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Review Article

A Holistic Approach to Understanding Epigenetics, its Mechanisms and Effects in the Biological System

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Abstract

Epigenetics is the study of inheritable changes in gene expression or the gene phenotype, which does not involve any change in the DNA sequence. The "epigenetics" word implies "in addition to changes in the gene sequence". It means that these changes are hereditary if the germ cells are involved that it usually involves transmission from the parents to the daughter cells. Originally it was meant for changes across generations, but now it includes changes due to cell differentiation in one organism. Epigenetic mechanisms aid in regulating gene expression within the cell as well as silencing of certain sequences.

In certain cases the effects of epigenetic changes can be reversed. These changes usually involve addition of chemical groups to the DNA sequence, such as methylation, acetylation (addition of acetyl group), ubiquitinylation (addition of ubiquitin protein, they tag protein for proteosomal degradation), phosphorylation (addition of phosphate group).

Keywords: Epigenetics; Mechanisms; Biological System

Enzymes such as DNA methyltransferases add methyl groups to the DNA at the Cytosine regions. The methyl group is donated by S-adenosyl methionine, as a result of which both folic acid as well as Vitamin B12 affects methylation and demethylation within the cell. Usually, these modifications occur in the dinucleotides called CpG. Methylation of the 5' end of cytosine with methyl group project it into the major groove of DNA (Figure 1).

DNA methylation have been implicated in:

- Histone modification and its post translation modification that is responsible for chromatin organization.
- DNA methylation also controls silencing of telomere, centromere.
- Prevents transposition of transposable elements and preventing mutagenesis
- Ensure attachment of microtubules to centromeres
- Since promoter regions contain CpG islands, which are methylated, they can control silencing of genes and even it's reverse.

Epigenetic changes have also been linked to various diseases. Especially their development. In cancer cells there is a massive loss of methylation on CpG dinucleotides in the promoter regions. This massive demethylation of repetitive sequences leads to chromosomal instability, mutations which can disrupt gene expressions and cause cancer. However, genomic demethylation can cause oncogenesis in some tissues and prevent malignancy in others. DNA methylation has also been linked to Lupus, Rheumatoid Arthritis, Type 1 Diabetes, Multiple Sclerosis and other various autoimmune diseases. Once researchers can analyze and determine etiology of these diseases on the basis of epigenetics, it will be easier to consider remedies based on therapeutics and drug development.

DNA folding

Chromosomal DNA are usually packaged and condenseddd further to fit inside the nucleus with histone proteins. A histone octamer is wrapped around by DNA to form units called nucleosomes, which are then connected with the help of Histone H1 or linker DNA.

Figure 1: Types of epigenetic modifications. (A) Histones can undergo phosphorylation (Ph), methylation (Me), and acetylation (Ac), among other chemical modifications. These modifications are involved in chromatin remodeling and transcriptional regulation. (B) DNA molecules are methylated by the addition of a methyl group to carbon position 5 on cytosine bases, a reaction catalyzed by DNA methyltransferase enzymes, which maintains repressed gene activity. (C) mRNA is translated into a protein product, but this process can be repressed by binding of microRNAs (miRNA), a class of noncoding RNA (ncRNA). Ref- Gómez-Díaz, Elena., et al. "Epigenetics of host–pathogen interactions: the road ahead and the road behind." PLoS pathogens 8.11 (2012): e1003007.

- Chromatin, which is accessible to transcriptional factors, is:
- Euchromatin and is loose and decondensed with methylated histones.
- Whereas chromatin that is condensed due to removal of methylation and not opens to transcription factors, thus being transcriptionally inactive is termed as heterochromatin.
- Heterochromatin is also composed of:
- Facultative heterochromatin: hypoacetylated nucleosomes such as inactive X chromosome or transcriptionally inactive genes
- Constitutive heterochromatin: hypoacetylated regions including highly repeated sequences such as centromere and telomere.

Gene expression and how epigenetics regulate it

Gene expression is when certain DNA sequences are transcribed successfully by certain transcription factors. This is followed by translation of the transcribed RNA sequences into fully functional proteins that regulate various biological functions within the organism. DNA methylation and demethylation can regulate gene expression. Their two main functions being:

- Prevent regulatory proteins from binding to DNA sequences that are methylated
- Allow binding of only certain proteins to the methylated DNA sequences.

Figure 2: Schematic diagram illustrating euchromatin and heterochromatin. Heterochromatin on the left is characterized by DNA methylation and deacetylated histones, is condensed and inaccesible to transcription factors (closed chromatin conformation), which is repressive regulation of transcription. On the contrary, euchromatin on the right is in a loose form and transcriptionally active; DNA is unmethylated and histone tails acetylated (open chromatin conformation), which is active regulation of transcription (adapted by Hatzimichael., et al. J Drug Deliv 2013;2013:529312 [90], and modified by author).

