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Contribution of epigenetics in diabetic retinopathy

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Diabetes has become the epidemic of the 21st century, and with over 90% patients with diabetes becoming at a risk of developing retinopathy, diabetic retinopathy has emerged as a major public health concern. In spite of cutting edge research in the field, how retina and its vasculature are damaged by the diabetic milieu remains ambiguous. The environmental factors, life style or disease process can also bring in modifications in the DNA, and these epigenetic modifications either silence or activate a gene without altering the DNA sequence. Diabetic environment up- or downregulates a number of genes in the retina, and emerging research has shown that it also facilitates epigenetic modifications. In the pathogenesis of diabetic retinopathy, the genes associated with important enzymes (e.g., mitochondrial superoxide dismutase, matrix metalloproteinase-9 and thioredoxin interacting protein) and transcriptional factors are epigenetically modified, the enzymes responsible for these epigenetic modifications are either activated or inhibited, and the levels of microRNAs are altered. With epigenetic modifications taking an important place in diabetic retinopathy, it is now becoming critical to evaluate these modifications, and understand their impact on this slow progressing blinding disease.

diabetic retinopathy, epigenetic modifications, histone acetylation, histone methylation, miRNA

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Diabetic retinopathy is the most feared complication as over 90% of the patients with diabetes for more than 20 years encounter some symptoms of retinopathy. With the number of diabetes increasing at an alarming rate, the number of people with retinopathy is expected to increase from 126.6 million in 2011 to 191 million by 2030, and the vision-threatening retinopathy during this period will increase from 37.3 million to 56.3 million [1]. Due to high circulating glucose, the tiny blood vessels that nourish the retina are damaged, and in the early stages of the disease, microaneurysms, hemorrhages, intra-retinal microvascular abnormalities result in bleeding. If not controlled, this non-proliferative stage could progress to proliferative stage where the new vessels begin to grow, ultimately resulting in retinal detachment and blindness [2].

The pathogenesis of diabetic retinopathy is complex; although hyperglycemia is considered as the leading cause of the development of diabetic retinopathy, however, hypertension and dyslipidemia are also some of the major risk factors associated with the disease [3–5]. A number of metabolic abnormalities initiated by hyperglycemia are implicated in the development of diabetic retinopathy. Oxidative stress is regarded as one of the leading mechanisms in its development, and increase in oxidative stress in diabetic milieu is associated with a number of other interlinking metabolic abnormalities including the accumulation of advanced glycation end products, activation of protein kinase C, polyol and hexosamine pathways [2,6–8]. Though many leading laboratories are involved in cutting edge research to understand the etiology of this complex disease, the exact mechanism responsible for its development remains elusive.

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1 Genetics and diabetic retinopathy

In addition to metabolic and physiologic factors, pathogenesis of a disease is also influenced by genetic factors. Due to variability in the severity of retinopathy among patients with diabetes with similar risk factors, however, genetic associations with diabetic retinopathy remain unclear. Genome-wide association studies have identified a number of genetic variants that could explain some of the inter-individual variations in the susceptibility of diabetes. A meta-analysis study has shown a significant variation in the *AKR1B1*gene, a gene encoding aldo-keto reductase family 1 member B1, and this rate limiting enzyme of the polyol pathway is strongly associated with diabetic retinopathy [9]. Another meta-analysis study with patients with type 2 diabetes has shown a protective role of Pro12Ala polymorphism in the peroxisome proliferator-activated receptor-γ2 gene in the incidence of retinopathy [10]. In contrast, recent studies have failed to find any associations between vascular endothelial growth factor (VEGF)-related single nucleotide polymorphisms (rs6921438 and rs10738760) and the risk of retinopathy and nephropathy in patients with diabetes [11]. Thus, the association between genetic factors and diabetic retinopathy needs further investigation.

2 Epigenetics and gene regulation

Epigenetics is now emerging as one of the important factors in many diseases 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. Epigenetic change is a regular and natural phenomenon, and can also be influenced by several factors including age, the environment, lifestyle and disease state [12,13]. Epigenetic modifications can act like switches helping to control gene activity and allowing alternations in genome function without altering the gene sequences. At least three major epigenetic modifications including DNA methylation, histone modification and non-coding RNA are considered to initiate and sustain changes in gene regulation [14,15].

