The Role of DNA Methylation in Ageing and Cancer

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Abstract

The aim of this review paper is to survey the literature related to DNA methylation, and its association with cancer and ageing. The review will outline the key factors, including diet, which modulate DNA methylation. Our rationale for conducting this review is that ageing and diseases, including cancer, are often accompanied by aberrant DNA methylation; a key epigenetic process, which is crucial to the regulation of gene expression. Significantly, it has been observed that with age and certain disease states, DNA methylation status can become disrupted. For instance, a broad array of cancers are associated with promoter-specific hypermethylation and concomitant gene silencing. This review highlights that hypermethylation, and gene silencing, of the EN1 gene promoter, a crucial homeobox gene, has been detected in various forms of cancer. This has led to this region being proposed as a potential biomarker for diseases such as cancer. We conclude the review by describing a recently developed novel electrochemical method which can be used to quantify the level of methylation within the EN1 promoter and emphasise the growing trend in the use of electrochemical techniques for the detection of aberrant DNA methylation.

1. Introduction

The epigenetic mechanism of DNA methylation controls gene expression and repression (1). DNA methylation refers to the covalent addition of methyl (CH_3) groups to the carbon 5 position of the pyrimidine ring of cytosines, typically in a CpG dinucleotide, of which there are approximately 28 million in the haploid genome of a human (2). Regions of DNA with a high CpG content are referred to as CpG islands (CGIs). There are approximately 45,000 CGIs per human haploid genome (3), which are typically between 200 and 1400bp in length (4) and generally located around transcription start sites (5). Saxonov, Berg and Brutlag determined that 72% of promoters are rich in predominantly unmethylated CpG (5). DNA methylation varies due to a number of factors including, age and disease status. Interestingly, hypermethylation of CpG sites in promoters or enhancers typically leads to transcriptional silencing, whereas hypomethylation of CpG sites in a gene body frequently results in an increase in gene expression (6, 7).

As outlined in Figure 1, the production of 5-methylcytosine is regulated by DNA methyltransferases (DNMTs) DNMT1, DNMT3A and DNMT3B, which transfer methyl groups from S-adenosyl-L-methionine. DNMT1 primarily acts as a maintenance methyltransferase, targeting hemimethylated DNA, formed after DNA replication, thus ensuring the re-establishment of the parental DNA methylation pattern in daughter DNA (8); while DNMT3A and DNMT3B act as *de novo* DNA methyltransferases (9). Additionally, there is another member of the DNMT3 family, DNMT3L. Although catalytically inactive, DNMT3L has been observed to markedly stimulate the *de novo* methylation by DNMT3A of DNA when coexpressed (10).

Demethylation can be either passive, through incorrect DNA replication, or be an active process regulated by ten-eleven translocation (TET) enzymes. TET catalyse the oxidation of 5-methylcytosine to 5-carboxylcytosine via the intermediates 5-hydroxymethlcytosine and 5-formylcytosine. Thymine DNA glycosylase (TDG) then removes 5-carboxylcytosine and 5-formylcytosine from the DNA strand allowing the insertion of an unmethylated cytosine into the deleted base site through base excision repair (BER) (11).

2. Impact of Ageing on DNA Methylation

During ageing, epigenetic drift can be used to describe the increase in methylation in CGI sites which are unmethylated in the young, and the decrease in methylation globally. These findings have been reported across species (12). Maegawa et al. (2017) showed that average methylation increased from 2±0.1% to 18±5%, 2±0.3% to 22±3%, and 3±0.5% to 20±4 with age in sites observed to be unmethylated in young mice, rhesus monkeys and human subjects respectively. When analysing highly methylated non-CGI sites, ageing resulted in a reduction in methylation from 94±0.4% to 78±4%, 94±0.3% to 73±4%, and 93±1% to 74±2% in the same three mammalian species. These data indicate that methylation drift associated with ageing is evolutionarily conserved. Interestingly, drift rates were calculated as 4.1±1.2%, 0.34±0.14% and 0.1±0.02% per year for mice, rhesus monkeys and human respectively, and an inverse relationship between the rate of methylation drift and longevity in these mammalian species was established (12). A similar finding was described by Wilson et al. (1987). In this work, ageing resulted in a global decrease in 5-methyldeoxycytidine in multiple tissues from 2 murine models and human bronchial epithelial cells obtained from autopsy donors, and an inverse relationship between lifespan and rate of loss of 5-methyldeoxycytidine was reported. An estimated loss of 5.6-8.9 $x10^5$ and 2.3-2.8 $x10^5$ per year was observed for *Mus musculus* and *Peromyscus leucopus* species which have lifespans of 3.5 and 8.0 years respectively, while a loss of $1.6x10^4$/year was observed in human cells (13). Their conclusion corroborates with the findings of Drinkwater et al. (1989), where it was determined that lymphocytes obtained from 20-30 year old volunteer donors contained 54.6±1.6% methylated CmCGG sites, while a statistically significant 7.1% reduction (47.5±2.6%) was observed in 65-80 year olds (14).

Maegawa et al. (2017) further examined if methylation drift is ubiquitous in differing tissue types. By analysing 12 genes which were associated with hypermethylation and 3 associated with hypomethylation with age, it was determined that tissue from kidney and liver generally exhibited lower levels of age related hypermethylation. In contrast, tissue from the intestines (small and large) and bone marrow showed reduced age associated hypomethylation. In further investigations by Maegawa et al. (2017) it was reported that there was a significant inverse relationship between methylation drift and the level of change in gene expression. It was noted that when looking at the methylation pattern of genes which had increased expression, a significant reduction in DNA methylation was observed, while conversely, silenced genes had a concomitant increase in methylation.