Histone modifications

The primary epigenetic modifications are DNA methylation and histone modifications, which can alter the accessibility of the chromatin in the genome and thereby altering gene expressions. The core histone proteins are basic in nature; the flexible tails that

protrude from the nucleosome are subjected to the covalent modifications, such as methylation, acetylation and phosphorylation [2]. Other modifications include ubiquitinylation, sumoylation, non-covalent proline isomerization in histone H3.

Nucleosomal positioning

Nucleosomes are a histone octamer surrounded by 147 base pairs of DNA. Greater the number of nucleosomes greater is the packaging in that loci, greater the inaccessibility of proteins and other complexes to interact with DNA, implying gene repression and of heterochromatin behaviour. DNA methylation is highly active in regions such as heterochromatin where gene is repressed but is depleted near active promoter sites. However it is not yet elucidated whether nucleosomal presence and DNA methylation affect each other or not. However it was observed from experimental analysis that nucleosomal DNA is more methylated than other regions. This proves that DNA methyltransferases also preferentially target regions with greater number of nucleosomes [2].

Deactivation of DNMTs can cause demethylation.

Demethylation can also be caused by 5-methylcytosine glycosylases and base excision repair pathway in plants. DNA demethylation also occurs when the methylated CpG is not propagated to daughter DNA during mitosis. For example-Presinilin (PSEN1) 5'- is a gene that has a specific methylation pattern which changes when exposed to an external metabolic stimuli. It has been connected to demethylation when overexpressed. During an induced Vitamin B deficiency in mice causes PSEN1 overexpression and DNA demethylation. It was observed that there was a reduction in the DNMT activity, CpG demethylation occurred and several genes, which were silenced due to methylation earlier, were reactivated. This example sheds light on the fact that both addition as well as removal of methyl groups from DNA can regulate gene expressions [2].

Methyl binding proteins (MBP)

These are proteins that bind to Methylated DNA sequences and in concert with other factors cause gene repression and also cause chromatin remodeling. There are three types of MBPs:

- Methylated CpG Binding Domain family. It comprises of MeCP2 and MBD1-4. MeCP2, MBD1, MBD2 bind to methylated DNA and they silence the gene of interest with transcriptional repression domain and other repressor complexes.
- Zinc Finger Domain containing Protein interacts with methylated DNA such as ZBTB33, ZBTB4 etc. They are known to have an apparent lack of specificity for methylated regions *in vitro*.
- SET and Ring finger associated domain proteins (SRA) comprising of UHRF1 and UHRF2. They recognize and bind semi methylated DNA sites with their SET and Ring finger domains [2].

Targeting of CpG methylation as a means of controlling gene expression

CpG methylation represses transcription of repeat elements and transposons, causes X chromosome inactivation and also causes imprinting in chromosomes (histone and DNA methylation, which is passed on via germ cells and is maintained through mitosis in somatic cells. It does not follow Mendel's law of inheritance). Methylation of the Imprinting Center controls the cluster of genes in the chromosomal region by repressing them in a coordinated manner. These imprinting centers are also called Differentially Methylated Regions (DMRs). These DMRs may often overlap with CpG islandsDuring embryogenesis, all of the methyl groups from the genome are removed and is re-established later except the CpG islands at promoter regions. Methylation of these CpG regions usually represses transcription in genes, which are specific to the germline that includes pluripotency genes. Thus differentiation of cells is brought about by repression of specific genes, which in turn is brought about methylation of DNA [7].

CpG dinucleotides of promoter regions are often targeted to repress expression of certain genes within the cell. Since these epigenetic marks are passed on from one progeny to another therefore excess or unwanted gene products are gotten rid of in this manner. Studying X chromosome inactivation and imprinting in chromosomes can help us obtain information about this targeting as they both involve repression of genes due to DNA methylation.

Imprinting inactivation

Imprinted genes are composed of only a single copy from either of their parents i.e. they are monoallelic. Methylated DNA marks the imprinted gene and its transcription is repressed. The methyl groups added to the parents based on the environment it was exposed to, determine the methyl groups that are present in the subsequent generations. However if imprinting is lost, it might be due to the fact that instead of one, both the gene copies starts expressing itself.

