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RESEARCH ARTICLE

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Impact of cytotoxic T-lymphocyte-associated protein 4 codon 17 variant and expression on vitiligo risk

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Abstract

Background: Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is one of the essential brakes expressed on T cells that prevent T-cell hyperactivation-associated autoimmune disorders. Several *CTLA4* polymorphisms were implicated in the regulation of gene expression. We aimed to explore the association of *CTLA4* expression and rs231775 (c.49A>G) variant with vitiligo risk and severity of the disease in a sample of the Middle Eastern population.

Methods: The *CTLA4* gene expression and genotyping for rs231775 (A/G) variant were assessed in 161 vitiligo patients and 165 controls using a real-time polymerase chain reaction. Vitiligo Area Severity Index (VASI) and Vitiligo Disease Activity score (VIDA) were evaluated.

Results: A higher frequency of rs231775 G allele was observed in vitiligo cases than controls (45% vs. 33%, p = 0.002). After adjustment of age, sex, family history of vitiligo, and *CTLA* expression level, using multivariate analysis, G/G carriers were associated with a higher risk of vitiligo under recessive (OR = 2.94, 95% CI = 1.61–5.35, p < 0.001), dominant (OR = 1.87, 95% CI = 1.14–3.06, p = 0.013), and homozygote comparison (OR = 3.34, 95% CI = 1.73–6.42, p = 0.001) models. Although the *CTLA4* relative expression levels were comparable to that of controls, G/G carriers exhibited a significantly lower expression profile (median = 0.63, IQR = 0.34–1.75) than A/A (median = 1.43, IQR = 0.39–4.25, p = 0.018) and A/G carriers (median = 1.68, IQR = 0.49–3.92, p = 0.007). No significant associations of *CTLA4* variant/expression with disease severity and/or activity were observed.

Conclusion: The CTLA4 rs231775 variant was associated with vitiligo susceptibility and gene expression; the risky genotype (GG) was associated with lower *CTLA4* relative expression levels than the other genotypes. Further large-scale studies in different populations are warranted.

KEYWORDS
CTLA4, gene expression, polymorphism, real-time PCR, rs231775, vitiligo

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1 | INTRODUCTION

Vitiligo is an autoimmune disease characterized by melanocyte loss, which results in patchy depigmentation of skin and hair.[1] Patients with vitiligo have elevated frequencies of other autoimmune diseases, suggesting that they share genetic components.[2] Various susceptibility genes and unknown environmental stimuli have been implicated in vitiligo pathogenesis, supporting its complex genetic makeup.[3] Although accumulating evidence has supported the association of cytotoxic T lymphocyte antigen 4 (*CTLA4*) gene with susceptibility to multiple autoimmune disorders; however, the literature on the genetic association between gene variants and vitiligo was inconsistent.[4]

According to the National Center for Biotechnology and Information, "*CTLA4* gene is a member of the immunoglobulin superfamily, which encodes a protein that transmits an inhibitory signal to T cells. Alternate transcriptional splice variants of this gene, encoding different isoforms, have been characterized. The membrane-bound isoform functions as a homodimer, while the soluble isoform functions as a monomer" (https://ghr.nlm.nih.gov/gene/CTLA4). Several mutations in this gene were associated with type 1 diabetes, systemic lupus erythematosus, Hashimoto thyroiditis, Graves' disease, thyroid-associated orbitopathy, celiac disease, among other autoimmune disorders.[5]

The single nucleotide polymorphism (SNP) rs231775 (A/G) is one of the SNPs implicated with many autoimmune diseases (https://www.ncbi.nlm.nih.gov/snp/rs231775). The high-risk G allele of this variant results in threonine (Thr) substitution by alanine (Ala) at codon 17 of the leader sequence of the CTLA-4 polypeptide, raising the possibility of affecting the conformation of the leader peptide resulting in altered intracellular CTLA-4 trafficking. This variant has been correlated with decreased negative regulation of T-cell proliferation and is thought to predispose to developing several autoimmune diseases and cancers.[4,6]

Although several case-control studies of *CTLA4* variant rs231775 in vitiligo have been reported,[4,7-9] the *CTLA4* expression in vitiligo patients has not been reported before, especially in a sample of the Middle East population. In this sense, the authors were inspired to explore the possible association between *CTLA4* expression and vitiligo and the potential association of rs231775 (A/G) variant and vitiligo risk with correlation to the available clinical and laboratory data of the patients.

2 | SUBJECTS AND METHODS

2.1 | Study population

This case-control study included 161 unrelated vitiligo patients who met strict clinical criteria reported by the "Vitiligo European Task Force"[10] and 165 sex- and age-matched blood donor controls. The participants were recruited from the dermatology outpatient clinics, Suez Canal University, and Mansoura hospitals. The controls have no vitiligo, any other autoimmune disease, chronic diseases, or cancers. Informed consent was obtained from all subjects. The study was conducted according to the Declaration of Helsinki Principles and its later amendments or comparable ethical standards. The Medical and Bioethics local committee of the Faculty of Medicine, Suez Canal University approval was taken (approval No. 3967).

2.2 | Patient assessment

Localized vitiligo includes focal and segmental patterns, while generalized vitiligo includes acrofacial, Vulgaris, and Universalis distribution. Vitiligo Area Severity Index (VASI) was evaluated by dividing the body into five regions (hands, upper limb, trunk, lower limb, feet). Each measured in hand unit. Residual depigmentation value is estimated within each region. The VASI is then calculated as the sum of all body sites [Hand Units] × [Residual Depigmentation].[11] Vitiligo Disease Activity score (VIDA) is a six-point scale for assessing vitiligo stability over time. It depends on the patient's report for disease activity.[12]

2.3 | Blood sampling

From all participants, a 5 ml venous blood sample was collected under complete aseptic conditions on trisodium EDTA (1 mg/ml) tubes. One tube was used for RNA extraction, and the other one was used for DNA genotyping studies.

