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Study of Nuclear Factor Erythroid Related Factor 2 Gene Polymorphism in Patients with Type 2 Diabetes Mellitus

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ABSTRACT
Background: The molecular mechanisms that downregulate the nuclear factor erythroid related factor 2 (NRF2) expression in addition to gene polymorphism among type 2 diabetes mellitus (T2DM) patients.
Objective: To investigating possible association between NRF2 gene polymorphism with T2DM development.
Patients and Methods: This case-control study included 81 persons who were categorized into three groups; The first group, made up of 27 healthy people, served as a control, group 2 consisted of twenty-seven type 2 diabetics without foot ulcers, and group 3 consisted of twenty-seven patients with diabetic foot ulcer who had type 2 diabetes. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was utilized to do genotyping of NRF2 (rs35652124) single nucleotide gene polymorphism.
Results: Concerning NRF2 genotyping in the investigated groups, 18 (67%) of controls possessed the CC genotype, while 7 (26%) possessed the CT genotype, and 2 (7%) possessed the TT genotype. Eight (30%) of cases in the cases group had the CC genotype, while 13% had CT genotype and 48% had TT genotype. Cases had a far higher frequency of the CT and TT genotypes than controls did. In terms of NRF2 allele distribution, we found that although 43 (80%) of controls and 11 (20%) of controls carried the C allele, 29 (54%) of patients carried the C allele and 25 (46%) of cases carried the T allele. T2DM patients had a significantly greater frequency of T alleles than control subjects.
Conclusion: Regarding NRF2 genotyping, our study revealed that TT genotypes and T alleles were statistically significantly higher among patients' group (diabetic with and without diabetic foot) compared to controls. NRF2 gene has a proper value in prediction of T2DM.
Keywords: Nuclear Factor Erythroid Related Factor 2, Gene Polymorphism, Type 2 Diabetes Mellitus.

INTRODUCTION

High rates of morbidity and mortality associated with type 2 diabetes mellitus (T2DM) and its consequences make this disease a global public health crisis [(1)]. Retinopathy, nephropathy, and neuropathy are examples of microvascular problems, while ischemic heart disease, peripheral vascular disease, and stroke are examples of macrovascular complications strongly linked to T2DM [(2)]. The prevalence of diabetic foot ulcers (DFUs) is increasing, and they are a leading cause of death among people with diabetes [(3)].

Gene NRF2 encodes nuclear factor erythroid 2-related factor 2, also known as NRF2. This protein interacts as an important part for keeping homeostasis. NRF2 activates a plethora of proteins like, UDP-glucuronosyltransferase (UGT), glutathione-S-transferase (GST), glutathione peroxidase (GPx) as well as nicotinamide adenine dinucleotide phosphate NAD(P), which play a role in detoxification and cellular protection [(4)]. NRF2 has been shown to be downregulated in a variety of inflammatory illnesses, according to studies [(5)]. Previous research has found that genetic variables like single-nucleotide polymorphisms (SNPs) control NRF2 expression [(6)].

In T2DM and DFU, the molecular processes that reduce NRF2 expression are unclear. SNPs are among the most common types of genetic changes that put a person at risk for diabetes and its consequences [(7)]. Genetic and epidemiological research have connected variations in the NRF2 promoter to oxidative stress-related disorders, providing strong evidence for a relationship between NRF2 polymorphisms and disease risk [(8)]. Therefore, we set out to see if there was any connection between a variation in the NRF2 gene and the onset of type 2 diabetes.

SUBJECTS AND METHODS

In a case control study at Medical Biochemistry and Molecular Biology, Medicine and Surgery Departments, Faculty of Medicine, Zagazig University, 81 subjects were included. The first group, made up of 27 healthy people, served as a control, group 2 consisted of twenty-seven type 2 diabetics without foot ulcers, and group 3 consisted of twenty-seven patients with diabetic foot ulcer who had type 2 diabetes.