X linked inactivation

X linked inactivation is the transcriptional silencing of the majority of genes on one of the X chromosomes of mammalian females, especially in some somatic cells. Sex chromosomes such as X and Y-chromosomes evolved due to acquiring the sex determining genes in one of the copies of a pair of chromosomes, there are reduced recombination between these two sex chromosomes and thereby have given rise to the two. This leads to formation of either heteromorphic (XY) or homomorphic (XX) allosomes (sex chromosomes).

RNA based mechanism of epigenetic regulation

RNA based mechanisms for epigenetic regulation is not well understood, however, they are heavily involved in X chromosome inactivation and imprinting of monoallelic genes. However some other forms of RNA have been implicated in other forms of epigenetic regulation within the cell. Non-coding RNAs such as tRNA, rRNA, small nuclear RNA (snRNA) and small nucleolar RNA (snoRNA).

These RNAs control translation, splicing, catalysis and a host of other infrastructural functions. They also regulate expressions of various genes. However, a vast region of the genome that is eventually transcribed to RNA is not translated into proteins. Non-coding long RNAs regulate both transcriptional as well as post transcriptional regulation. Non coding RNAs has been known for several decades, its biological functions were known to scientists for close to 60 years. Later it was found that most of the genome (almost 80%) is transcribed whereas the rest of it is termed as junk RNA. It is difficult to provide a clean line of distinction between ncRNAs and junk RNA.

There are two types of non-coding RNAs:

- Small non coding RNAs with transcripts ranging less than 30 nucleotides
- Long non coding RNAs with transcripts ranging greater than 200 nucleotides.
- Both the two classes of non-coding RNAs were involved in gene silencing, heterochromatin formation, histone modifications etc (Figure 3).

Small non coding RNA

RNAse III enzymes such as Drosha and Dicer cleave larger RNA molecules into smaller ones like, microRNA (miRNA), short interfering RNA (siRNA), repeat associated RNA (rasiRNA), PIWI interacting RNA (piRNA).

Figure 3: Types of noncoding RNAs (ncRNAs). Noncoding RNAs are classified into housekeeping and regulatory noncoding RNAs. Ref- Losko, Magdalena, Jerzy Kotlinowski, and Jolanta Jura. "Long noncoding RNAs in metabolic syndrome related disorders." Mediators of inflammation 2016 (2016).

- miRNAs are formed from hair pin loops present in long noncoding RNAs and introns of coding or non coding genes, which are cleaved by Dicer or Drosha. They complementary base pair with target 3' UTR mRNA and prevents translation, this is in case if they base pair imperfectly. It brings in the RISC complex that further degrades the mRNA in case their base pairing is perfect. miRNAs also affect gene regulation by histone modifications and methylation of DNA at promoter sites. The RNA Induced Transcriptional Silencing (RITS) complex binds to miRNA to modify histone tails post translation. For example, miRNA was also found to regulate de novo DNA methylation in mouse embryo stem cells.
- Small interfering RNA or siRNA have the same length as miRNA (~21nt). Unlike the miRNA, siRNA is derived from double stranded RNA that is cleaved and processed by Dicer. siRNA base pairs perfectly with their complimentary RNA and target them for degradation, but if the base pairing is weak then they repress the translation. siRNA perform transcriptional gene silencing, especially in plants. For example, siRNA generates heterochromatin and transcriptional gene silencing by employing DNA methyltransferases in animals as well as in plants such as Saccharomyces pombe

- piRNA are 28-32nt in length. They are found in oocytes and male germ cells and are associated with PIWI family of proteins. piRNA are responsible to transfer the epigenetic marks from the parents to the progeny that alters the phenotype in a heritable manner. This is regulated by the piRNA derived from the maternal chromosome. They control the activities of transposable elements in the germ lines of mammals, fish, Coenorhabditis elegans etc. Germ line viability is also dependent on piRNAs.

Long non-coding RNA (lncRNA)

lncRNA are a heterogeneous group of non-coding RNAs. They are ~200nt in length with no discernable coding function within the genome. They are transcribed by RNA polymerase II and processed accordingly: splicing, capping and polyadenylation. Genes transcribing lncRNA are more rapidly evolving and tend to be restricted while expressing in certain tissues as compared to others. lncRNA tend to affect expression of neighboring genes in cis despite being in low quantities. Mechanisms such as antisense mediated repression, genomic imprinting can affect the genes present nearby [3].

LncRNA can complex with chromatin modifying genes and target both chromatin conformation as well as gene regulation. They also serve as precursor to siRNA. One specific group of lncRNA is the long interspersed non-coding RNA (lincRNA). They interact with chromatin modifying complexes that target specific sequences and modify their epigenetic states. For example Xist and its negative regulator Tsix, which are responsible for X linked inactivation. Due to H3K4 dimethylation of the Xist gene, Xist and Tsix is actively transcribed prior to its differentiation. As a result, Xist's activity is increased; Xist RNA coats the X chromosome that leads to histone methylation and eventually to chromosome inactivation. Recent studies suggest that a large number of lcRNA interact with these protein complexes that add epigenetic marks to the genome, which regulates specific loci using these modifications.