2.4 | *CTLA4* gene expression analysis

2.4.1 | RNA extraction

The total RNA was extracted from the peripheral mononuclear cells by Ficoll-Paque as a density-gradient medium using "ABIOpure Total RNA (AllianceBio, Catalog no. M541RP50-B)" following the manufacturer's protocol. RNA concentration and purity were determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Tech., Inc.). RNA integrity has been checked by gel electrophoresis.

2.4.2 | Reverse transcription (RT)

"High Capacity cDNA Reverse Transcription" Kit was used as detailed in our previous work.[13] The RT was carried out in a Mastercycler Gradient Thermocycler (Eppendorf) at 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min, then hold at 4°C. Non-template and no-enzyme negative controls were applied in each run.

2.4.3 | Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Relative expression levels of *CTLA4* were quantified using the TaqMan gene expression assay specific for the *CTLA4* gene (Applied

TABLE 1 Demographic and clinical characteristics of the study population

Variables	Categories	Controls N = 165	Vitiligo N = 161	*P*-value
Demographics				
Age, years	Mean ± SD	39.4 ± 15.0	33.5 ± 17.6	0.19
	≤20 years	38 (23)	54 (33.5)	
	≤40 years	61 (37)	48 (29.8)	
	≤60 years	52 (31.5)	46 (28.6)	
	>60 years	14 (8.5)	13 (8.1)	
Sex	Female	111 (67.3)	102 (63.4)	0.48
	Male	54 (32.7)	59 (36.6)	
Residence	Rural	34 (20.6)	71 (44.1)	**<0.001**
	Urban	131 (79.4)	90 (55.9)	
Obesity	Normal weight	102 (61.8)	84 (52.2)	0.16
	Overweight	43 (26.1)	48 (29.8)	
	Obese	20 (12.1)	29 (18)	
Smoking	Negative	165 (100)	148 (91.9)	**<0.001**
	Positive	0 (0)	13 (8.1)	
Clinical data				
Family history	Vitiligo	23 (13.9)	40 (24.8)	**0.017**
	AD	5 (3)	2 (1.2)	0.44
Risk factors	Stress	36 (21.8)	78 (48.4)	**<0.001**
	Sunburn	35 (21.2)	7 (4.3)	**<0.001**
Autoimmune disease	Thyroid disease	29 (17.6)	6 (3.7)	**<0.001**
	Alopecia	2 (1.2)	0 (0)	0.49
	Halo nevi	0 (0)	1 (0.6)	0.49
	RA	0 (0)	21 (13)	**<0.001**
Comorbidities	Hypertension	1 (0.6)	20 (12.4)	**<0.001**
	CAD	6 (3.6)	2 (1.2)	0.28
	T2DM	15 (9.1)	23 (14.3)	0.16
Drug history	Anti-hypertensive drugs	1 (0.6)	12 (7.5)	**0.001**
	Cholesterol-modifying drugs	13 (7.9)	6 (3.7)	0.15
	Anti-diabetic (oral/insulin)	22 (13.3)	14 (8.7)	0.21
	Anti-rheumatic drugs	0 (0)	10 (6.1)	**0.002**
	Allergic drugs	11 (6.7)	2 (1.2)	**0.020**
	Anti-thyroid drugs	4 (2.4)	1 (0.6)	0.37
	Oral contraceptive drugs	3 (1.8)	0 (0)	0.24

Data are shown as number (percentage) or mean ± standard deviation (SD). Chi-square and Fisher's exact tests were used for qualitative variables; and Student's *t* test was used for quantitative variables. OR (95% CI): odds ratio and confidence interval. Bold values indicate statistically significant at *P*-value <0.05.

Abbreviations: AD, Autoimmune disease; CAD, Coronary artery disease; RA, Rheumatoid arthritis; T2DM, type 2 diabetes mellites.

Biosystems, Hs00175480_m1), TaqMan Endogenous control assay for *GAPDH* (Applied Biosystems, Hs02758991_g1), and Taqman® Universal PCR master mix II, No UNG (2×; Applied Biosystems). The PCR reactions were carried out according to the "minimum information for publication of quantitative real-time PCR experiments (MIQE)" guidelines[14] in final volumes of 20 µl, including 1.33 µl RT product, 2× TaqMan Universal PCR Master Mix, 1 µl TaqMan assay. All reactions were run in duplicate and included a no-template control (with water instead of cDNA) and a no-RT control. The PCR was performed on the StepOne™ Real-Time PCR System (Applied Biosystems), as follows: 95°C for 10 min followed by 40 cycles of 92°C for 15 s and 60°C for 1 min. The fold change of mRNA expression in each sample relative to the average expression in healthy controls was calculated based on the quantitative cycle (C_q) value using

TABLE 2 Disease characteristics of vitiligo patients according to distribution pattern (*n* = 161)

