Chapter 6

Methylomes

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/57535

1. Introduction

Epigenetic modifications stably influence gene expression without changing the underlying DNA sequence [1]. The epigenomic era has revealed a well-connected network of molecular processes. These processes comprise abnormal methylomes, transcriptosomes, genome-wide histone post-transcriptional modifications patterns, histone variants, and noncoding RNAs [2]. The genome-wide DNA methylation status of cells exists in a methylated, hydroxymethylated or unmethylated state, collectively referred to as the DNA methylome [3]. Methylation of the DNA (DNAme) occurs in position 5 in cytosine residues. In mammals, the vast majority (98%) of DNA methylation occurs in CpG (cytosine-phosphate-guanine) dinucleotides in somatic cells [4]. In embryomic stem (ES) cells, however, about one-quarter of all DNA methylation occurs in non-CpG context [4]. The haploid human genome contains approximately 29 million CpGs, DNA methylation involves the transfer of a methyl group to cytosine in a CpG dinucleotide through DNA methyltransferases that creates or maintains methylation patterns [3]. Hydroxymethylation of cytosines has also been reported, though its biological functions are unknown. Several methods have been developed which enable capture of genome-wide profiling of DNA methylation. The complete DNA methylomes for several organisms are now available, helping clarify the evolutionary story of this epigenetic mark and its distribution in key genomic elements. The variation of DNA methylome plays an important role in regulating normal development and differentiation. DNA methylation patterns can be inherited and influenced by the environment, diet and aging, and disrupted in diseases [5]. From a functional perspective, the DNA methylome variation is a stable change in a transcriptional regulatory element, which changes the expression of a gene without any change in DNA sequence or in the intracellular environment. However, a change in environmental conditions could perturb the stability profile of the methylome at a locus, changing the probability of variant states arising, even making a variant state more stable than the previous reference state [6].

2. Methylomes and evolution

DNA methylation is a typical characteristic of most eukaryotes and some of its features are conserved in many species. The methylation states that present in the germline are heritable and participate in evolution. Boffelli D and Martin DI [6] combined phylogenomic and somatic methylation data to infer germline methylation states. Methylated CGs undergo mutation to TG much more frequently than unmethylated CGs, but only CG decay that occurs in the germline results in heritable sequence changes that can become fixed within a species. The predominant trend within the genome is to lose methylated cytosines and this destrucrion of a CpG dinucleotide by a SNP has been shown to lead to significant cis-methylation effects [7]. The loss and gain of CpGs over time is proposed to be a significant evolutionary device, the polymorphic nature of CpG-SNP dinucleotide sequences will help define human population epigenomics [8].

Currently, by the base-resolution sequencing, the complete methylomes for 25 organisms are available (10 animals, 8 plants, 2 insect and 5 fungi). These eukaryotic methylomes have provided initial insights into the evolutionary history of DNA methylation (Figure 1). Since the density of possible methylation sites, and the distribution of 5-methyl-cytosines (5meC) is not uniform in the genome contexts, the role of DNA methylation in promoters, gene bodies, regulatory features, and transposable and repetitive elements can be remarkably different. Such as, the evolutionary history of DNA methylation in gene bodies and transposons is independent. Zemach et al. quantified DNA methylation in 17 eukaryotic genomes and found that gene body methylation is an ancient property of eukaryotic genomes, and is conserved between plants and animals, whereas selective methylation of transposons is not [9]. The transposon methylation is only conserved in fungi [10], and appears to be related to the degree of sexual outcrossing [11]. In general, the DNA methylation landscape can be either continuous along the genome, or constituted by a series of heavily methylated DNA domains interspersed with domains that are methylation free, and the methylation pattern is quite conserved [5].

3. Human methylomes

Because of its association with human development and disease, DNA methylation has stayed in the research focus for almost half a century. The development of methods for whole-genome methylation profiling has now enabled acquisition of the complete human methylomes, especially powerful are methylome profiling techniques with single-base resolution sequencing. Genome-wide, tissue-specific or cell type-specific DNA methylation profiling has begun to shift the focus of DNA methylation research from mostly promoters and immediate upstream enhancers to including intragenic regions and distal intergenetic regions [12]. These revealed that methylation of gene bodies is more frequent than in promoters in the vertebrate genomes [13]. Shen et al [14] characterized the methylome in purified peripheral blood monocytes (PBMs) by using methylated DNA immunoprecipitation combined with high-throughput sequencing, and found that promoters were commonly (58%) found to be

