Pathogenesis of Type 2 Diabetic Retinopathy by the Epigenetic Modification of 5-Carboxylcytosine DNA Methylation in the CpG Island of Promoter Region of Brain Derived Neurotrophic Factor

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Abstract

Worldwide Type 2 Diabetic Retinopathy is one of the leading causes of blindness. It is the most frequently occurring complication of diabetes mellitus and remains a leading cause of vision loss in many countries. Although some potential functions of DNA methylation have been demonstrated already, many questions remain in terms of unveiling the role of 5caC; whether it serves either merely as an intermediate of DNA demethylation or as a stable epigenetic marker. 5-carboxylcytosine (5caC) is proved to be not merely serving as an intermediate of DNA demethylation, but also acts as a stable epigenetic marker. This review define how to control the gene expression and DNA Methylation in the CpGIsI and, The DNA Demethylation leads to convert the 5mc to 5hmc by the TeTprotein and again the 5hmc transfer to 5fc and further 5caC by the TeT enzyme in the BDNF gene. This review show the method to analyze the DNA Methylation at the 5caC region for the BDNF gene and the fully mechanism which show the demethylation from 5hmc to 5fc and the pathway of DNA Demethylation from cytosine to 5-caC (5-Carboxylcytosine).

Keywords: Epigenetic Modification, DNA methylation, BDNF gene, 5-caC, CpGIs, Diabetic Retinopathy.

Introduction

Pathology of diabetic retinopathy has been studied for half a century, yet there are disappointingly few therapeutic options. Though some new treatments have been made known for Diabetic Macular Edema (DME) (e.g. anti-VEGFs' (intravitreal vascular endothelial growth factor inhibitors) and other novel steroids), only up to 50% of patients could respond. Laser photocoagulation remains a mainstay therapy (even though it is inherently a destructive procedure)for people with proliferative diabetic retinopathy (PDR).[¹] In many diseases epigenetics is now emerging as one of the important factors as it can regulate the complex interplay between genes and the environment, and these heritable changes can occur without any change in the DNA sequence. Influenced by several factors
including age, environment, lifestyle and disease state epigenetic changes are regular and natural phenomenon. Epigenetic changes can work like switches helping to control gene activity and allowing alternations in genome function without altering the gene sequences. DNA methylation, histone modification and non-coding RNA is considered to initiate and sustain changes in gene regulation are major epigenetic modifications.\(^2\)

### Epigenetic mechanisms in the pathogenesis of type 2 diabetes

Methylation of cytosine to 5-methyl cytosine (5mC) is considered as one of the major epigenetic modifications; methylation of the CpG islands, a CG rich region in the promoter of many genes, changes protein-DNA interactions leading to alterations in chromatin structure, and this interferes with the binding of transcriptional machinery, resulting in gene suppression.\(^3\) DNA methylation is catalyzed by DNA methyltransferases (Dnmts), a family with five members-Dnmt1, Dnmt2, Dnmt3a, Dnmt3b and Dnmt3L; out of which only Dnmt1, Dnmt3a and Dnmt3b are catalytically active, and Dnmt3b are de novo methyltransferases, and Dnmt1 is a maintenance enzyme important in regulating tissue-specific patterns of methylated cytosine residues.\(^4\)

**Figure 1.** Role of epigenetic mechanisms in the pathogenesis of type 2 diabetes proposed by model.\(^5\)

### DNA Demethylation Pathways

By a reduction in activity or absence of DNA methyltransferases (Dnmts) (black) Passive DNA demethylation has long been known to occur. For de novo DNA methylation Dnmt3A and 3B are responsible. Whereas Dnmt1 maintains DNA methylation patterns through successive rounds of cell division. Three enzyme families have been implicated in active DNA demethylation via DNA repair recently.

1. Ten eleven translocation (TET) family of enzymes (blue) can hydroxylate 5-methylcytosine (5mC) to form 5-hydroxymethylcytosine (5hmC) or further oxidized to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC).

2. AID/APOBEC (Activation-induced Cytidine Deaminase /apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) family members (purple) can deaminate 5mC (or 5hmC) to form 5-methyluracil (5mU) or 5-hydroxymethyluracil (5hmU)

3. Uracil-DNA Glycosylase (UDG) family of base excision repair (BER) glycosylases (green) initiate replacement of these intermediates (i.e., 5mU, 5hmU, or 5caC) like TDG or SMUG1, culminating in cytosine replacement and DNA demethylation.\(^6\)