Variables	Localized	Generalized	*P*-value
Demographics			
Age, year			
Mean ± SD	29.4 ± 18.7	35.1 ± 17.1	0.91
Median (quartiles)	27.0 (14.5–44.0)	35.0 (18.0–50.0)	
≤20 years	18 (48.6)	36 (29)	0.16
≤40 year	8 (21.6)	40 (32.3)	
≤60 years	9 (24.3)	37 (29.8)	
>60 years	2 (5.4)	11 (8.9)	
Sex			
Female	20 (54.1)	82 (66.1)	0.24
Male	17 (45.9)	42 (33.9)	
Obesity			
Normal weight	21 (56.8)	63 (50.8)	0.43
Overweight	12 (32.4)	36 (29)	
Obese	4 (10.8)	25 (20.2)	
Clinical data			
Family history			
Vitiligo	7 (18.9)	33 (26.6)	0.39
Disease duration, year			
Mean ± SD	3.61 ± 4.6	7.98 ± 6.1	**<0.001**
Median (quartiles)	2.0 (0.33–4.5)	7.0 (3.0–10.0)	
≤1 years	15 (40.5)	17 (13.7)	**0.001**
≤10 years	18 (48.6)	79 (63.7)	
>10 years	4 (10.8)	28 (22.6)	
Premature graying of hair			
Positive	12 (32.4)	63 (50.8)	0.06
Fitz Patrick skin typing			
Type I	0 (0)	1 (0.8)	0.89
Type II	13 (35.1)	41 (33.1)	
Type III	12 (32.4)	46 (37.1)	
Type IV	12 (32.4)	36 (29)	
Lesion characteristics			
Trichrome	14 (37.8)	29 (23.4)	0.09
Depigmentated	37 (100)	117 (94.4)	0.35
Follicular repigmentation	6 (16.2)	31 (25)	0.37
Hypopigmentation	4 (10.8)	40 (32.3)	**0.011**
Koebner phenomena	4 (10.8)	45 (36.3)	**0.004**
Location of the initial site			
Face	12 (32.4)	44 (35.5)	0.32
Trunk/back	7 (18.9)	24 (19.4)	
Upper limb	8 (21.6)	38 (30.6)	
Lower limb	10 (27)	18 (14.5)	
Location of affected lesions			
Mouth	2 (5.4)	20 (16.1)	0.10
Eyes	5 (13.5)	23 (18.5)	0.62

(Continues)

TABLE 2 (Continued)

Variables	Localized	Generalized	*P*-value
Face	10 (27)	49 (39.5)	0.18
Trunk	10 (27)	69 (55.6)	**0.003**
Arms	8 (21.6)	68 (54.8)	**<0.001**
Hands	7 (18.9)	42 (33.9)	0.10
Legs	14 (37.8)	63 (50.8)	0.19
Feet	5 (13.5)	43 (34.7)	**0.014**
Localized distribution			
Focal	23 (62.2)	–	NA
Segmental	14 (37.8)	–	
Generalized distribution			
Acrofacial	–	6 (4.8)	
Vulgaris	–	113 (91.1)	
Universalis	–	5 (4)	
Severity assessment			
VASI score			
Mean ±SD	13.6 ± 6.5	27.0 ± 20.9	**<0.001**
Median (IQR)	10.0 (1.0–17.5)	25.0 (10.0–25.0)	
VIDA score			
−1	9 (24.3)	27 (21.8)	0.71
0	4 (10.8)	6 (4.8)	
+1	3 (8.1)	12 (9.7)	
+2	5 (13.5)	20 (16.1)	
+3	6 (16.2)	15 (12.1)	
+4	10 (27)	44 (35.5)	
Treatment history			
Treatment modalities			
No TTT	1 (2.7)	7 (5.6)	0.68
Combined PVT	36 (97.3)	117 (94.4)	
Duration treatment, year			
Median (Q1-Q3)	1.0 (0.16–3.0)	4.0 (1.0–9.0)	**<0.001**
Range	0–10	0–30	
Course treatment			
Compliant	28 (75.7)	58 (46.8)	**0.002**
Interrupted	9 (24.3)	66 (53.2)	

Data are shown as number (percentage), mean ± SD, or median (quartiles). Chi-square and Mann-Whitney *U* tests were used. Bold values indicate statistically significant at *p*-value <0.05. Localized vitiligo includes focal and segmental patterns, while generalized vitiligo includes acrofacial, Vulgaris, and Universalis distribution. Fitzpatrick scale for skin type: only four subtypes were found in the study population. Vitiligo Area Severity Index (VASI): the body is divided into five regions (hands, upper limb, trunk, lower limb, feet). Each measured in hand unit. Residual depigmentation value is estimated within each region. The VASI is then calculated as the sum of all body sites [Hand Units] × [Residual Depigmentation]. Vitiligo Disease Activity score (VIDA) is a six-point scale for assessing vitiligo stability over time. It depends on the patient's reports of disease activity. PVT: combined topical treatment of PAUVA, vitamin, and topical treatment.

the equation of relative quantity $=2^{-\Delta\Delta qT}$ method, where $\Delta\Delta C_q = (C_q\ CTLA4 - C_q\ GAPDH)_{\text{vitiligo}} - (C_q\ CTLA4 - C_q\ GAPDH)_{\text{mean control}}$.[15]

2.4.4 | *CTLA4* rs231775 (c.49A>G) variant analysis

Genomic DNA was purified from the whole blood using the QIAamp DNA Blood Mini kit (Qiagen) following the manufacturer's protocol. Extracted DNA purity and concentration were assessed, as mentioned above. Genotyping for the selected variant was assayed using real-time PCR allelic discrimination assay, as described in our previous work.[16,17] PCR reactions were run in duplicates in a 25-μl final volume containing 20 ng/μl genomic DNA, TaqMan Universal PCR Master Mix, and TaqMan SNP Genotyping Assay Mix according to the standard protocols in StepOne™ Real-Time PCR System (Applied Biosystems). Allelic discrimination was called by the SDS software version 1.3.1

TABLE 3 Allele and genotype frequencies of rs231775 (c.49A>G) polymorphism of the *CTLA4* gene in vitiligo patients and controls

Parameter	Allele/ Genotype	Total	Controls	Cases	p-value
Allele frequency	A	399 (61)	221 (67)	178 (55)	**0.002**
	G	253 (39)	109 (33)	144 (45)	
	Total	652	330	322	
Genotype frequency	A/A	139 (43)	80 (48)	59 (37)	**0.017**
	A/G	121 (37)	61 (37)	60 (37)	
	G/G	66 (20)	24 (15)	42 (26)	
	Total	326	165	161	

Values are shown as the number (%). Two-sided chi-square (χ^2) test was used. OR (95% CI), odds ratio, and confidence interval; calculated as a present versus absence. Bold values are statistically significant at $p < 0.05$.

