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Mini Review

Sci Vis Vol 13 Issue No 1
January-March 2013
ISSN (print) 0975-6175
ISSN (online) 2229-6026

On DNA methylation: an introductory review

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Received 1 October 2012 | Revised 6 December 2012 | Accepted 10 December 2012

ABSTRACT

DNA methylation is an epigenetic phenomenon whereby a methyl ($-CH_3$) group is added to cytosine. There are two basic types of methylation: hypomethylation in which the level of DNA methylation is greatly reduced, and hypermethylation in which there is an increased level. A methyl group is added and removed by DNA methyltransferases (DNMTs) and DNA demethylases respectively. The methyl group is transferred from a universal methyl donor S-adenosyl methionine. DNA hypermethylation in the promoter element can repress gene expression and therefore is crucial for a wide range of cellular activities such as genome stability and protection, imprinting, X-chromosome inactivation, paramutation, tissue specific gene regulation, carcinogenesis and aging. Global DNA hypomethylation has been known to play significant role in carcinogenesis. Studies in the field of DNA methylation have yielded promising potential disease biomarkers in terms of therapeutic functions. It has been concluded that the possible utilization of the degree of DNA methylation of specific genes as biomarkers for the prognosis and diagnosis of diseases is a matter which demands consideration for researchers all over the world. Canonical information on DNA methylation is highlighted in this review.

Key words: Biomarkers; diagnosis; disease; epigenetic; methylation; prognosis.

INTRODUCTION

DNA methylation is an epigenetic event whereby a methyl ($-CH_3$) group is generally added to the nitrogenous base cytosine, specifically on the fifth carbon position in the pyrimidine ring and is often called 5-methylcytosine (5-mC), or 5-methyl deoxycytosine to point out specifically that it takes place in DNA.[1] Occasionally it is also called as the fifth nucleotide of DNA.[2] In 1904, Wheeler and Johnson described the method of preparation and complete chemical properties of 5-mC. However, its existence in nature was not known until 1925 when Johnson and Coghill of the Department of Chemistry, Yale University, discovered it in tuberculinic acid, the nucleic acid of the tuberculosis-causing bacterium *Tubercle bacillus*.[3]

Approximately 2-8% of all cytosines in mammals and up to 50% in higher plants are consid-

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ered methylated, but 5-mC is undetectable in budding and fission yeasts and nematodes.[2] Vertebrates have the highest levels of 5-mC in the animal kingdom.[4] DNA methylation can occur in three different sequence contexts CG, CHG, and CHH, where H=C, T, or A.[5] In most animals, cytosines, whose downstream neighbour is guanine, are particularly a common target for methylation. This cytosine joined with guanine by phosphate is termed CpG dinucleotide. A stretch of over hundred nucleotides having a high composition of CpG dinucleotides, i.e. GC -rich (typically >50% GC) is called CpG island. 5-mC is chemically unstable and is highly subjected to deamination to thymine, which is usually not recognized by the host DNA repair machinery, which on the other hand generally removes abnormal bases due to deamination. Consequently, over a long evolutionary period, the number of CpG dinucleotides in vertebrates has declined as a result of the conversion of CpG to TpG.[4] It was known that the approximate number of CpG islands per haploid genome is 45,000 in human and 37,000 in mouse.[6]

Basically two enzymes are responsible for DNA methylation, namely DNA methyltranferases (DNMTs) and DNA demethylase.[7] Methyl group is transferred from a universal methyl donor S-adenosyl methionine to cytosines by DNA methyltransferases in a reaction that involves base flipping, whereby a cytosine base is swung completely out of the DNA helix into an extra-helical position so that the enzyme can access and methylate the cytosine.[2] The methyl group lies in the major groove on the outside of the DNA double helix and so does not interfere with the base pairing.[4] Interestingly, DNA methylation in promoter elements can repress gene transcription directly by interfering with the binding of transcriptional activators and indirectly by favouring the formation of repressive chromatin by methyl DNA-binding proteins. In higher eukaryotes, DNA methylation is crucial for a wide range of cellular activities such as genome stability and protection, imprinting, X-chromosome inactivation, paramutation, tissue specific gene regulation, carcinogenesis and aging.[2,7,8] The pattern of DNA methylation can be inherited when cell divides.[9]

