

Epigenetics: Principles and Practice

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Abstract

Epigenetics is defined as heritable changes in gene expression that are, unlike mutations, not attributable to alterations in the sequence of DNA. The predominant epigenetic mechanisms are DNA methylation, modifications to chromatin, loss of imprinting and non-coding RNA. Epigenetic regulation of gene expression appears to have long-term effects and wide-ranging effects on health. Diet and environmental exposures may potentially alter the level and scope of epigenetic regulation, thus interesting developments in the study of epigenetics might explain correlations that researchers have found between lifestyle and risk of disease. Aberrant epigenetic patterns have been linked to a number of digestive diseases including Barrett's esophagus, cirrhosis, inflammatory bowel disease, and numerous gastrointestinal malignancies. In fact, many exciting discoveries about epigenetics in general have been made by studying diseases of the gastrointestinal tract and hepatobiliary tree. Epigenetic modifications of DNA in cancer and precancerous lesions offer hope and the promise of novel biomarkers for early cancer detection, prediction, prognosis, and response to treatment. Furthermore, reversal of epigenetic changes represents a potential target of novel therapeutic strategies

and medication design. In the future, it is anticipated that innovative diagnostic tests, treatment regimens, and even lifestyle modifications will be based on epigenetic mechanisms and be incorporated into the practice of medicine.

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Introduction

Epigenetics is defined as heritable changes in gene expression that are, unlike mutations, not attributable to alterations in the sequence of DNA. The predominant epigenetic mechanisms are DNA methylation, modifications to chromatin structure, loss of imprinting, and non-coding RNA [1]. An important feature of epigenetic modifications is that they are heritable between mother and daughter cells (mitotic inheritance) and between generations (meiotic inheritance). Epigenetics is one of the explanations how cells and organisms with identical DNA can have such dramatic phenotypic differences. Diet and environmental exposures may potentially alter the level and scope of epigenetic regulation, thus interesting developments in the study of epigenetics might explain the correlations the researchers have found between lifestyle and risk of disease [2]. In addition, epigenetic regulation of gene expression has emerged as a fundamental pathway in the pathogenesis of numerous diseases, and in particular malignancies [3, 4]. Diseases of the digestive

system are no exception, in fact many exciting discoveries about epigenetics in general have been made by studying cancers of the gastrointestinal tract, liver, and hepatobiliary tree. Epigenetic modifications of DNA offer hope and the promise of novel biomarkers for early cancer detection, prediction, prognosis, and response to treatment. Furthermore, reversal of epigenetic changes represents a potential target of novel therapeutic strategies and medication design [5]. There has been an explosion of data and studies published in the last decade regarding the epigenetic mechanisms and their involvement in the development of phenotypes and diseases. The current report aims to provide an introduction to the field of epigenetics, with examples of some the most salient and provocative studies related to this topic.

DNA Methylation

DNA methylation is the covalent addition or subtraction of a methyl group to a cytosine nucleotide in a sequence of DNA. Methylation is controlled by a family of specific enzymes known as DNA methyltransferases (DNMTs). In vertebrates, addition of a methyl group only occurs at a cytosine preceding a guanine (CpG dinucleotide) [1]. Regions of the genome rich in sequences of a cytosine preceding a guanine are known as CpG islands. In fact, CpG islands exist in the promoter regions of approximately half of all genes.

Hypomethylation

Approximately 80% of CpG dinucleotides outside of promoter regions are methylated under normal physiologic circumstances. Genome-wide decreases in methylation, or hypomethylation, are most functionally relevant when they occur in coding regions of genes, leading to alternative versions or levels of messenger RNA. Global hypomethylation of the DNA from the tumors of patients with colon cancer was one of the first epigenetic abnormalities to be described [6]. It is theorized that hypomethylation contributes to carcinogenesis by favoring mitotic recombination, leading to deletions, translocations, and chromosomal rearrangements – collectively known as genomic instability. DNA hypomethylation (also known as demethylation) is also associated with activation of proto-oncogenes, such as c-Jun, c-Myc, and c-Ha-Ras [7]. Overall, most DNA in tumors is hypomethylated, with occasional gene-specific hypermethylation (fig. 1) [8]. In general, hypomethylation increases as a tumor progresses. Accordingly, hypomethylation of repeti-

