ASSOCIATION BETWEEN FOXP3 (RS3761548) PROMOTER POLYMORPHISM WITH VITILIGO PATIENTS, THEIR CLINICAL DATA AND RESPONSE TO PHOTOTHERAPY: A STUDY FROM EGYPT

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Background: *The rs3761548 polymorphism (3279 C> A) of the FOXP3 gene is associated with several autoimmune disorders. Its role in vitiligo has not been well studied.We sought to investigate whether rs3761548 polymorphism is associated with vitiligo in Egyptian subjects.* ***Methods:****Case-control study where genomic DNA was isolated from blood samples of 100 patients and 100 control subjects and genotyping was done by allele-specific primers. Given that FOXP3 is an X-linked marker, data analysis was done for the entire cohort and then stratified based on the gender.* ***Results:****The genotype frequencies differed significantly from patients to control subjects showing that AC genotype was significantly higher in the patient group than control subjects (risky genotype) despite of the protective nature of CC genotype which observed in our study. According to the alleles , the A allele was higher in the patient than in the control group .Insignificant results were reported according to the association between FOXP3 (rs3761548) promoter polymorphism and clinical data of patients and their response to phototherapy.****Conclusions:*** *The rs3761548 of FOXP3 gene may be associated with susceptibility to vitiligo because of altered expression.Both the A allele and AC genotype were significantly associated with vitiligo .*

Keywords: *FOXP3, Vitiligo, Autoimmunity, Egypt.*

INTRODUCTION

Vitiligo is a pigmentary disease in which immune imbalance has been approved to play a role[1]. The skin, hair, and nails are covered by white patches of different sizes and shapes[2]. It usually affects areas exposed to the sun like elbows, the back of the hands, and wrists and it affects the areas around the body orifices[3]. The depigmentation occurs due to the destruction of melanocytes responsible for melanin production[4].

Multiple observations strongly support the role of autoimmune processes in vitiligo. First,

the strong association between vitiligo and other autoimmune disorders like Addison's disease[5], Alopecia Areata[6], Rheumatoid arthritis[7], Type 1 Diabetes Mellitus[8], Systemic Lupus[9], Pemphigus Vulgaris[10], Graves' disease[11], Autoimmune thyroiditis[12] and pernicious anaemia[13]. Second, it strongly runs in families with a positive history of vitiligo and other autoimmune disorders[14]. Third, autoantibodies in patients with depigmentation may be positive in up to 50% of patients with small areas and 90% of patients with larger areas[15]. Fourth, T cells[16] and B cell infiltration[17] were found in skin biopsies taken from the lesion. Fifth, the strong association of vitiligo with generic loci related to autoimmune disorders, especially that related to class I and class II major histocompatibility complexes located on the sixth chromosome[18&19]. Sixth, the response of vitiligo to immunosuppressant agents like tacrolimus[20] and topical steroids[21].

The regulatory T cells (Tregs) are members of the CD4 T helper cell family that play a pivotal role in the prevention of the immune response against self-antigens and also in the down-regulation of an excessive immune response against other foreign antigens[22&23]. The role of T regs in the development of the autoimmune process in vitiligo has been investigated in different studies[24-27]. The flow cytometric analysis of Tregs in vitiligo patients compared to controls shows a reduction in their percentage[28]. Moreover, histopathological analysis of skin biopsies increased the numbers of cytotoxic T cells and reduced Treg numbers[29]. In addition to the number of Treg defects in vitiligo, function reduction has also been detected[30].

Forkhead box protein 3 (FOXP3) is the critical transcription factor that regulates Tregs' development, proliferation, and function. The *FOXP3* gene is 1296bp in length, related to the winged-helix transcription factor family, and located on the X chromosome (Xp 11.23) within an area linked to autoimmune disorders[31]. It is formed of 11 exons which encode a protein formed of 431 amino acids; its weight is 43KD and it is formed of 4 domains: forkhead, leucine zipper, zinc finger, and proline-rich residues domains[29]. Although Tregs are the primary cells expressing FOXP3, some tumour cells were found to express it, like pancreatic cancer cells, melanoma cells, and cancer breast cells, which may represent a mechanism for tumour evasion[32]. Moreover, the regulation of T cell subsets and the expression of some cytokines have been mediated by FOXP3[33]. The qualitative or quantitative defect in FOXP3 leads to a fatal, aggressive, and fatal autoimmune disorder named immune dysregulation, polyendocrinopathy, enteropathy, or X-linked syndrome[34]

Different single nucleotide polymorphisms (SNPs) have been studied, especially those that lie within the promotor region, as they affect the gene expression and, as a result, affect Tregs function and differentiation. In this study, rs3761548C> A was selected as it lies within the promotor region, and its allele was found to be associated with decreased *FOXP3* gene expression[35].

