DNA methylation profiles of cancer-related fatigue associated with markers of inflammation and immunometabolism

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DNA methylation profiles of cancer-related fatigue associated with markers of inflammation and immunometabolism

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Key words: fatigue, epigenetics, DNA methylation, gene expression, inflammation

Running Title: DNA methylation and cancer-related fatigue

Conflicts of interest to disclose: none
Abstract

Cancer patients are commonly affected by fatigue. Herein, we sought to examine epigenetic modifications (i.e., DNA methylation) related to fatigue in peripheral blood among patients during and after treatment for head and neck cancer (HNC). Further, we determined whether these modifications were associated with gene expression and inflammatory protein markers, which we have previously linked to fatigue in HNC. This prospective, longitudinal study enrolled eligible patients with data collected at pre-radiotherapy, end of radiotherapy, and six months and one-year post-radiotherapy. Fatigue data were reported by patients using the Multidimensional Fatigue Inventory (MFI)-20. DNA methylation (Illumina MethylationEPIC) and gene expression (Applied Biosystems Clariom S) arrays and assays for seven inflammatory markers (R&D Systems multiplex) were performed. Mixed models and enrichment analyses were applied to establish the associations. A total of 386 methylation loci were associated with fatigue among 145 patients (FDR<0.05). Enrichment analyses showed the involvement of genes related to immune and inflammatory responses, insulin and lipid metabolism, neuropsychological disorders, and tumors. We further identified 16 methylation-gene expression pairs (FDR <0.05), which were linked to immune and inflammatory responses and lipid metabolism. Ninety-one percent (351) of the 386 methylation loci were also significantly associated with inflammatory markers (e.g., interleukin 6, c-reactive protein; FDR<0.05), which further mediated the association between methylation and fatigue (FDR<0.05). These data suggest that epigenetic modifications associated with inflammation and immunometabolism, in conjunction with relevant gene expression and protein markers, are potential targets for treating fatigue in HNC patients. The findings also merit future prospective studies in other cancer populations as well as interventional investigations.
Fatigue is among the most commonly reported symptoms in cancer patients, and has a significant adverse impact on quality of life and survival.\(^1,2\) Patients diagnosed with head and neck cancer (HNC), who typically receive radiotherapy (RT) with or without chemotherapy, have high rates of fatigue after receiving treatment.\(^3\) Our studies, along with others, have found that fatigued cancer patients exhibit elevated peripheral inflammation, as represented by protein inflammatory markers, such as interleukin (IL)-6 and tumor necrosis factor (TNF)-alpha, as well as inflammatory gene expression signaling pathways, compared to those without fatigue.\(^4,5\) Moreover, both fatigue and inflammatory markers peak at the end of RT and chemotherapy, suggesting potential impact of cancer treatment as an environmental stimulus in fatigue development. Epigenetic modifications, key regulatory processes between gene and environment interactions, may provide a plausible mechanism for cancer and treatment-related fatigue and inflammation.

DNA methylation, a well-studied epigenetic modification, is an enzyme-mediated chemical modification of DNA at cytosine-guanine dinucleotides (i.e. CpG sites). The level of DNA methylation, particularly within regulatory genomic regions such as the promoter region, correlates with related gene expression and thereby modifies expressed products.\(^6\) Environmental stimuli, such as diet, infection, and cancer treatment, have profound effects on DNA methylation that can trigger susceptibility to disease, including psychological disorders\(^7\) and inflammatory conditions.\(^8\) However, whether methylation changes during cancer treatment are related to fatigue remains unclear, and the potential functional consequences of altered methylation on gene expression have not been assessed.
Here, we examined the longitudinal association between genome-wide DNA methylation and fatigue during and after cancer therapy up to one-year post-treatment. We also evaluated the relationship between methylation modifications associated with fatigue and gene expression as well as peripheral blood inflammatory markers.

**Methods**

HNC Patients were evaluated at pre-RT, the end of RT, and six months and one year post-RT (Supplemental Figure 1). The study was approved by Emory University Institutional Review Boards; all participants provided written informed consent.