**Figure 2: 5-caC methylation pathway.**\(^6\)
With neurodegeneration pathological Dnmt activity and aberrant 5mC formation have been linked. 5mC can be oxidized to 5-hydroxymethylcytosine (5hmC) by ten-eleven translocation enzymes, and 5hmC can also be further oxidized to generate 5-formylcytosine (5fc) and 5-carboxylcytosine (5caC). By removing the 5-methylcytosine base a subfamily of DNA glycosylases are assumed to promote active DNA demethylation, followed by cleavage of the DNA backbone at the abasic site, and an unmethylated cytosine can replace methylated cytosine. In contrast, the passive process involves absence/inactivation of Dnmt1 resulting hypomethylated DNA.[7]

Epigenetic modifications (DNA methylation) and diabetes
Glucose is critically important for the organisms for survival, but sustained levels of high glucose are detrimental, and can initiate a number of metabolic, biochemical and genetic abnormalities. It affects regulation of genes throughout the body. Recent work has suggested that diabetic milieu favors epigenetic modifications in various organs associated with micro- and macro-vascular complication[8] S-Adenosyl methionine (SAM), the donor of methyl group for DNA methylation, is shown to influence the expression of genes related with diabetic complications, and blood deficiency of SAM is reported in the patients with diabetic nephropathy, and leukocytes from diabetic patients have reduced Dnmt1 levels. Global hypomethylation and reduced level of SAM are also observed in Zebrafish with chemically induced diabetes.[9] In contrast, in Zucker fatty rat, a model of Type 2 diabetes, sustained global DNA hypermethylation is observed in the liver, and this is associated with abnormal metabolism of the methyl group. These divergent patterns of DNA methylation in diabetes suggest that various tissues could be responding differently to diabetes.[10-11]

Epigenetic modification in diabetic retinopathy
Diabetic retinopathy, a slow progressing disease, is associated with a number of metabolic abnormalities. However, the role of epigenetic modifications in diabetic retinopathy is still not clear. In a Finnish study, an association between the polymorphism in SUV39H2, a gene that encodes histone methyltransferases and microvascular complications, including retinopathy has been observed in patients with diabetes. In a cross-sectional study with over 1,000 patients having type 2 diabetes, analysis of their family history has suggested a possible genetic and epigenetic basis for the development of diabetic retinopathy. Laboratory proof using in vitro and in vivo models of diabetic retinopathy have shown that the actions of HDACs are increased and that of HATs are decline in the retina and its capillary cells in diabetes, and global acetylation of histones is decreased.[12-13] DNA methylation, an important epigenetic modification, is closely associated with the regulation of gene transcription. A case control study using patients having type 2 diabetes has shown significantly higher levels of global DNA methylation in patients having diabetes with retinopathy compared to those with no retinopathy, and although global DNA methylation appears to be independent of retinopathy risk factors, e.g., hyperglycemia, dyslipidemia and hypertension, in these patients, the methylation status of DNA shows a correlation with the progression of retinopathy.[14-15]

Type 2 Diabetic Retinopathy
Type 2 Diabetic Retinopathy is microvascular complications of polygenic disease, and hyperglycemia remains because the major instigator of its development. Changes in the blood vessels of the retina can cause Type 2Diabetic Retinopathy. When these blood vessels area get injured, they leak blood and grow fragile new vessels. Once the nerve cells area gets damaged, vision is impaired. Diabetic retinopathy can cause vision loss in two ways: Macula Edema
and Proliferative retinopathy.\textsuperscript{[16-17]} Type 2 Diabetic Retinopathy classified into two clinical forms they are non proliferative diabetic retinopathy, proliferative diabetic retinopathy. Nonproliferative diabetic retinopathy subdivided into 3 different stages they are mild, moderate and severe.\textsuperscript{[18-19]} Nonproliferative diabetic retinopathy is being characterized by the quantity of microaneurysms, dot and blot hemorrhages, cotton wool spots and blood vessel abnormalities.\textsuperscript{[20]}

5. Ocular complications of diabetes Diabetic retinopathy

**Figure 3 (A)** Non-proliferative diabetic retinopathy. Wide-field fundus photo of 65-year-old female patient (right eye) showing several retinal hemorrhages

**Figure 3 (B)** Non-proliferative diabetic retinopathy. Color fundus photo of a 51-year-old male patient with micro-aneurysms and lipid exudates.\textsuperscript{[22]}

**Figure 4 (A)** Proliferative diabetic retinopathy with neovascularization at disk.

**Figure 4 (B)** Advanced proliferative diabetic retinopathy with neovascularization and limited vitreous hemorrhage.\textsuperscript{[22]}
Figure 5 (A) Advanced proliferative diabetic retinopathy with tractional retinal detachment.

