Kuwait J.Sci., Vol.50, No.(1A),January.2023,pp(1-18)

Sequence analysis of tumor necrosis factor-α (TNF-α) promoter, 5'UTR and exon1 and association of rs361525 (-238 G>A) with BMI

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Abstract

Obesity is among the most common complex diseases with a high rate of morbidity and mortality globally and locally in Kuwait. Tumor Necrosis Factor-α(TNF-α) is a pro-inflammatory cytokine that is primarily secreted by monocytes/macrophage. Increased expression of TNF-α has been observed in the adipodse tissue of obese subjects that could disrupt lipid metabolism and lead to and lead to sustained obese state and obesity-related diseases. The human *TNF-α* promoter exhibits a high number of genetic variants, mainly single nucleotide polymorphisms (SNPs) that have been shown to influence the level of transcription in association with diseases. The human *TNF-α* genetic variants have never been fully reported in Arabs, therefore, we aimed to identify these variants by sequencing the *TNF-α* promoter, 5' UTR, and exon 1 in 290 Kuwaiti Arabs. As a result, we identified 14 genetic variants, including one novel SNP. Two promoter SNPs; rs1800750 (-376G>A) and rs361525 (-238G>A) were found to be in strong linkage disequilibrium ($r^2 = 0.73$) and ($D' = 1$, LOD = 32). To investigate the association of rs361525 (-238G>A) with obesity, we genotyped an additional 573 samples of the general Kuwaiti population by Real-time PCR (total n=863). Linear and logistic regression analysis have not shown any significant association in carriers of the A allele of rs361525 with continuous and categorical BMI, respectively. This is the first study in the Middle East and Kuwait that has sequenced and identified the common, rare and novel genetic variants of *TNF-α* promoter, 5'UTR and exon 1 in Arabs.

Keywords: BMI; Kuwait; obesity; sequencing; TNF-α.

1. Introduction

Obesity is a chronic metabolic disease that is characterized by the storage of excessive amounts of triglycerides in the adipose tissue (Herrera & Lindgren, 2010). Obesity is commonly defined with body mass index (BMI) of 30.0 kg/m² or greater according to WHO Expert Committee (1995). According to Global Burden of Diseases (GBD) report in 2015, obesity pandemic affected over 700 million individuals with estimate of 4 million deaths globally (Afshin *et al.,* 2017). Kuwait has the second highest obesity prevalence of 41% in men and 49% in women in

2015 within the Eastern Mediterranean Region (EMR), thus marking obesity as significant risk factors in Kuwait (Mokdad *et al.,* 2018).

Tumor necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine that is primarily secreted by monocytes/macrophages, including those that are found in adipose tissue, making adipose tissue a major source of TNF-α (de Ferranti & Mozaffarian, 2008; Russo & Polosa, 2005; Skibola *et al.,* 2005). The pleiotropic biological responses of TNF-α such as regulation of inflammation, cytokine production and energy metabolism are mediated through two types of TNF-α membrane receptors (TNFR1 and TNFR2) that trigger different signal transduction pathways, thus activating the expression of different genes (Parameswaran & Patial, 2010). It has been suggested that TNF-α regulates obesity through TNFR2, that was found to be overexpressed in obese individuals in comparison to lean subjects (Hotamisligil *et al.,* 1997). Transcriptional profiling studies have revealed that inflammatory and stress-response genes are among the most abundantly regulated gene sets in adipose tissue of obese animals (Wellen & Hotamisligil, 2005). Elevated levels of *TNF-α* mRNA was found in the adipose tissue of obese rodents, and was associated with obesity-induced insulin resistance (Alcalá *et al.*, 2017; Hotamisligil *et al.*, 1993). In obese vs lean humans, the expression level of adipose tissue TNF-α (Hotamisligil et al., 1995), and the level of systemic serum TNF-α (Berberoğlu, 2001; Moon *et al.*, 2004) were positively correlated with body fat percentage and BMI.

The human *TNF*-α gene locus is located on chromosome 6p21.31 spanning 3 kb and consisting of 4 exons that encode a 233 amino acid protein (Feldman et al., 2000; Laddha *et al.,* 2012). *TNF-α* promoter is about 1.3 kb upstream from the transcription start site (TSS) (Baena *et al.,* 2002). Some SNPs within the promoter region have been constantly reported such as rs1800630 (-863C>A,) rs1799724 (-857C>T), rs1800750 (-376G>A), rs1800629 (-308G>A), rs673 (-244G>A), and rs361525 (-238G>A), whereas others were less reported such as rs4645838 (C insertion that can be located at +68 - +71) at 5'UTR (Bayley *et al.,* 2004; Hajeer & Hutchinson, 2001; Posch *et al.,* 2003). This high density of promoter SNPs have been shown to influence the rate of transcription and protein production of TNF-α in association with diseases by affecting the binding of transcription factors (Elahi *et al.,* 2009). The genetic variants of the promoter at positions: −238, −308, −857, and −1031 (relative to TSS) may upregulate *TNF*-α gene transcription whereas, −863 downregulates the transcription (Laddha *et al.,* 2012).

