

Association of CTLA-4 Polymorphisms with Type 1 Diabetes in the Egyptian Population

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Abstract

Background: Polymorphisms in the cytotoxic T-lymphocyte antigen 4 (CTLA-4) are associated with the risk of type 1 diabetes (T1D). Here, we investigated the most associated variants CT60 and +49 A/G and five other putative promoter SNPs for their association with T1D in the Egyptian population, a multi-ethnic group. The comparison of disease association between populations can provide further evidence for putative disease variants.

Methods: Association of seven SNPs (-1722,-1661,-651,-319, +49, -819 and +6230G>A) in the CTLA-4 gene with T1D was investigated in 396 patient and 396 control subjects of Egyptian origin. The diagnosis of T1D was made based on ketoacidosis or ketosis with acute onset and severe symptoms of diabetes mellitus at presentation and continuous dependence on insulin. Controls were negative for anti-GAD antibodies and were older than 24 years of age. Genotyping was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

Results: Five of the seven CTLA-4 gene SNPs were associated with T1D with the highest association for +49 A/G in exon 1 (P=0.0002; odds ratio: 1.6, 95% CI 1.3-1.9). Association conditional on SNP +49 A/G was further tested, revealing some independent association for SNPs -1661 and -318. Haplotype analysis of these SNPs demonstrated that no single haplotype was indicative of T1D risk.

Conclusion: The results further support the association of T1D with +49 A/G SNP in the CTLA-4 gene in the Egyptian population. The pattern of association specifically differed from that observed in European and other North African populations, providing further opportunity for fine mapping of genetic disease variants of type-1 diabetes.

Keywords: CTLA-4; T1D; RFLP; Children; Egyptian; Race

Introduction

Type 1 diabetes (T1D) is a genetically complex disorder of glucose homeostasis caused by autoimmune mediated self-destruction of insulin-secreting cells of the pancreas. The disease is thought to be the result of exposure to environmental factors combined with genetic susceptibility that contribute to the development of T1D in a complex manner [1]. To date, genetic predisposition to T1D has been associated to more than 20 confirmed genetic loci [2].

The strongest risk for T1D is conferred by the major histocompatibility complex, class II, DQ beta 1 gene (HLA-DQB1) also named as Insulin Dependent Diabetes Mellitus gene (IDDM1). It has been estimated that about 40% of heritable risk can be explained by IDDM susceptibility genes [3]. The Cytotoxic-T-lymphocyte antigen (CTLA-4, CD-152) encoding gene (IDDM12) is one of the most important non-HLA susceptibility genes of T1D and other autoimmune diseases. IDDM12 maps to chromosome 2q33 [4]. The role of CTLA-4 in the autoimmune process has been emphasized by its association with systemic lupus erythematosus, celiac disease, rheumatoid arthritis, and autoimmune thyroid diseases [5]. CTLA-4 is a receptor, that down-regulates the immune response. The IDDM12 gene contains a cluster of T lymphocyte-regulating genes including CTLA-4, CD28, and inducible T-cell co-stimulator (ICOS). CD28 and CTLA-4 are co-stimulatory receptors that together with the antigen specific T-cell receptor (TCR) bind the B7 family molecules on the surface of antigen presenting cells (APC). CD28 enhances whereas CTLA-4 inhibits T-cell proliferation. Binding of CTLA-4 to the B7 limits the proliferation of T-cells and terminates the ongoing immune response [6]. CTLA-4 knockout mice

develop severe lymphoproliferative disorder; die within a few days after birth, highlighting the importance of this gene in the negative regulation of the immune response [7]. Recently, the susceptibility for T1D was remapped to the 3' untranslated region (UTR) of the CTLA-4 gene [8]. The previously implicated +49 and CT60 variants were confirmed to have the strongest association with T1D but the mapping of the causal variant remained ambiguous and did not exclude the possibility of association with multiple functional variants. Apart from re-sequencing, association of CTLA-4 variants in different populations is a strategy for further fine mapping. In general, the compelling data on the association of CTLA-4 polymorphisms with T1D is convincing in some populations including Belgian [4,9], French, Italian, Korean, Mexican, Spanish-American [10], Japanese [11-15], Chinese [16], Russian [17], British [18], Moroccan [19], Estonian [20], and Croatian [21] populations.