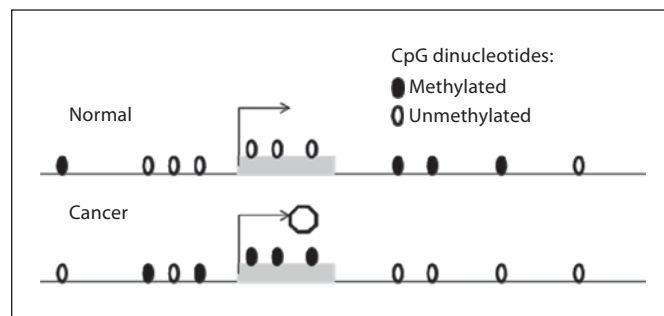


Fig. 1. Hypo- and hypermethylation of DNA. The black line represents a gene and the grey rectangle the promoter. Under normal conditions, CpG outside the promoter region are methylated. In cancer, methylation of CpG in the promoter region may occur and hypomethylation of the CpG outside the promoter region may occur. Hypermethylation of CpG in the promoter region may result in transcriptional repression. Adapted from Herman and Baylin [10].

tive DNA elements such as SAT2, LINE1, and ALU occur in the multistep process of hepatocarcinogenesis, and correlates with a poor prognosis [9].

Hypermethylation

The addition of methyl groups, or hypermethylation, can be highly specific to a particular gene. Hypermethylation of CpG islands in the promoter region of a gene can result in transcriptional silencing of the gene, and subsequent loss of protein expression. Thus, hypermethylation of tumor suppressor genes is now recognized as a means of gene-silencing alternative to mutation or allelic loss [10]. The methyl groups project into the major groove of DNA, therefore changing the biophysical properties of DNA. These changes can positively or negatively affect the binding capabilities of DNA and certain proteins. For example, if RNA polymerase 1 is unable to bind to DNA correctly, then the process of transcribing DNA into RNA is altered. During mitosis, methylation patterns are copied from the mother strand of DNA to the daughter strand of DNA by DNMT1.

Hypermethylation of genes involved in the cell cycle, DNA repair, angiogenesis, metabolism of carcinogens, apoptosis, and cell-cell interaction have been implicated in carcinogenesis. It should be noted that hypermethylation also occurs as a normal physiologic process, for example during inactivation of the second X chromosome (Barr body) in females. In addition, hypermethylation is a physiologic process associated with aging and methylation-induced transcriptional repression of repetitive DNA elements helps to prevent chromosomal instability.

Techniques Used to Detect Methylation

In the laboratory, DNA methylation can be measured by many different methods in tissues, and occasionally in peripheral blood or other body secretions such as bile. One of the major advantages of attempting to detect methylation is the inherent stability of DNA. Before the advent of DNA methylation sequencing methods, isoschizomers with different methylation sensitivities were used to detect DNA methylation. A major disadvantage with this method is that less than 5% of the methylated cytosines can be assessed in any given DNA sequence. Since the early 1990s, a key method by which methylation levels are determined requires an initial bisulfite conversion of DNA. This bisulfite treatment converts unmethylated but not methylated cytosines to uracils. Subsequent gene-specific methylation can be determined by qualitative or quantitative methylation-specific PCR (MSP) using primers and probes specific to the corresponding methylated DNA sequence [11]. The advantages of MSP is that it gives a positive display of methylated cytosines and provides the entire profile of methylation for a defined DNA sequence rather than assessment of just a few cytosines within a sequence. Real-time quantitative MSP is preferred by the author, as it determines the actual percentage of methylated alleles in a given sample.

DNA sequencing can also be performed on bisulfite-converted DNA in order to determine specific regions of hyper- or hypomethylation. This is a particularly useful technique to determine regions of differential methylation, and aid in primer and probe design for the more specific MSP. Pyrosequencing is a method of real-time DNA sequencing that is based upon the activity of DNA polymerase and relies on the luminometric detection of pyrophosphate release after nucleotide incorporation [12]. An advantage of pyrosequencing is that it combines the high-throughput nature of PCR-based technologies with the ability to analyze all of the individual CpGs of a given region [12].

