

Forkhead Box P3 (FOXP3) Gene Polymorphisms Association with its Serum Levels in Egyptian Vitiligo Patients

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ABSTRACT

Background: Vitiligo is an autoimmune disorder characterized by loss of pigmentation from the skin due to selective destruction of cutaneous melanocytes. Its pathogenesis is linked to regulatory T-cell (Treg) dysfunction. Forkhead box P3 (FOXP3) is a specific Treg marker and a master regulator of its activity.

Objective: The aim of this study was to examine the relationship between serum levels of FOXP3 in Egyptian vitiligo patients and single-nucleotide polymorphisms (SNPs) at two distinct loci (rs3761548) A/C and (rs2232365) A/G situated in the promoter region of the FOXP3 gene.

Patients and methods: The case control study comprised 50 untreated vitiligo patients who attended the dermatology clinic at Benha University Hospitals, and 30 age- and sex-matched healthy controls. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to identify FOXP3 gene polymorphism, and a FOXP3 enzyme linked immunosorbent assay (ELISA) was used to assess the quantity of FOXP3 in the blood.

Results: Highly significant difference ($P < 0.001$) was found between patients and control groups in rs3761548 A/C, rs2232365 A/G and combined genotypes as well as in serum FOXP3 levels measurements. The SNPs (rs3761548 and rs2232365) of FOXP3 gene were significantly associated with susceptibility to vitiligo.

Conclusion: In our sample of Egyptian patients, analysis of FOXP3 protein levels and its gene SNPs demonstrates their substantial correlation with vitiligo. As the number of mutant alleles rises, the risk of vitiligo tends to follow. Patients with vitiligo have lower blood levels of FOXP3, which is indicative of the disease's pathophysiology and progression.

Keywords: Forkhead Box P3 (FOXP3), Gene Polymorphisms, PCR-RFLP, Egyptian, Vitiligo.

INTRODUCTION

Vitiligo is cutaneous acquired depigmentation brought on by melanocyte loss from the epidermal basal layer [1]. It is unclear exactly how vitiligo manifests its pathogenesis. There are several leading theories about the aetiology of vitiligo, with autoimmune pathogenesis being one of the most well-established and well-liked [2].

According to **Ben Ahmed et al.** [3], functional deficiencies or a lack of T regulatory cells (Tregs) have been firmly postulated to be involved. Treg cells, also known as CD4+CD25+ T regulatory cells, are crucial for avoiding autoimmunity, upholding immunological homeostasis, and regulating T cell responses [4].

Patients with vitiligo had significantly less Treg cells in their peripheral blood, nonlesional, perilesional, and lesional skin. The inability of Treg cell migration may be the cause of the diminishing periphery Treg cell. These results suggested that the pathophysiology of vitiligo is directly tied to Treg cells [5,6]. The only master regulator of these cells and the only marker exclusively produced by Treg cells is forkhead box P3 (FOXP3). On chromosome Xp11.23, the human FOXP3 gene produces FOXP3, a transcription factor that belongs to the forkhead/winged helix family. A leucine zipper-like motif, a single C2H2 zinc finger, and a carboxyl

terminal forkhead domain are the three functional domains that make up FOXP3. The forkhead domain is required for nuclear localization and DNA binding, which result in transcriptional activation and/or repression [7,8]. By binding to the relevant target genes, FOXP3 can regulate the production of important molecules that mediate suppression. In addition to helping to transform naïve T cells into Treg-like cells with inhibition activity through these modulation methods, FOXP3 also supports Treg development and function [9].

According to **Wu et al.** [10] and **D'Amico et al.** [11], vitiligo is caused by an autoimmune mechanism that involves impaired FOXP3 function. It is known that FOXP3 gene polymorphisms modify the expression and function of the FOXP3 protein, which may result in Treg cell shortage or malfunction and consequently cause autoimmune illness. Regulatory SNPs in the promoter regions are particularly likely functional polymorphisms since they are located at or near the transcriptional start point [12].