http://dx.doi.org/10.5772/57535

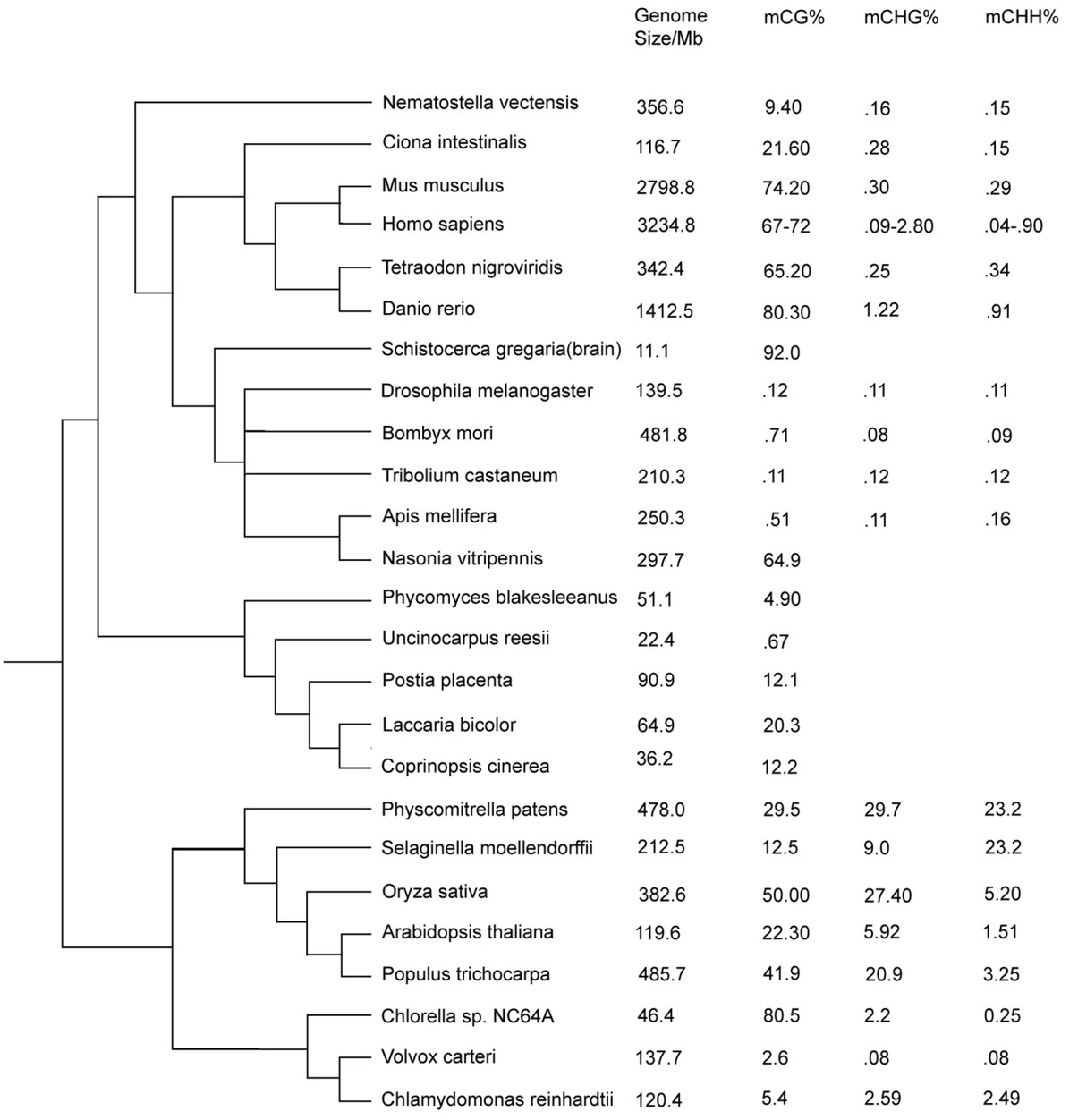

Figure 1. Methylation levels in 25 eukaryotic organisms. The organisms are organized according to their evolutionary distance. Tree topology is determined from the NCBI Taxonomy (http://www.ncbi.nlm.nih.gov/guide/taxonomy/) and displayed using TreeView X (http://darwin.zoology.gla.ac.uk/~rpage/treeviewx/index.html). The genome size is indicated together with the percentage of methylated sites within three sequence contexts: CpG, CHG and CHH (H being A, C or T).

unmethylated; whereas protein coding regions were largely (84%) methylated. Zilbauer et al [15] provide detailed functional genome-wide methylome maps of five primary peripheral blood leukocyte subsets including T-cells, B-cells, monocytes/ macrophages and neutrophils obtained from healthy individuals and identified important cell-type specific hypomethylated regions (HMRs) that strongly correlate with gene transcription levels. SNPs associated with

immune-mediated disease in genome-wide association studies (GWAS) preferentially localised to these cell-specific regulatory HMRs, offering insight into the pathogenesis role of DNA hypomethylation in regulating immune mediated disease. Recent insights into tissue-specific intra-and intergenic methylation and into cancer methylomes suggest that both cancer-associated DNA hypomethylation and hypermethylation are found throughout the genome [16]. The hypermethylation includes promoters of tumor suppressor genes whose expression becomes repressed, thereby facilitating cancer formation. Cancer-associated DNA hypomethylation from intergenic enhancers, promoter regions, silencers, and chromatin boundary elements may alter transcription rates. Whereas, the intragenic DNA hypomethylation might modulate alternative promoter usage, production of intragenic noncoding RNA transcripts, cotranscriptional splicing, and transcription initiation or elongation [16].The new discoveries that genomic 5-hydroxymethylcytosine is an intermediate in DNA demethylation and exhibits cancer-associated losses.

4. Methylomes in cell differentiation and reprogramming

During embryonic development, cells become gradually restricted in their developmental potential and start elaborating lineage-specific transcriptional networks to ultimately acquire a unique differentiated state [17].That is, cell differentiation is a process characterized by the progressive loss of developmental potential and gain in functional specialization. During this process, DNA methylation plays an important role in epigenetic programming by silencing developmental genes and activating tissue-specific genes, thus establishing a cellular memory that defines both cell lineage and cell type.

4.1. DNA methylation remodeling in the embryo development

Over the course of mammalian development, the genome undergo nearly complete remodeling of DNA methylation patterns. Primordial germ cells begin with very low DNA methylation levels, then with gametogenesis parental imprinting tags are established, with substantially methylated but differing methylomes in the sperm and egg. In the preimplantation early embryo there is a wave of genome-wide demethylation that occurs, which is rapid in the paternal genome, except for centromeric, repetitive and paternally imprinted genes, with a comparative slow process occurring in the maternal genome [18].This is then followed by heavy de novo methylation, DNA methylation patterns are then progressively re-established, marking gradual commitment towards lineage-specific differentiation [19].

4.2. DNA methylomes in embryonic stem cells

Embryonic stem cells (ESCs) are a special population of pluripotent cells derived from the inner cell mass (ICM) of a blastocyst during mammalian development. ESCs retain the ability to indefinitely self-renew and differentiate into all cell types found in the adult body. The importance of DNA methylation in ESC stemness maintenance and differentiation is indicated by diverse studies demonstrating the following: (1) DNA methylation is essential for pluripo-

tency but not self-renewal in ESCs. Embryonic stem cells deficient in Dnmt1 and/or Dnmt3a/3b or lacking CpG-binding proteins show a loss of pluripotency and severe impairment of differentiation potential, but still maintain self-renew [20-23]. (2) CpG methylation contributes to differentiation of ESCs. In a search for differentially methylated (DM) regions (DMRs) by reduced-representation bisulfite sequencing(RRBS), Meissner et al [24] found that approximately 8% of CpGs that were unmethylated in ESCs became methylated in ESC-derived neural progenitor cells and approximately 2% of CpGs methylated in ESCs were unmethylated in the neural progenitor derivatives; Genomic analysis provides supporting evidence for the CpG methylation of gene promoters to selectively silence differentiation genes in ESCs, and global DNA demethylation is mostly linked with the upregulation of tissue-specific genes [25]. (3) ESCs are enriched in non-CpG methylation. A recent major study using Methyl-Seq technology reports significant non-CpG methylation in human ESCs, estimating nearly 25% of total cytosine methylation to be non-CpG sites, with CHG and CHH as the major motifs (where H=A, C, or T). Genomic regions enriched in non-CpG methylation are associated with genes involved in RNA processing, RNA splicing and RNA metabolic processes. Interestingly, enrichment of non-CpG methylation in gene bodies correlates with significantly more intronic RNA.