Altered expression of the enzymes responsible for DNA methylation and demethylation have been repeatedly reported to be a contributing factor to changes observed in DNA methylation patterns with age. For instance, Sun et al. (2014) observed the expression of genes encoding for the DNA methyltransferases dnmt1, dnmt3a, and dnmt3b, considerably declined between the ages of 4 months and 24 months in C57BL/6 male mice. Interestingly, the expression of demethylation enzymes tet1 and tet3 were also reduced with age (15). In humans, a reduction in TET1 and TET3 expression was observed with age and a correlation between TET1 and DNMT1, DNMT3B and TDG was determined in peripheral blood mononuclear cells obtained from 188 volunteers, aged 34-74, from eight European countries. Interestingly, while a global reduction in 5-hydroxymethylcytosine (5hmC) was also detected with age, a statistically significant increase in the methylation of the CpG islands within the TET1 gene was found in 69-74 year olds when compared to 34-41 year olds (16). These findings are consistent with the observation that hypermethylation within certain gene regions is often associated with gene silencing.

In contrast to the findings of Sun et al. (2014), Lopatina et al. (2001) showed that although dnmt1 declined with age in WI-38 fibroblast cells, the activity of *de novo* methylation enzymes decreased in middle age, compared with young cells, and rose slightly with senescence. This resulted in the ratio of *de novo* to maintenance methylation enzymes increasing with age. The authors postulate the decline in dnmt1 could lead to the global hypomethylation, and add that the rise in the ratio of *de novo* to maintenance methylation enzymes with age could be responsible for the regional hypermethylation associated with gene silencing (17). Similarly, Casillas Jr et al. (2003) observed that Dnmt1 expression declined significantly with age in foetal human WI-38 fibroblasts, with old aged cells having 75.44% of the expression exhibited by young cells. Furthermore, the activity of this maintenance methyltransferase declined from 83.2cpm/μg protein in young cells to 52.1cpm/μg protein in middle aged cells, and to 28.1cpm/μg protein in old lung WI-38 fibroblast cells. Conversely, the activity of the *de novo* methyltransferases increased from 21.4cpm/μg protein in young cells to 59.0 and 75.0cpm/μg protein in middle aged and old cells respectively. Interestingly, ageing appeared to have an opposing effect on the expression of the *de novo* methyltransferases, with Dnmt3a declining to 60.61% that of young cells in old age, while expression of Dnmt3b in young cells was 75.21% compared to that expressed by old cells. Thus a change in the ratio between maintenance methyltransferases and *de novo* methyltransferases could be a key factor in the aberrant DNA methylation associated with ageing.

3. DNA Methylation and Cancer

Germline cells have specific DNA patterns to enable suitable gene regulation during embryonic development. Importantly, within a small proportion of genes, one parental allele is exclusively expressed, due to DNA methylation regulated gene imprinting (18). Inappropriate methylation during development can result in imprinting failures and diseases including Beckwith-Wiedemann, Prader-Willi, Silver-Russell and Angelmans's syndromes (19). Epigenetic modifications are also frequently seen in diseases with later onsets; including cancer (20), neurodegeneration (21), and autoimmune disease (22). With a focus on the effect of epigenetic modification on cancer pathogenesis, both gene silencing, due to hypermethylation in gene promoters (23), and oncogene activation or chromosomal instability due to global hypomethylation (24, 25) will be discussed.

In one study of promoter hypermethylation, it was reported that in 7/9 non-small cell lung cancers the tumour suppressor gene *p16* was fully methylated, while the CpG islands in samples of healthy lung, kidney, and blood lymphocytes the CpG sites were found to be unmethylated (23). Interestingly, Christensen et al. (2010) discovered through locus-by-locus analysis, a trend between loci methylation and cancer characteristics, including tumour grade and size, oestrogen and progesterone status and triple negative status in invasive breast cancer specimens, from 162 women from Northern California. Interestingly, at all 74 CpG loci which were associated with tumour size, there was a positive correlation between the level of methylation and tumour size. Moreover, increased methylation was observed in all 5 CpG loci associated with lymph node infiltration, when disease-positive lymph nodes were reported. Array validation revealed CpGs within the promoters of P2RX7, a gene encoding for a receptor which mediates apoptosis, and HSD17B12, a gene coding for an enzyme involved in oestrogen metabolism and fatty acid elongation, had statistically elevated methylation levels as tumour size increased, while methylation of CpGs within the promoter of GSTM2, which reduced mRNA expression of the detoxifying enzyme GSTM2, was correlated with tumour grade (26).