By negatively expressing FOXP3, FOXP3 functional polymorphisms may contribute to vitiligo through the dysregulation of Treg cells. The likelihood of developing vitiligo tends to rise as the number of variant alleles rises, indicating that these variations work in concert to raise the likelihood of developing vitiligo. Additionally, they proposed that

there could be some interactions between the clinical traits of individuals with vitiligo and the total number of risk alleles [1].

The aim of current study was to explore the relationship between serum levels of FOXP3 and its gene single nucleotide polymorphisms at two loci (rs3761548 A/C, and rs2232365 A/G) in vitiligo Egyptian patients.

PATIENTS AND METHODS

This study was carried out at Dermatology, Venerology and Andrology Department and Microbiology & Immunology Department of Benha University Hospital, Benha Faculty of Medicine, Egypt.

The study included 50 vitiligo patients who attended the Dermatology Out-patient Clinic of Benha University Hospitals and had not received treatment for their condition for 6 months, and 30 healthy controls matched for age and sex, and did not have any clinical signs of vitiligo or other autoimmune diseases.

Each patient and control was subjected to medical history taking, full clinical examination, Vitiligo extent tensity index (VETI) score, blood sample taking, molecular detection of FOXP3 gene poly-morphism by PCR-RFLP and measurement of serum FOXP3 level by FOXP3 ELISA.

Vitiligo extent tensity index (VETI) score: the score combines examination of the severity and extensity of vitiligo. The rule of nines is used to measure the proportion of extension participation (p). The body is divided into five sites, head (h), upper limbs (u), trunk (t) and lower limbs (l) and genitalia (g).

Each is separately scored by using five stages of disease tensity (T): Normal skin is stage 0, hypopigmentation is stage 1 (including trichrome and homogeneous lighter pigmentation), complete depigmentation with black hair and with perifollicular pigmentation is stage 2, complete depigmentation with black hair and without perifollicular pigmentation is stage 3, complete depigmentation with a mixture of white and black hair is stage 4, and complete depigmentation with significant hair whitening is stage 5. $VETI = (Ph \times Th) + (Pt \times Tt)4 + (Pu \times Tu)2 + (Pl \times Tl)4 + (Pg \times Tg)0.1$ with maximum score of VETI is 55.5 [13].

Samples: The skin over the vein was sterilized by 70% alcohol and 6 ml of peripheral venous blood sample were collected from both patients and controls and processed as follows:

A) Three millilitres of venous blood were drawn and immediately treated for buffy coat separation in tubes containing ethylene diamine tetraacetic acid (EDTA). Human peripheral

blood was centrifuged using a Ficoll Hypaque gradient to separate mononuclear cells [14].

B) Three millilitres of blood were collected in sterile, empty tubes, and left to coagulate for 10 to 20 minutes at room temperature. They were then centrifuged at a speed of 2000 rpm for 20 minutes. For the Eliza test, the supernatant was collected and kept at -80°C.

Materials:

I) Materials used for FOXP3 PCR-RFLP:

A) Quick-gDNA™ MiniPrep for genomic DNA extraction (Thermo-scientific, USA).

B) Materials used for FOXP3 amplification and endonuclease digestion:

1. MyTaq™ red Mix (2X) (Thermo scientific, U.S.A).

2. Primer pairs for FOXP3 gene [10, 12], (Biosearch technologies ,U.S.A):

- FOXP3(rs3761548); F(5'-GCCCT TGTCTACTCCACGCCTCT-3'), R(5'-CAGCCTTCGCC AATACAGA GCC-3').

- FOXP3 (rs2232365);F(5'-AGGAGA AGGAGTGGGCATTT-3'),R(5'-TGT G AGTGGAGGAGCTGAG G-3').

3- Restriction endonucleases (Thermo scientific, New England):

- Pst I time-Saver™ for FOXP3(rs3761548).

- BsmBI time - Saver™ for FOXP3 (rs2232365).

II) Materials for FOXP3 ELISA: Determination of serum FOXP3 levels were done for all the collected serum samples using a commercially available FOXP3 ELISA kits (Pilibiotech, Germany).

METHODS

I) Genomic DNA extraction from buffy coat samples: DNA was extracted by quick gDNA™ MiniPrep and according to the manufacturer's instructions.

