Using DNA Methylation to Understand Biological Consequences of Genetic Variability

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Key Words

Methylation • Genetic variability • Quantitative trait loci • Genome-wide association

Abstract

The advent of high-content genomic mapping technologies has provided numerous clues about the genetic architecture of complex disease and the tools with which to understand the biological framework resulting from this architecture. We believe that understanding and mapping epigenetic marks, in particular DNA methylation, which is suited to such assays, offers a timely opportunity in this context. Here, we make an argument for this work, describing this opportunity, the likely path ahead, and the problems and pitfalls associated with such work.

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Introduction

Single-nucleotide polymorphism genotyping technologies have afforded the opportunity to examine common genetic variation in a genome-wide, high-throughput manner. Hundreds of loci associated with complex disease and traits have been identified through genome-wide association (GWA) studies [1]. The study of mende-

lian disease, where pathogenic mutations in underlying genes result in changes in protein sequence or gene copy number, gave rise to the idea that risk loci identified by GWA would likely be confined to regions of the genome capable of altering the protein code. However, this idea became obsolete as many GWA loci mapped to noncoding regions of the genome. Although GWA studies have successfully identified loci capable of altering the risk for common complex disease by factors from 1.1- to 5.0-fold [1, 2], the biological basis for disease associations identified through GWA remains mostly unsolved. An approach widely used by us and others to better understand this phenomenon, centers on correlating risk variants with expression of proximal genes, effectively determining which risk loci are also expression quantitative trait loci (eQTL). There are several limitations to this method. Although eQTL analyses have offered a practical and useful path into disease etiology, this method offers resolution of only part of the picture. Many limitations of eQTL analyses have been discussed by us and others, and include the technical limitations of assays, availability of tissue of interest, inherent heterogeneity of tissue, and the cross-sectional nature of tissue acquisition. In the context of neurological disease, where human brain tissue is often used, these limitations may be particularly detrimental. The dynamic range and somewhat targeted nature of many expression methods are beginning to be addressed

by the application of RNA sequencing, rather than an array-based assay. Further, the confounding of assaying heterogeneous tissues and alterations in cellular composition associated with age and disease are also beginning to be addressed with methods that aim to isolate and assay single cell types. One limitation of expression assays in postmortem tissue that is difficult to solve is the cross-sectional nature of sample collection. One can imagine that many expression and splicing effects important in disease, and perhaps mediated by disease-linked risk variants, may not be constitutive, but rather induced. In this respect, we believe that examining the epigenetic modification of DNA methylation offers valuable additional insight, because DNA methylation reveals local transcriptional potential rather than the current transcriptional profile, and as such may reveal the extent to which expression can plausibly change in response to an acute stimulus.

In this article, we discuss the investigation of DNA methylation as a quantitative trait influenced by genetics, particularly in the context of complex disease. This review is not meant to serve as a comprehensive review of epigenetics or quantitative trait mapping, nor is it meant to exclude other biological mediators of genetic risk loci, but rather to highlight what we see as an interesting opportunity to gain insight into complex biology.

Understanding Pathobiology in Complex Disease

Using traditional gene cloning to find genetic mutations provides a penetrant, often coding mutation with which to model disease. The path of biological investigation in these cases, while difficult, is clear. Creation of model systems that recapitulate some aspect of the aberrant gene has traditionally been used as a tool to study the disease mechanism. In contrast to highly penetrant alleles associated with monogenic disease, the risk loci implicated by GWA are associated with small effect sizes and, in the majority of cases, are not linked to nonsynonymous protein coding variants. A substantial gap still exists between single-nucleotide polymorphism associations from GWA studies and understanding how loci contribute to disease; however, clues are emerging through the study of gene expression. Given that many GWA loci do not map to coding changes or protein open reading frames, it is likely that a great deal of biologically and clinically important genetic variation exerts a pathobiological effect through differential gene expression and/or splicing, rather than point mutations in protein

sequence. In this manner, genetic variability can have a direct impact on gene expression either quantitatively or qualitatively. Gene eQTL mapping has been used in an attempt to catalog, map and understand these effects. However, an additional, intermediate and plausible effect of genetic variability is on transcriptional potential through varying levels of local DNA methylation.