Inclusion criteria:

The World Health Organization's (WHO) criteria was used to diagnose persons with type 2 diabetes [(9)]:

- Over 126 mg/dL in the blood while fasting.
- more than 200 mg/dL in the blood within two hours after eating
- Patients with HbA1c levels greater than 6.5% who are experiencing hyperglycemic symptoms or a hyperglycemic crisis.
- Cases who had abnormal oral glucose tolerance test (OGTT).
- WBC 4000-12000 cells /µL and wound size (2 cm) were used to determine which DFU would be used.

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Exclusion criteria:

- This study did not include participants who had any form of peripheral nerve damage due to infectious diseases, peripheral vascular disease, autoimmune disorders, or hematological diseases, or due to vitamin B12 deficiency, use of neurotoxic medicines, or hereditary neuropathy.
- Lack of permission and uncooperative patient.

All subjects were undergone to the following:

Full history: factors such as age, gender, race, and family history of type 2 diabetes. With particular focus on chronological and sexual.

Complete physical and clinical examination, paying particular attention to hypertension (systolic and diastolic).

The following Routine lab:

- Blood sugar during fasting.
- 2 Hour's post-prandial plasma glucose level.
- HbA1c.
- CBC.

Specific investigation:

The NRF2 (rs35652124) gene was analyzed for an SNP using polymerase chain reaction-restriction fragment length polymorphism.

For assessment of specific SNP NRF2 **(rs35652124**), we performed PCR-RFLP test by employing a specific forward primer for the **NRF2** gene (5- CCTTGCCCTGCTTTTATCTC-3) and reverse primer (5- CTTCTCCGTTTGCCTTTGAC-3). Ten microliters of 2X TOPsimpleTM DyeMIX-nTaq (enzynomics Biotechnology, Korea), one microliter of each primer, five microliters of genomic DNA, and three microliters of deionized water made up the final volume of 20 microliters for the PCR.

PCR Protocol:

A PERKIN ELMER DNA thermal cycler 480 (Norwalk, CT 06856, USA, Serial No. P16462) was 4085tilized for the amplification, and the process was as follows: Start with a 5-minute denaturation at 95 °C, then repeat 30 times with shorter denaturation at 95 °C, shorter annealing at 57 °C, shorter extension at 72 °C (1 min and 10 min, respectively).

Restriction Digest Reaction:

Following the manufacturer's protocol, BseRI restriction endonuclease (Neb enzyme, USA) was used to digest the PCR products.

The samples of digested PCR products were incubated at 37 degrees Celsius for 15 minutes. Incubation at 65 degrees Celsius for 20 minutes rendered BseRI inactive.

Electrophoresis on a 2% agarose gel was used to separate the samples, which were then stained with ethidium bromide and seen under a UV transilluminator. Individuals with the CC genotype had one band of 264 bp in length, those with the CT genotype had three bands of 264 bp, 192 bp, and 72 bp in length, and those with the homozygous TT genotype had two bands of 192 bp and 72 bp in length.

