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ORIGINAL ARTICLE

Comprehensive gene expression analysis of histone deacetylases and the transcription factor Nrf2 in the progression of diabetic nephropathy

Kannan Harithpriya[1] · Udyama Juttada[2] · Ravichandran Jayasuriya[1] · Satyavani Kumpatla[2] · Vijay Viswanathan[2] · Kunka Mohanram Ramkumar[1]

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Abstract

Background Nuclear factor erythroid-2-related factor 2 (Nrf2) is a crucial transcription factor in maintaining cellular homeostasis. The regulation of Nrf2 expression is an essential target for treating diabetic nephropathy (DN), and this regulation has been reported to be influenced by epigenetics. Few studies highlighted that Nrf2 regulation is associated with epigenetic markers such as histone deacetylases (HDAC) and DNA methyltransferase (DNMTs).

Objective This cross-sectional study aimed to investigate the association of all isoform-specific HDACs and their correlation with Nrf2 expression with the development of DN among south Indian people with type 2 diabetes (T2DM).

Methods A case–control cross-sectional study was performed using 108 T2DM individuals, comprising 23 participants with only T2DM and 60 participants with DN without any other complications, and 25 healthy volunteers. The gene expression of Nrf2, its downstream targets, and all HDAC targets involved in this study were assessed using qPCR.

Results We observed a significant decrease in the gene expression of Nrf2 in the DN group compared to healthy controls. In parallel, a significant downregulation of HDAC3/7/8/9/10/11 and SIRT1/2/3/4/7 has been observed in DN subjects compared to T2DM. On the other hand, HDAC1/2/4/5/6 showed a significant upregulation in DN groups compared to T2DM. A significant negative correlation between Nrf2 and HDAC1/2/4/5 was observed, which infers an imbalance in the Nrf2-HDAC axis.

Discussion In summary, our study findings provide compelling evidence of the association between HDACs and Nrf2 in the pathogenesis of DN, shedding light on potential therapeutic avenues for this condition.

Keywords Nrf2 · HDACs · Microalbuminuria · Macroalbuminuria · Epigenetic regulation

Introduction

Diabetes is characterized by chronic hyperglycemia resulting from defects in insulin secretion, insulin action, or both and rising to an alarming epidemic level [1]. The chronic hyperglycemia of diabetes is associated with an increased risk of microvascular complications, including diabetic nephropathy (DN), which has been identified as the most common cause of chronic kidney failure or end-stage kidney disease (ESKD) that affects 20–40% of the diabetic population in their lifetime [2]. The prevalence of DN that turns into ESKD continues with an increasing number of dialysis and renal transplants worldwide. The indisputable contribution of oxidative stress to the pathogenesis of DN highlights the deficit of antioxidant defense in the body. Nuclear factor erythroid 2-related factor 2 (Nrf2) is one such master regulator of redox homeostasis that triggers a battery of antioxidant genes enzymes like catalase (CAT), NAD(P)H quinone oxidoreductase-1 (NQO1), heme oxygenase-1 (HO-1), glutathione peroxidase (GPx), and superoxide dismutase (SOD) through its nuclear translocation [3]. Previous reports have highlighted that activation of Nrf2 prevents the progression

✉ Vijay Viswanathan
drvijay@mvdiabetes.com

✉ Kunka Mohanram Ramkumar
ramkumak@srmist.edu.in

[1] Department of Biotechnology, School of Bioengineering, SRM Institute of Science and Technology, Kattankulathur, Tamil Nadu 603203, India

[2] Department of Biochemistry and Molecular Genetics, Prof. M. Viswanathan's Diabetes Research Center, M.V. Hospital for Diabetes, Royapuram, Chennai, Tamil Nadu 600013, India

of DN by inhibiting Reactive oxygen species (ROS) production [4]. Dysregulated Nrf2 signaling that cannot combat the prevalence of chronic oxidative stress in the progression of DN is documented.

Individuals with good glycemic control can evade hyperglycemic stress, although metabolic memory is a problem that worsens more with further complications like DN [5]. While much research has been focused on the genetic modifications of Nrf2, recent studies have revealed that substantial epigenetic alterations within the Nrf2 gene play a pivotal role in driving the progression of DN [6]. Epigenetic alterations, including aberrant DNA methylation of Nrf2 and alterations in Nrf2-associated histone modifications, are likely to play a role in the progression of DN. Histone deacetylases (HDACs) are certain classes of epigenetic regulators that remove the acetyl group from histone proteins [7]. It is known that the human body contains 18 HDAC enzymes categorized into four classes: class I comprises RPD3-like proteins (HDAC1, HDAC2, HDAC3, and HDAC8); class II consists of Hda1 proteins (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10); class III encompasses Sir2-like proteins known as sirtuins (SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, and SIRT7); and class IV includes HDAC11. HDACs are recognized for their ability to modulate the expression of numerous cell cycle regulators. Though the association of HDACs has been recently investigated, its involvement in the control of Nrf2 in DN remains unknown. Hence, in the present study, we aimed to profile various classes of HDACs (classes I/II/IV and sirtuins–class III) among people with different grades of DN. Furthermore, we intend to use correlation analysis to identify the potential that dysregulates Nrf2 in the progression of DN, shedding light on the novel treatment strategy by describing the critical pathological mechanisms of DN.