**Study participants:**

As described in more detail elsewhere,\textsuperscript{9} inclusion criteria were: 1) newly diagnosed with histologically proven squamous cell carcinoma of the head and neck region, 2) no distant metastasis, and 3) without uncontrolled major organ disease. Exclusion criteria included: 1) primary tumors at other anatomic sites, 2) major psychiatric disorders such as schizophrenia, and 3) chronic health conditions involving the immune system or regular use of immunosuppressive medications.

**Symptom, demographic, and clinical measurements:**

Fatigue was measured using the patient-reported Multidimensional Fatigue Inventory (MFI)-20 at all four time points. The MFI-20 is a well-validated, 20-item fatigue tool\textsuperscript{10} that has been used in a variety of cancer populations, including HNC.\textsuperscript{9} The total score of MFI-20 ranges from 20 to 100, with higher scores indicating greater fatigue.
The following demographic and clinical characteristics were collected by a standard patient-reported questionnaire or medical chart review: age, sex, race, marital status, history of smoking (defined as cigarette smoking ≥ one year), history of alcohol use (defined as ≥one drink/week during the past year), body mass index (BMI), functional status (Eastern Cooperative Oncology Group [ECOG] Performance), medical comorbidities (Charlson Comorbidity Index), primary cancer site, cancer stage (TNM), radiation dose, chemotherapy, surgery, feeding tubes, and human papillomavirus (HPV) status (defined as either HPV or p16 positive).

**Laboratory measurements:**

Whole blood was collected into chilled EDTA tubes at the four measurement times. Samples were then processed for the isolation of peripheral blood leukocytes (buffy coat) and plasma and stored at -80°C until batched assay for DNA and RNA extraction and protein inflammatory markers.

**Genome-wide DNA Methylation:** To obtain purified genomic DNA, we used the QIAamp DNA mini kit (Qiagen). DNA quantification was determined using the Quant-iT dsDNA broad range assay kit (ThermoFisher) and was assessed on a 2% agarose gel. DNA of greater concentration than 20μg/ml was used for methylation assays (MethylationEPIC BeadChip, Illumina, San Diego, CA) at the Emory Integrated Genomics Core (EIGC). The MethylationEPIC BeadChips, covering >850,000 methylation sites, were imaged on an Illumina iScan System, following manufacturers’ protocols.

DNA methylation data were reprocessed with R package minfi.\(^\text{11}\) Methylation-level values for each CpG site were estimated as beta-values: the ratio of methylated probe intensity over total intensity (methylated plus unmethylated probe intensities). Beta values range from 0 to 1 and are
equivalent to the percentage of methylation of the CpG site. The normalized probes were filtered according to the following criteria: probes with a detection p-value > 0.01 in more than 5% of total samples; probes with SNPs at CpG site; probes on sex chromosomes; and cross-reactive probes\textsuperscript{12}. A total of 807,104 probes were left for the following analysis. The quality of methylation data for each sample was further controlled by removing samples with the percentage of low-quality probes (detection p-value > 0.01) > 1% and outliers in principal component analysis and clustering. Four data points from one patients were removed, and 481 data points from 145 patients were left for the following analysis. Functional normalization methods were adopted to correct batch effects of the data.\textsuperscript{13} Finally, we computed M-values using the logit transformation $M = \log_2(\beta/(1-\beta))$, as these have been shown to provide better performance in terms of true detection rate.\textsuperscript{14}

**Genome-wide RNA gene expression:** Total RNA was isolated from blood leukocytes according to manufacturer’s protocol (Qiagen RNeasy Mini Kit: Qiagen; Valencia, CA). RNA integrity was determined by scanning with an Agilent 2100 Bioanalyzer using the RNA 6000 Nano LabChip. Samples with RNA integrity number (RIN) <5 were excluded. Isolated RNA was kept at -80 C until microarray analysis. The EIGC analyzed RNA samples for gene expression using Clariom S Assay for human (Applied Biosystems; San Diego, CA).