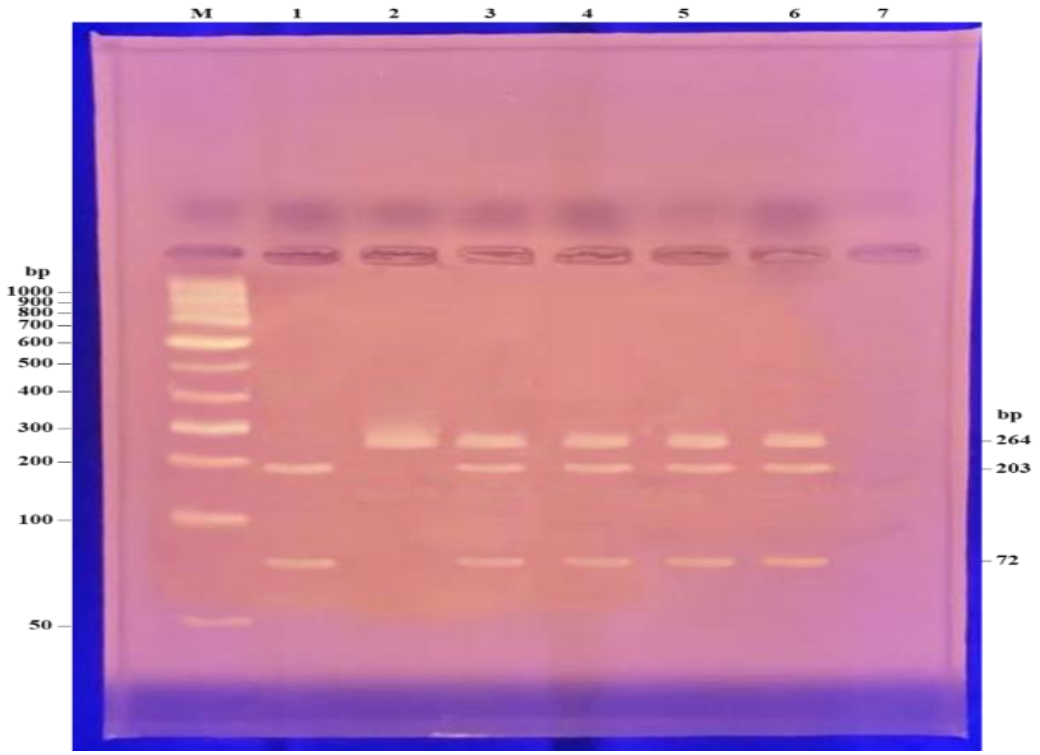

Figure (1): Analysis of the NRF2 gene (rs35652124) gene polymorphism was depicted on an ethidium bromide-stained, control agarose gel electrophoresis. The DNA ladder (100 bp in size). First set of lanes: 192+72 bp bands present, indicating a TT genotype. Lanes (2): a single band present in a CC genotype (264 bp). Three bands are present in the CT genotype, as shown in lanes 3, 4, 5 and 6. 264+192+72 bp.