II) Genomic DNA amplification: The PCR mixture was carried in microcentrifuge tube and placed on ice. It contained 25 µl of MyTaq red PCR Master Mix (2X) , 1 µl of forward primer (20 Mmol), 1 µl of reverse primer (20 Mmol), 5 µl of template DNA and 18µl of nuclease free water.

PCR was performed using the recommended thermal cycling conditions as following: initial de-naturation at 95°C for 1 min and 1 cycle, then denaturation at 95°C for 15 s and 25-35 cycles, the Annealing was at (63°C for for FOXP3 rs3761548) and (55°C for FOXP3 rs2232365) for 15 s and 25-35 cycles, the Extension was at 63°C for 10 s and 25-35 cycles.

III) Restriction endonuclease digestion: 10 µL of amplified DNA was restricted with 1 µL of Pst I and BsmBI restriction endonucleases and incubated at 37°C and 55°C for FOXP3 (rs3761548 & rs2232365) respectively (Thermo-scientifics, New England).

IV) DNA was detected by agarose gel stained with ethidium bromide (10mg /ml) according to **Primrose et al.** [15] in comparisons with known ladder (Hyperladder 50bp DNA ladder, ThermoScientific, U.S.A) bands as follows:

- FOXP3 (rs3761548) gives a band at 487 bp [10].
- FOXP3 (rs2232365) gives a band at 249 bp [12].

After addition of restriction endonucleases, FOXP3 genotypes were determined by the site of amplified product in comparisons with known ladder bands as follows:

- PstI gives bands at 487 bp for A allele and 329 bp and 158 bp for C allele [10].
- BsmBI gives bands at 249 bp for A allele and 117 bp and 132 bp for G allele [12].

V) Estimation of serum FOXP3 levels: The procedures were done according to the manufacturer’s instructions (Pilobiotec, Germany).

Ethical consent:

The Ethical Institutional Review Board at Benha University approved the study. After explaining our research objectives, written informed consent was obtained from all study participants. This study was conducted in compliance with the code of ethics of the world medical association (Declaration of Helsinki) for human subjects.

Statistical analysis

The collected data were coded, processed and analyzed using the SPSS version 24 for Windows. The collected data were summarized in terms of mean and standard deviation (SD) and range for quantitative data, and frequency and percentage for qualitative data. Comparisons between the different study groups were carried out using Chi-square (χ^2) and Fisher’s Exact Test (FET) to compare proportions as appropriate. The student’s t-test (t) and the F-test (F) were used to compare differences in the mean between two and more than two groups respectively regarding normally distributed data. After the calculation of each of the test statistics, the corresponding distribution tables were consulted to get the “P” (Probability value). P value <0.05 was considered significant. The Receiver Operating

Characteristics (ROC) curve analysis was carried out for serum FOX3 level as a predictor for Vitiligo. The best cutoff point and the corresponding sensitivity, specificity and area under the curve (AUC) were calculated. The sensitivity of a test is the probability that the test is positive given a patient has the condition. Sensitivity = Probability (T+ | D+).

The specificity of a test is the probability that the test is negative given a patient does not have the condition. Specificity = Probability (T- | D-)All statistical analyses were carried out using STATA/SE version11.2 for Windows (STATA corporation, College Station, Texas)

RESULTS

Vitiligo patients’ group were 38 (76%) females and 12 (24%) males. Mean age of patients was 22.68 (SD 14.58) years and average disease duration at the time of hospital visit was 3.2 (SD 3.27) years. Generalized vitiligo was the commonest form of the disease in 17 (34%) patients followed by focal vitiligo in 13 (26%) patients and least common was segmental vitiligo in 1 (2%) patient. Koebner’s phenomenon was observed in 5 (10%) patients, family history of vitiligo was positive in 7 (14%) patients. The VETI score of studied patients ranged from (0.15-13.8) with mean 3.79 (SD 3.42). The control group was composed of 17 (56.67%) females and 13 (43.33%) males, and the mean age was 26.8 (SD 12.57) years. No statistical significant difference was found between cases and controls as regards age and sex. As regards rs3761548, highly statistical significant difference (P <0.001) was found between cases and controls in frequency of AA; AC and CC genotypes. There was also a highly statistical significant difference (P <0.001) between cases and controls in (A & C) alleles frequency (**Table 1 and Figure 1**).