2.5 | Statistical analysis

Continuous data were expressed as mean ± standard deviation (SD). Categorical variables were presented as frequency counts and compared using the chi-square test, while Student's *t* tests, Mann-Whitney *U*, and Kruskal-Wallis tests were used to compare continuous variables according to data distribution and variance homogeneity that will be checked by Shapiro-Wilk test and Levene test, respectively. Allele and genotype frequencies were estimated as detailed previously.[18] Hardy-Weinberg equilibrium (HWE) was calculated by SNPstats (https://www.snpstats.net/).[19] Genotype-specific adjusted odds ratios (ORs), and the 95% confidence intervals (CIs) were calculated by logistic regression analyses, and all the association analyses under the five genetic associations models were tested.[20] For selecting the best genetic model, the Akaike information criterion (AIC) mathematical method was estimated based on using the maximum likelihood estimate and the number of parameters. The smaller the AIC value, the better the model fit.[21]). The "G*Power version 3.0.10" was applied to calculate the study power. The estimated power was 96% at total sample size = 326, calculated effect size = 0.25, and alpha error probability = 0.05. A two-tailed *p*-value <0.05 was considered statistically significant. Statistical Package for Social Science (SPSS) software version 27.0 was used for the general statistical analyses. Spearman's correlation test was performed for genotype-phenotype correlation. Multivariate analysis was employed for identifying predictor risk factors for the severe disease phenotype.

3 | RESULTS

3.1 | Demographic and clinical characteristics of the study population

The study included 326 participants. Females accounted for 65.3% of the study population. Their mean age was 36.6 ± 16.6 years, and more than half (57.1%) had normal weight. Table 1 showed a comparison between 161 vitiligo patients and 165 controls. There was no significant difference in their age, sex, and body weight. A higher frequency of patients was living in rural areas (44.1% in the vitiligo group vs. 20.6% in controls; $p < 0.001$). None of the controls were smokers, while 13 patients (8.1%) were active smokers. A higher proportion of patients reported a positive family history of vitiligo (24.8% vs. 13.9%, $p = 0.017$). While stress and rheumatoid arthritis were more prevalent in patients, sunburn and thyroid diseases were less reported in that group (all $p < 0.001$). Hypertension was a frequent comorbid condition among vitiligo patients compared with only one individual in the control group ($p < 0.001$; Table 1).

3.2 | Disease characteristics of vitiligo patients

Localized vitiligo, categorized as focal and segmental patterns, accounted for 37 participants, while 124 patients presented with generalized vitiligo disease classified into acrofacial, Vulgaris, or Universalis distribution. As depicted in Table 2, hypopigmentation lesions (32.3% vs. 10.8%) and Koebner phenomena (36.3% vs. 10.8%) were more frequently seen in generalized vitiligo. This latter group had a prolonged duration of the disease (7.98 ± 6.1 years) than the localized vitiligo group (3.61 ± 4.6 years, $p < 0.001$), longer duration of treatment (2.12 ± 2.59 years vs. 5.37 ± 5.1 years, $p < 0.001$), and less compliant treatment course (46.8% vs. 75.7%, $p = 0.002$; Table 2).

3.3 | Allelic discrimination of *CTLA4*

A total of 326 participants were genotyped, including 161 vitiligo patients and 165 controls. Genotype distribution slightly deviates from Hardy-Weinberg equilibrium in controls ($p = 0.036$). In the study population, the minor allele frequency (MAF; G allele) was 0.33, consistent with those reported in 100Genome Project in a population with South Asian and European background (Figure S1). Compared with the control group, a higher G allele prevalence was observed in vitiligo cases (45% vs. 33%, $p = 0.002$). Similarly, G/G was the most prevalent genotype among patients (26% vs 15%, $p = 0.017$; Table 3).

Homozygote carriers of G variant were nearly two times more likely to develop vitiligo than A allele carriers under recessive model (OR = 2.94, 95% CI = 1.61–5.35, $p < 0.001$), dominant model (OR = 1.87, 95% CI = 1.14–3.06, $p = 0.013$), and homozygote comparison (OR = 3.34,

95% CI = 1.73–6.42, $p = 0.001$). After adjusting age, sex, family history of vitiligo, and expression level of *CTLA4* gene using multivariate analysis, G/G was associated with a higher risk of vitiligo (Table 4).

3.4 | *CTLA4* gene expression

As depicted in Figure 1A, median fold change of *CTLA4* gene in vitiligo was 1.40 (IQR: 0.37–2.89) compared to controls, ($p = 0.17$). Receiver Operative Characteristics analysis revealed a poor diagnostic performance with accuracy of 62.3%, sensitivity of 57.1%, and specificity of 67.3%. Area Under the curve was 0.63 ± 0.03 (Figure 1B). On comparing the expression levels in patients with different genotypes, G/G carriers (median = 0.63, IQR = 0.34–1.75) exhibited a significant lower expression profile than A/A (median = 1.43, IQR = 0.39–4.25, $p = 0.018$) and A/G carriers (median = 1.68, IQR = 0.49–3.92, $p = 0.007$; Figure 1C).

3.5 | Genotype-phenotype correlations

Table 5 demonstrated no significant associations of both *CTLA4* expression profile and rs231775 polymorphism with clinical characteristics of vitiligo patients. However, lower gene expression showed significant association with the site of vitiligo (the eye type; $p = 0.018$), and the specified variant AG genotype showed a higher frequency of patients presented with premature graying of the hair (66.7%) vs. the homozygous wild and mutant genotypes (44.1% and 47.6%, respectively; $p = 0.032$).