In many cases, it is difficult to study the effect of a single SNP in isolation especially that in some populations, such as Caucasians, some SNPs are in linkage disequilibrium, i.e. the -376 A, -308 G and -238 A alleles (Hajeer & Hutchinson, 2001). Thus, functional genetic variants may act in a cooperative manner and interact together to determine the overall activity of the *TNF-α* promoter. That is an important reason to justify our approach in choosing to re-sequence the promoter, 5'UTR and exon 1 region in our study cohort. In our study we aimed to sequence *TNF-α* promoter in 290 Kuwaiti Arabs whose both parents are settlers from the Arabian Peninsula (Al-Bustan *et al*., 2005). This is the first sequencing of this region that has been performed on Arabs and Kuwaitis in particular. Furthermore, we investigated the

association of rs361525 (-238G>A) in 863 samples of the general Kuwaiti population which is an admixture between settlers from the Arabian Peninsula and other populations (Al-Bustan *et al*., 2005).

2. Materials and methods

2.1. Studied Samples

This study was conducted within the guidelines of the Declaration of Helsinki, and the protocol was approved by the Local Ethical Committee at Kuwait University. The samples recruited for sequencing were 290 Kuwaiti Arabs, while for validation, 573 Kuwaitis of the general population. All samples were recruited from volunteers who attend the regional polyclinics or the major hospitals in Kuwait. A written informed consent was obtained from all 863 participants of the Kuwaiti general population. BMI, medical and family medical history of hypertension, hypercholesterolemia, hypertension, type 2 diabetes mellitus (T2DM) and coronary heart disease (CHD) were documented. Ethnicity was verified by tracing both maternal and paternal lineages at least four generations using pedigree analysis. The cohort consisted of 425 females and 279 males with age range 18-80 years old. Subjects were divided into two groups according to their BMI. Non-obese group (n=558) with BMI < 30 kg/m^2 and obese group (n=305) with BMI ≥ 30 kg/m^2. Phenotypic variables including BMI and medical history in the studied cohort (n=863) are summarized in Table 1.

Table 1. Demographic and clinical features of study cohort (n = 863).

Variable	Descriptive Statistic
Age (years)	35 ± 17
Sex	
Male	43% (n=370)
Female	57% (n=493)
BMI (kg/m^2)	28.94 ± 7.61
<30	65% (n=558)
≥30	35% (n=305)
Medical History of:	
T2DM	11.8% (n=102)
Hypercholesterolemia	7.4% (n=64)
Hypertension	10.2% (n=88)
CHD	14.3% (n=123)

2.2. DNA Analysis

Total genomic DNA was extracted from whole blood using the salting-out procedure described by Miller *et al.* (1988). Three sets of primers were custom designed to cover 1394 bp target region at nucleotide position 31574504 to 31575900 spanning the *TNF-α* promoter, 5'UTR and

exon 1 based on the reference sequence NG_007462.1 (NCBI Genbank). Primer 3 Input software version 0.4.0 (//Frodo.wi.mit.edu/) was used in primer design (Supplementary Table S1). Amplification reactions were performed by PCR in an Applied Biosystems Fast thermal cycler (Version 1.01, Life Technologies, USA) (Supplementary Table S2 and Table S3). PCR products were purified using Nucleospin® extract II column Kit (Clontech Laboratories, Inc., Version No. PR48598) following the suggested protocol (Macherey-Nagel, Germany). Cycle sequencing was then performed according to the manufacturer's instructions using the BigDye Terminator v.3.1 in Fast Thermal Cycler (Life Technologies, Applied Biosystems, USA) (Supplementary Table S4). The extension products were purified using BigDye® XTerminator™ Kit (Life Technologies, Applied Biosystems, USA). Capillary electrophoresis was then performed in ABI-3130xl Genetic Analyser (GS01/02) (Life Technologies, Applied Biosystems, USA).

2.3. Sequence Analysis and Variants Identification

The obtained DNA sequences for each sample were analysed using the AB DNA Sequencing Analysis Software version 5.3.1 (Life Technologies, Applied Biosystems, USA). Genetic variants were detected by scanning the resulting chromatograms and aligning the obtained sequence from two reactions for quality assurance with the reference sequence (NG_007462.1) using ClustalW software (Multiple Alignment Tool). The common, rare and novel variants in each sample were reported and compared with NCBI and Ensembl databases (NG_007462.1, ENSG00000232810).

2.4. Association of the identified Genetic Variants

One SNP; rs361525, was selected for validation in 573 randomly selected samples of the Kuwaiti general population and were genotyped by real-time PCR [ABI 7800HT (GS01/02)] (Life Technologies, Applied Biosystems, USA) with commercially available pre-designed primer and probe sets (Applied Biosystems, Assay # C__2215707_10 # 4351379); [VIC/FAM]:GGCCCAGAAGACCCCCCTCGGAATC[A/G]GAGCAGGGAGGATGGGGA GTGTGAG. The genotyping assay and protocol were followed based on the manufacturer's recommendations for Taqman™ Genotyping Master Mix (Applied Biosystems # 4371355).