4.3. DNA methylation remodeling in multipotent stem cells

Multipotent stem cells provide a unique intermediate between pluripotent ESCs and unipotent differentiated cells. Multipotent stem cells also show significant reprogramming variability, including somatic memory and aberrant reprogramming of DNA methylation [26].

4.3.1. Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent stem cells of mesodermal origin that can be isolated from various sources and induced into different cell types [27]. Adipose tissue-derived stem cells (ADSCs) are isolated from liposuction material, which provide an abundant source of MSCs. Studies show that select adipogenic and nonadipogenic promoters in MSCs, ADSCs and hESCs are hypomethylated and hypermethylated, respectively, suggesting DNA methylation controls adipogenic differentiation by activating adipogenic-related genes and silencing nonspecific lineage genes[27]. In addition, epigenomic changes in DNA methylation and chromatin structure have been hypothesized to be critical in the determination of lineage-specific differentiation and tumorigenesis of MSCs [27]. Leu et al [27] applied a targeted DNA methylation method to methylate a polycomb group protein-governed gene, Trip10, in MSCs, which accelerated the cell fate determination of MSCs. However, targeted methylation of HIC1 and RassF1A, both tumor suppressor genes, transformed MSCs into tumor stem cell-like cells.

4.3.2. Hematopoietic stem cells

Hematopoietic stem cells (HSCs) are a special population of multipotent stem cells that are derived from the bone marrow and give rise to a subset of mature blood cells that directs all the immune responses. Accumulating evidence suggests that DNA methylation play critical roles in the maintenance of both self-renewing hematopoietic stem cells and leukemic

stem cells [29]. Changes in the DNA methylation profile have a critical role in the division of these stem cells into the myeloid and lymphoid lineages and in the establishment of a specific phenotype and functionality in each terminally differentiated cell type [30]. HSCs deficient in both Dnmt3a and 3b show a loss of proliferative ability but retain differentiation potential, suggesting de novo methylation is important for self-renewal in HSCs [28]. The aberrant DNA methylation has been associated with several immune deficiencies and autoimmune disorders [30].

4.3.3. Multipotent neural progenitor cells

The central nervous system (CNS) is composed of three major cell types-neurons, astrocytes, and oligodendrocytes-which differentiate from common multipotent neural stem cells (NSCs). Comparisons between ESCs, NSCs and terminally differentiated neurons demonstrate that the majority of de novo methylated genes are already present in NSCs, suggesting that the bulk of DNA methylation changes during differentiation is associated with a loss of pluripotency and a commitment to a multipotent state, rather than terminal differentiation [28]. Interestingly, when NSCs further differentiate into astroglial lineage, selective promoter demethylation occurs in glial marker genes, including GFAP and S100B [31, 32]. Loss of methylation using 5-azacytidine (5-azaC) also triggers premature glial differentiation [31]. Consistently, Dnmt1-deficient NSCs precociously differentiate into astroglial cells, which have been linked to increase JAK-STAT signaling and demethylation of the STAT1 and GFAP promoters [32].

4.4. DNA methylations in cell reprogramming

Epigenetic marks can be reset and usually result in the gain of developmental potential, called epigenetic reprogramming. Researches indicated that mammalian somatic cells can be directly reprogrammed into induced pluripotent stem cells (iPSCs) by introducing defined sets of transcription factors Oct4, Sox2, Klf4 and c-Myc [33-35]. The ultimate aim of research on cell reprogramming is to create iPSC that is identical to embryonic stem cells (ESC) and differentiates into tissue specific cell types with intact function, which will pave the way for great advances in regenerative medicine in the future.

4.4.1. 5-hydroxymethylcytosine-mediated epigenetic modifications during reprogramming to pluripotency

Although 5-hydroxymethylcytosine (5hmC) was discovered several decades ago, it became a major focus of epigenomic research only after it was recently identified in murine brain and stem cell DNA. 5hmC is an oxidative product of 5-methylcytosine (5mC) which catalyzed by the ten eleven translocation (TET) family of enzymes [36]. TET1-mediated 5hmC modification could contribute to the epigenetic variation of iPSCs reprogramming and iPSC-hESC differences [37]. Wang et al [37] found that 5hmC levels is increased significantly during reprogramming to human iPSCs mainly owing to TET1 activation, and this hydroxymethylation change is critical for optimal epigentic reprogramming, but does not compromise primed pluripotency.

4.4.2. Non-CpG cytosine methylation in cells reprogramming

Although methylation mainly occurs on the cytosines in the CpG dinucleotide context, non-CG methylation (mCH:DNA methylation targeting CpA, CpT, and CpC dinucleotides) is prevalent in brain, oocytes and pluripotent stem cell [38-40]. Compared to non-growing oocytes (NGOs), germinal vesicle oocytes (GVOs) were over four times more methylated at non-CG sites, indicating that non-CG methylation accumulates during oocyte growth. Widespread methylome reconfiguration occurs during fetal to young adult development, coincident with synaptogenesis. During this period, highly conserved non-CG methylation (mCH) accumulates in neurons, but not glia, to become the dominant form of methylation in the human neuronal genome[38]. Shirane et al [39] found that nearly two-thirds of all methylcytosines occur in a non-CG context in GVOs. The distribution of non-CG methylation closely resembled that of CG methylation throughout the genome and showed clear enrichment in gene bodies. Ziller et al [40] reported a comprehensive analysis of non-CpG methylation in 76 genome-scale DNA methylation maps across pluripotent and differentiated human cell types, and confirm non-CpG methylation to be predominantly present in pluripotent cell types and observe a decrease upon differentiation and near complete absence in various somatic cell types. Non-CpG cytosine methylation has been identified at a high level in stem cells and reprogrammed progenitor cells, indicating that loss of this form of methylation may be critical in the path from pluripotency to differentiation. The total level of global methylation and the degree of non-CpG methylation is inversely proportional to the level of differentiation.