DNA is not a static, fixed entity, but instead it is 'highly dynamic', and it responds to the environmental stimuli by modifying its properties in adapting to the changes [16]. Methylation of cytosine to 5-methyl cytosine (5mC) is considered as one of the major epigenetic modification; 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 [17,18]. The hypomethylated DNA re-activates the repetitive genomic sequences, resulting in chromosomal instability and abnormal gene expression [19,20]. DNA methylation is catalyzed by DNA methyl transferases (Dnmts), a family with five members—Dnmt1, Dnmt2, Dnmt3a, Dnmt3b and Dnmt3L; out of which only Dnmt1, Dnmt3a and Dnmt3b are catalytically active. Dnmt3a and Dnmt3b are *de novo* methyltransferases, and Dnmt1 is a maintenance enzyme important in regulating tissue-specific patterns of methylated cytosine residues [21,22]. Pathological Dnmt activity and aberrant 5mC formation have been linked with neurodegeneration [23]. 5mC can be oxidized to 5-hydroxymethylcytosine (5hmC) by ten-eleven translocation enzymes, and 5hmC can also be further oxidized to generate 5-formylcytosine and 5-carboxylcytosine [24]. A subfamily of DNA glycosylases are considered to promote active DNA demethylation by removing the 5-methylcytosine base, followed by cleavage of the DNA backbone at the abasic site, and the methylated cytosine is replaced by an unmethylated cytosine [25]. In contrast, the passive process involves absence/inactivation of Dnmt1 resulting hypomethylated DNA [26].

Chromatin, a composite structure of histones and nucleic acid, instructs the expression pattern of different genes, and conformational changes in the DNA, by altering the binding of transcription factors and its machinery, can change the gene expression. Among the 4-histone proteins, histone 2 (H2) exists in 2-subtypes, H2A and H2B which along with H3 and H4 forms a tetrameric structure, wrapped with ~146 bp of nucleic acid [27]. This sophisticated DNA packaging still allows N-terminal sequences of histones to undergo modifications including acetylation, methylation and phosphorylation. These modifications can either open or restrict access to DNA by directly altering the electrostatic potential and/or the structure of the local chromatin environment or indirectly alter the recruitment of the effector proteins [28]. Histone modifications are regulated by a balance between the enzymes inserting or removing a group [29,30]. Acetylation of histones is one of the most common modifications; acetylation opens up the chromatin structure, which allows recruitment and binding of the transcription factor and RNA polymerase II [31]. A group of enzymes with opposing functions maintain the required acetylation status, while histone acetyltransferases (HATs) inserts an acetyl group on a lysine of the histone; histone deacetylases (HDACs) remove the acetyl group [29]. In contrast to acetylation, methylation of histone, depending on the target site, can turns "off" or "on" the genes [32]. While trimethylation of histone H3 at lysine 4 (H3K4me3) is generally considered as an active mark for transcription, dimethylation of histone H3 at lysine 9 (H3K9me2) as a transcriptional silencing mark. In addition, depending on the degree of residue methylation, different functions can be expected; monomethylated H4K20 (H4K20me1) and H4K20me3 are considered as transcriptional repressors, H4K20me2 is largely considered as an activator [32–35]. Histone methyltransferases (HMT) catalyze the transfer of one, two, or three methyl groups to lysine (and arginine) residues of his-

tone proteins. There are two major types of HMTs, lysine-specific, which can be SET (Su(var)3-9, Enhancer of Zeste, Trithorax) domain containing or non-SET domain containing. Contrary to a relatively well defined histone methylating system, histone demethylation machinery is not well studied; LSD1, a lysine-specific histone demethylase, specifically removes methyl group from H3K4me and H3K9me [36,37].

Recent studies have shown that DNA methylation and histone modifications could function in concordance [38]; for example, Dnmt1, methyl CpG binding protein 2 (MeCP2) and methyl-CpG-binding domain proteins (MBDs) could associate with HDAC (39) or CpG methylation could cooperate with euchromatic histone methyltransferase SETDB1/ESET in histone H3 lysine 9 trimethylation, resulting in gene regulation. In addition, histone modifying enzymes, SUV39h1 and EZH2 lysine histone methyltransferases can interact with Dnmt and regulate their functions [40–42], and PRMT5-mediated methylation of arginine could silence gene expression via recruiting Dnmt3A [43]. Thus, both histone modification and DNA methylation could regulate the same gene. Although these epigenetic modifications do not have to be permanent, their response to the changing environment, and passing to the successive generations, makes them one of the most important drug targets for a chronic disease like diabetic retinopathy.

