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The association between multiple sclerosis and genetic variations in tumor necrosis factor alpha and interleukin-6 genes in a sample of Iraqi patients

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ABSTRACT

Multiple sclerosis (MS) is a neurodegenerative autoimmune disease that is suggested to be triggered by genetic, epigenetic and environmental factors. Pro-inflammatory cytokines such as Tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) are stated to play a pivotal role in the pathogenesis of MS. The aim of the current study is to highlight the association between *TNFA*$_{-308, -238}$ and *IL6*$_{-174, -597}$ single nucleotide polymorphisms (SNPs) and MS predisposition in a sample of multiple sclerotic Iraqi patients. Sixty eight Iraqi Arab relapsing-remitting multiple sclerosis (RRMS) patients and forty eight healthy individuals were enrolled. *TNFA*$_{-308, -238}$ and *IL6*$_{-174, -597}$ SNPs were detected *via* sequence-specific primers polymerase chain reaction (SSP-PCR) technique. The results showed a significant variation for homozygous and heterozygous genotypes with *A* allele of *TNF*$_{-238}$ SNP between patients and controls (75.0% *vs*. 93.8%, $P = 0.02$, OR = 0.2, CI 95% = 0.06-0.7; 23.5% *vs*. 6.3%, $P = 0.03$, OR = 4.6, CI 95% = 1.3-16.7; 13.2 *vs*. 3.1%, $P = 0.02$, OR = 4.7, CI 95% = 1.4-16.4). AA genotype was absent in controls. Neither *IL6*$_{-174, -597}$ SNPs nor haplotypes showed a considerable variation between patients and controls, but a strong linkage disequilibrium between these two loci was observed through Haploview software analysis. Conclusively, heterozygous and minor allele (*A*) of *TNF*$_{-238}$ SNP comprised risk factors for MS development whereas none of studied *IL6* SNPs influenced the susceptibility to MS in this sample of Iraqi population.

KEYWORDS: multiple sclerosis, single nucleotide polymorphism, TNF-α, IL-6, haplotype

INTRODUCTION

Multiple sclerosis (MS) is a neurodegenerative disease characterized by chronicity and it specifically targets young adults, causing a demyelination of myelin sheath surrounding the axon leading to plaque formation in the central nervous system (CNS), especially the white matter, with multiple neurological disorders [1]. Many genetic, epigenetic, and environmental factors trigger MS infection [2, 3]. Pro-inflammatory cytokines (such as TNF-α and IL-6) are known to play a pivotal role in disease exacerbation; therefore, a body of evidence has highlighted the impact of cytokine gene variation on MS development. TNF-α modulates both innate and adaptive immune response and regulates cell proliferation, differentiation, and cell death [4]. It is expressed by natural killer (NK) cells, phagocytes, and microglia of CNS, and is responsible for many biological activities such as promoting the expression of adhesion molecules in endothelial cells in addition to inducing oligodendrocyte apoptosis; besides it is one of the biomarkers of inflammation and demyelination in both experimental autoimmune

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encephalomyelitis (EAE) mice model and MS [5]. Upon infection, microglia release a large amount of this cytokine as neuroinflammatory response to infection that leads to a bunch of neurological disorders [6]. *TNFA* gene is located on chromosome no.6. Two polymorphisms, particularly at loci -308 and -238 of promoter region, were detected by many researchers, but inconsistent results were generated from these trials [7-9]. Furthermore, the influence of these variations on modulating gene expression was suggested to be associated with various autoimmune diseases [10]. IL-6 is a pleiotropic pro-inflammatory cytokine produced mainly by macrophages and monocytes. It is known as a B-cell antibody stimulating factor which contributes to inflammatory responses and development of nervous system [11]. In combination with TGFB, IL-6 induces the differentiation of CD4 T-cells into Th-7 cells and any imbalance between these two cytokines could result in autoimmune diseases [12]. In MS patients, IL-6 induces the proliferation and trafficking of T-cells to CNS by stimulation of adhesion molecules on endothelial cells [13]. Animal experiments have revealed the pivotal role of IL-6 in autoimmune encephalomyelitis development [14]. *IL6* is located on chromosome no.7 and variations in its promoter region have been intensively studied to prove if there is any association between these variations and some autoimmune diseases including MS but the results were inconsistent which may be due to ethnicity variations [15-17].