Similarly to the aberrant DNA methylation associated with ageing, disease associated changes to the methylome could be due to changes in DNMT expression. For instance, in a study of 76 women with primary cervical cancer, DNMT1 was on average observed in 77.5% of cancer cells, in comparison to only 16% of normal cells. In addition, the intensity score was calculated as 1.0 for cancerous cells compared with a reduced figure of 0.2 for normal cells. Interestingly,

individuals with >77.5% DNMT1 positive cells were 4.3 times more likely to die prematurely compared with individuals who exhibited <77.5% DNMT1 positive cells, while those with an intensity score >0.9625 were 4.9 times more likely to die earlier than those <0.9625 (27). Furthermore, Mizuno et al. (2001) determined that in 33 patients with acute myeloid leukaemia (AML), DNMT1, DNMT3A and DNMT3B exhibited an average 5.3, 4.4 and 11.7 fold increase in comparison to levels observed in control bone marrow cells (28). Interestingly, $p15^{INA4B}$, a tumour suppressor gene commonly silenced by methylation in AML, was methylated in 72% of AML patients, and in these 24 cases, DNMT1 was statistically higher than those without $p15^{INA4B}$ methylation. Further examination of chronic myeloid leukaemia cells revealed that DNMT expression was phase dependent. During the chronic phase, expression of these three methyltransferases was comparable to normal bone marrow cells, however, with advancement to the acute phase, DNMT1, DNMT3A and DNMT3B expression was raised with an average 3.2, 4.5 and 3.4 fold increase respectively (29). Conversely, Gaudet et al. (2003) reported that mice exhibiting 10% of DNMT1 compared with wild type mice, exhibited a 30% reduction in birth weight, and 80% developed aggressive T cell lymphoma within 8 months (24). While examining hypomethylated tumours, it was determined that 10/12 exhibited chromosomal instability (gain of chromosome 15), in comparison to only 2/12 Moloney murine leukaemia virus induced tumours, thus indicating that global hypomethylation can also play a role in the pathogenesis of cancer through chromosomal instability.

3.1. EN1 Gene and Disease

The EN1 gene encodes for the protein homeobox protein engrailed-1. First characterised in *Drosophila*, EN1 mutation results in abnormal development including posterior-anterior duplications and malformation of the wings (30). Within humans, the EN1 gene has been associated with pattern formation within the central nervous system during development (31). Wilson et al. (2011) detail that expression of EN1 is observed within multiple neuronal cell types within the cerebellum, and that significant changes to its distribution occurs during gestation, with expression remaining until >21 days (32).

Hypermethylation of this gene has been observed in multiple cancer types including colorectal (33), prostate (34), and breast cancer (35). For instance, Bell et al. (2011) reported that the EN1 gene transcriptional start site exhibited significant hypermethylation in human salivary gland adenoid cystic carcinoma when compared with normal tissue, with a 59% difference in

methylation across the EN1 promoter. Furthermore, the extent of hypermethylation was correlated with tumour grading, location and patient outcome. Significantly, it was observed that out of 32 loci, the EN1 gene displayed the greatest difference in methylation between normal and diseased tissue, and little variation in hypermethylation across 9 CpG islands, thus emphasising its possible use as a biomarker in cancer (36). Similarly for prostate cancer, differential methylation between normal and cancerous cells was greatest in the EN1 gene (34). In addition, the EN1 gene was most frequently methylated in colorectal cancer when compared to the SCTR and INHBB genes. Interestingly, EN1 was more likely to be methylated in colorectal carcinoma compared to colorectal adenoma, with 73% (66/90) colorectal carcinomas and 40% (4/10) adenomas showing hypermethylation, and result in gene silencing (33). Similarly, Frigola determined that the EN1 gene was hypermethylated in 70% of colorectal tumours, and found hypermethylation resulted in suppression of the EN1 gene (37). Importantly, Mayor et al. (2009) outlined that only 1.12% (1/89) of EN1 genes in normal samples exhibited hypermethylation, an important factor when searching for a cancer biomarker. Interestingly, EN1 methylation resulted in approximately a 30% reduced survival rate after 5 years compared to patients without hypermethylation of the EN1 gene (33).

4. Effect of Poor Diet on DNA Methylation and Disease

There is a strong association between poor diet, obesity, and cancer (38). For instance, Zhang et al. (2017) examined the effect of DNA methylation in rats fed a high fat diet for 14 weeks, and reported that within 1000bp of transcriptional start sites of known genes, 7 genes exhibited differentially methylated CpGs. Differential CpG methylation ranged from 5-22% difference, in addition to altering gene expression, in animals which gained approximately 90% body mass from the high fat diet in comparison to rats fed a standard chow diet. When expanding to CpGs within 10,000bp of transcriptional start sites, 147 genes were differentially methylated and expressed. One of the genes of note, *Phlda1*, became hypermethylated with a high fat diet, which was associated with reduced expression, and in turn steatosis, a contributor to the pathophysiology of obesity (39). Furthermore, Vucetic, Kimmel, and Reyes (2011) outlined that mice fed a high fat diet (60% fat) from weaning at 3 weeks, until 18-20 weeks, showed significant hypermethylation in the μ-opioid receptor (MOR) promoter in reward-related brain regions, and repression of the MOR gene, which was related to an increase in binding of the transcriptional repressor methyl CpG binding protein 2 (MeCP2). It was suggested that

repression of the MOR gene was responsible for a significantly reduced preference for sucrose; thus indicating that animals on a high fat diet exhibit reward hypofunctioning, which may contribute to difficulties reversing obesity after long term exposure to highly palatable foods (40). As mentioned, obesity is strongly linked with cancer (41). For instance, it has been found that many of the 31 differentially methylated CpGs in obese children, and 151 differentially methylated CpGs in severely obese children discussed by Fradin et al. (2017) are also associated with cancer, thus warranting concern regarding the risk for cancer pathogenesis in later life (42). Similar results were observed by Xu et al. (2013), who examined differentially methylated CpG sites in 48 obese African American participants aged 14-20 years old compared to their non-obese counterparts (43). It is important to note that the type of ingested fat may differentially methylate DNA. Garcia-Escobar et al. (2017) examined the effect of different fats on TNFα promoter methylation, and reported reduced methylation in animals who were fed coconut oil (high SFA), which was inversely correlated with the pro-inflammatory cytokine TNFα in adipocytes (44).