Small noncoding RNAs (~22 nucleotides) are also considered to regulate gene expression as these microRNAs (miRNA) bind to the complementary sequences in the 3′ untranslated region of mRNAs, and cleave mRNA resulting in decreased protein synthesis and expression of the targeted gene [44,45]. Methylation of DNA and histone, and miRNA appear to work in concordance as the function of Dnmts depends on histone modification patterns, such as H3K9 methylation and histone deacetylation and inhibition of Dnmts reactivates some of the miRNAs [46]. Since these epigenetic modifications are mainly caused by the local environment and can be passed on to the next generation, they are now being considered as some of the attractive targets for chronic disease, including diabetes and cancer [47].

3 Epigenetic modification 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 complications [8,48,49]. Genome-wide DNA methylation study using blood cells from patients with type 1 diabetes has identified 19 potential CpG sites that are prone to DNA methylation in diabetes. *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 [50–53], and leukocytes from diabetic patients have reduced Dnmts levels [54]. Global hypomethylation and reduced level of SAM are also observed in Zebrafish with chemically induced diabetes [55]. 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 [53]. These divergent patterns of DNA methylation in diabetes suggest that various tissues could be responding differently to diabetes.

Hyperglycemia is also associated with aberrant changes in H3K4me2 and H3K9me2 in human monocytic THP-1 cells and increases histone acetylation in the chromatin region containing the promoter of the transcription factor, nuclear factor kappa B (NF-κB)-p65 [56]. In vascular smooth muscle cells derived from type 2 diabetic mice, irreversibly decreased levels of both H3K9me3 and methyltransferase Suv39H1 at the promoters of interleukin-6 and monocyte chemoattractant protein1 are observed, and SUV39H1 gene silencing irreversibly elevates the expression of inflammatory genes and decreases H3K9me3 at their promoters [57]. Monocytes from case subjects enrolled in landmark the Diabetes Control and Complications Trial and the follow up Epidemiology of Diabetes Interventions and Complications study have shown a significant association between H3K9 acetylation and hemoglobin A1C levels [58].

In addition to histone modifications and DNA methylation, miRNAs are also associated with various diabetic complications. Increased levels of miR-377 and miR-21 are observed in the human and mouse mesangial cells exposed to hyperglycemic milieu [59,60]. Increased miR-377 levels have been linked to the induction of fibronectin, which contributes to the renal fibrosis in hyperglycemia [59]. Glucose-induced miR-146a downregulation is mediated through the HAT p300, suggesting an interrelationship between histone acetylation and miR-146a mediated fibronectin expression and a possible functional link between miRNA expression and histone modification in diabetes [61]. Thus, epigenetic modifications appear to play an important role in the development of diabetes and its complications.

4 Epigenetic modification in diabetic retinopathy

Diabetic retinopathy, a slow progressing disease, is associated with a number of metabolic abnormalities [2,7,8], however, the role of epigenetic modifications in diabetic retinopathy is still not clear. In a Finnish study, an associa-

tion between the polymorphism in *SUV39H2*, a gene that encodes histone methyltransferases and microvascular complications, including retinopathy has been observed in patients with diabetes [62]. 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 [63,64]. Experimental evidence using *in vitro* and *in vivo* models of diabetic retinopathy have shown that the activities of HDACs are increased and that of HATs are decreased in the retina and its capillary cells in diabetes, and global acetylation of histones is decreased [65]. However, contrary to this, others have shown significant increase in retinal histone acetylation in diabetes [66]; undermining the importance of further investigation into the role of histone modifying enzymes in the development of diabetic retinopathy.

Mitochondrial superoxide are considered as the unifying molecules connecting many metabolic abnormalities associated with diabetic retinopathy [7,67,68], and in diabetes, retinal mitochondria are damaged and superoxide scavenging enzyme (superoxide dismutase) is dysfunctional [69]. We have shown that diabetes epigenetically modifies *Sod2*, the gene encoding mitochondrial superoxide dismutase, and H4K20me3, acetyl H3K9 and p65 subunit of NF-κB (p65) are increased at its promoter/enhancer, H3K4 is demethylated and LSD1 binding is increased. These results have clearly suggested that epigenetic modifications have a major role in the regulation of superoxide levels, and thus in the development of retinopathy [70,71]. Furthermore, epigenetic modifications are also implicated in the function of Nrf2, a master regulator which regulates the expression of stress responsive gene. Due to epigenetic modifications at the promoter of Kelch-like ECH associated protein 1 (*Keap1*, an intracellular inhibitor of Nrf2), the binding of transcriptional factor Sp1 is increased. This results in increased expression of Keap1 and Keap1 tries to restrain the redox sensitive transcription factor in the cytosol, impairing its transcriptional activity and increasing oxidative stress. Furthermore, due to increased H3K4me1, Nrf2 binding at its glutamate-cysteine ligase-antioxidant response element region 4 *Gclc-ARE4* is decreased, resulting in decreased transcripts of the catalytic subunit of glutamate-cysteine ligase, an important enzyme responsible for biosynthesis of the intracellular antioxidant, glutathione [72–74].

