

**Nuclear factor - Erythroid 2 - related factor2 Gene Polymorphisms in vitiligo patients**

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**Conflicts of interest:** The authors have no conflict of interest to declare.

**Funds:** This is an author's own work and no funds were received.

## **Abstract**

**Background:** Oxidative stress is now one of the accepted theories of vitiligo development. Nuclear factor - Erythroid 2 - related factor 2 (Nrf2) regulates the expression of anti-oxidant proteins.

**Aim of the work:** This work aimed to evaluate the association of Nrf2 gene polymorphisms with the susceptibility to vitiligo among a sample of Egyptian vitiligo patients.

**Participants and methods:** This case-control study was performed on 100 patients suffering from vitiligo and 50 apparently healthy matched volunteers as a control group. Genotyping was carried out by Real Time PCR.

## **Results**

The frequency of TT, CT, combined (TT+CT) genotypes and T allele of Nrf2 (rs35652124) were significantly increased in the studied vitiligo patients when compared with healthy controls ( $P < 0.001$ , 0.012,  $< 0.001$  and  $< 0.001$ , respectively). There was non-significant difference between patients and controls regarding Nrf2 (rs6721961) genotypes. However, T allele of Nrf2 (rs6721961) was significantly predominant in the studied patients when compared to the controls ( $P = 0.029$ ). Among the studied criteria, T allele of Nrf2 (rs6721961) was predominant in patients with marginal type of repigmentation ( $P = 0.022$ ), while G allele of the same SNP was associated with higher BMI ( $P = 0.034$ ). Hundred percent of vitiligo patients with Nrf2 (rs6721961) GT genotype had progressive course of the disease ( $P = 0.015$ ).

## **Conclusion**

Nrf2 (-617 T/G) and (-653 T/C) polymorphism may have a role in the susceptibility to vitiligo and modify the clinical presentation of the disease.

**Keywords:** Gene polymorphism, Nuclear factor-Erythroid 2-related factor 2, vitiligo.

## **Introduction**

Vitiligo is the commonest acquired depigmenting disorder affecting genetically predisposed individuals (1). It is not yet well established how vitiligo develop, however, autoimmune, oxidative stress, neural, and biochemical-based theories are accepted (2).

Reactive oxygen species (ROS) created via different pathways may contribute to melanocytes destruction either via direct damaging effect or through increased melanocyte specific autoantibodies production (3).

The Nrf2 is a basic leucine zipper protein which plays a role in regulating the expression of anti-oxidants proteins which protect the body cells against the damaging effects of the oxidative stress (4,5).

Nrf2 gene polymorphisms especially those affecting the promoter region can impair Nrf2 proteins expression and activity. Two polymorphisms in the promoter region – a T to C substitution at position \_653 (Nrf2 rs35652124) and a G to T substitution at \_617 (Nrf2 rs6721961) – are described and known to be associated with different pathologic conditions (6).

This study aimed at investigating the association between Nrf2 (rs35652124) and (rs6721961) gene polymorphisms and the susceptibility to vitiligo, and to assess the effect of different genotypes on the clinical aspects of the disease.

## **Subjects and Methods**

The study included one hundred patients suffering from different clinical presentations of vitiligo, in addition to fifty apparently healthy vitiligo free individuals from the Dermatology Outpatient Clinic of Benha University Hospital. The study was approved by the ethical committee of human research of Faculty of Medicine, Benha

University. After discussing the study's risks, benefits and other aspects with all participants, written informed consent was obtained from each participant before sample collection. History of the present illness and the related medical conditions and the associated diseases was discussed in details with all patients. Subjects with other inflammatory or autoimmune cutaneous or systemic disorders were excluded from this study.

