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Diabetes Microvascular Complications: An Overview of Epigenetic Modifications

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Abstract

Diabetic nephropathy (DN) and diabetic retinopathy (DR) are two serious and long-standing microvascular complications of type 2 diabetes mellitus (T2DM) whose burden is increasing worldwide due to increasing burden of T2DM. Several factors which may predispose to the development of DN and DR are persistent hyperglycemia and its consequences such as formation of advanced glycation end products (AGEs), activation of hexosamine pathway, polyol pathway, uncontrolled blood pressure, increased oxidative stress, age, family history of kidney disease or hypertension, ethnic background etc. However, the pathophysiological mechanisms of these complications are complicated and not completely understood yet. Hence it is the demand to discover newer approaches to treat these devastating complications completely. Recently, various epigenetic modifications, which are the transmissible alterations in the expressions of a gene, are being studied to understand the pathophysiology of diabetic vascular complications. Metabolic and environmental factors may lead to dysregulated epigenetic mechanisms which might further affect the chromatin structure and related expressions of a gene, which may lead to diabetes-associated complications. Therefore, it is the need to explore its role in vascular complications in the current scenario. In this chapter, various epigenetic studies with regard to DN and DR, epigenome-wide association studies (EWAS) approach, and starting clinical material for such studies have been discussed. We have also summarized the better understanding of epigenetic alterations and their role in microvascular complications of diabetes through this chapter. The better understanding of epigenetic mechanisms and their role in diabetic microvascular complications could be used in clinical management of DN as well as DR or could be helpful to improve the available therapies for these complications.

Keywords: diabetes, epigenetics, methylation, histone modification

1. Introduction

Diabetes is a chronic metabolic disorder in which blood glucose levels upsurge more than normal. Type 2 diabetes mellitus (T2DM) contributes to the majority of diabetes cases accounting for more than 90% of them. An imbalance of insulin supply and demand results in type 2 diabetes [1]. Decrease in insulin sensitivity accompanied by deficiency of insulin are the two primary pathogenetic defects underlying type 2 diabetes and together explain 85–90% of diabetes [2]. Long term

diabetes instigates vascular diseases affecting almost all blood vessels of the body, which further results in increased morbidity and mortality in diabetic populations. Among well-known risk factors of diabetes, non-changeable factors include genetics, age and ethnicity while others are changeable, for example physical activity, adiposity, environmental exposures and diet, via combination of treatment at both individual as well as population level [3]. Type 2 diabetes is frequently seen in older adults, but now-a-days, may be, as a result of increasing physical inactivity, obesity and/or the absence of healthy diet it is also being seen increasingly among children, teenagers and younger adults. Diabetes is globally affecting 425 million people or 8.8% of adult population. By 2045, diabetes is projected to affect about 629 million of adult population in the world [3].

India, now-a-days, is becoming the diabetes capital of the world with estimated prevalence of diabetes as 7.3% and that of pre-diabetes as 10.3% [4]. In the current report (2017), 72.9 million Indians were suffering from diabetes and this is expected to rise to 134.3 million by the year 2045 [3]. Prolonged hyperglycemia is the foremost cause of kidney disease, cardiovascular disorders, retinopathy and neuropathy [5], which are the main vascular complications of diabetes.

2. Diabetes mellitus-associated vascular complications

Hyperglycemia triggers damage to the vasculature and thus, leads to the failure of various organs including kidney, heart, retina of eyes and nerves; usually develop after many years of diabetes. This gives rise to the development of vascular complications which are categorized into micro- and macrovascular complications. Microvascular disease or microangiopathy is actually the thickening of walls of small blood vessels so that they bleed and leakage of protein occurs. This narrowing of blood vessels results in decreased blood flow and impairment of oxygen flow throughout the body which leads to the damage of tissues or organs that are extremely sensitive to oxygen levels i.e., kidney cells, nerve cells and retina. On the other hand, macrovascular disease or macroangiopathy is the disease of large blood vessels due to clot formations that further results in the decreased blood flow all through the body. This may cause heart diseases, peripheral vascular diseases or stroke. Both micro- and macrovascular complications are the result of hyperglycemia and it seems that they both may be interconnected but who precedes whom or whether they progress together, it is not clear. Complications of T2DM keep on increasing due to increasing burden of diabetes, thus deteriorating the quality of human life. Smoking, age factor, increased weight, lack of physical activity and high-fat diet are the common risk factors to diabetes complications. Now-a-days diabetic kidney disease (DKD) or DN and diabetic retinopathy (DR) are among the most frequent complications of diabetes. Improved and maintained glycemic control may reduce risk of some of the diabetic complications, but it is not the only factor which, if under control, may reduce the progression of all vascular complications. In this segment, we have elaborated two major microvascular complications of diabetes, i.e., DN and DR.

2.1 Diabetic nephropathy

Diabetic nephropathy is the major microvascular complication of diabetes affecting 20–30% of patients with type 2 diabetes mellitus [6], which weaken the quality of life leading to increased morbidity and mortality. Symptoms of DN are less evident in the early years of diabetes, usually develops after many years of diabetes. In India approximately 48% cases of CKD are caused by diabetes [7].

DN is defined as a clinical syndrome characterized by persistent proteinuria, a moderate deterioration of eGFR and an increasing arterial blood pressure [8]. Being the foremost cause of end-stage renal disease (ESRD), it results in considerable morbidity and mortality and incurs massive burden of cost on patient and the society as well. Pathways, specifically renin-angiotensin-aldosterone system (RAAS), have been known to play a central role in the development and progression of nephropathy which eventually triggers numerous inflammatory factors directing to the development of fibrosis in the kidney, hypertension/hyperfiltration in the glomerulus and increased permeability to macromolecules leading to proteinuria [9]. It has been seen that some patients with good glycemic control may develop DN at later stages and patients with poor glycemic control may not always develop DN. This may partly be due to genetic predisposition among various ethnic populations. Presence of diabetic nephropathy within families and the large differences in its incidence among diabetic populations with different ethnicity suggests the contribution of several genetic and epigenetic factors in the development and progression of DN. Till date several candidate genes, that are susceptible to DN, have been recognized with the advancements of molecular techniques via linkage studies, GWAS or candidate gene studies. The important candidate genes includes ADIPOQ [10] and ACAC β [11] from lipid metabolism, GCKR [12] and TCF7L2 [13] from glucose metabolism, transforming growth factor- β 1 (TGF- β 1) involved in inflammation [14], genes associated with angiogenesis i.e., VEGF-A [15] and RAAS genes i.e., ACE [16] and AGTR1 [17], and recently SLC12A3 [18] whose various polymorphisms are reported to be associated with DN. Genes involved in RAAS have been most extensively investigated in the context of DN. Among RAAS genes, angiotensin converting enzyme (ACE) gene is found to be strongly correlated with DN. For this reason, ACE inhibitors and angiotensin receptor blockers (ARBs) are the first line of drugs for the treatment of diabetic nephropathy that aims to reduce proteinuria. Though these drugs have shown to reverse the progression of albuminuria from macroalbuminuria or microalbuminuria to normoalbuminuria [19], thereby slows down the progression of disease, but are not able to provide a stable renoprotective effect. The response of DN patients to ACE inhibitors or ARBs alone or in combination is also not uniform despite several studies. Moreover, there are some limitations regarding their usage based on particular patient to be treated. Hence, these drugs along with strict glycemic control contribute to some degree of renoprotection, but not complete. Therefore, it is the urge to discover new pathways leading to the development of more specific therapies/treatments to help DN patients and improving their life.