On the other hand, further studies found no association in Chinese

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[10], Japanese [22-24], Czech [25], Iranian [26], Argentinean [27], Chilean [28], Portuguese [29] and Turkish [30] children.

These distinct association patterns in different populations may provide important clues to clarify the causal variants by exploiting recombination events in the past that may generate distinct CTLA-4 haplotypes, whose frequencies may vary between races [31]. In this study, we investigated whether the seven most implicated Single Nucleotide Polymorphism (SNPs) of the CTLA-4 gene are associated with T1D in the Egyptian population.

Materials and Methods

Patients and controls

All individuals who participated in this study gave informed written consent. The study was approved by the Egyptian Bioethics Review Committee for Bioethics (BERD) VACSERA. Three hundred and ninety six unrelated patients with T1D from different governorates of Egypt were recruited consecutively. The diagnosis of T1D was based on ketoacidosis or ketosis with severe symptoms of acute onset at presentation and continuous dependence on insulin. Moreover, patients included in the study had a Glycated Hemoglobin (HbA1c) $\geq 8.5\%$ [32,33].

All patients were non-obese and ≤ 14 yr of age at enrolment. Patients were separated into two groups for statistical analysis: younger than 6 yr of age (0-5 age group) and 6 to 14 yr of age (6-14 age groups).

Healthy individuals older than 24 years of age - as recommended by the DiaMond protocol [34], with normal blood glucose ($n=396$) were selected randomly from the Egyptian population to serve as controls. This age restriction is placed on the controls to exclude subjects from the high-risk period of 0-14 yr of age, during which T1D is most likely to develop [18]. None of the controls had a family history of T1D. A standardized medical history questionnaire was completed by all patients and controls (Table 1).

The diagnosis of T1D was confirmed by elevated anti-glutamic acid decarboxylase (GAD) antibodies and low C-peptide levels. In controls, anti-GAD antibodies were absent and C-peptide levels were within the reference range (0.4-2.2 ng/ml). Individuals who were suspected to have Maturity-Onset Diabetes of the Young (MODY) or Wolfram syndrome were excluded from the study.

SNP genotyping

Blood (1 ml) was collected in EDTA-tubes, and the DNA was extracted by a salting out method [35]. The DNA was dissolved in 50 μ l Tris-EDTA (20 mM Tris, 2 mM EDTA, pH 7.5) buffer and stored at -20°C until use.

Genomic DNA was amplified using PCR with different primers (forward and reverse) as shown in Table 2. Reaction volume was 50 μ l:

Patients	All patients 1-14 years old	Controls >24 years old
Number of Patients	396	396
Age (yrs) \pm Mean Range	7.28 \pm 1.9 1-14	31.5 \pm 4.7 25-39
Weight (kg)	39.0 \pm 9.67	75.4 \pm 11.22
Sex, N (%)	Female 198 (50) Male 198 (50)	Female 198 (50) Male 198 (50)
Glycated Hemoglobin (HbA1c) (Mean \pm SD)	9.7 \pm 1.45	5.6 \pm 0.25

Table 1: Demographics of the Study for Egyptian Population.

4 μ l DNA at 100 ng/ μ l, 25 μ l DreamTaq Green master mix (Fermentas, Lu), 3 μ l of each primer (30 pmol/ μ l), and 15 μ l H₂O. Reaction conditions were carried out using a model PTC100 thermal cycler (M&J research, Watertown, MA, USA)

Following a PCR reaction carried out at 94°C for 5 min followed by 35 cycles of 94°C for 30 s. Finally two heating cycles were applied at ca. 50°C for 30 or 45 seconds and then 72°C for 30 sec (Table 3). Next, 10 μ l PCR product from each sample was resolved in 2% agarose gel to check the PCR products at the given bp fragment (Table 3). Restriction fragment length polymorphism (RFLP) analysis was done using the restriction enzymes given (Table 3) in 20 μ l total volume by mixing: 10 μ l of PCR product + 1.0 μ l restriction enzyme + 2.0 μ l 10 X restriction enzyme's buffer + 7 μ l nuclease-free water. The mixture was incubated at the given temperatures (Table 3) for 1 hour. DNA fragments were resolved in 3.0% agarose gel visualized by Ethidium Bromide (EtBr) staining. The digested alleles yielded the fragments listed in Table 3.

