Nuclear Factor Erythroid Related Factor2 Gene Polymorphism in Patients with Diabetic Foot Ulcer

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Abstract

Background: Recent research is now focusing on the role of epigenetic factors, which by themselves and/or in combination with classical genetic factors, may be the major causative factor for progression of diabetic foot ulcer (DFU). The molecular mechanisms that down regulate the NRF2 expression in Type 2 diabetes mellitus (T2DM) and DFU remain unresolved.

Aim: to investigate potential association of Nrf2 gene polymorphism with development of DFU.

Methods: This case-control study included 81 subjects who were categorized into three groups; group 1 included 27 healthy individuals as a control group, group 2 included 27 type 2 diabetic patients without diabetic foot ulcer and group 3 included 27 type 2 diabetic patients with diabetic foot ulcer. Genotyping of NRF2 (rs35652124) single nucleotide gene polymorphism was done by by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

Results: In the diabetic foot ulcer group, CC genotype was present in 1 (4%) cases, CT and TT genotypes were in 13 (48) and 13 (48%) cases. CT and TT genotypes of NRF2 were significantly higher in diabetic foot ulcer group compared to controls. There was significant difference between diabetic foot ulcer cases and controls regarding to T allele of NRF2 that was higher in diabetic foot ulcer cases than control. There was significant difference regarding C and T allele that was higher in diabetic foot ulcer group than T2DM group.

Conclusion: TT genotypes of NRF2 were also higher in case of DFU compared to T2DM. T allele is significantly higher in DFU patient group than only diabetic groups, Nrf2gene has a proper value in prediction of T2DM and development of diabetic foot ulcer in patients with T2DM.

Keywords: Nuclear Factor Erythroid Related Factor2, Diabetic Foot Ulcer.

1. Introduction

Diabetes mellitus (DM) is characterized by chronic hyperglycemia and impaired carbohydrates, lipids, and proteins metabolism caused by complete or partial insufficiency of insulin secretion and/or insulin action. There are two primary forms of diabetes, insulin-dependent diabetes mellitus (type 1 diabetes mellitus, T1DM) and non-insulin-dependent diabetes mellitus (type 2 diabetes mellitus, T2DM). T2DM is the most common form of DM, which accounts for 90% to 95% of all diabetic patients and is expected to increase to 439 million by 2030. **(1).**

T2DM is strongly associated with both microvascular (retinopathy, nephropathy, and neuropathy) and macrovascular complications, including ischemic heart disease, peripheral vascular disease, and stroke **(2).** Diabetic foot ulcer (DFU) is the fastest growing chronic complication of diabetes and major cause of mortality in the diabetic population **(3).**

The progression of DFU is often complicated by wide range diabetic changes such as neuropathy and vascular disease. Recent research is now focusing on the role of epigenetic factors, which by themselves and/or in combination with classical genetic factors, may be the major causative

factor for progression of DFU. Nuclear factor erythroid 2-related factor 2 (Nrf2), encoded by the gene NRF2, is a main redox homeostasis mediator. Nrf2 triggers an array of proteins such as Glutathione-S-transferase (GST), glutathione peroxidase (GPx), UDP-glucuronosyltransferase (UGT), nicotinamide adenine dinucleotide phosphate NAD(P)H: quinone oxidoreductase 1 (NQO1), multidrug resistance associated protein (MRP), heme-oxygenase-1(HO-1), peroxiredoxin (Prx), Sulfiredoxin 1 (SRXN1), and Thioredoxin reductase 1 (TXNRD1) involved in cytoprotection & detoxification **(4).**

the molecular mechanisms that down regulate the NRF2 expression in T2DM and DFU remain unresolved. One of the most prevalent genetic variations that predispose an individual to diabetes and its complications is SNPs **(5).**

Nrf2 auto regulates its activity through its antioxidant resistant elements (ARE), and the consequence of a polymorphism in ARE could be the decline of transcriptional activity of Nrf2-dependent cytoprotective genes **(6).**

Thus, this study aimed to investigate potential association of Nrf2 gene polymorphism with development of DFU.

1. Subjects and Methods

This case-control study included 81 subjects who were categorized into three groups; group 1 included 27 healthy individuals as a control group, group 2 included 27 type 2 diabetic patients without diabetic foot ulcer and group 3 included 27 type 2 diabetic patients with diabetic foot ulcer.