In the pathogenesis of diabetic retinopathy, activation of matrix metalloproteinase-9 (MMP-9) is shown to damage mitochondria, and this initiates the apoptotic machinery [75,76]. *MMP-9* is regulated by NF-κB and the activation of NF-κB is modulated by the acetylation of its p65 subunit. Sirt1, a deacetylase, plays an important role in the acetylation-deacetylation of p65, and the activity of retinal Sirt1 is decreased and the acetylation of p65 is increased in diabetes [77]. Consistent with this, retina from human donors with diabetic retinopathy have decreased H3K9me2 at *MMP-9* promoter, acetyl H3K9 levels are elevated, and this facilitates the recruitment of p65 at its promoter and upregulates *MMP-9*, damaging mitochondria and increasing superoxide levels [78]. In addition, epigenetic modifications of thioredoxin interacting protein, an endogenous inhibitor of antioxidant thioredoxin, are associated with sustained *Cox2* expression seen in the retina in diabetes [79,80].

DNA methylation, an important epigenetic modification, is closely associated with the regulation of gene transcription [17,18]. 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 [63,64]. Experimental studies using *in vitro* and *in vivo* models of diabetic retinopathy have shown that the regulatory region of DNA polymerase gamma, an enzyme important in mitochondrial DNA biogenesis, is hypermethylated, and due to increased CpG methylation, its expression is reduced and its binding to the regulatory region of mtDNA is attenuated, resulting in subnormal mtDNA biogenesis [51]. These studies have clearly suggested that both the histone modifications and DNA methylation have important roles in maintaining mitochondrial homeostasis and regulating superoxide levels, which has an important role in the development of diabetic retinopathy.

In addition to modifications of histones and DNA, the small non-coding RNAs can also regulate post-transcriptional gene expression by binding to their target messenger RNAs, resulting in alterations in gene transcription [44,45]. These miRNAs are stable, and this makes them as ideal biomarkers in several diseases, including diabetes. Their function is somewhat complex as the same miRNA can target a number of genes, and the same gene can be targeted by a number of miRNA [81,82]. Studies with experimental models of diabetic retinopathy have revealed a number of miRNAs with either increase or decrease in their expressions. Experimental models of diabetic retinopathy have shown an association between the downregulation of miR-126, miR-146a and miR-200b and upregulation of VEGF, and downregulation of miR-146a is also associated with fibronectin production, and upregulation of miR-195 with downregulation of deacetylase Sirt1. Upregulation of miR-29b in the early stages of diabetes is considered to be protective against apoptosis of the retinal ganglion cells [83,84]. These studies have clearly suggested the important role for miRNAs in regulating various aspects of diabetic retinopathy, including blood retinal breakdown and neovascularization.

Thus, diabetic environment favors epigenetic modifica-

tions in the retina; DNA methylation and miRNAs are altered and histones are modified. DNA methylation and histone modifications could also affect miRNA levels. Due to epigenetic modifications, the binding of transcription factors (e.g., NF-κB and Nrf2) and the expression of genes (*Sod2*, *MMP-9*, *Keap1*, *TXNIP*, *VEGF*, etc.) become abnormal, resulting in the metabolic, physiological and structural abnormalities, and the development of diabetic retinopathy (Figure 1, Table 1).