MATERIALS AND METHODS

Subjects

The G*power software version 3.1.9.7 was used for sample size calculation[36]. The software parameters were adjusted to be: the power level (1-ßerror probability), the alpha error probability was 0.05 (two-tailed), the effect size was 0.5, and the allocation ratio (N2/N1) was 1. The minimum sample size for each group was 86 subjects, and we increased both groups to 100 people to avoid missing values probability.

The patients' group involved 44 males and 56 females. The age of vitiligo patients ranged from 5-67 years old, with a mean of 33.2 16.9. A dermatologist diagnoses all patients subjected to careful history taking and clinical examination, both general and dermatological. Localized vitiligo was observed in 85% of patients, while generalised distribution was observed in 15%. Also, non-segmental vitiligo was observed in 84% of patients, while segmental was observed in 16%. The vitiligo disease activity score (VIDA) is a six-point score used to evaluate disease activity[37]. All patients who were pregnant, lactating, had a neoplasm, or had received either photo or systemic vitiligo therapy in the previous 4 weeks of the study were excluded.

The control group involved 50 males and 50 females with an age range from 5-66 years old, and the mean was 33.9 15.9. All controls were clinically examined for any dermatological problems and asked for a family history of autoimmune disorders.

Ethical issues

Written consent was signed by each individual participating in this study, either patient or control. For the children, a written consent signed by the caregiver was collected. The study protocol was reviewed and approved by the Institutional Review Board of Mansoura Faculty of Medicine, Mansoura University (R.20.08.968).

Genotyping

A 5 ml blood sample was collected from each individual and preserved at -20 ℃ until use. Thermo scientific DNA extraction min kit (cat number: K0781) was used for DNA extraction. The genotyping for the *FOXP3* rs3761548C< A was done using the following set of primers as mentioned in previous study[38]:
Outer forward:
5'GACTTAACCAGACAGCGTAG3',
Inner forward:
5'TTCTGGCTCTCTCCCCAACTGC3',
Inner reverse:
5'TGAGGGGTAAACTGAGGCCTT3' and
Outer reverse:
5'CTGGTGTGCCTTTGGTCT3'

The polymerase chain reaction (PCR) condition was as the follows: the number of cycles was 30 cycles, Initial denaturation for 7 minutes at 95 ℃, denaturation for 30 seconds at 94 ℃, annealing for 45 seconds at 53.5 ℃, elongation for 1 min at 72℃ and final elongation for 5 min at 72℃. The final volume consisting of (ten μL of polymerase chain reaction mix consists of 1.25 μL of 1X complete buffer, 1.5 μL of genomic DNA, 0.3 μL of five U of Taq polymerase, 0.3 μL of dNTPs, and 0.15 L of each control primer) then check by running on 2% agarose gel electrophoresis with ethidium bromide at 100 V for 20 minutes[39]. On 209 bp, 397 bp, and 564 bp bands, the A allele, C allele, and general product were detected, respectively (Figure 1).

Statistical analyses

Continuous data is expressed by standard deviation (SD) in the expression of continuous data, but the expression of number and percentage is done by categorical data. The comparison of the continuous between patients and controls and the comparison of the continuous data between the different alleles were made by independent sample Student's t-test. Nevertheless, the comparison of the continuous data between the different genes was made by ANOVA. The chi-square test makes categorical data comparisons. The odds ratio and the 95% confidence interval (CI) were calculated for the proportions of the genes and alleles between patients and controls, with a p-value 0.05 being considered significant. The Statistical Package for Social Science (IBM-SPSS) version 20 (Chicago, IL, USA) for Windows was used for this statistical analysis of data.
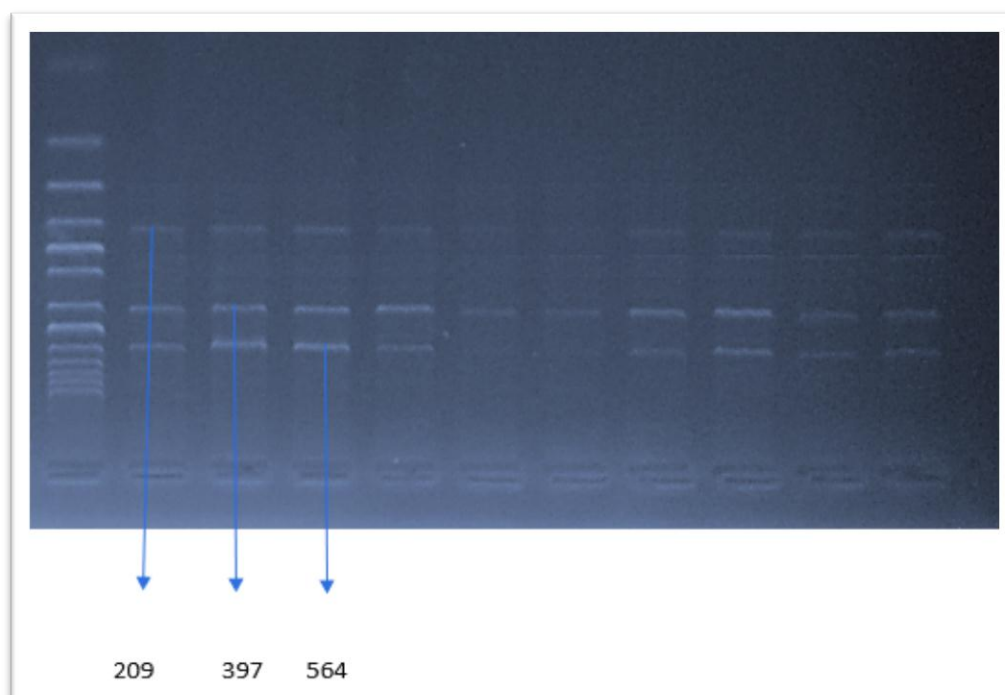