Figure a: Examples of lncRNAs implicated in epigenetic regulation of gene expression. Ref-Gibney, E. R., and C. M. Nolan. "Epigenetics and gene expression". Heredity 105.1 (2010): 4.

History of epigenetics

Epigenetics was unknown to scientists in the early years and was mostly associated with evolution and development. It was mostly associated with how a fertilized zygote can develop into a whole organism; the process was very poorly understood. However it was a field that received much attention since the 1970s. Moreover the meaning associated with the word epigenetics has also undergone a sea of changes, as a lot of new information was unearthed in the past 50-60 years. People were able to put a name to the phenomena where there is no change in the DNA sequence, but the chemical modifications attached to it is still capable of bringing about an entire spectrum of changes. It also includes phenotypic changes.

In the recent years it was unearthed that epigenetics is responsible for a wide variety of diseases- cancer, developmental, pulmonary, autoimmune etc. It was affected by the environment, diet, lifestyle, and fossil fuels, exhaust fumes. This brought this field of study into wide scrutiny. The fact that it could be studied to prevent the diseases made it such a field of interest. Conrad H. Waddington, a biologist from Cambridge University in 1942, coined the term. However it was not really linked to the concept of DNA methylation, as not much was known to people about genes and its role. Rollin Hotchkiss at the Rockefeller Institute of Medical Research first detected methylated cytosine in 1948, during preparation of calf thymus. The function of methylated cytosine was not yet clear to biologists and how it affected gene regulation. One of the first instances of assigning a biological function to it was in 1969. John S Griffith and Henry R Mahler from Indiana University hypothesized that methylated cytosine might be related to the memory storage in brain.

In 1975 further development was made when it was suggested that methylated cytosine might be associated with switching on and off biological functions of the gene. This was brought forward by Arthur Riggs from the City of Hope National Medical Center, California, Robin Holliday at the National Institute of Medical Research, London, and Ruth Sager from Harvard Medical School.

In the 1970s Marianne Frommer and a group of scientists from Australia developed bisulphite sequencing, this made it possible to study DNA methylation and its relation to gene regulation. In 1985 Adrian bird and his lab made one of the most important discoveries from Edinburgh University [1]. They demonstrated that DNA methylation occurs on specific sequences, albeit randomly, on CpG islands. These methylated CpG dinucleotides can determine the regulation of a gene within the nucleus of the cell. This major discovery connected that DNA methylation and methylated Cytosines are indeed related and are used by the biological system to turn on and off genes in concert to control various aspects of the organism (Figure 4).

Figure 4: Timeline of milestones in the history of epigenetics. Ref- Prachayasittikul, Veda., et al. "Exploring the epigenetic drug discovery landscape." Expert opinion on drug discovery 12.4 (2017): 345-362.

Transgenerational inheritance

Transgenerational epigenetics is the study of transmission of non-genomic traits from parents to the progeny [44]. Lamarck had hypothesized that the lifestyle and habits of parents can affect the progeny; those traits are inherited by a generation from the previous one. His theory that environment and interaction between individuals also contribute to evolution of species was strongly refuted. However, the theory is slowly coming back with the study of epigenetics. Variations developed out of mutations and were later not selected due to natural selection; it was later determined that epigenetic marks were also transmitted from parents to progeny. This form of variation that did not affect the DNA sequence, also contributed to the evolutionary diversity [8,42]. There are several ways of transmission of these epigenetic marks. They are transmitted between somatic cells, from germline cells, between somatic and germline cells and vice versa. It was observed that the early stages of embryonic development are most affected by the environmental effects and eventually changes the genomic profile. Environmental influences such as exposure to chemicals that are hallucinogenic, toxic, pharmacological or unknown can act as an influence.

Examples of transgenerational inheritance as found in studies

Several studies indicated that environment influences the program of the genome, which in turn gives rise to a change in gene expression. It usually affects in the early stages of embryogenesis and after birth. They affect immunity, metabolism, and behavior. IGF2 (Insulin Like Growth factor) is necessary for growth and development and is normally regulated by maternal genomic imprint. It was found that IGF2 has low DNA methylation in individuals exposed to the Dutch Famine during World War II. This was yet another example linking environmental factors to DNA methylation. Especially in individuals exposed to such conditions during the early stages of their pregnancy [45].