3.6 | Predictors for severity

Multivariable analysis, including *CTLA4* gene expression and polymorphism, is shown in Figure 2. Linear regression analysis revealed that a higher risk of severe phenotype were evident in elder patients (beta = 0.06, 95% = 0.03–0.09, $p < 0.001$) and those presented with Keobner phenomenon (ie, the appearance of new skin lesions on previously unaffected skin secondary to trauma; beta = 0.30, 95% CI = 1.09–3.26, $p < 0.001$).

4 | DISCUSSION

As one of the immune activation inhibitors, the T-cell brakes (CTLA-4) protein represents one of the vital players that prevent T-cell hyperactivation-associated autoimmune destruction.[22] In the present study, the authors explored one of the T-cell brakes variants, rs231775 (c.49A>G), and the gene expression with vitiligo susceptibility and phenotype. The study findings indicated that after covariate adjustment, the G allele homozygote carriers were nearly two times more likely to develop vitiligo than A allele carriers under the

TABLE 4 Risk of vitiligo according to genetic association models

Genetic association model	Comparison group	Model 1 OR (95% CI)	Model 1 *p*-value	Model 1 AIC	Model 2 OR (95% CI)	Model 2 *p*-value	Model 2 AIC	Model 3 OR (95% CI)	Model 3 *p*-value	Model 3 AIC
Heterozygote comparison	A/G vs. A/A	1.33 (0.82–2.18)	0.07	449.8	1.35 (0.81–2.23)	0.06	437.4	1.32 (0.76–2.30)	0.87	385.4
Homozygote comparison	G/G vs. A/A	**2.37 (1.30–4.34)**	**0.002**	451.2	**2.37 (1.28–4.41)**	**0.022**	438.6	**3.34 (1.73–6.42)**	**0.001**	391.0
Dominant model	A/G-G/G vs. A/A	**1.63 (1.05–2.53)**	**0.030**	449.1	**1.64 (1.04–2.59)**	**0.033**	436.8	**1.87 (1.14–3.06)**	**0.013**	384.3
Recessive model	G/G vs. A/A-A/G	**2.07 (1.19–3.62)**	**0.009**	455.9	**2.07 (1.17–3.67)**	**0.012**	443.1	**2.94 (1.61–5.35)**	**<0.001**	396.9
Over-dominant model	A/G vs. A/A-G/G	1.01 (0.65–1.59)	0.96	448.1	1.02 (0.64–1.63)	0.92	435.7	0.87 (0.53–1.44)	0.60	384.9

OR (95% CI): odds ratio, and the confidence interval. Bold values indicate statistically significant at *p*-value <0.05. Binary logistic regression analysis was performed. The Akaike information criterion (AIC) mathematical method was estimated based on using the maximum likelihood estimate and the number of independent variables for selection of the best genetic model. The smaller the AIC value, the better the model fit.

Model 1 represented univariate regression analysis, and crude odds ratio and confidence interval were shown.

Model 2 was adjusted by age, sex, and family history of vitiligo.

Model 3 was adjusted by age, sex, family history of vitiligo, and expression level of *CTLA* gene.

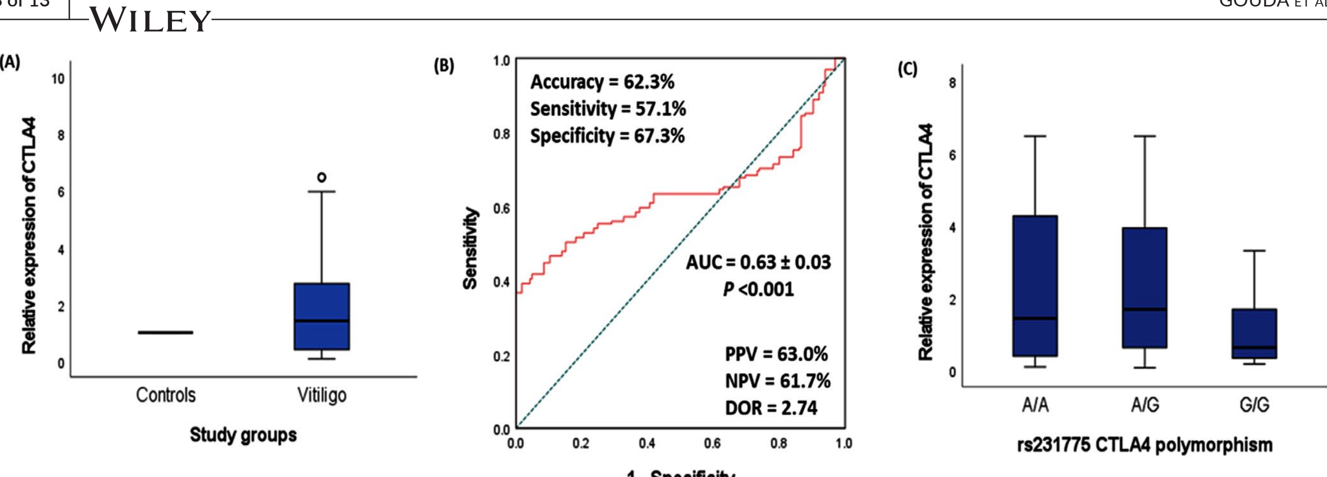

FIGURE 1 Relative expression level of *CTLA4* in the study population. (A) Fold change in patients and controls. Mann-Whitney *U* test was used. (B) Diagnostic performance using *CTLA4* relative expression levels. (C) Association between *CTLA4* genotype and expression level in vitiligo patients. Kruskal-Wallis followed by multiple comparison tests were used