A limitation of the PCR-based technologies is that they specifically target candidate genes of interest. More recently, the development of high-throughput, genome-wide, microarray platforms have been developed in attempt to define the global methylation pattern of tumors. Methylated DNA immunoprecipitation (MeDIP) is an immunologic approach that enriches methylated DNA, and is based upon the principle that genomic DNA is randomly sheared by sonication and can be immunoprecipitated with an antibody that specifically targets 5-methylcytidine. This technique can be used to generate comprehensive, genomic DNA methylation profiles and to

identify abnormally (hyper- or hypo-)methylated genes [13].

The methylated CpG island amplification (MCA) method is based on digestion of genomic DNA with the methylation-sensitive restriction enzyme, *SmaI*, which cuts only unmethylated sites, leaving blunt ends between the C and G. The DNA is then digested with the methylation-insensitive, *SmaI* isoschizomer, *XmaI*, which leaves a four-base overhang. These two serial digests are followed by ligation of adaptors to the overhang, and finally performing adaptor-specific PCR amplification [14]. Thus, this method results in the enrichment and amplification of methylated DNA fragments only. These methylated DNA fragments are then used for interrogations with microarrays platforms.

An additional method by which to discover novel targets of methylation-induced transcriptional silencing is to treat cancerous cell lines with agents that reverse epigenetic events, and then perform gene expression microarrays to determine which genes become upregulated.

Chromatin Modification

Chromatin is comprised of histones and DNA. Histones are the protein components of chromatin, the structure around which DNA is wound. Histones are octamers with variable tails that extend out of the DNA/histone complex (nucleosome). There are several types of post-translational modification that can affect the histone tails, including methylation, acetylation, phosphorylation, and ubiquitination. These modifications can affect interactions between DNA and histones, leading to alterations in gene transcription, DNA repair, DNA replication, and even the organization of chromosomes [4].

One of the best studied histone modifications is the acetylation of the lysine residue. In general, histone acetylation is associated with transcriptional activation, and deacetylation is linked with transcriptional repression (fig. 2). Acetylation neutralizes the positively charged lysine residue in the histone tail, reducing the strength of the bond between the histone tails and DNA. This phenomenon opens up the DNA/histone complex such that it is accessible to transcription factors [1]. Methylation of histones can positively or negatively affect transcription. Collaboration between DNA methylation and histone modifications may also occur. With all the different modifications and combinations of alterations, the complexity of chromatin modifications is remarkable, and is still an area of intense investigation [15].

Methods to Detect Chromatin Modifications

In the laboratory, histone modifications are readily and accurately detectable by mass spectrometry. However, this technique is laborious and requires highly specialized training and equipment [16]. In order to determine the true biological significance of histone modification, DNA sequence information is also required. The optimal technique, chromatin immunoprecipitation (ChIP), combines sequencing technology with DNA that has been immunoprecipitated with antibodies against specific histone modifications. Genome-wide studies of histone modifications are now possible through the use of so-called 'ChIP on chip' studies that couple immunoprecipitation with microarray sequencing platforms. Current limitations of this technique are the quality of the polyclonal antibodies engineered against the histone modifications [16].

Loss of Imprinting

Imprinted genes have mono-allelic expression – there is only 1 copy (from one of the parents) instead of 2. Approximately 1% of autosomal genes are imprinted. DNA methylation and histone acetylation marks the imprinted gene, and keeps the gene from being transcribed. Because expression is dependent upon only 1 parent, the expression in the current generation is dependent upon the environment in which the previous generation resided and any epigenetic marks that may have occurred [2]. Loss of imprinting means that there is either bi-allelic expression of the gene, or both copies are not expressed. The functional haploid state of these genes makes them exquisitely susceptible to further epigenetic alterations or mutations [3]. Several developmental disorders are associated with imprinted genes, such as Angelman syndrome, Prader-Willi syndrome, and Beckwith-Wiedemann syndrome. Based on a parent-of-origin pattern of inheritance, it is theorized that bipolar disorder, autism, schizophrenia, and Tourette's syndrome may also be associated with loss of imprinting [2].