Abusive behavior, stress, neglect can cause also epigenetic modifications. For example maternal mice that groom their pups do not have stress and do not have heightened hypothalamus-pituitary-Adrenal response. In comparison, pups that are not exposed to loving care of their mothers are fearful and have a high endocrinal response/HPA response to stress [46]. This behavior changes the genomic profile due to DNA methylation and is transmitted to the progeny. Maternal care changes methylation patterns enough to change the chromatin structure, due to differential methylation of the GR promoter region. Pups that receive less care show an increase in DNA methylation, reduction in the synthesis of GR, increase in the acetylation of GR H3K4 histone, and an increase in the synthesis of corticosterone as a response to stress [48]. Even in humans, a similar behavior is observed where exposure to stressful conditions leads to late onset of disorders such as schizophrenia, bipolar disorder, autism, diabetes, and obesity [48]. In schizophrenia, DNA methylation is linked to protein renelin that promotes neuronal migration that promotes brain plasticity and development [9,49].

Environmental epigenetics

It is the study of environmental effects on epigenetic state of organisms. Exposure to chemicals and pollutants can change the synthesis of S-adenosyl methionine and therefore change DNA methylation profiles across the genome. Cigarette smoke, exhaust smoke from fossil fuels, particulate matter is some of the environmental factors. In order to understand how environmental factors can change methylation profiles across genomes and cause diseases, Epigenome Wide Association Studies (EWAS) was developed. It analyzed methylation status against environmental factors such as diet, lifestyle, exposure to pollutants etc. [8].

Maternal smoking during pregnancy was analyzed against DNA methylation in in over 450,000 CpG sites in about 6685 newborn infants. They found genes that were differently methylated and were linked to orofacial cleft in infants. The effects of this maternal smoking were found to persist in the infants several years beyond childhood [41]. In another study studies were done on the association of maternal plasma folate and methylation in cord blood of newborns. Thus interaction of the environment on the epigenome can be used as a marker to study diseases over generations, plus help us create remedies or drugs to counteract it (Figure 5).

Figure 5: Effects of environmental factors on epigenetics. Ref- Norouzitallab, Parisa., et al. "Can epigenetics translate environmental cues into phenotypes?". Science of the Total Environment 647 (2019): 1281-1293.

Techniques used to study epigenetics

Epigenetics is one of the most rapidly growing fields of study. It is found to be associated with a wide plethora of biological functions. Adding to the fact that DNA methylation is also being associated with studies of several diseases such as cancer, cardiovascular diseases, autism, diabetes etc. This makes epigenetics an important field of study for therapeutics sand development of drugs. There has been a shift from studying specific modifications on DNA and histones to the alterations ensuing from the epigenetic marks on the genome as a whole. Some of the techniques/assays developed monitor epigenetic changes, changes in the methylation patterns. These assays help us keep track of the modifications, create a map of these modifications on the various sequences and most importantly help us understand gene regulations. Two most important techniques used to study these modifications are Methylated DNA Immunoprecipitation (MeDIP) and Bisulphite Modification of DNA.

Bisulphite sequencing

It is regarded as the standard to detect DNA methylation as it provides one of the most sensitive and efficient single base pair resolutions of methylated bases. It is based upon the exposure of Cytosine and 5-methylcytosine (5'-mC) to bisulphite reaction. It undergoes a deamination reaction where unmethylated cytosine is deaminated to uracil, whereas, 5'-mC is unreactive. This is primarily used to differentiate between methylated and unmethylated regions on the DNA. This is followed by treatment with primers specific to methylated bases and PCR to amplify the modified sequences for further analysis.

The amplified sequences are then further analyzed by Sanger sequencing, pyrosequencing or mass spectrometry. It can be used to study the extent of methylation on each cytosine. Bisulphite sequencing can be used to study methylation not only in single stranded DNA but also in double stranded DNA strands. To optimize the results of bisulphite sequencing, various adjustments and modifications are done depending upon the need.

This technique has a lot of other versions, however it always remain the choice for studying DNA methylation due to its efficiency and high sensitivity to the detection of modified bases. One of the best ways to determine whether the reaction is successful or not is by checking methylated cytosines, if they are not in abundance in CpG regions then there might be incomplete conversion [10,11].