Statistical analysis

Genotype and allele distribution differences between patients and controls were tested by the chi-squared test with Yates' continuity correction. Statistical significance was defined as $p < 0.05$, when the calculated $\chi^2 > 3.841$ for 1 degree of freedom, and $\chi^2 > 5.991$ for 2 degrees of freedom. Hardy-Weinberg equilibrium (HWE) was tested by comparing the expected and observed genotypes in $2 \times 3 \chi^2$ tables. None of the SNPs in patient or control groups deviated from the HWE. Power calculation was used the Genetic Power Calculator (<http://pngu.mgh.harvard.edu/~purcell/gpc/>) [36]. Our sample had sufficient power (80%) to detect significant association of the studied SNPs for Odds ratios (OR) ranging between 1.6 and 1.05 for minor allele frequencies of 0.07 till 0.48, respectively, assuming a co-dominant model of inheritance as has been suggested for CTLA-4. OR calculation, haplotypes and conditional analysis were calculated using the statistical program Unphased (<https://sites.google.com/site/fdudbridge/software/>) [37], which uses a logistic regression model to estimate effect sizes that are expressed as ORs [37]. The method is equivalent to calculating ORs and corresponding confidence intervals from 2×2 tables using the Woolf-Haldane's method [38]. Adjustments to the level of significance were made for multiple analyses by Bonferroni correction of 7 independent tests [39].

Results

In the present study, we analyzed seven SNPs spanning the entire DNA sequence of the CTLA-4 gene for association with T1D using a case/control study of an Egyptian population. All controls were negative for anti-GAD antibodies and were 25 yr of age or older. Furthermore, no control had a family history of T1D (Table 1).

Genotype and allele distributions of the seven SNPs were tested for association with disease status (Tables 4 and 5). We observed significant association after correction for multiple testing for SNPs -1722, -1661, +49 A/G, -819, and CT60. The +49 A/G SNP had the strongest influence on disease state. Therefore, we proceeded to test all SNPs for independent association conditional on +49 A/G, and vice versa, +49 A/G conditional on all other SNPs (Table 6). This analysis demonstrated a +49 A/G independent association of SNPs -1661 and -318 with T1D. Furthermore, evidence of +49 A/G independent associations were found for all other investigated SNPs except SNP -318.

SNP haplotype analysis

The observation that the association of +49 A/G had a stronger

No	SNP Name	Forward primer	Reverse Primer	Alleles registered in NCBI
1	-1722C>T	5'CAAGCTTTGTCTGTGACCA3'	5'AAGCGCCAACAAGCATAAC3'	FJ013949 FJ013950
2	-1661A>G	5'GCCACTGCTGTGTTCCT3'	5'ATGTGCCTGTTGGTGTGAT3'	FJ013951 FJ013952
3	-651C>T	5'TCCTTCTGCAAACCCAGAGGCAGCT3'	5'AGTACAAGGGTCTCCTAAATCCCA3'	GQ370246 GQ370247
4	-318C/T	5'-AAGGATGCCAGAAAGATTGA3'	5'AGCCGTGGGTTTACTGTGTTA3'	Q390244 Q390245
5	+49A>G	5'-AAGGCTCAGCTGAACCTGGT-3'	5'CTGCTGAAACAAATGAAACCC3'	DQ534199 DQ534200
6	-819C>T	5'GGAGAGGGGCTGGTTAGTTACA3'	5'AGAGAGGCAGCGGTG GTGTCA3'	EU103999 EU104000
7	CT60 (+6230G>A)	5'-CACCACTATTTGGGATATACC-3'	5'AGGTCTATATTTTCAGGAAGGC3'	GQ370006 GQ370007

Table 2: The primers used in PCR-RFLP technique for genotyping the SNPs of CTLA-4 in this study.