5. Aberrant DNA Methylation Therapy

There is increasing evidence suggesting the influence of lifestyle factors such as diet, physical activity, weight, and smoking status, on the methylome and age related disease. Due to the ability to somewhat modulate and reverse methylation using lifestyle factors, targeting the methylome more rigorously with chemotherapy could provide a promising avenue to treat diseases such as cancer.

5.1. Diet

The role of diet in modulating metabolic health throughout lifespan has long been known. For instance, a significant amount of insight has been gained from analysing the impact of being born during the Dutch Hunger Winter, which took place in the Netherlands during world war two. It is now emerging that changes to DNA methylation could be a central player in directing how the deleterious effects of the Dutch Hunger Winter unfold. A recent genome-scale analysis of differential DNA methylation in whole blood after periconceptional exposure to famine during the Dutch Hunger Winter emphasises this phenomenon (45). Following a thorough assessment of prenatal malnutrition-associated differentially methylated regions (P-DMRs), it

was found that P-DMRs which preferentially occur at regulatory regions, are characterized by intermediate levels of DNA methylation, and map to genes enriched for differential expression during early development. Moreover, it was revealed differential methylation of P-DMRs was associated with pathways which are defined by growth and metabolism. P-DMRs found in the insulin receptor precursor gene and the carnitine palmitoyltransferase 1A gene (involved in fat metabolism) were found to have enhancer activity *in vitro* and differential methylation was interconnected with birth weight and serum low density lipoprotein-cholesterol levels. In addition to the findings from studying those exposed to the Dutch Hunger Winter it has also been recognised by Barker since the mid-1990s that exposure to a suboptimal intrauterine environment has deleterious metabolic consequences for later life (46). Similar to the Dutch Hunger Winter, recent studies have revealed that this phenomenon is underpinned by epigenetic regulation. For instance, it has been shown that placental leptin gene DNA methylation levels were correlated with glucose levels (2-hours post-oral glucose tolerance test) in women with impaired glucose tolerance and with decreased leptin gene expression in the whole cohort (47). The methylome is not simply a nutrient sensor during the intrauterine period. Strikingly, in a recent study DNA methylation changes were correlated with body composition in pre-school children as part of the epigenome-Wide-Analysis in the European Childhood Obesity Project (CHOP)-Study. It was found DNA methylation variants were identified to be associated with BMI, fat-mass, fat-free-mass, fat-mass-index and fat-free-mass-index (48). Specific aspects of diet have also been associated with DNA methylation changes. As discussed, the effect of poor diet on aberrant DNA methylation and disease pathogenesis can be significant, therefore it is conceivable that a healthy diet could play a role in the prevention of aberrant DNA methylation. For instance, plant polyphenols, originating in fruit and beverages often associated with healthy diets, have been associated with reduced oxidative stress, inflammation and risk of cancer (49), which could be mediated through modulation of DNA methylation (50). In one example, polyphenols associated with the Mediterranean Annuraca apple, reportedly increased p53, reduced methylation in the promoters of hMLH1, p14ARF, and p16INK4a, restoring normal expression of silenced tumour suppressor genes in colorectal cancers (51). In another example, it was observed that 2 weeks of 6g/day of cocoa, a rich source of polyphenols, led to 2.991±0.366 %5mC in comparison to 3.909±0.380 %5mC in peripheral leukocyte DNA from control participants with pre-hypertension, type 1 hypertension, or hypercholesterolaemia in a randomised controlled trial. Furthermore, *in vitro* treatment of subject peripheral blood mononuclear cells, revealed cocoa significantly lowered DNMT1, 3a and 3b mRNA expression in addition to

methylenetetrahydrofolate reductase (MTHFR) and 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (MTRR) gene expression (52). Similar results were observed by Nandakumar et al. (2011) who reported that green tea polyphenols epicatechin-gallate and epigallocatechin-3-galate significantly lowered DNMT1, 3a and 3b activity and expression in a dose dependent manner, reduced global methylation, and reactivated the silenced tumour suppressor genes $p16^{INK4a}$ and Cip1/p21 in human skin cancer A431 cells (53).

5.2. Folate Feeding Studies

One of the most studied supplements in regards to DNA methylation is folate. This is because, folate plays a key role in one carbon metabolism through its conversion to N-5-methyltetrahydrofolate (5-methylTHF), which in turn is converted to SAM, the global methyl donor in DNA methylation (54). Methyl group deprivation can lead to changes in one carbon folate metabolism, metabolites, which can irreversibly perturb DNA methylation and interestingly, induce lesions associated with the pathogenesis of cancer. For instance, Pogribny et al. (2006) reported that male F344 rats fed a diet deficient in methyl groups (low methionine and choline, and folic acid negative) for 9 weeks, exhibited a 70% reduction in SAM when compared to mice on a control diet, while SAH was unaffected, thus a significant decrease in the SAM/SAH ratio, an important predictor of methylation capacity, was observed. The methyl-deficient diet also led to a 60% increase in unmethylated CCGG sites obtained from liver tissue. Interestingly, the reintroduction of a methyl-sufficient diet resulted in normalised DNA methylation in rats who were fed a methyl-deficient diet for 9 weeks. However, in rats fed a methyl-deficient diet for 18, 24 or 36 weeks, the reintroduction of a methyl-sufficient diet could not reverse the hypomethylation induced. Significantly, the appearance of glutathione-S-transferase π (GSTπ), a characteristic of hepatocarcinogenesis was observed despite the reintroduction of the methyl-sufficient diet, even after 9 weeks of exposure to a methyl-deficient diet (55). Additionally, Jung et al. (2011) reported that 800µg/day for 3 years of folic acid did not influence DNA methylation in moderately hyperhomocysteinemic Dutch males and females aged 50-70 years (56).