5. Methylomes in cancer

Carcinogenesis is a complex multifactorial process of the transformation of normal cells into malignant cells, and is characterized by many biologically significant and interdependent alterations triggered by the mutational and/or non-mutational (i.e., epigenetic) events. One of these events, specific to all types of cancer, is alterations in DNA methylation. Aberrant DNA methylation is frequently observed and considered to be a hallmark of cancers.

5.1. DNA methylome alterations induced by carcinogens

According to the mechanism of cancer causation, all carcinogenic agents may be divided into genotoxic (carcinogenic agents that interact with DNA) and non-genotoxic (carcinogenic agents causing tumor by mechanisms other than directly damaging DNA) carcinogens (Figure 2). Exposure to genotoxic carcinogens induces genotoxic and non-genotoxic effects in the DNA methylome, whereas exposure to non-genotoxic carcinogens causes non-genotoxic effects only. Genotoxic effects on the DNA methylome are consist of increased carcinogen–DNA-adduct formation at methylated CpG sites and subsequent elevated mutation rates; however, genotoxic effects may cause also non-genotoxic events in the DNA methylome by compromising ability of the DNA methylation machinery to methylate DNA accurately. Nongenotoxic effects of both genotoxic and non-genotoxic carcinogens consist of global loss of DNA methylation, gene-specific hypermethylation, and gene-specific hypomethylation. Non-

genotoxic global DNA hypomethylation leads to genotoxic events such as elevated mutation rates and genome instability. Gene specific DNA hypermethylation of critical tumor suppressor genes causes transcriptional repression and the loss of gene function. In contrast, gene specific DNA hypomethylation induces activation of oncogenes and tumor-promoting genes. Silencing of DNA repair genes, e.g., MGMT, BRCA1 and MLH1, or activation of xenobiotic metabolizing genes, e.g., CYP1A1, may elevate mutation rates indirectly.

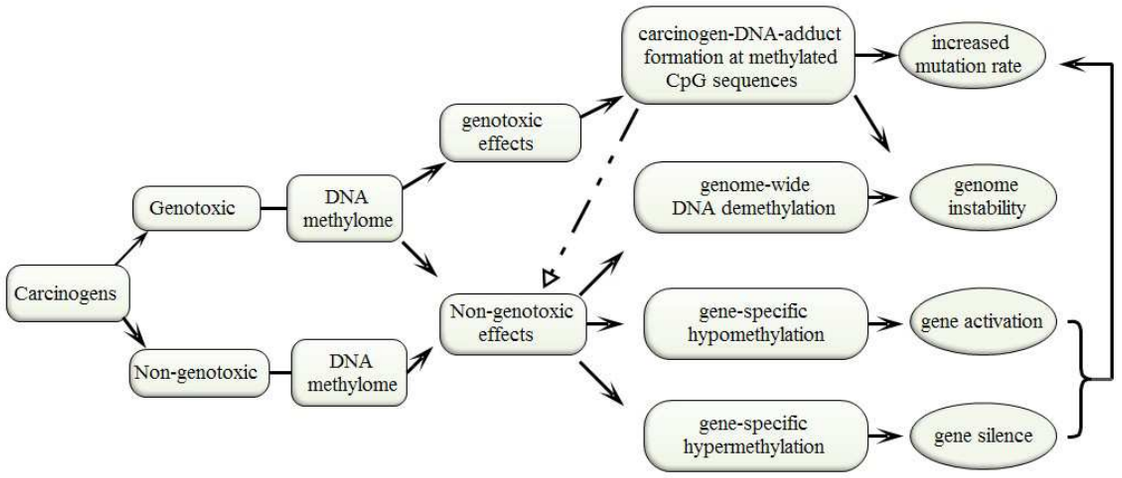

Figure 2. Alterations of the DNA methylome induced by carcinogens

5.1.1. Global DNA hypomethylation in cancer

DNA hypomethylation arises mainly from the loss of methylation at normally heavily methylated areas of genome. The loss of global DNA methylation is one of the most common DNA methylome alterations in human cancers, which is closely related to carcinogenesis. First, genomic demethylation causes a significant elevation in mutation rates] and aberrant activation of"normally"silenced tumor promoting genes [41-43]. Second, hypomethylation of DNA results in the loss of genomic imprinting (LOI), which is currently considered as one of the earliest and most frequent alterations in human tumors [44-46]. Third, demethylation of repetitive sequences, such as long interspersed nucleotide elements (LINE)-1, short interspersed nucleotide elements (SINE), and retroviral intracisternal. Alu elements may cause chromosomal abnormalities and genomic instability via the induction of permissive transcriptional activity of repetitive elements [47-48]. Finally, the recent research indicated that the loss of 5hmC has been found in a broad spectrum of solid tumors, including lung, breast, brain, gastric, and colorectal cancers [49-50].

5.1.2. Cancer-linked gene-specific DNA hypermethylation or hypomethylation

DNA hypermethylation is the most extensively studied epigenetic abnormality in cancer, and the hypermethylation of promoter CpG islands causes permanent and stable transcriptional silencing of a wide range of protein-coding genes and non-coding RNA genes. There is also

growing evidence for the importance of non-CpG island-promoter methylation in cancer, including methylation of CpG island shores [51], non-CpG promoters [52], and coding regions [53], which results in gene silencing. A mechanistic link between DNA hypermethylation and carcinogenesis is epigenetic silencing of critical tumor-suppressor genes, such as cyclin-dependent kinase inhibitor 2A (CDKN2A; p16INK4A), secreted frizzled-related protein genes (SFRPs), adenomatous polyposis coli (APC), Ras association (RalGDS/AF-6) domain family member 1 (RASSF1A) [54], et al. It has been established unequivocally that role of epigenetically-driven gene silencing has been the main mechanism favoring tumor development and progression. This overshadowed the importance of gene-specific hypomethylation in cancer; however, accumulating evidence indicates that the hypomethylation of "normally" methylated CpG island-containing genes also plays a significant role in tumor development. Currently, several hypomethylated tumor-promoting genes, including S100 calcium binding protein A4 (S100A4), plasminogen activator, urokinase (UPA), heparanase (HPA), synuclein, gamma (SNCG), trefoil factor 3 (TFF3), and flap structure-specific endonuclease 1 (FEN1), have been identified in major human cancers [55].