Inclusion criteria:

Type 2 diabetic patients will be diagnosed according to World Health Organization (WHO) criteria :

- Fasting plasma glucose >126 mg/dL.

- 2-h post prandial blood. glucose levels >200mg/dL

- HbA1c >6.5% in patients with classic symptoms of symptoms of hyperglycemia or hyperglycemic crisis.

- Patients with abnormal oral glucose tolerance test (OGTT).

- DFU were selected based on their symptoms of systemic inflammatory responses (WBC 4000-12000 cells / µL) and wound size (≥2 cm).

Exclusion criteria:

. Subjects with infectious diseases, peripheral vascular disease, autoimmune diseases, and hematological diseases and subjects with other reasons of harm to the peripheral nerves, such as vitamin B12 insufficiency, use of neurotoxic drugs, and inherited neuropathy were not considered for this investigation.

. Patient refuses to give consent and lack of cooperation.

All participants were subjected to all the following:

Full history: including a family history of T2DM and duration of diabetes. With special emphasis on age, gender.

Complete physical and clinical examination: Weight in kilograms (Kg), height in meters (m), and body mass index (BMI= Kg/m^2).

with special emphasis on blood pressure (systolic and diastolic).

The following Routine investigations:

- Fasting blood glucose level.
- 2Hour's post-prandial plasma glucose level.
- HbA1c.
- CBC.

Specific investigation

Detection of NRF2 (rs35652124) single nucleotide gene polymorphism by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

***Genotyping of NRF2 (rs35652124) single nucleotide gene polymorphism by* polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP):**

To assess the specific SNP NRF2 **(rs*35652124***), a PCR-RFLP test was performed using a specific forward primer for the ***NRF2*** gene (5- CCTTGCCCTGCTTTTATCTC-3) and reverse primer (5- CTTCTCCGTTTGCCTTTGAC-3). The PCR was performed in a final volume of 20 µl including 10 µl of 2X TOPsimple^{TM} DyeMIX-nTaq (enzynomics Biotechnology, Korea), 1 µl of each primer ,5 µl of genomic DNA, and 3 µl of deionized water.

PCR Protocol:

The amplification was performed using DNA thermal cycler 480, PERKIN ELMER (Norwalk, CT 06856, USA, Serial No. P16462) according to the following protocol*:* initial denaturation at 95°C for 5 minute, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C for 30 seconds, extension at 72°C for 1 minute, and final extension at 72°C for10 minutes.

Restriction Digest Reaction:

The PCR products were digested using BseRI restriction endonuclease (Neb enzyme, USA).) as the manufacturer's instructions.

Samples were loaded on 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized via UV transilluminator. The presence of only one band of 264 bp length indicated individuals with CC genotype, the presence of three bands of 264 bp, 192 bp, and 72 bp length indicated individuals with CT genotype, and the presence of two bands of 192 bp and 72 bp length indicated individuals with homozygous TT genotype.