Raw data were processed with R package oligo\textsuperscript{15} first and then normalized with the log scale robust multi-array analysis (RMA).\textsuperscript{16} A total of 19,556 genes were left after the preprocessing. Samples were further filtered by using the median of the signal (<90) and the area under the curve (AUC; <0.9). AUC was calculated from negative and positive control probes in each sample.
**Statistical Analysis**

Descriptive statistics were used for demographic and clinical characteristics.

**Association analysis between DNA methylation and fatigue**

A linear mixed-effect model described below was used to determine the association between fatigue and methylation data, using the methylation M-values as the outcome.

\[
M_{ij} = \beta_0 + \beta_1 \cdot r_{ij} + \beta_2 \cdot t_{ij} + f(CellType_{ij}) + f(X_{ij}) + \beta_F \cdot F_{ij} + e_{ij}
\]

where \( M_{ij} \) is the methylation level of patient \( i \) at time point \( j \), \( j \in (1, 2, 3, 4) \), indicating Pre-RT, end of RT, six months post-RT, and one year post-RT. \( r_{ij} \) indicates whether patient \( i \) took radiotherapy at time point \( j \). It is 0 when \( j = 1 \) and 1 when \( j > 1 \). \( t_{ij} \) is the time after radiotherapy (in year) of patient \( i \) at time \( j \), which modeled the recovery process after the radiotherapy. The value of \( t_{ij} \) is shown below

\[
t_{ij} = \begin{cases} 
0 & j = 1,2 \\
0.5 & j = 3 \\
1 & j = 4 
\end{cases}
\]

\( f(CellType_{ij}) \) is a linear combination of cell type proportion, which show the influence of different proportion of cells in blood to the methylation level. The proportion of six cell types (CD8+ T cell, CD4 + T cell, NK cell, B cell, monocytes, and neutrophils) was estimated with R package “FlowSorted.Blood.EPIC”\(^{17} \). \( f(X_{ij}) \) is the linear effects from other covariates, \( b_i \) is the random effects of patient \( i \), and \( e_{ij} \) is the random error.
We included the following covariates in the regression models: age, sex (0: female; 1: male), race (0: non-Black; 1: Black), BMI, smoking (0: never; 1: ever), HPV (unassociated vs. associated), surgery (no vs. yes), and chemotherapy (cisplatin vs. carboplatin/paclitaxel vs. others). We categorized race into Black and non-black because multidimensional scaling\textsuperscript{18} of methylation data showed that patients were clustered into Black and non-Black groups. \( F_{ij} \) is the fatigue level for patient \( i \) at time point \( j \). Age ranges between 35 and 83 in our data. We scaled age between 0 and 1, \( \text{scaled age} = \frac{\text{age} - \text{age}_{\text{min}}}{\text{age}_{\text{max}} - \text{age}_{\text{min}}} \), to avoid a singularity issue while during solving the mixed-effects model. The same scaling method was applied to BMI. We tested association between all CpGs and fatigue level with the model above and calculated a \( p \) value for each CpG.

**Results**

**Demographic**

Table 1 (N=145) shows baseline demographic and clinical characteristics. The sample was majority older, white, and male with approximately 50% having a history of alcohol or tobacco use. Two-thirds were diagnosed with advanced cancer stage (stage III and IV), and 52% were with oropharyngeal cancer. Among those with oropharyngeal cancer, 91% were HPV-associated. Seventy-eight percent of the patients received concurrent chemoradiation with or without surgery.

**Differential methylation at single CpG sites for fatigue**

We observed 386 methylated loci significantly (FDR<0.05) associated with fatigue over time after correcting for cell type composition and covariates (e.g., age, race, sex, BMI, tobacco,
radiotherapy, chemotherapy, etc.; Figures 1 and 2 and Supplemental Table 1). For 263 of these loci, the probes were annotated to a known protein coding gene according to the UCSC Genome Browser. Among them, 45% were in the gene body, and 17% were found in regions relevant to gene regulatory elements (combined Transcription Start Site [TSS]1500, TSS200, and 1st Exon). Regarding the location relation to CpG islands, out of the 263 methylated loci, 5% were in CpG islands, 19% mapped 2 kb upstream and downstream of CpG islands (N, S Shores), and 4% localized to regions 2 kb upstream and downstream of CpG shores (N, S Shelves), whereas 72% were found in open sea regions not annotated to gene bodies, CpG islands, shores, or shelves. Overall, 63% of the 386 methylated loci were negatively associated with fatigue, while 37% were positively associated with fatigue.