Non-Coding RNA

The best studied non-coding RNA are microRNA (miRNA). These are approximately 22 nucleotide long sequences that are coded by long non-coding RNA or introns of genes. miRNA are transcribed in the nucleus and undergo several modifications prior to their maturation.

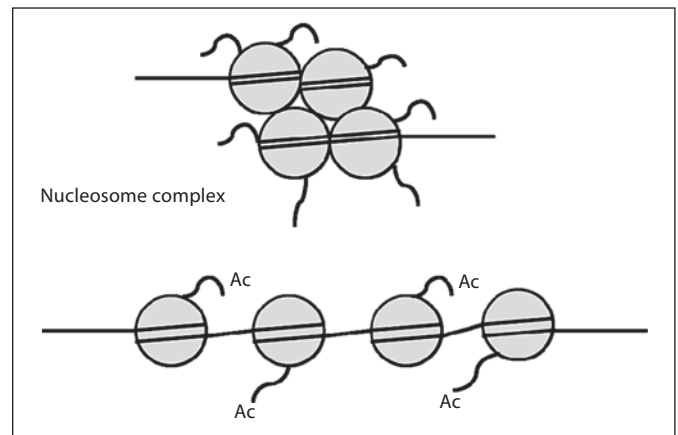


Fig. 2. Histone acetylation. In the top frame, DNA (black lines) is tightly wound around the histones (circles) forming the nucleosome complex. The histone tails (curvy lines) are not acetylated. In the bottom frame, acetylation (Ac) of the histone tails 'opens up' the DNA for access to transcription factors.

tion. miRNA can inhibit translation of mRNA into protein by two methods. If the miRNA is a direct sequence complement to the mRNA, then the miRNA binds to the mRNA and degrades it through actions of the RISC complex. If the miRNA is an imperfect match to the mRNA, then the miRNA partially binds to the 3' end of the mRNA and prohibits the actions of transfer RNA [1]. MiRNA have been found to be abnormally expressed in a host of gastrointestinal disease, including inflammatory bowel disease [17, 18], cholangiocarcinoma [19], esophageal adenocarcinoma [20], and hepatocellular carcinoma [5, 15].

Environmental Effects on Epigenetics

There is increasing evidence suggesting that environmental exposures early in development play a role in disease later in life. It is epigenetics that provides a plausible link between environmental exposures and disease risk [2]. Epigenetics may also explain how the risk of a certain disease is passed down through generations. A classic example of how environmental exposures can effect epigenetic change is the story of the agouti mouse. The murine agouti allele *Avy* has a transposable element (named IAP) upstream of its promoter, and can be regulated by DNA methylation. The wild-type *a* allele encodes brown coat color and the *Avy* allele encodes yellow coat color, obesity and diabetes. Under basal conditions, the agouti mouse

displays a chimeric phenotype with mixed brown and yellow coat color. The natural methylation of the IAP element results in increasing brown coat color and lean body mass. In one experiment, *a/a* mothers were bred with *a/Avy* males, and supplemented with methyl donors betaine, choline, vitamin B₁₂, and folate. The offspring of the supplemented mothers were more lean and had more brown in their coats. Furthermore, the IAP elements of the supplemented animals were found to have increased methylation than the offspring of the unsupplemented mothers [2]. This experiment showed that exposure *in utero* could affect the epigenetic status of the offspring, and affect the phenotype as well. This same research group was able to induce methylation of the IAP element with the plant estrogen genistein at similar doses to humans that consume soy-based diets. Remarkably, the genistein supplementation of the mothers protected the offspring from diabetes [2].