DNA Methylation in Mammals

DNA methyltransferases

In mammals, CpG methylation is maintained by DNA methyltransferase family that comprises of three active members *viz*. DNMT1, DNMT3A and DNMT3B. DNMT1 is responsible for maintaining CpG methylation during DNA replication. It acts on the hemimethylated sites of DNA which are produced as a result of replication, thereby maintaining the pattern of CpG methylation in the newly synthesized DNA strand. It gets localized at the site of replication during S-phase via the help of a cofactor called ubiquitin-like containing PHD and RING finger domains 1 (UHRF1, also known as NP95) and PCNA which recognize hemimethylated DNA at the replication foci.[5,10]

DNMT3A and DNMT3B are highly homologous enzymes responsible for *de novo* DNA methylation during development. DNMT3B is most prevalent in the early stages of embryonic development and is responsible for the accomplishment of DNA methylation during implantation. DNMT3A acts in the later part of embryonic development and is also responsible for determining the methylation pattern in mature gametes. DNMTs contain a conserved methyltransferase catalytic domain in the c-terminal regions.[2] A fourth member DNMT2B is also present but with a function that is still enigmatic.[11]

In normal development

The pattern of DNA methylation of the entire genome is markedly reprogrammed during early embryonic development in mammals.[10] Early studies by restriction enzymes have specified that most of the methylation inherited from the parental gametes have been deleted in preimplantation blastula and morula.[12] In mouse,

soon after fertilization, the level of methylation declined as a result of demethylation. Remethylation of the demethylated genome is believed to occur after implantation. In adult mouse, there are ~5,000 tissue specific differentially methylated regions and ~10,000 developmental stage specific differentially methylated regions (DS-DMR). Many of the DS-DMRs are also found to be methylated in the earlier stages of development but are unmethylated in the adult stage.[13]

DNA methylation also plays a critical role in one of the most disturbing clinical inconveniences during the course of gestation, i.e. miscarriage. The reason behind early pregnancy loss (EPL) has been attributed to the low expression of DNMT1 in the villous of a woman with EPL in comparison with the normal villous. A study in mouse model also showed that a disorder in maintenance of methylation with DNMT1 inhibitor may eventually result in global decrease in DNA methylation and an impaired embryonic development.[14] The housekeeping genes on the X chromosome are one of the best examples of some of the genes that undergo *de novo* methylation at certain stages of development. In female, it is because of this methylation pattern that one of the X chromosomes is maintained in an inactive state in all cells for the lifetime of the organism. It seems that most tissue-specific genes are methylated at least at the later stages of development and in a large variety of cell types.[15]

In cancer

The pattern of DNA methylation is one of the most consistent hallmarks of cancer.[11] Cancer shows a paradoxical change in methylation pattern such as global hypomethylation that mostly takes place in DNA repetitive elements and a concurrent hypermethylation at the promoter CpG islands of tumor suppressor genes such a hMLH1 (human Mut-L-Homolog 1), BRCA1 (breast cancer gene), VHL (von Hippel-Lindau), p16Ink4a, etc.[16] The first known CpG island methylation of tumor suppressor genes in human is the retinoblastoma (Rb) gene by Greger *et al.* in 1989.[17] Based on Esteller (2002),[18] Table 1 shows some of the selected genes that undergo CpG island hypermethylation in human cancer.