In contrast, results from Pufulete et al. (2005) suggest DNA hypomethylation brought about by a low dietary intake of folate could be reversed by folate supplementation. It was reported that a 400µg/day for 10 weeks supplement of folic acid, in patients with colorectal adenoma, increased serum folate from 7.4 to 13.4µg/l, and plasma homocysteine decreased 12%, while

DNA methylation increased by 31 and 25% in leucocytes and colonic mucosa respectively (57). Interestingly, Park et al. (2017) found folate supplementation produced distinct differences in DNA methylation patterns dependent on body weight. In this study, supplementation of 800µg/day for 8 weeks in normal weight and obese females aged 18-35 increased serum folate by 86.2 and 109.6% respectively. Before supplementation, 10.7% of CpG sites differed between the different weight categories; this rose to 15.2% after supplementation. Higher levels of methylation were observed in 52.9% and 55.0% of obese women before and after treatment respectively. After treatment, CpG sites were more likely to have reduced levels of methylation; 67.9 and 75.8% for normal weight and obese females respectively. Interestingly, while the supplementation induced methylation changes in genes associated with neural tube closures in women of normal weight, overweight women exhibited changes in methylation in genes associated with folate metabolism, methylation and vitamin B metabolism (58).

Conversely, a 3 month 100µg/day, 400µg/day, and a 4000µg/day supplement of folate resulted in an 11.5, 11.7 and 18.9% reduction in % 5 methyl-deoxycytidine respectively in coagulated blood samples from Chinese women of reproductive age, who showed an average % methyl-deoxycytidine level of 4.42±0.12% at enrolment. Interestingly, it was observed that genotype can influence the DNA methylation response to dietary folate. When analysing the effect of a 3 month 4000µg/day folate supplement in the presence of the MTHFR 677C→T variant, it was found that there was an 11.6, 18.8 and 19.5% reduction in % 5 methyl-deoxycytidine for the CC, CT and TT genotypes respectively compared to baseline results (59).

5.3. Caloric Restriction

Recent evidence has suggested that caloric restriction (CR), even in the short term, could potentially ameliorate aberrant methylation in disease associated genes as observed in age related methylation drift (60). Maegawa et al. (2017) detailed that CR was able to counteract hypermethylation associated with ageing without producing novel methylation patterns. In both DNA from whole blood of mice and Rhesus monkeys, a significant inverse relationship was observed between CR and the rate of methylation drift. Specifically, there was an average DNA methylation, across 24 genes, of 26±2% and 27±0.7% for aged mice and Rhesus monkeys fed ad libitum, compared to 17±0.7 and 24±0.9% in aged mice and Rhesus monkeys respectively who underwent CR (12). Hahn et al. (2017) showed that a 40% dietary restriction

in mice resulted in a reduced number of differentially methylated regions in DNA extracted from the liver. In aged mice fed ad libitum, age differentially methylated 3176 regions, of which 1945 became hypermethylated and 1231 became hypomethylated, whereas aged mice who underwent 40% DR exhibited only 2250 differentially methylated regions were identified, of which 1512 became hypermethylated and 738 became hypomethylated (61). To further this, Wang et al. (2017) reported that 40% CR resulted in a predicted 9.4 month reduction in epigenetic age within the livers of 22 month old mice compared with age-matched controls (62), therefore it appears that CR may provide a promising treatment strategy for aberrant DNA methylation.

5.4. Drug Therapy

Due to the association of hypermethylation of promoters and tumour suppressor gene silencing in cancer, the use of drugs which ameliorate this change may provide a successful method for reducing DNA methylation in these regions and enable the re-expression of these genes. Hypomethylating agents 5-azacytidine (azacytidine) or 5-Aza-2'-deoxycytidine (decitabine) are 2 such examples, which were approved for use by the European Medicines Agency (EMA) in 2008 and 2012 respectively for patients with myelodysplastic syndromes (MDS).

Following the cellular uptake of decitabine, it is phosphorylated to 5-aza-2'-deoxycytidine-triphosphate, and becomes incorporated into DNA strands in place of cytosines within CpG sites. The substitute nucleotide binds DNA methyltransferases similarly to cytosine, however due to the substitution of carbon-5 in the cytosine ring for nitrogen, β-elimination is inhibited and thus covalent bonding is irreversible. This results in enzyme inhibition and eventual degradation of the bound enzyme, and thus a reduction in DNA methylation. Azacytidine acts in a similar way, however it acts upon RNA. Interestingly, during phosphorylation, approximately 10-20% is converted to a 5-aza-2'-deoxycytidine-triphosphate precursor and thus acts upon DNA (63).