Materials and Methods

Study design

A total of 108 patients were recruited for the cross-sectional study with the age ranging from 30 to 70 years. The experimental groups were categorized as NGT—participants having normal glucose tolerance; T2DM—participants diagnosed with type 2 diabetes mellitus without any complications; and DN—participants diagnosed with diabetic nephropathy based on eGFR and albumin ratio. The DN group was screened based on KDIGO classification on both eGFR (G) and albumin (A) stages. The DN group was further grouped as microalbuminuria (micro) comprising participants falling under the categories G1A2, G2A2, G1A3, G2A3, G3Aa1, G3Aa2, and G3Ba1. On the other hand, the macroalbuminuria (macro) group included subjects categorized as G4A1, G4A2, G4A3, G5A1, G5A2, G5A3, G3bA2, G3bA3, and G3aA2. The inclusion criteria for the recruitment of study participants for DN were adults with the normal range of white blood cells to minimize the confounding effect of infections, duration of diabetes more than 5 years, Hb1Ac 8–10%, and the estimated eGFR should be in the range ≥ 80 to ≤ 15 (mL/min/1.73 m^2). Those with urinary tract infection, other renal disease, rheumatological, neoplastic, and other endocrine diseases (except T2DM) and with abnormal erythrocyte sedimentation rate (ESR) and total lymphocyte count (TLC) were excluded from this study to ensure the presence of infection did not affect the study variables. For enrolling participants under NGT, the participants were screened for no traces of diabetes with parameters, HbA1c < 6.5%, FPG < 100, and PPG < 130 and also screened for ruling out any secondary complications by restricting the participants with eGFR ratio > 80 and creatinine < 0.1. For enrolling participants under T2DM, the participants were screened for no traces of other complications with parameter HbA1c 6.5–10%, FPG > 120, PPG > 140, eGFR > 80, and creatinine < 1, and the duration of diabetes mellitus ranged between 2 and 12 years; biothesiometer test negative; diabetic nephropathy, retinopathy eye check-up (both NPDR &OPDR) – negative; no foot ulcers; and normal LET levels SGOT < 40U/L, and SGPT < 50 U/L. For enrolling participants for DN, the participants having diabetes for at least 10 years were recruited having eGFR rate > 30 mL/min, albumin to creatinine ratio: < 30 mg/mmol under microalbuminuria, having eGFR rate < 30 mL/min; and albumin to creatinine ratio: > 30 mg/mmol under macroalbuminuria.

Isolation of peripheral blood mononuclear cells (PBMCs)

The venous blood was collected from each participant in EDTA-coated blood collection tubes (BD, USA). The fresh blood was layered over 3 mL of Histopaque solution (Sigma, USA). The contents were centrifuged at 3500 rpm for half an hour at room temperature, and the buffy coat containing PBMCs was carefully separated into a sterile microcentrifuge tube. The buffy coat was washed twice with about 500 µL of 1X PBS, and the resultant pellet was stored in TRIzol (RNA isoplus Reagent, Takara, Japan) at – 80 °C until further use.

Total RNA isolation

About 300 µL of TRIzol was added to the PBMC pellet and lysed by vortex for about 30 min on ice. After complete lysing of cells, 200 µL of chloroform (Himedia, India) was added to the homogenate and mixed on ice. The contents were centrifuged at 12,500 rpm at 4 °C for 20 min. The upper aqueous layer was carefully separated and transferred to a new sterile microcentrifuge tube. An equal volume of isopropanol (Himedia, India) was added to the aqueous layer, mixed, and stored at − 20 °C for 3 h. After the incubation, the contents were centrifuged at 12,000 rpm at 4 °C for 20 min. The pellet obtained was washed twice with 70% ethanol and dissolved in RNase-free water.

cDNA synthesis and quantitative real-time PCR (q-RT-PCR)

The quality and concentration of RNA were estimated using a Nano quant photo spectrometer (Tecan, Switzerland). The RNA with purity 2.0 was considered and reverse transcribed using an iScirpt cDNA synthesis kit (Bio-Rad, USA). The cDNA obtained was used to assess target gene expression levels using SSO Advanced SYBR premix (Bio-Rad) on a CFX connect RT-PCR instrument (Bio-Rad). The expression of Nrf2, its downstream targets (NQO1, CAT, SOD, HO-1), angiogenic markers (HIF1-α, SDF, VEGF), and all HDACs (class I- IV) was calculated using $2-\Delta\Delta Ct$, normalized to GAPDH housekeeping gene.

Statistical analysis

Statistical calculations were performed using SPSS (version20.0; SPSS, Chicago, IL, USA). Statistical significance was evaluated by one-way analysis of variance, and the individual comparison was obtained by Tukey's multiple comparisons test. All analyses for graphical plots were performed on GraphPad Prism software (v.8.4.2). Multiple binary logistic regression analysis was performed to determine the association of age and BMI with other study variables. Pearson's correlation analysis was used to determine the correlation between HDACs and Nrf2 used in this study. A statistically significant result was defined as a p value < 0.05.