In rs2232365 genotype, highly statistical significant difference (P =0.001) was detected between cases and controls as regards AA; AG and GG genotypes. There was also a highly statistical significant difference (P <0.001) between cases and controls as regards (A & G) alleles frequency (**Table 2 and Figure 2**).

Table (1): Frequency of rs3761548 A/C genotypes and alleles in studied groups.

rs3761548 genotypes	Cases (No.=50)		Controls (No.=30)		Test	P value	OR (95% CI)
	No.	%	No.	%			
AA	16	32.0	25	83.33	$\chi^2 = 19.79$	<0.001	1.00
AC	15	30.0	2	6.67			11.72 (2.17-114.26)
CC	19	38.0	3	10.0			9.89 (2.28-58.35)
Alleles	A	47/100	47.0	52/60	$\chi^2 = 25.01$	<0.001	7.33
	C	53/100	53.0	8/60			13.33

χ^2 :Chi-square test. OR: Odd Ratio. CI: Confidence Interval.

Table (2): Frequency of rs2232365 A/G genotypes and alleles in studied groups

rs2232365 genotypes	Cases (No.=50)		Controls (No.=30)		Test	P value	OR (95% CI)
	No.	%	No.	%			
AA	18	36.0	24	80.0	$\chi^2 = 14.68$	0.001	1.00
AG	14	28.0	2	6.67			9.33 (1.73-91.48)
GG	18	36.0	4	13.33			6 (1.56-27.85)
Alleles	A	50/100	50.0	50/60	$\chi^2 = 17.78$	<0.001	5 (2.18-12.21)
	G	50/100	50.0	10/60			

χ^2 : Chi-square test. OR: Odd Ratio. CI: Confidence Interval.

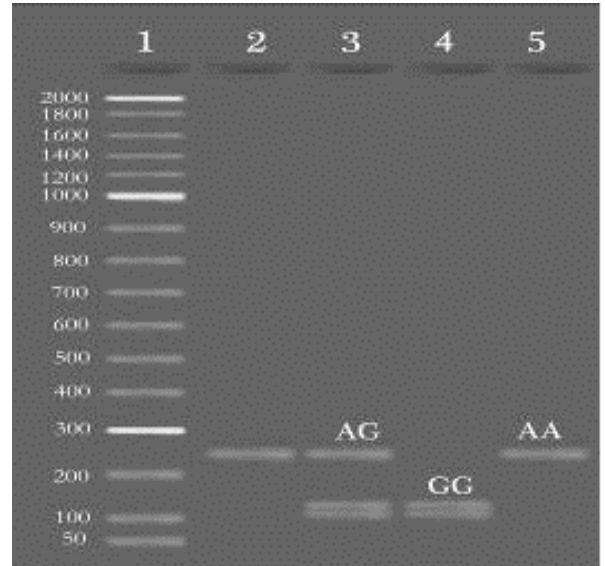
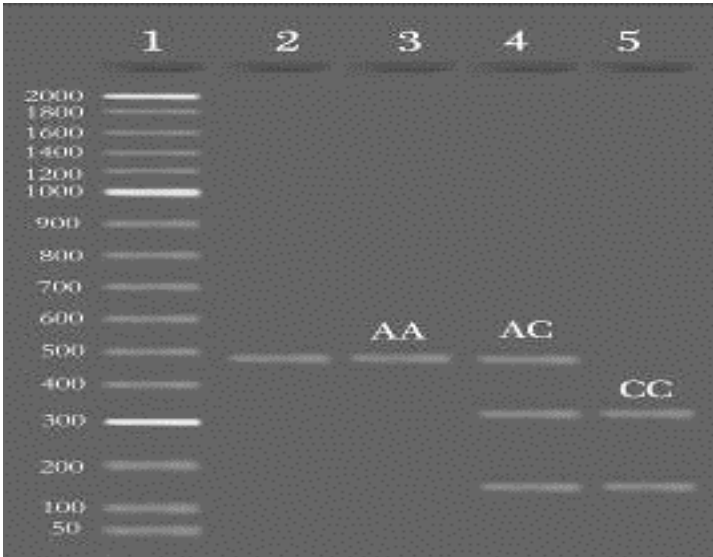


Figure (1): Agarose gel showing PstI restriction profiles of the FOXP3 rs3761548 A/C polymorphic sites in studied groups.