2.5. Linkage Disequilibrium and Haplotype Analysis

Linkage disequilibrium (LD) between the selected SNPs with minor allele frequency (MAF) ≥ 0.05 was analysed using Haploview (Barrett *et al.,* 2005) (version 4.2.)**.** Squared coefficient of correlation (r^2) and the standard color scheme of D'/LOD were calculated for each pair of SNPs. In this study, r^2 above 0.7 and (D′=1; LOD ≥2) with bright red color indicate strong linkage disequilibrium. Haplotype block was created according to Gabriel *et al.,* (2002) criteria that require 95% of informative comparisons to be in strong LD defined by the confidence bounds of D'.

2.6. Statistical Analysis

Genotype and allele frequencies were estimated using a simple gene counting method in which MAF was determined for each genetic variant in the total cohort and studied groups. Hardy-Weinberg equilibrium (HWE) was tested using online calculator and confirmed using the GENEPOP software (Version 4.2) (Rousset, 2008). One-way analysis of variance (ANOVA) was used to test the statistical difference in BMI (mean ± standard deviation) between the genotype groups of selected variants that showed MAF ≥ 0.05. Genetic modeling of rs361525 was performed in multiple linear regression and logistic regression adjusting for age, sex and ethnicity in the studied cohort (n=863). The statistical significance level for all tests was set at $p < 0.05$ using SPSS software (version 25; SPSS Inc., Chicago, IL, USA). The power of study in 863 samples was analysed using the Power and Sample Calculation Program (version 3.1.6) (Dupont & Plummer, 1990) assuming an odd ratio (OR) of 2.5.

3. Results

3.1. Identification of Genetic Variants

Sequenicng of the *TNF-α* promoter, 5'UTR and exon 1 regions (1394 bp) at nucleotide position 31574504 to 31575900 in 290 samples of Kuwaiti Arabs allowed the identification of 14 genetic variants among all the samples sequenced. Across the sequenced region, ten variants were found in the promoter, two in the 5'UTR and two, including a novel SNP, in exon 1 **(Figure 1)**. A new reference for *TNF-α* promoter, 5'UTR and exon 1 for Kuwaiti Arabs was defined and deposited in GenBank with an accession number: MH287061.

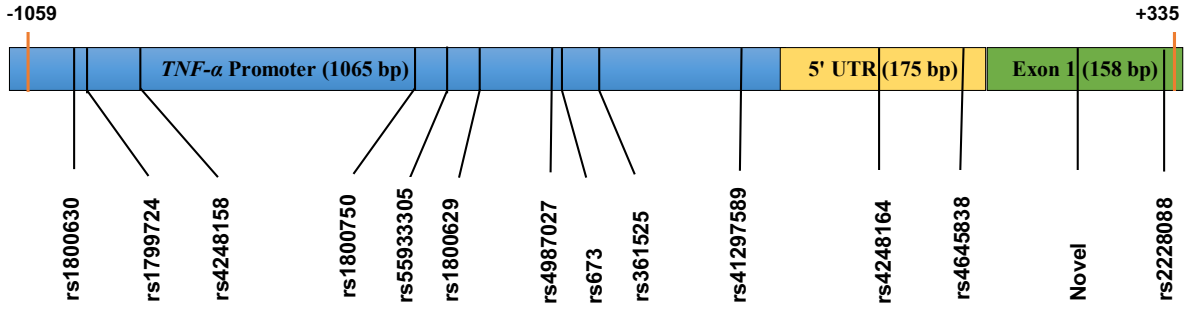

Fig. 1. The fourteen identified genetic variants in TNF-α target region on chromosome 6p21.31 form nucleotide (nt) -1059 to +335 (ch6: 31574504:31575900).

Among the detected genetic variants (Table 2), one SNP in exon 1, rs2228088 (c.87G>T), was defined as a synonymous mutation causing a G to T transversion at nucleotide position 267. Another identified variant rs4645838 (c.-108_-107 InsC); C nucleotide insertion in the 5' UTR that apparently does not cause a frame shift mutation due to its position in a non-coding region. The novel SNP in exon1 (c.39C>T) at position 6:31575780 is a synonymous mutation that causes a C to T transition at nucleotide position 219 that does not change the Alanine (Ala) at

residue 13. The sample with the novel SNP belongs to a 21 years old female whose ancestry was traced to the Arabian Peninsula (AP). The case is overweight (BMI=29.6 kg/m^2) and had been diagnosed with T2DM, hypertension and hypercholesterolemia, but had no history for Cardiovascular diseases (CVD) while her family medical history is positive for T2DM and CVD. A heterozygote C insertion of rs4645838 was also identified in an upstream position (6:31575634) in the same sample, thus making the novel SNP only detectable in the reverse sequence of primer 3 as G>A transition (Supplementary Figure S1).

Table 2. Characterization of the identified genetic variants at *TNF-α* promoter and exon 1 region among Kuwait Arabs.

