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Review Article

DNA methylation and Cancer: Identifying and targeting epigenetic modifications may be the future of cancer therapy.

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Abstract

DNA methylation has been recognized as one of the most important epigenetic mechanisms regulating the expression and inhibition of genes giving rise to an organism’s phenotype. It is hence of no surprise that when DNA methylation mechanisms are disrupted by intrinsic or extrinsic causes, the likelihood of tumourigenesis increases. Both hypermethylation and hypomethylation may predispose to cancer formation through aberrant inhibition or expression of particular genes and this is seen in different types of cancers, such as laryngeal squamous cell carcinoma and acute myeloid leukaemia. By increasing our knowledge and understanding of these epigenetic mechanisms, we will be able to develop diagnostic techniques such as methylation profiling, to screen for and detect aberrant methylation patterns which may predispose to cancer formation in our patients. This would enable early diagnosis and treatment which may also involve the use of drugs developed to provide directed epigenetic therapy, shifting away from the current trend which involves the use of radical anti-cancer therapy. These diagnostic and treatment options may be the future of cancer management.

Keywords
Epigenetics, DNA Methylation, Cancer, Epigenetic therapy, methylation profiling.

Introduction

The term ‘epigenetics’ was first defined by Conrad Waddington as the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being. (1) Over time, as our understanding of epigenetics increased, the definition was narrowed down to the one recently proposed, which states that “An epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence.”(2)

The study of Epigenetics is not a new field, dating back to the 1980s. However, it has recently attracted a lot of attention after studies started to indicate that these modifications can be targeted by pharmacological agents, resulting in the potential treatment of numerous diseases, including cancers.

Over time, two main epigenetic modifications were identified: a) processes that involve the addition of methyl groups (CH₃) to DNA bases, known as DNA methylation and b) binding of histones and other DNA-binding proteins which may or may not be modified (3). This review however, will focus exclusively on DNA Methylation.

DNA methylation

DNA Methylation is one of the most studied types of epigenetic modifications and is a covalent modification which involves the addition of a methyl group (–CH₃) from S-adenosylmethionine (SAM) to position 5 of a cytosine molecule which is followed by a guanine molecule and together form a cytosine–phosphate–guanine (CpG) dinucleotide. This addition of the –CH₃ group is replication-dependent and occurs during S-phase of the cell cycle through the action of DNA methyltransferases (DNMTs), which are present at the replication fork (4). The addition of the methyl group may be performed on isolated or clustered CpG dinucleotides, anywhere along the genome. Yet, CpG dinucleotides are not very common in the eukaryotic genome, except in the regions known as CpG islands, making such regions major sites for DNA methylation.
(5). CpG islands are very often found in sequences which have been identified as being gene promoters and in fact, it is known that CpG islands are present in about 60 per cent of all human gene promoters (6). Elevated methylation levels at gene promoter CpG sites results in gene inhibition by preventing the binding of transcription factors to their relative sites. This is done through the recruitment of methyl–binding domain proteins (MBDs) (4). MBDs are part of a family of transcription–repressor proteins which bind to methylated CpG islands within promoters. These MBDs, along with other proteins involved in epigenetic modifications such as Histone Deacetylases (HDACs) and Histone Methyltransferases, form corepressor complexes and result in chromatin reconfiguration and gene silencing (7).

The effects brought about by promoter CpG methylation have shown that, in itself, DNA methylation does not only happen as part of a pathogenic process. This is evidenced by its role in a number of normal developmental processes. In fact, silencing of certain genes through the methylation of promoter CpG islands is a vital step in many tissue–specific and germline–specific processes such as X chromosome inactivation in females, inactivation of transposable elements and repetitive sequences, and genomic imprinting (8). However, disturbances in the patterns of DNA methylation brought about either by environmental factors (such as smoking) or as a result of a genetic mutation affecting the genes coding for DNMTs, may result in the initiation or progression of cancer.