In other diseases

De novo methylation has been known to play a very imperative role in the development of fragile X syndrome, whereby the FMR1 gene containing a triplet repeat becomes repressed.[12] Chestnut *et al.* (2011) have shown that neurons destined for apoptosis showed a marked increase in DNA methylation and apparently in the level of DNMTs such as DNMT1and DNMT3A. It was also believed that the participation of DNMTs and DNA methylation in neuronal cell death could be related to the development of human neurodegenerative diseases such as spinal muscular atrophy and amyotrophic lateral sclerosis.[19]

A study on the seven CpG loci proximal to the insulin gene (INS) promoter showed that there is a lower level of methylation in CpG-19, -135 and -234, and a higher level of methylation in CpG-180 in patients suffering from type 1 diabetes than controls.[20] A comprehensive profiling of DNA methylation of pancreatic islets in type 2 diabetic and control individuals by Volkmar *et al.* (2012) revealed a significantly different DNA methylation in 276 CpG loci linked to the promoter of 254 genes of which more than 250 genes have been found to be affected by aberrant methylation.[21] A study on monozygotic twins also indicated that there is an association between global DNA hypomethylation and insulin resistance, a hallmark of type 2 diabetes.[22] Other mammalian diseases such as Silver-Russell syndrome, Beckwith-Wiedemann syndrome, Angelman's syndrome and Prader-Willi syndrome have been known to be caused as a result of failure to methylate and imprint. In the brains of patients suffering from Parkinson's disease, hypomethylation of the intron-1 of alpha-synuclein (SNCA) gene was observed. Global DNA and RNA hypomethylation was also observed in the

Table 1. Selected genes undergoing CpG island hypermethylation in human cancer (Esteller 2002).[18]

Gene	Function	Location	Tumor profile	Consequences
$P16^{INK4a}$	Cyclin-dependent kinase inhibitor	9p21	Multiple types	Entrance in cell cycle
P14ARF	MDM2 inhibitor	9p21	Colon, stomach, kidney	Degradation of p53
P15INK4b	Cyclin-dependent kinase inhibitor	9p21	Leukemia	Entrance in cell cycle
hMLH1	DNA mismatch repair	3p21.3	Colon, endometrium, stomach	Frameshift mutations
MGMT	DNA repair of 06-alkyl-guanine	10q26	Multiple types	Mutations, chemosensitivity
GSTP1	Conjugation to glutathione	11q13	Prostate, breast, kidney	Adduct accumulation?
BRCA1	DNA repair, transcription	17q21	Breast, ovary	Double strand-breaks?
P73	P53 homologue	1p36	Lymphoma	Unknown (cisplatin?)
LKB1/STK11	Serine/threonine kinase	19p13.3	Colon, breast, lung	Unknown
ER	Estrogen receptor	6q25.1	Breast	Hormone insensitivity
PR	Progesterone receptor	11q22	Breast	Hormone insensitivity
AR	Androgen receptor	Xq11	Prostate	Hormone insensitivity
RARβ2	Retinoic acid receptor β2	3p24	Colon, lung, head and neck	Vitamin insensitivity?
RASSF1	Ras effector homologue	3p21.3	Multiple types	Unknown
VHL	Ubiquitin ligase component	3p25	Kidney, hemangioblastoma	Loss of hypoxic response?
Rb	Cell cycle inhibitor	13q14	Retinoblastoma	Entrance in cell cycle
THBS-1	Thrombospondin-1, anti-angiogenic	15q15	Glioma	Neovascularization
CDH1	E-cadherin, cell adhesion	16q22.1	Breast, stomach, leukemia	Dissemination
HIC-1	Transcription factor	17p13.3	Multiple types	Unknown
APC	Inhibitor of β-catenin	5q21	Aerodigestive tract	Activation b-catenin route
COX-2	Cyclo oxygenase-2	1q25	Colon, stomach	Antinflamatory resistance?
SOCS1	Inhibitor of JAK/STAT pathway	16p13.13	Liver	JAK2 activation
SRBC	BRCA1-binding protein	1p15	Breast, lung	Unknown
SYK	Tyrosine kinase	9q22	Breast	Unknown
RIZ1	Histone/protein methyltransferase	1p36	Breast, liver	Aberrant gene expression?
CDH13	H-cadherin, cell adhesion	16q24	Breast, lung	Dissemination?
DAPK	Pro-apoptotic	9q34.1	Lymphoma, lung, colon	Resistance to apoptosis
TMS1	Pro-apoptotic	16p11	Breast	Resistance to apoptosis
TPEF/HPP1	Transmembrane protein	2q33	Colon, bladder	Unknown

entorhinal cortex layer II neurons of persons with Alzheimer's disease.[23-25]