2.2 Diabetic retinopathy

Diabetic retinopathy is a medical condition where damage to retina, as a result of high glucose, occurs. It is the most frequent cause of blindness in patients with diabetes. Patients with DR usually does not develop any major symptoms at an early stage but during later stages physiological and metabolic abnormalities can appear leading to blindness, if left untreated. The risk factors associated with DR includes high blood glucose [20], duration and type of diabetes [21], high B.P. [22] and, lipids [23]. Presently it is being diagnosed with the identification of microvascular lesions in the retina. It has been differentiated clinically in 2 categories on the basis of ophthalmic observation: proliferative DR (PDR), the advance stage and; non-proliferative DR (NPDR), the early stage. NPDR can be identified by fundus where hard exudates, microaneurysms or hemorrhages are seen. NPDR is further categorized into mild, moderate and severe NPDR. On the other hand, detection of retinal neovascularization confirms PDR. The risk of progression of DR can

be reduced by early detection, but it is difficult to achieve as there is little or no symptoms at early stages. Several molecular mechanisms are thought to involve in the development and progression of DR including polyol pathway, enhanced expression of vascular endothelial growth factor (VEGF), production of advance glycation end products (AGEs), activation of RAAS, hemodynamic alterations, etc. Current treatment involves conventional laser therapy and anti-VEGF or other anti-angiogenic, anti-inflammatory, non-steroidal anti-inflammatory drugs (NSAIDs) treatment. Despite this, reading is also difficult in patients with severe retina loss. Some treatments are precise but they are associated with high cost or side effects. Hence, the discovery of fundamental molecular mechanisms involved is required for the development of more specific interventions. Among genetic predisposition to the disease, several candidate genes have been identified in the past few decades with contradictory findings, although few genes have been found to be associated with DR in mostly studies. Among them, aldose reductase (AKR1B1) is important enzyme in polyol pathway. Activation of this pathway and AKR1B1 polymorphisms are incriminated in the pathogenesis of DR [24]. VEGF is another the most important growth factor activated by hyperglycemia and implicated in the development and progression of DR [25]. Various polymorphisms of VEGF were found to be associated with DR with conflicting results [24]. ACE I/D polymorphism was found to be associated with PDR in a meta-analysis [26]. Receptor for advance glycation end products (RAGE) gene polymorphisms is also reported to be associated with DR in Indian population [27].

The pathophysiology of complications of diabetes is very complex as depicted in **Figure 1**.

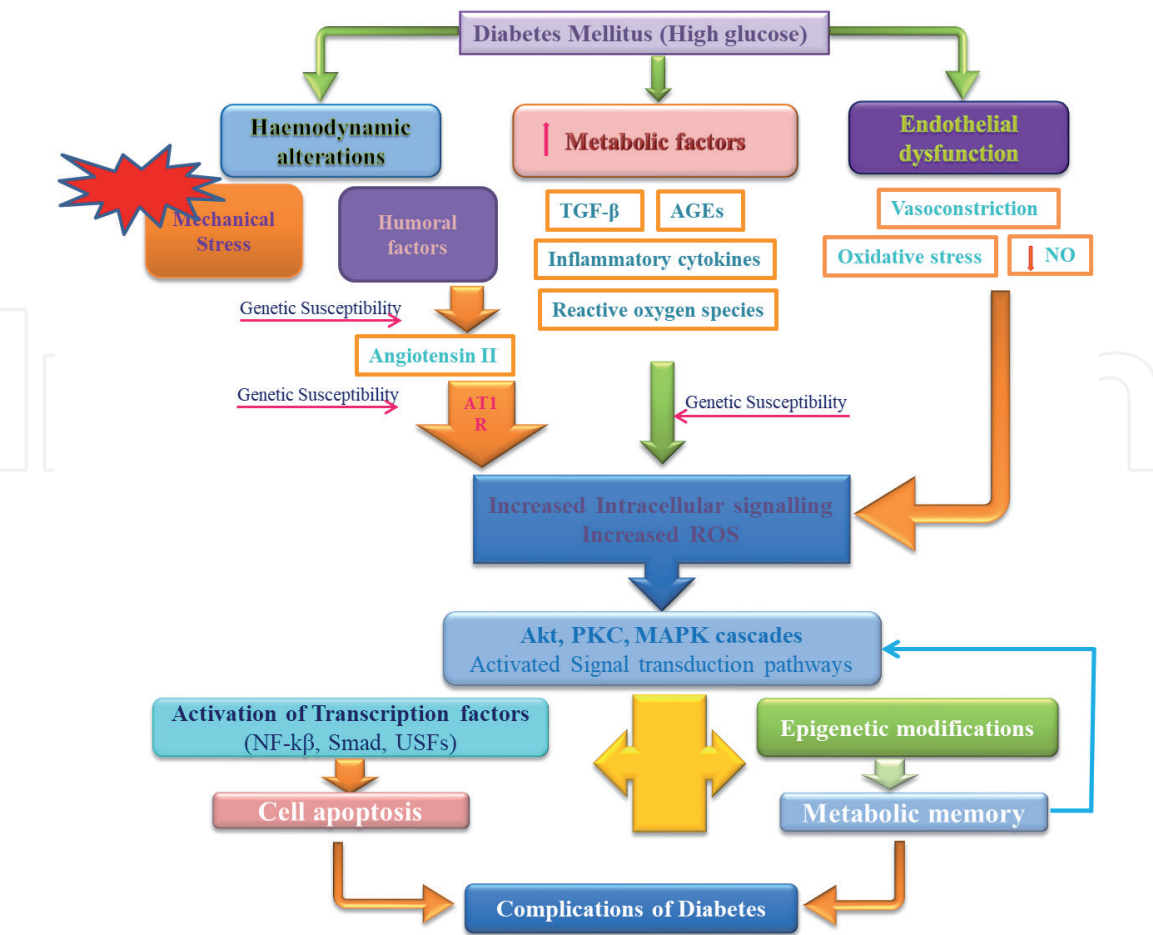


Figure 1. Signaling pathways facilitating the pathogenesis of microvascular complications of diabetes mellitus.