Anyway, cancer-linked gene-specific DNA hypermethylation and hypomethylation are associated with the well-established hallmarks of cancer, including the acquisition of persistent proliferative signaling, resistance to cell death, evasion of growth suppression, replicative immortality, inflammation, deregulation of energy metabolism, induction of angiogenesis, and activation of invasion, complement and enhance each other in the disruption of cellular homeostasis favoring cancer development [56]. Next, as examples, the role of DNA methylation alterations in carcinogenesis was demonstrated in chronic lymphocytic leukemia and glioma.

5.2. Methylome in chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL) is the most frequent leukemia of adults in western countries. Next-generation sequencing of whole genomes, exomes and DNA methylomes in CLL has provided the first comprehensive view of somatic mutations and methylation changes in this disease. Here, we mainly elaborate the change of DNA methylomes in CLL.

5.2.1. Hypomethylation contributes to the genomic instability and oncogene activation in CLL

Two decades ago, high-pressure liquid chromatography (HPLC) analysis revealed the genome DNA of CLL to be globally hypomethylated relative to healthy controls. The subsequent study found that aberrant hypomethylation of repetitive sequences, such as ALU, LINE and SATα leading to genomic instability may be a contributing factor in the increased propensity of TP53-deleted/mutated cases to acquire CLL genomic alterations [57]. Recently, next-generation sequencing of the DNA methylome has also noted gene body hypomethylation to be particularly widespread within enhancer regions in CLL patients [57-59]. After the CLL genome was discovered to be hypomethylated, hypomethylation of oncogene is found to correlate with increased protein expression in CLL. Such as, BCL2, a key anti-apoptotic gene, MDR1, the multiple drug resistance gene, and TCL1, an activator of NF-κB, were subsequently found to be hypomethylated and upregulated in CLL [57].

5.2.2. Cells origin and subtypes of CLL on basis of genome and methylome

Because of the differences in the immunoglobulin heavy variation (IGHV) mutational status and B-cell receptor reactivity, chronic lymphocytic leukemia was classified into two subtypes: chronic lymphocytic leukemia lacking significant somatic IGHV mutation (uCLL) and chronic lymphocytic leukemia with significant somatic IGHV mutation (mCLL), which was derived from naive B cells and memory B cells [60]. Most mutated genes cluster in a few molecular pathways that are also differentially represented in the two subtypes of CLL. Mutations in NOTCH1 signaling, mRNA splicing, processing and transport, and DNA damage response pathways are more common in uCLL, whereas mutations in the innate inflammatory pathway occur predominantly in mCLL.

B cells at different maturation stages require a different level of DNA methylation reprogramming to give rise to uCLL and mCLL. Microarray analysis of a large series of patients indicates that uCLLs acquire approximately seven times more DNA methylation changes than mCLL compared with their respective cells of origin. In particular, two-thirds of the DNA methylation changes that take place in the transformation of naive B cells into uCLL can also be detected in their physiological differentiation into memory B cells [58].

By genome-wide differential DNA methylation profile analysis in uCLL and mCLL, CLL was derived from three different B-cell subpopulations: uCLL resembles both native B cells (IgD $^{+}$and CD27^{-}) and CD5^{+} pregerminal center mature B cells (CD5^{+}, IgD^{+}, and CD27^{-}), whereas mCLL is more similar to non class-switched and class-switched memory B cells (IgM/ D^{+} or IgA/G^{+}, CD27^{+}). The third group of CLL was accompied with an intermediate DNA methylation pattern and enriched for mCLLs with a significantly lower level of somatic IGHV mutations [58]. This group might be derived from a third B-cell type, for example, an antigen-experienced, germinal center-independent B cell that has acquired low levels of somatic hypermutations [60].

5.3. Methylomes in glioma

Glioma is the most frequent and devastating primary brain tumor in adults. Aberrant DNA methylation contributes to glioma development and progression.We employed MeDIP-chip to investigate the whole-genome differential methylation patterns between glioma and normal brain samples. We identified 524 hypermethylated and 114 hypomethylated differential regions in the primary gliomas. Intriguingly, some of the human genome differential methylation regions (DMRs), 199 hypermethylation and 30 hypomethylated differential regions were mapped to genomic regions without any gene annotation (Fig 3A). Only 325 hypermethylation and 74 hypomethylation differential regions were mapped to annotated genes regions, including promoter, intragenic and downstream of genes (Fig3A). A great percentage of DMRs, 63.0% (216) of hypermethylated and 79.0% (60) of hypomethylated differential regions, was mapped to promoter regions of known genes (Fig 3B). 53 hypermethylated and 27 hypomethylated differential regions were mapped to the regions which were both promoter of known genes and CpG islands (Fig 3C). Thus, we identified many novel DMRs that reside in promoters, intragenic, downstream of known genes and unannotated genomic regions in primary gliomas. Since change of promoter methylation status may have close related with

http://dx.doi.org/10.5772/57535

gene expression and involved in tumor development, we focused on the analysis of DMRs in glioma which were mapped to gene promoter regions. The 216 promoter hypermethylated genes and 60 promoter hypomethylated genes identified by MeDIP-chip were analyzed according to their chromosomal location and the physical distribution of these loci was further analyzed (Fig 3D). Except that there were intensive promoter hypermethylation genes in 1, 2, 3, 17 and X chromosomes, the promoter hypermethylated genes were found to be distributed evenly in other chromosomes. While promoter hypomethylated genes mainly distributed in 1, 11, 16, 19, 20 and 22 chromosomes, the number of genes in these chromosomes account for the majority of the total promoter hypomethylated genes [61].