209   397   564

Fig. 1: The genotypes of *FOXP3* rs3761548C<A on agarose gel 2%.

RESULTS AND DISCUSSION

Results

This study involved 200 individuals, 100 patients, and 100 healthy control subjects with a mean age of 33.2 16.9 and 33.9 15.9, respectively. The patients' age range was 5-67 years, and for the control, the age range was 5-66 years. The patients' group consisted of 45 (45%) males and 55 (55%) females, while the control group had 50 (50%) males and 50 (50%) females. The mean age of onset for the disease was 26.0 14.8, and when stratified according to gender, it was 28.5 14.2 and 23.9 15.1 in male and female patients, respectively. The HWE was applied to the control group, and it showed deviation (p 0.002).

The distribution of *FOXP3* rs3761548C<A AA, AC, and CC genotypes showed in Table 1 was 14%, 78%, and 8% in the patient group and 23%, 33% 44% in the control group, respectively. For alleles, A allele was 53% in patients and 39.5% in control, while the C allele was 47% in patients and 60.5% in the control group. A significant association was observed when both groups were compared with each other. The AC genotype and A allele were more frequent in the patient group than in control, and a significant association was observed ($p< 0.001$ and 0.007), respectively. The CC genotype was more frequent in the control group, with a significant association ($p< 0.001$) than other genotypes. The correlation between the genotypes and alleles (Table 2) of the studied polymorphism with demographic and clinical data like age, age of onset, VIDA score, sex, course, psychic trauma, alopecia, family history of vitiligo, family history of another autoimmune disease, remission with therapy and phototherapy was investigated. The only significant relation detected was between the genotype and the coursed of the disease (p 0.019).

Discussion

Autoimmunity is achieved by breaking the immune tolerance mechanism, which is the balance between autoreactive cells and regulatory mechanisms. A defect in regulatory mechanisms or an increase in autoreactive cells leads to a shift toward an autoimmune response. The Tregs are the key regulators that maintain this counterbalance[40].The prominent landmark that is expressed uniquely by Treg is FOXP3. This study demonstrated that *FOXP3* gene polymorphism rs3761548C< A was closely associated with vitiligo in Egyptian patients. The results showed that patients who carry the A allele have a higher risk for vitiligo than these cases carry the C allele. However, when the clinical characteristics of the patient group like age, sex, family history, and others were examined for the association with the genotypes and alleles of the selected variant, no association was detected (Table 2).

Table 1: Distribution of *FOXP3* alleles and genotypes Vitiligo patients and healthy controls.

FOXP3 polymorphism	Case (N—100) N (%)	Control (N=100) N (%)	OR (95% CI)	P/Pc
Rs3761548C<A				
AA	14 (14)	23 (23)	0.55 (0.26-1.13)	0.101
AC	78 (78)	33 (33)		<0.001
CC	8 (8)	44 (44)	7.20 (3.83-13.5)	<0.001
A	106 (53.0)	79 (39.5)		
C	94 (47.0)	121 (60.5)	0.11 (0.05-0.25)	0.007
			1.63 (1.61-2.57)	
HWE		0.002		

Table 2: The association between the demographic and clinical findings and the *FOXP3* rs3761548C<A genotypes and alleles.