Diabetes-induced hyperglycemia promotes various growth factors which play influential roles in the progression of diabetic complications. These factors act by binding to their specific receptors to initiate multiple downstream signaling cascades involved. Subsequently, these signaling pathways trigger transcription factors and promote their crosstalk with epigenetic mechanisms that lead to diabetic microvascular complications. Transcription factors also interact with epigenetic factors that further participate in metabolic memory. AT1R, Angiotensin II type 1 receptor; AGEs, Advance glycation end products; TGF- β , Transforming growth factor- β 1; ROS, Reactive oxygen species; NO, Nitric oxide; Akt, Serine/threonine-specific protein kinase; PKC, Protein kinase C; MAPK, Mitogen-activated protein kinase; NF- κ B, Nuclear factor- κ B; USFs, Upstream stimulatory factors.

Several genetic factors and gene polymorphisms have been extensively discovered, studied and implicated in DN as well as DR, but no report is able to provide strong evidence regarding uneven response to available treatment. No drug or treatment is able to provide a stable and long-term protective effect against these complications.

In past few years, a lot of interest has been generated in gene–environment interactions as they seem to be involved in the pathophysiology of diabetes mellitus. Recently, epigenetic mechanisms have been linked to various complications of diabetes, as altered gene expressions are the results of several post-transcriptional modifications (PTMs) of chromatin. Accomplishing complete control of blood glucose is also not sufficient to stop or retard the development and progression of diabetes complications; this proposes the involvement of initial glycemic ‘metabolic memory’ in various complications of diabetes. So, how epigenetic modifications play the role in diabetic vascular complications is still not completely understood, therefore, we have described the understanding about epigenetic mechanisms and their role in the pathophysiology of diabetic microvascular complications.

3. Epigenetics-an addition to current treatment strategy

In mammalian cells, expression of a gene is known to be controlled by genetic as well as epigenetic mechanisms. In the recent times, epigenetic mechanisms have been shown to play substantial roles in the development and progression of diabetes and its microvascular complications. In this section, epigenetic mechanisms have been elaborated in the complications of diabetes.

Epigenetic mechanisms are deprived of any modification in the principal DNA structure which involves vibrant switching within ‘active’ (euchromatin) and ‘inactive’ (heterochromatin) positions of chromatin; that determined ‘gene activation’ and ‘gene repression’ states and thus biological outcomes [28]. Fundamentally, any change, in the expression of a gene without variation in its nucleotide (DNA) sequence, unlike genetic variations, is known as epigenetic variation. Subsequently, epigenetic studies in diabetic complications may help us to understand the role of epigenetic mechanisms in the alteration of expressions of genes involved in various complications. Hypermethylation at CpG Island in promoter region of a gene is likely to silence its expressions. In contrast, when CpG turns out to be hypomethylated, reverse can takes place [29]. At first, ‘epigenetics’ term was described by Waddington as ‘the casual interaction between genes and their products which bring the phenotype into being’ [30]. Epigenetic mechanisms maintains the structure of chromatin to confer transcription memory important for the faithful transmission of gene expression pattern across multiple cell divisions even in the absence of signals that initiated them [31]. Such a control of gene expression by the epigenetic modifications elucidates the mechanisms which triggers our cells with

the same DNA to differentiate into numerous cell types with various phenotypes [32, 33]. That's why phenotype of a person is not only decided by its genome but by its epigenome too.

3.1 Factors associated with epigenetic mechanisms

The suggested mechanism behind altered expression of a gene was the activation of an intracellular signal by environmental factors, which sequentially specifies the accurate chromatin position for epigenetic alterations [34, 35]. Certain environmental aspects takes place during the course of formation and development of embryo (such as maternal diet and intrauterine nutrition) and such an initial development could influence health and disease conditions even at later stages [36]. Additionally, several other environmental exposures accelerate alterations in epigenetic mechanisms, such as heavy metal exposure, smoking, revelation of pesticides, even insufficiencies of nutrients (such as folate and methionine) [37]. Moreover these mechanisms are also appeared to be altered by age and obesity which may possibly cause type 2 DM [38].

3.2 Epigenetic mechanisms in diabetes mellitus and its complications

In past few years, environment has shown a significant role in activating diabetes, although diabetes has a trend to run in family due to intense genetic component. Obesity, older age and sluggish routine with absence of physical doings are the pronounced risk factors for getting hyperglycemia. Diabetes may cause altered epigenetic mechanisms which can direct diabetes-associated complications such as diabetic nephropathy, by altered expression of genes in target cells as depicted in **Figure 2** [39].

Diabetes mellitus results in the activation of several signaling pathways following activation of alterations in DNA and histone proteins and transcription factors including NF- κ B. These mechanisms via chromatin remodeling resulted in regulated target genes expression in targeted tissues along with activation of several ncRNAs including miRNAs, lncRNAs and circRNAs. Such post transcriptional alterations in target tissues resulted in specific key pathological changes in specific tissues and promote the development of specific vascular complication of diabetes. Even after blood glucose control, synchronized crosstalk between various transcription factors and altered epigenetic mechanisms contribute to the metabolic memory and increased expression of ncRNAs that is embroiled in risk of development of microvascular complication of diabetes.

High glucose can also stimulate abnormalities in DNA at key genes which are well-known to be involved in endothelial dysfunction as evident by sequencing studies in endothelial cells [40]. Augmented DNA methylation at the promoter region of peroxisome proliferator activated receptor gamma coactivator-1 alpha (PPARGC1A) was reported to be associated with decreased expression of PPARGC1A in pancreatic islets [41]. Tewari et al. [42] have demonstrated the decreased transcriptional activity owing to decreased binding of mitochondrial DNA (mtDNA) to DNA polymerase as a result of hypermethylation at regulatory region of DNA polymerase. Global DNA hypomethylation and thereby, anomalous gene expression due to hyperglycemia was observed in the animal model of diabetes, which was further correlated with inadequate wound healing process [43]. Glucose-induced insulin secretion was shown to be influenced by hyper-acetylation of H4 (histone) at promoter region of insulin gene [38]. Hyperglycemic environment exposure to endothelial cells showed the increased expression of p65 subunit of NF- κ B along with other inflammatory genes that correspond with increased H3K4me1 alterations on promoter region of p65 subunit [44].

