DNA methylome modifications and protection by sulforaphane in UVB-induced skin carcinogenesis

# Abstract

Exposure to UVB irradiation results in DNA damage and is responsible for non-melanoma skin cancers. Although the genetic mutation is well documented, the epi-mutation, the alteration in epigenetics, remains to be elucidated. DNA methylation is a stable and heritable epigenetic changes in the control of gene expression. We adapted Methyl-seq, which was recently developed approach to identify a genome-wide DNA methylation, to profile the DNA methylation in collected specimens of ultraviolet B (UVB)-irradiated skin epidermis and non-melanoma skin cancers at different stages. We used the Agilent mouse SureSelect Methyl-seq method of bisulfite sequencing as the single-ended for 75 bases and sequencing on the Illumina HiSeq2000. The majority of differentially methylated cytosines were observed were presented in introns, intragenic regions, near CpG islands, and transcriptional enhancers. We also identified genes involved in skin carcinogenesis that were not previously reported to be differentially methylated.

# Keywords

Non-melanoma skin cancer, sulforaphane, DNA methylation, Methyl-seq, ultraviolet-B (UVB)

# Introduction

Exposure to ultraviolet B (UVB) is one of the major causative factors for NMSCs. Long-term exposure to UVB radiation induces inflammation, oxidative stress, DNA mutation, and damage, which are involved in initiation, promotion and progression of NMSCs (1). The early UV-exposure induces inflammatory responses with the increased blood flow, vascular permeability, expression of cyclooxygenases-2 (COX-2), and production of prostaglandin (PG) metabolites (2). UVB-induced inflammation is an important event in all three stages of NMSCs (2), showing the importance of controlling the UVB-induced inflammation to prevent the skin cancer risk.

DNA methylation can be altered by environmental influences and provides a mechanism to affect the phenotypes in skin aging and cancer; the role of methylation as a molecular link between aging and cancer is complex and remained to be elucidated (3). Currently, there are a few approached commonly used with the next-generation sequencing platforms to profile the genome-wide DNA methylation. We have previous applied protein-affinity enrichment of methylated regions in two selected representative carcinogenesis models (4). This method reports the varying enrichment CpG density but not show the base-pair resolution of methylated cytosine (5, 6). An unbiased method to detect methylated CpGs at base-pair resolution is whole genome bisulfite sequencing. However, 70% - 80% of the sequenced reads provide useful DNA methylation information (7), and a more cost-effective method is to detect the methylation at base-pair resolution without bias of CpG dense and poor regions for the genome-wide DNA methylation. This study describes the application of Methyl-Seq for the mouse methylome and it reliably detects the DNA methylation in different stages of skin carcinogenesis. This study provides the insights on UV-induced alterations in DNA methylation in understanding the genome-wide DNA methylation signatures in UVB-induced skin carcinogenesis mouse model.

Sulforaphane (SFN) is an important cancer prevention agent and also a Nrf2 activator with no known side effects. SFN inhibits Nrf2 proteasomal degradation and enhances Nrf2 transcriptional activation of ARE genes. SFN suppressed Bmi-1 and Ezh2 level leading to the reduced H3K27me4 formation in SCC-13, A431, and HaCaT cells, and suppress tumor formation in the epidermis (8, 9). Recently, we reported that SFN inhibited TPA-induced JB6 cellular transformation, and decreased the methylation level of the CpG sites of Nrf2, and enhanced the translocation of Nrf2 into the nucleus. In addition, SFN reduced the protein expression of some epigenetic modifying proteins such as DNA methyltransferase and histone deacetylases (10). It has been shown that SFN reduced tumor growth in A375 melanoma cell – derived melanoma cancer stem cells in immune-compromised mice by reducing Ezh2 level and H3K27me3 formation (11).

In this study, we used the UVB-induced skin cancer mouse models to examine the epigenetic and epigenomic changes during different stages of skin cancer from imitation, promotion, to later progression. Furthermore, we investigated the roles of SFN in reversing the epigenetic changes resulting in the cancer prevention. These studies contributed to the development of a safe and efficient strategy using natural phytochemicals chemopreventive compounds to prevent skin cancers and to identify potential epigenetic and epigenomic biomarkers during skin carcinogenesis to provide novel therapeutic strategies.