	AA	AC	CC	A	C
Age (years)	31.0 ±18.4	32.9 ±17.2	39.5 ±12.1	32.5 ±17.4	34.1 ±16.5
P		**0.514**		**0.498**	
Age at onset (years)	24.4 ±18.3	25.8 ±14.5	31.6 ±12.2	25.4 ±15.4	26.8 ±14.2
P		**0.519**		**0.521**	
Vida score	3.0 ±1.3	2.2 ±1.6	2.5 ±1.4	2.4 ±1.6	2.3 ±1.6
P		**0.226**		**0.454**	
Sex					
Females	7 (50%)	45 (57.7%)	3 (37.5%)	59 (55.7%)	51 (54.3%)
Males	7 (50%)	33 (42.3%)	5 (62.5%)	47 (44.3%)	43 (45.7%)
P		**0.507**		**0.842**	
Course					
Regressive	0 (0%)	6 (7.7%)	0 (0%)	6 (5.7%)	6 (6.4%)
Stable	0 (0%)	2 (2.6%)	2 (25%)	2 (1.9%)	6 (6.4%)
Progressive	14 (100%)	70 (89.7%)	6 (75%)	98 (92.5%)	82 (87.2%)
P		**0.019***		**0.258**	
Psychic trauma	4 (28.6%)	20 (25.6%)	2 (25%)	28 (26.4%)	24 (25.5%)
P		**0.972**		**0.887**	
Alopecia	1 (7.1%)	11 (14.1%)	2 (25%)	13 (12.3%)	15 (16%)
P		**0.509**		**0.452**	
Family history of vitiligo	0 (0%)	8 (10.3%)	0 (0%)	8 (7.5%)	8 (8.5%)
P		**0.293**		**0.802**	
Family history of autoimmune diseases	2 (14.3%)	7 (9%)	0 (0%)	11 (10.4%)	7 (7.4%)
P		**0.530**		**0.470**	
Remission with therapy	8 (57.1%)	50 (64.1%)	5 (62.5%)	66 (62.3%)	60 (63.8%)
P		**0.884**		**0.819**	
Phototherapy	14 (100%)	77 (98.7%)	8 (100%)	105 (99.1%)	93 (98.9%)
P		**0.867**		**0.932**	

A hospital-based case-control study involved 682 Han Chinese patients with vitiligo. A strong association of AA genotype was detected (OR 1.82, 95%CI 1.10-3.01, *P* 0.033). Also, in the same study, a combined analysis of this variant with the other 2 variants (rs2232365 and rs5902434) found increases in the risk of the disease in patients who had 2–6 variant alleles[41]. In agreement with this study, another study from India involved 303 patients with non-dermatomal vitiligo who found the AA genotype incriminated in vitiligo susceptibility. The A allele was frequent in the patient group, while the C allele was frequent in the control group. The patient group was further stratified according to gender. The CC genotype was protective (OR 0.39, *P* 0.001), and the CA genotype increased the vitiligo risk in the female patient by 3-fold (OR 2.63, *P* 0.002)[39].

Besides vitiligo, FOXP3 rs3761548C< A was found to be associated with other autoimmune disorders like multiple sclerosis[42], systemic lupus erythematosus[43], Graves' Disease[44], acute cellular rejection after liver transplantation[45], Autoimmune thyroid disease (Fathima *et al.,* 2019), Psoriasis vulgaris (Elsohafy *et al.,* 2019), allograft rejection episodes in kidney transplantation (Khuja *et al.,* 2018), aplastic anaemia (In *et al.,* 2017), Pemphigus foliaceus (Ben Jmaa *et al.,* 2017),

ulcerative colitis[46] and idiopathic recurrent miscarriages[47].

The exact role of this variant in FOXP3 gene regulation is still unexplained. However, a study found that this variant is located in the "GGGCGG" sequence of the specificity protein 1 transcription factor, and this mutation C> A might interfere with the binding site of this factor and hence affect FOXP3 expression[41]. Another explanation is that in the AA genotype there is a loss of binding sites for 2 transcription factors, C-Myb and E47. E47 is necessary for FOXP3 gene transcription in the G1 phase of the cell cycle, while C-Myb is essential in the G2/M phase; both of these transcription factors overlap the rs3761548 at CANNTG and T/CAACG/TG sequences, respectively. So, any disruption of these 2 binding sites leads to failure of gene transcription[29].