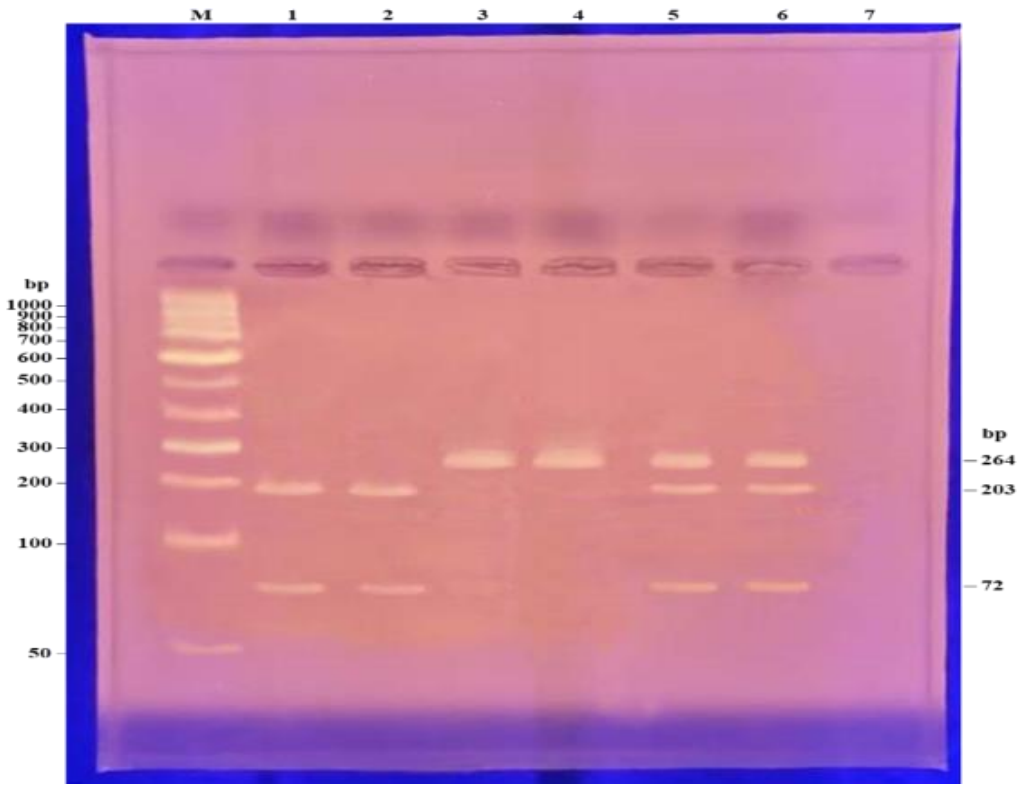

Figure (2): The PCR result from studying the NRF2 gene (rs35652124) gene polymorphism was seen on an ethidium bromide-stained DM agarose gel electrophoresis. Size of DNA ladder (in 100 bp increments). The TT genotype has two bands, totaling 192+72 bp, as seen in lanes 1 and 2. Banding for the CC genotype is present in lanes 3 and 4. (264 bp). Three bands seen in lanes 5 and 6 indicate a CT genotype. 264+192+72 bp.

Ethical consent:

This study was ethically approved by the Institutional Review Board of the Faculty of Medicine, Zagazig University. Written informed consent was taken from all participants. The study was conducted according to the Declaration of Helsinki.

Statistical analysis

Data were analyzed using SPSS 21 (Statistical Package for the Social Sciences) (SPSS). Qualitative data were presented as means, standard deviations, and confidence intervals (Cis) and were compared by one-way ANOVA (F) test, and if the difference was significant then; Tukey's test was used as a post-hoc test. Qualitative data were presented as frequency and percentage and were compared by Pearson Chi-Square (X^2) test. P v/alue of 0.05 or less was judged statistically significant.

RESULTS

This table shows that patients who had diabetes type 2 and diabetic foot ulcers had significantly higher mean age, BMI, FBG, 2h-PPBG, and HBA1C levels than the control group. Diabetic foot ulcer patients had significantly longer durations of DM, FBG, 2h-PPBG, and Hba1C. Patients with diabetic foot ulcers received much more care than those with type 2 diabetes.

Table (1): Demographics and clinical statistics among the studied groups

Variables	Control (N= 27)	Type 2 diabetes mellitus (N=27)	Diabetic Foot Ulcer (N=27)	F/X^2	P Value	
Age (Years)	51.6 ± 5.8	58.8 ± 8.8	57.1 ± 6.4	5.87	0.005*	P1<0.005* P2=0.039* P3=0.72
Sex **Female** **Male**	 12 (40%) 15 (60%)	 12 (40%) 15 (60%)	 15 (60%) 12 (40%)	X^2= 0.89	0.64	
BMI (kg/m^2)	26.8 ± 1.8	31.7 ± 5.1	31.8 ± 4.8	13.1	<0.0001*	P1<0.0001* P2<0.0001* P3=0.99
Duration of DM (Years)		7.94 ± 3.02	9.88 ± 3.5	2.18	0.03*	
SBP (mmHg)	121.1 ± 3.2	121.2 ± 4	121.5 ± 3.1	6.2	0.09	
DBP (mmHg)	78.1 ± 3.7	77.2 ± 3.5	77.8 ± 2.1	39	0.57	
Fast blood g lucose(mg/dl)	102.1 ± 5.1	174.3 ± 40.1	184.2 ± 45.6	45.5	<0.0001*	P1<0.0001* P2<0.0001* P3=0.52
2 HR PPBG (mg/dl)	123.1 ± 6.9	282.6 ± 45.9	288.3± 38.8	200.3	<0.0001*	P1<0.0001* P2<0.0001* P3=0.8
HbA1c (%)	4.9 ± 0.33	8.1 ± 1.21	8.06 ± 0.81	126.6	<0.0001*	P1<0.0001* P2<0.0001* P3=0.91
Treatment **Nil** **Insulin** **Oral**	 27 (100%) 0 (0%) 0 (0%)	 0 (0%) 11 (41%) 16 (59%)	 0 (0%) 15 (60%) 12 (40%)	X^2= 84.8	<0.0001*	

Data are presented as means ± standard deviation or as frequency (Percent), *: Significant, P1: Significance between control Vs type 2 diabetes mellitus, P2: Significance between control Vs diabetic foot ulcer, P3: Significance between diabetic foot ulcer Vs type 2 diabetes mellitus.