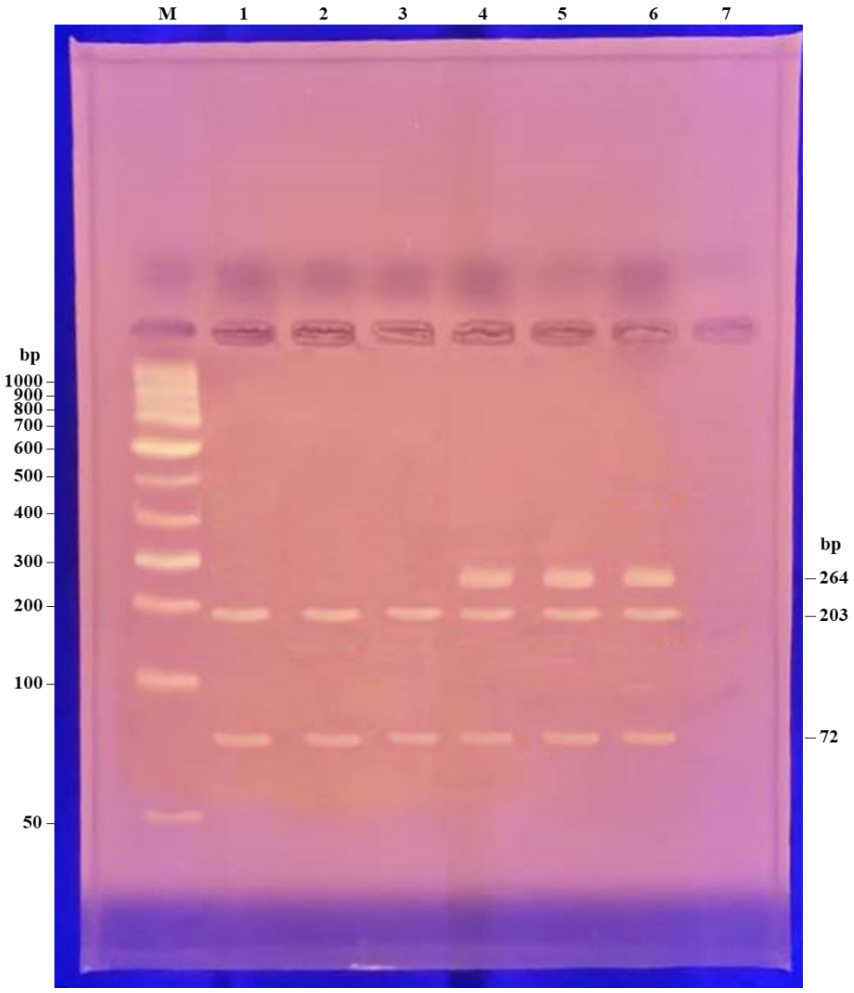


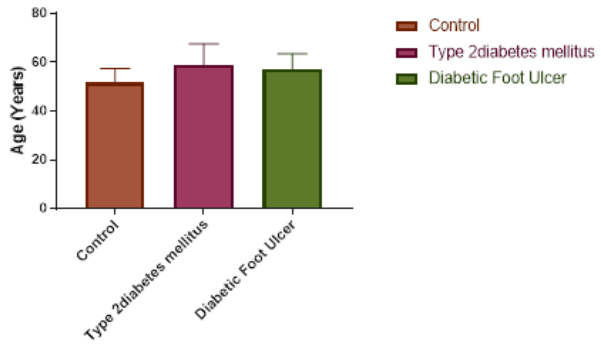
Figure 1: DFU Agarose gel electrophoresis picture stained with ethidium bromide showing the PCR product in which there was the analysis of the Nrf2gene (rs35652124) gene polymorphism. DNA size marker (100bp) ladder Lanes (1,2,3): TT genotype showing the presence of two bands 192+72bp. Lanes (4,5,6)CT genotype showing the presence of three bands264+192+72 bp

Statistical Analysis

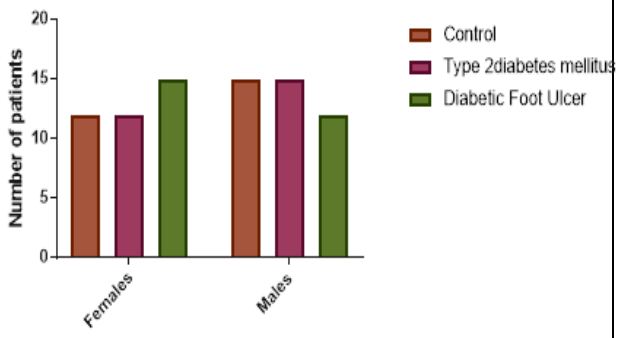
Data was analyzed using SPSS 21 (Statistical Package for the Social Services) (SPSS). The findings were displayed using both tabular and graphical formats. Results were displayed using standard statistical measures such as means, medians, standard deviations, and confidence intervals. The accuracy of the data was demonstrated with the help of statistics. The student's t test (T) is utilized. Pearson Chi-Square and Chi-Square for Linear Trend were used to analyse the quantitatively diverse data (X2). In this example, a P value of 0.05 or less was judged statistically significant

2. Results

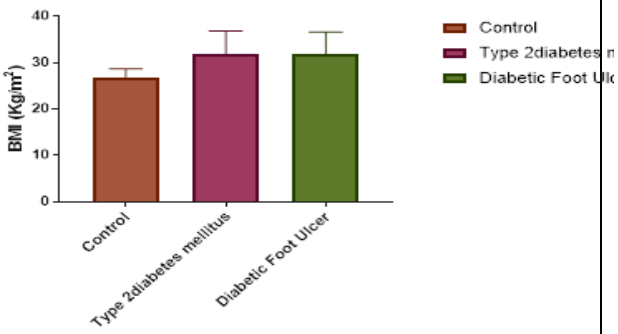
The mean age, BMI, FBG, 2h-PPBG and HBa1C were significantly higher in type 2 diabetic patients and diabetic foot ulcer patients than control group. The duration of DM, FBG, 2h-PPBG and HBa1C were significantly higher in diabetic foot ulcer patients than type 2 diabetic patients. The treatment was significantly higher in diabetic foot ulcer patients than type 2 diabetic patients. **(Figures 2)**