# Materials and methods

## Chemicals and reagents

D, L - Sulforaphane (SFN) was purchased from Toronto Research Chemicals (S699115; Toronto, Ontario, Canada. Acetone (HPLC grade) and 10% phosphate-buffered formalin were obtained from Fisher Scientific (Hampton, NH, USA). UV lamps that emit UVB (280 – 320 nm; 75-80% of total energy) and UVA (320-375 nm; 20-25% of total energy), as described in previous studies (12). These UV lamps (FS72T12-UVB-HO; National Biological Corp., Twinsburg, OH, USA) emit little or no radiation < 280 nm and > 375 nm. The lamps emit UVB (280-320 nm; 75-80% of total energy) and UVA (320-375 nm; 20-25% of total energy), as described in our previous studies (13, 14). The dose of UVB was quantified using a UVB Spectra 305 dosimeter (Daavlin Co., Bryan, OH, USA). The radiation was calibrated with an IL-1700 research radiometer/photometer from International Light Inc. (Newburyport, MA, USA).

## UV-induced skin carcinogenesis model

The UV-induced model was generated as previously described (15) and the procedure is presented in Figure 1. Mice were randomly assigned to three experimental groups and were topically applied from 7-8 weeks of age until the end of the experiment. All of the animals except those in the control group received UV-irradiation twice per week and followed by one immediate toplical application of acetone the vehicle control, or 200 µmol sulforaphane (SFN).

Female SKH-1 hairless mice at the age of 6-week were randomly divided into five groups and the tattoo of mouse ID number was placed on a tail of each mouse. UVB-irradiation and topical application started at the mouse age of 8-week. Topical application of acetone (vehicle), SFN at the dorsal region was right after a single dose of UVB-irradiation at the strength of 60 mJ/cm2, and at every following day. This topical application after UVB-irradiation avoided the potential sunscreen effects of SFN. Two times of UVB-irradiation per weeks worked as both a tumor initiator and promoter. The body weight of mice was monitored biweekly, and we found that it increased steadily with or without UVB-irradiation. The health condition was monitored every three days, especially the skin condition including UVB-induced epidermal hyperplasia, and the actinic keratosis as pre-cancers.

## Animals and sample preparation

Female SKH-1 hairless mice were purchased from Charles River Laboratories ([Wilmington, MA](https://www.google.com/search?biw=1536&bih=734&q=Wilmington+Massachusetts&stick=H4sIAAAAAAAAAOPgE-LSz9U3MC4wLLY0VuIEsQ1zjQoqtLSyk63084vSE_MyqxJLMvPzUDhWGamJKYWliUUlqUXFAFLbgtRFAAAA&sa=X&ved=0ahUKEwjq8uD7hrnUAhWJ7D4KHa_wA4wQmxMIrQEoATAT), USA), as described in previous studies (16). Mice were housed at the Rutgers Animal Facility, maintained under 12 hours light and dark cycles, and provided ad libitum access to food and water. These mice were housed in the animal facility for at least one week before experiments. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUCs). Mice were sacrificed followed by immediately extraction of skin epidermis. For Methyl-Seq experiments 2 biological replicates of these tissues were used. For subsequent bisulfite-pyrosequencing or bisulfite-sanger sequencing validation, 2 identical specimens and another 4 biological replicates of these tissues were used. DNA extraction was performed using the DNA extraction kit (QIAGEN Cat. No. 80204) as described in the manufacturer’s protocol and previously (17).

## Histopathological analysis

The histopathological analysis was performed as described previously (18). Tissue blocks were serially sectioned (4 µm) and mounted on glass slides. The sections were stained with hematoxylin and eosin and were carefully evaluated by a histopathologist. Images of H&E stained sections were captured at 400x total magnification.

## Methyl-Seq library preparation

Methyl-seq was performed using Agilent SureSelect Methyl-Seq kit (Cat. No. G9651A) as described in the manufacturer’s protocol (Methyl-seq protocol, Version X, August 2017). 3 µg of DNA was used in the library preparation. 550 ng of adaptor ligated DNA was used for the hybridization capture and the final concentration of indexed library was 8 to 15 nM. The following changes to minimize the loss of DNA in the process of enzymatic reactions. AMPure XP beads (Cat. No. A63880, Beckman Coulter, USA) were incubated with DNA reaction mix for 10 min at room temperature prior to pelleting by magnetization. AMPure XP beads were then washed twice with 80% ethanol and dried at 37ºC for 5 min. DNA was dissolved for 10 min at at 37ºC. AMPure beads were retained in the solution for adenylation and end-repair reaction. An equal volume of binding buffer was added to this reaction mix at 1:1 ratio to enable the AMPure XP beads to rebind to DNA, incubated at room temperature for 10 min. DNA was further purified as described above. Concentration and size of DNA fragments were determined by Agilent Bioanalyzer 2100 to be in the range suggested in the protocol. Adaptor ligated DNA > 550 ng was used for the hybridization capture in the manufacturer’s protocol.