DNA METHYLATION IN *DROSOPHILA MELANOGASTER*

For over a long time, it was thought that there is no 5-mC in *Drosophila melanogaster*. In 1999, Tweedie *et al.* unexpectedly found two proteins that have strikingly high resemblance with cytosine DNA methyltransferase and methyl-CpG-binding-domain (MBD) protein of

mammals.[26] The transcript of this hypothetical dDNMT has been found to be present in the embryo, larva and adult. The carboxy-terminal amino acid sequences of the proteins dMBD2 and dMBD3 showed significant similarities to that of mammalian MBD2 and MBD3. It was also found that dMBD2/3 associates with histone deacetylases (dHDACs) as mammalian MBD2 and MBD3 associates with HDAC. MBD proteins have bifunctional role in that it binds specifically to methylated DNA and at the same time recruits HDAC complexes to their site of binding.[11] It was latter unveiled that the hypothetical dDNMT belongs to DNMT2 family and was renamed as dDNMT2.[27]

In 2000, Gowher *et al.*[28] and Lyko *et al.*[29] independently showed that there is in fact 5-mC in the genome of *D. melanogaster* as known in many other insects. Approximately one methylcytosine appears in 1,000-2,000 cytosine residues. DNA methylation in *Drosophila* has been shown to be evenly distributed throughout the entire genome and have a direct effect on the structure of chromatin. It was also revealed to play role in the delaying of cell cycle progression and reduced DNA replication in tissues with high DNA methylation. Salzberg *et al.* (2004) suggested that DNA methylation in *Drosophila* is also a defense mechanism.[27]

DNA Demethylating Agents

DNA demethylase has been known to demethylate both fully and hemi-methylated DNA. The shortest form of methyl-CpG binding domain (MBD2), MBD2b, has been proposed to be a DNA demethylase and has already been cloned and characterized.[7] Currently, five nucleosides are known to have DNMTs inhibiting potential. These drugs are all chemically cytosine analogues: 5-azacytidine, 5-aza-2'deoxycytidine, dihydro-5-azacytidine, arabinosyl-5-azacytosine and zebularine. 5-aza-cytidine is also known as 5-azaCR and azacytidine (Vidaza®); 5-aza-2'deoxycytidine as 5-azaCdR and decitabine (Dacogen®); dihydro-5-azacytidine as DHAC; arabinosyl-5-azacytosine as Ara-C, fazarabine and kymarabine. Non-nucleoside DMNT inhibitors such hydralazine, procainamide, procaine, epigallocatechin 3-gallate (EGCG), MG98 and RG108 have also shown to be very promising for future usage in the treatment of different diseases including cancer.[9,30-35]

Conclusion

Although the role of DNA methylation in the initiation and development of cancer and other diseases has been known only very recently, recognizing its importance in clinical research and therapy, it has gained a vast interest among scientists all over the world. Even as DNMT inhibitors such as 5-azacytidine, 5-deoxy-2'-azacytidine, etc. have already been utilized in the treatment of certain cancers, studies have also been directed towards the use of DNA methylation as disease biomarker.[34] Al-Moundhri *et al.* (2010) indicated that DNA methylation may serves as a non-invasive prognostic procedure since the blood of gastric cancer patients showing differences in level of DNA methylation has a diverse survival rate and blood can be easily obtained without producing much damage.[36] The release of cancerous cells containing such aberrant hypermethylation or free methylated DNA into the blood stream may thus allows the early detection of cancer or the identification of high risk individuals from developing such disease.

However, not much research which is conclusively utilitarian has been done in the area of DNA methylation analysis or the discovery and invention of possible disease biomarkers which may not only have prognostic significance but also therapeutic implications when aided with another desirable remedy. For this cause, the possible utilization of the degree of DNA methylation of specific genes as biomarkers for the prognosis and diagnosis of diseases is a matter which demands consideration for researchers all over the world. However, study design and data analysis should be considered fastidiously as data could represent level of methylation, me-

thylation content, methylation pattern, etc.

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