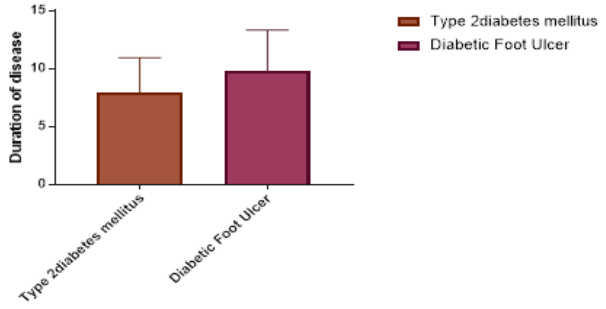
(A) Age of the studied groups



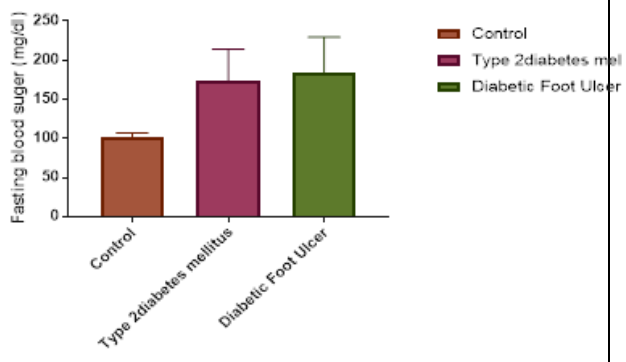
(B) Gender of the studied groups



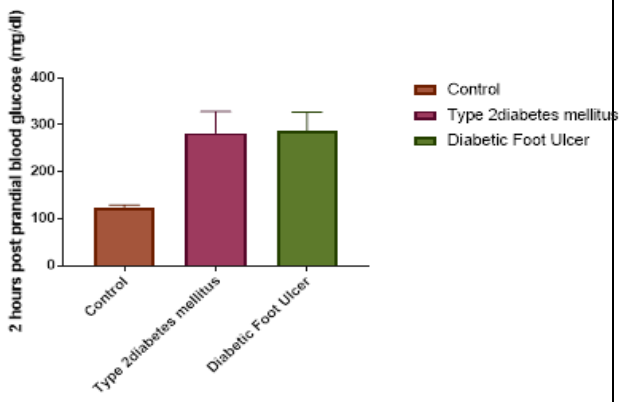
(C) BMI of the studied groups



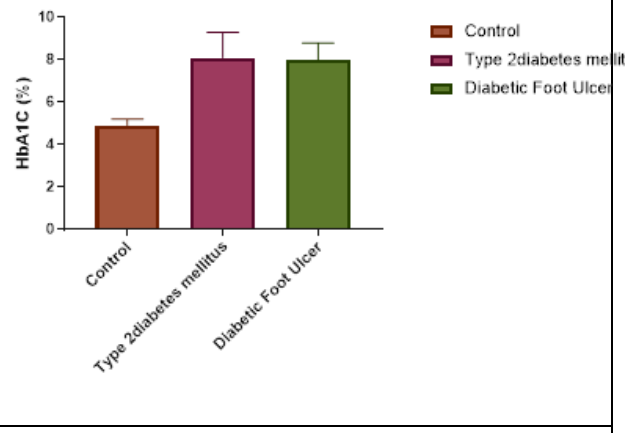
(D) Duration of disease of the studied groups