Figure (2): Agarose gel showing BsmBI restriction profiles of the FOXP3 rs2232365 A/G polymorphic sites in studied groups.

Statistical significant difference was detected between cases and controls as regards combined rs3761548 A/C and rs2232365 A/G genotypes with AA+AG (P =0.02) ; AA+GG (P =0.04); AC+AA (P =0.049); AC+AG (P =0.002); CC+AA (P <0.003) and CC+AG (P <0.006) and a highly statistical significant difference was detected as regards AA+AA (P <0.001) ; CC+GG (P <0.001) and AC+GG (P =0.001) (**Figure 3**).

There were statistical significant differences between FOXP3 serum levels and rs3761548 AC, rs2232365 AG and combined genotypes frequencies in studied cases. P values (P =0.05, P =0.007, P <0.001) respectively (**Table 3**).

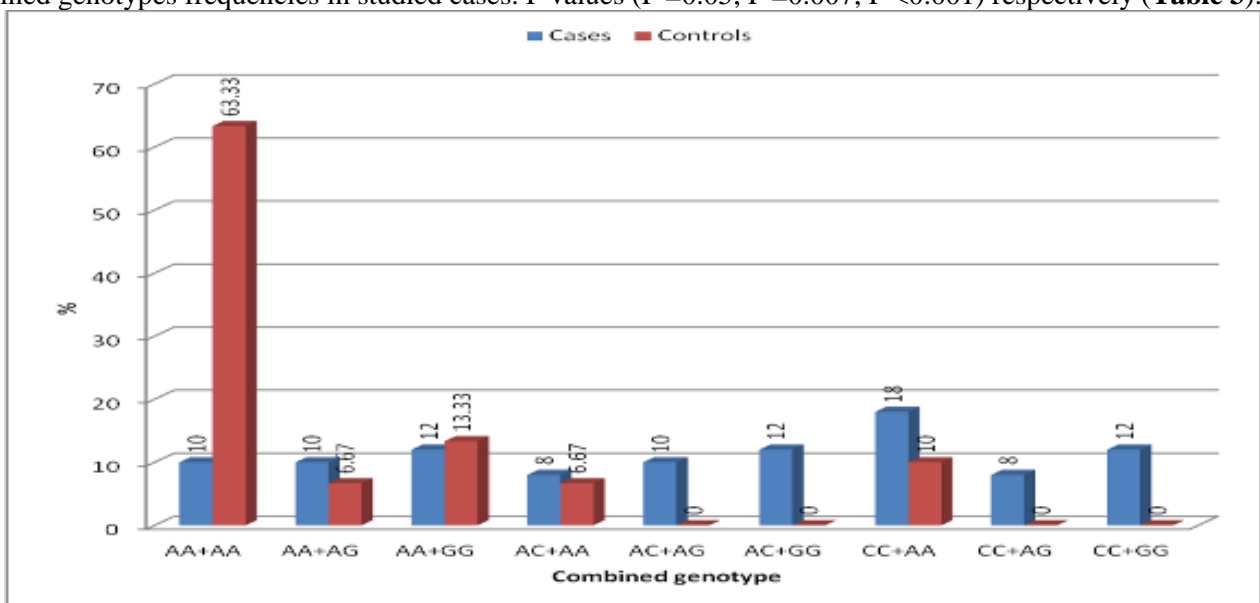


Figure (3): Frequency of combined genotypes.

Table (3): FOXP3 serum levels in relation to genotypes frequency in studied cases.