### **DNA extraction and Genotyping**

Genomic DNA was extracted from peripheral blood samples from all participants. Genomic DNA was isolated using GeneJET Genomic DNA Purification Kit cat. No K0721 (Qiagen- Germany). The purity and concentration and of DNA was evaluated spectrophotometrically using Nanodrop (Thermo Scientific, Wilmington). The quality of DNA was assessed with the A260/280 ratio. Genotyping was performed using the Taqman SNP ready-made assay (Qiagen- Germany) which includes TAQMAN UNIVERSAL MMIX II (Cat. NO 4440043) and TAQMAN SNP ASSAYS MTO HUMAN SM (Cat. NO 4351379). The TaqMan genotyping assay used forward primer, reverse primer and two probes as follow rs35652124 forward, 5'-CCT TGC CCTGCT TTT ATC TC-3 and reverse 5'-CTT CTC CGT TTG CCT TTG AC-3, rs6721961 forward 5'-GAA AGG CGT TGG TGT AGG AG-3 and reverse 5'-GAA TGGAGA CAC GTG GGA GT-3. Normal probe (5'-3') VIC- ACTCCTTTCACCTATTCCCAAGGCCT-MGB-NFQ, Mutant probe (5'-3') FAM- CAGCTACACCTGTATGTAGGCTAGA MGB-NFQ. The probes were designed with minor groove binder (MGB) and non-fluorescent quencher (NFQ) at the 3' end, whereas the 5' end contained the fluorescence reporter dyes 2'-chloro 7'-phenyl-1,4-dichloro-6-carboxyfluorescein(VIC) or 6 carboxyfluorescein (FAM). The wild type probe labeled with VIC dye while the variant probe labeled with FAM dye. PCR was

performed in a volume of 20<sub>ul</sub> by Rotorgene real time PCR system (Qiagen- S.Korea). Thermal cycling conditions were as following: 60C for 30 sec, 95C for 10 min, 40 cycles of denaturation 95°C for 15 sec and 60°C for 1 min.

### **Statistical analysis**

Data were coded and entered using the statistical package SPSS (Statistical Package for the Social Sciences) version 25. Data was summarized using mean, standard deviation, median, minimum and maximum in quantitative data and using frequency (count) and relative frequency (percentage) for categorical data. Comparisons between quantitative variables were done using the non-parametric Kruskal-Wallis and Mann-Whitney tests.

For comparing categorical data, Chi square ( $\chi^2$ ) test was performed. Exact test was used instead when the expected frequency is less than 5. Genotype and allele frequencies were compared between the disease and the control groups. Odds ratio (OR) with 95% confidence intervals was calculated using binary logistic regression. P-values less than 0.05 were considered as statistically significant.

### **Results**

Patients and control subjects groups were matching regarding age ( $23.79 \pm 15.87$  versus  $24.46 \pm 9.12$  years,  $P=0.147$ ), sex (male: female ratio 68: 32 of the patients and 30:20 of the control subjects,  $P= 0.332$ ) and body mass index ( $24.41 \pm 5.57$  kg/m<sup>2</sup> versus  $25.49 \pm 4.40$  kg/m<sup>2</sup>,  $P= 0.094$ ).

The mean disease duration was  $6.68 \pm 6.39$  years. Stressful condition was associated with disease onset in 51% of cases. Koebnerization was positive in 36% only of the vitiligo patients. Hearing and vision impairment were positive in 10% and 8% of patients respectively. Regarding the clinical type of vitiligo in the studied sample, 86 patients had non-segmental vitiligo, while 14 patients presented with segmental

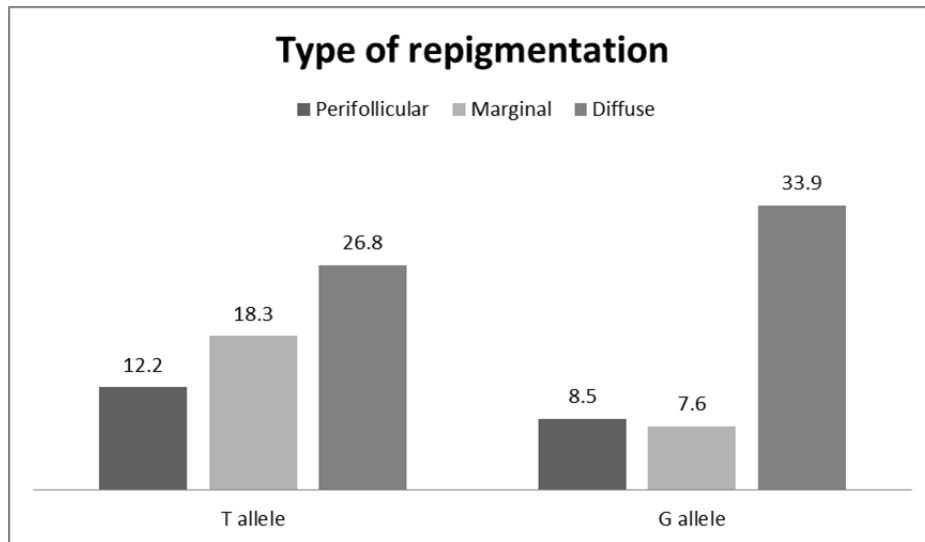
vitiligo. Leukotrichia was observed in 37 cases only. The mean VASI score was (13.16 ± 24.16). Applying Hardy Weinberg equation, revealed that Nrf2 genotypes and alleles frequency in patients and control groups in both studied SNPs were in Hardy Weinberg equilibrium. The TT and CT genotypes and T allele of Nrf2 gene (rs35652124) and T allele of Nrf2 (rs6721961) increase risk of vitiligo significantly