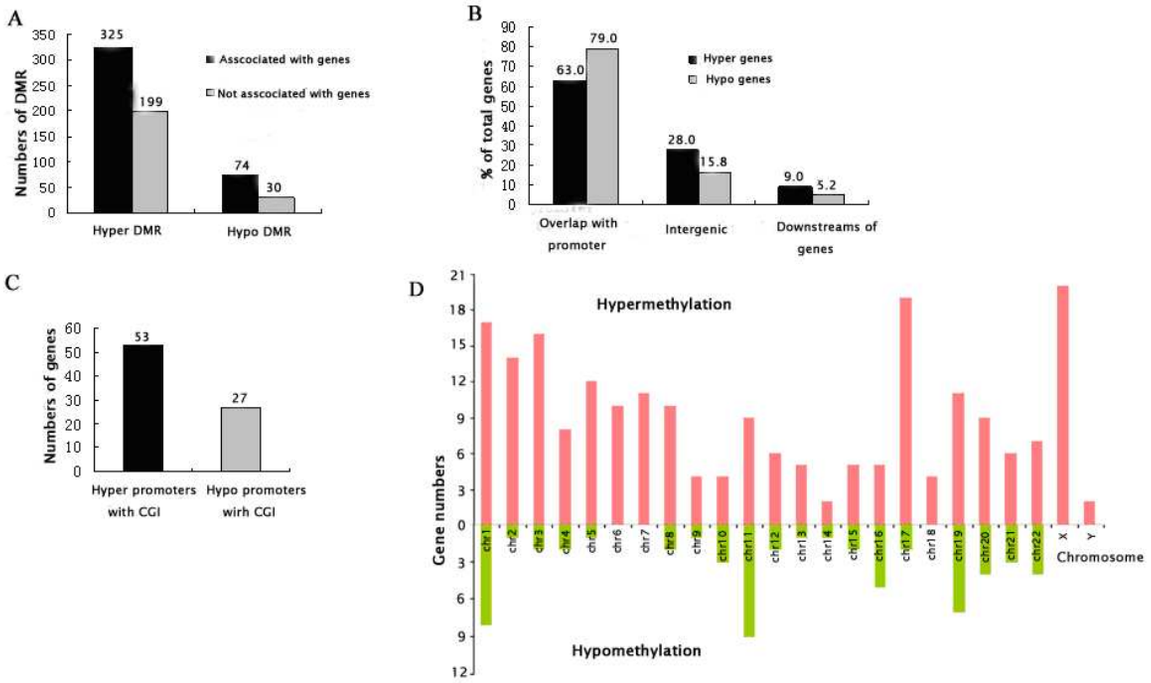

Figure 3. Genome-wide analysis of DMRs in primary glioma. (A) Number of differentially methylated regions that are associated with or without genes. (B) Distribution of diifferentially methylated regions associated with genes. Most of the identified DMRs associated with genes were mapped to gene promoters. (C) Number of DMRs which were both gene promoters and CpG islands. (D) Chromosomal distribution of 216 promoter hypermethylated genes and 60 promoter hypomethylated genes.

6. The connected networks of DNA methylation, histone modifications, transcript factor and miRNAs

Epigenetic mechanisms include: DNA methylation; histone tail modifications; chromatin remodeling; and noncoding RNA expression. DNA methylation is essential for a properly functioning genome through its roles in the maintenance of chromatin structure, chromosome stability and transcription. Histones are the protein moiety around which DNA is packaged within the chromatin, and they can suffer a variety of post-translational modifications of their

N-terminal tails, including acetylation, methylation, phosphorylation, sumoylation, ubiquitination and ADP ribosylation [62,63]. miRNAs are ~20–22 nucleotide non-coding RNA molecules that tend to negatively regulate genes by binding to the 3' untranslated region of the target mRNA via the RNA-induced silencing complex causing mRNA destabilization and/or translational inhibition [64,65]. Growing evidence supports a role for miRNAs as both targets and effectors in aberrant mechanisms of DNA methylation [66,67]. Meanwhile, miRNAs are also invovlved in the control of DNA methylation by targeting the DNA methylation machinery [68, 69]. In this section, we combined with our own work to demonstrate the connected networks of DNA methylation, histone modifications, transcript factor and miRNAs in glioma. On the basis DNA methylome of glioma, we identified fifteen new methylated genes including 9 hypermethylated genes(ANKDD1A, GAD1, SIX3, SST, PHOX2B, PCDHA8,PCDHA13, HIST1H3E and LRRC4) and 6 hypomethylated genes (F10, POTEH, CPEB1, LMO3, ELFN2 and PRDM16) were validated by the Sequenom MassARRAY platform and bisulfite sequencing (BSP) in glioma. Aberrant promoter methylation and changed histone modifications were associated with gene abnormal expression in glioma. miR-185 targets the DNA methyltransferases 1 and regulates global DNA methylation [61], however, miR-101 regulates histone methylation modication of hypomethylated gene CPEB1 by targeting EZH2 and EED, and DNMT3A, and affected their methylation level and expression in glioma [70].

6.1. The regulation networks between hypermethylated gene, miRNA, transcript factor and target gene in glioma

Leucine-rich repeat C4 (LRRC4) gene, a new hypermethylated gene identified by DNA methylome of glioma, are highly specific to brain tissue and it behaved as a tumor suppressor gene in the pathogenesis of gliomas. Methylation of the LRRC4 promoter has been considered as one of the important mechanisms inactivating LRRC4 in gliomas. Exogenous overexpression of LRRC4 could inhibit glioma cells growth and arrest glioma cells in the G0/G1 phase of the cell cycle. Induction of LRRC4 expression inhibited glioma cell proliferation and invasion by downregulating the ERK/MAPK and PI-3K/AKT signaling pathways [71-75].