## Bisulfite conversion and next-generation sequencing

Bisulfite conversion was performed using EZ DNA Methylation-Gold kit from Zymo Research, USA as described in the manufacturer’s protocol. Sequencing was performed by 75 bp paired-end sequencing by Illumina HiSeq2000 as described in the manufacturer’s protocol.

## Bioinformatics analyses of SureSelect Methyl-seq

The reads were aligned to the *in silico* bisulfite-converted mouse genome (mm9) with the Bismark (version 0.15.0) alignment algorithm (19). After alignment, DMRfinder (version 0.1) was used to extract methylation counts and cluster CpG sites into DMRs (20). Each DMR contains at least five CpG sites. Methylation differences greater than 0.10 and with a *P* value smaller than 0.05 were considered significant. Genomic annotation was performed with ChIPseeker (version 1.10.3) in R (version 3.4.0) (21).

## Bisulfite-Pyrosequencing and Sanger sequencing

The bisulfite-treated DNA was amplified by PCR using Platinum PCR Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) with the forward and reverse primers listed in Supplementary Table 2. Specifically, the reverse primers were biotinylated at the 3’ end. The PCR product was separated by agarose gel electrophoresis and was visualized by ethidium bromide staining using a Gel Documentation 2000 system (Bio-Rad, Hercules, CA, USA) to ensure pure PCR products. Later, the biotinylated PCR product was captured using streptavidin-coated beads (GE Healthcare, Piscataway, NJ, USA) and PyroMark Q24 Advanced CpG Reagents (Qiagen) and was washed in a vacuum prep workstation (Qiagen). After annealing with the sequencing primer at 80°C for 5 min, the single-stranded PCR product was pyrosequenced on a PyroMark Q24 advanced instrument (Qiagen).

## PCA enrichment analysis

Clustering of the samples with principal components analysis (PCA) calculated using methylation levels. [Davit, we may need to expand the statistical method?].

## Statistical analysis

The data are presented as means ± SD. Comparisons of multiple groups were analyzed using one-way analysis of variance (ANOVA) with Tukey’s multiple comparison test, and simple comparisons between two groups were analyzed using Student’s t-test. Tumor incidence was examined by Fisher’s exact test. Methylation differences were analyzed by Mann-Whitney U test. A *P* value less than 0.05 was considered statistically significant.

# Results

## DNA methylation profiling

Topical application of SFN reduced tumor incidence (percentage of tumor bearing mice), average tumor volume, and tumor multiplicity (tumor number per mouse) after 24-week treatment (Figure X, optional but Anne will add a graphic and histo images).

To identify DNA methylation changes in UVB-induced skin non-melanoma skin carcinogenesis, we collected DNA methylation data obtained with the Mehtylation450 BeadChip (Illumina) and compared the DNA methylation status of a total of 235,310 CpG clusters, annotated using ChIPseeker (v 1.14.2) and TxDb.Mmusculus.UCSC.mm10.knownGene (v 3.4.0). Sequencing data was aligned to the bisulfite-converted mouse genome and deduplicated. Globally, methylation changes were found to be more pronounced in later stage compared to the early stages, while comparison of hyper- and hypo-methylation changes were variable and tissue type dependent. The genomic distribution of the CpG clusters revealed that hypermethylated CpG sites were preferentially found at low-density CpG DNA regions. These hypermethylated CpG sites were enriched at intergenic regions, CpG islands and gene promoters.

## Distribution of differentially methylated cytosines in genomic and CpG contexts

To determine the genomic distribution of differentially methylated cytosines among different regions, differentially methylated cytosines were assigned to their targeted capture baits that has been mapped to these functional genomic contexts. Differentially methylated cytosines in the CpG context were present in significantly greater numbers than expected in the introns, intergenic and promoter regions downstream of genes (Figure 1).

Average methylation levels were present in promoter, 3’ untranslated regions (3’ UTR), 5’ untranslated regions (5’ UTR), body and downstream in the control group, UVB (the model), and SFN+UVB (the treatment group with SFN). For the promoter regions, differentially methylated cytosines in the CpG context were present in significantly smaller numbers compared to other regions (Figure 2). However, there was no significant difference among the control, model and treatment groups (Figure 2).

Further, the clustering of all samples with Euclidean distances calculated using methylation levels (Figures 3&4). In Figure 3, it’s shown that tumor samples from the model and treatment groups and normal skin samples from the control group at 25-week were presented separately from samples from other time points at the methylation level. Further, aside from tumor or skin samples at 25-week, we compared the skin epidermis samples from time points of 2-week, 15-week and 25-week, suggesting the time-dependent changes in methylation. UVB-irradiation and treatment by SFN groups were observed with significantly changes in CpG methylation compared to the control groups per time point.