Concerning NRF2 genotyping in the control and type 2 diabetes mellitus group, cases had a far higher frequency of the CT and TT genotypes than controls did. In terms of NRF2 allele distribution, T2DM patients had a significantly greater frequency of T alleles than control subjects.

Table (2): Genotype of NRF2 gene polymorphism among type 2 diabetes mellitus versus control

	Control (N= 27)	Type 2 diabetes mellitus (N=27)	OR	95% CI	P value
CC	18 (67%)	8 (30%)		Ref	
CT	7 (26%)	13 (48%)	4.1	1.13-13.3	0.02*
TT	2 (7%)	6 (22%)	6.7	1.5-36.5	0.02*
C allele	43 (80%)	29 (54%)		Ref	
T allele	11 (20%)	25 (46%)	3.3	1.39-37.2	0.004*

*: Significant

This table (3) shows that neither demographic data nor clinical findings differed significantly between NRF2 genotypes in the control group.

Table (3): Demographic and clinical data among control subjects with different genotypes

	CC (N=18)	CT (N=7)	TT (N=2)	F	P
Age (Years)	49.8 ±6.1	51.7 ± 5.7	54 ± 7.07	0.45	0.63
Sex **Female** **Male**	 9 (50%) 9 (50%)	 3 (43%) 4 (57%)	 0 (0%) 2 (100%)	**X^2=** 1.3	0.51
BMI (kg/m^2)	27.1 ± 1.7	26.9 ± 1.85	26.6 ± 2.4	0.06	0.94
SBP (mmHg)	120.5 ± 3.1	119.8 ± 3.5	122.5 ± 0.7	0.62	0.54
DBP (mmHg)	73.4 ± 4.5	74.8 ± 3.4	70 ± 0	1.71	0.2
Fast blood glucose (mg/dl)	101± 7.8	102.3 ± 4.29	105.5 ± 0.7	0.56	0.57
2 HR PPBG (mg/dl)	124 ± 8.12	122.4 ± 6.7	128.5 ± 3.5	0.71	0.5
HbA1c (%)	4.85 ± 0.41	4.92 ± 0.32	5.05 ± 0.07	0.26	0.77

Data are presented as means ± standard deviation or as frequency (Percent)

This table (4) shows that a significant difference was found between different NRF2 genotypes among type 2 diabetes patients regarding duration of disease, FBG, PPBG, HbA1C that were higher in TT than CT and CC and higher in CT than CC.

Table (4): Demographic and clinical data among type 2 diabetes mellitus patients with different genotypes

Type 2diabetes mellitus	CC (N=8)	CT (N=13)	TT (N=6)	F	P
Age (Years)	67 ± 0	59.1 ± 8.1	53.7 ± 8.7	1.84	0.18
Sex **Female** **Male**	 4 (50%) 4 (50%)	 5 (38%) 8 (62%)	 3 (30%) 3 (30%)	**X^2= 0.36**	0.83
BMI (kg/m^2)	40.9 ± 0.03	30.5 ± 5.1	32.3 ± 4.4	2.27	0.12
Duration	6.5 ± 0.04	7.8 ± 0.5	9.5 ± 0.9	52.4	<0.0001* P1<0.0001* P2<0.0001* P3<0.0001*
SBP (mmHg)	120.8 ± 2.7	121.3 ± 3.1	121.1 ± 2.5	0.22	0.8
DBP (mmHg)	75 ± 1.1	77.1 ± 5.3	78.2 ± 4.1	0.25	0.77
Fast blood glucose (mg/dl)	129 ± 2.1	152.4 ± 26.7	173.2 ± 37.5	17.8	<0.0001* P1=0.006* P2<0.0001* P3=0.01*
2 HR PPBG(mg/dl)	216 ± 1.6	261.1 ± 21.5	295 .5± 46.7	16.4	<0.0001* P1=0.002* P2<0.0001* P3=0.03^
HbA1c(%)	6.5 ± 0.3	8.2 ± 0.9	9.1 ± 0.5	26.3	<0.0001* P1<0.0001* P2<0.0001* P3=0.03*
Treatment **Insulin** **Oral**	 5 (63%) 3 (37%)	 4 (31%) 9 (69%)	 2 (34%) 4 (66%)	**X^2=2.21**	0.32