	-1722C>T	-1661A>G	-651C>T	-318C>T	+49 A>G	-819C>T	+6230G>A (CT60)
Temp 1 (°C)	50.5	53	57.5	58	55	62	59
Duration 1 (s)	45	30	30	30	30	40	30
Temp 2 (°C)	72	72	72	72	72	72	72
Duration 2 (s)	40	30	30	30	30	30	30
bp fragment	398	214	330	212	152	180	216
Restriction enzyme	APek1	DraI	Aci1	Tru1	EcoR911	CviKI-I	NcoI
Source	NEB	FL	NEB	FL	FL	NEB	FL
Incubation temp (°C)	37	65	37	65	37	37	65
Digested allele	CC	AA	CC	TT	AA	CC	AA
Yielded fragments (bp)	266, 132	162	300	93, 119	131	69, 111	162
Digested allele	TC	AG	CT	CT	AG	CT	AG
Yielded fragments (bp)	132, 266, 398	52, 162, 214	300, 330	93, 119, 212	131, 152	69, 111, 180	54, 162, 216
Digested allele	TT	GG	TT	CC	GG	TT	GG
Yielded fragments (bp)	398	214	330	212	152	180	216

Abbreviations: NEB: New England Biolabs, UK; FL: Thermo Fisher Scientific, Inc (Fermentas), USA

Table 3: Details of SNP genotyping.

SNP (major/minor allele)	Cases (%)		Controls (%)		Pc-value	OR	95%CI
-1722(T>C) (rs733618)	669 (84)	123 (16)	710 (90)	82 (10)	0.0294*	1.6	1.2-2.1
-1661 (A>G) (rs4553808)	503 (64)	289 (36)	574 (72)	218 (28)	0.0182*	1.5	1.2-1.9
-651 (C>T) (rs11571318)	714 (90)	78 (10)	736 (93)	56 (7)	0.658	1.4	1.0-2.1
-318 (C>T) (rs5742909)	717 (91)	75 (9)	743 (94)	49 (6)	0.21	1.6	1.1-2.3
+49 (A>G) (rs231775)	498 (63)	294 (37)	576 (73)	216 (27)	3.724E-04*	1.6	1.3-1.9
-819 (C>T) (rs231726)	540 (68)	252 (32)	592 (75)	200 (25)	0.0532	1.4	1.1-1.7
CT60 (+6230G>A) (rs3087243)	347 (44)	445 (56)	409 (52)	383 (48)	0.0252*	1.4	1.1-1.7

The table shows counts and percentages between brackets of the alleles of seven CTLA-4 SNPs studied. P-value (Pc) is corrected for multiple testing of seven SNPs. Odds Ratio (OR) and corresponding 95% confidence intervals are given for the minor allele

Table 4: Allelic association.

influence on T1D outcome than CT60 may indicate different CTLA-4 haplotypes in the Egyptian population. Therefore, the pattern of LD and CTLA-4 haplotypes in the Egyptian sample was calculated. In order to test LD between SNPs typed, the D' values among seven SNPs were calculated using the estimated haplotype frequencies. All SNP combinations had a high absolute value of D' except the combination of -651 with -319 SNPs. This observation may explain the lower association of these two SNPs with T1D. To further investigate this, all possible CTLA-4 haplotypes of all seven SNPs were calculated

(Table 7). This analysis showed thirty three different haplotypes in 396 Egyptian patients. The TACCACG haplotype (where the nucleotide is indicated for each of the seven SNPs, as shown in Table 7) was the most frequent in the Egyptian population (22.9% vs. 30.8% in cases and controls respectively). This haplotype was treated as the 'wild type' or reference haplotype. Odds Ratio's of all other haplotypes were consequently calculated against this reference. As expected from the single SNP analysis, most haplotypes occurring more frequently in cases as compared to controls carried the susceptible G-allele at +49

A/G. Interestingly, the most frequent haplotype carrying a G-allele at +49 A/G (haplotype TACCGCA) did not confer risk. Significantly increased risk relative to the wild type haplotype was found for six haplotypes of which two were carrying the A-allele at +49 A/G.

These analyses demonstrated that the combination of these SNPs tag the undetected causal variant in a more efficient way than the single SNPs, confirming previous fine mapping studies.

