, 1.54)	0.351	0.99 (0.74, 1.32)	0.945
Weak transcription	7	0.70 (0.06)	0.480	<0.001	1.17 (0.93, 1.48)	0.174	1.26 (0.96, 1.65)	0.096	1.11 (0.88, 1.41)	0.379
Enhancers	3	0.78 (0.03)	-0.161	0.042	0.96 (0.77, 1.20)	0.720	0.91 (0.71, 1.16)	0.445	0.99 (0.79, 1.23)	0.922

†Fully adjusted models adjusted for age, sex, race, stage and six surrogate variables.
‡Both CpG loci for *IL8* were associated with the Quiescent/Low chromatin state.
Models were run using complete case analysis. Maximum sample sizes were 181 for overall survival, 174 for disease-specific survival and 181 for progression-free interval.
TSS: Transcription start site.

cancer survival [17,21,58]. In our study, we observed that higher methylation in ten loci across *TGFβ1*, *TGFβ2* and *TGFβ3* was associated with increased hazards of overall and disease-specific mortality, while methylation in three loci was associated with decreased disease-specific mortality. DNA methylation in TGF-β genes and pancreatic cancer survival have been evaluated in two previous studies, which actually found longer survival among individuals with greater methylation in *TGFβ2* [34,35]. Both of these studies, however, had patient samples and methods that were dissimilar from the present analysis. In the first study, which used a subset of TCGA pancreatic tumor samples, the authors evaluated overall *TGFβ2* gene methylation and separated individuals into short (<1 year) and long (>2 year) survival groups [35]. In the other study, the researchers found that higher methylation in one

unspecified CpG site in *TGFβ2* was associated with improved survival, but the results were not fully adjusted and were only based on a small sample of eleven pancreatic cancer tumors [34]. Given these differences in study design, future research with more consistent methods are needed to validate the current findings.

Among the *TGFβ1*, *TGFβ2* and *TGFβ3* CpG sites that were associated with mortality in our study, the strongest associations were observed among loci located near regions of weak transcription and TSS. However, the methylation of the CpG loci in these regions had weak to moderate correlations with expression in their respective genes. One possible explanation for this scenario could be that methylation at these particular loci may not account for all epigenetic regulation of gene expression. Additionally, there has been limited literature on the association between TGF-β gene expression and pancreatic cancer mortality. The only previous study examining this relationship, also using TCGA data, found enhanced survival among patients with *TGFβ1* overexpression [22].

TNF-α is a pro-inflammatory cytokine that has been observed to be involved in the progression and invasiveness of pancreatic tumors [13,59]. Past literature has shown that pancreatic cancer patients have higher blood levels of both *TNF* expression and TNF-α concentrations compared to controls [12,60]. In our study, higher methylation in five CpG loci in *TNF* was associated with reduced overall and pancreatic cancer-specific survival. Methylation at all of these sites was also negatively correlated with *TNF* expression. Previous DNA methylation studies on *TNF* are sparse and have focused on genes in the TNF receptor superfamily, which observed higher methylation in *TNFRSF1A* in the leukocyte DNA of pancreatic cancer patients [61] and lower methylation for *TNFRSF10C* in pancreatic cancer cell lines [62]. Research on *TNF* expression and pancreatic cancer mortality has also been somewhat inconsistent. While one prior study found no relationship between tumor *TNF* expression and mortality [63], another study found that increased expression was associated with decreased survival [18]. To our knowledge, this is the first study to evaluate DNA methylation in *TNF* and survival in pancreatic cancer patients.

IL-10 is an anti-inflammatory cytokine that is released by pancreatic cancer cells and contributes to the development of a tumor-tolerating microenvironment [13,59]. Current evidence has suggested that higher serum concentrations of IL-10 is associated with poorer survival in pancreatic cancer patients [14,20]. We found that increased methylation in one CpG site in *IL10*, which had a weak inverse correlation with expression, had a protective association for all three clinical outcomes. Though there has been no other literature on *IL10* DNA methylation and survival, previous studies have found lower *IL10* methylation in leukocyte DNA [61] and increased *IL10* expression [17] in the tumor tissue among pancreatic cancer cases. Furthermore, *IL10* has been shown to be hypomethylated in other malignancies, such as colon, kidney, stomach, lung and breast cancer [64].

IL-8 is a pro-inflammatory cytokine that has been shown to promote the proliferation and invasiveness of pancreatic cancer [13,16,59]. In the present study, higher methylation in one CpG locus in *IL8* had protective associations for pancreatic cancer-specific mortality and disease progression, but no association for overall survival. While methylation in *IL8* and pancreatic cancer outcomes have not been previously investigated, three prior studies also found no association between IL-8 concentrations and overall survival from pancreatic cancer [14,20,23]. Another study, however, observed improved survival among patients with lower concentrations of IL-8 [15]. Our results suggest that epigenetic modification of *IL8* could have a greater impact on disease-specific outcomes rather than overall survival.

IL-6 is a pleiotropic cytokine that is involved in several pathways in pancreatic cancer tumorigenesis [65,66]. Many prior studies have found poorer survival among pancreatic cancer patients with higher serum levels of IL-6 [14,17,20,23]. In our analysis, we detected a decreased risk for overall mortality for increased methylation at one CpG locus in *IL6*. The relationship between DNA methylation in *IL6* and pancreatic cancer has not been well evaluated in the current literature. While a previous study observed lower leukocyte DNA methylation in *IL6* among pancreatic cancer patients [61], no prior study has evaluated DNA methylation in this gene in the context of pancreatic cancer survival. Additionally, this CpG site was located near the *IL6* enhancer region, but there was no significant correlation between methylation and *IL6* expression. It is possible that DNA methylation in this region does not have a strong influence on genetic expression.

