CHAPTER

20 COMPUTATIONAL ANALYSIS OF EPIGENETIC MODIFICATIONS IN MELANOMA

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INTRODUCTION

Epigenetic information is coded in the form of modifications on our genetic material. DNA bases and histone proteins are heavily modified with various chemical moieties (such as methylation, acetylation, ubiquitination, SUMOylation, and phosphorylation). Some of these modifications are laid in specific manner on certain kinds of epigenetic elements and therefore can act as markers for identification of such elements in a genome-wide manner. For example, acetylation on histone H3 at lysine 27 marks active enhancers or promoters [1,2]. Since the advent of genomic technologies in the past decade, such as DNA microarrays and next-generation sequencing, a number of approaches have been developed to map these modified bases or histones at the genome-wide scale. These studies have yielded wealth of epigenomic data in various biological contexts including various solid and hematological malignancies. This chapter focuses on epigenomic alterations in cutaneous melanoma, the most aggressive form of skin cancer. We briefly summarize the current knowledge on alterations in various epigenetic processes, mention the methods for genome-wide analyses of these processes, and provide a brief description of computational analyses of most popular methods (Table 20.1).

Melanoma is a highly aggressive disease, which primarily arises from melanocytes present in the skin. Rate of melanoma occurrence is increasing every decade with an alarming rate [3]. As such, melanoma represents 5% and 4% of cancer cases in men and women, respectively. In 2017, an estimated 87,100 new cases of melanoma will be diagnosed and approximately 9730 people will die of this disease [4]. Treatment strategies for melanoma patients have been rapidly improved with the advent of immune checkpoint blockade agents and adaptive T-cell therapies, which have been approved by FDA in the past 2−3 years [5,6]. These therapies have provided remarkably durable responses in melanoma. However, response rates remain low [6−11]. Therefore, a major unmet need in melanoma therapy is to identify biomarkers of responses to immunotherapies. Epigenetic marks could be potentially used as biomarkers. Hence, there is a need for comprehensive understanding of epigenomic aberrations in melanoma, especially those associated with response to immunotherapies.

Melanoma arises from melanocytes that produce melanin for absorption of reactive oxygen species (ROS) derived from cellular response to ultraviolet (UV) radiation [12]. Due to its ability to induce cyclobutane pyrimidine dimers, UV irradiation causes a large number of somatic mutations in the

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Table 20.1 Available Computational Tools for Analysis of Epigenomic Data Sets

Epigenomic Elements	Methods	Tools	References
DNA methylation (5mC)	Infinium HM450K beadchip array	Minfi, ChAMP (R)	[117,118]
DNA methylation (5mC)	RRBS	Methylkit, BiSeq (R)	[36,38]
DNA methylation (5mC)	WGBS	Bsseq (R)	[119]
DNA methylation (5mC)	MeDIP-Seq	MEDIPS (R)	[120]
DNA methylation (5hmC)	OxBS-Seq	oxBS-MLE, MLML	[121,122]
DNA methylation (5hmC)	TAB-Seq	MLML	[122]
Histone modification	ChIP-Seq	MACS, HOMER	[75,123]
Transcription factor binding	ChIP-Seq, ChIP-exo	MACS, MACE	[75,124]
Chromatin accessibility	DNaseI-Seq	F-Seq, HotSpot	[125,126]
Chromatin accessibility	MNase-Seq	DANPOS	[127]
Chromatin accessibility	FAIRE-Seq	HOMER, ZINBA, F-Seq	[123,125,128]
Chromatin accessibility	ATAC-Seq	MACS, nuceloATAC	[75,110]
High-order chromatin structure	Hi-C	HiC-pro, HiCCUPS, HOMER, diffHic	[97,123,129,130]
High-order chromatin structure	Capture Hi-C	CHiCAGO	[131]
High-order chromatin structure	4C	w4CSeq, FourCSeq	[132,133]
High-order chromatin structure	5C	HiFive	[134]
Long-range chromatin interaction	ChIA-PET	Mango, CHIA-PET2	[135,136]
Long-range chromatin interaction	HiChIP	HiC-pro	[130]

DNA [13]. Hence, melanoma is typified by a large number of somatic mutations that harbor UV signature [14]. This poses challenges in interpretation of epigenomic aberrations observed in melanoma as well as in determining their functional roles.