Figure 5 (B) Neovascularization of the iris. These neovascular vessels may block the trabecular meshwork and cause neovascular glaucoma. [22]

BDNF (Brain Derived Neurotrophic Factor)

Nerve growth factor (NGF) was discovered in the early 1950s due to its trophic (survival- and growth-promoting) effects on sensory and sympathetic neurons. To support survival of a subpopulation of dorsal root ganglion neurons, and subsequently purified from pig brain, in 1992, Brain-derived neurotrophic factor (BDNF), the second member of the “neurotrophic” family of neurotrophic factors, was demonstrated. [23] Since then, other members of the neurotrophin family such as neurotrophin-3 (NT-3) [24] and neurotrophin-4/5 (NT-4/5) [25-26] have been described, each with a distinct profile of trophic effects on subpopulations of neurons in the peripheral and central nervous system. [26]

BDNF gene structure

The human BDNF gene is located on chromosome 11, region p13–14 and it spans ~ 70 kb. The gene has a complex structure as it consists of 11 exons (I–IX, plus Vh and VIIIh) in the 5′ end and of nine functional promoters that are used in tissue and brain regions specifically, namely exon I, II (with the transcripts Ia, Ib and Iic), III, IV, V (with the transcripts Va, Vb, Vc and V–VIII–VIIIh), VI (with the transcripts VIb, VIb–IXabd and VIb–IXbd), VII (with transcripts VIIa and VIIb) and IX (with transcripts IXabd and IXabcd; Figure 6). [27]
Human BDNF gene structure. In the figure the structure of the human BDNF gene is shown; the gene contains 11 exons (I–IX, plus Vh and VIIIh), which combine to many different transcripts. In exon IX has Coding DNA sequence (CDS). CpG islands and Alternative splice-donor sites are also reported BDNF. [27] Although the BDNF gene comprises nine exons, the coding sequence resides in exon 9, with eight upstream exons that encode promoters regulating regional and cell-type-specific expression. [27] Among these, exon IV has been the most extensively characterized, as this exon, containing promoter elements, regulates activity-dependent BDNF expression. [27]

**BDNF and Diabetic Retinopathy**

In the animal model with Type 2 Diabetic Retinopathy, few studies suggest that reduced level of BDNF in the Type 2 Diabetic Retinopathy may damage neurons, thereby leading to neuro degeneration. [28] Another study reported that through endothelial progenitor cells activation neuroic factors may induce neovascularization, leading to the pathological retinal neovascularisation found that BDNF was effective in protecting retinal neurons from hyperglycemia in vitro. The patients with Type 2 Diabetic Retinopathy were found to have a drastic reduction in the level of BDNF in retina. [29] If any alteration occurs in any signaling molecule such as BDNF, which in turn alter TrkB (receptor of BDNF) it may lead to cause Type 2 diabetic retinopathy. BDNF levels are lower in individuals with Type 2 Diabetes mellitus compared to non-diabetic individuals, both in plasma and serum, and in different populations. [30] It has been observed that difference in serum BDNF levels, DNA methylation patterns and genetic variants, across different glucose metabolic state advice that BDNF may be involved in the patho physiological process of insulin resistance and Type 2 Diabetes which may cause Type 2 Diabetic Retinopathy. [31]

**5-caC (5-Carboxylycytosine) DNA methylation**

When Tet enzymes oxidize 5-methylcytosine, 5-hydroxymethylcytosine and subsequently 5-formylcytosine5-Carboxylycytosine has been identified as one of the DNA methylation variants. It is believed that the oxidation of 5-methylcytosine through to 5-carboxylycytosine represents a mechanism of DNA demethylation, and that this demethylation pathway has a function during development and germ cell programming [32]. It has been suggested that 5-caC is excised from genomic DNA by thymine DNA glycosylase (TDG), which returns the cytosine residue back to its unmodified state. 5-Carboxylycytosine has been identified in mouse embryonic stem (ES) cells. [32-33] This DNA modification appears in the paternal pronucleus post-fertilization, concomitant with the disappearance of 5-methylcytosine, further lending support that this variant is part of a DNA demethylation pathway. Unmodified DNA standard and 5-carboxylycytosine DNA standard, which can be used as negative and positive controls, respectively, in the analysis of 5-carboxylycytosine. [34]

**Detection of 5’caC (5-Carboxylycytosine)**

MAB (methylation-assisted bisulfite sequencing) Method Enables Highly Efficient Detection of 5caC.