The current research aimed to investigate if there is any association between promoter SNPs of $TNFA_{-308,\ -238}$ and $IL6_{-174,\ -597}$ and MS predisposition in a sample of multiple sclerotic Iraqi patients.

PATIENTS AND METHODS

Study groups

Sixty eight Iraqi Arab patients were diagnosed with relapsing-remitting multiple sclerosis (RRMS) by physicians according to McDonald criteria 2010 revision [18] at the Multiple Sclerosis Clinic at Baghdad Teaching Hospital. The mean age of the patients was 34.8 ± 1.18. The patients were also distributed according to their ambulation capability parameter, i.e. the so called EDSS (Expanded Disability Status Scale), which ranges from 0 to 10 (from normal neurological patient to risk of death) [19]. All these details are illustrated in Table 1. On the other hand, the control group included forty eight healthy subjects (distributed as 16 males and 32 females) from Teaching Laboratories of Medical City personnel who had no history of any autoimmune disease, and were apparently healthy. They matched the patients in ethnicity, age, and gender and had a mean age of 34.2 ± 1.3.

Sample collection and DNA extraction

The blood of 68 relapsing-remitting multiple sclerosis (RRMS) patients and forty eight healthy subjects was withdrawn, aliquoted into K2-EDTA tubes and stored at -20 °C until use. Genomic DNA was extracted from frozen whole blood contained in EDTA tubes using ReliaPrep™ gDNA MiniPrep System Kit, manufactured by Promega Company, USA.

The concentration and purity of the DNA samples were estimated by NanoDrop technology at optical densities of 260/280 nm wavelength.

Genotyping method

Single nucleotide polymorphisms of *TNFA* and *IL6* genes were analysed by using Cytokine CTS-SSP-

Table 1. Details of multiple sclerosis patients and their distribution.

Characteristics		Multiple sclerosis patients (No. = 68)	
		Males (No. = 23)	Females (No. = 45)*
Mean age ± SE (Years)		34.7 ± 2.6	34.7 ± 2.2
Extended Disability Status Score (EDSS)	< 3	16	24
	≥ 3	7	17

*EDSS was missing in four cases.

PCR Tray kit provided by Collaborative Transplant Study (CTS), Heidelberg University, Germany. The genotyping was performed according to the instructions supplied with the kit and the thermocycling conditions were set as follows: initial denaturation at 94 °C for 2 minutes, followed by 10 cycles of denaturation (94 °C/15 seconds), and annealing/extension (65 °C/60 seconds). This was followed by 20 cycles of denaturation (94 °C/15 seconds), annealing (61 °C/50 seconds) and extension (72 °C/30 seconds). Finally, the PCR products were held at 4 ºC for 15 minutes. Agarose gel electrophoresis of PCR products was run for 25 minutes at 170 volts using 2% agarose. The pattern of bands was observed under UV transilluminator (312 nm), and interpreted according to the manual supplied with the kit that was based on internal control bands.

Statistical analysis

IBM Statistical package for Social Sciences (SPSS) 23 windows version (LEAD technology, Inc, USA.) was the software of choice to perform statistical analysis of the study including the difference between means of age, gender, expanded disability status scale (EDSS) and genotype distribution between the study groups. The significant difference was adjusted at P-value ≤ 0.05. Genotype and allele frequencies were calculated by direct counting. Pearson's Chi-square(X^2) goodness-of-fit test was used for the comparison between observed and expected frequencies of Hardy-Weinberg equilibrium (HWE) equation at significant level 0.05. WINPEPI freeware package program for epidemiologists was used to estimate Pearson Chi square(X^2) test of independence, the odds ratio (OR) with the corresponding confidence intervals (CI at 95%), and the etiological/protective fractions (EF/PF) [20]. Haploview software version 4.2 was used to estimate haplotypes, haplotype frequencies and linkage disequilibrium (LD) which depends on correlation coefficient (r^2), delta coefficient of LD (D`) and logarithm of odds (LOD) statistics; $r^2$ = 1, D`= 1 and LOD ≥ 3 refer to complete LD [21].