DNA MODIFICATIONS

5-Methylcytosine

5-Methylcytosine is the most abundant DNA base modification present in the eukaryotic cells that is associated with gene repression. The distribution and aberrations in 5-methylcytosine have been studied extensively during normal development and in cancer including melanoma (reviewed in Refs. [15,16]). Historically, it was found that cancers display hypomethylation at the global level and hypermethylation on promoters of specific tumor suppressor genes (leading to their silencing) [17]. In melanoma, pregenomic era studies identified several important genes' promoters to be hypermethylated including *CDKN2A*/*CDKN2B* (cyclin dependent kinase inhibitor 2A/2B), *PTEN* (phosphatase and tensin homolog), *MGMT* (O-6-methylguanine-DNA methyltransferase), *RASSF1A* (Ras association domain family member 1), *RAR-β2* (retinoic acid receptor beta 2), *TBC1D16* (TBC1 domain family member 16), and *FES* (tyrosine protein kinase FES) [18–24]. The largest study

defining 5-methylcytosine patterns in melanoma is the TCGA (The Cancer Genome Atlas Group) study, which profiled 333 melanoma samples using Infinium Human Methylation 450K beadchip array [14]. Clustering analysis of differentially methylated probes in the TCGA study identified four subgroups of melanoma samples: "normal" like, hypomethylated, hypermethylated, and super-hypermethylated CIMP (CpG-island methylator phenotype) clusters [14]. Here, CIMP cluster showed marginally significant enrichment of *IDH1/2* (isocitrate dehydrogenase 1/2) and *ARID2* (AT-rich interaction domain 2) mutations. Another recent study compared DNA methylation profiles derived from MIRA-Seq of 27 melanoma tumors with normal melanocytes and identified *KIT* (KIT proto-oncogene tyrosine kinase receptor), *PAX3* (paired box 3), and *SOX10* (SRY-box 10) as hypermethylated and downregulated genes [25]. Finally, another study followed methylation patterns in premalignant nevi (n = 14), primary tumors (n = 33), and metastatic tumors (n = 28) using HumanMethylation450 BeadChip array, leading to identification of some other developmental genes such as *HoxA9* (homeobox-A9) as well as potential biomarkers in methylation of *PON3* (paraoxonase 3) and *OVOL1* (ovo-like transcriptional repressor 1) [26]. For detailed information on aberrant methylation in melanoma, please refer to the review article by Micevic et al. [15].

Most popular methods to generate DNA methylation profiles remain Infinium HM450K beadchip array (replaced with Infinium Methylation EPIC 850K beadchip array), MeDIP-Seq (methylated DNA immunoprecipitation followed by sequencing), RRBS (reduced representation bisulfite sequencing), and WGBS (whole genome bisulfite sequencing) depending on the need of depth and economic considerations. Infinium HM450K array is a probe-based technology that covers about 450,000 CpGs that are present in the promoter region 5′UTR, CpG island, CpG shores, CpG shelves, first exon, gene body, and 3′UTR, providing a comprehensive view of methylation on 99% of RefSeq genes. MeDIP-Seq is dependent on immunoprecipitation of methylated DNA using 5-methylcytosine specific antibody followed by next-generation sequencing [27]. The gold standard however, for determining the DNA methylome remains bisulfite-based. In essence, WGBS is whole-genome resequencing, preceded by treatment of genomic DNA with sodium bisulfite [28,29]. Excluding repetitive regions, this technique is capable of determining the state of virtually all cytosines in the genome. But given the costs and bioinformatic challenges, WGBS is used less than other methods. Therefore, we focus here on RRBS, the "reduced" form of WGBS, which is low cost, yet single-base resolution, at the expense of genome coverage.

Reduced Representation Bisulfite Sequencing (RRBS)

RRBS is a cost-efficient method for genome-wide DNA methylation profiling [30,31]. Genomic DNA is first digested by a methylation-insensitive restriction enzyme (e.g., BglII, MspI) and size selected to produce a small subset of the genomic DNA enriched for CpG sites in most of the promoters and CpG islands. Bisulfite conversion is performed and sequencing library is constructed subsequently.