Risk factors	Unstandardized Beta [95% CI]	p value
Age	0.07 [0.03, 0.98]	**<0.001**
Sex	-0.21 [-1.37, 0.96]	0.72
Obesity	-0.45 [-1.17, 0.27]	0.22
Skin type	-0.91 [-1.62, -0.20]	**0.012**
Trichrome	0.58 [-0.53, 1.70]	0.30
Depigmentated	-0.70 [-3.21, 1.82]	0.58
Follicular repigmentation	-0.17 [-1.41, 1.07]	0.78
Hypopigmentation	0.69 [-0.51, 1.89]	0.25
Koebner phenomena	2.18 [1.10, 3.26]	**<0.001**
CTLA4 variant	-0.25 [-0.88, 0.37]	0.42
CTLA4 expression	0.00 [-0.10, 0.11]	0.92

FIGURE 2 Multivariable analysis to predict independent factors for high Vitiligo Area Severity Index score. Bold values indicate statistically significant at *P*-value <0.05

recessive, dominant, and homozygote comparison models. Notably, when analyzing whether the rs231775 variant was in HWE, the authors found a very small deviation from HWE ($p < 0.036$). Although it is difficult to speculate the main source of violation of this equilibrium, some of the potential causes of population differences shown in the present study are "selection (as the study participants were hospital-based selected individuals), relatively small sample size, population stratification, and genetic drift".[23] Also, the allele frequencies described in the present study are comparable to others as presented in Figure S1. Moreover, the high quality of the genotyping assays (ie, the genotyping call rate = 100%, with unambiguous allelic discrimination plots) suggested a violation of HWE assumptions in the study groups rather than technical genotyping errors as proposed by Esser et al.[23] and experienced in our previous publication.[18]

The *CTLA4* rs231775 variant has been reported to be associated with various autoimmune diseases such as Graves' disease in adults[24] and autoimmune hypothyroidism in children of the Han Chinese population,[25] multiple sclerosis,[26] vitiligo,[7] vasculitis,[27] systemic lupus erythematosus,[28-30] rheumatoid arthritis,[31,32] latent autoimmune diabetes in adults,[33] primary biliary cirrhosis,[34] primary biliary cholangitis,[35] and Pemphigus Vulgaris.[36,37] Additionally, a more recent meta-analysis by Wang and colleagues indicated a significant association of *CTLA4* rs231775 with autoimmune diseases' susceptibility under different genetic models, highlighting its potential implication as a diagnostic genetic biomarker in both Asian and Caucasian populations.[38] However, our results are not consistent with Song et al. and Liang et al. meta-analyses[8,9] and partially agree with Birlea et al.[4] The latter study showed no association of the specified variant with vitiligo in the overall analysis; however still, a significant one was evident in vitiligo patients with other concomitant autoimmune diseases. It is worth noting that these previous meta-analyses were ethnic-specific types of analysis, including only data from Asian and European patients; thus, the results apply to only these ethnicities. In this sense, the present study in a sample of the Middle Eastern population is reasonable to be conducted to further add to the previous ones.

Although *CTLA4* expression levels were similar in vitiligo patients and the control groups, interestingly, the GG genotype carriers showed a significantly lower expression profile than A/A and A/G ones. This finding is congruent with the earlier functional

TABLE 5 Association of *CTLA4* rs231775 polymorphism and gene expression with clinical features in vitiligo patients

Variables	Categories	No.	Fold change	*P*-value	A/A	A/G	G/G	*P*-value
Demographics								
Age, years	≤20 years	54	1.41 (0.33–3.29)	0.63	16 (27.1)	26 (43.3)	12 (28.6)	0.43
	≤40 years	48	1.16 (0.40–2.98)		18 (30.5)	16 (26.7)	14 (33.3)	
	≤60 years	46	1.63 (0.45–3.36)		18 (30.5)	14 (23.3)	14 (33.3)	
	>60 years	13	1.19 (0.34–1.76)		7 (11.9)	4 (6.7)	2 (4.8)	
Sex	Female	102	1.68 (0.38–3.53)	0.12	37 (62.7)	40 (66.7)	25 (59.5)	0.75
	Male	59	0.96 (0.36–2.32)		22 (37.3)	20 (33.3)	17 (40.5)	
Residence	Rural	71	1.43 (0.34–3.07)	0.97	27 (45.8)	26 (43.3)	18 (42.9)	0.94
	Urban	90	1.28 (0.43–2.72)		32 (54.2)	34 (56.7)	24 (57.1)	
Obesity	Normal weight	84	1.09 (0.34–2.98)	0.33	31 (52.5)	33 (55)	20 (47.6)	0.83
	Overweight	48	1.43 (0.37–2.92)		18 (30.5)	15 (25)	15 (35.7)	
	Obese	29	1.88 (0.66–3.13)		10 (16.9)	12 (20)	7 (16.7)	
Smoking	Negative	148	1.41 (0.40–3.08)	0.33	54 (91.5)	56 (93.3)	38 (90.5)	0.86
	Positive	13	0.90 (0.30–1.94)		5 (8.5)	4 (6.7)	4 (9.5)	
History								
Family history of vitiligo	Negative	121	1.41 (0.37–2.89)	0.91				
	Positive	40	1.38 (0.38–2.98)					
Pregnancy	Negative	116	1.38 (0.35–3.08)	0.98	42 (71.2)	48 (80)	31 (73.8)	0.52
	Positive	45	1.42 (0.40–2.47)		17 (28.8)	12 (20)	11 (26.2)	
Stress	Negative	83	0.96 (0.34–2.69)	0.11	28 (47.5)	35 (58.3)	20 (47.6)	0.41
	Positive	78	1.63 (0.58–3.36)		31 (52.5)	25 (41.7)	22 (52.4)	
Sunburn	Negative	154	1.42 (0.40–3.07)	0.28	58 (98.3)	56 (93.3)	40 (95.2)	0.40
	Positive	7	1.26 (0.26–1.41)		1 (1.7)	4 (6.7)	2 (4.8)	
Thyroid disease	Negative	155	1.41 (0.40–2.72)	0.89	56 (94.9)	57 (95)	42 (100)	0.33
	Positive	6	1.44 (0.30–6.45)		3 (5.1)	3 (5)	0 (0)	
RA	Negative	140	1.31 (0.34–2.98)	0.37	53 (89.8)	51 (85)	36 (85.7)	0.70
	Positive	21	1.59 (0.82–2.88)		6 (10.2)	9 (15)	6 (14.3)	
Hypertension	Negative	141	1.41 (0.34–3.53)	0.35	52 (88.1)	52 (86.7)	37 (88.1)	0.96
	Positive	20	1.28 (0.43–1.80)		7 (11.9)	8 (13.3)	5 (11.9)	
T2DM	Negative	138	1.41 (0.39–3.36)	0.23	48 (81.4)	55 (91.7)	35 (83.3)	0.24
	Positive	23	1.36 (0.34–1.89)		11 (18.6)	5 (8.3)	7 (16.7)	