6.1.1. LRRC4-AP-2-miR-182-LRRC4 loop played important role in the pathogenesis of glioma

It's known that miRNAs took part in proliferation and growth in glioma cells. miR-182 or miR-381 overexpression could promote glioma cell growth in vivo and in vitro. Therefore, they were considered to be potential therapeutic biomarker in glioma. miR-182 or miR-381 silencing could arrest glioma cells in the G0/G1 phase of the cell cycle and inhibit glioma cells growth by upregulating phosphorylated Rb and suppressing E2F3. LRRC4 was the co-target gene of miR-182 and miR-381. The expression of miR-182, miR-381 and BRD7 were inversely correlated with LRRC4 expression in gliomas. miR-182 and miR-381 silencing was found to inhibit the expression of BRD7, upregulate phosphorylated Rb, suppress E2F3, arrest glioma cells in the G0/G1 phase of the cell cycle, inhibit glioma cells growth and induce differentiation of glioma cells to astrocyte-like cell by upregulating LRRC4 and suppressing LRRC4-mediated binding of AP-2/SP1/E2F6/c-Myc to BRD7 in ERK/MAPK and PI-3K/AKT signal pathways [76].

Transcription of miR-182 was induced by transcription factor AP-2 predicted by online softwares and confimed by ChIP. According to our previous results, miR-182 was verified to inhibit the expression of LRRC4, and LRRC4 might inhibit the expression and transcription of AP-2 through negatively regulating the ERK/MAPK and PI-3K/AKT signaling pathways. It's indicated that the LRRC4-AP-2-miR-182-LRRC4 loop formed among LRRC4, miR-182 and AP-2 was involved in glioma development [77].

6.1.2. LRRC4-miR-185/SP1-DNMT1-LRRC4 loop played an important role in glioma

miR-185 could function as a tumor suppressor gene. It's certified that miR-185 could inhibit glioma cell growth, motility and invasion identified by MTT, scratch test and transwell test [78-80]. DNMT1 is one of the most important DNA methyltransferase which maintains methylation. Our research showed that overexpression of miR-185 could inhibit DNMT1 and reduce global methylation by HPLC-DAD, and decreased the expression of nine new hyper-methylated genes (LRRC4, ANKDD1A, GAD1, HIST1H3E, PCDHA8, PCDHA13, PHOX2B, SIX3 and SST) [61]. Hence, miR-185 was considered to inhibit glioma cells growth and migration by targeting DNMT1, reducing global methylation and recovering expression of such hypermethylation genes as LRRC4.

miR-185 also was predicted to participate in Rho GTPase activity based on GO analysis, while CDC42 and RhoA were the main elements regulating Rho GTPase. Then CDC42 and RhoA were identified to be the direct targets of miR-185[61]. Further, CDC42 and RhoA were inversely correlated with miR-185 expression in gliomas. miR-185 was clarified to mediate glioma cell growth and migration by inhibiting CDC42 and RhoA and VEGFA indirectly [61].

It's verified that overexpressing LRRC4 could increase the expression of miR-185, while miR-185 could regulate global methylation by inhibiting DNA methyltranferase DNMT1 and increasing the expression of such hypermethylation gene as LRRC4. There may be form LRRC4-miR-185-DNMT1-LRRC4 loop which LRRC4 are to be as core the loop, miR185 and DNMT1 that participating in glioma development. In addition, DNMT1 was positively regulated by SP1, and it could increase the expression of LRRC4, while LRRC4 could also inhibit SP1 by negatively regulate ERK/MAPK and PI-3K/AKT signal pathway. So that the LRRC4-SP1-DNMT1-LRRC4 loop formed among LRRC4, SP1 and DNMT1 took part in the glioma formation.

In conclusion, development of glioma is the pathological processing with multiple genes and multi-stages. Genes, miRNAs and DNA methylation play an important role in glioma formation. They may support or antagonize each other and construct complicated network in glioma. In sum of above study, at the time of LRRC4 regulating miRNAs as a tumor suppressor, those miRNAs regulated by LRRC4 were found to regulate the binding of transcription factors to DNA in their targets mediated signaling pathways by directly targeting genes (such as LRRC4), or regulate methylation and expression of such hypermethylation genes as LRRC4 by directly targeting DNA methyltransferase and controlling global methylation. And multipases regulation loops which the core was LRRC4 were formed. They were LRRC4-AP-2-miR-182-LRRC4, LRRC4-miR-185-DNMT1-LRRC4 and LRRC4-SP1-DNMT1-LRRC4. These

loops participated in glioma development with multiple positive feedback formation among them (Fig 4).

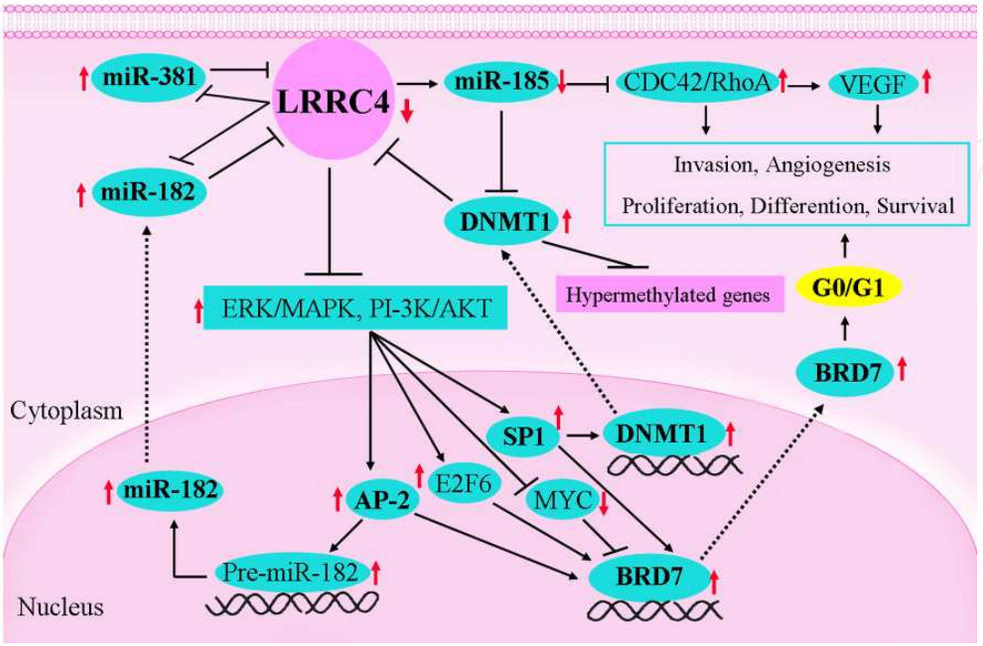

Figure 4. The regulation networks of hypermethylated genes, miRNA, DNMT, transcript factors and targets genes in glioma

6.2. miR-101 regulates the expression of hypomethylated/hpermethylated genes by different histone protein methylaiton modification

It's well-known that miRNAs play significant role by regulating gene expression in tumors. We assumed to analyze the upregulation mechanism of hypomethylation genes in the extent of gene regulation by miRNA. Subsequently, we predicted miRNAs which could regulate hypomethylation genes CPEB1. Interestedly, CPEB1was predicted to be a target of miR-101 by online software Targetscan6.0. It's confirmed that miR-101 could bind to the 3'UTR of CPEB1 and inhibit their expression [70].