To process RRBS data, the raw sequencing fastq files undergo quality control first. Low quality bases and adapters are trimmed off by tools such as *Trim Galore* [32]. Lambda spike-in DNA is used as a control for bisulfite conversion rate. The lambda DNA used in the spike-in is unmethylated. If the bisulfite conversion is efficient, high percentage of cytosines (C) in the lambda DNA should be converted to thymine (T). Then, quality controlled fastq reads are aligned to reference genome using aligners such as *Bismark* [33], *BSMAP* [34], and *BWA-meth* [35]. *Bismark* or *MethylDackel* can be used to extract the methylation calls from the aligned bam files.

For each CpG site, the number of reads supporting the C (methylated) and the number of reads supporting the T are extracted from the bam files. Methylation level of each CpG is calculated as the number of C divided by the total number of reads covering that site. This ratio is also known as the beta value. Differentially methylated loci are then identified by statistical tests assuming the methylation level of a CpG site follows a beta distribution [36,37]. Downstream differential methylation analysis can be carried out by packages such as *methylKit* [38], *Biseq* [36], and *DMAP* [39]. For a full list of packages, one can refer to this review [40].

So far, not many studies have used RRBS in studying global DNA methylation changes in melanoma. A recent study used RRBS to profile paired primary and metastatic melanoma cell lines identified *EBF3* (early B cell factor 3) to be hypermethylated in the metastatic cell lines [41]. Unexpectedly, hypermethylation of *EBF3* promoter associated with increased gene expression, which is somewhat contradictory to what is known for promoter methylation. Given that RRBS is cost effective, more studies can be carried out using it to study global DNA methylation changes in melanoma. Moreover, RRBS profiling on the tumor samples rather than the cell lines will give more insight on how DNA methylation contributes to melanoma tumorigenesis. However, the computational challenge of analyzing tumor RRBS data is that tumor samples contain a lot of microenvironment cells. Teasing apart the normal stromal cell contribution in DNA methylation is critical. One algorithm, *MethylPurify* [42], has been developed to deconvolute the normal cell composite and analyze differential methylation for RRBS data and WGBS data.

5-Hydroxymethylcytosine

5-Hydroxymethylcytosine is a recently discovered (in 2009) modification of DNA that is derived from oxidation of 5-methylcytosine by TET (Tet methylcytosine dioxygenase) enzymes [43]. It is subsequently converted to 5-formylcytosine and 5-carboxylcytosine during the consecutive oxidation processes [44,45]. We have limited information on distribution and aberrations in these marks in melanoma. A prominent study in melanoma showed that 5hmC levels are reduced during transition to metastasis, consistent with downregulation of TET2 enzyme [46]. They found strong correlation between loss of 5hmC and poor prognosis of patients, thereby suggesting 5hmC levels as a potential biomarker in melanoma. Further studies are required to determine the patterns of 5hmC, 5fmC, and 5camC in melanoma tumors to determine heterogeneity among patients and global patterns for their downstream regulatory pathways. In this space, multiple methodologies have been recently developed including OxBS-Seq (oxidative bisulfite sequencing), TAB-Seq, and hMeDIP-Seq (5-hydroxymethylated DNA immunoprecipitation followed by sequencing) (reviewed in Ref. [47]).

HISTONE MODIFICATIONS AND CHROMATIN STATES

DNA wraps on histone proteins to form nucleosomes, which are the fundamental units of chromatin [48,49]. Nucleosome is composed of an octamer of the four core histones (H3, H4, H2A, H2B) [50,51]. The N-terminals of the histones are subject to various posttranslational modifications including acetylation, methylation, and phosphorylation [52,53]. A specific combination of histone modifications is termed “histone code”, which correlates with the gene expression pattern. For example, histone H3 lysine 27 acetylation (H3K27ac) is usually found at active promoters or enhancers while H3K27me3 is usually located at the repressive promoters [54,55].