Results

Clinical and biochemical characteristics of the study participants

The clinical and biochemical characteristics of the study participants are presented in Table 1. Among the 108 participants recruited for this study, we specifically excluded

Table 1 Clinical and biochemical characteristics of the study groups

Clinical parameters	NGT ($n=20$)	T2DM ($n=20$)	Microalbuminuria ($n=25$)	Macroalbuminuria ($n=27$)
Gender (M/F)	8 M/12F	10 M/10F	19 M/6F	18 M/9F
Age (years)	53.05 ± 6.92	50.8 ± 6.97 ^{ns}	54.5 ± 6 ^{ns}	54.92 ± 6.21 ^{ns}
BMI (kg/m^2)	28.58 ± 7	28.26 ± 5.28 ^{ns}	28.15 ± 4.4 ^{ns}	28.33 ± 4.57 ^{ns}
SBP (mm Hg)	124.15 ± 14.2	122.3 ± 16.76 ^{ns}	129.16 ± 13.3 ^{ns}	136.96 ± 22.44 $^{ns\ \#}$
DBP (mm Hg)	79.3 ± 9.34	74.75 ± 8.55 ^{ns}	79 ± 8.9 ^{ns}	77.44 ± 11.97 ^{ns}
FPG (mg/dL)	93.41 ± 36.18	127.5 ± 37.24 ^{ns}	168.16 ± 40.06***	167.8 ± 77.01***
PPG (mg/dL)	133.13 ± 64.56	204.1 ± 75.88 ^{ns}	286.2 ± 88.8***$^{\#}$	285.6 ± 139.1***$^{\#}$
HbA1c (%)	5.53 ± 0.5	7 ± 1.48***	9 ± 0.92*** $^{\#\#\#}$	8.77 ± 0.86*** $^{\#\#\#}$
TSC (mg/dL)	186.88 ± 66.28	160.25 ± 47.05 ^{ns}	149.72 ± 37.85 ^{ns}	155.42 ± 49.17 ^{ns}
HDL-c (mg/dL)	52.9 ± 18.55	51.45 ± 8.53 ^{ns}	48.92 ± 6.7 ^{ns}	48.04 ± 18.91 ^{ns}
LDL-c (mg/dL)	103 ± 38.93	81.8 ± 30.6 ^{ns}	74.12 ± 21.63*	77.54 ± 34.43*
Urea (mg/dL)	17.5 ± 3.98	21.6 ± 5.2 ^{ns}	24.32 ± 6.24 ^{ns}	48.61 ± 32.06*** $^{\#\#\#}$
Creatinine (mg/dL)	0.93 ± 0.18	0.9 ± 0.15 ^{ns}	1.04 ± 0.15 ^{ns}	2.08 ± 1.1*** $^{\#\#\#}$
WBC (10^9/L)	6.8 ± 2.7	8 ± 3.2	7.39 ± 1.7	9.7 ± 3.2**
eGFR rate (mL/min/1.73 m^2)	82.35 ± 16.8	86.35 ± 16	76.76± 14.75	39.81 ± 21.79***,$^{\#\#\#}$
Diabetes duration (years)	0	6.55 ± 5.61***	14.96 ± 4.92***$^{\#\#\#}$	17.06 ± 6.68***$^{\#\#\#}$

Data represented as mean ± SD. The statistical significance was analyzed using SPSS software (v.20.0)

ns non-significant

*Comparison between NGT and experimental groups

$^{\#}$Comparison between T2DM and DN study groups

$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, and $^{\#\#\#}p<0.001$

5 NGT, 3 T2DM, 5 microalbuminuria, and 3 macroalbuminuria participants in order to ensure age matching across the groups. Hence, further analyses were carried out using a total of 92 participants (Figure S1). BMI was found to be non-significant between the study groups. Duration of diabetes in the T2DM group was 6.55 ± 5.61 years, whereas it was significantly increased to 14.96 ± 4.92 years and 17.06 ± 6.68 years for the microalbuminuria and macroalbuminuria groups, respectively. Our analysis within the DN subjects revealed no significant difference in duration of diabetes between micro and macroalbuminuria groups. Likewise, fasting blood glucose (FBG) was significantly higher in T2DM, microalbuminuria, and macroalbuminuria groups, when compared to healthy volunteers. As mentioned earlier, significant increase in HbA1c was observed in DN participants, compared to NGT participants. Along with this, we also noted a significant increase in the levels of urea and creatinine, with lowered eGFR rate among the DN participants, compared to healthy volunteers. Furthermore, to account for potential variations in age and BMI among the groups and their impact on epigenetic alterations of Nrf2 by HDACs, we conducted multivariable regression analysis. The logistic model table predicted that age ($p = 1.000$) and BMI ($p = 1.000$) were not confounding to the epigenetic changes in the participants with DN with respect to NGT case and T2DM (Table S1 and S2).

Expression of Nrf2 and its downstream targets among the study groups

To check the status of Nrf2 in PBMCs of DN study groups, we analyzed the mRNA (Fig. 1a) using q-RT-PCR analysis. We observed the mRNA expression of Nrf2 was decreased in T2DM, followed by an average of 4.5-fold decrease in micro and macroalbuminuria of DN compared to the control. The mRNA expression of Nrf2 downstream targets such as NQO1 (Fig. 1b), CAT (Fig. 1c), HO-1 (Fig. 1d), and SOD-1 (Fig. 1e) was found to be decreased by an average fold change of 1.5 in individuals with T2DM when compared to NGT. Further, we observed a downregulated expression of these targets with a fold change of 2.4 in microalbuminuria compared to the NGT. Further, when compared to NGT, T2DM, and microalbuminuria subjects, the macroalbuminuria cases are observed with a significant decrease in expression of NQO1 with 1.5-fold, CAT with twofold, HO-1 with 3.1-fold, and SOD-1 with 1.9-fold.