(E) Fasting blood glucose level of the studied groups



(F) Post prandial blood glucose level of the studied groups

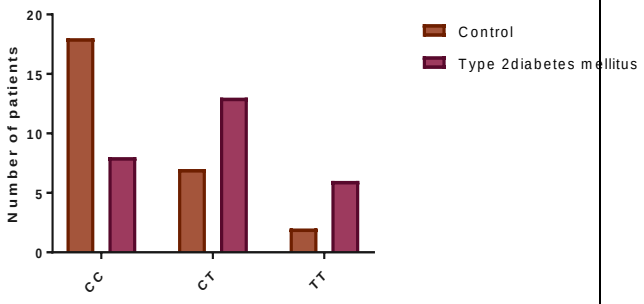


(G) Glycated hemoglobin level of the studied groups

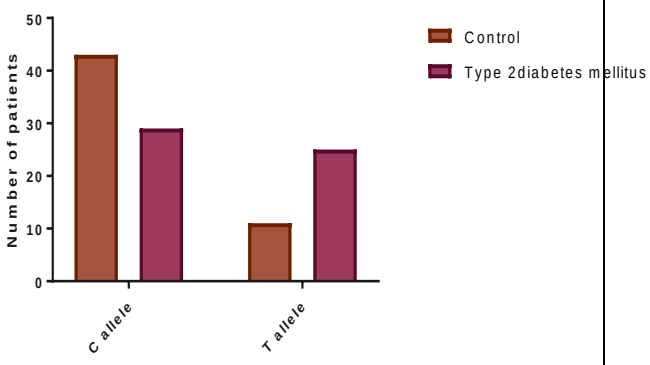
Figure 2: Demographic and clinical data among the studied groups

Regarding NRF2 genotyping in the studied groups, in the control group, CC genotype was present in 18 (67%) of controls, CT and TT genotypes were in 7 (26%) and 2 (7%) of controls. In cases group, the CC genotype was present in 8 (30%) of cases; CT and TT genotypes were in 13 (48%) and 6 (22%) respectively. CT and TT genotypes were significantly higher in cases compared to controls.

Regarding NRF2 allele distribution, in the control group, C allele was present in 43 (80%) controls and T allele in 11 (20%) controls, while in the studied cases, C allele was present in 29 (54%) cases and T allele in 25 (46%) cases. T alleles were statistically higher in the T2DM group compared to controls. **(Figure 3)**



(A) Genotype of NRF2 gene polymorphism among Type 2diabetes mellitus versus control



(B) Alleles of NRF2 gene polymorphism among Type 2diabetes mellitus versus control

Figure 3: Genotype of NRF2 gene polymorphism among Type 2diabetes mellitus versus control

Table 1: Genotype of NRF2 gene polymorphism among Diabetic Foot Ulcer patients versus control

	Control (N= 27)	Diabetic Foot Ulcer (N=27)	OR	95% CI	P value
CC	18 (67%)	1 (4%)		Ref	
CT	7 (26%)	13 (48%)	33.4	4.5-371	0.0001*
TT	2 (7%)	13 (48%)	117	9.7-1275	<0.0001*
C allele	43 (80%)	15 (28%)		Ref	
T allele	11 (20%)	39 (72%)	10.1	4.1-25.6	<0.0001*

Regarding NRF2 genotyping in studied groups; in the healthy group, the CC genotype was present in 18 (67%) controls; CT and TT genotypes were in 7 (26%) and 2 (7%) controls respectively. In the diabetic foot ulcer group, CC genotype was present in 1 (4%) cases, CT and TT genotypes were in 13 (48) and 13 (48%) cases. CT and TT genotypes of NRF2 were significantly higher in diabetic foot ulcer group compared to controls.

Regarding NRF2 allele distribution, in the control group, C allele was present in 43 (80%) controls and T allele in 11 (20%) controls, while in the diabetic foot ulcer cases, C allele was present in 15 (28%) cases and T allele in 39 (72%) cases. There was significant difference between diabetic foot ulcer cases and controls regarding to T allele of NRF2 that was higher in diabetic foot ulcer cases than control.

Table 2: Genotype of NRF2 gene polymorphism among Type 2diabetes mellitus versus Diabetic Foot Ulcer

	Type 2diabetes mellitus (N=27)	Diabetic Foot Ulcer (N=27)	OR	95% CI	P value
CC	8 (30%)	1 (4%)		Ref	
CT	13 (48%)	13 (48%)	8	1.07-94.2	0.04*
TT	6 (22%)	13 (48%)	17.3	1.9-203.2	0.004*
C allele	29 (54%)	15 (28%)		Ref	
T allele	25 (46%)	39 (72%)	3.1	1.35-6.68	0.006*

Regarding NRF2 genotyping in studied groups; in type 2 diabetic group, the CC genotype was present in 8 (30%) case; CT and TT genotypes were in 13 (48%) and 6 (22%) cases respectively. In the diabetic foot ulcer group, CC genotype was present in 1 (4%) cases, CT and TT genotypes were in 13 (48%) and 13 (48%) cases. There was significant difference regarding CT and TT between that was higher in diabetic foot ulcer group than T2DM group.