Recently, alterations in enhancers have been shown in multiple malignancies by H3K27ac profiles [56]. However, more than 100 epigenetic modifications have been identified [57,58] without clear understanding of their biological roles and interdependence. Furthermore, there are an even larger number of possible combinatorial patterns of these histone and DNA modifications, and it is these combinatorial patterns—not individual modifications—that dictate epigenetic states [53]. Hence there is tremendous need to identify alterations in these chromatin states during cancer progression. Comprehensive knowledge of epigenome alterations in cancers has been lagging in part due to technical (e.g., generation of large-scale data), analytical (e.g., algorithms to define combinatory states), and biological (e.g., lack of "germline normal" equivalence) challenges. However, with the recent development of high-throughput ChIP-sequencing methods, computational approaches to predict combinatorial patterns, and a surge in epigenome profiling studies [59–63], it is now possible to determine epigenetic states associated with different stages of tumorigenesis and therapeutic resistance.

Indeed, we have recently exploited these advances to define chromatin state changes associated with nontumorigenic to tumorigenic transition in melanoma using an optimized high-throughput ChIP-Seq protocol [64,65] for 35 epigenomic marks [66] and cutting-edge computational algorithms such as *ChromHMM* [67]. ChIP-Seq is short for chromatin-immunoprecipitation followed by sequencing, which is a gold standard approach to study protein and DNA interaction in vivo. The basic steps are as follows: First, the DNA-binding protein in vivo is crosslinked with formaldehyde and the chromatin is sheared into 200–600 bp short fragments. Then, the DNA–protein complex is immunoprecipitated with an antibody specific to the protein of interest. Finally, the DNA is purified and made to a library for sequencing [68,69].

Chromatin-Immunoprecipitation Followed by Sequencing (ChIP-Seq)

Analysis of ChIP-Seq data involves a series of steps of quality control and preprocessing. Briefly, the quality of the sequencing fastq reads is assessed by *fastqc*, and then the reads are aligned to the reference genome using aligners such as *bowtie* and *bwa*. *Bowtie* has two versions: *bowtie1* and *bowtie2*. *Bowtie2* is better for read length greater than 50 bp and it can tolerate indels. It is still very common to use 36 bp single-end sequencing library for ChIP-Seq, so *bowtie1* is preferred for ChIP-Seq with short reads and if one does not care about the indels. The aligned bam files can be used to call peaks. Finally, the resulting peak files and raw signal (bigwig) files are visualized in a genome browser such as IGV.

To accommodate great need for defining chromatin states in melanoma tumor samples, we have established an integrated platform for high-throughput ChIP-Seq which constitutes a wet-lab module and a computational module [70]. The computational module utilizes a processing pipeline [71] based on snakemake [72]. *Snakemake* is a workflow management system, an extension of python language by adding declarative code to define rules. Rules describe how to create output files from input files, which is very similar to GNU Make. *Snakemake* greatly reduces the complexity of creating workflows by providing a fast and comfortable execution environment, together with a clean and modern specification language in python style. We incorporated all the preprocessing steps in this pipeline including quality control of raw fastq reads, aligning to genome by *bowtie1* [73], assessing ChIP quality by *phantompeakqual* following ENCODE standard [74], down-sampling all samples, making RPKM normalized bigwig tracks by deepTools, calling peaks using both *macs1* and *macs2* [75],

generating superenhancer calls by *Rose* [76], and running *ChromHMM* [67] models. We added a *multiQC* [77] module in the pipeline, which generates an HTML output aggregating all the quality control results: *fastqc* [78], *samtools* [79] *flagstat*, and *bowtie* alignment report.

The pipeline was implemented in a way that all jobs are submitted to the computing cluster; independent jobs are run in a parallelized manner; dependent jobs start after the upstream jobs finish. In this way, computing capacity can be maximized by taking advantage of a multicore computing cluster. Moreover, there is a configuration file that can be used to fine-tune the running parameters. One can use a different genome (e.g., mouse) to align reads; set peak calling *P* value cutoffs; change the number of reads to one that all samples are downsampled to; choose different number of chromatin state for *ChromHMM*; etc. If certain files failed, rerunning the whole pipeline will only rerun the files that failed. Of course, one can force rerun any desired step for any files. Our pipeline ensures computation reproducibility as *snakemake* records exact commands that are used to generate the outputs and the versions of the tools that are used to run the commands.