Gene expression of class I HDACs and its correlation with Nrf2 among DN groups

The expression of class I HDACs such as HDAC1, 2, 3, and 8 is represented in Fig. 2a–d. As depicted in the figure, we observed an increase in the expression for HDAC1 and 2 in T2DM participants without DN compared to NGT groups. Further, we observed a gradual rise in HDAC1

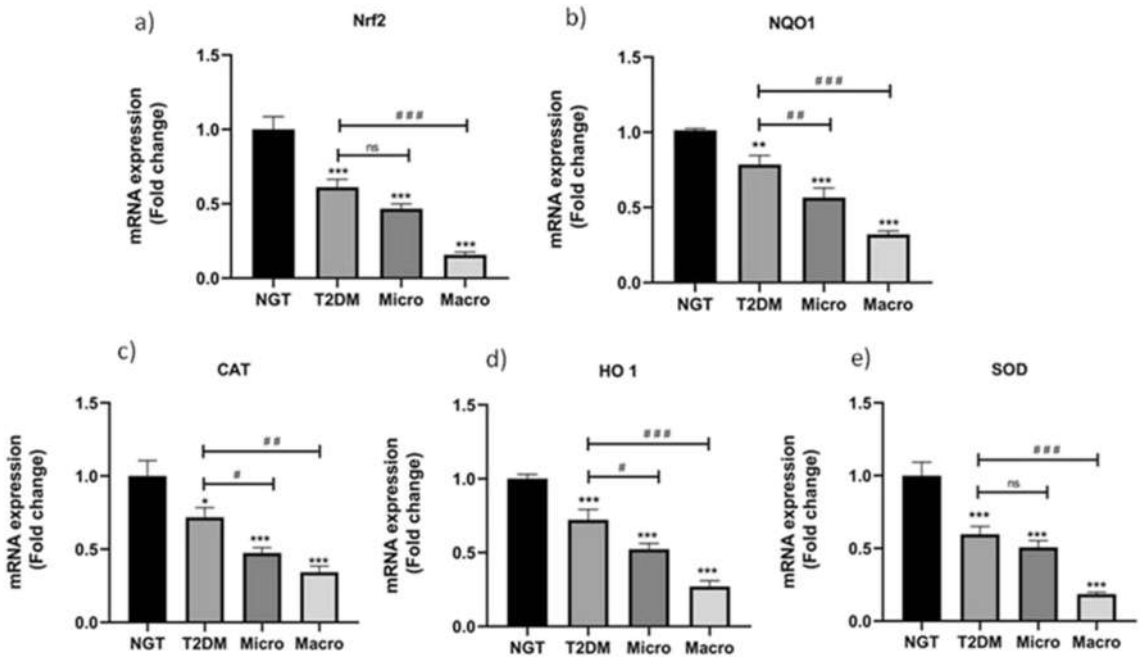

Fig. 1 mRNA expression of **a** Nrf2, **b** NQO1, **c** CAT, **d** HO-1, and **e** SOD among the study subjects. Data are represented as mean ± SEM. $^{*,\#}p < 0.05$, $^{**,\#\#}p < 0.01$, and $^{***,\#\#\#}p < 0.001$; NGT, normal glucose tolerance; T2DM, type 2 diabetes mellitus; Micro, microalbuminuria; Macro, macroalbuminuria. * means comparing with control; # means comparison with T2DM