(A) Whereas the 5-methyl and 5-hydroxymethyl cytosine are protected Bisulfite treatment of DNA leads to deamination of cytosine, 5fC, and 5caC. Treatment of DNA with the 5-Adenosyl-methionine-dependent CpG methyltransferase M.SssI prior to bisulfite treatment leads to the conversion of unmodified cytosines to 5-methyl cytosine, thus enabling the specific detection of 5fC and 5caC residues. (B) Containing only unmodified, 5-methyl, 5-hydroxymethyl, 5-formyl, or 5-carboxyl cytosine residues, Validation of MAB on five DNA standards. Dot blot analysis reveals complete conversion of unmodified cytosines to 5-methyl cytosines following M.SssI treatment. After bisulfate treatment, Sanger sequencing detects 5fC and 5caC as T, whereas unmodified C (methylated by M.SssI), 5mC, and 5hmC are detected as. [35]
7. DNA Methylation Analysis
By adding methyl or hydroxymethyl group to the C5 position of cytosine, DNA methylation in vertebrates was characterized, which occurs mainly in the context of CG dinucleotides. Non-CpG methylation in a CHH and CHG context (where H = A, C or T) exist in embryonic stem cells.\[36\]

Figure 7. MAB Method Enables Highly Efficient detection of 5caC
(A) Bisulfite treatment (B) Validation of MAB \[35\]

Figure 8. A graphical guide for choosing the right method for a specific project using a simple algorithm. \[36\]
Serum Level of BDNF in Diabetic and PDR Patients

Measured systemic level of BDNF in the serum of nondiabetic, diabetic with no retinopathy, and PDR patients. The mean serum levels of BDNF in diabetic patients and PDR patients were 21.8 ± 4.9 and 10.01 ± 8.1 ng/ml, respectively (Fig. 9). In the serum of control patients, the mean BDNF level was 25.5 ± 8.5 ng/ml. Statistical analysis suggests that in PDR patients, the serum level of BDNF is significantly reduced (p < 0.001) compared to that of non-diabetic controls and diabetic patients (ANOVA). The level of BDNF in diabetic serum was modestly reduced to 15% compared to that in nondiabetic control; however, the difference was not significant (p = 0.38). Serum levels of BDNF in control, diabetic, and PDR patients were measured by ELISA kit. To the level from non-diabetic controls (n = 19) and early diabetic patients (n = 22) Reduced levels of BDNF in PDR groups (n = 47) were compared. Mean non-diabetic BDNF (25.5 ± 8.5 ng/ml), diabetic (21.8 ± 4.7 ng/ml), and PDR (10.0 ± 8.1 ng/ml). Mean values were compared across groups by one-way ANOVA and post hoc test for multiple comparisons. Values are mean ± SD, *#p < 0.001 compared with control and diabetic. \[37\]

Conclusions

Epigenetic mechanisms play an important role in the case of Type 2 Diabetic Retinopathy because they provide a mechanism for continued altered gene expression. It has been noted that hyperglycemia can induce site-specific DNA methylation. This literature reviewed thoroughly concluded that level of DNA methylation in BDNF in retinal cells may affect the microvasculature of human retina that can be named as neovascularization or any retinal microvasculopathy. The Expression level of DNA methylation of BDNF gene between the patient and a healthy person can be important in the establishment of cause of Type 2 Diabetic Retinopathy and hints about their unique role in the pathogenesis of disease. The serum BDNF levels were significantly higher in the Type 2 Diabetic mellitus patients compared to the healthy controls. BDNF has a central role in Diabetic Retinopathy. The rapid pace of research in the epigenetic modifications and mechanisms controlling BDNF gene expression indicates that progress in BDNF epigenetics will have wide spread applications in diagnosis, prognosis and bio markers for the Diabetes. This is hypothesized that in the Diabetic retinopathy Epigenetic mechanisms play a role because they provide a mechanism for continued altered gene expression without the presence of the initiating HG (H=A,C,T) stimulus. It has been reported that HG can induce site specific DNA methylation. Present study concluded that level of DNA methylation in BDNF in retinal endothelial cells affect the microvasculature of human retina that can named as neovascularization or any retinal microvasculopathy. The Expression level of DNA methylation of BDNF gene between the patient and a healthy person can be important in the establishment of cause of type 2 Diabetic Retinopathy and hints about their unique role in the pathogenesis of disease. Previous studies suggested that 5caC merely serve as a DNA demethylation intermediate but functions as a stable epigenetic mark. 5caC enriches in gene

![Figure 9. BDNF serum concentration in healthy controls, diabetic patients, and PDR patients.\[37\]](image-url)
bodies, promoters, and transcription factor binding sites and mounting evidence suggest roles of 5hmC in regulating gene expression and controlling cell identity. The dysregulation of 5hmC levels may lead to Diabetes Retinopathy and neurodegenerative diseases. These discoveries collectively provide novel insights in understanding the function of this epigenetic modification including DNA demethylation in eyes. The current evidences mainly indicate a correlative relationship between 5caC enrichment and gene expression. With a view to allow better understanding the function of 5cac finally it may be very helpful for therapeutic purpose in the Diabetes Retinopathy.

References


5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science*, 333(6047), 1300-1303.