Data are presented as means ± standard deviation or as frequency (Percent), *: Significant, P1: Comparison between CC and CT, P2: Comparison between CC and TT, P3: Comparison between CT and TT.

DISCUSSION

Cancer is just one of several diseases for which variations in the nuclear factor erythroid 2-related factor 2 (NRF2) gene are employed as diagnostic tool. NRF2 plays a crucial role in wound healing by regulating new blood vessel formation. The effect of the NRF2 SNP rs35652124 on function and its association with type 2 diabetes and diabetic foot ulcers have been studied previously [(10)].

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.

Tao *et al.* [(11)] disagreed with us and reported that the frequency of the NRF2 rs6721961 genotype was not substantially different between the MetS and control groups, and this genetic variant was not shown to be associated with any metabolic characteristics. This study found that those with the AA genotype had a 2.41-fold higher chance of developing MetS than those with the GG genotype, while those with the AG genotype had a 1.94-fold higher risk.

Sireesh *et al.* [(12)] revealed that NRF2, a redox regulator, was investigated in relation to inflammatory cytokines and clinical remission in people with newly diagnosed type 2 diabetes (DM). We found that the plasma levels of NRF2 in DM patients were significantly lower than those in healthy controls (1.8 pg/mL vs. 0.79 pg/mL). Compared to healthy controls, the expression of NRF2 in DM PBMC was reduced by a factor of 0. 43.

In the current study, those with type 2 diabetes and diabetic foot ulcers were shown to have significantly higher mean values for age, body mass index, fasting blood glucose, and hemoglobin A1c than the control group.

Sireesh *et al.* [(12)] agreed with us and reported that SBP, DBP, FPG, PPG, HbA1c, total blood cholesterol, and LDL-c were all significantly higher in the DM group than in the control group. When comparing DM subjects to healthy controls, however, neither BMI nor HDL-c showed statistical significance.

Long *et al.* [(13)] indicated the therapeutic effects of activating NRF2 in this condition, as well as NRF2's central role in diabetic wound healing, lay the groundwork NRF2 activators for the treatment of diabetic skin ulcers: a target for future clinical research. Using human clinical specimens, they demonstrated that the oxidative stress on the perilesional skin tissues of diabetic patients is greater than that of normoglycemic patients, and that there is a greater activation of the nuclear factor-E2-related factor 2 (NRF2)-mediated antioxidant response in the diabetic patients. Using a mouse model of diabetes caused by streptozotocin, the delayed wound closure rates in NRF22/2 mice are caused by an increase in oxidative DNA damage, a decrease in transforming growth factor-b1 (TGF-b1) expression, an increase in matrix metalloproteinase 9 (MMP9) production, and an increase in apoptosis. Pharmacological activation of NRF2 also significantly improves wound healing in diabetics. High glucose levels in vitro enhanced the expression of transforming growth factor beta 1 (TGF-b1) and downregulated the expression of matrix metalloproteinase 9 (MMP9) in human immortalized keratinocyte cells, showing that NRF2 promotes wound healing.

Wang *et al.* [(14)] agreed with us and reported revealed that the NRF2 rs6721961 polymorphism was substantially associated with oxidative stress, anti-oxidative state, and risk of newly diagnosed type 2 diabetes. When compared to control participants, those with T2DM had a much greater prevalence of the allele T (29.4%). People with the TT genotype had a significantly increased chance of developing type 2 diabetes compared to those with the CC genotype, even after accounting for other risk factors.