6.2.1. miR-101 indirectly suppressed expression of CPEB1 and affected their methylation levels by targeting EZH2, EED and DNMT3A and regulating histone methylation in glioma cells

As miR-101 regulated the methylation status and expression of gene through histone modification, it may regulate the methylation status of CPEB1 in the same way. Hence, the effect of miR-101, EZH2 siRNA, EED siRNA and DNMT3A siRNA on histone methylation and expression of CPEB1 was detected. ChIP combining with qRT-PCR and BSP was used to verify that miR-101 decreased the H3K4me2 and H3K27me3 occupancy at CPEB1 core promoter and increased the H3K9me3 and H4K20me3 occupancy at CPEB1 core promoter by targeting

EZH2, EED and DNMT3A, then it recovered the methylation levels of CPEB1 gene promoter, and indirectly down-regulating the expression of these hypomethylation genes.

6.2.2. miR-101 recovered the expression of hypermethylation gene LRRC4 by down-regulating H3K27me3 occupancy and hypomethylation level of LRRC4 in glioma cells

LRRC4 has been a glioma suppressor gene and its hypermethylation and down-expression is common in glioma. In order to clarify the mechanism of LRRC4 regulation, miRNAs regulating LRRC4 were predicted. miR-101 was predicted to target LRRC4. Here, we indicated that miR-101 could not bind to 3'UTR of LRRC4, but it remain to upregulate the expression of LRRC4 in glioma cells. miR-101 decreased the occupancy of H3K27me3 at LRRC4 core promoter and induced hypomethylation of LRRC4 by targeting EZH2, EED and DNMT3A [81].

Token together, deregulation of gene methylation including hypermethylation and hypomethylation plays an important role in carcinogenesis of glioma. Hypermethylation or hypomethylation of genes and their deregulated expression could be applied to predict the early diagnosis and prognosis of glioma. miRNAs are small noncoding RNA, around 22-24 nucleotides in size. They could not only directly regulate expression hyper/hypo-methylation genes by binding to 3'-UTR of genes, but also regulate the methylation level and gene expression through histone and DNA methylation modification by targeting histone and DNA methyltransferases (Fig 5).

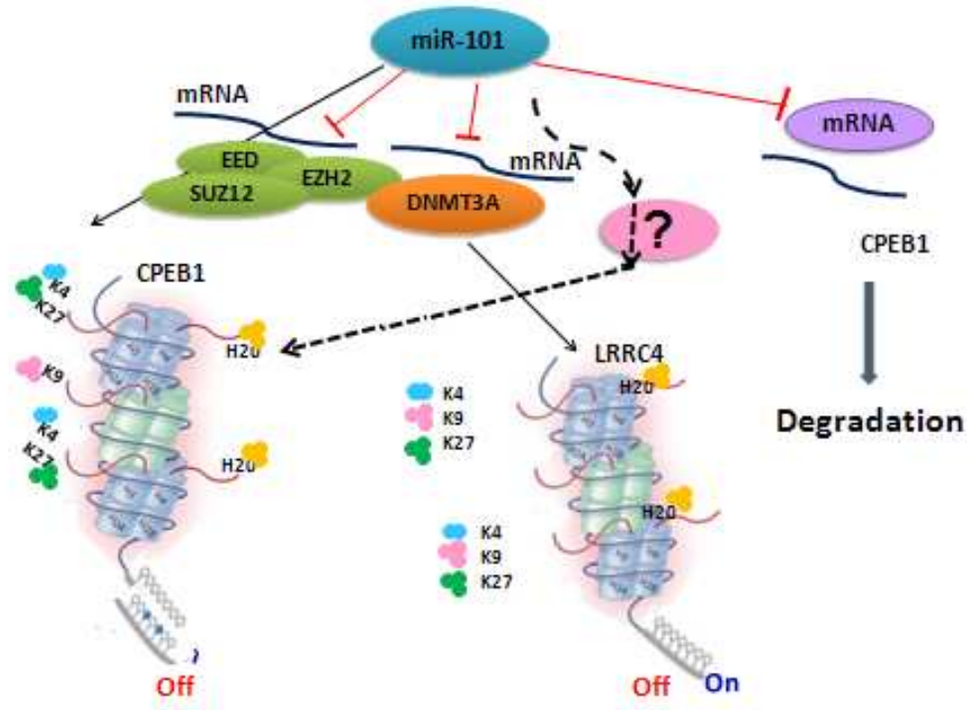

Figure 5. The regulation networks of miRNA, genes methylation and histone protein modification in glioma

7. Conclusion

The advances in next-generation sequencing technologies have allowed for mapping of DNA methylation and its derivatives: 5hmC and 5fC at base-pair resolution. These studies have provided key new insights into the function, dynamics and distribution of DNA methylation in vertebrate genomes. In the near future, studies of DM sites and focal DMRs will aid the discovery of transcription factors and transcription regulatory elements involved in controlling the expression of specific genes in vivo. More experiments in model systems will be done to directly test the functionality of DMRs or individual DM sites identified in epigenomic profiles. It is likely that intragenic and distant intergenic changes in DNA methylation will be studied much more than at present for their contribution to diseases involving epigenetic deregulation, especially cancer, immunological diseases and neurological diseases.

Acknowledgements

This work was supported by grants from National Natural Science Foundation of China (81272297; 81171932) and Hunan Province Natural Sciences Foundations (11JJ1013); Research Fund for the Doctoral Program of Higher Education of China (20110162110037). There are no financial or other relationships that might lead to a conflict of interest.

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