Various findings using ChIP-Seq have been reported in studying melanoma progression. ChIP-Seq against two important histone modifications representing activated (H3K27ac) and repressed (H3K27me3) chromatin marks was performed, which identified *TEADs* (TEA domain transcription factor) as the regulators of the invasive state of melanoma [80]. Another study used ChIP-Seq to identify MITF (melanogenesis associated transcription factor) and SMARCA4 (SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 4) binding sites and showed that MITF interacts with chromatin remodeling complex comprising SMARCA4 and CHD7 (chromodomain helicase DNA binding protein 7). Laurette et al. further showed that *SMARCA4* is essential for melanoma cell proliferation in vitro and for normal melanocyte development in vivo [81]. More recently, using H3K27ac as a surrogate for superenhancers, a study showed activation of superenhancers at neural crest progenitor (NCP) genes in both zebrafish and human melanomas, identifying an epigenetic mechanism for control of this NCP signature leading to melanoma [82]. Using ChIP-Seq, *CTCF* (CCCTC-binding factor) was shown to have the reduced binding at the mutant alleles in melanoma. Topologically associating domains with mutated *CTCF* anchors contained differentially expressed cancer-related genes [83].

HIGHER-ORDER CHROMATIN STRUCTURE

Human genome DNA is ~2 m long if it is stretched. How a ~2 μm nucleus harbors this much longer DNA? Eukaryotic DNAs are not linear inside of the nucleus. The conventional model is that DNA wraps on the histone proteins to form nucleosomes with 11 nanometer (nm) in diameter [48,50]. These "beads on a string" structures are then further folded into several different scales of high-order structures until they form chromosomes. Recent studies found that chromosomes are spatially segregated into sub-megabase scale domains: topologically associating domains (TADs) [84,85]. TADs are quite conserved across species [84] and tissue types [86]. Importantly, the high-order chromatin structure has been showed to be critical in regulating gene expression and determining cell identity. Misregulation of high-order chromatin structure can cause diseases [87,88] including cancer [89]. However, this view is challenged by a recent electron microscopy tomography (EMT) with a labeling method (ChromEM) study in living cells [90], in which the authors showed that no such high-order structure was observed. Instead, DNA and nucleosomes assemble into disordered chains that have diameters between 5 and 24 nm, with different particle arrangements, densities, and structural conformations.

Chromosome Conformation Capture Based Methods

There are two ways to study higher-order chromatin structures: microscope-based [91] and genomic sequencing-based assay [92]. Here, we will focus on the genomic approaches. To study long-range chromatin interaction, several methods are developed including Hi-C [92], capture Hi-C [93], ChIA-PET [94], and HiChIP [93]. The resolution of the interaction map can range from megabase [92] to kilobase [95,96]. Architectural proteins such as CTCF and cohesin are shown to be enriched in the TAD boundaries [84,97]. Interestingly, different studies show discrepant observations on whether CTCF and cohesin are required for the TAD formation [98–102].

All these methods have a similar experimental procedure. First, chromatin is crosslinked by formaldehyde, a restriction enzyme is used to digest the chromatin, and then free-ends of the DNA are ligated in a diluted volume and the ligated DNA fragment pairs are subject to high-throughput sequencing. Interaction of the genomic sites is then identified by computational methods. The difference between ChIA-PET, HiChIP, and Hi-C is that the interaction pairs are enriched by applying an antibody to the protein of interest, which mediates the looping confirmation, while Hi-C is capturing all the interactions in the genome. Paired-end sequencing of the Hi-C library results in fastq reads files similar to other high-throughput sequencing assays. Raw reads are quality controlled and then aligned to the reference genome. Note that the paired-end reads are aligned separately because the insert size of the Hi-C ligation product can vary drastically. The data are then binned into fixed genomic interval sizes, to aggregate data and remove noise. Data are further normalized by mappability, GC content, and fragment length [103]. Lastly, the interactions and TADs are predicted by various tools [104].