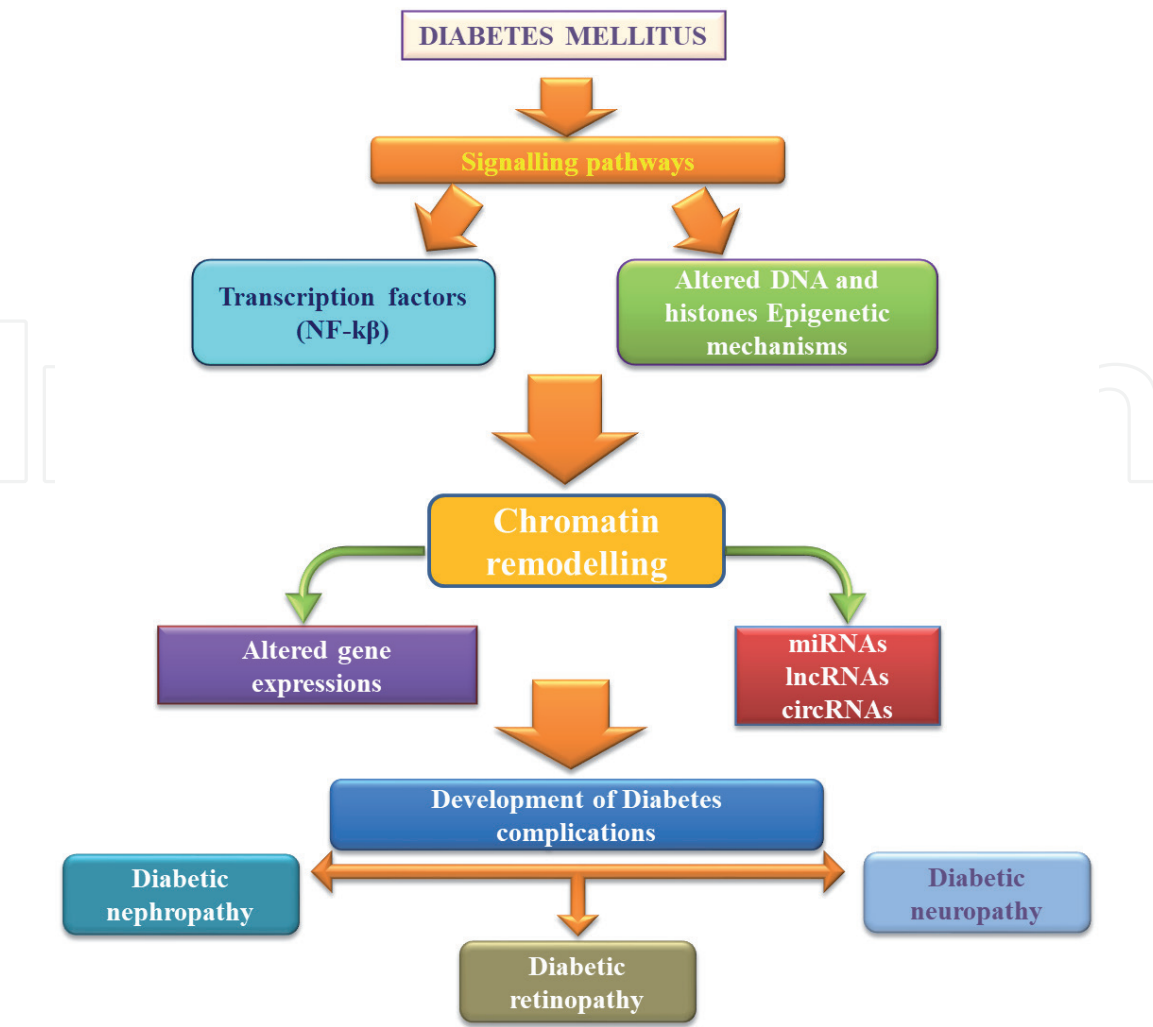


Figure 2.
Pathways preceding the development of microvascular complications of diabetes.

Hyperglycemia has also shown to alter micro RNA (miRNA), a mechanism of epigenetic modifications, which is also implicated in complications of diabetes. The alteration in miRNA-133a has been reported in cardiomyocyte hypertrophy in diabetes patients [45]. miRNA-320 upregulation was also observed in myocardial microvascular endothelial cells in rat model with type 2 diabetes [46]. The elementary epigenetic modifications viz., (a) methylation of promoter sites in DNA, (b) modifications in histone proteins and, (c) non-coding RNAs facilitated pathways, as illustrated in **Figure 3**, known to modify the expressions of a gene are described as below:

DNA in chromosomes is packed round the histones to form nucleosomes. Unwrapping and accessibility of nucleosomes is regulated by alterations in histone proteins. DNA methylation involves addition or removal of methyl groups to cytosine residues in CpG islands via DNA methylating enzymes (DNMT) or DNA demethylases, thus, preventing the binding of transcription factors and suppressing respective gene expression. Histone modifications include acetylation, methylation and phosphorylation. HATs/HDACs regulates the acetylation and deacetylation of histone tails, whereas histone methylation is regulated by HMTs/HDMs. Alterations in histone tail coupled with DNA methylation and control the chromatin accessibility or inaccessibility, hence, regulating the expression of various genes. ncRNAs can be act as housekeeping molecules or regulatory molecules. miRNAs act as regulatory molecules among epigenetic mechanisms and are most widely studied mechanism regulating gene expressions at post-transcriptional level. This dynamic condition of chromatin is exposed to modifications by external

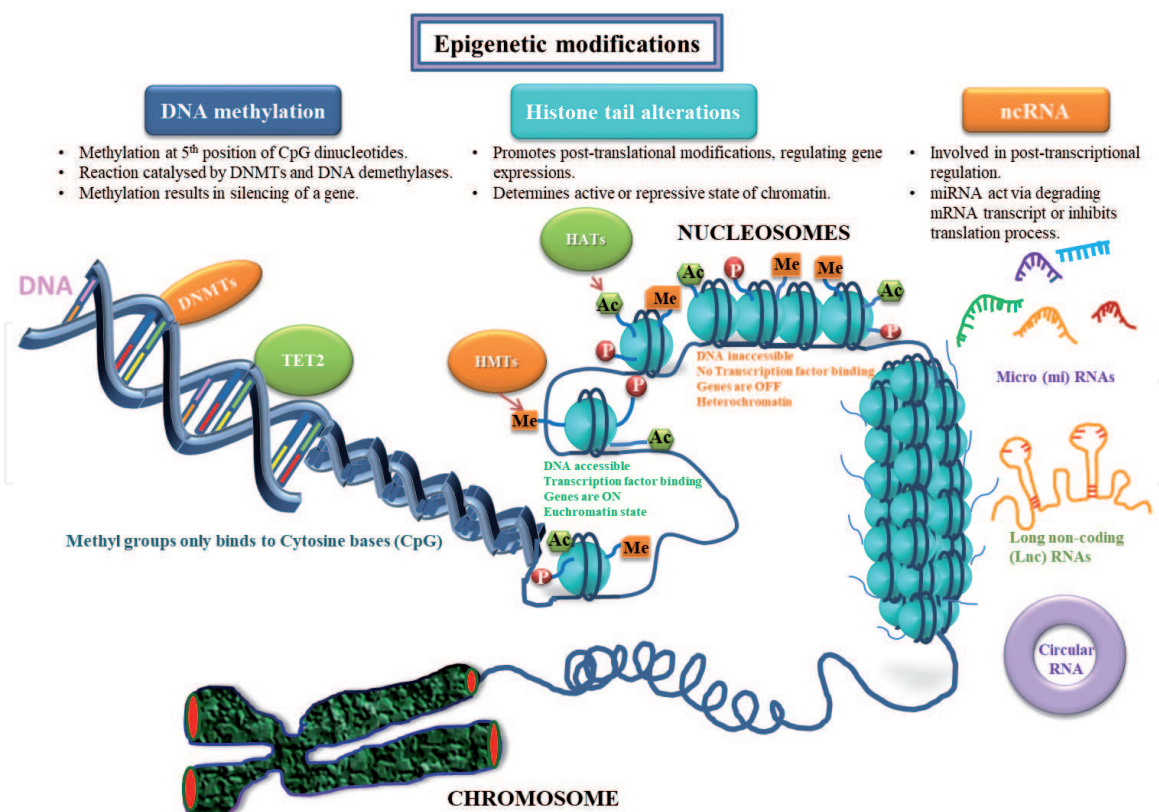


Figure 3.
Framework of inheritable epigenetic modifications.