Enrichment GO analysis showed that response to alkaloid, cellular response to insulin stimulus, response to insulin, cellular response to cytokine stimulus were among the top ten significant biological processes (p= 6.88E-03 to 1.34E-02). We also performed IPA analyses for genes with fatigue-related methylation loci. Endotoxin lipopolysaccharide (LPS)/IL-1 Mediated Inhibition of RXR (p= 3.48E-03) was the top canonical pathway. Lipid metabolism was the top Molecular and Cellular Function pathway (p=1.58E-02 to 7.33E-04). Top diseases and disorders were Cancer (p=2.05E-02 to 8.35E-11), Gastrointestinal Disease (p=1.69E-02 to 8.35E-11), Organismal Injury and Abnormalities (p=2.06E-02 to 8.35E-11), and Neurological Disease (p=2.06E-02 to 2.97E-09).

**Differential methylation at single CpG sites within promoter regions of genes for fatigue**

Promoter regions were defined as being TSS1500, TSS200, and 1st Exon. Of the identified significant methylated loci, 17% (N=67) were found within promoter regions with known protein
coding genes (Supplemental Table 1). Among them, 9% were mapped to CpG island, 44% localized to shores, 1% to shelves, and 46% to open sea. Fifty-one percent of the significant loci at the promoter regions were negatively associated with fatigue, and 49% were positively associated with fatigue. Among the top ten loci (Figure 3), four were near genes related to cancer (YPEL3, MAP1LC3A, KIAA0125, TEX12),19-21 and others were related to immune processes and inflammation (CACNA2D3, ZNRF2, AIM2)22-24 and neuro systems (KCNE1, DBNDD1).25,26

**Association of fatigue-related DNA methylation with blood gene expression**

We next tested for associations between the 386 fatigue-associated methylation loci with blood gene expression and identified 16 methylation-gene expression pairs at FDR <0.05 controlling for covariates (Supplemental Table 2). Of the 16 significant pairs, 7 (44%) displayed negative correlations between methylation and mRNA levels. Six of the significant pairs were related to immune and inflammatory responses (SULF2, ADGRE3, AIM2, STAT4, TMED7-TICAM2);22,27-29 three were associated with mitochondrial function and lipid metabolism(SDHD, TIMM8B, LAPTM4A);30-32 others were tumor-related. Two of the methylation sites (cg10636246, cg24145401) were in the promoter region of AIM2, which is linked to immune and inflammatory responses.22

**Association of fatigue-related DNA methylation with blood inflammatory markers**

Finally, we examined associations of the fatigue-associated methylation loci with blood inflammatory markers (Supplemental Table 3). Among the 386 methylation loci, 91% of them were significantly associated with inflammatory cytokines (FDR<0.05): 310 were correlated to IL6, 303 to CRP, 296 to sTNFR2, 288 to IL10, 178 to IL1ra, 60 to IL1β, and 38 to TNFα. Sixty-
eight percent of the methylation loci were negatively associated with the blood concentration of inflammatory markers. In addition, 13% of the methylation sites were correlated with more than 5 inflammatory markers at an FDR <0.05 level (Supplemental Table 3). The top 10 significantly correlated pairs of inflammatory markers and methylation loci are indicated in Figure 4. cg10636246 and cg24145401 (AIM2) were among the top 10 methylation sites in the promoter region associated with inflammatory markers, and each of them was significantly and negatively associated with 5 inflammatory makers.

In the mediation analysis, 4 inflammation markers (sTNFR2 with 281 CpGs, CRP with 273 CpGs, IL6 with 258 CpGs, and IL1ra with 136 CpGs) in 948 CpG were found to significantly mediate the association between these inflammatory marker-associated CpG sites and fatigue. (FDR <0.05; Supplementary Table 4).