DNA methylation patterns and cancer development

It is now widely accepted that carcinogenesis results due to a combination of both genetic and epigenetic changes which occur within the individual’s genome. Being one of the most studied aspects of epigenetics, it is not surprising that changes in DNA methylation patterns have been described in a number of cancer–types such as colorectal cancer and also in Acute Myeloid Leukaemia (AML). The role of the environment in DNA methylation has been thoroughly studied and has led to the conclusion that DNA methylation is also a reason behind the fact that different individuals have different susceptibilities to a number of diseases, including cancer. A study conducted by Fraga et al. in 2005 showed how the DNA methylation levels in monozygotic twins exposed to different environments varied over time. Upon analysis, Fraga and colleagues showed that the younger the twins, the greater the similarity in the levels and patterns of DNA methylation within their genomes. However, as the twins grew older and became exposed to differing environments, DNA methylation patterns within their genomes started to change resulting in genomic and phenotypic differences between monozygotic twins of a given set (6). This means that even individuals with the exact same genome can be susceptible to different diseases such as cancer as a result of the environmentally–induced changes in their DNA methylation patterns.

The characteristic epigenetic picture which is present in most cancer types involves the hypomethylation of Oncogenes and the hypermethylation of Tumour Suppressor Genes (TSGs), as well as increased chromosome instability which is brought about by hypomethylation of repetitive elements and retrotransposons which may lead to chromosomal rearrangement through their translocation (10). Hypomethylated oncogenes include certain growth–promoting genes such as the R-ras gene which is typically activated in gastric cancer and the S-100 gene in colon cancer. In contrast, hypermethylation of DNA leads to inactivation of genes. This is of particular importance when the gene affected is a TSG since loss of TSG activity promotes cancer formation. TSGs are not the only genes which may be affected by hypermethylation since this epigenetic alteration may also result in the repression or inhibition of genes coding for transcription factors or genes coding for proteins involved in DNA repair mechanisms. Examples of affected genes are BRCA1, p16 and p53. (11)

While both hypomethylation and hypermethylation have been identified and studied, more knowledge is required in order to fully understand the mechanisms behind such epigenetic changes. Attempts are still being made to determine whether such processes occur passively upon exposure to certain environmental factors, such as when DNMTs are unable to access the newly replicated DNA, or whether these environmental factors trigger active processes which are characterised by selective recruitment of enzymes (12). Whatever the process, DNA methylation plays a role in a number of cancers, two of which are discussed next.

The Role of DNA Methylation in Laryngeal Squamous Cell Carcinoma

Laryngeal squamous cell carcinoma (LSCC) is known to account for over 90% of all laryngeal cancer and is also a common candidate for surgery and radiotherapy (13). Studies conducted by Fu et al. in 2011 found that the transcription of one particular gene is decreased in LSCC. This gene is MYCT1 and is regulated by means of c–Myc, a transcription factor which binds to E–box elements in the MYCT1 promoter region (14).

Studies show that increased or aberrant levels of methylation in the promoter region can prevent the binding of c–Myc to the E–box elements, resulting in decreased expression of the gene which is normally transcribed when c–Myc is bound to its promoter. This mechanism
was confirmed further when studies performed on HL–60 myeloid leukaemia cells with highly methylated promoters and E–box elements showed that in the absence of a demethylating agent such as 5–aza–2–deoxycytidine, the expression of genes which are normally regulated by c–Myc is decreased, while when the demethylating agent was added and hence the hypermethylation of E–box regions was removed, the genes regulated by c–Myc were expressed (15).

In 2012, Yang et al. conducted a study with a particular focus on the role of MYCT1 in LSCC. It was believed that MYCT1 might act as a TSG and that its low expression in LSCC may be due to aberrant hypermethylation of E–box elements in the MYCT1 promoter region, resulting in a decrease in the gene’s expression by preventing the binding of c–Myc. The results obtained by Yang et al. actually confirmed that 11 of the 12 sites present in MYCT1 promoter region do become methylated in cancer, indicating that there is a significant difference between methylation levels of the MYCT1 promoter in normal cells when compared to that in cancer cells. This study also examined the relationship between promoter methylation and mRNA levels of MYCT1, noting that promoter hypermethylation results in a significant decrease in MYCT1 mRNA levels, while treatment of these methylated promoters with 5–aza would then result in an increase in mRNA levels of MYCT1. One final observation derived from this study was that methylation around the non–canonical E–box (–695 to –692) in MYCT1’s promoter interferes with the binding of the transcription c–Myc, hence affecting expression of MYCT1. (13).