Genotypes		FOXP3 Serum level (ng/ml)			F test	P value
		No.	Mean	SD		
rs3761548 genotype	AA	16	8.48	2.11	3.19	0.05
	AC	15	5.35	1.21		
	CC	19	6.12	1.42		
rs2232365 genotype	AA	18	6.04	1.33	5.48	0.007
	AG	14	4.76	1.22		
	GG	18	8.71	2.11		
FOXP3 combined genotypes	AA+AA	5	6.26	1.21	5.23	<0.001
	AA+AG	5	4.89	1.03		
	AA+GG	6	13.31	3.31		
	AC+AA	4	6.88	1.31		
	AC+AG	5	4.63	1.11		
	AC+GG	6	4.93	1.12		
	CC+AA	9	5.54	1.31		
	CC+AG	4	4.76	1.14		
	CC+GG	6	7.89	1.87		

F: One way Analysis Of Variance (ANOVA).

Measurement of FOXP3 serum levels detected a highly statistical significant difference ($P < 0.001$) between cases and controls groups (Table 4 and Figure 4). The best cut off point of FOXP3 serum level is (9.933) with sensitivity (86.0%) and specificity (93.33%). The area under curve (AUC) is (0.9607) (Fig. 5). There was no statistical significant difference detected as regards genotypes frequency of rs3761548, rs2232365 or combined genotypes as well as serum FOXP3 levels in relation to VETI score in studied patients. The spearman correlation was ($\rho = -0.08$) (Table 5 and Figures 6 and 7).

Table (4): FOXP3 serum levels in studied groups.

Variable	Cases (No. =50)		Controls (No. =30)		Test	P value
	No.	%	No.	%		
Serum level (ng/ml) Mean \pm SD	6.64 \pm 1.63		20.62 \pm 5.01		t= 11.28	<0.001

t: Independent Student t-test.

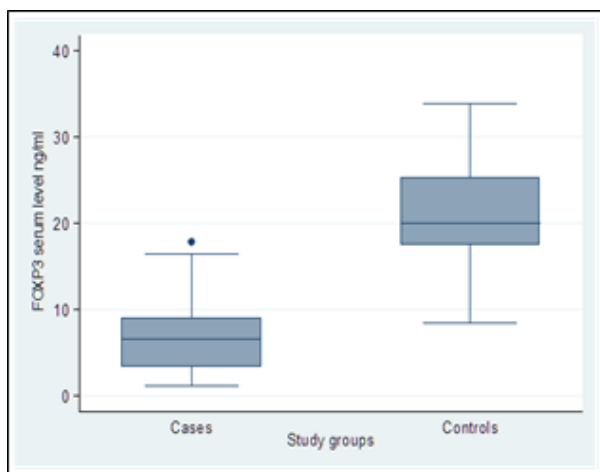


Figure (4): FOXP3 serum levels.

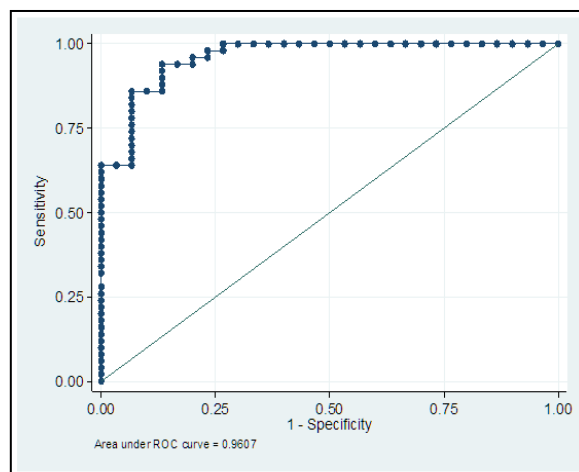


Figure (5): ROC analysis of serum FOXP for the prediction of vitiligo.

Table (5): Spearman correlation between FOXP3 serum level and VETI score in studied patients.

No.=50	VETI scores	
	Spearman correlation coefficient (ρ)	P value
FOXP3 serum levels	-0.08	0.55

Rho: Spearman correlation coefficient.