Figure 6: Principles of methylation analysis using bisulfite genomic sequencing. After treatment with sodium bisulfite, unmethylated cytosine residues are converted to uracil whereas 5-methylcytosine (5mC) remains unaffected. After PCR amplification, uracil residues are converted to thymine. DNA methylation status can be determined by direct PCR sequencing or cloning sequencing. Ref- Li, Yuanyuan, and Trygve O Tollefsbol. "DNA methylation detection: bisulfite genomic sequencing analysis." Methods in molecular biology (Clifton, N.J.) vol. 791 (2011): 11-21. doi:10.1007/978-1-61779-316-5_

Figure 7: Bisulphite sequencing- Interpretation of methylation sequencing results. After bisulfite treatment, all unmethylated cytosines (C) convert to thymine (T) and the presence of a C-peak indicates the presence of 5mC in the genome. Total methylation or complete conversion of a single residue shows a single peak. The presence of both C- and T-peaks indicates partial methylation or potentially incomplete bisulfite conversion. Ref- Methods in molecular biology (Clifton, N.J.) vol. 791 (2011): 11-21. doi:10.1007/978-1-61779-316-5.

Methylated DNA immunoprecipitation (MeDIP)

It was an assay that was basically created to isolate the methylated fractions from the sample. The procedure made it possible to differentiate between methylated and non-methylated regions and thereby build an accurate methylation profile. Sample as low as 200 ng is used for the assay, it can be used for the immunoprecipitation reaction. The sample is sonicated so the DNA shears down to a size of 300-1000 bp. It is then divided into input (IN) and immunoprecipitated (IP) fractions. Heating then denatures IP DNA; it is then treated with anti-5-mC antibodies and incubated so that these antibodies bind specifically to the methylated regions. The magnetic beads containing the secondary antibody, which binds to the first antibody, is added to the mixture and incubated. A magnet is then used to separate these antibodies, which in turn bound to the magnetic beads. These antibodies are already bound to the methylated segments of DNA, after several washes with the IP buffer the unbound non-methylated DNA is washed out, leaving us with the desired regions. The methylated regions are then digested with Proteinase K that breaks down the antibodies without affecting the methylated regions. The DNA is then treated with phenol:chloroform mixture to further purify the DNA. The protein is removed and the DNA is then precipitated and re-suspended in water for later use. The purified IN and IP fractions can then be amplified with PCR, using primers against known methylated regions. This is basically used to validate the efficiency of the PCR technique. Since there is a growing awareness regarding the role DNA methylation plays in various diseases, the techniques to detect these methylation segments have also gained importance around all the labs. MeDIP can provide rapid results of the DNA methylation profiles using only a small quantity of the starting material. It is difficult to provide quantification of single stranded DNA product is difficult, however various techniques such as spectrophotometry works best for double stranded DNA. It helps us to draw easy comparisons of the methylation profiles between various sources. It is also easy to modify the process based on the requirements of the experiment. If the starting material in really small amounts then the entire process can be scaled to increase the yield [9] (Figure 8).

Figure 8: Procedure/Workflow for Methylated DNA Immunoprecipitation assay for detection of methylated DNA fragments from the given sample; Ref- Karpova, Nina N., and Juzoh Umemori. "Protocol for methylated DNA immunoprecipitation (MeDIP) analysis." Epigenetic Methods in Neuroscience Research. Humana Press, New York, NY, 2016. 97-114.

Although MeDIP is faster than the bisulphite sequencing process but MeDIP is also dependent on CpG density, upon its composition within the sequence and on the presence of repetitive elements in the genome.

Special care needs to be taken throughout the MeDIP procedure:

- To ensure proper fragmentation of DNA after sonication so that the DNA is completely denatured
- There should be safe lab practices while handling chemicals as in this case phenol is used.
- Use of siliconized tubes so that it does not allow non-specific binding of the DNA to the tube walls [6,9].

Diseases caused by epigenetics

Cancer

Cancer is caused due to due to hypermethylation of certain regions of a promoter, or of the first exon leads to inactivation of certain tumor suppressor genes, or protooncogenes. A number of genes are silenced due to DNA methylation, in different types of cancer. However hypomethylation leads to genomic instability, causes cell transformation, activates the proto-oncogenes [36]. CpG dinuclotides are also hotspots for mutation in certain genes, in cancer [37]. Some of these mutations include spontaneous deamination of 5-methylcytosine to thymine, DNMT inducing enzymatic deamination from 5-methylcytosine to thymine, DNMT inducing enzymatic deamination of unmethylated cytosine to uracil, DNMT methylating of uracil to thymine [38].