All these studies, along with others focusing on this type of cancer, have confirmed that the aberrant epigenetic component plays a central role in the development of LSCC and proposed that treatments targeting these changes may play a role in the development of a better overall prognosis.

The Role of DNA Methylation in Acute Myeloid Leukaemia

Acute Myeloid Leukaemia (AML) is a tumour which is characterized by an increase in the number of myeloid cells in bone marrow arrested in a blast phase, often leading to hematopoietic insufficiency (16). Like many other cancers, studies on hematologic malignancies have shown both defects in the classical cytosolic signalling molecules and in transcription factors, which usually regulate haematopoiesis. (17) Aberrant changes in the epigenetic regulatory pathways which are known to establish stem cell function and differentiation are also seen, along with mutations in a number of epigenetic regulators such as DNMT3A and isocitrate dehydrogenase 1 and 2 (IDH1 and IDH2), amongst others (18). Hence, the accepted model is that for full–blown leukaemia (such as AML) to occur, there must be a mixture of Class I mutations (i.e. mutations in genes coding for proteins involved in signal transduction pathways and/or proteins which are important for human stem cell survival), Class II mutations (i.e. mutations in genes which code for transcription factors or mutations which inhibit differentiation and/or in apoptosis) (19, 20) and Class III mutations (i.e. mutations in genes which are involved in epigenetic regulation e.g. genes coding for DNMT3A) (21).

Recent studies were focused on the role of DNMTs in aberrant methylation patterns observed in hematologic malignancies such as AML. All human blood cells are derived from hematopoietic stem cells (HSCs) which have two important characteristics: self–renewal and the ability to differentiate. The balance between these two characteristics of HSCs is key to correct development and it is thought that methylation of DNA within the HSCs, which is brought about by DNMTs, plays a crucial role in maintaining this balance (21).

Recent studies have shown that HSCs lacking functional DNMTs exhibited a number of genes with markedly changed expression. The study found that genes involved in self–renewal showed increased expression, while genes responsible for differentiation appeared to suffer a decrease in expression when compared to a control (22). In support to these findings, another study reported that cells with mutated DNMT3A, and which exhibit hypomethylation in regions corresponding to genes of the HOXB gene family (especially HOXB4), appear to be more inclined towards the process self–renewal as a response to multiple extrinsic signals (23). Other genes such as STAT1, CCND1, MYC and RUNX1 were also found to be hypomethylated in HSCs with a mutated DNMT3A (22). Hence, these findings suggest that DNMT3A malfunction may result in activation of genes which promote self–renewal of HSCs, blocking them in the HSC or Myeloid blast cell stage, but further studies are required in order to understand the exact underlying mechanisms (24).

Other studies conducted on leukaemia stem cells (LSCs) have shown that DNMT1 is another important factor in self–renewal of LSCs and hence contributes to the development of haemological malignancies such as AML (25). LSCs are leukaemia cells which are able to initiate tumorigenesis by self–renewal and differentiation processes like those seen in HSCs. The identification of the important role played by DNMT1 in these cells resulted in the initiation of studies, such as that conducted by Trowbridge et al. in 2012, which aim to uncover the potential therapeutic effects which may arise if the DNMT1 in LSCs is inhibited. This study showed that
mice with DNMT1 haploinsufficiency exhibited lower LSC self-renewal and decreased leukemogenesis without changes in normal haematopoiesis (26). This finding may set the foundation for further studies which would focus the development of DNMT1 inhibitors which may be used in haematological malignancies such as AML.

Hence, the understanding of DNA methylation and other epigenetic processes in carcinogenesis is of vital importance as it would allow researchers to focus on both diagnostic and therapeutic techniques which may improve prognosis of certain cancer-types. A number of studies have already adopted this idea and researchers are actively working to come up with these diagnostic and therapeutic techniques, some of which are outlined in the next section.