Epigenetics and Its Importance in Complex Disease

Epigenetics is the study of heritable changes in gene function caused by mechanisms other than changes in the underlying DNA sequence. Epigenetic modifications, such as DNA methylation, are heritable but potentially reversible, may alter throughout life and can be affected by the environment, such as lifestyle, diet and toxin exposure [3]. The study of epigenetics is an expanding field of research where technical breakthroughs have recently allowed the success of large-scale epigenomic studies. For example the discovery of CpG island shores was made [4], the human methylome was characterized at single nucleotide resolution [5], the putative identification of non-CpG methylation was made [6], and the roles of novel histone variants and modification have been defined [7–9].

Until recently, the majority of epigenetic research focused on the study of cancer. Not only has global DNA hypomethylation consistently been observed in many cancers but all three types of normal epigenetic modifications of DNA, including chromatin modifications, DNA methylation and genomic imprinting, are altered in cancer cells [10, 11]. As the field of epigenetics research has expanded over the last few years, epigenetic alterations have been found to be linked to disorders such as metabolic disorders [12] cardiovascular diseases [13–15] and myopathies [16].

There is also strong evidence suggesting a relationship between epigenetic alterations and neurological disorders. For example, hypermethylation of the FMR1 promoter has been described in fragile X syndrome patients [17], along with hypermethylation of gene promoters FXN in Friedreich's ataxia, SMN2 in spinal muscular atrophy, and neprilysin (also known as membrane metalloendopeptidase) in Alzheimer's disease [18]. Conversely, the overexpression of tumor necrosis factor α in the substantia nigra of Parkinson's disease patients is associated with promoter hypomethylation, inducing apoptosis in neuronal cells [19]. Neurodegenerative disorders such as Alzheimer's and Parkinson's disease are believed to have

a multifactorial origin arising from a combination of risk factors and susceptibility genes, where age, diet, lifestyle and level of education are all correlated with the onset and severity of the sporadic forms [20, 21]. The mode(s) in which environmental factors and susceptibility genes interact to cause disease are not fully understood; however, epigenetic mechanisms may provide a link between genes and environment.

There are three major categories of epigenetic modifications: DNA methylation, histone modification and nucleosome positioning [22]. When studying epigenetics, it is important to understand that the observed outcome of epigenetic modifications is the sum of their interactions and feedback mechanisms. However, the study of DNA methylation as a single modification has the ability to convey important epigenetic information by distinguishing regions of transcriptional silence or transcriptional potential. Because subsets of potential target CpG sites are methylated within the genome, the signature of methylated sites can easily be distinguished. This fact makes the study of CpG methylation attractive, especially on a genome-wide level.

CpG Methylation

DNA methylation is believed to be an important epigenetic regulator of chromatin structure and function making it a key regulator of gene expression, splicing, growth and differentiation in virtually all tissues, including brain [22]. DNA methylation is perhaps the most widely studied epigenetic modification and is the oldest epigenetic mechanism known to correlate with gene expression [23]. In its most fundamental form, DNA methylation consists of the addition of a methyl group at the cytosine residue of CpG dinucleotides. Such additions to DNA are associated with the repression and silencing of gene expression and are central to genomic imprinting and X chromosome inactivation. In general, the CpG sites within the landscape of genomic DNA in mammals tend to be methylated [24]. The distribution of DNA methylation throughout the genome shows enrichment at noncoding regions and interspersed repetitive elements, but not in CpG islands of active genes [25]. CpG islands are clusters of CpG dinucleotides that have a strong association with gene promoters and housekeeping genes [26]. CpG islands are largely unmethylated throughout the genome in normal cells, allowing access to the transcriptional machinery, facilitating transcription. Thus, while an unmethylated CpG island in a gene

promoter does not necessarily mean active expression of the associated gene, it does suggest there is transcription potential. There are approximately 30,000 CpG islands in the human genome, and recent studies have identified a growing number of methylated islands in nonpathological somatic tissues [27].