The qualitative and quantitative defect of FOXP3 in vitiligo has been proved in different studies. A study involving patients with vitiligo found that in PBMNCs, there was decreased expression of FOXP3 in comparison with healthy controls[48&49]. The histopathological examination of lesional skin biopsies from patients and normal skin from healthy control found decreased FOXP3 expression in lesional samples[50-52].FOXP3 mRNA expression has been studied with varying results; one study found decreased expression in the lesional area when compared to healthy individuals[53], while another study found increased expression in the lesional area when compared to non-lesional area, implying recruitment of Tregs to balance the immune response in the area of the lesion[25].

To the best of our knowledge, this is the first study that involves FOXP3 rs3761584C<A gene polymorphism in Egyptian patients with vitiligo. However, different studies from Egypt involved the relation between FOXP3 and vitiligo. A study found a marked reduction in FOXP3+ Treg cells in marginal skin lesions when compared with lesional and non-lesional skin of the same patient[54]. mRNA 164a and FOXP3 expression were studied in patients with non-segmental vitiligo, and there was increased mRNA expression while FOXP3 expression was decreased. Moreover, flow cytometric analysis of 80 patients with segmental vitiligo shows a significant decrease in FOXP3+ Treg cells, and their number correlates negatively with the VIDA score (Hegab and Attia, 2015).The assessment of cytotoxic T cells and FOXP3+ Treg cells showed increases in cytotoxic cells and defects in Treg cells in marginal skin of stable and active lesions[55].

The Hardy–Weinberg equilibrium (HWE) states that the frequencies of alleles and genotypes in a population remain constant as if there were no evolutionary events. Theoretically, in case-control studies where the controls are free from the disease, the HWE and HWE should be followed[56]. In our study, there was a deviation in the control group from HWE. The gaining or loss of heterozygosity can explain this deviation. The gain issue is usually related to genotyping errors. On the other hand, the loss may be due to inbreeding, deletion polymorphism, copy number variation, or purifying selection. The reduction of heterozygosity may be due to the prevalence of consanguineous marriage in the Egyptian community[57], and this may explain the deviation from HWE in this study.

Conclusion

Both the A allele and the AC genotype were significantly associated with vitiligo in the Egyptian population. Furthermore, this study provides additional evidence for the role of autoimmunity in the pathogenesis of vitiligo. Further research with a larger sample size, different geographical regions, multiple SNPs, and different techniques is encouraged to help support the role of the immune system in vitiligo pathogenesis and may lead to novel effective immune-therapy for vitilgo patients.

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نشرة العلوم الصيدليـــة

جامعة أُسيوط

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الارتباط بين (FOXP3 (rs٣٧٦١٥٤٨ تعدد الأشكال المحفز مع مرضى البهاق ، وبياناتهم السريرية والاستجابة للعلاج بالضوء: دراسة من مصر

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الخلفية: يرتبط تعدد الأشكال rs٣٧٦١٥٤٨ (C > A ٣٢٧٩) لجين FOXP3 بالعديد من اضطرابات المناعة الذاتية. لم يتم دراسة دوره في البهاق جيدًا ، لذا سعينا إلى التحقق مما إذا كان تعدد الأشكال rs ٣٧٦١٥٤٨ مرتبطا بالبهاق في المصريين.

الطريقة: دراسة الحالات والشواهد حيث تم عزل الحمض النووي الجينومي من عينات الدم لـ ١٠٠ مريض و ١٠٠ من الأشخاص الضابطين وتم إجراء التنميط الجيني بواسطة بادئات خاصة بالأليل. نظرًا

لأن FOXP3 عبارة عن علامة مرتبطة بـ X ، فقد تم إجراء تحليل البيانات للمجموعة بأكملها ثم تم تقسيمها على أساس الجنس.

النتائج: اختلفت ترددات النمط الجيني بشكل كبير من المرضى إلى الأشخاص الضابطين مما يدل على أن النمط الجيني AC كان أعلى بشكل ملحوظ في مجموعة المرضى من الأشخاص الضابطين (النمط الوراثي المحفوف بالمخاطر) على الرغم من الطبيعة الوقائية للنمط الجيني CC الذي لوحظ في دراستنا. وفقًا للأليلات ، كان الأليل A أعلى في المريض منه في المجموعة الضابطة. تم الإبلاغ عن نتائج غير مهمة وفقًا للعلاقة بين تعدد الأشكال المحفز(rs٣٧٦١٥٤٨) FOXP3 والبيانات السريرية للمرضى واستجابتهم للعلاج الضوئي.

الاستنتاجات: قد يترافق rs٣٧٦١٥٤٨ من جين FOXP3 مع القابلية للإصابة بالبهاق بسبب التعبير المتغير ، وقد ارتبط كل من الأليل A والنمط الجيني AC بشكل كبير بالبهاق.

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