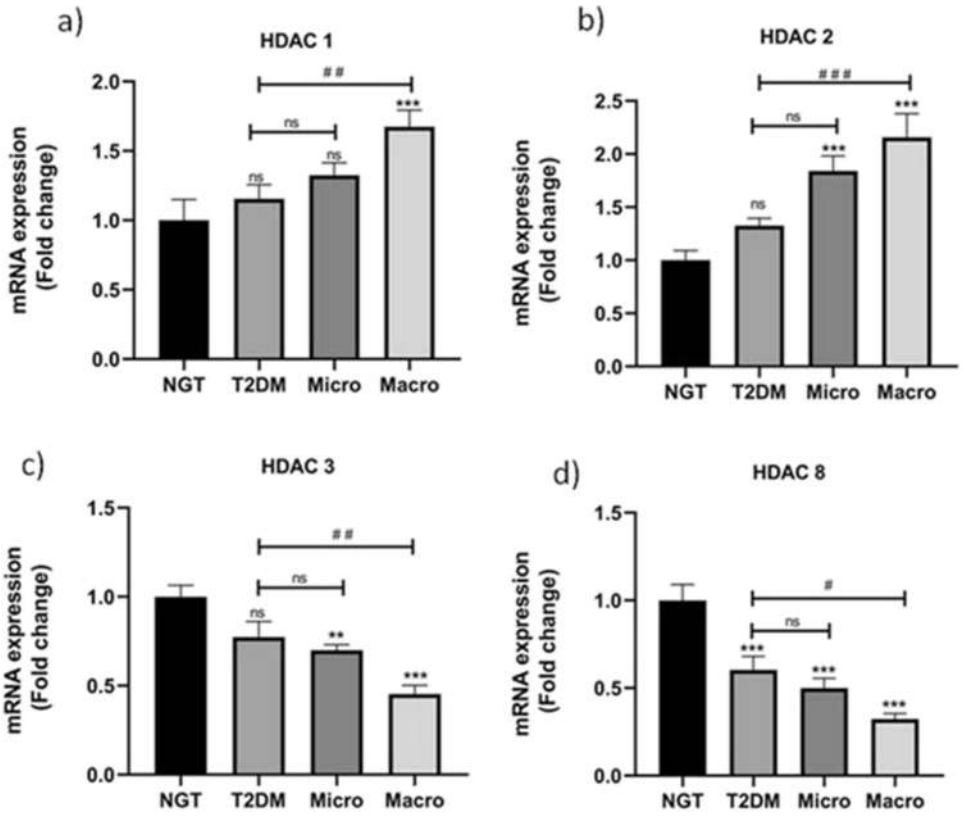

Fig. 2 mRNA expression of class 1 HDACs **a** HDAC1, **b** HDAC2, **c** HDAC3, and **d** HDAC8 among the study subjects. Data are represented as mean ± SEM. $^{*,\#}p < 0.05$, $^{**,\#\#}p < 0.01$, and $^{***,\#\#\#}p < 0.001$; NGT, normal glucose tolerance; T2DM, type 2 diabetes mellitus; Micro, microalbuminuria; Macro, macroalbuminuria. * means comparing with control; # means comparison with T2DM

Table 2 Pearson's correlation coefficient of class I HDACs with Nrf2 among DN subjects

HDACs	Micro		Macro	
	r value	*p* value	*r* value	*p* value
HDAC1	-0.843^{**}	0.001	-0.867^{*}	0.001
HDAC2	-0.633^{*}	0.027	-0.865^{**}	0.001
HDAC3	0.795^{**}	0.006	0.846^{**}	0.002
HDAC8	0.742^{**}	0.006	0.674^{*}	0.033

The statistical significance was analyzed using SPSS software (v.20.0)

micro microalbuminuria, *macro* macroalbuminuria, *r* Pearson's coefficient, *p* significance

$^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$

and 2 in participants with microalbuminuria and macroalbuminuria compared to NGT. On the other hand, a significant downregulation were seen in HDAC3 and 8 in microalbuminuria of DN groups. Also, the macroalbuminuria study population showed a further decrease in the expression of these HDACs, with an average fold change ranging from 2.2 to threefold. Upon correlating HDACs with Nrf2 expression, HDAC1 and 2 showed a negative correlation, and HDAC3 and 8 showed a positive correlation, as given in Table 2.

Gene expression of class II HDACs and its correlation with Nrf2 among individuals with DN

Along with this, the expression of class II HDACs such as HDAC4, 5, 6, 7, 9, and 10 was profiled using qRT-PCR and represented in Fig. 3a–f. We observed a gradual increase in HDAC4, 5, and 6 expression with an average fold change of 1.3 in T2DM compared to NGT without diabetes. As depicted in Fig. 3, as the disease progressed, the expression of these HDACs showed a further increase with an average fold change of 1.4 and 2.5 in patients with micro and macroalbuminuria compared to T2DM and NGT. On the other hand, among class II HDACs, HDAC7, 9, and 10 showed a decrease in the expression of T2DM when compared to healthy individuals. Further, we observed a gradual reduction in the expression as the disease progressed, with an average change of twofold in microalbuminuria patients and a 3.5-fold decrease in macroalbuminuria compared to T2DM and NGT. Overall, HDAC4, 5, and 6 showed a negative correlation with Nrf2, whereas HDAC7, 9, and 10 showed a positive correlation with Nrf2 (Table 3).

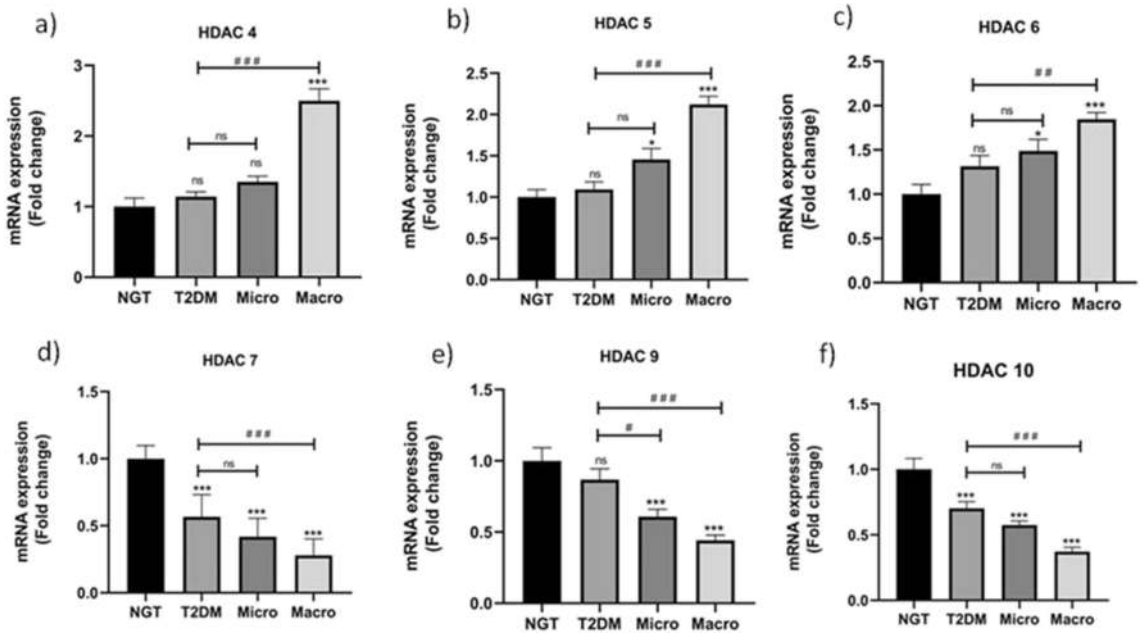

Fig. 3 mRNA expression of class II HDACs **a** HDAC4, **b** HDAC5, **c** HDAC6, **d** HDAC7 **e** HDAC9, and **f** HDAC10 among the study subjects. Data are represented as mean ± SEM. $^{*,\#}p < 0.05$, $^{**,\#\#}p < 0.01$, and $^{***,\#\#\#}p < 0.001$; NGT, normal glucose tolerance; T2DM, type 2 diabetes mellitus; Micro, microalbuminuria; Macro, macroalbuminuria. * means comparing with control; # means comparison with T2DM