Another example of environmental exposure and its effect on epigenetics is the Dutch Winter Hunger Study of 1944–45. During this time in occupied Holland, the Dutch were under strict rations. The daily meals consisted of two potatoes, two slices of bread, and a piece of beetroot. Six decades later, when compared to their same sex siblings, the offspring descended from the pregnant mothers of the famine continued to have biallelic expression of the imprinted gene *IGF2* [2]. It is presumed that the folate deficiency incurred by the famine resulted in this phenomenon. Diets rich in processed foods, such as those enjoyed in the Western world, are thought to be deficient in folate, betaine, and choline, thus potentially predisposing humans to epigenetic abnormalities.

Numerous other environmental exposures have been linked with altering epigenetic patterns during a lifetime, and subsequent risk of disease. Specific examples include tobacco smoke, alcohol, viral hepatitis, industrial pollutants, and carbon emissions. One study looked at the methylation and histone modifications in monozygotic twins at different stages in life. Early in life, the epigenetic signatures are nearly identical. However, at age 50, dramatic differences were found, suggesting that environmental exposures altered the epigenome [3].

Epigenetics and the Practice of Medicine

The epigenetic events that regulate gene expression have clearly emerged as a fundamental mechanism in developmental biology and in the pathogenesis of hu-

man disease. For example, multiple genes that affect numerous cellular pathways are silenced by hypermethylation in cancer, and studying these genes has increased our understanding of how cancer develops and progresses. A majority of studies, however, focus on methylation of a single gene or panel of genes, without detailed investigation of the functional relevance of the gene silencing. With the advent of genome-wide microarray platforms, the ‘methylome’ will be further defined. The molecular information gained from epigenetic studies, in conjunction with other genetic information, could be used to develop a novel classification system for tumors and diseases. This theoretical classification system could be designed to reflect so-called ‘tumor biology’ that could predict clinical outcomes such as overall prognosis, risk of recurrence after surgery, or response to chemotherapy.

Naturally, with the increased knowledge regarding the pathogenesis of disease, there is hope for the development of a new era of novel therapeutic agents that may effectively treat patients. The beauty of epigenetic modifications to DNA is that they are potentially preventable or even reversible. For example, blocking DNA methylation by inhibiting DNMTs results in demethylation of CpG islands in daughter cells, with subsequent restored expression of tumor suppressor genes and abrogation of tumor growth. There are several DNA demethylating compounds that are actively being investigated, including 5-azacytidine (AzaC), 5-aza-2'-deoxycytidine, zebularine, procainamide, and procaine [8]. Unfortunately, toxicity has been a major limiting feature of these medications, and they are currently only indicated in patients with advanced myelodysplastic syndromes [21]. Rather than systemically administered therapeutics, specialized delivery systems that specifically target aberrant methylation in tumors are actively being developed and studied. Abnormal promoter methylation has also been shown to correlate with chemotherapy and radiation resistance [22]. In the future, it is conceivable that demethylating agents could be used to enhance the effectiveness of traditional chemotherapy [21].

There are also a host of histone deacetylase (HDAC) inhibitors that are being studied for the treatment of cancer. Examples of these HDAC inhibitors include suberoylanilide hydroxamic acid, trichostatin A, valproic acid, and sodium butyrate [8]. These agents result in an increase of histone acetylation by blocking the action of multiple HDACs, and are commonly used in laboratory experiments to reverse epigenetic-induced gene silencing.

Many of the epigenetic events described have been detected in human samples in preneoplastic tissues. In particular, some hypermethylated genes may even be detected in the serum of patients prior to clinical detection of malignancy. Another example is the prediction of the development of dysplasia progression in Barrett's associated adenocarcinoma of the esophagus. In a randomized, double-blinded, multicenter study, a panel of eight hypermethylated genes predicted the progression of dysplasia better than traditional clinical risk factors [23]. For this reason, DNA methylation, hypomethylation, or histone modification are potential candidates for biomarkers for the early detection of disease.

In summary, epigenetics has emerged as a crucial link between nature and nurture. With an incredible degree of complexity, epigenetic phenomena exert a profound influence on the regulation of how genetic information is

transcribed and translated into proteins and phenotypes. With advanced technologies and knowledge, future discoveries regarding the pathogenesis of multifactorial and previously idiopathic diseases are possible.

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