(Continues)

TABLE 5 (Continued)

Variables	Categories	No.	Fold change	*P*-value	A/A	A/G	G/G	*P*-value
Clinical data								
Disease duration	≤1 years	32	0.95 (0.33–4.17)	0.62	12 (20.3)	13 (21.7)	7 (16.7)	0.35
	≤10 years	97	1.43 (0.43–3.07)		32 (54.2)	40 (66.7)	25 (59.5)	
	>10 years	32	1.36 (0.56–2.65)		15 (25.4)	7 (11.7)	10 (23.8)	
Premature graying of hair	Negative	86	1.38 (0.34–2.72)	0.68	26 (44.1)	40 (66.7)	20 (47.6)	**0.032**
	Positive	75	1.41 (0.43–3.30)		33 (55.9)	20 (33.3)	22 (52.4)	
Fitz Patrick skin typing	Type II	54	1.31 (0.40–2.63)	0.91	17 (28.8)	20 (33.3)	17 (40.5)	0.41
	Type III	58	1.51 (0.43–2.80)		20 (33.9)	24 (40)	14 (33.3)	
	Type IV	48	1.16 (0.34–3.42)		22 (37.3)	16 (26.7)	10 (23.8)	
Lesion characteristics	Trichrome	43	1.19 (0.34–2.48)	0.25	16 (27.1)	16 (26.7)	11 (26.2)	0.99
	Depigmentated	154	1.41 (0.40–3.07)	0.42	58 (98.3)	56 (93.3)	40 (95.2)	0.41
	Foll repig	37	1.41 (0.34–4.20)	0.74	16 (27.1)	11 (18.3)	10 (23.8)	0.51
	Hypopigmentation	44	1.16 (0.34–2.45)	0.68	15 (25.4)	15 (25)	14 (33.3)	0.59
	Koebner phenomena	49	0.96 (0.40–3.72)	0.95	22 (37.3)	15 (25)	12 (28.6)	0.33
Location of the initial site	Face	56	1.01 (0.33–2.63)	0.24	24 (40.7)	15 (25)	17 (40.5)	0.29
	Trunk/back	31	1.41 (0.43–2.48)		11 (18.6)	11 (18.3)	9 (21.4)	
	Upper limb	46	1.30 (0.52–2.70)		12 (20.3)	23 (38.3)	11 (26.2)	
	Lower limb	28	1.88 (0.93–5.34)		12 (20.3)	11 (18.3)	5 (11.9)	
Location of affected lesions	Mouth	22	1.64 (0.63–3.02)	0.44	9 (15.3)	7 (11.7)	6 (14.3)	0.84
	Eyes	28	0.40 (0.26–1.97)	**0.018**	11 (18.6)	7 (11.7)	10 (23.8)	0.26
	Face	59	1.36 (0.43–3.53)	0.61	26 (44.1)	17 (28.3)	16 (38.1)	0.20
	Trunk	79	1.43 (0.43–2.69)	0.49	30 (50.8)	25 (41.7)	24 (57.1)	0.28
	Arms	76	1.41 (0.40–2.64)	0.62	29 (49.2)	28 (46.7)	19 (45.2)	0.92
	Hands	49	1.59 (0.50–2.60)	0.24	19 (32.2)	17 (28.3)	13 (31)	0.89
	Legs	77	1.43 (0.40–3.07)	0.53	27 (45.8)	32 (53.3)	18 (42.9)	0.53
	Feet	48	1.68 (0.91–3.42)	0.06	25 (42.4)	12 (20)	11 (26.2)	0.24
Localized distribution	Focal	23	0.43 (0.24–1.59)	0.18	6 (10.2)	11 (18.3)	6 (14.3)	0.21
	Segmental	14	0.34 (0.24–1.99)		4 (6.8)	4 (6.7)	6 (14.3)	
Generalized distribution	Acrofacial	6	0.98 (0.83–1.86)	0.52	4 (6.8)	0 (0)	2 (4.8)	0.07
	Vulgaris	113	1.68 (0.59–3.91)		42 (71.2)	45 (75)	26 (61.9)	
	Universalis	5	1.43 (0.84–1.83)		3 (5.1)	0 (0)	2 (4.8)	

(Continues)

TABLE 5 (Continued)