Table 3 Pearson's correlation coefficient of class II HDACs with Nrf2 among DN subjects

HDACs	Micro		Macro	
	r value	*p* value	*r* value	*p* value
HDAC4	-0.911^{**}	0.001	-0.952^{**}	0.001
HDAC5	-0.644^{*}	0.024	-0.798^{**}	0.010
HDAC6	-0.844^{**}	0.001	-0.798^{**}	0.006
HDAC7	0.697^{*}	0.025	0.859^{**}	0.001
HDAC9	0.697^{*}	0.012	0.882^{**}	0.001
HDAC10	0.810^{**}	0.001	0.752^{*}	0.012

The statistical significance was analyzed using SPSS software (v.20.0)

micro microalbuminuria, *macro* macroalbuminuria, *r* Pearson's coefficient, *p* represents significance

$^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$

Gene expression of class III (Sirtuins) and class IV HDACs and its correlation with Nrf2 individuals with DN

The HDAC11 (class IV) (Fig. 4a) showed a gradual decrease in expression as the disease progressed with the fold change of 1.1 to 2.4 from T2DM and DN with micro- and macroalbuminuria compared to NGT without diabetes.

The class III HDACs known as sirtuins, such as SIRT1, 2, 3, 4, and 7, are represented in Fig. 4b–f. We observed a gradual decrease in the expression of these HDACs in T2DM compared to NGT without diabetes. As depicted in Fig. 4, HDACs such as SIRT1, 2, 3, 4, and 7 showed an average 1.5–twofold reduction in expression compared to T2DM and NGT. Further, it was evident that as the disease progressed, the expression of these class III HDACs in macroalbuminuria study population seemed to decrease with the fold change ranging from 1.5 to fourfold compared with T2DM participants and NGT. Upon correlating, all the class III and class IV HDACs showed a positive correlation with Nrf2 expression (Table 4). The heat map depicted in Fig. 5 summarizes the differential expression of HDACs analyzed in the study.

Discussion

Prolonged exposure to hyperglycemia may increase oxidative stress, which could play a role in the pathogenesis of diabetic nephropathy [8]. To combat such stress, increasing the level of one important redox regulator transcription factor, Nrf2 has gained interest. The regulator of Nrf2 is said to be regulated by epigenetic modulation, which includes DNA methylation and modified histone proteins [9, 10]. Regarding this, we are resolute in studying the expression of epigenetic modulation in regulating the Nrf2-mediated disease pathogenesis.

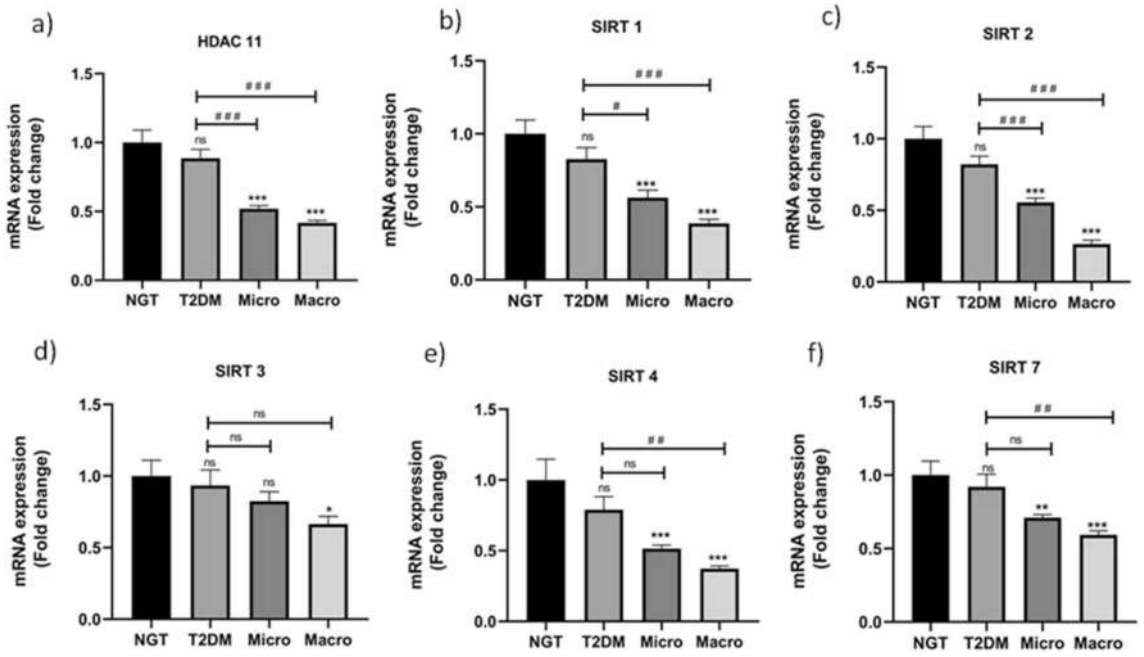

Fig. 4 mRNA expression of class IV and III HDACs **a** HDAC11, **b** SIRT 1, **c** SIRT 2, **d** SIRT 3, **e** SIRT 4, and **f** SIRT 7 among the study subjects. Data are represented as mean ± SEM. $^{*,\#}p < 0.05$, $^{**,\#\#}p < 0.01$, and $^{***,\#\#\#}p < 0.001$; NGT, normal glucose tolerance; T2DM, type 2 diabetes mellitus; Micro, microalbuminuria; Macro, macroalbuminuria. * means comparing with control; # means comparison with T2DM