CpG sites have been shown to act as hot spots for germline mutations, contributing to 30% of all point mutations in the germ line, and for acquired somatic mutations that lead to cancer. For example, methylated CpG sites in the p53 tumor suppressor-coding region contribute to almost 50% of all inactivating mutations in colorectal cancer and to 25% of cancers in general [39]. In human cancers there is an overall increase in the DMNT1, 3a and 3b activity. DNMT1 may promote tumorigenesis by its link to activation of the oncogenic ras signaling pathway, it binds to cell nuclear antigen and increases the cellular proliferation and also by reducing cellular p21 (belongs to the cyclin-dependent kinase (CDK) inhibitor family), it prevents of p53-dependent apoptosis, it also promotes carcinogenesis by methylation of CpG islands on promoters of tumor suppressor and mismatch repair genes [1,40] (Figure 9).

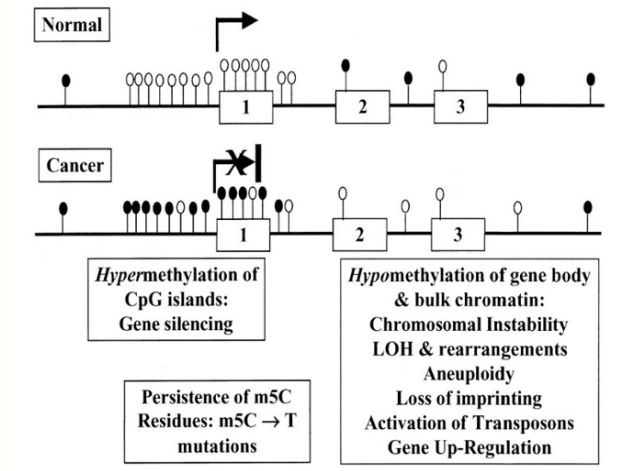

Figure 9: Distribution of cytosine-guanine (CpG) dinucleotides in the human genome and CpG methylation patterns in normal and tumor cells. In contrast to methylated CpG sites in the CpG-poor bulk of the genome and unmethylated CpG islands in normal cells, cancer cells simultaneously harbor widespread loss of methylation in the CpG-depleted regions where most CpG dinucleotides should be methylated and gains in methylation of CpG islands in gene promoter regions. Open circles represent unmethylated CpG sites whereas filled circles are methylated CpG sites. Boxes 1, 2, and 3 represent exons and line between exons are introns. X at the transcription start site represents transcriptional silencing.

Ref- Kim, Young-In. "Nutritional epigenetics: impact of folate deficiency on DNA methylation and colon cancer susceptibility." The Journal of nutrition 135.11 (2005): 2703-2709.

Systemic lupus erythematosus

It is an autoimmune disorder where the body's immune system attacks healthy tissues and leads to swelling, chest pain, facial rash, body and joint pain. It is characterized by an autoantibody response to the cytoplasmic or nuclear antigens. Genes such as ITGAL, CD40LG, PRF1, CD70, IFGNR2, MMP14, LCN2 and rRNA gene promoter (18S, 28S) [12-16] are overexpressed, and their promoters show global hypomethylation. Global hypomethylation also affect chromatin structure of T cells, leading to overexpression of these genes. This overexpression causes inflammatory response, cell hyperactivity and the constant immune response [17,18].

Drugs that act as DNA methylation inhibitors such as procainamide and hydralazine, also hypomethylates DNA [19]. Procainamide [20] competitively inhibits DNMT1, whereas hydralazine [21] inhibits The B- and T- cell signal regulated kinase pathways. They reduce action of DNMTs and instead cause expression of adhesion molecules of lupus-drug-induced-lymphocytes [22-24].

Rheumatoid arthritis (RA)

It is an autoimmune disorder that leads to swollen, painful joints and stiffness throughout the body. It is caused by progressive destruction of joints by invasive synovial fibroblasts, which initiates and eventually perpetuates the disease [25]. The overexpression of inflammatory cytokines in the synovial fluid is caused by global hypomethylation of these synovial fibroblasts [26,27]. L1 is one of the classes of repetitive elements that are spread throughout the genome and they also serve as markers for being methylated in the synovial tissue. In RA patients, their synovial tissue contains hypomethylated L1 due to reduced expression of DNMTs. However the hypomethylation of promoters in inflammatory response genes causes increase in expression of cytokines, adhesion molecules and in the end causes irreversible changes to the fibroblasts [28].

Type I diabetes

Type I diabetes (juvenile diabetes) is a form where little or no insulin is produced from the pancreas. There is an increased of glucose or sugar levels detected in blood, marked by symptoms such as increased thirst, increased urination, weight loss, increase in hunger. However unlike the previous autoimmune disorders, this is caused by modified metabolism of homocysteine leading to the hypermethylation [29]. Both glucose and insulin inhibit the trans-sulfuration and increases the homocysteine production [30]. Increase in homocysteine levels DNMTs catalyzes methionine to S-adenosyl Methionine. This leads to genome wide DNA methylation. In pregnant females, increase in homocysteine can lead to loss of vascularity in the fetus and a lack of glucose tolerance throughout its adult life [31].