RESULTS

TNFA gene SNPs at -308, -238 loci

TNFA gene SNPs at positions -308, -238 presented with three genotypes (GG, GA and AA), showed no significant differences between the observed and expected genotype frequencies in MS patients and controls; therefore, both groups showed no departure from HWE (Table 2). Despite the non-significant variations between genotypes/alleles of patients and controls, some differences were recorded for $TNFA_{-308}$ SNP. GG genotype showed a decreased frequency in patients compared to controls (67.7% *vs*. 75.0%; OR = 0.7; PF = 0.2; CI 95% = 0.3-1.6). In contrast, the heterozygous genotype (GA) showed an increased frequency in patients as compared to controls (30.9% *vs*. 22.9%; OR = 1.5; EF = 0.1; CI 95% = 0.7-3.5). Significant variations between patients and controls were recorded for dominant homozygous and heterozygous genotypes at locus -238. GG genotype frequency showed a significant decrease in patients as compared to controls (75.0 *vs*. 93.8%; P = 0.02; OR = 0.2; PF = 0.8; CI 95% = 0.06-0.7), while GA genotype frequency increased significantly among patients compared to controls (23.5% *vs*. 6.3%; P = 0.03; OR = 4.6; EF = 0.2; CI 95% = 1.3-16.7). AA genotype was absent in the control group with a significant increased frequency of *A* allele among the patients as compared to controls (13.2 *vs*. 3.1%; P = 0.02; OR = 4.7; EF = 0.1; CI 95% = 1.4-16.4) (Table 2).

IL6 SNPs at -174, -597 loci

The results revealed that *IL6* SNPs at both loci -174 and -597 showed no significant variations between patients and controls and their observed and expected frequencies were compatible with the HWE principle. CC and AA genotypes of -174, and -597 SNPs, respectively were absent in the control group whereas GG genotype for both SNPs was recorded as the highest genotype in both patients and controls (Table 3).

Haploview analysis

The results indicated that the two SNPs of *TNFA* at -308 and -238 loci were not in linkage disequilibrium (D` = 0.19, LOD = 0.3, and r^2 = 0.03) since these values did not match the standard parameters for complete LD (Figure 1A). For its absence in the control group, AA haplotype showed a significant increase in its distribution among patients as compared to controls (3.9 vs. 0.0%; P = 0.05; OR = 8.1; EF = 0.04; CI 95% = 0.5-145.6) and GA haplotype showed a higher increase in frequency in patients than in the control group and the difference was nearly significant (9.3% *vs*. 3.1%; P = 0.06, OR = 3.3; EF = 0.07; CI 95% = 0.9-11.8) (Table 4).

Table 2. Observed and expected genotype and allele frequencies of $TNFA_{-308,-238}$SNPs according to Hardy-Weinberg equilibrium (HWE) equation for both MS patients and controls with the corresponding epidemiological parameters.

MS patients (68)				Controls (48)			Epidemiological parameters			
Interleukin SNP	**Observed frequency (%)**	**Expected frequency (%)**	**HWE P-value ≤0.05**	**Observed frequency (%)**	**Expected frequency (%)**	**HWE P-value ≤0.05**	**OR**	**EF/PF**	**Pearson X^2 *P*- value (Yates correction)**	**CI 95%**
TNFA -308										
GG	46 (67.7)	46.9 (69.0)	0.4	36 (75.0)	35.9 (74.8)	0.9	0.7	0.2	0.5	0.3-1.6
GA	21 (30.9)	19.1 (28.1)		11 (22.9)	11.2 (23.4)		1.5	0.1	0.4	0.7-3.5
AA	1 (1.5)	1.9 (2.9)		1 (2.1)	0.9 (1.8)		0.7	0.01	1.0	0.04-11.2
G	113 (83.1)	-		83 (86.4)	-		0.8	0.2	0.6	0.4-1.6
A	23 (16.9)	-		13 (13.6)	-		1.3	0.04	0.6	0.6-2.7
TNFA -238										
GG	51 (75.0)	51.2 (75.3)	0.8	45 (93.8)	45.1 (93.9)	0.8	0.2	0.8	**0.02***	0.06-0.7
GA	16 (23.5)	15.6 (23.0)		3 (6.3)	2.9 (6.1)		4.6	0.2	**0.03***	1.3-16.7
AA	1 (1.5)	1.2 (1.8)		0 (0)	0.05 (0.1)		2.2	0.01	1.0	0.09-52.42
G	118 (86.8)	-		93 (96.9)	-		0.2	0.8	**0.02***	0.06-0.7
A	18 (13.2)	-		3 (3.1)	-		4.7	0.1	**0.02***	1.4-16.4

*Bold numbers refer to significant difference at *P* -value ≤0.05.