While meta-analysis data suggest that both Azacitadine and Decitabine are superior to best supportive care in patients with MDS (64), there is conflicting evidence on the superiority of these drugs. For instance, Lee et al. (2013) conducted a comparative analysis of Azacytidine, given for 7 days in a 28 day cycle, and Decitabine, given for 5 consecutive days in a 28 day cycle, in patients with myelodysplastic syndromes, and response rates of 46 and 52%, a median peak response at 4.2 and 4.0 months, and median survival time of 23.3 and 22.9 months were

reported respectively. While these parameters were not statistically different from one another, it was established that the survival rate was significantly improved in patients >65 taking Azacitidine, and patients showed reduced vulnerability to infection, in addition to a lower incidence of grade 3/4 of cytopenia (65). Xie, Jiang and Xie (2015) conducted a meta-analysis of 11 trials, containing 1392 MDS patients and similarly found that while there was no significant difference between the rate of complete response in patients undertaking Decitabine or Azacytidine treatment (13 vs. 12%), Azacitidine treatment resulted in a significantly higher overall response rate compared with Decitibine (73 vs. 42%). Moreover, a statistically significant improvement in overall survival was observed for Azacytidine treatment when compared to best supportive care, while no statistical difference was observed for Decitabine treatment (66).

In contrast, results from a randomised phase II trial in patients with low/intermediate-risk MDS or chronic myelomonocytic leukemia indicated an overall response rate of 70 and 49% for patients who received intravenous Decitabine or Azacytidine for 3 consecutive days, on a 28 day cycle, respectively. Furthermore, the one year event-free survival rate was significantly greater in patients who received Decitabine (74 vs. 55%). In addition, haematological improvements were observed in 24% of patients treated with Decitabine compared with 8% of Azacytidine patients, and of the patients who were transfusion dependent at the start of the trail, 32 and 16% became transfusion independent following Decitabine or Azacytidine treatment (67).

However, it is important to note the use of such hypomethylating agents should be used with caution due to selectivity concerns. For instance, in one study which used Azacytidine to treat the non-invasive breast cancer cell lines MCF-7 and ZR-75-1, the drug lowered DNMT1 and DNMT3b and methylation within the promoters of several pro-metastatic genes, including uPA and MMP2, leading to gene expression. Furthermore, it was shown that treatment increased the invasiveness of both cell lines (68).

6. Detecting DNA Methylation

In recent years a systems orientated approach has become common place in bioscience research (69-73). The essence of this approach is to utilise novel approaches to study molecules, cells, or entire organisms. Nutrition research is no different and is beginning to benefit from this new paradigm (74-80). It can be argued electrochemical techniques come under this umbrella of

systems techniques. For instance, recently, there has been heightened interest in using electrochemical techniques to detect DNA methylation as it can be a rapid, easy to use and cost effective solution to many of the challenges posed by more conventional methods and enables the quantitative analysis of complex biochemical systems (81).

There are several techniques that can be employed to analyse DNA methylation, many of which require prior bisulphite conversion, which converts unmethylated cytosines to uracil, while methylated cytosines remain unchanged. These techniques include bisulphite sequencing (82), methylation specific PCR (83), pyrosequencing (84) and immuno-based recognition (85). Methods which don't require prior bisulphite conversion include high performance liquid chromatography (86), mass spectrometry (87), microarray analysis (88), surface plasmon resonance (89) and surface enhanced Raman spectroscopy (90). Many of these methods have several draw backs including the need for expensive laboratory equipment and/or biological molecules coupled with long analysis times and the requirement for highly skilled operators.

An example of an elegant electrochemical DNA-methylation sensor, is the eMethylsorb method of Koo et al. (2014). The method consists of two steps. First, a gold electrode is exposed to a solution of bisulfite modified DNA. This exploits the findings of Kimura-Suda et al. (2003), who demonstrated that single stranded homo-oligonucleotides adsorbed onto gold with the following affinity $A > C \geq G > T$ (91). The DNA adsorption essentially blocks (or passivates) the gold surface, decreasing its reactivity. The lower the methylation level of the original DNA, the higher the number of adenines present in the bisulfite treated sample. Consequently, unmethylated DNA results in a more passivated and less reactive surface than methylated DNA. In the second step of the emethylsorb method, the reactivity of the gold electrode surface is measured in an electrochemical reaction. Initially, Koo et al. developed the eMethylsorb method using disposable gold screen printed electrodes (consisting of a 4mm diameter gold working electrode, gold counter electrode and silver reference electrode) exposed to solutions of synthetic oligonucleotides diluted in 5X SSC buffer, designed to represent bisulphite modified and asymmetrically amplified methylated and unmethylated versions of a 53 base section, containing 8 CpG sites, of the EN1 gene promoter. After the adsorption step, the reactivity of the modified gold service was measured by performing differential pulse voltammetry (DPV) in an electrolyte containing 2.5 mM ferrocyanide, 2.5 mM ferricyanide and 100mM KCl, where the peak current for the reduction of Fe^{3+} to Fe^{2+} inversely correlated with the level of DNA adsorption on the gold electrode.

Optimisation of the adsorption step revealed the greatest current response difference (between methylated and unmethylated samples) was observed when 50nM of synthetic oligonucleotides was adsorbed for 10 minutes (in quiescent solution) at pH 7.0. The method was used to successfully detect 10% methylation in heterogeneous samples of synthetic oligonucleotides. Furthermore, the technique was able to detect 10% methylation in heterogeneous samples containing various combinations of MCF-7 and whole genome amplified (WGA) DNA. Interestingly, the sensitivity of the method was significantly greater for these 140 base DNA samples in comparison to the 53 base synthetic oligonucleotides (92).