stimuli via regulation of miRNAs, thus directing several pathophysiological outcomes. DNMTs, DNA methyl transferases; HATs, Histone acetyl transferases; HDACs, Histone deacetylases; HMTs, Histone methyl transferases; HDMs, Histone demethylases; ncRNAs, non-coding RNAs, miRNAs, micro RNAs.

a. Methylation of DNA

It is the renowned epigenetic modification that is well studied in cancer, and lot of interest has been generated in DNA methylation in the framework of diabetes and its related complications. In detail, DNA undergoes methylation at 5th position of CpG dinucleotides and form 5-methylcytosine, which is a post-replicative mechanism. DNA methylation is extremely dynamic process in the progress of a disease, which tends to alter related gene expressions. These alterations can be reversed by external stimuli. Commonly repression of a gene takes place due to addition of methyl groups at promoter region on DNA, while methylation at gene bodies may regulate their transcription during elongation and also during alternative splicing [31].

DNA methyl transferases (DNMTs) are known to catalyze DNA methylation reaction which, in freshly synthesized DNA, methylates CpG dinucleotides. Hence, to sustain DNA methylation in proliferating cells, DNMTs are vital. Throughout the embryonic development, for *de novo* methylation, presence of DNMT-3a and -3b enzymes is obligatory [47]. The molecular effects of DNA methylation were interceded by a group of methyl binding domain (MBD) proteins. Out of these, merely MBD2 alone is identifiable for methyl-CpG positions, which guides the interaction of methylated DNA to a multifaceted complex encompassing nucleosome remodeling and histone deacetylases (HDACs) bustles, thereby conducting silencing of a gene [48]. DNA methylation is commonly studied by various methods including methylation specific PCR (MS-PCR), methylation sensitive high resolution melt

curve (MS-HRM), immunoprecipitation or sequencing approaches. Advantage of MS-HRM is that it offers a low-cost and rapid method for the detection of even low levels of methylation at gene promoters.

Earlier exposure of target cells to high glucose can result in a 'metabolic memory' which results in persistence of its detrimental effects long after glucose stabilization. Diabetes-induced altered epigenetic mechanisms, resulting in modified gene expression in target cells can lead to diabetes-associated complications, such as diabetic nephropathy [39]. In the pathogenesis of DN and ESRD, DNAm (DNA methylation) has been explored by several studies via studying differentially methylated genes related to DN [31, 49, 50]. In a genome-wide methylation analysis (GWAS), significant alterations in DNA methylation in DN patients as compared to control were reported at 19 CpG sites that were found to be associated with the risk of DN. They also correlated the degree of methylation with time to development of DN [50]. In DNA isolated from the saliva of type 2 diabetic patients with end-stage kidney disease (ESRD), differentially site-specific methylation of DNA was recorded at 187 gene targets in comparison to those without ESRD [49]. DN patients have altered DNA methylation at important key gene promoters in comparison to those without DN [50]. However, studies in DN animal models or in renal cells under hyperglycemic conditions were not competent to show any significant changes in DNA methylation patterns [51]. In patients having type 2 diabetes with diabetic nephropathy, global DNA methylation variations were also observed to be associated with albuminuria in a recent study [52]. Noteworthy alterations in histone and DNA methylation patterns were observed to be present in peripheral blood mononuclear cells (PBMCs) of patients with membranous nephropathy [53]. Genome-wide DNA methylation study also depicted modifications in differential DNA methylation profiles among type 1 diabetes patients with or without nephropathy, where degree of methylation is linked with time towards the progression of DN [50].

It has been demonstrated that the promoter of human ACE gene, the most important and widely studied gene in pathophysiology of DN, harbor CpG islands. ACE transcription and expression levels were also observed to be influenced by methylation in its promoter region both in vivo and in vitro [54]. The magnitude of epigenetic alterations, particularly DNA methylation, has been shown to correlate with ACE activity levels [54, 55]. These studies demonstrated an increase in ACE activity with hypomethylation of ACE gene promoter. Also, a relation between epigenetics of ACE gene and I/D polymorphism has been suggested, where decreased DNA methylation in 3 CpG sites of ACE gene was observed in low birth weight (LBW) children with DD genotype although this has not been reported directly in DN patients [55]. Global DNA methylation variations were also observed to be associated with albuminuria in a recent study [52]. Additionally alterations in DNA methylation of ACE promoter are suggested to be a fundamental cause of major depression (MD) and a shared pathogenic factor for bi-directional connection between MD and cardiovascular disorders [56].

Apart from the importance of DNA methylation in DN, their role in DR is not clear, however, DNA methylation has been shown to control the expressions of many genes associated with retinal homeostasis. Previous studies have shown the link of DR development and DNA methylation, which indicates that DR may be associated with epigenetic alterations. In this connection, a GWAS between PDR and healthy controls was conducted in PBMC'S sample and out of 349 identified methylated sites, only 17 genes were observed to be hypermethylated [57]. They assumed that PBMCs could be used as a predictor for diabetic retinopathy. Another study evaluated global DNA methylation levels in blood leukocytes in persons with and without retinopathy [58]. They found a significantly higher global methylation levels in patients with DR than those without DR. These changes were seen to be

progressive from non-DR stage to NPDR and eventually to PDR and were independent of hyperglycemia, dyslipidemia, diabetes duration and person's blood pressure. Binding of polymerase gamma 1 (POLG1) to mtDNA (mitochondrial DNA) also results in compromised transcriptional activity as a result of hypermethylation at promoter region of DNA polymerase gamma 1 (POLG1) in the hyperglycemic environment [42]. This study was conducted in rat model of diabetes which showed that the mitochondrial damage in retina of diabetic rats could be diminished/controlled by maintaining stable glycemic control for longer time periods or therapy that targets directly DNA methylation. However, it does not benefit DNA methylation machinery by the reversal of hyper-glycemic environment for shorter duration [59]. In people with diabetes mellitus, it has been seen that activity of Dnmt1 enzyme was elevated in retinal and its capillary cells. However, this was not observed with Dnmt-3a or Dnmt-3b [60, 61]. Similar differential DNA methylation patterns were also observed in persons with PDR [57].

b. Histone modifications:

It is the interesting and emerging mechanism that exhibits the addition of methyl groups at histones related to a gene. As DNA is structured into chromosomes in eukaryotic cells, it is tightly wrapped onto series of nucleosomes (the basic unit of chromatin), which are the octamer complexes of small core (a H3-H4 tetramer and two H2A-H2B dimers) linked by linker histone proteins (H1) [62]. These histones are involved in post-translational modifications (PTMs) which may regulate gene expressions. The gene activation and repression are determined by dynamic chromatin structure that directly depends upon these PTMs, as they will allow transformation of inactive or repressive chromatin to euchromatin, the active condition of chromatin. These modifications, like DNA methylation, are able to regulate the gene expression without any change in its DNA sequence. Hence, histone tails can be acetylated, methylated, or phosphorylated. Histones with methylated (Kme) or acetylated (Kac) lysine residues, mostly at amino terminal tails, have been identified. Generally, these modifications are correlated with either gene activation or repression. Like, on one hand, histone lysine acetylation (H3K9ac, H3K14ac and H4K5ac) is generally associated with gene activation that opens the chromatin for the binding of transcription machinery [63]. Histone acetylation is tightly controlled by the equilibrium between acetylation (HATs) and deacetylation (HDACs) enzymes that add or deletes acetyl group. On the other hand, methylation on lysine or arginine residues can be correlated with both, gene activation or gene repression, depending on the residue to be modified. For example, mono- or tri-methylation of Histone 3 at lysine 4 residue (H3K4me, H3K4me3), H3K79me2 [64] and at lysine 36 residue (H3K36me) facilitated by lysine methyl transferases (KMTs) such as SET1/7/9 are associated with gene activation [63]. Although, mono-methylation of histone 3 at lysine residue 9 (H3K9me) mediated by suppressor of variegation 3-9 homolog 1 (SUV39H1) is correlated with gene activation whilst, its trimethylation (H3K9me3) is linked with gene repression [65]. Additionally, H3K27me3 and H4K20 were associated with gene repression. Afterwards, lysine demethylases (LSD1) are there to reverse such steady modifications at H3K4 and H3K9 [66, 67] as a co-repressor or co-activator respectively. Their nomenclature has already been changed from LSD1 to lysine demethylases (KDMs) [68]. In a study in lymphocytes from type 1 diabetic patients, as compared to controls increased H3K9me2 levels were reported to be correlated with immune and inflammatory pathways associated with diabetes and its complications including DN [69]. Such histone modifications at N-terminal are two key mechanisms that may alter development and progression of diabetes and its related complications; they are noteworthy as discussed below.

In DN pathogenesis, expressions of a gene that are associated with DN are regulated by post-translational modifications of histone proteins, apart from DNA methylation. Smad2/3/4 (transcription factors) are activated by TGF- β and also team up with HATs and other chromatin remodeling factors. Alterations in DNA methylation and H3K9Ac at gene promoters were found to be associated with endothelial dysfunction in endothelial cells cultured in hyperglycemic conditions. Among various epigenetic mechanisms, methylation among core histone tails is considered to be the highly stable PTM that could be a key factor in the pathogenesis of various complications of diabetes. Previous studies have studied the role of histone modifications in cultured cells as well as animal model in the presence of TGF- β and high glucose environment, the two key factors in diabetes [70]. They reported an increased H3K9/14Ac at PAI-1 and p21 promoters near Smad/SP1 binding sites. Cultured rat mesangial cells (RMCs), obstructed by TGF- β antibodies, displayed increased levels of p21 and PAI-1 under hyperglycemic conditions. Also in glomeruli of diabetic animal model, increased expressions of PAI-1 and p21 were found to be linked with increased promoter H3K9/14Ac. In the model of DN, TGF- β stimulated expressions of key fibrotic genes were found to be associated with enrichment of histone active chromatin marks (H3K4me1/2/3) and reduced repressive chromatin marks (H3K9me2/3) at their promoters [71]. Collectively, TGF- β plays as an intermediary in hyperglycemia induced histone modifications of promoters of key genes in mesangial cells leading to kidney damage. In glomeruli of diabetic mice, increased chromatin active marks along with decreased repressive marks were observed at PAI-1 and receptor for AGE (RAGE) gene promoters as compared to control, which showed the regulation of histone modifications in kidney in the presence of hyperglycemia [72]. In addition, AT1R inhibitor decreased key indicators of DN and also reversed some of the epigenetic changes in diabetic mice including reduced H3K9/14Ac at PAI-1, RAGE and MCP-1 promoters in diabetic mesangial cells. In the animal models of DN, increased histone active marks (H3K4me2) and decreased repressive marks (H3K27me3) were observed to be associated with the expression of genes related to DN [73]. In the kidney of uninephrectomized db/db mice model, H3K4me2 levels were increased in association with albuminuria, glomerular filtration rate and glomerular cell proliferation, which can be reversed by MCP-1/CCL2 antagonist [74]. In diabetic kidneys, HDAC inhibitor (Trichostatin A) has been observed to block the induction of TGF- β at essential fibrotic genes, both *in vitro* and *in vivo*. This implies major role of HDACs in TGF- β facilitated kidney fibrosis and ECM accumulation [75]. In another study, treatment of renal epithelial cells with Trichostatin A (TSA) resulted in downregulated TGF- β mediated epithelial-to-mesenchymal transition (EMT) [75, 76]. Taken as a whole, these studies demonstrate the involvement of HDACs in renal injury via TGF- β .

Histone post-translational modifications have also been studied extensively in the context of DR. Increased oxidative stress and simultaneous decreased levels of retinal superoxide dismutase (SOD2) are the key features of DR. Increased histone repressive mark (H4K20me3) along with increased NF- κ B p65 in association with decreased SOD2 mRNA levels and decreased activation marks (H3K4me1/2) at SOD2 promoters were observed in retinal endothelial cells cultured in high glucose. Acetylation of core histone protein on lysine residues is thought to opens up the DNA, thereby, increased availability for binding of transcription factors. Afterwards, activated proinflammatory transcription factors, for instance NF- κ B, binds to particular sequence in DNA and activates and bind coactivators (like p300) having intrinsic HAT activity to the target promoters of target gene. These coactivator molecules then, regulate the expressions of target gene owing to their HAT activity [77]. Contrary to this, recruitment of HDACs results in compact

chromatin, coiled DNA and less accessibility for binding of transcription factors to DNA, thereby decreased expression of target gene. Hence, the balance between acetylation and deacetylation of histones regulates the transcription of the gene. Increased HDACs and decreased HATs along with decreased global histone acetylation activities were also found in diabetic retinal cells in the models of diabetic retinopathy [78]. However, reversal of hyperglycemic conditions did not able to restore changes in histone activities. This is in contrast to a study in diabetes where activation of histone acetylation was observed in retinal cells [79]. Pro-apoptotic enzyme, MMP-9, is also observed to be associated with epigenetic alterations in DR [80, 81]. Lysine of histone 3 was reported to be methylated by SUV39H1 resulted in H3k9me3 [82]. Another methyl transferase gene i.e., SUV39H2 is involved in the onset of disease, when methylates histone H3K9 results in the inception of DR [83]. Moreover under hyperglycemic conditions, recruitment of Set7 (HMT) at promoter region of NF-KB p65 unit was linked with its enhanced transcription [44]. Western blotting and mass spectrometry studies in diabetic rat model also confirmed the acetylation of several lysine residues on histones due to hyperglycemia leading to increased expressions of proinflammatory proteins in retina and associated with DR [79].