Table 3. Observed and expected genotype and allele frequencies of *IL6*$_{-174,-597}$ SNPs according to Hardy-Weinberg equilibrium (HWE) equation for both MS patients and controls with the corresponding epidemiological parameters.

MS patients (68)				Controls (48)			Epidemiological parameters			
Interleukin SNP	**Observed frequency (%)**	**Expected frequency (%)**	**HWE *P*-value ≤0.05**	**Observed frequency (%)**	**Expected frequency (%)**	**HWE *P*-value ≤0.05**	**OR**	**EF/PF**	**Pearson X^2 *P* -value (Yates correction)**	**CI 95%**
IL6 -174										
GG	45 (66.2)	42.9	0.1	31 (64.6)	32.5	0.1	1.1	0.05	1.0	0.5-2.3
GC	18 (26.5)	22.2		17 (35.4)	14.0		0.7	0.1	0.4	0.3-1.5
CC	5 (4.3)	2.9		0 (0.0)	1.5		8.4	0.07	0.1	0.5-151.2
G	108 (79.4)	-		79 (82.3)	-		0.8	0.1	0.7	0.43-1.6
C	28 (20.6)	-		17 (17.7)	-		1.2	0.04	0.7	0.6-2.3
IL6 -597										
GG	45 (66.2)	42.9	0.1	35 (72.9)	35.9	0.3	0.7	0.2	0.6	0.3-1.6
GA	18 (26.5)	22.2		13 (27.1)	11.2		1.0	0.01	1.0	0.4-2.2
AA	5 (4.3)	2.9		0 (0.0)	0.9		8.4	0.07	0.1	0.5-151.2
G	108 (79.4)	-		83 (86.5)	-		0.6	0.3	0.2	0.3-1.2
A	28 (20.6)	-		13 (13.5)	-		1.7	0.08	0.2	0.8-3.4

Table 4. Estimation of *TNFA*$_{-308, -238}$ and *IL6*$_{-174, -597}$ haplotype frequencies with corresponding epidemiological parameters.

***TNFA* haplotypes**	**Patients (136 chromosomes)**	**Controls (96 chromosomes)**	**Epidemiological parameters**			
	Observed frequency (%)	**Observed frequency (%)**	**OR**	**EF/PF**	**X^2P- value**	**95% CI**
GG	100 (73.8)	80 (83.3)	0.6	0.4	0.09	0.3 - 1.1
AG	18 (13.0)	13 (13.5)	1.0	0.004	0.9	0.5 - 2.1
GA	13 (9.3)	3 (3.1)	3.3	0.07	0.06	0.9 -11.8
AA	5 (3.9)	0 (0.0)	8.1	0.04	**0.05***	0.5 - 145.6
***IL6* haplotypes**						
GG	104 (76.4)	78 (81.2)	0.8	0.2	0.4	0.4 - 1.4
AC	24 (17.6)	12 (12.4)	1.5	0.06	0.3	0.7 - 3.2
GC	4 (3.0)	5 (5.3)	0.6	0.02	0.4	0.2 - 2.1
AG	4 (3.0)	1 (1.1)	2.9	0.02	0.3	0.3 - 25.9

*Bold numbers refer to significant difference at *P* -value ≤0.05.