Table 4 Pearson's correlation coefficient of class III and IV HDACs with Nrf2 among DN subjects

HDACs	Micro		Macro	
	r value	*p* value	*r* value	*p* value
HDAC11	0.797**	0.002	0.747**	0.013
SIRT1	0.733**	0.007	0.756*	0.011
SIRT2	0.764*	0.004	0.780**	0.008
SIRT3	0.700*	0.011	0.679*	0.031
SIRT4	0.621*	0.031	0.708**	0.022
SIRT7	0.613*	0.034	0.696*	0.025

The statistical significance was analyzed using SPSS software (v.20.0)

micro microalbuminuria, *macro* macroalbuminuria, *r* Pearson's coefficient, *p* significance

*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$

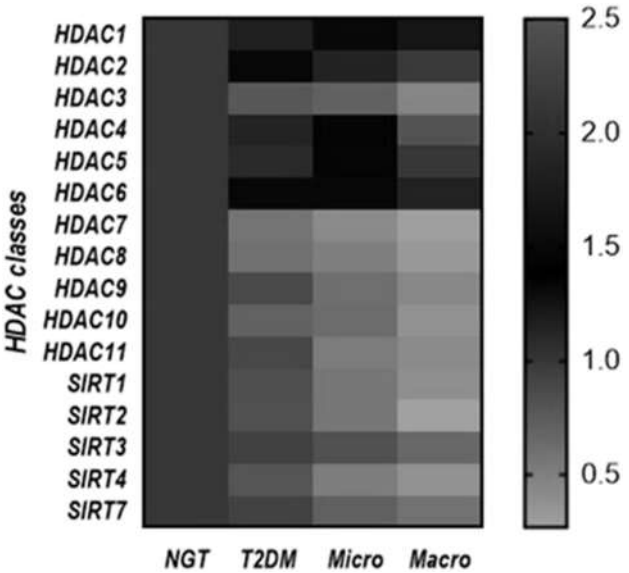

Fig. 5 Heat map representing the differential expression of HDACs among study subjects using GraphPad Prism 8.4.0. NGT, normal glucose tolerance; T2DM, type 2 diabetes mellitus; Micro, microalbuminuria; Macro, macroalbuminuria

Our expression study found that as the disease progresses, the expression of Nrf2 and its downstream targets are reduced gradually in individuals diagnosed with DN. At the later stage of diabetic nephropathy, the expression of these antioxidant defense mechanism enzymes and Nrf2 get saturated as an increase in OS is seen, which leads to end-stage renal damage. Tao et al. have demonstrated that stress-mediated activation of Nrf2 neutralizes ROS and improves renal function [4]. Liu et al. also showed that an increase in the expression of pro-inflammatory cytokines and chemokines in Nrf2 − / − mice exacerbates renal function and survival [11]. This evidence suggests that Nrf2 is a

chief regulator in maintaining redox homeostasis, and thus, we assessed the role of epigenetic markers in altering the expression of Nrf2 in DN individuals.

Epigenetic alterations of Nrf2 involve inheritable changes in the gene function with respect to no change in its nucleotide [12]. Unlike genetic changes, these epigenetic changes are essential in maintaining redox homeostasis at the cellular level. In this regard, a previous study by Pergola et al. identified that Nrf2 reduces macrophage infiltration by reducing the oxidative load [13]. Also, evidence suggests that activation of Nrf2-mediated ARE elements in the liver fibrosis mouse model was shown to inhibit the function of pro-inflammatory cytokines by inhibiting the ROS, thereby preventing DN disease progression [14]. Yet the contributing factors behind the Nrf2 dysregulation in DN individuals are unclear. Epigenetic alteration was reported to be involved in ameliorating the expression of Nrf2 in *in-vitro* gained interest [15, 16]. Yet another epigenetic signature, HDACS, helps in silencing the gene function by eliminating the acetyl group from lysine residue, causing a conformational change in chromatin [17, 18]. Hence, it is necessary to unravel the role of HDACs in regulating the expression of Nrf2 to observe the molecular events puzzling the disease pathogenesis.

Our study showed a progressive increase in the expression of HDAC1 and HDAC2 under the class I zinc-dependent HDACs. The activated Nrf2 associates with sMaf protein and facilitates the transcription of ARE-driven antioxidant enzyme pockets. The Binding of HDAC1 with maf makes a complex with Bach1 a primary leucine zipper transcription factor 1, thus inhibiting the Nrf2-ARE-driven antioxidant signaling. In line with this, a negative correlation was observed between Nrf2 and HDAC1 and 2 in micro- and macroalbuminuria, further supporting the deficit in initiating antioxidant genes among DN study groups [19]. HDAC2 negatively correlates with Nrf2 by inducing hypoacetylation, binding to the ARE site, and repressing ARE-dependent gene transcription. Studies have revealed that HDAC1 knockout resulted in overexpression of HDAC2, resulting in a negative correlation. Among class I HDACs, HDAC3 and 8 showed a progressive decrease in micro and macroalbuminuria DN in healthy volunteers [20]. The finding by Jeong et al. further validates our findings, in which the expression of Nrf2 is negatively correlated with HDAC1 and HDAC2. HDAC3 and 8 are reported to be positively correlated to poor glycemic control, pro-inflammation, and insulin resistance. As reported in this study, the diabetes group also had poor glycemic control and hence decreased expression of HDAC3 and 8 in DN participants compared to healthy individuals [21].