Regarding NRF2 allele distribution, in type 2 diabetic group, C allele was present in 29 (54%) controls and T allele in 25 (46%) cases, while in the diabetic foot ulcer cases, C allele was present in 15 (28%) cases and T allele in 39 (72%) cases. There was significant difference regarding C and T allele that was higher in diabetic foot ulcer group than T2DM group.

Table 3: demographic and clinical data among Diabetic Foot Ulcer patients with different genotypes

Diabetic Foot Ulcer	CC (N=1)	CT (N=13)	TT (N=13)	F/ X^2	P
Age (Years)	57 ± 0	56.2 ± 6.04	58 ± 7.42	0.22	0.8
Sex **Female** **Male**	 1 (100%) 0 (0%)	 7 (54%) 6 (46%)	 7 (54%) 6 (46%)	X^2= 0.83	0.66
BMI (kg/m^2)	39.5 ±0	32.4 ±4.4	31.1 ± 5.3	1.37	0.27
Duration	7.9± 0	9.7 ± 0.8	11.3 ± 1.1	20.3	<0.0001* P1=0.01* P2<0.0001* P3=0.0005*
SBP (mmHg)	123 ± 0	124.1 ± 3.8	123.9 ± 4.7	0.03	0.97
DBP (mmHg)	82 ± 0	82.3 ± 2.06	83 ± 2.34	0.3	0.74
Fast blood glucose (mg/dl)	140 ±0	184.2 ± 22.4	220.5 ± 28.4	15.7	<0.0001* P1=0.02* P2<0.0001* P3=0.002*
2 HR PPBG(mg/dl)	255 ±0	295.7 ± 14.4	325.2 ± 31.5	12.8	0.0001* P1=0.03* P2=0.0002* P3=0.006*
HbA1c(%)	6.9 ± 0	8.5± 0.78	9.6 ± 0.7	19.5	<0.0001* P1=0.005* P2<0.0001* P3=0.001*
Treatment **Insulin** **Oral**	 1 (100%) 0 (0%)	 8 (62%) 5 (38%)	 6 (46%) 7 (54%)	X^2= 1.45	0.48

Data are represented as mean ±SD. Data are analyzed with One way ANOVA followed by tukeys test or chi square test.

P1: Comparison between CC and CT, P2: Comparison between CC and TT, P3: Comparison between CT and TT.

This table showed that there was significant difference between different NRF2 genotypes among diabetic Foot Ulcer patients regarding duration of disease, FBG, PPBG, HbA1C that were higher in TT than CT and CC and higher in CT than CC.

3. Discussion

Regarding **NRF2 genotyping**, our study revealed that **TT genotypes** and **T alleles** were statistically significant higher in the patients' group (**diabetic with and without diabetic foot**) compared to controls. **TT genotypes of NRF2** and **T alleles** were also significantly higher in case of DFU compared to T2DM.

Teena et al (7) investigated the genetic association of NRF2 single-nucleotide polymorphism rs35652124 with T2DM and DFU and assessed its functional impact. They agreed with us and reported that rs35652124 (TT) is a harmful genetic variant that predisposes to insulin resistance and impaired angiogenesis. Hence, it may serve as a diagnostic genetic marker for T2DM and DFU in combination with different inflammatory markers. The "TT" genotype of rs35652124

was associated with a significant risk for T2DM and DFU. A significant decrease in transcriptional levels of NRF2 and IL-10 and a remarkable increase in TNF-α and IL-6 were observed in subjects with TT genotype.

Sireesh et al (8) investigated the association of redox regulator Nuclear factor erythroid 2-related factor 2 (Nrf2) and inflammatory cytokines as well as clinical remission in patients with recent onset type 2 diabetes (DM). They agreed with us and reported that plasma Nrf2 levels were found to be lowered (0.79 pg/mL) in DM subjects when compared to control subjects (1.8 pg/mL). They found 0.43-fold lower level expression of Nrf2 in PBMC of DM compared with control subjects.

Finally, our study reported that **mean age** and **BMI**, **FBG** and **Ha1c** were **significantly higher in type 2 diabetic patients and diabetic foot** ulcer patients than control group and the **duration of DM , FBG** and **Ha1c** were significantly **higher in diabetic foot ulcer patients** than type 2 diabetic patients.