**Methylation Profiling – The future of early cancer diagnosis?**

Methylation profiling has been studied intensively over the past couple of years and is based on the fact that, by determining the DNA methylation patterns within an individual’s genome, one might be able to discover what diseases that individual is susceptible to either due to the inherited or due to environmentally acquired patterns of DNA methylation.

There are two main approaches towards methylation profiling: genome-wide methylation profiling or profiling of methylation patterns at specific CpG sites, and both approaches are brought about using the same methods which involve fluorescence, colorimetric testing, restriction enzymes which are methylation sensitive and Polymerase chain reaction (PCR) based techniques. (27)

To date, a number of studies have been conducted in order to analyze global DNA methylation patterns observed in white blood cells (WBCs) from patients with cancers of the stomach (28) and of the head and neck (29), amongst others. The results of these studies noted a difference in risk for cancer between those individuals which fell into the lowest quantile of global DNA methylation and those which fell into the highest quantile, with those in the lowest quantile of global DNA methylation exhibiting an increased risk (30).

A few other studies, including one conducted by Langevin et al. in 2012 (31) focused inspecting the DNA methylation patterns on individual genes rather than those of the whole genome. The idea is to identify genes which are typically hypermethylated or hypomethylated in the cancer type under review and use them as biomarkers which will aid in establishing the individual’s risk of developing that cancer. While most of the studies have focused on finding biomarkers in breast and colon, Langevin and colleagues set out to discover blood-based biomarkers for Head and Neck Squamous Cell Carcinomas (HNSCCs). In the study, six CpG loci were identified, (regions associated with the FGD4, SERPINF1, WDR39 (CIAO1), IL27, HYAL2 and PLEKHA6 genes), and the methylation levels of these loci were compared between individuals with HNSCC and controls. The results of this study proved quite promising. Langevin and colleagues have managed to distinguish HNSCC cases from control subjects accurately in most cases, showing that using gene methylation as a biomarker for cancer may set the path for future diagnostic techniques.

Despite the numerous developments in the field of biomarkers and our vast understanding of the role of DNA methylation in cancer initiation and progression, a lot of research needs still to be done in order to determine which genes to look for in a particular cancer type, what better methods are available over the ones currently used and, most importantly, we have to find a way how to make methylation profiling cheaper and more accessible so that the results obtained can be used by clinicians in the diagnosis of disease.

Directed Epigenetic therapy – Could this replace the conventional methods of radical chemotherapy?

The knowledge acquired over the years, starting in 1940 when Waddington first described epigenetics, have led to new fields of research which focus on finding drugs which target and reverse aberrant epigenetic changes in patients with a predisposition to or with established cancers which are due to these changes in the epigenetic aspect of DNA.

At present, DNA methyltransferase inhibitors (DNMTIs) that are nucleoside analogues of cytidine with a modified cytosine ring are the most commonly used drugs. These DNMTIs include 5–azacytidine, 5–aza–2′–deoxycytidine, 5–fluoro–2′–deoxycytidine, 5,6–dihyrod–5–azacytidine and zebularine (32). Despite the existence of a number of DNMTIs, only two of these were approved by the Food and Drug Administration (FDA) as of 2011: 5–azacytidine (Vidaza®) and 5–aza–2′–deoxycytidine (Decitabine®) (33), while the others were still undergoing clinical trials.

The mode of action of these drugs is relatively straightforward: once these DNMTIs are taken up by the cell, kinases convert them to nucleotides which are either incorporated directly into the cell’s DNA or first undergo ribose reduction and then become incorporated (34). Once incorporated into the cell’s DNA, the only step left is that initiated when a DNMT comes into close proximity to the nucleotide. Due to its modified cytosine ring, the nucleotide is able to form a covalent bond with the DNMT, resulting in its inactivation. As more DNMTs are inactivated, the cells...
would not be able to maintain their methylation patterns after every division, hence resulting in hypomethylated DNA (34, 35).