Traditionally, a CpG island is defined as having a G + C content greater than 50%, an observed versus expected ratio for the occurrence of CpGs of more than 0.6 and a minimum size of 200 bp. However, the definition of CpG island continues to evolve. A recent study revised the traditional rules of CpG island prediction in order to exclude other GC-rich genomic sequences such as Alu repeats. In comparison to previous definitions, it was shown that DNA regions immediately 5' to genes with a G + C content >55% and an observed versus expected ratio of CpG dinucleotides of >0.65, both in a track of 500 bp or longer, are more likely to be true CpG islands [28]. Seventy-five percent of transcription start sites and 88% of active promoters are associated with CpG-rich sequences [29]. Although research has typically focused on CpG islands spanning the 5' end of the regulatory region of genes, it is now evident that variation in methylation occurs more often in the 'shores' of CpG islands versus within the islands themselves. It appears that around 76% of methylated sites occur a short distance away from CpG islands, with only 6% found within the islands themselves. Interestingly, most tissue-specific DNA methylation occurs in these CpG island shores, up to 2,000 base pairs away from CpG islands [4]. Additionally, a recent study revealed an important role for intergenic DNA methylation in the regulation of alternative promoters within gene bodies [30]. Intergenic methylation appears to modulate gene expression and splice variants, and CpG islands in introns can serve as promoters for non-coding RNA regulatory functions [31]. As research focuses on the most widely studied epigenetic modification, the complexity and significance of DNA methylation will continue to be highlighted.

DNA Methylation as a Quantitative Trait

The expression profile of a cell and its response to environmental signals effectively define its overall phenotype. Using gene expression as a quantitative trait has successfully identified genetic modifiers of gene expression. However, assaying DNA methylation as an intermediary between genetic variation and gene expression patterns provides a new and, unlike gene expression, stable mea-

sure of the cellular phenotype. Quantitative measures of DNA methylation provide a chromatin signature of cellular transcriptional potential that is preserved and can be regenerated during cell division. DNA methylation has been shown to influence gene expression in an age-dependent and tissue-dependent manner [32, 33], characteristics that are potentially important for the study of neurodegenerative disease, where distinct regions of brain tissue and/or cell types are compromised in an age-dependent manner. Therefore, the study of QTL influencing epigenetic regulators of gene expression such as the covalent modifications of DNA are highly attractive as a means to further explore the molecular pathology of neurodegenerative disease beyond RNA quantification.

With a focus on the study of the genetics of age-related neurodegenerative disease [34–37], one of our critical goals is to determine the immediate biological consequences of disease-associated common genetic variation in the human brain. As discussed above, two functional, quantitative variables that can efficiently be investigated from a genome-wide perspective are mRNA expression and DNA methylation. Combining these data with QTL analysis allows a systematic, genome-wide, and relatively hypothesis-free investigation into the effect of common genetic variability on important functional variables.

DNA Methylation and Aging

Neurodegenerative diseases are frequently late onset, implying there is a biological characteristic that changes as a person ages. One unvarying factor for each disease is that neurons steadily lose function as the disease progresses with age. The biochemistry of aging is complex with significant alterations occurring in proteins, lipids and nucleic acids. Differential DNA methylation has been shown to be age related [38, 39], and methylation of DNA sequences within or near regulatory elements has been shown to suppress gene expression through effects on DNA binding proteins and chromatin structure [40]. Indeed, both increases and decreases in DNA methylation have been shown to occur with aging contingent upon the tissue and the gene [40]. It is possible that dysregulation of DNA methylation with age promotes or exacerbates pathobiological consequences. Therefore, it is an important undertaking to understand the effects of DNA methylation on the normal aging brain. This new understanding will provide a foundation for gaining biological insight into age-related neurodegenerative diseases such as Parkinson's and Alzheimer's diseases.

DNA Methylation Analysis Tools

The methylation signature in a genomic DNA sample is complex as it represents the CpG methylation levels from a compilation of cells within the DNA sample. These cells are likely to have varying levels of DNA methylation that contribute to the overall signal. Assessing CpG methylation can be done for the pattern of methylated CpG sites along a sequence for individual DNA molecules or as an average methylation signal at a single genomic locus across many DNA molecules. Techniques to comprehensively characterize DNA methylation patterns are the most highly developed of the epigenetic methods.

Standard molecular biology techniques such as PCR and cloning erase DNA methylation marks; therefore, DNA must be pretreated to reveal the presence or absence of the methyl group at cytosine residues. There are three different initial treatments that can be used: endonuclease digestion, affinity enrichment and bisulfite conversion.

Techniques designed to pretreat DNA for methylation analysis were initially confined to localized regions of the genome; however, many methods now enable DNA methylation analysis on a genome-wide scale, including the bisulfite treatment of DNA. Bisulfite conversion is the most conventional approach for pretreatment and is considered the gold standard for determining DNA methylation status because it offers single CpG resolution [41].