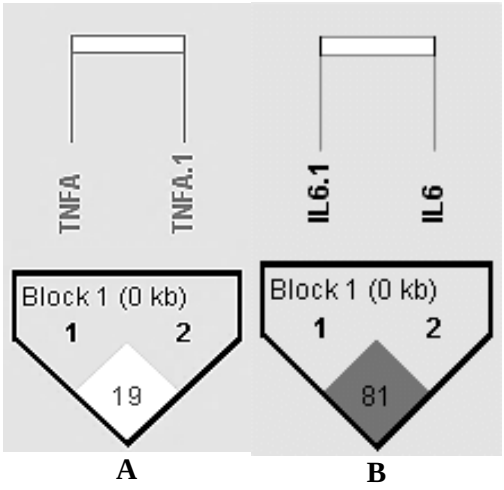

Figure 1. Linkage disequilibrium analysis for (A) *TNFA*$_{-203, 238}$ and (B) *IL6*$_{-174,-579}$ SNPs. (A): Haploview Pairwise analysis of *TNFA* showed that linkage disequilibrium was absent in the two loci. (B): pairwise analysis of *IL6* SNPs showed a strong linkage disequilibrium between these two loci. The number displayed inside the box represents LD coefficient (D`) value. White box represents D`<1 and logarithm of odds LOD<3, while gray box demonstrates D`~1 and LOD>3. White and gray boxes represent the absence of linkage disequilibrium and strong linkage disequilibrium, respectively according to the Haploview manual. TNF and TNF.1 are designated for -308 and -238 SNPs, respectively, while IL6 and IL6.1 are designated for -174 and -597 SNPs, respectively as designed by Haploview software.

Concerning Haploview analysis of *IL6* $_{-174, -597}$ SNPs, a strong linkage disequilibrium emerged between these two loci (D` = 0.8, LOD = 13, and r^2= 0.7) (Figure 1B). None of the *IL6* haplotypes showed a significant variation between patients and controls. However, AC and AG haplotypes showed a slight increase in distribution among patients as compared to the control group (17.6% *vs.* 12.4%, OR = 1.5, EF = 0.06, CI 95% = 0.7-3.2; 3.0% *vs.* 1.1%, OR = 2.9, EF = 0.02, CI 95% = 0.3-25.9) (Table 4).

DISCUSSION

Multiple sclerosis is a chronic autoimmune disease, in which cytokines have been suggested to play a pivotal role in the disease progression, severity, or amelioration. In this context, the pro- inflammatory cytokine TNF-α has been landmarked by its contribution to various MS pathogeneses; for instance, demyelination and oligodendrocyte apoptosis [22]. However, with the relatively recent knowledge on cytokine gene polymorphisms, differences between individuals have been discovered that influence not only cytokine gene expression, but also susceptibility to diseases, their progression, severity, and clinical outcomes [23]. Therefore, studying the frequency of cytokine SNP variations at the population level has become one of the important biomarkers for diseases. These genetic variations are due to natural selection of related

alleles that influence the individual resistance and/or susceptibility to diseases [23].

Correspondingly, single nucleotide polymorphisms (SNPs) in *TNFA* gene promoter have been suggested to be essential in modulating *TNFA* gene expression and also to be positively associated with different diseases (infectious, autoimmune, and cancerous diseases). $TNFA_{-308}$ and $TNFA_{-238}$ are among these SNPs that have been extensively investigated due to their impact on *TNFA* gene expression at the transcriptional level, as well as their association with susceptibility to different diseases, especially autoimmune diseases [4].

In the current study, no association between $TNFA_{308}$ SNP alleles/genotypes and MS risk was observed. However, Hajifathali *et al*. (2012) reported contradicting results among Iranian patients [24]. The authors reported that GG genotype and *G* allele frequencies were significantly increased in MS patients. They concluded that this allele and the corresponding homozygous genotype are risk factors associated with MS predisposition in Iranian population. Two earlier studies also reported that such SNPs might be associated with MS [25, 26], but such findings gained no support from others [27]. Such a conflict has also been a subject of a recent meta-analysis data that emerged from 21 studies with 2880 MS patients and 3579 healthy controls [28]. This analysis revealed that GA genotype has no influence on MS susceptibility in European populations in general compared to the homozygous genotype. In addition, genome wide association studies (GWAS) referred to the negative correlation between $TNFA_{308}$ SNP and susceptibility to MS; therefore, depending on these data, the study reached the conclusion of absence of such an association [28].