Jiménez-Osorio *et al.* [(15)] agreed with us and reported that patients with prediabetes and diabetes had reduced amounts of NRF2 in their PBMC nuclear extracts. This is surprising because oxidative stress is thought to increase NRF2 levels in diabetes people. These results show that NRF2 response is reduced in prediabetic and diabetic patients. Total antioxidant status and GSH levels were reduced, whereas lipid peroxidation and SOD activity were elevated, in diabetes patients. Therefore, oxidative stress and redox status imbalance result from the low levels of NRF2 observed in pre-diabetic and diabetic patients. It is possible that NRF2 will become a therapeutic target for reducing oxidative stress in pre-diabetic patients.

Based on the recent studies, **Wang *et al.*** [(14)], **Jiménez-Osorio *et al.*** [(15)] and **Bhakkiyalakshmi *et al.*** [(16)] expression regulating protein therapeutic targeting of NRF2 has been proposed for the management of diabetes and its complications. Blood NRF2 levels have been shown to correlate with both oxidative stress and inflammatory cytokines in type 2 diabetics, and to the best of our knowledge, this is the first clinically significant piece of data linking these two factors.

Rockwell *et al.* [(17)] and **Kikuchi *et al.*** [(18)] claimed that activation of NRF2 decreases production of Th1 cytokines while increasing production of Th2 cytokines. Additionally, **Rockwell et al** [(17)] also evidenced that, tBHQ, a NRF2 activator, upregulates IL-4, IL-5, and IL-13 transcription while downregulating IFN- IFN-γ production in T helper 2 (Th2) cells. They also reported that dendritic cells lacking NRF2 were found to have increased oxidative stress, which confers a Th2-like immunological response and alters the balance between Th1 and Th2 cells.

Our results matched those of a small but growing body of literature, including a study by **Jiménez-Osorio *et al.*** [(15)] on the Mexican population, which showed that

compared to diabetics whose blood sugar was under control, those whose blood sugar was out of control had significantly lower NRF2 levels.

Wang *et al.* [14] found that a polymorphism in NRF2 (comparing those with the CC genotype and those with the AA genotype) is substantially associated with lower levels of NRF2 and its antioxidant status in DM sufferers, showing that these people are especially vulnerable to oxidative stress.

When we compared DM participants to healthy controls, we found that the DM subjects had a higher amount of NRF2 downstream antioxidant genes. The importance of NRF2 and its downstream target genes in a wide range of clinical disorders was recently highlighted by **Al-Sawaf *et al.* [19].**

Meakin *et al.* [20] and He and Ma [21] tested the effects of NRF2 activators on oxidative and inflammatory stress in both cultured cells and living animals, finding that they have a beneficial effect on diabetes. The inability of NRF2-deficient animals to deal with oxidative and inflammatory stresses is also a major problem.

Carrasco-Pozo *et al.* [22] and **Sireesh *et al.* [23]** showed that activation of NRF2 rescues insulin production in pancreatic-cells after they had been damaged by cytokines and glucose.

The strength points of our study:

This study's strengths lie in its cross-sectional design and the fact that no patients were lost to follow-up. It was the first study of its kind to examine the role of the NRF2 gene as a predictive genetic marker in type 2 diabetes and the onset of diabetic foot ulcers in patients with the disease at Zagazig University Hospitals. All possible measures were taken to ensure that all follow-up data were recorded and that only full data sets were used for analysis. The same group of clinicians performed all of the study's assessments and analyses.

The limitations of our study:

Important limitations include the study's hospital setting, which meant a smaller sample size relative to the study's results; the lack of a multicentric design, which increases the likelihood of publication bias; and the study's failure to provide a representative sample of the general population.

CONCLUSION

Regarding NRF2 genotyping, our study revealed that TT genotypes and T alleles were statistically significantly higher in the patients' group (diabetic with and without diabetic foot) compared to controls. NRF2 gene is advised to be used as a genetic marker of significant value in prediction of T2DM. It is accurate with no side effects. The findings of the current study add to the body of knowledge and provide new insight into the role of the NRF2 gene genetic polymorphism in the prediction of various medical illnesses.

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Competing interests: Nil.

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