So far, no studies have used Hi-C to investigate higher-order chromatin structure changes during melanoma progression. Integrating Hi-C data in a different cell type and mutation data from melanoma, a recent study showed that genomic regions display similar mutation profile if they are in close spatial proximity to late-replicating domains [105]. Hi-C and other types of long-range interaction assays in melanoma are urgently needed to investigate how high-order chromatin changes contribute to melanomagenesis.

NUCLEOSOME POSITIONING

Nucleosome packaging has a significant effect on the availability of DNA sequences to proteins such as transcription factors, which are central players in regulating gene expression. Thus, open or accessible regions of the genome are considered as the places where regulatory elements reside. Characterizing the accessible regions of the genome is critical in studying how the transcription is regulated. There are several methods that are developed to locate the accessible regions in a genome-wide scale utilizing the high-throughput sequencing techniques [106]: DNase I digestion (DNaseI-Seq), formaldehyde-assisted isolation of regulatory elements (FAIRE-Seq), and the more recent assay of transposase-accessible chromatin (ATAC-Seq) [107]. All of the assays probe the open chromatin regions harboring regulatory elements such as enhancers and promoters. For example, ENCODE project has used DNaseI-Seq to profile the accessible chromatin landscape of the human genome [108]. A complementary method micrococcal nuclease digestion (MNase-Seq) can be used to identify nucleosome positioning.

ATAC-Seq

Of all the methods, ATAC-Seq is gaining popularity due to its less laborious steps and less number of cells as starting materials. This method uses hyperactive Tn5 transposase, which inserts sequencing

adapters into accessible regions of chromatin, to detect accessible regions of the genome [107]. Sequencing reads are mapped to reference genome to infer open regions, to footprint the transcription factor motifs and to infer nucleosome positioning.

ATAC-Seq analysis is thoroughly described in Ref. [107]. Briefly, the sequencing reads are quality controlled with *fastqc* and the adapters are trimmed. Then the reads are aligned back to reference genome using *bowtie2* [109]. It is quite common to have mitochondrial sequences in the reads, so the mitochondrial reads are removed from the aligned bam files. Peaks are called using *MACS2* [75]. Nucleosome positioning is inferred by *nucleoATAC* [110]. We have developed a *snakemake* [72] based pipeline [111] to preprocess ATAC-Seq data.

Surprisingly, few studies have assayed chromatin accessibility during melanoma progression. Using DNase-Seq data from melanocyte generated by ENCODE, it is reported that DNA mutation rate in melanomas is highly increased at active transcription factor binding sites and nucleosome embedded DNA, compared to their flanking regions [112]. Genome-wide DNA accessibility data are scarce in melanoma. A recent study in zebrafish model of melanoma identified superenhancers (using ChIP-Seq) and open chromatin regions (using ATAC-Seq) near neural-crest identity defining *crestin* and *sox10* genes in melanoma tumors. It will be quite interesting to investigate the chromatin accessibility change during melanoma progression. Assaying on the primary tumors is possible because only a small number of cells are required for ATAC-Seq.

FUTURE PERSPECTIVE

Although we have begun to understand the epigenetic aberrations in the cancer, we are still in the early stages of our understanding of cancer epigenome. We need better methods to define epigenome from tumors rather than cell lines as cell lines have been cultured for a long time under artificial conditions. Further, given the heterogeneity within patient samples, we need to profile a large number of patient samples to determine the subsets where specific epigenetic therapies may be useful. Hence high-throughput versions of the existing methodologies are needed. On the other hand, to get a better understanding of intratumor heterogeneity, we need to define epigenome in tumor cells as well as those in the microenvironment. This is especially important in melanoma given the durable effects of immunotherapy. Importantly, in the light of precision medicine efforts, this needs to be done in a large set of patient samples. Here, methodologies that profile epigenome at the single cell level will be highly useful. Indeed, significant advances have been made in determining DNA methylation, higher-order chromatin structure, and ATAC-Seq profiles from single cells [113–116]. These need to be applied in melanoma. Finally, it needs to be determined if specific genetic mutations have differential effects on the epigenome as it could identify specific patient populations that may be benefited from epigenetic therapy.

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