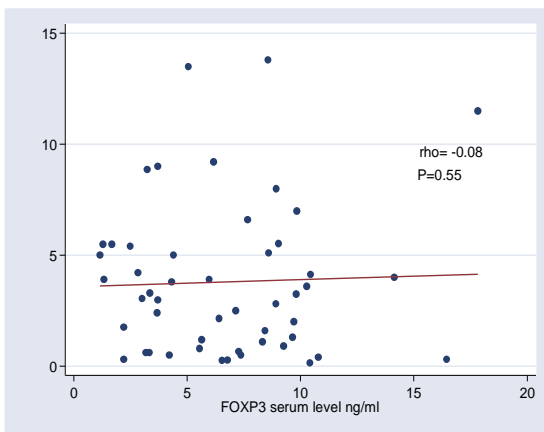


Figure (6): Spearman correlation between FOXP3 serum level and VETI score.



Figure (7): Focal (a) and generalized (b) vitiligo.

DISCUSSION

Among the various markers that are expressed by Treg cells, only FOXP3 is a particular master regulator of Treg cells. By binding to the relevant target genes, FOXP3 can regulate the production of important molecules that mediate immune homeostasis. Through these modulation techniques, FOXP3 not only aids in the conversion of naive T cells into Treg-like cells with suppression activity, but it also promotes the growth and operation of Treg cells [9]. Through the dysregulation of Treg cells by FOXP3 that is adversely expressed, FOXP3 functional polymorphisms may be linked to vitiligo [1]. The connection between SNPs at two separate loci, rs3761548 A/C and rs2232365 A/G, situated in the FOXP3 gene promoter area and the serum levels of

FOXP3 in Egyptian patients with vitiligo was examined in this study.

According to **Jahan et al.** [16], the FOXP3 rs3761548 gene poly-morphism may be linked to vitiligo susceptibility. The FOXP3 rs3761548 A/C polymorphism may also affect the risk to other autoimmune disorders (AD), according to **He et al.** [17]. The current study revealed presence of rs3761548 A/C genotypes (A&C) alleles with CC genotypic variant and C allele at higher frequency in vitiligo patients than controls. This could be explained by the fact that risk genotypes contributed to a decrease in Treg cells and a reduction in the immunosuppressive capacity. Additionally, it may result in FOXP3 gene transcription that is flawed [10].

In the Han's Chinese population, there were statistically significant differences in rs3761548 genotypes between cases and controls, with people with the rs3761548 A variant allele having a higher risk for vitiligo than those with the rs3761548 C variant allele, according to a study by **Song et al.** [1].

Our study found that individuals with vitiligo had genotype and allele distributions that were substantially different from control participants' distributions for the rs2232365 A/G FOXP3 gene

polymorphism. When compared to rs2232365 A alleles of the wild type, statistical analysis showed that rs2232365 G alleles considerably enhanced the risk of vitiligo in the Egyptian population.

These findings are in line with those of **Jahan et al.** [16] and **Song et al.** [1], who discovered that there were statistically significant differences between patients and controls for the genotypes of rs2232365 and the frequency of the G allele. They demonstrated that in the Han Chinese community, those with the rs2232365 G variant allele had a greater chance of developing vitiligo than individuals with the rs3761548 A variant allele. Also, the GG genotype of rs2232365 decreases FOXP3 expression and modifies Treg cell activity, upsetting the Th1/Th2 balance and resulting in the proinflammatory disease vitiligo, according to **Song et al.** [18] and **Wu et al.** [10].

The FOXP3 rs2232365 G allele was substantially related with an elevated risk of rheumatoid arthritis (RA) in the Polish population (P=0.004) as compared to the wild type rs2232365 A alleles, according to statistical analysis of the **Paradowska Gorycka et al.** [12] study. Additionally, it was shown by **Chen et al.** [19] and **Jaber and Sharif** [20] that the rs2232365 FOXP3 polymorphisms were strongly linked to recurrent pregnancy loss (RPL). The rs2232365A/G polymorphism is located in the putative DNA-binding site of the transcription factor GATA-3, which directly controls FOXP3 production and regulates Treg cell activity by interacting with regulatory regions. Therefore, this allele mutation may prevent GATA-3 from interacting with the FOXP3 promoter's binding area, which is likely to impact FOXP3 production, Treg cell activities, and have a role in autoimmune disease [21-23].