**Table 1: Genotypes and alleles of the studied SNPs.**

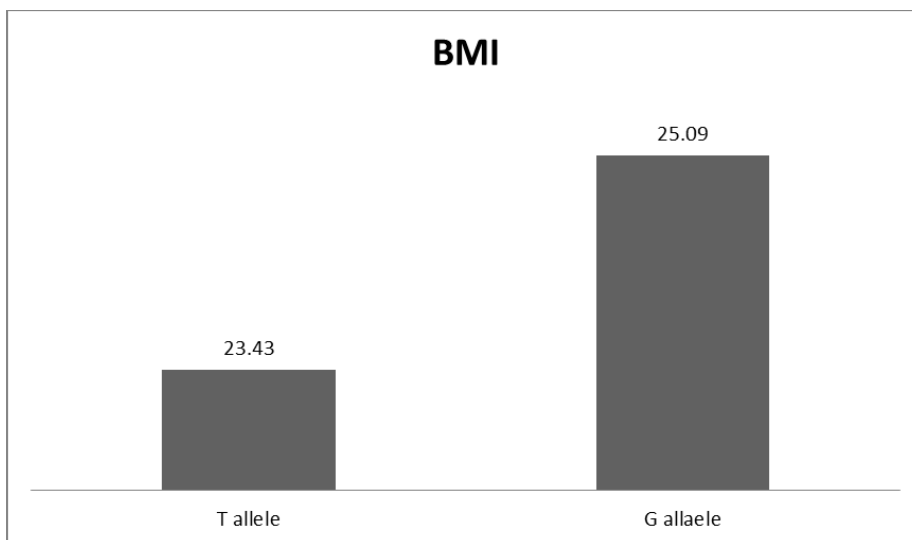
Genotypes and alleles	Cases (n=100)	Control (n=50)	P value	OR	95% CI	
	n(%)	n(%)			lower	Upper
<b>Nrf2 (rs35652124)</b>						
<b>TT (mutant)</b>	48 (48.0)	10 (20.0)	<0.001	7.040	2.734	18.130
<b>CT (heterozygous)</b>	37 (37.0)	18 (36.0)	0.012	3.015	1.27	7.159
<b>CC (wild)</b>	15 (15.0)	22 (44.0)	Reference			
<b>TT(mutant) + CT(heterozygous)</b>	85 (85.0)	28 (56.0)	<0.001	4.452	2.035	9.742
<b>allele T</b>	133 (66.5)	38 (38.0)	<0.001	3.239	1.966	5.336
<b>allele C</b>	67 (33.5)	62 (62.0)	Reference			
<b>Nrf2 (rs6721961)</b>						
<b>TT(mutant)</b>	20 (20.0)	5 (10.0)	0.062	2.842	0.949	8.514
<b>GT(heterozygous)</b>	42 (42.0)	18 (36.0)	0.181	1.658	0.791	3.476
<b>GG(wild)</b>	38 (38.0)	27 (54.0)	Reference			
<b>TT+GT</b>	62 (62.0)	23 (46.0)	0.064	1.915	0.963	3.808
<b>allele T</b>	82 (41.0)	28 (28.0)	0.029	1.787	1.063	3.004
<b>allele G</b>	118 (59.0)	72 (72.0)	Reference			
<b>Haplotype</b>						
<b>rs35652124 allele T + rs6721961 allele T</b>	71 (35.5)	18 (18.0)	<0.001	3.663	1.931	6.948
<b>rs35652124 allele T + rs6721961 allele G</b>	62 (31.0)	20 (20.0)	0.001	2.879	1.534	5.403
<b>rs35652124 allele C + rs6721961 allele T</b>	11 (5.5)	10 (10.0)	0.965	1.021	0.401	2.604
<b>rs35652124 allele C + rs6721961 allele G</b>	56 (28.0)	52 (52.0)	Reference			