Discussion

Our study is the first to examine genome-wide methylation changes during and after cancer treatment and their longitudinal associations with fatigue in patients with HNC. Significant methylated loci associated with fatigue were in genes related to immune and inflammatory responses, insulin and lipid metabolism, neuropsychological disorders, and tumors. These significantly methylated loci were further linked to relevant mRNA expression changes and peripheral inflammatory protein markers, which in previous studies have been associated with fatigue in HNC patients.33

Substantial changes in DNA methylation at a genome-wide level were observed for their association with cancer-related fatigue. Approximately 1/5 of these differential methylation changes were mapped to the promoter regions, methylation of which could directly regulate gene
expression. For approximately half of the differentially methylated loci, decreased methylation (hypomethylation) was linked to increased fatigue, elevated gene expression, and increased protein markers of inflammation, as expected. Additionally, the preponderance of the differentially methylated positions did not reside at CpG islands but involved shore and open sea regions. Fatigue-associated differential methylation was even more prominent at CpG shores when only considering loci at promoter regions (44% vs. 19% for the promoter regions and whole genome, respectively). Furthermore, in our data of differential methylation loci associated with peripheral inflammatory markers, 39% of the methylation loci in the promoter region were located on CpG shores and 51% in open sea regions, while only 7% were located on CpG islands. This higher number of differential methylated loci on shores and open sea than islands is in line with findings from cancer and other genome-wide methylation studies, supporting that the genomic location of a methylation site provides functional insight into regulatory features. Indeed, studies have shown that methylation on shores is especially relevant and has a higher correlation with gene expression than methylation on CpG islands.

Supporting our previous observations, our data revealed a notable relationship between methylation states and fatigue, as well as inflammation. Enrichment analyses using both GO and IPA showed that immune and inflammation-associated pathways (cellular response to cytokine stimulus and endotoxin LPS/IL-1 Mediated Inhibition of RXR) were involved in fatigue. Moreover, 88% of the 386 methylation loci associated with fatigue were significantly associated with peripheral inflammatory markers, with hypomethylation of these loci leading to increased peripheral concentrations of the inflammatory markers. Our mediation analysis further suggested the direct regulatory function of the methylation sites in the expression of peripheral inflammatory markers in the context of fatigue. Additionally, three genes (CACNA2D3, ZNRF2,
AIM2) near the top ten methylation sites at the promoter region were immune and inflammation related.22-24 Two methylation sites in the promoter region of AIM2 (cg10636246 and cg24145401) likely regulate the mRNA expression of AIM2, as the hypomethylation of AIM2 was significantly associated with increased expression of AIM2 in our data. Notably, AIM2 with cg10636246 was the most significant methylation locus at the promoter region associated with the inflammatory markers, IL6, CRP, IL10, sTNFR2, IL1ra, and IL1B. A large epigenome-wide association study of 12,974 participants also demonstrated that cg10636246 in the promoter of AIM2 was the most significant methylation locus associated with chronic inflammation as represented by CRP.22 AIM2 plays a crucial role in the innate immune system by assembling inflammasome and allowing IL1B and IL18 release and participates in host antiviral and antibacterial defenses.38 Given the scarcity of epigenetic research on cancer-related fatigue, future studies may verify our findings on AIM2 and other immune and inflammation related pathways.

Our analyses also highlighted the potential role of insulin and lipid metabolism in fatigue among cancer patients. Enrichment analysis (GO) of the significant methylation loci associated with fatigue showed that response to alkaloid, cellular response to insulin stimulus, and response to insulin were among the top ten significant biological processes. Results from IPA also revealed that lipid metabolism was the top molecular and cellular function involved. Furthermore, three out of the 16 significant methylation-gene pairs were associated with lipids and energy (SDHD, TIMM8B, LAPT4A)30-32, suggesting the influence of our significant methylation loci on the expression of lipid and energy-related genes. These insulin and lipid-related metabolic alterations are similar to findings from depression and are consistent with metabolic reprogramming in activated immune cells involving a shift from the energy efficient oxidative
phosphorylation to the energy expedient glycolysis. Of note, dysregulated insulin and lipid metabolism may play a role in chronic fatigue syndrome or general fatigue.\textsuperscript{39} Indeed, two small diet interventional studies observed that increased high-density lipoprotein and decreased low-density lipoprotein and triglycerides were associated with decreased fatigue among patients with multiple sclerosis consuming high fruit and vegetable diets.\textsuperscript{40,41} Given the energy demands associated with immunometabolic changes during cellular inflammatory responses,\textsuperscript{42} our findings suggest a novel mechanism for cancer-related fatigue and a new management strategy relevant to insulin and lipid regulation, immunometabolism, and inflammation.