## Methylation levels of control group in aging

Moreover, to identify differentially DNA methylation changes in aging, we compared the proportion of Methylated Reads in the control groups among the comparison of 2-week, 15-week, and 25-week time points with FDR < 0.1 (Figure 5). The control groups at 15-week and 25-week showed increased accumulation of changes in methylation in skin epidermis (Figure 5). Genes with CpG regions with at least 20% difference in methylation and FDR < 0.01 for each comparison were leased in Figure 6, with the comparison of 15-week to 2-week at outer layer, 25-week to 2-week at the middle layer, and 25-week to 15-week at the innermost layer. 51 [To be confirmed/ revised in a table] genes were identified with increased methylation with at least 20% at 15-week and 25-week compared to 2-week, while 23 [To be confirmed/ revised in a table] genes with decreased methylation. Interestingly, these genes did show smaller changes in the comparison between 25-week to 15-week (Table 1 or Figure 6B), suggesting that DNA methylation changes related to aging accumulated more in the earlier before 15-week, but bot after until 25-week.

## Effects of SFN in DNA methylation

Furthermore, we analyzed the methylation of the skin epidermis and collected tumor samples in the comparison of UVB vs control, and UVB+SFN vs UVB at week-2, 15 and 25 (Figures 7-9, Davit will add another tumor figure).From week 2, UVB induced siginificantly hyper- or hypo-methylation (FDR < 0.1) compared to the control group, while the SFN treatment induced smaller but significant changes in the DNA methylation compared to the UVB model group (Figure 7). Moreover, 16 genes were identified with increased methylation in UVB vs Control, and decreased in SFN+UVB vs UVB, while 27 genes were observed with reversed changes at week 2, as shown in Figure 8B or Table 2. At week 15 (Figure 10 or Table 3), 12 differentially methylated regions (DMR) were identified with increased methylation in UVB vs Control and decreased in SFN vs UVB with FDR < 0.1. Oppositely, methylation of 7 DMRs were decreased in UVB vs Control and increased in UVB+SFN vs UVB. At week 25 (Figure 11 or Table 4), 16 differentially methylated regions (DMR) were identified with increased methylation in UVB vs Control and decreased in SFN vs UVB with FDR < 0.1; 16 DMRs were decreased in UVB vs Control and increased in UVB+SFN vs UVB.

# Discussion and conclusions

This present study will provide unique new insights into alterations induced by UVB in initiation, promotion, and progression stages of skin inflammation and carcinogenesis. Importantly, we will identify the anti-oxidative stress, anti-inflammation and epigenetic modifying effects of UA in the prevention of skin carcinogenesis.

Methyl-seq was developed to study the patterns of the mouse methylome after exposure to UV-irradiation. In addition, after validation, genes with differentially methylated also tend to show decreased gene expression. In this study, we have identified genes with altered methylation in the different stages of UVB-induced skin carcinogenesis. For example, [Add discussion of the major pathways and molecules, related references.]

There are several limitations of this current study. First, the magnitude of difference in DNA methylation is among the Methyl-seq, bisulfite-pyrosequencing or sanger sequencing. The Agilent’s SureSelect Methyl-Seq assay is designed to detect DNA methylation on the negative strand. Further validation by bisulfite-pyrosequencing or sanger sequencing is not always possible because of the limitations in the primer design on the sequence with low complexity on the negative strand. The discrepancy may be caused by the effect of hemi-methylation. Moreover, an external control can be spiked into the Methyl-seq library preparation to provide accurate detection of bisulfite conversion error rates, although this is not presently performed in the standard Agilent Methyl-seq protocol.

In summary, we have demonstrated the application of Methyl-seq to detect DNA methylation in different stages of UVB-induced skin carcinogenesis. We have also shown that the intervention of SFN by topical application partially reversed the DNA methylation differences introduced by chronic UVB-exposure. Further studies will try to understand the correlation of DNA methylation to the gene silencing and expression of these selected genes using RNA-sequencing, and how SFN introduce the protection against UVB damage by targeting at DNA methylation of these genes.

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# Conflict of interest

The authors declare that there are no conflicts of interest.

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# Legend

**Table 1**: Up and down regulated top 10 genes in all time points and tumors.

**Figure 1**: Distribution of CpG clusters by gene region and cluster size. Majority of clusters are made of 3 CpGs.

**Figure 2**: Heatmap and cluster for all samples (4 samples from Control, 4 samples from UVB, 4 samples from SFN with UVB).

**Figure 3**: PCA or column chart of all samples (samples are same in 3)

**Figure 4**: MA ploting (DEseq2)

**Figure 5**: Gene heatmap and Venn diagram.

**Figure 6**: Time vs CpG region with ??? (still working on this figure)