Wang et al (9) agreed with us and reported that *NRF2* rs6721961 polymorphism was significantly associated with oxidative stress, anti-oxidative status, and risk of newly-diagnosed T2DM. The frequency of allele T was significantly higher in T2DM subjects (29.4%), compared to control subjects (26.1%). Individuals with the TT genotype had a significantly higher risk of developing T2DM, relative to those with the CC genotype, even after adjusting for known T2DM risk factors.

Based on the recent studies **Bhakkiyalakshmi et al (10)**, **Jiménez-Osorio et al (11)** and **Wang et al (9)** it has been reported that Nrf2 is a promising therapeutic target for diabetes and its late complications. However, under clinical settings to the best of our knowledge, this is the first line of evidence to explain the correlation of circulatory levels of Nrf2 with both oxidative stress and inflammatory cytokines in DM subjects.

Kurrer et al (12) and **Padgett et al (13)** reported that Th1 and Th2 cytokine balance plays a key role in upholding of normal immune response. Association of Th1 cytokines has been reported to be involved in the destruction of pancreatic β-cells through the release of cytotoxic mediators such as nitric oxide, oxygen radicals, serine esterases, etc.

Azar et al (14) also highlighted the importance of Nrf2 activators in skewing of Th2 mediated immunity but the exact mechanism is still unclear. Overall this study highlighted that Nrf2 signaling showed a regulatory mechanism in Th1 and Th2 mediated immunity.

Moreover, **Rockwell et al (15)** reported that Nrf2 deficient dendritic cells showed elevated levels of oxidative stress that confers a Th2-like immune responsiveness, which leads to alter in Th1 and Th2 balance.

Our findings coincided with few reports including **Jiménez-Osorio et al (11)**, who studied on Mexican population, a significant decrease in the levels of Nrf2 in subjects with DM having uncontrolled blood sugar levels, when compared to subjects having DM with controlled blood sugar.

Another study from Chinese population by **Wang et al (9)** reported that Nrf2 polymorphism (compared individuals with the CC genotype, those with the AA genotype) in DM subjects has significantly associated with decreased Nrf2 and its antioxidant status and also demonstrated that those subjects are highly susceptible to oxidative stress.

Moreover, **Jiménez-Osorio et al (11)** and **Wang et al (9)** demonstrated that significant decrease in antioxidant genes (HO-1, GPx, SOD, CAT) of DM compared with control subjects.

Moreover, several *in vitro* and *in vivo* studies **Meakin et al (16)** and **He and Ma (17)**

emphasized the anti-diabetic role of Nrf2 activators by attenuating both oxidative and inflammatory stress. Moreover, Nrf2 knockout mice are found to be highly prone to oxidative and inflammatory insults.

Carrasco-Pozo et al (18) and **Sireesh et al (19)** demonstrated that, insulin secretion was inhibited in pancreatic β-cells due to cytokine and glucose insult and restored by Nrf2 activation.

Further, **Rockwell et al (15)** stated that Nrf2 activation by tBHQ in CD4+ T cells, inhibits Th1 cytokine production, whereas it was concurrently stimulating the secretion of Th2 cytokines but not in Nrf2 knockout cells.

Finally, **Madhumitha et al (20)** and **Ahmad et al (21)** reported that reduced levels of Th2 cytokines and higher levels of Th1 cytokines in progression of metabolic diseases were reported in Kuwait and south Indian populations.

The strength points of our study:

The strength points of this study are that it was cross-sectional study design and having no patients who were lost during the study period. It was the first study in Zagazig University Hospitals to assess the use of Nrf2gene as predictive genetic marker in T2DM and development of diabetic foot ulcer DFU in patients with T2DM. Every effort was made to ascertain that all follow-up data were documented, and only complete information was included in data analysis. All clinical assessment and evaluation of study outcomes were done by the same team.

The limitations of our study:

The limitations of the study are worthy of mention, this study was a hospital-based study, hence there was a limited number of cases with relatively smaller sample size relative to study outcomes, not being a multicentric study and this represents a significant risk of publication bias and did not represent a particular community.

4. Conclusion

Nrf2gene is advised to be used as a genetic marker of significant value in prediction of T2DM and development of diabetic foot ulcer in patients with T2DM. It is accurate with no side effects. The present study can burden the knowledge and shed some light on future prospective studies with to reevaluation of our results and conclusions and assess the role of Nrf2gene genetic polymorphism in prediction of other medical disorders.

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