A major strength of this study is that it is one of the larger cohorts to evaluate DNA methylation in proximity to cytokine genes and pancreatic cancer survival using a candidate gene approach. Past research on this topic has been evaluated primarily in epigenome-wide association studies with much smaller sample sizes. We also incorporated chromatin states and expression in our analyses, which enhanced the interpretability of our results. However, our sample size was not sufficiently powered to perform subgroup analyses, so we do not know if the observed associations differ by certain patient characteristics. In addition, TCGA had limited clinical information, so we could not adjust for patient-related covariates, such as body mass index or comorbidities, that could have

potentially influenced our findings. Generalizability of our results may also be an issue since the patient population was comprised of mainly white individuals with early-stage tumors. However, the age and sex distributions of the cohort are fairly similar to the observed rates among pancreatic cancer patients in the USA [54]. Furthermore, the methylation assays were performed using DNA from surgically resected tumor samples [53], so the timing of the sample collection and methylation assays may not exactly correspond to the time of diagnosis. Lastly, the pancreatic tissue samples may likely be within a mixture of other cells that could have impacted some of the associations. As the pancreatic cancer tumor microenvironment involves the interaction of many different cell types, we did not adjust for cellular heterogeneity because it could have perhaps overcorrected and removed true biologic signals.

Each cytokine gene in this study was selected specifically because increased expression or serum concentrations of the cytokine was associated with pancreatic cancer in prior literature. Though this was a nonagnostic analysis, using the false discovery rate to correct for multiple comparisons led to three remaining significant hits (cg14789529 in *IL10*, cg24767336 in *TGFβ1* and cg04392234 in *IL8)* in the fully adjusted models. However, many loci in the minimally adjusted models would still be significant, indicating a potential loss of statistical power when adjusting for additional covariates. Ultimately, our observed findings are still helpful for describing associations and generating hypotheses on the relationship between the immune system and pancreatic carcinogenesis.

Conclusion

In this study of pancreatic cancer patients from TCGA, we report several associations between pancreatic cancer outcomes and methylation in CpG loci across *TGFβ1*, *TGFβ2* and *TGFβ3*, poorer survival for higher methylation in *TNF* probes and reduced death and progression for methylation in loci in *IL10*, *IL8* and *IL6*. Our findings suggest that the interactions between cytokines and pancreatic cancer outcomes are complex and may potentially be influenced by epigenetic modifications. As epigenetics research on cytokine-related genes and pancreatic cancer is still quite sparse, future studies should evaluate these associations in larger samples to further elucidate the immune-associated mechanisms behind pancreatic cancer development and mortality.

Future perspective

Pancreatic cancer remains a challenging malignancy due to its immunosuppressive nature and poor prognosis. Our study sought to identify potential epigenetic biomarkers of survival and progression by investigating DNA methylation in immune-regulatory cytokines genes. Future studies should aim to validate these findings in larger, more racially heterogeneous populations to examine whether these associations are similar among understudied minority populations. A deeper epigenetic exploration using the 850k MethylationEPIC Array would also be warranted to identify additional immune-related CpG loci of interest. Furthermore, we plan to perform a more integrative analysis that synthesizes information on single nucleotide polymorphisms, DNA methylation, microRNA and protein expression to better elucidate the genomic and molecular pathways involved in pancreatic cancer outcomes. Together, these efforts should provide a clearer understanding of the genomic landscape of pancreatic cancer and help to establish improved biomarkers and prognostic models of survival and disease progression.

Summary points

- Using data from 184 pancreatic cancer patients from The Cancer Genome Atlas, we investigated the association between DNA methylation in loci across seven cytokine genes and overall survival, pancreatic cancer-specific survival and progression-free interval.
- We also examined whether DNA methylation influenced clinical outcomes through changes in genetic expression.
- Over ten CpG loci across *TGFβ1*, *TGFβ2* and *TGFβ3* were associated with both increased and decreased hazards of overall mortality, pancreatic cancer-specific mortality and disease progression.
- Increased methylation in five CpG probes in *TNF* (cg17741993, cg20477259, cg09637172, cg05952498, cg02137984) was associated with elevated risks of overall and disease-specific death.
- Higher methylation at cg14789529 in *IL10* was associated with decreased hazards of overall mortality, disease-specific mortality and disease progression.
- One CpG site in *IL8* (cg04392234) had protective associations for disease-specific mortality and disease progression.
- Elevated methylation at cg26061582 in *IL6* was associated with a reduced hazard of overall death.
- There was no clear relationship between DNA methylation, genetic expression and clinical outcomes.
- Our findings may shed some light on underlying biological mechanisms and help guide the development of prognostic models and the identification of potential treatment targets.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2019-0335

Disclaimer

The results of this study are based upon data generated by the TCGA Research Network: https://www.cancer.gov/tcga. This work used computational and storage services associated with the Hoffman2 Shared Cluster provided by UCLA Institute for Digital Research and Education's Research Technology Group.

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