In a related study, the same research group, used a 2 mm gold disk working electrode (Pt counter electrode and Ag/AgCl reference electrode) to detect methlaytion levels in the same synthetic oligonucleotides (in 5X SSC buffer). The electrochemical reactivity of the modified gold surface was measured via DPV in a solution of 2.5 mM ferrocyanide, 2.5 mM ferricyanide and 10 mM PBS. Using the two-step eMethylsorb procedure, the greatest relative current difference was observed between methylated and unmethylated DNA, when 200nM DNA was adsorbed for 10 minutes (in quiescent solution) at pH 7.0. Again a negative linear relationship between % methylation in heterogeneous samples of synthetic methylated and unmethylated oligonucleotides and relative current response was observed ($R^2 = 0.99398$). Sina et al. (2014) also investigated the effect of the number of methylated CpG sites within the 53 base synthetic oligonucleotide (0, 1, 4, and 8). A negative linear relationship was observed between the number of methylated CpG sites and relative DPV current response ($R^2 = 0.971411$). Finally it was determined that only 20µl of secondary PCR product (from real DNA samples) in 200µl buffer was required to produce a considerable difference in relative current. Once again, the sensitivity of the method greatly improved on moving from synthetic to real DNA samples (89).

Our project set out to improve the repeatability and sensitivity of the eMethylsorb method via a new approach to the adsorption step and the electrochemical technique. The new procedure was optimised using 30 base synthetic oligonucleotides, containing 6 CpG sites, designed to represent bisulphite modified and asymmetrically amplified methylated and unmethylated versions of a region downstream of the transcription site of the EN1 gene promoter (93).

It was also imperative to test if % methylation could be determined using these optimised electrochemical procedures in a heterogeneous sample. This was to reflect biopsy samples gained in a clinical setting, as tumours are often found to contain cells exhibiting diverse

phenotypic features; including methylation status. This phenomenon, termed intra tumour heterogeneity, has been observed in multiple cancers including breast (94), lung (95), endometrial (96) and prostate cancer (97). To test the applicability of the procedure in detecting methylation in DNA derived from humans, the procedure was repeated using bisulphite modified and asymmetrically amplified 140 base ssDNA from the EN1 region of DNA extracted from the breast cancer cell line MCF-7 (methylated), and WGA DNA (unmethylated). Our method successfully detected high methylation levels in the breast cancer cells (93).

7. Conclusion

This review has highlighted a wide variety of dietary components which can influence DNA methylation status during life. Based on our survey of the literature it can be argued many instances of aberrant DNA methylation are the direct result of diet. Nowhere is this more apparent than cancer, because the methylation changes which are a hallmark of many cancers are influenced by dietary factors such as folate consumption, calorie intake, and polyphenol content. This review has reinforced the idea that identifying DNA methylation changes early could be an effective means of predicting cancer risk. An important take home message from our review is that the early detection of cancer could be achieved by monitoring methylation levels within biomarker genes such as the EN1 gene. Finally, this review has revealed that the goal of early cancer detection could be achieved by using novel electrochemical techniques to quantify DNA methylation levels. There is little doubt that techniques such as this will prove to an invaluable tool in the detection of cancer in the future.

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Conflicts of Interest

None.

Authorship

M.T.MA and T.J.D conceived the project. A.E.M and M.T.MA drafted the manuscript.

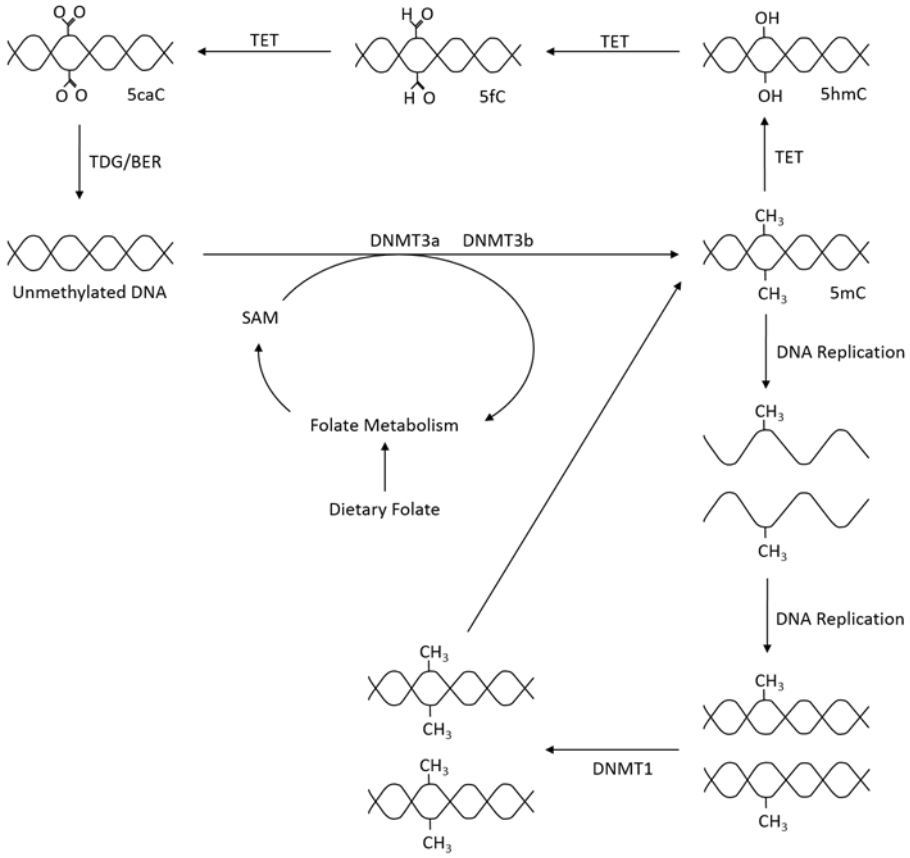

Figure 1 Overview of DNA Methylation.