Genetic Variants Ref. No.	Global MAF	Position at Ref. Seq.	*Chromosomal Position	mRNA Position**	Type	Amino Acid Change
rs1800630	C	-863C>A	g.31574699C>A	NR	Upstream	NR
rs1799724	C	-857C>T	g.31574705C>T	NR	Upstream	NR
rs4248158	C	-806C>T	g.31574756C>T	NR	Upstream	NR
rs1800750	G	-376G>A	g.31575186G>A	NR	Upstream	NR
rs55933305	C	-347C>T	g.31575215C>T	NR	Upstream	NR
rs1800629	G	-308G>A	g.31575254G>A	NR	Upstream	NR
rs4987027	C	-245C>T	g.31575317C>T	NR	Upstream	NR
rs673	G	-244G>A	g.31575318G>A	NR	Upstream	NR
rs361525	G	-238G>A	g.31575324G>A	NR	Upstream	NR
rs41297589	T	-76T>A	g.31575485T>A	NR	Upstream	NR
rs4248164	C	+4C>T	g.31575571C>T	c.-171C>T	5' UTR	NR
rs4645838	-	+68Ins -/C	g.31575634dupC	c.-108_-107insC	5' UTR	NR
Novel	UR	+213C>T	g.31575780C>T	c.39C>T	Synonymous	p.Ala13=***
rs2228088	G	+261G>T	g.31575828G>T	c.87G>T	Synonymous	p.Arg29=

*Chromosomal position is based on chromosome 6 published reference sequence using GRCh38.p7 primary assembly (NC_000006.12) in the NCBI GenBank database.
**mRNA position is based on the TNF-α mRNA sequence (NM_000594.3) in GenBank database.
***change in amino acid was predicted using TNF-α protein sequence (CCDS4702.1) in CCDS database.
NR: Not-Reported.

3.2. Genotype and Allele Frequencies

The population homogeneity was tested for HWE in the studied cohort for sequencing (n = 290) and in the two divided groups based on BMI; non-obese (n = 211; BMI<30) and obese (n = 79; BMI ≥ 30) (Table 3). Ten genetic variants in the studied population were in HWE (*p*-value > 0.05) except for four SNPs. A significant deviation was found with rs1800630 in the total population ($p = 0.002$) and non-obese group ($p = 0.010$). Similarly, rs4248158 was deviated in the total population ($p = 0.006$) and non-obese ($p = 0.001$). Whereas rs1800750 and rs361525 were deviated from HWE in the total population at $p = 0.002$ and 0.004, respectively, and in obese group at $p < 0.001$ for both (Table 3).

In the studied population (n=290), six genetic variants were found to be common with MAF ≥ 5%, four variants were considered less frequent with MAF 1-5%, whereas the remaining 4 SNPs were rare (MAF < 1%) (Table 3). The most common genetic variant was rs1800630C>A with MAF of 0.17 in the total samples followed by rs1800629G>A with MAF of 0.157. Within the common variants, the most common genotype was the homozygous wild type GG of rs361525 and TT of rs41297589 with a frequency of 0.890 for both followed by the homozygous CC of rs1799724 (0.852).

Table 3. Genotypic and allelic frequencies of the identified genetic variants of *TNF-α* in the studied Kuwaiti Arabs population (n=290) and in two groups divided based on BMI. The MAF in our study and the global MAF were reported.

Genetic Variants	Genotypes and Alleles	Non-obese (n=211)	Obese (n=79)	Total Sample (n=290)	Global MAF
	CC	0.740 (156)	0.650 (51)	0.714 (207)	
rs1800630	CA	0.210 (45)	0.280 (22)	0.231 (67)	
	AA	0.050 (10)	0.070 (6)	0.055 (16)	
MAF	A=	0.150	0.215	0.170	0.1542/772
HWE *p*-value		**0.010***	0.120	**0.002***	
	CC	0.830 (175)	0.911 (72)	0.852 (247)	
rs1799724	CT	0.170 (36)	0.089 (7)	0.148 (43)	
	TT	0	0	0	
MAF	T=	0.085	0.044	0.074	0.0990/496
HWE *p*-value		0.175	0.680	0.173	
	CC	0.934 (197)	0.937 (74)	0.934 (271)	
rs4248158	CT	0.057 (12)	0.063 (5)	0.059 (17)	
	TT	0.009 (2)	0	0.007 (2)	
MAF	T=	0.038	0.032	0.036	0.0258/129
HWE *p*-value		**0.001***	0.771	**0.006***	
	GG	0.910 (192)	0.937 (74)	0.917 (266)	
rs1800750	GA	0.085 (18)	0.038 (3)	0.072 (21)	
	AA	0.005 (1)	0.025 (2)	0.013 (3)	
MAF	A=	0.047	0.044	0.047	0.0112/56
HWE *p*-value		0.422	**<0.001***	**0.002***	