The combined rs3761548 A/C and rs2232365 A/G genotypes frequency differences between patients and controls were statistically significant, according to our study. According to **Song et al.** [1], this conclusion may be explained by the fact that the risk of vitiligo tends to rise as the number of variant

alleles rises, indicating that these variations have a synergistic and cumulative influence on the risk for vitiligo. Additionally, they proposed that there could be some interactions between the clinical traits of vitiligo patients and the total number of risk alleles.

The FOXP3 rs3761548 and rs2232365 polymorphisms have been associated with psoriasis, allergic rhinitis, autoimmune thyroid illness, and susceptibility to SLE, according to studies by **Gao et al.** [24], **Inoue et al.** [25] and **Fodor et al.** [26].

According to our study, there was a highly statistically significant difference in the blood levels of FOXP3 between patients and controls. FOXP3 levels were observed to be lower in cases compared to controls. **Elela et al.** [27] research demonstrating patients with non-segmental vitiligo had markedly lower peripheral blood FOXP3 levels than controls which supports this result.

Contrarily, FOXP3 serum levels were much greater in RA patients than in healthy people, according to **Paradowska-Gorycka et al.** [12], correlating with the continuous inflammatory process in patients.

In this study, statistical significant differences were found between FOXP3 serum levels and rs3761548 A/C, rs2232365 A/G and combined genotypes in studied cases. The rs3761548 AA genotype appears to be protective as patients with AA genotype had the highest production of FOXP3 with mean 8.48 (SD 4.92) ng/ml.

According to **Paradowska-Gorycka et al.** [12], there were no correlation between FOXP3 rs2232365 A/G polymorphism and variations in FOXP3 blood levels between RA patients and controls. They proposed that rather than only one genotype, the quantity and activity of Treg cells, which participate in inflammatory processes, would have a significantly greater impact on the FOXP3 level in serum.

Our results showed that there was no statistical difference detected between rs3761548 & rs2232365 genotypes & alleles and sex, age and type of vitiligo in studied patients.

According to **Song et al.** [1], subgroups with the following characteristics had a higher probability of developing vitiligo: age >20 years, male sex, nonsegmental vitiligo, and other concomitant autoimmune disorders. **Li** [28] also noted that non-segmental vitiligo was more common in males with the FOXP3 rs3761548 AA and rs2232365 GG genotypes, independent of the age or type of the patients.

A statistical significant difference was found between rs2232365 A/G genotypes and alleles and duration of active diseases in our studied patients. P values were 0.04 and 0.05, respectively. This finding is in agreement with **Song et al.** [1] who reported that the increased vitiligo risk was more pronounced

among patients with active vitiligo. They made the claim that there are interactions between a patient's genetic make-up and their clinical characteristics.

CONCLUSIONS

The SNPs (rs3761548 and rs2232365) of FOXP3 gene are significantly associated with being susceptible to vitiligo especially patients carrying the rs3761548 C and rs2232365 G variant alleles. As the number of variant alleles rise, the risk for vitiligo tended to rise as well, indicating that these variations are becoming more and more important in vitiligo risk. Understanding the relationship between vitiligo and the FOXP3-mediated regulation of Treg activity is made easier thanks to FOXP3 polymorphisms. Patients with vitiligo have lower serum levels of FOXP3, which is indicative of the disease's pathophysiology and progression. Future research may identify regulatory FOXP3 polymorphisms as brand-new treatment targets for vitiligo.

RECOMMENDATIONS

Analyzing the impact of the FOXP3 polymorphisms rs3761548 A/C and rs2232365 A/G on FOXP3 mRNA expression in the skin of vitiligo patients from Egypt. Find out if the FOXP3 polymorphisms rs3761548 A/C and rs2232365 A/G are linked to vulnerability to other autoimmune diseases.

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Conflict of interest: Nil.

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