Oxidative stress also plays a central role in diabetic complications and has been shown to control histone acetylation or deacetylation in diabetic conditions. High blood glucose is known to increase ROS production, which further activates important pathways that are required for the development of DR [84]. ROS is observed to inhibit acetylation of histones by increasing HDAC activity and decreasing HAT activity [85]. Hence, it was believed that there is involvement of ROS in regulating acetylation and deacetylation. Usually, oxidative stress was found to be increased in retina and capillary cells [86]. Thus, it is possible that diabetes via increased ROS production may regulates histone acetylation and deacetylation in retina. Ischemia and hypoxia are also known to promote the process of histone deacetylation [87] and hypoxia in diabetes is the leading cause for neovascularization in retina [88] which indicates the role of retinal hypoxia in diabetic retinopathy via stimulating retinal histone deacetylases. Thus, in hyperglycemia, epigenetic alterations may be involved at a larger level in modulating the expressions of various important genes in pathogenesis of DR.

Various researches on histone protein alterations may suggests that chromatin state is likely to be affected by multiple histone code modifications and hence, screening of various histone alterations at key genes promoters and/or bodies related to DN is crucial. The role of DNA methylation, histone code modifications and changes in epigenetic marks in response to various therapies is not well studied and would be of great concern to see whether these modifications could be altered in response to therapy. In future, more epigenome studies are required to elucidate the mechanisms of pathogenesis of DN that could help in developing better treatment strategies for people suffering from this devastating complication.

c. Micro RNAs (miRNA):

Whole transcriptome studies (RNA-sequencing) have uncovered that majority of the transcribed genome (into RNA) is non-coding part, apart from the coding mRNA [89]. Non-coding RNA refers to the RNA that does not code for any protein. These non-coding RNAs are also a part of epigenetic mechanisms that are of immense interest in the context of diabetic complications as they are observed to repress the expressions of target genes via regulating transcription and post-transcription mechanisms. Non-coding RNAs includes small non-coding RNAs (miRNAs approx. 20-22 bp long), circular RNAs (circRNAs) as well as long

non-coding RNAs (lncRNAs approx. 200 bp long). They are reported to control the expressions of important genes associated with diabetic complications. In contrast to miRNAs, few studies have observed the role of lncRNAs in DN [90, 91]. miRNAs are usually single stranded RNA of approximately 20–25 nucleotides long. They are well-known non-coding RNAs that involved in post-transcriptional regulation by means of either suppression of translation or degradation of mRNA transcript by binding 3' UTR of target sequences [92, 93]. lncRNAs, instead, are usually longer (>200 bp) than miRNAs (20–22 bp). They function as scaffolds [94] and may regulate miRNA due to their antisense activity [95] and have tissue specific expressions [96]. Similar to mRNA, lncRNAs are formed due to transcription in the presence of RNA polymerase II and undergo splicing, although they are slightly polyadenylated [96]. lncRNAs also participate in modifications of epigenetic marks as they harbor histone methylation marks at H3k4 and H3K36 [97]. They are also reported to be involved in the development and progression of diabetic microvascular complications [98–101]. Recently circular RNAs, the next level of epigenetic regulation, are holding our interest in addition to lncRNAs as they are generated from mRNA via its back-splicing and later on both 5' and 3' spliced ends ligated together to form a circular structure. They regulate miRNAs, thereby regulating the expressions of miRNAs targetted genes. They also act as sponge for various miRNAs. Several circRNAs are observed to stimulate the pathogenesis of diabetes-related microvascular complications [102, 103].

On the other hand miRNAs, at first, were portrayed in *C. elegans*, a nematode, during early 1990s. Over 1000 miRNAs in human genome have been identified; lin-4 was the first described miRNA [104]. Various miRNAs are found in humans, algae, plants, animals and viruses [105]. miRNAs, unlike other small RNAs, are derived from the transcripts that themselves can rapidly fold back to form a hairpin-like structure. RNA polymerase II transcribed miRNA as primary transcript (pri-miRNA) in nucleus, where they are later spliced into precursor miRNAs (pre-miRNA) [106, 107] by the action of endonuclease complex. Exportin-5, a protein transport pre-miRNA into the cytoplasm from nucleus where they are further processed to mature miRNA duplex (~ 22 nucleotides) by the action of ribonucleases [107]. One strand of mature miRNAs is selected and loaded on RNA induced silencing complex (RISC) and other stand undergoes the process of degradation [106, 108]. This complex binds to their complementary sequence on mRNA for post-transcriptional suppression. Initially, lin-4 RNA was observed to have complementarity with conserved sites in mRNA of lin-14 [109] within untranslated (3'-UTR) site. But how to find their targets was the primary question in initial times. Algorithm tool, at first, identifies the perfect Watson-Crick pairing to 2–8 nucleotides of miRNAs starting from 5' region [110]. This 7 seven nucleotide sequence (at 5'-end) was termed as 'miRNA seed'. This finding was clearly in agreement with the earlier study which showed that 5' end is the most conserved region in metazoan miRNAs [111]. Afterwards extending seed match with adding more base pairs to the miRNA continues in both directions, but stopping at discrepancies [110]. Therefore, the silencing effect of target gene by miRNA is via binding of seed sequence at miRNA with the complementary sequence at mRNA in 3'-UTR. miRNAs based therapies would have a better lead in that they can target multiple genes of a particular pathway or process [112]. Because, one miRNA can suppress expression of many genes and subsequently one gene can also be targetted by more than one miRNAs. Another advantage is that these miRNAs can cross blood-retina barrier so as to get into the target tissue, which is the foremost obligation with this therapy. In past years, several studies have linked miRNAs with diabetic complications. Henceforth we have, now described the role of miRNAs in the pathogenesis of diabetes complications.