5 Therapeutic implications

As mentioned above, epigenetic appears to play a major role in the development of diabetic retinopathy. Histone modifications have an important dynamic role in the regulation of gene expression; acetylation status of histones can directly influence the transcription of a gene. There is clearly a growing interest in the therapeutic use of HDAC inhibitors in the treatment of abnormalities in histone acetylases and deacetylases. Epigallocatechin-3-gallate is a strong histone acetylase inhibitor, and is shown to inhibit NF-κB activation [85] and in the pathogenesis of diabetic retinopathy, activation of NF-κB is considered to accelerate apoptosis of capillary cells, suggesting that inhibitor has potential to inhibit the development of diabetic retinopathy. Resveratrol, a naturally occurring compound found in grapes, wine and eucalyptus, is a potent activator of Sirt1, and it also inhibits some histone deacetylases [86]. In addition, curcumin [87] and genistein [88] are also shown to activate histone acetylases and inhibit deacetylases [89]. Vorinostat (suberanilohydroxamic acid), a HDAC inhibitor, is now approved by FDA for Cutaneous T-cell lymphoma [90]. As with histone acetylating-deacetylating enzymes, histone methyltransferases are also being considered as targets for therapeutics. Enhancer of Zeste Homolog 2 (EZH2), important for H3K27 methylation, has been shown to be inhibited by 3-deazaneplanocin, and specific inhibitors of EZH2, e.g., GSK126, seem to be showing promising results for the treatment of cancer. Epigenomic-based therapies targeting histone modifications are also being developed, and they offer new approaches for the treatment of ovarian cancer [91].

DNA methylation also plays a key role in gene regulation, and DNA methyltransferases are the key enzymes for DNA methylation. Nucleoside analogs incorporate into the DNA and trap all DNA methyltransferases, and the US Food and Drug Administration has already approved Dnmt inhibitors 5-azacytidine (5-Aza-CR; azacitidine; Vidaza) and 5-aza-20-deoxycitidine (5-Aza-CdR; decitabine; Dacogen) for myeloid cancers and cutaneous T cell lymphoma. Non nucleotide analogue RG108 is now in pre-clinical trials, and MG98 in phase I/II clinical trials [92]. VEGF receptor promoter methylation is considered an important factor in determining the efficacy of the VEGF-targeted drugs on the proliferation of cancer tissue [93], and this has tremendous

Table 1 Epigenetic modifications in diabetic retinopathy

Enzymes/miRNAs	Targets/modifications
KDM5A, LSD1	*Gclc* promoter histone modification (H3K4me3, H3K4me1)
SetD7/9	*Keap1* promoter histone modification (H3K4me1)
LSD1	*MMP-9* promoter histone modification (H3K9me2, H3K9-Ac)
LSD1, SUV420h2	*Sod2* promoter histone modification (H3K4me1, H3K4me2, H4K20me3, H3K9-Ac)
HAT (p300)	*TXNIP* promoter
Dnmts	*PolG1* promoter DNA methylation
miR-200b	*Oxr1*, *VEGF*
miR-129b	*Rax*
miR-146	*NF-κB*

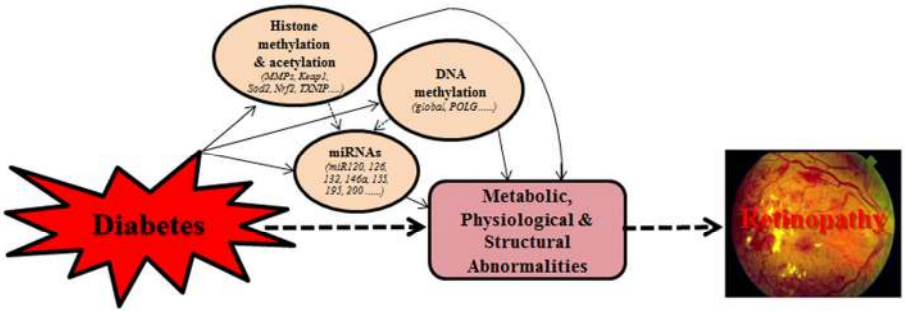

Figure 1 (color online) Sustained hyperglycemic insult results in a number of metabolic (e.g., PKC, AGEs, polyol pathway, oxidative stress), physiological (vascular permeability etc.) and structural (capillary cell loss, hemorrhages etc.) abnormalities in the retina that culminate in the development of retinopathy. In addition, diabetic environment also favors epigenetic modifications in the histones and DNA, and alters miRNA levels. These epigenetic modifications also fuel into the metabolic/physiological/structural abnormalities associated with the pathogenesis of diabetic retinopathy.

clinical implications for diabetic retinopathy.

Since miRNAs are considered as potential diagnostic biomarkers for disease, double-stranded miRNA mimics and anti-mRNA antisense oligo-deoxyribonucleotide are being used to target specific miRNA [84]. Also, since one miRNA can have a number of targets, a therapy targeting a specific miRNA can also alter other pathways associated with the disease [81,82]. However, one of the major caveats with the miRNA-based therapy could be their access to the posterior part of the eye and trouble with crossing the blood-retina barrier.

As a number of epigenetic modifications are now being associated with the development of diabetic retinopathy, there is a great potential for therapeutics targeted towards these modifications to be applied for this sight-threatening disease.

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