Our results suggest neurological disorders’ involvement in cancer-related fatigue. IPA analyses showed that genes near fatigue-associated methylation sites were linked with neurological disease. Additionally, \textit{KCNE1}, located near the top methylation site associated with fatigue, is recognized for its participation in dopamine-DARPP32 feedback onto the cAMP pathway.\textsuperscript{25} Dopamine is a primary neurotransmitter and, along with other neurotransmitters, regulates DARPP-32 activation, all of which have been implicated in a number of psychiatric and neurological disorders.\textsuperscript{43} Peripheral inflammation also likely contributes to fatigue through inhibitory effects on dopaminergic circuits in the basal ganglia.\textsuperscript{44} Indeed, our data also showed that two methylation loci in the promoter region of \textit{KCNE1} that were associated with fatigue were significantly associated with 12 inflammatory markers (6 overlapping inflammatory markers). However, the exact mechanisms of how neurological systems and neurotransmitters may impact fatigue warrant further investigations.

\textbf{Limitations:}
While this is a large, longitudinal study among patients with HNC, some limitations need to be noted. The studied population included mainly white males, thus limiting the generalizability of study findings to non-white males and females. In addition, the project was conducted at a single institution and only included patients with HNC, and verification of our study findings at other institutions and in other cancer populations are warranted. Despite the relatively large sample size for patients with HNC, our study could be underpowered for a genome-wide study with a sample of 145. However, this sample size is commonly observed in DNA methylation studies of complex diseases, including depression. Furthermore, with a longitudinal design and data collected at four time points, our findings provide informative evidence on the association between DNA methylation and fatigue.

**Conclusion:**

Findings from this large, longitudinal study suggest that genome-wide DNA methylation patterns during cancer treatment play a vital role in fatigue. Methylation patterns related to immune and inflammatory responses and insulin and lipid metabolism are likely critical elements of fatigue pathophysiology among patients with HNC. Our study also provides a first step toward integrating the functional significance of DNA methylation with gene expression and inflammatory protein markers for cancer-related fatigue. Given the potent role of insulin and lipids in the immunometabolic shifts in cellular inflammatory responses, our findings may suggest a novel mechanism in cancer-related fatigue and a new management strategy relevant to immunometabolism and inflammation.

**Table 1**
Baseline demographic and clinical characteristics of the participants (N=145)
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<th>Variables</th>
<th>Mean ± SD or N (%)</th>
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*Note. BMI = Body Mass Index, HPV = Human papillomavirus, IMRT = Intensity-Modulated Radiation Therapy, SD = Standard deviation.

*Having missing data: History of alcohol use (2); Antidepressants (2); Feeding tubes (5).

*aMarried includes patients married or living as married; Unmarried includes patients single, separated, divorced, or widowed.

*bComorbidities was assessed using the Charlson Comorbidity Index excluding tumor.
References


32. database Gthg. LAPTMA4A. 2022.


Figure Legends

Figure 1. Manhattan plot

Figure 2. Heatmap of CpG sites and fatigue

Figure 3. Scatter plots of the top 10 significant CpG sites in the promoter region associated with fatigue

Figure 4. Scatter plots of the top 10 significant CpG sites in the promoter region associated with inflammatory markers
Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementalTable1methylationsignificantlyassociatedwithfatigueFINAL.xlsx
- SupplementalTable2significantlyassociatedmethylationandgeneexpressionFINAL.xlsx
- SupplementalTable3methylationsignificantlyassociatedwithinflammatorymarkersFINAL.xlsx
- SupplementalTable4mediationanalysis.xlsx