De novo methylation is regulated by DNMT3a and DNMT3b and uses SAM as a methyl donor. The new methylation pattern is passed on to daughter cells through DNMT1, which acts on hemi-methylated DNA. DNA can become demethylated through the TET and TDG enzymes and BER. DNMT, DNA methyltransferase; TET, ten-eleven translocation enzymes; BER base excision repair, 5mC, 5-methylcytosine; 5hmC, 5-hydroxymethlcytosine; 5fC, 5-formylcytosine; 5caC, 5-carboxylcytosine.

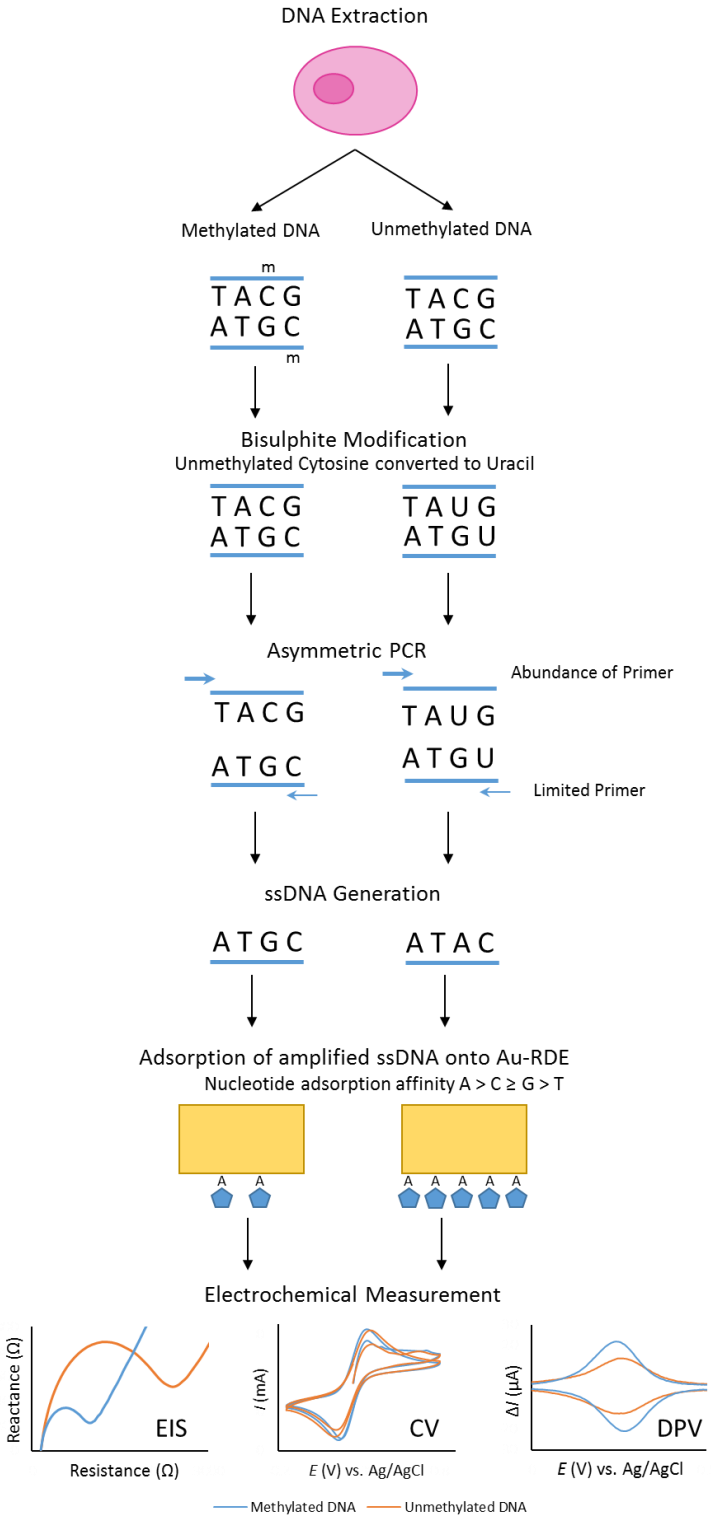
DNA Extraction
Methylated DNA
Unmethylated DNA
m
TACG
ATGC
m
TACG
ATGC
Bisulphite Modification
Unmethylated Cytosine converted to Uracil
TACG
ATGC
TAUG
ATGU
Asymmetric PCR
Abundance of Primer
TACG
ATGC
TAUG
ATGU
Limited Primer
ssDNA Generation
ATGC
ATAC
Adsorption of amplified ssDNA onto Au-RDE
Nucleotide adsorption affinity A > C ≥ G > T
A A
A A A A A
Electrochemical Measurement
Reactance (Ω)
EIS
Resistance (Ω)
i (mA)
CV
E (V) vs. Ag/AgCl
Δi (μA)
DPV
E (V) vs. Ag/AgCl
Methylated DNA
Unmethylated DNA

Figure 2 Overview of bisulphite treatment, asymmetric PCR and electrochemical measurement.

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