Variables	Categories	No.	Fold change	*P*-value	A/A	A/G	G/G	*P*-value
Severity assessment								
VIDA score	−1	36	1.94 (0.48–3.76)	0.48	17 (28.8)	11 (18.3)	8 (19)	0.55
	0	10	1.51 (0.40–1.90)		4 (6.8)	2 (3.3)	4 (9.5)	
	+1	15	0.89 (0.26–2.72)		5 (8.5)	5 (8.3)	5 (11.9)	
	+2	25	1.77 (0.42–5.04)		12 (20.3)	9 (15)	4 (9.5)	
	+3	21	0.90 (0.34–1.80)		6 (10.2)	8 (13.3)	7 (16.7)	
	+4	54	1.30 (0.35–2.78)		15 (25.4)	25 (41.7)	14 (33.3)	
Treatment history								
Treatment modalities	No TTT	8	1.38 (0.28–1.62)	0.31	4 (6.8)	1 (1.7)	3 (7.1)	0.33
	Combined PVT	153	1.41 (0.40–3.07)		55 (93.2)	59 (98.3)	39 (92.9)	
Course treatment	Compliant	86	1.31 (0.34–2.81)	0.28	34 (57.6)	32 (53.3)	20 (47.6)	0.61
	Interrupted	75	1.68 (0.45–3.07)		25 (42.4)	28 (46.7)	22 (52.4)	

Data are shown as number (percentage) or mean ± standard deviation (SD). Chi-square and Fisher's exact tests were used for qualitative variables, and Student's *t* test and Mann-Whitney *U* test were applied for quantitative variables. Bold values indicate statistically significant at *p*-value <0.05.

Abbreviations: AA% affected, percentage of the scalp affected by alopecia areata; BMI, body mass index; DLQI, Dermatology Life Quality Index questionnaire; FH of AA/AD, family history of alopecia areata or autoimmune disorder; Foll repig, Follicular repigmentation; SALT, Severity of Alopecia Tool score for severity assessment; Stress, emotional stress as a predisposing factor.

work of Ligers et al.,[26] who reported similarity of mRNA and protein expression levels in multiple sclerosis/myasthenia gravis patients and controls; however, there was a clear association between *CTLA4* expression and genotype. Specifically, GG homozygous carriers showed significant downregulation of "*CTLA4* mRNA" in non-stimulated peripheral blood cells.[26] Although Dwivedi and colleagues reported no significant associations were observed between rs231755 variant different genotypes and the *CTLA-4* expression for its two transcripts; the full-length and the soluble forms, in vitiligo patients, however, a decrease in the soluble form of *CTLA-4* transcript was found in patients carriers of (+49A: CT60G) haplotype which includes the studied variant.[39] This could support the positive correlation of the studied polymorphism and expression in vitiligo pathogenesis. Others showed *CTLA4* (soluble form transcript) downregulation in patients with type 1 diabetes, suggesting that "lower expression of this gene may directly affect the suppressive capacity of Treg cells and thereby modulate disease risk".[40]

The *CTLA4* rs231775 (c.49A>G) variant changes "threonine to alanine at position 17" in CTLA-4 signal peptide, resulting in the decline of T-cell *CTLA4* expression, thus interfering with its normal function as T-cell downregulator.[41] Although CTLA-4 protein is constitutively expressed on T cells, it only overexpressed on active T and Treg cells to inhibit its activation and maintain immune tolerance.[42] Thus, it is plausible that deregulated CTLA4 expression and/or function are associated with autoimmune diseases, including vitiligo.

To further explore whether the specified polymorphism could have an additional effect rather than merely change the gene expression, online bioinformatics tools, HaploReg [43] and SNPinfo,[44] were applied in an attempt to predict other potential functions of this missense variant. Interestingly, it has been predicted that *CTLA4* rs231775 can lead to AP (adaptor protein)-4 and Rad-21 (double-strand-break repair protein) motifs changes. This variant also shows linkage disequilibrium ($r^2 \geq 0.8$) with other *CTLA4* polymorphisms (shown in Table S1). These changes and associations could, in turn, mediate some effects on the molecular pathogenesis of vitiligo disease.

On correlating the gene expression and the studied variant with the clinical phenotype, there were no significant associations of both *CTLA4* expression profile or rs231775 variant with the clinical characteristics of vitiligo patients, except, lower gene expression showed a modest significant association with the site of vitiligo and the AG genotype showed a higher frequency of patients presented with premature graying of the hair. This latter observation supports the general role of CTLA-4 plays in autoimmunity, which is implicated as one of the etiopathogeneses of the premature graying of the hair.[45] The apparent non-significant associations of *CTLA4* gene expression and variants with the disease phenotype, activity, and severity could indicate that the major role this gene plays is in disease etiology and pathogenesis rather than progression.[46]

Collectively, the above findings could support the role of *CTLA4* rs231775 played in vitiligo pathogenesis to be related, in part, to

the low gene expression and/or function with a subsequent uncontrolled T cells proliferation and autoimmunity augmentation that may increase the risk of disease when appropriate environmental triggers are present. Some limitations of our study merit consideration. Firstly, the relatively small sample size, small deviation from HWE, and the study design as a hospital-based case-control study can limit the causal relationship. Secondly, other potentially functional variants of the *CTLA4* which can impact the gene expression and disease susceptibility were not explored in the study cohort. Finally, functional studies are required to confirm the potential impact of the study rs231775 variant on gene expression in vitiligo cases. In this sense, future studies in large multi-ethnic cohorts investigating the functional relevance of the *CTLA4* studied variant would be warranted for fully establishing the impact on susceptibility to vitiligo.

5 | CONCLUSION

This study indicated that the *CTLA4* (rs231775*GG) genotype conferred increased susceptibility to vitiligo among the study population with a potential decrease in gene expression as one mechanism implicated in disease risk. However, no significant associations of *CTLA4* variant and expression with VASI and/or VIDA scores were found. The authors have to confirm that although the risk conferred by individual loci is small, in combination with other genetic and non-genetic data, this could be helpful in refining the genetic profile of vitiligo patients and could be one of the promising molecular targets for individualized therapy in the near future.

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CONFLICT OF INTEREST

The authors have declared no conflicting interests.

AUTHORS CONTRIBUTIONS

All authors shared equally in this study and approved the final version of the article to be submitted.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/supplementary material.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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