Multiple sclerosis

It is characterized by progressive neurodegeneration due to destruction of the myelin sheath, insulating the neurons and the spinal cord. It can cause blindness in one eye, loss of coordination, muscle weakness etc. Peptidyl Arginine Deiminase II (PAD2) helps in the citrullination of Myelin Basic Protein (MBP). This helps in the protein auto-cleavage, which creates new epitopes that helps in modulating the immune response.

Systemic sclerosis

It is characterized by deposition of collagen all over skin and internal organs. On skin it can cause hardening and scarring with blood vessels getting exposed. The Fli1 gene (transcription factor that prevents collagen production) promoter with CpG islands re hypermethylated. The silencing of Fli1, increases collagen synthesis and ultimately tissue fibrosis occurs [32].

Neurodegenerative disorders

There are numerous studies that have linked epigenetic marks and histone modifications being associated with neurodegenerative diseases. Inactivation of demethylase enzymes can cause autism, X-linked mental retardation. Regulation of H3K4 methylation histone proteins is found to influence and develop neurodegeneration. DNA methylation has been identified in schizophrenia and Alzheimer's disease as a decrease in DNA methylation is observed in the repetitive regions of the genome [5,33].

Epigenetics can influence brain development due to various factors [5]

- Disordered chromatin organization in both early and adult neurodegenerative diseases in patients
- Chromatin modifying drugs that are therapeutic to neurodegenerative disorders.
- Flexibility of epigenetics during all stages of brain development and ageing as well as regulation in neurons.

Epigenetics and its clinical implications

Epigenetic clearly regulate gene expression and with the aid of new techniques, not only the regions that undergo epigenetic modification can be detected, but they also can be used to detect diseases and help us to understand disease pathogenesis. One of the earliest diseases to be studied under epigenetics was cancer. In the 1970s Holliday and Pugh demonstrated that hypermethylation of silenced tumor suppressor genes leads to cancer [34]. Multiple pathways are silenced or activated due to DNA methylation, which causes cancer. Analysis and study of these genes with the knowledge of epigenetics has added to our understanding of the disease. As a result, there is hope for effective treatment of diseases through

proper development of drugs. However identification of cancer at an early stage is not an easy task. Tests are being developed to identify epigenetic markers to identify lesions or cancer at an early stage. They look for abnormal methylation patterns, histone modifications.

The advantage of epigenetic modifications is that it can be reversed and hence can be prevented. For example, blocking DNA methylation by inhibiting DNMTs results in demethylation of CpG islands in daughter cells, resulting in restoration of expression of tumor suppressor genes and abrogation of tumor growth [6]. There are several DNA demethylating compounds that are actively being investigated, such as zebularine, procainamide, and procaine. However major limiting factor is drug toxicity.

Abnormal promoter methylation increases resistance to chemotherapy and resistance to radiation, thus demethylating agents can be used to enhance the efficiency of chemotherapy [35]. Based on epigenetics, a number of cancer drugs were developed. The first cancer drug was Azacytidine [34], which was licensed by FDA in 2004 for treatment of blood cancer. Despite the necessity of epigenetics to identify and understand diseases several scientists have raised their doubts. They are concerned with the capacity to identify the risk, to determine when a cell or tumor is so altered that it progresses to cancer, eventually to resistance of treatment.

Conclusion

Epigenetics is gradually becoming an important field of study due to its widespread outlook of the biological systems. Although the field of study is not well known, however a lot of studies have analyzed biological systems from an epigenetic perspective. This field has now widened into other mechanisms involving those of RNA. Research in epigenetics picked up a lot of momentum with the start of Human Epigenome Project (HEP). It was launched to mainly map the DNA methylation and the various epigenetic markers located throughout the genome; it furthered our understanding of epigenetics in gene regulation and disease development. Since epigenetics was linked to so many diseases, it became critical for us to understand how DNA methylation patterns are important for normal gene functioning. Many factors are said to affect the epigenetics of an organism such as his diet, sleeping patterns, exposure to pollutants/chemicals. Environmental triggers can cause these epigenetic changes that can be passed from generation to another. However, epigenetic modifications are reversible and therefore preventable when associated with any disease. Based on the flexibility of epigenetic modification it seems that they can be easily treated with proper drugs. Epigenetics opened up an entire new strategy for developing therapeutic drugs and provide diagnosis to diseases. This heralds an amazing future for Indian research on disease development and eventually cure.

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