Bisulfite treatment converts unmethylated cytosines to uracil while leaving methylated cytosines unconverted [42–44]. DNA can then be amplified or hybridized to arrays [45, 46]. One microgram of bisulfite-converted DNA can now be used to ascertain quantitative measurements of DNA methylation for up to 450,000 CpG dinucleotides on genome-wide methylation microarrays, such as the Illumina Infinium Human Methylation 450 array. The Infinium Methylation Assay uses two different bead types to detect CpG methylation. One bead type matches the unmethylated CpG site, and the other type matches the methylated site. The level of methylation for the interrogated locus is determined by calculating the ratio of the fluorescent signals from the methylated versus unmethylated sites. The field of epigenomics has flourished with the use of microarray hybridization techniques adopted from gene expression and genome-based assays to profile histone modifications and whole-genome DNA methylation patterns [47–50].

Bisulfite treatment of DNA in conjunction with next-generation sequencing can be used to decode the methylation status of the entire genome [6]. However, due to

the high costs currently associated with large-scale sequencing, other methods concentrating on more limited sequencing of genomic regions have been developed, such as reduced representation bisulfite sequencing [51], which is a random approach for large-scale high-resolution DNA methylation analysis, where only a subset of the genome is analyzed. DNA is digested with methylation-insensitive restriction enzyme (*MspI*) to remove much of the unmethylated regions of the genome, then only DNA fragments of a specified length are bisulfite sequenced, consisting mostly of methylated DNA.

Although bisulfite treatment of DNA has long been considered a superior technique for measuring DNA methylation status, it does have disadvantages. Bisulfite conversion typically calls for larger quantities of sample DNA which can degrade following chemical treatment, it can be limited by incomplete conversion of all unmethylated cytosines to uracils, and bisulfite conversion cannot discriminate between methylcytosine and hydroxymethylcytosine. Alternative assessments of methylation status are based on enrichment of methylated DNA with immunoprecipitation (MeDIP-seq) or affinity purification (MethylCap-seq) and subsequent analysis of enriched sequences using microarrays or sequencing [52]. The HELP assay (*HpaII* fragment enrichment by ligation-mediated PCR) pretreats DNA with methylation-sensitive and insensitive restriction endonuclease digestion. Subsequent comparative analysis of the resulting fragments using microarray or sequencing is used for the determination of the methylation state of restriction sites [53]. Several methods have been developed to map DNA methylation on a genome-wide scale. These methods, while diverse in technique, have been shown to produce concordant results [54].

Practical Application of DNA Methylation QTL Mapping

One of the particularly compelling factors of generating large-scale data to map and understand genetic variability and DNA methylation is that these data can be rapidly and repeatedly mined and augmented by the community. We have performed initial experiments in this regard, combining both genome-wide genotyping and array-based assays of DNA methylation that examine methylation levels at approximately 27,000 CpG sites in 4 distinct regions of neurologically normal brains [55, 56]. Similarly to previous work in the eQTL field, this showed an abundance of QTLs for DNA methylation, and an ex-

treme enrichment of signals when the genetic variability linked to methylation status was close to the CpG site in question. This effort and the public posting of these data have allowed us and others to examine the effects of disease-linked variants on both gene expression and DNA methylation in the human brain [34, 57–59]. One would expect that an expansion of this work, to include more brain regions, and a larger series of samples will bring a greater level of resolution in the mapping of genetic loci that control local DNA methylation levels. Further, the use of methods to isolate cell types, such as neuronal enrichment by NeuN-based cell sorting offers the ability to assay DNA from more homogenous populations of cells extracted from human brain [60].

Conclusions

Understanding the genetic control of biological processes is an important goal in the postgenome era. DNA methylation signatures are key in determining cellular differentiation and tissue-specific expression patterns. Moreover, assaying DNA methylation provides a window into the transcriptional potential of a cell type or tissue, rather than the cross-sectional view of transcription generally offered by expression analysis. We believe that comprehensive analysis of CpG methylation levels and chronological age has the potential to provide insight into coordinated changes in DNA methylation during aging, perhaps providing a starting point for understanding the underlying mechanisms of aging and age-related diseases.

Much of the technology required to comprehensively and accurately assay and link genetic variation and DNA methylation is now in place, and we believe that significant progress in understanding the relationship between genetics and epigenetics can be made in the near future. Further, we believe that the future refinement of these methods to allow their use on single cell types and perhaps even in situ within tissue will offer even clearer insight into this relationship.

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