	CC	0.995 (210)	1 (79)	0.997 (289)	
rs55933305	CT	0.005 (1)	0	0.003 (1)	
	TT	0	0	0	
MAF	T=	0.002	-	0.002	0.0004/2
HWE *p*-value		0.972	-	0.977	
	GG	0.711 (150)	0.722 (57)	0.713 (207)	
rs1800629	GA	0.251 (53)	0.278 (22)	0.259 (75)	
	AA	0.038 (8)	0	0.028 (8)	
MAF	A=	0.164	0.139	0.157	0.0903/452
HWE *p*-value		0.235	0.150	0.702	
	CC	0.991 (209)	0.987(78)	0.990 (287)	
rs4987027	CT	0.009 (2)	0.013 (1)	0.010 (3)	
	TT	0	0	0	
MAF	T=	0.005	0.006	0.005	0.0002/1
HWE *p*-value		0.944	0.955	0.929	
	GG	0.962 (203)	0.937 (74)	0.955 (277)	
rs673	GA	0.038 (8)	0.063 (5)	0.045 (13)	
	AA	0	0	0	
MAF	A=	0.019	0.032	0.022	0.0192/96
HWE *p*-value		0.778	0.771	0.696	
	GG	0.872 (184)	0.937 (74)	0.890 (258)	
rs361525	GA	0.118 (25)	0.038 (3)	0.100 (28)	
	AA	0.010 (2)	0.025 (2)	0.010 (4)	
MAF	A=	0.069	0.044	0.062	0.0609/305
HWE *p*-value		0.280	**<0.001***	**0.004***	
	TT	0.886 (187)	0.899 (71)	0.890 (258)	
rs41297589	TA	0.104 (22)	0.101 (8)	0.103 (30)	
	AA	0.010 (2)	0	0.007 (2)	
MAF	A=	0.062	0.051	0.060	0.0106/53
HWE *p*-value		0.153	0.635	0.286	
	CC	1 (211)	0.987(78)	0.997 (289)	
rs4248164	CT	0	0.013 (1)	0.003 (1)	
	TT	0	0	0	
MAF	T=	-	0.006	0.002	0.0012/6
HWE *p*-value		-	0.955	0.999	
	-/-	(165)	(63)	0.787 (228)	
rs4645838	-/Ins C	(43)	(16)	0.203 (59)	
	Ins C/Ins C	(3)	0	0.010 (3)	
MAF	Ins C=	0.116	0.082	0.112	0.009/44
HWE *p*-value		0.917	0.316	0.931	
	CC	0.995 (210)	1 (79)	0.997 (289)	
Novel	CT	0.005 (1)	0	0.003 (1)	
	TT	0	0	0	
MAF	T=	0.002	-	0.002	-
HWE *p*-value		0.972	-	0.999	
	GG	0.967 (204)	0.962 (76)	0.966 (280)	
rs2228088	GT	0.033 (7)	0.038 (3)	0.034 (10)	
	TT	0	0	0	
MAF	T=	0.017	0.019	0.017	0.0176/88
HWE *p*-value		0.806	0.863	0.956	

*Significant at $p<0.05$

3.3. Linkage Disequilibrium and Haplotype

A total of 182 pairs were analysed and one SNP pair was found in strong LD with $D' = 1$, LOD = 32 and r^2 value of 0.73 between rs1800750 (-376G>A) and rs361525 (-238G>A) that indicate their co-segregation **(Figure 2)**. These two SNPs are located within 138 bp where four SNPs are found in between; rs55933305 (-347C>T), rs1800629 (-308G>A), rs4987027 (-245C>T) and rs673 (-244G>A). No haplotype block could be created applying Gabriel *et al*., 2002 criteria, since the majority of the pairs were non-informative.