Discussion

Genetic mapping of causal variants of susceptibility genes for complex diseases such as T1D remains complicated. Several aspects may be responsible for this complexity. First, each gene variant accounts for only a small proportion of susceptibility, necessitating large samples to acquire sufficient power for dissection of the association signal. Furthermore, the association signal may be caused by multiple causal variants causing synthetic association [37]. Furthermore, conflicting

SNP (genotype)	Cases (%)			Controls (%)			P-value
-1722 (TT/TC/CC)	282 (71)	105 (27)	9 (0.03)	318 (0.80)	74 (19)	4 (1)	0.084
-1661 (AA/AG/GG)	159 (40)	185 (47)	52 (13)	208 (53)	158 (40)	30 (7)	0.0098*
-651 (CC/CT/TT)	318 (80)	78 (20)	0 (0)	343 (86)	50 (13)	3 (1)	0.112
-318 (CC/CT/TT)	325 (82)	67 (17)	4 (1)	347 (88)	49 (12)	0 (0)	0.84
49 (AA/AG/GG)	157 (40)	184 (46)	55 (0.14)	211 (53)	154 (39)	31 (8)	0.0028*
-819 (CC/CT/TT)	181 (46)	178 (45)	37 (9)	214 (54)	164 (41)	18 (5)	0.098
+6230 (AA/AG/GG)	76 (19)	195 (49)	125 (32)	107 (27)	195 (49)	94 (24)	0.112

The table shows counts and percentages between brackets of the genotypes of seven CTLA-4 SNPs studied. P-values are corrected for multiple testing of seven SNPs

Table 5: Genotype association.

Table 3. SNPv	p-value of SNP conditional on 49A/G	p-value of 49 A/G conditional on SNP
-1722	0.8	0.0002
-1661	0.005	0.001
-651	0.3	0.0002
-318	0.0008	0.11
-819	0.01	9.50E-05
+6230	0.09	0.002

The table shows Bonferroni corrected p-values for conditional testing of each SNP conditional on +49 A/G SNP (first column), and of +49 A/G SNP conditional on each SNP (second column)

Table 6: Results of test of conditional association.

Haplotype	Case	Control	Ca-Freq	Co-Freq	Ca-D'	Ca-r ²	Co-D'	Co-r ²	Odds-R	95%Lo	95%Hi	Pc
TACCACG	181	244	22.9%	30.8%	0.8825	0.03714	0.9021	0.04237	1	1	1	
TGCCACA	64	78	8%	9.8%	-0.009883	7.75e-5	-0.03606	0.001167	1.11	0.76	1.62	
TACCGCA	59	108	7.4%	13.6%	1	0.01344	1	0.01829	0.74	0.51	1.07	
TACCATA	52	80	6.5%	10.1%	1	0.01227	1	0.01291	0.88	0.59	1.31	
CGCCGCA	41	34	5.2%	4.3%	1	0.4007	1	0.384	1.63	1.00	2.67	
TGCCATG	41	33	5.2%	4.2%	-0.02038	0.000205	0.5242	0.001439	1.68	1.03	2.76	
TACCGCG	31	6	3.9%	0.76%	1	0.01066	1	0.000914	6.28	2.62	15.06	0.00003
TACCACA	22	28	2.8%	3.5%	-0.002983	2.422e-6	0.6362	0.001765	1.09	0.61	1.95	
TACCATG	21	5	2.7%	0.6%	1	0.004763	1	0.0007987	4.65	1.84	11.76	0.004
TGCCGCG	19	17	2.4%	2.1%	-0.1551	0.005829	0.179	8.718e-5	1.50	0.76	2.95	
TACTGTG	19	1	2.4%	0.13%	1	0.004202	1	9.68e-5	15.76	2.44	101.70	0.00007
TATCACA	16	29	2%	3.7%	1	0.003412	1	0.00432	0.76	0.40	1.43	
TACTGCA	14	0	1.8%	0%	1	0.003412	1	0.00432	16.04	1.61	159.83	0.0007
CGCCGTG	13	20	1.6%	2.5%	1	0.005063	1	0.004677	0.87	0.43	1.79	
TACCGTA	12	2	1.5%	0.25%	1	0.1223	0.7455	0.1629	6.99	1.56	31.37	0.02
TATCATG	11	15	1.4%	1.9%	1	0.002475	1	0.002209	1.03	0.47	2.26	
TATCATA	10	2	1.3%	0.25%	1	0.002279	1	0.0002206	5.82	1.29	26.19	
CGCCACA	10	12	1.3%	1.5%	0.009883	7.75e-5	0.03606	0.001167	1.10	0.47	2.58	
TACTACA	10	31	1.3%	3.9%	1	0.0025315	1	0.002338	0.46	0.22	0.96	
TGCCGTA	9	5	1.1%	0.6%	1	0.001878	1	0.000662	2.32	0.77	7.04	
CGTCGTG	7	7	0.9%	0.9%	0.6348	0.0373	0.7977	0.05621	1.36	0.47	3.92	
TACCGTG	7	5	0.9%	0.5%	1	0.00137	1	0.0007303	1.73	0.56	5.34	
TACTATG	0	13	0%	1.6%	1	5.987e-13	1	0.001956	0.10	0.01	1.61	0.05
Other	123	17	15.5%	2.1%	-----	-----	-----	-----	-----	-----	-----	-----