**OR:** odds ratio; **CI:** confidence interval

Among the studied variables, there was non-significant relation between the genotypes and alleles of the studied SNPs except for the significant association between the T allele of rs6721961 and the marginal type of repigmentation (**Figure 1**), and between the G allele of the same SNP and the BMI (**Figure 2**).



**Figure 1:** Relation between different Nrf2 (rs6721961) alleles and type of repigmentation.



**Figure 2:** Relation between Nrf2 (rs6721961) alleles and BMI.

## **Discussion**

Nuclear factor - Erythroid 2 - related factor 2 (Nrf2) and its negative regulator protein Keap1 play a crucial antioxidant role protecting all body cells against the harmful effects exerted by the oxidative stress (7). Moreover, activating Nrf2 may be a promising therapeutic option in managing many inflammatory conditions in which oxidative stress may be involved in its pathogenesis (8).

Many single nucleotide polymorphisms (SNPs) in Keap 1 and Nrf2 have been investigated and their association with many inflammatory diseases are now documented (9). Of particular importance, the two promotor region of the gene SNP, the rs35652124 and the rs6721961 polymorphisms. The ancestral alleles at Nrf2 (rs35652124) and (rs6721961) are the C allele and the G allele respectively. Both ancestral alleles are associated with greater Nrf2 activity (6,10).

In the present work, a significant link between Nrf2 (rs35652124) polymorphism and vitiligo was observed, while there was non-significant difference between patients and controls regarding Nrf2 (rs6721961) genotypes. However, T allele of Nrf2 (rs6721961) increased the risk of vitiligo.

**Song et al. (6)** investigated the association of the same gene SNPs with vitiligo in a sample of Chinese population. In accordance to the present work, they reported an association between the Nrf2 (rs35652124) TT and CT genotypes and increased risk of vitiligo. But they didn't observe a significant difference between patients and controls regarding Nrf2 (rs6721961) genotypes or alleles. The discrepancy between results of the two studies may be explained by the difference in sample size [1136 patients and 1200 control in **Song et al. (6)** study], the ethnic variations and different genetic and racial background.



In the present study, T allele of Nrf2 (rs6721961) was significantly more predominant in vitiligo patients with marginal type of repigmentation. **Selvan et al. (11)** reported that the marginal pattern was the predominant in both narrowband ultraviolet B and excimer laser-treated patients. Moreover, **Yang et al. (12)** reported that vitiligo patients treated with topical therapies mostly developed marginal repigmentation. However, **Gan et al. (13)** reported that the most common pattern of repigmentation was the perifollicular, followed by diffuse, combined, and marginal repigmentation. But during the six months follow up after cessation of the used therapeutic lines, **Gan et al. (13)** detected that marginal repigmentation was the most stable form. From these findings, we could predict that carriers of T allele of Nrf2 (6721961) have a good prognosis.

The G allele of Nrf2 (rs6721961); known to be associated with high Nrf2 activity, was associated with higher BMI in the studied patients. This finding was interesting because **Shin et al. (14)** demonstrated the effect of Nrf2 in preventing fat accumulation and body weight gain. However, this finding could be explained by the exhaustion of Nrf2 function due to its constant activation **(15)**.

The incidence of gastrointestinal diseases (ulcerative colitis and celiac disease) in vitiligo patients is higher than that in normal population **(16)**. **Arisawa et al. (17)** reported that Nrf2 (rs35652124) C allele is protective against gastrointestinal diseases, and the patients from the Japanese population heterozygous at locus Nrf2 (rs35652124) CT were more susceptible to ulcerative colitis. This could provide a genetic explanation to the association between these conditions.

## **Conclusion**

Nrf2 (-617 T/G) and (-653 T/C) polymorphism may have a role in the susceptibility to vitiligo and modify the clinical presentation of the disease.

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