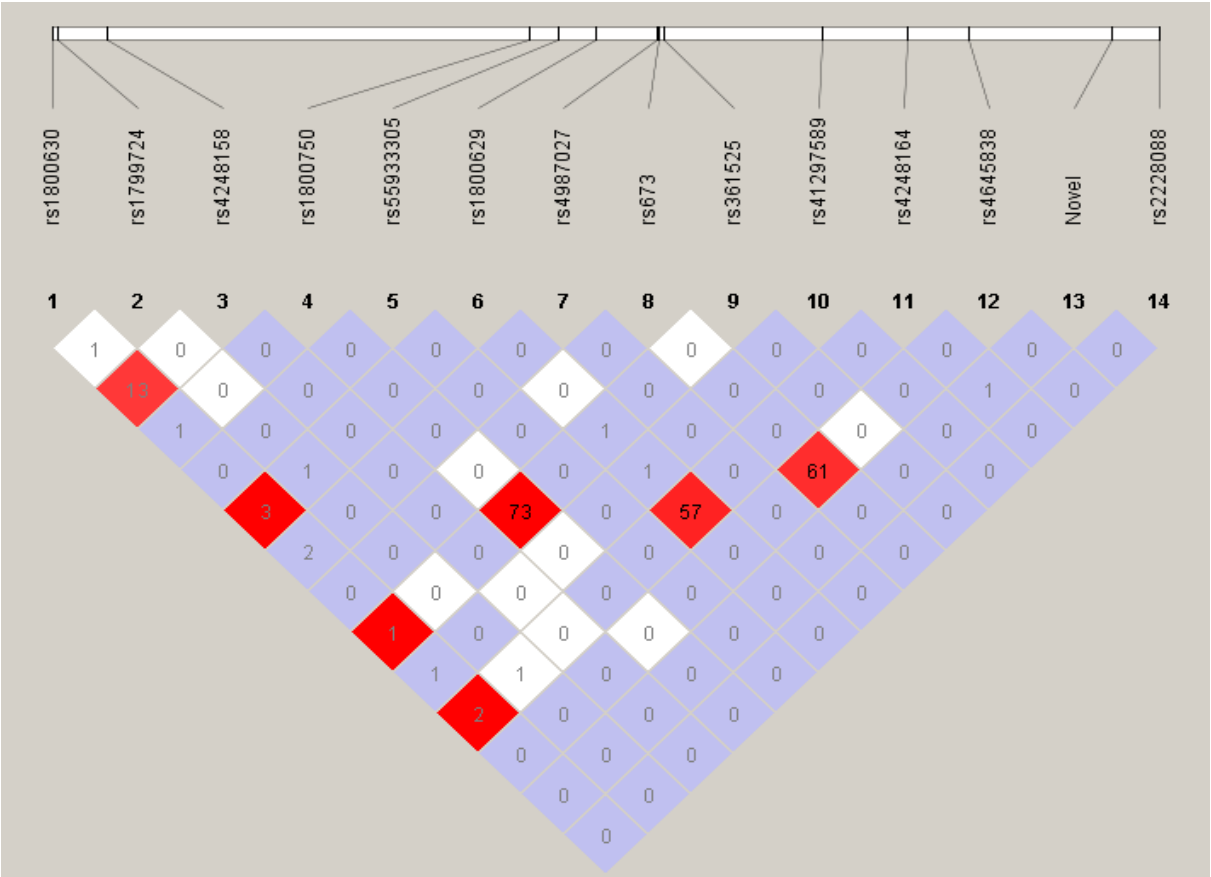

Fig. 2. Schematic representation of linkage disequilibrium (LD) between the fourteen genetic variants of TNF-α in 290 Kuwaiti Arabs. LD was defined by r^2 value expressed as percentile inside each pair and the standard D'/LOD color scheme with bright red indicates strong LD (D'=1; LOD ≥2), shades of pink/red indicate intermediate LD, and white color means no LD.

3.4. Association of Common Variants with BMI and obesity

Six genetics variants with MAF ≥ 0.05 were selected for analysing the distribution of mean BMI between genotype groups in 290 samples using one way-ANOVA **(Table 4)**. A significant (p = 0.003) increase in BMI has been found in carriers of the minor homozygous genotype of rs361525 -238 AA (39.60 kg/m^2 ± 17.14) in comparison to carriers of the heterozygous genotype GA (27.03 kg/m^2 ± 7.34), and the major homozygous genotype GG (28.87 kg/m^2 ± 6.57).

Table 4. Distribution of the mean BMI in the three genotype groups of the six selected genetic variants of *TNF-α* in 290 Kuwaiti Arabs.

Genetic variants	Genotype	Mean BMI ± S.D	*p*-value
rs1800630	CC (n=207)	28.47 ± 6.73	
	CA (n=67)	29.51 ± 7.45	0.284
	AA (n=16)	30.84 ± 7.59	
rs1799724	CC (n=247)	29.09 ± 7.15	
	CT (n=43)	27.40 ± 5.58	0.142
	TT (n=0)	-	
rs1800629	GG (n=207)	29.01 ± 7.23	
	GA (n=75)	28.64 ± 6.48	0.549
	AA (n=8)	26.35 ± 2.49	
rs361525	GG (n=258)	28.87 ± 6.57	
	GA (n=28)	27.03 ± 7.34	**0.003***
	AA (n=4)	39.60 ± 17.14	
rs41297589	TT (n=258)	28.94 ± 6.85	
	TA (n=30)	28.23 ± 8.07	0.669
	AA (n=2)	25.30 ± 2.63	
rs4645838	-/- (n=228)	28.87 ± 7.10	
	-/Ins C (n=59)	25.64 ± 3.72	0.726
	InsC/Ins C (n=3)	28.91 ± 6.56	

*Significant result at *p*-value<0.05

Analysis of rs361525 correlation with continuous BMI in 290 samples in linear regression recessive model had shown a significant positive association with carriers of the minor homozygous genotype -238 AA (β = 12.57; 95% CI = 5.98-19.16; $p < 0.001$) adjusting for age and sex (Supplementary Table S5). All samples with the minor homozygous genotype (n = 4) were Kuwaiti Arab male with BMI (28.30 - 64.80 kg/m^2) (Supplementary Figure S2).