The table shows counts and percentages of estimated haplotypes. P-values (Pc) are corrected for 23 haplotype comparisons. Odds Ratio (OR) and corresponding 95% confidence intervals are given, whereas Global D' for cases and controls are 0.7182 and 0.7922, respectively

Table 7: Haplotypes of seven CTLA-4SNPs in the Egyptian T1D and control samples.

results from different populations may be caused by certain mutations that may not exist in all racial/ethnic groups or geographical populations. Therefore, screening of hitherto uninvestigated populations may help elucidating genetic complexities [31].

We have previously investigated the prevalence of C-819T and A+49G SNPs of the CTLA-4 gene in male and female Egyptian children [40]. In the present paper we analyzed SNP associations and performed haplotyped analysis of 7 SNPs of the CTLA-4 gene in T1D. This study confirmed association of five SNPs of the CTLA-4 gene with T1D in the Egyptian population and demonstrated that distinct susceptibility haplotypes exist in this patient cohort of Egyptian origin. The current analysis confirmed the association of CTLA-4 polymorphism with T1D in the Egyptian population. In contrast to previous fine mapping in European samples where the CT60 SNP had the strongest influence on disease state [8], in the present study, the strongest association mapped to the +49 A/G SNP located in the first exon and not to the more 3-prime region of the CTLA-4 gene. The +49 A/G SNP has been extensively studied in several ethnic populations of mostly European ancestry with conflicting results [11,41,42]. However, our haplotype analysis demonstrated that haplotypes not carrying the susceptible G-allele at +49 A/G were also detected in two susceptible haplotypes. This observation suggested that the +49 A/G were not the only variant influencing T1D. Conditional analysis further demonstrated that it is unlikely that either +49 or any of the other associated SNPs alone can be responsible for T1D risk. Rather a network of SNPs seems to have a prognostic relevance.

The pattern of LD and CTLA-4 haplotypes in the Egyptian sample was compared to previously published data of another North African population in Morocco. In comparison with the haplotypes found in Morocco, several differences illustrated the genetic diversity in this region. Bouqbis et al. published frequencies for four SNP haplotypes (SNPs -1722,-1661,-319 and +49) and reported that the TACA haplotype was the most frequent at 54.4% in the South Moroccan population [19]; similarly to the Egyptian population (54%) in our study. Moreover, Bouqbis et al. reported that haplotype TGCA had the strongest association with T1D [19]. In our sample, this was clearly different regarding the -1661 and +49 SNPs: the TACG haplotype had the strongest association with T1D in the investigated Egyptian population. However, given the limited sample size of our study and that of Bouqbis et al. these differences could represent stochastic variation due to sampling error [19]. Our results do not confirm the absence of association of the +49 A/G variant in Egyptian population as in the West African and the Moroccan populations [18]. Therefore, the finding reported by Bouqbis et al. may be unique for the population studied, and again points to the fact that it is unlikely that the +49 A/G SNP alone is a causal variant [19]. The samples studied by Bouqbis et al. were derived from Agadir town in south Morocco, which is geographically near to West Africa, but far from Egypt [19].

Furthermore, the +49 A/G allele has been found to be associated with T1D in Mediterranean populations, which are related to the North African populations generally and to the Egyptian specifically. Therefore, further studies comparing North and West African populations may clarify differences in the pattern of association of the CTLA-4 gene region and further fine map the disease variant in CTLA-4.

In summary, a strong association of the +49 A/G SNP in the CTLA-4 gene with T1D was found in a sample of Egyptian origin. The pattern of association was distinct from that observed previously in other populations, providing further evidence to the clarification of demographic and disease associations of different CTLA-4 SNPs.

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