Several miRNAs including miR-29, miR-192, miR-194, miR-200b/c, miR-204, miR-215, miR-216a, miR-217, miR-377 etc. have been found to be associated with DN. Characteristics of DN includes fibrosis, accumulation of extracellular matrix (ECM), podocyte dysfunction and proteinuria [113, 114]. TGF- β has been implicated in the pathogenesis of DN and is found to be upregulated during the progression of DN, which in turn, induce fibrotic events, kidney deterioration and dysfunction [114]. TGF- β has shown to upregulate several miRNAs including miR-192, miR-216a, miR-217 in mesangial cells as well as in kidneys of diabetic mouse models as compared to control group [115–117]. ZEB2, a translation repressor that suppress fibrotic gene collagen type 1 Alpha 2 (*Col1a2*), was observed to get suppressed by miR-192, thus, resulted in an increased expression of *Col1a2* gene and contribute to matrix accumulation and kidney fibrosis in DN model [115]. In diabetic mice, increased expressions of p53, TGF- β and miR-192 was reported in renal cortex and was found to be associated with augmented fibrosis and glomerular expansion as compared to control. Moreover, knockout of miR-192 gene resulted in decreased markers of DN. However, conflicting reports to these results are also described. One of such reports observed that TGF- β decreased the expression of miR-192 in cultured proximal tubule cells and concluded that decreased miR-192 levels are associated with increased fibrogenesis in PTCs [118]. Another study also showed that kidney fibrosis was associated with the loss of miR-192 [119]. These contradictory studies showed that the interconnection between DN and miR-192 is much more complicated than it seems. Also, a decreased expression of miR-21 was found in DN and albuminuria was decreased in diabetic mice due to ectopic expression of miR-21 [120]. miR-377 expression was found to be upregulated in DN [121]. It actually alters the levels of MnSOD and PAK1, which in turn, resulted in augmented fibronectin expression in mesangial cells in streptozotocin (STZ)-induced diabetic model, thus contributing to DN progression indirectly. TGF- β induced miR-216a expression has been shown to increased collagen (*Col1a2*) expression [116] and subsequently participates in the fibrogenesis in proximal tubular cells (PTCs) [122]. Another important contributor to DN is VEGF and treatment with anti-VEGF showed to improve kidney functions in diabetic animal model [123]. Earlier miRNA-93 was considered as ‘signature miRNA’ in both *in vivo* as well as *in vitro* hyperglycemic environment [124]. Long et al. also demonstrated that increased expression of miR-93 resulted in reduced high glucose-stimulated VEGF-A levels via downregulation of the host *MCM7* gene promoter.

Earlier studies have also reported the role of miRNAs in diabetic retinopathy. Neovascularization is the hallmark of DR and several studies have confirmed the importance of miRNAs in neovascularization regulation in retina [125]. Microarray studies recognized increased (miR-146, miR-106a, miR-181, miR-199a, miR-214, miR-424 and miR-451) as well as decreased expressions of various miRNAs (miR-31, miR-150, miR-184) in model of ischemic retinopathy [126]. In retina and retinal endothelial cells (RECs), increased miRNAs corresponding to NF- κ B, p53 and VEGF were identified reflecting pathological changes of early DR by means of functional analysis, thus, revealing the role of miRNA in pathogenesis of DR [127]. In diabetes, downregulated miR-200b was detected in retina of diabetic rat model with simultaneous elevated levels of VEGF mRNA and protein. In addition, *in vitro* miR-200b antagonist transfection resulted in elevated VEGF expression [128]. This demonstrates VEGF to be the direct target of miR-200b. During early stage of diabetes, miR-29 shown to be anti-apoptotic for retinal ganglion cells (RGCs) and inner nuclear layer (INL) cells through pro-apoptotic RNA dependent (PKR) signaling pathway [129].

Therefore, this chapter has enlightened the role and contribution of epigenetic mechanisms in the pathogenesis of two major diabetic vascular complications i.e.,

DN and DR. Together all, it indicates the important connection of miRNAs with microvascular complications of diabetes; hence, it would be worth to explore the role of these alterations in the pathophysiology of DN as well as DR. As reviewed in this chapter, methylation in DNA, histone tail alterations and variable expressions of miRNAs are found to be altered in hyperglycemic environment either upregulated or downregulated affecting directly or indirectly. Current treatment for DN and DR is not able to stop the progression of these devastating complications, henceforth, focusing treatment approaches via targeting epigenetic alterations alone or in combination with conventional therapy could provide a new approach to combat or retard the progression of these diabetic complications. However, the fact that a particular miRNA can have multiple targets made it difficult and challenging with few limitations, still it will increase our understanding about the disease pathophysiology.

4. Targeting diabetic complications via targeting epigenetic marks

Heritable epigenetic alterations are the results of interactions between environmental (momentary) and genetic (long-standing) components and thus, may play a decisive role in the pathophysiology of diabetic complications. They are able to alter the gene expression, thereby, gene function, the underline mechanism in the pathogenesis of vascular complications of diabetes. Reversible attribute of epigenetic marks provides immense opportunity of developing restorative interventions for treating patients with these complications. Till date, some of the drugs targeting epigenetic marks are already being clinically used for cancer therapy including HDAC inhibitors [130] and DNA methylation inhibitors [131, 132]. However, preclinical studies targeting histone as well as DNA methylation are still in progress [133–135]. Metformin, the current line of drug for treating hyperglycemia, upregulates sirtuin 1 (SIRT1) expression along with downregulating NF- κ B expression [136], SIRT1 has been shown to possess NAD⁺-dependent protein deacetylase activity [137]. In glomerular mesangial cells, SIRT 1 induces antioxidant genes and simultaneously downregulates TGF- β 1 and the expression of AGEs-induced fibronectin [138]. In diabetic mice glomeruli, BF175, a SIRT1 agonist, ameliorates hyperglycemia-induced podocyte loss, proving the protective role of SIRT1 against diabetes-induced kidney damage [139]. Recently, angiotensin II (Ang II) of RAAS has been reported to induce the expressions of few non-coding RNAs including miRNAs [140] and lncRNAs [141] as well. Enhancers, the elements that affect transcription of genes and are associated with specific histone modifications [142], when blocked by JQ1, a Bromodomain (an epigenetic reader) inhibitor, also obstructs enhancer functions along with attenuation of Ang II-mediated hypertension and inflammation *in vivo* in vascular smooth muscle cells (VSMCs) [143], hence, strongly supporting the importance of targeting enhancers in Ang II-mediated actions for treating vascular complications. This, in turn, could reveal evidence directing new therapeutic interventions for treatment of diabetic vascular complications. In addition, the modified inhibitor of miR-192 i.e., Locked nucleic acid (LNA) not only downregulates key fibrotic markers of kidney damage but also shown to reduce proteinuria in diabetic mice [144], favoring miRNAs based therapeutic interventions for DN. Several studies have also reported the amelioration of kidney-injury parameters via targeting miR-21 [145–147] implying that its inhibition could be a promising therapeutic intervention in DN. Recently with the use of latest and advanced approach of genome editing i.e., CRISPR-Cas9, locus-specific changes in epigenetic alterations could be generated owing to the fusion of Cas9 proteins with various DNMTs or TETs or histone modification proteins [148–150],

thus, reversing the epigenetic marks of important genes involved in the pathogenesis of s disease. Despite extensive ongoing research, more detailed epigenetics-targeted approach is required to combat diabetic microvascular complications.

5. Conclusion

In conclusion, discovering specific role and targeting pathways related to epigenetic alterations for the development of therapeutic interventions in T2DM patients with microvascular complications could be promising. Moreover, this will certainly be helpful in increasing our knowledge and developing tools for better and early diagnosis and subsequent more effective treatment of these distressing complications in clinical practice.

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