Additional 573 Kuwaiti samples were added to the final cohort to increases statistical power and validate our preliminary results. The genotyping of rs361525 by real time PCR resulted in 506 samples with the major allele homozygous genotype GG (0.883), 66 with heterozygous genotype GA (0.115) and 1 with the minor homozygous genotype AA (0.002). The carrier the -238 AA was a Kuwaiti Arab female with a BMI of 23.80 kg/m^2.

The association of *TNF-α* rs361525 (-238G>A) with BMI was further investigated in the extended studied cohort (n = 863) and the allele frequencies were found in HWE ($p = 0.262$) with MAF of 0.060. Analysis of variance (ANOVA) revealed no significant difference in BMI distribution between the three genotype groups of rs361525 at $p = 0.060$ (Table 5). In multiple linear regression, a significant positive correlation with BMI was found in carriers of the minor homozygous genotype AA (β = 7.21; 95% CI = 0.53-13.88; $p = 0.034$) but not with carriers of the A allele (Table 5). However, the low number of samples in the recessive model is not

sufficient to solely accept these results. Logistic regression between obese (n = 305) vs non-obese (n = 558) groups showed no significant association of rs361525 with obesity in any models after adjusting for age, sex and ethnicity (Table 6).

Table 5. Multiple linear regression of *TNF-α* rs361525 (-238G>A) with BMI in 863 Kuwaiti samples adjusting for age, sex and ethnicity.

Genetic Model	Allele/Genotype	BMI	ANOVA p-value	β-coefficient	95% CI	*p*-value
G vs A (n=1748)	G (n=1622)	28.94 ± 7.50		1		
	A (n=104)	29.03 ± 9.59	0.902	0.08	-1.42-1.59	0.913
Recessive	GG+GA (n=858)	28.99 ± 7.56	**0.030***	1		
	AA (n=5)	39.44 ± 16.44		7.21	0.53-13.88	**0.034***
Dominant	GG (n=764)	29.08 ± 7.44	0.604	1		
	GA+AA (n=99)	28.66 ± 9.09		-0.38	-1.98-1.21	0.637
Additive (0,1,2)	GG (n=764)	29.98 ± 7.44	0.060	0.02	-1.47-1.50	0.982
	GA (n=94)	28.24 ± 8.49				
	AA (n=5)	36.44 ± 16.44				

-BMI is reported as the mean value ± S.D
-ANOVA stands for analysis of variance
**p*-value<0.05 is significant

Table 6. Logistic regression of *TNF-α* rs361525 (-238G>A) with obesity in 863 Kuwaiti samples adjusting for age, sex and ethnicity.

Genetic Model	Obese (n=305)	Non-obese (n=558)	OR (95% CI)	*p*-value
Codominant				
GG	90.8% (277)	87.3% (487)	1	
GA	8.5% (26)	12.2% (68)	0.68 (0.42-1.09)	0.106
AA	0.7% (2)	0.5% (3)	1.18 (0.19-7.11)	0.860
Dominant				
GG	90.8% (277)	87.3% (487)	1	
GA+AA	9.2% (28)	12.7% (71)	0.70 (0.44-1.10)	0.125
Recessive				
GG+GA	99.3% (303)	99.5% (555)	1	
AA	0.7% (2)	0.5% (3)	1.22 (0.20-7.40)	0.825
Additive Model (0,1,2)	-	-	0.74 (0.48-1.14)	0.168

**p*-value<0.05 is significant

4. Discussion

This is the first study on an Arab population in the Middle East and Kuwait to sequence the *TNF-α* promoter, 5'UTR and exon 1 regions and report fourteen genetic variants; ten in the promoter region (1065 bp), two in the 5'UTR (175 bp) and two, including a novel SNP, in exon 1 (158 bp). Genetic variants at the *TNF*-α promoter were never fully reported in any Middle Eastern population prior to this study, thus identifying and selecting essential common variants for our association study was limited to data from other ethnic groups that may be different from Arabs. The MAF for most of the identified genetic variants in our study were consistent with the global MAF reported in the 1000 genomes project. The MAF of rs41297589 was found to be closer to the South Asian (SAS) population while MAF of rs1800629 was similar to the European (EUR) and African (AFR) population. In our study, the MAF of rs4645838 Ins C was identified as a common variant in Arabs but rare in other populations. Interestingly, our number of detected SNPs in *TNF-α* promoter was also consistent to the reported level of variation in the human genome where 10 SNPs were estimated to be found per 1 Kb in this region (Baena *et al.,* 2002).

One interesting finding from this study was the unlikely separation by genetic recombination of the rare allele of rs1800750 (-376A) indicating it probably arose as a mutation within the haplotype carrying the rare allele of rs361525 (-238A) which is supported by their close proximity (Knight *et al.,* 1999). Our study further supports the strong LD between rs1800750 (-376G>A) and rs361525 (-238G>A) with r^2 value of 0.73 and D' of 1. The relationship between these SNPs has been preserved in East African, West African and European populations (Bănescu *et al*., 2019; Georgescu *et al*., 2020; Knight *et al.,* 1999), and apparently in the Arab population as reported in this study.