With respect to $TNFA_{-238}$ SNP, the present results suggest that *A* allele and GA genotype might be predisposing genetic markers for MS in Iraqi population while *G* allele and the homozygous genotype were associated with a decreased risk for MS. Moreover, Amirzargar *et al*. (2007) [29] demonstrated a significant variation in the distribution of $TNFA_{-238}$ *A* allele, GG and GA haplotypes between Iranian MS patients and controls, and their MS risk effect was suggested and this consequence was consistent with our current result. However, these findings were not reported in Turkish MS patients [30]. In addition, a collection of data generated from 21 studies that included 2639 MS patients and 3303 controls were enrolled in a meta- analysis conducted by Xu *et al*. (2011) [9], and the final conclusion was in favor of no association between each of -308 and -238 *TNFA* SNPs and susceptibility to MS. Simultaneously, another study combined with meta-analysis data were conducted to reveal the status of $TNFA_{-308,\ -238}$ SNPs and their related haplotype combinations in MS patients. The overall analysis showed there was no significant impact of these polymorphisms and haplotypes on *TNFA* expression [31].

The results generated from Haploview software indicated that the estimated haplotype frequencies revealed that only AA haplotype showed a significant variation between MS patients and controls, and its impact on the disease susceptibility could be established. It has been suggested that *TNFA* haplotype estimation between alleles of cytokine gene SNPs on the same chromosome may provide a comprehensive approach for understanding the role of cytokine gene SNPs in etiopathogenesis of MS [29]. A cohort study was performed by Watson *et al*. (2012) [32] using GWAS that covered 400,000 SNP variants for MS. They concluded that rare variants could not be explained due to the imperfect linkage disequilibrium between rare causative variants and the genotyped SNPs that are likely to be associated with MS predisposition, but haplotype phasing is more important than genotypes in association studies and pedigree analysis.

Regarding *IL6* polymorphisms, -174 G>C and -597 G>A variations were mostly detected by researchers and were found to be associated with several autoimmune diseases like type2 diabetes [15], systemic lupus erythematosus [33], and rheumatoid arthritis [34].

A meta-analysis performed by Hu *et al*. (2014) [35] demonstrated the absence of association between -174 G/C SNP and MS risk in Caucasian and African ethnic groups and the presence of such association in Asians. Fedetz *et al*. (2001) [14] referred to a lack of association between -174 and -597 SNPs and susceptibility to MS which was consistent with our present results. Izad *et al*. (2010) [25] failed to establish the association between $IL6_{-174}$ SNP and MS and also there was no significant difference in genotype/allele frequencies among different MS courses. An interesting study showed a significant association between $IL6_{-572}$ *C* allele and MS progression but failed to find any association

between *IL6*$_{-174, -597}$ SNPs and susceptibility to MS [36]. Another association study showed a correlation between *G* allele of -174 SNP and MS development which was suggested to be strengthen by *HLA-DRB11501** allele [37]. Two research papers published in 2010 that studied two different populations indicated the lack of association between development of relapsing-remitting MS patients and *IL6*-174 G/CSNP [17, 26].

Unlike previous studies, CC genotype of -174 G/C SNP was suggested to be a risk factor for MS predisposition in Polish population [16]. Our current findings indicate a strong linkage disequilibrium between -174 and -597 SNPs (D` = 0.8 and LOD = 13) and this was compatible with Fedetz *et al*. (2001) [14]. None of the *IL6* pairwise haplotypes of SNPs showed any association with MS risk in this sample of Iraqi population and this was consistent with Yan *et al*. (2012) findings [36].

However, these contradictory observations derived from various studies might be explained by differences in the sample size used and the discrepancies among individuals in the context of genetic makeup, environmental factors, and ethnicity.

CONCLUSION

Since multiple sclerosis is an "illusive" disease that affects mostly young adults, we sought to establish an association between some genetic epidemiological risk factors and predisposition to multiple sclerosis in Iraqi population and this is the first genetic study in Iraq in this regard. In spite of the small sample size, we could put a spotlight on the association between some pro-inflammatory cytokine gene variations and MS. The study revealed that heterozygous genotype and *A* allele of *TNFA* SNP at promoter locus -238 might comprise risk factors in Iraqi population. Further genetic studies with larger sample size and various Iraqi ethnic groups should be accomplished for best evaluation of the effect of these variations on gene expression in MS patients.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

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