Other DNMTIs known as non–nucleoside DNMT inhibitors have also been described. Unlike the nucleoside DNMT inhibitors mentioned above, these drugs do not incorporate themselves into the cell’s DNA but are thought to function either by disrupting the interaction between DNMTs and their target sites or by blocking the catalytic site found on DNMTI. Examples of these non–nucleoside DNMT inhibitors are Procaine, which acts by disrupting the binding of DNMTs to their binding site (36), and hydralazine, which acts by blocking the catalytic site on DNMTI (37).

Following the discovery of DNMTI’s mode of action, many wondered whether such therapy leading to hypomethylation could activate genes which are normally silenced in healthy cells, giving rise to tumourigenesis. This was reviewed by Jones and Baylin in 2002 who stated that this issue is not of major concern since DNA methylation is only one of the many methods cells use in order to silence genes. Hence, inhibition of DNA methylation in a cell would not inhibit gene silencing completely since other mechanisms are still functional (38).

However, treatment with DNMT inhibitors seems to have one major drawback: discontinuation of treatment appears to result in reactivation of inhibited DNMTs and remethylation of genes, leading to their silencing (39). This, along with some other minor concerns related to dosage or drug interactions, are areas which pharmaceutical companies will shift their focus to in order to improve the quality and efficacy of these commonly used DNMT inhibitors.

Drugs acting on aberrant DNA methylation patterns or DNMTs are only the tip of the iceberg. A vast number of drugs which act on epigenetic changes in cancer are already undergoing clinical trials and are expected to be available for use by physicians in the coming years, to aid in the fight against the epigenetic aspect of cancer development and progression. Examples of such drugs include: SS110 which is a DNA methyltransferase inhibitor, Sirtinol and Salermide which are SIRTI protein inhibitors. These three appear to be effective against various tumour types. More specific drugs include Entinostat (MS–275) which is a Benzamide histone acetylase inhibitor effective against Lung tumours, Valproic Acid (Depakote®) which is a histone deacetylase inhibitor effective against Multiple Myelona, Melanoma and Glimonas and DZNep (Deazaneplanocin®) a histone methyltransferase inhibitor effective against AML (40).

Conclusion

The field of epigenetics is very vast and a lot is left to be explored and to be discovered. However, the discoveries made so far have led to a new perception on the mechanisms behind the development and progression of diseases such as cancer. Past studies have already opened up a large number of fields which scientists can explore in order to uncover more of the hidden mysteries which lie in the epigenetic modification of our genome. Our understanding of DNA methylation is continuously growing and ongoing studies are shedding a lot of light on the potential this particular epigenetic modification may have on the development of new diagnostic techniques based on methylation profiling of the genome and by using DNA methylation patterns observed in specific genes as biomarkers for cancer. Treatment modalities such as DNMT inhibitors have already been described and approved, while many other therapeutic agents are undergoing clinical trials. These advances in our comprehension of epigenetic modifications, specifically DNA methylation, will give hope to patients and their relatives, as well as to physicians and improving the chance that patient–tailored therapy is possible.

List of abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CpG</td>
<td>Cytosine–phosphate–guanine</td>
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<td>DNMT</td>
<td>DNA methyltransferase</td>
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<td>MBD</td>
<td>Methyl–binding domain protein</td>
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<td>AML</td>
<td>Acute Myeloid Leukaemia</td>
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<tr>
<td>TSG</td>
<td>Tumour Suppressor Gene</td>
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<td>LSCC</td>
<td>Laryngeal squamous cell carcinoma</td>
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<td>HSC</td>
<td>Haemopoietic stem cell</td>
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<tr>
<td>LSC</td>
<td>Leukaemia stem cell</td>
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<tr>
<td>HNSCC</td>
<td>Head and Neck Squamous Cell Carcinoma</td>
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<tr>
<td>DNMTi</td>
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Author’s contributions

1. **G. Maresca**, Medical Student, University of Malta Medical School, Msida, Malta – performed the literature research, analysis of data from the literature and wrote the manuscript.

2. **P. Schembri Wismayer**, Doctor and Senior Lecturer, Department of Anatomy of the University of Malta Medical School, Msida, Malta – Contributed towards the literature research and reviewed the manuscript prior to submission.
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