Hotamisligil *et al.* (1993) provided the first evidence of functional link between obesity and TNF-α that was elevated in models of obese rodents. The increased expression of TNF-α level associated with obesity could affect the metabolic status of adipose tissue, disrupt lipid metabolism, regulation of fatty acid uptake, lipogenesis and lipolysis that can lead to the development of obesity-related complications such as, insulin resistance and atherosclerosis (Jung & Choi, 2014; Sethi & Hotamisligil, 1999; Zietek & Rath, 2016). TNF-α as a powerful pro-inflammatory cytokine has never been sequenced in Kuwaiti and Arab population. Therefore, it was found to be a suitable candidate gene to investigate metabolic and cardiovascular diseases in Arabs focusing on obesity in this study. Two genetic variants of *TNF-α* promoter; rs1800750 and rs361525 were found to be significantly deviated in the obese group compared to other variants, thus could indicate an association with obesity. The *TNF-α*-238G>A has been annotated within a 25 bp region known as the *TNF-α* repressor site (TRS) which is localized between base pairs -254 and -230 in the promoter (Fong *et al*., 1994). The functional significance of rs361525 at TRS remains controversial between increasing the repression activity or blunt it or having no effect at all. Previous studies showed that the A allele of -238G>A was found to be associated with either an increase in TNF-alpha transcript levels (Baylel *et al*., 2001; Laddah *et al*., 2012), a decrease in the transcriptional activity (Huizinga *et*

al., 1997; Kaluza *et al*., 2000), or has no effect (Kaijzel *et al*., 1998). Furthermore, a construct mutant (-376A) was found to induce the expression level of TNF-α by 35% in comparison with the wild type -376G through altering the general topology of the region and creation of a binding site for the transcription factor organic cation transporter 1 (OCT-1) (Knight *et al*., 1999).

Five out of the six common genetic variants showed no significant association with BMI including; rs1800629 (-308G>A), this is consistent with what was previously reported in a sample of Kuwaiti individuals (Alrashid *et al*., 2021), which supports our findings. Our preliminary analysis showed significant association between carriers of the minor homozygous genotype of rs361525 (-238 AA, n=4) and BMI in Kuwaiti Arabs. However, considering the low number of carriers and to validate our results, the size of the cohort was increased to 863 samples from the Kuwaiti general population; which is an admixture between settlers from the Arabian Peninsula and other populations (Al-Bustan *et al.,* 2005). Although, linear regression confirmed the significant association between rs361525 and BMI in the recessive model, it was disregarded because of the persisting low number of carriers that could be misleading. Moreover, adjusting the significance level using Bonferroni correction has resulted in non-significant association. To confirm our finding, logistic regression between rs361525 and obesity had revealed no significant association in any of the genetic models after adjusting for age, sex and ethnicity. The sample size has been a critical factor in this study as the power has significantly increased from 74% to 96% with larger cohort (assuming OR of 2.5 and MAF of 0.66) supporting our results. Few studies had investigated the genetic association between obesity and rs361525 (-238G>A). Consistent with our results, studies in an Iranian population and a population from Johns Hopkins Weight management center reported no significant association between rs361525 (-238G>A) and obesity *(Hedayati et al.,* 2012; Walston *et al.,*1999). Contradictory to these findings, one study had reported a significant association between carriers of the -238 A allele (GA+AA) and higher body fat percentage in black South African woman (p<0.001) and in the combined group of black and white South African women (p=0.004) revealing an increased risk of obesity (Joffe *et al.,* 2012). On the other hand, the G Allele of -238G>A had presented a significant association in a Korean population with overweight/obesity as the frequency of the G/G genotype in the overweight/obese group was 9.3% higher than that in the control group (p= 0.0046) (Yu *et al.,* 2011).

5. Conclusion

This is the first study in the Middle East and Kuwait that sequenced and identified the common, rare and novel variants of *TNF-α* promoter, 5'UTR and exon 1 in Arabs. The common variants showed no significant association with BMI. Similarly, the selected rs361525 (-238G>A) has shown no significant genetic association with either continuous or categorical BMI in 863 Kuwaitis of the general population. The findings in this study do not rule out the association of *TNF-α* with obesity and may suggest a potential for *TNF-α* variants to interact with other genes

variants to predispose to obesity under epigenetic control. Moreover, the findings merit the importance of sample size in establishing a genetic association.

Data Availability

The authors declare that all data supporting the findings of this study are available within the article or from the corresponding author upon reasonable request.

Conflict of interest

The authors declare that there are no known conflicts of interest associated with this publication and the institutions where the work has been carried out.

Acknowledgment

This Study was supported and funded by Kuwait University Research Administration and College of Graduate Studies, Project Grant (YS01/16). The authors would also like to acknowledge the General Facility Project (GS01/02) for the use of the ABI 3130xl Gene Analyser with deep gratitude to Mrs. Sheela Thankakkon for her technical assistance. An extended sincere gratitude to Mrs. Babitha Annice for her guidance, designing the primers and technical support.

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Submitted: 18/04/2021
Revised: 26/08/2021
Accepted: 02/11/2021
DOI: 10.48129/kjs.13771