Considering class II, HDAC4 is increased, as observed in our study. As per the finding of Wang et al., HDAC4 activates NF-kB by increasing ROS accumulation. It is not surprising that NF-kB suppresses Nrf2 and activation of NK-kB signaling through upregulation of HDAC4 arrests the action of Nrf2 [22], as seen in our current study. Also, HDAC5 and 6 are negatively correlated to Nrf2 expression in micro and macroalbuminuria DN individuals compared to T2DM and NGT. In contrast, HDAC7, 9, and 10 are positively associated with Nrf2 expression among DN individuals compared to the NGT.

Sirtuins (SIRTs) are widely classified as class-III HDACs. Similar to other HDAC classes, they maintain homeostasis in cellular functions. SIRT1, whose expression is relatively low in DFU cases compared to controls, has been reported to have a direct association with Nrf2. This was confirmed by Hong and the group when increased SIRT1 activity protects against diabetes-induced podocyte injury in vitro [23]. The Nrf2-specific role of SIRT1 was reported by Huang et al., where SIRT-1 was negatively correlated with IL-6, a pro-inflammatory cytokine that disturbs Nrf2 signaling. Moreover, this research group also suggested a positive association between Nrf2 and SIRT1, similar to our study. Yet another essential sirtuin, the SIRT2, was observed to be low in DN cases. This correlates with the previous research where SIRT2 was reported to act as a central regulator of defense mechanism against ROS-induced stress in the diabetic environment [24–27]. An overview of Nrf2-mediated SIRT3 induction has been provided by Kim et al., according to one of his earlier reports. It has also been found that ARE binding site in the SIRT3 gene protects patients with liver disease against endoplasmic reticulum stress [27]. SIRT4 and SIRT7 were downregulated with decreased levels of Nrf2, suggesting a positive correlation among micro and macroalbuminuria DN individuals. To our knowledge, we did not find any line of evidence for sirtuins-mediated Nrf2 regulation in DN individuals.

Taking into account, HDACs are one of the key elements in regulating Nrf2 gene expression, which involves cellular homeostatic mechanisms. Altered expression of these epigenetic markers plays a potential role in disease progression, as seen in DN, and targeting them would help foresee defined homeostasis in disease conditions like DN [28]. While there exists a shred of evidence for HDAC4-mediated podocyte injury in diabetes-induced mice kidneys [29], we also put forth HDAC4 as a signature epigenetic candidate in the progression of disease associated with Nrf2 dysregulation in the progression of DN in humans. To date, four HDACi have been approved by the FDA against cancer and cardiovascular diseases, including vorinostat, romidepsin, panobinostat, and belinostat. However, there are still ongoing pre-clinical trials for understanding the HDACi mechanism in treating diabetes and its complications [30]. Inhibition of such potent HDAC may result in a widespread alternation of Nrf2 gene regulation and help to gain homeostasis for a controlled cellular response, which will be considered further. Moreover, this

study provides preliminary evidence that among 18 isoforms of HDACs, HDAC4 specifically contributes to dysregulated Nrf2 expression and disease pathogenesis. Therefore, it is essential to elucidate the role of these HDACs in diabetic kidneys, paving the way to develop novel HDAC inhibitors as one of the therapeutic strategies to combat disease pathogenesis. While this study presents preliminary evidence of the potential involvement of HDACs in dysregulating Nrf2 expression and contributing to disease pathogenesis within the South Indian population, it also reveals certain limitations. The findings presented here are confined to this specific demographic, which might restrict the broader applicability of the findings. Future investigations involving larger sample sizes and diverse populations are warranted to validate these findings across different ethnic groups.

Furthermore, while our study focused on HDAC expression, exploring additional epigenetic markers such as DNMT could offer valuable insights into the progression DN. Incorporating assessments of DNMT activity and its interactions with Nrf2 signaling pathways may provide a more comprehensive understanding of the underlying mechanisms driving DN pathogenesis. Such investigations could pave the way for the development of novel therapeutic strategies, including the exploration of HDAC inhibitors, to target epigenetic dysregulation and mitigate the progression of DN.

Conclusion

In summary, our study highlights the significance of Nrf2 dysregulation and its downstream targets in DN progression. These findings underscore the pivotal role of antioxidant defense signaling in DN, contributing to our understanding of its pathogenesis. Additionally, our comprehensive profiling of HDAC classes provides insights into the complex mechanisms governing the regulation of Nrf2 in the context of DN. Specifically, our investigation identified HDAC4 as a key epigenetic player, suggesting its potential as a distinctive candidate implicated in the progression of DN and its close association with Nrf2 dysregulation in human subjects.

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Data availability Data will be made available on request.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical clearance and consent of patient Approximately 4 mL of venous blood was drawn from each participant from a Tertiary care center for Diabetes, Chennai, where an institutional ethical clearance was approved (IEC/N-002/10/2021) and the recruitment of study participants for a period of 3 to 5 months.

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