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The Influence of Single Nucleotide Polymorphism of Tumor Necrosis Factor Alpha Gene on The Response to Etanercept in Iraqi Rheumatoid Arthritis Patients

Basman F. Abbas[1] *MSc*, **Nidhal A. Mohammad**[2] *PhD*, **Hassan M. Naif**[3] *PhD*

[1]Dept. of Molecular and Medical Biotechnology, College of Biotechnology, Al-Nahrain University, Baghdad, Iraq, [2]Dept. of Medical Technical, Al-Farahidi University, Baghdad, Iraq, [3]Dept. of Medical Laboratories Technology, College of Health and Medical Technology, AlShaab University, Baghdad, Iraq

Abstract

Background Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease that is characterized by the presence of autoantibodies and joint inflammation, which consequently leads to articular cartilage damage. Due to the tumor necrosis factor-alpha (TNF-α) crucial role in the pathogenesis of RA, TNF-α inhibitors have revolutionized this disease treatment.

Objective To evaluate the influence of the single nucleotide polymorphism (SNP) of TNF-α on the response to etanercept in RA patients.

Methods The study comprised of 60 RA patients and 25 healthy individuals. The age range of the participants was between 20 and 69 years. DNA sequencing was carried out by using conventional polymerase chain reaction (PCR) and sequencing. The blood samples were taken from the RA patients at the baseline before the administration of anti TNF-α therapy. The level of TNF-α in the sera was measured using an enzyme-linked immunosorbent assay (ELISA).

Results The TNF-α-308 G/G genotype was the most prevalent in Iraqi RA patients (78%), while there was no significant difference of TNF-α-308 G/A between responders and non-responder group. TNF-α -308 G/G or G/A genotype have no influence on the response to etanercept. The results indicate that the level of TNF-α was significantly different ($P < 0.05$) between RA patients (190.82±12.23 SEM pg/ml) and the control group (106.62± 4.15 SEM pg/ml), while there was no significant difference of the level of TNF-α between responders (188.53 ±15.81 SEM pg/ml) and non-responders (194.25±19.45 SEM pg/ml).

Conclusion The level of TNF-α was elevated in RA patients in comparison to healthy individuals. The level of TNF-α at baseline have no influence in the response to etanercept in RA patients. TNF-α-308 G/G or G/A gene polymorphism has no influence on the response to etanercept.

Keywords Rheumatoid arthritis; TNF-α; Etanercept, TNFR, ELISA, PCR.

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List of abbreviations: ELISA = Enzyme-linked immunosorbent assay, HWE = Hardy-Weinberg equation, OD = Optical density, OR = ODD ratio, PCR = Polymerase chain reaction, RA = Rheumatoid arthritis, SEM = Standard error of the mean, SNP = Single nucleotide polymorphism, sTNF-α = Soluble Tumor necrosis factor-alpha, TNF-α = Tumor necrosis factor-alpha, TNFR1 = Tumor necrosis factor receptor 1, TNFR2 = Tumor necrosis factor receptor 2

Introduction

Rheumatoid arthritis (RA) is a type of chronic disease which leads to human joint damage because of an autoimmune abnormality [(1)]. However, the prognosis of RA patients in the last decades has

been enhanced due to the knowledge expansion of the disease pathophysiology and etiology which lead to development of main active drugs [2]. RA can be generically developed by main risk factors, host and environmental factors. Host factors can be divided into genetic, epigenetic, neuroendocrine, reproductive, hormonal and comorbid host factors. While environmental risk factors comprise airborne exposures, smoking, infectious agents, microbiota, diet and socioeconomic factors [3]. The pathogenesis and the causation of RA are complicated, which require non-conventional therapeutic methods that involve the application of more than a single pharmaceutical intervention and it is highly dependent on the patient and the progression stage of the disease [4]. The B cells immunopathogenic role during RA onset represents by promote the proliferation, activation and differentiation of the other cells like T cells, osteoclasts in the synovium and monocytes by providing autoantibodies, cytokines and other mediators [5].

Tumor necrosis factor-alpha (TNF-α) is a cytokine that has pleiotropic effects on various cell types [6]. The diverse effects of TNFα depend on its two receptors, TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2), and their distinct downstream signaling pathways [7].

The RA drug can be generally classified into three main categories, which include nonsteroidal anti-inflammatory drugs, glucocorticoid and non-biological (synthetic origin)/biological disease-modifying anti-rheumatic drugs [4]. Polymorphic variants of the gene encoding TNF-α, like 308G/A (rs1800629) is associated with TNF inhibitor (Etanercept, Infliximab, and Adalimumab) sensitivity [8]. Etanercept is a biological inhibitor of TNF-α, which acts as a soluble TNF receptor and binds TNF-α and TNF-β. Etanercept structure consists of two TNFR2 fused to the human IgG fragment crystallizable portion [9].

This study aimed to investigate the influence of rs1800629 and TNF-α serum level at baseline with the response to etanercept in RA patients.

Methods

Sample Collection

Blood samples were collected from 60 RA patients aged between 20-69 years old, they were diagnosed and treated at least for one year in the RA Clinics, Teaching Hospital in Medical City of Baghdad and 25 unrelated healthy individuals through the period from September 2022 to March 2023. The sex of the 60 patients were 12 Males and 48 Females, who were chosen at baseline (before the administration of biological anti TNF therapy) and followed up for 6 months.

Genetic analysis

A total of genomic DNA was extracted from 85 frozen blood samples. DNA extraction was carried out by using a genomic DNA extraction kit supplied by Favorgen (Taiwan). Polymerase chain reaction (PCR) was carried out by using SimpliAmp thermal cycler, Applied biosystems (Singapore) in a total volume of 25 µl to amplify the single nucleotide polymorphism (SNP) flanked region which was 369 bp. The primers used and the reaction components are indicated in table (1).

Table 1. Primers and reagents used in PCR to amplify TNFα rs1800629 flanked region

Component	Volume (µl)
Master Mix: *Taq*DNA Polymerase, dNTPs, MgCl2, and reaction buffer.	12.5
Forward Primer (CCCTCCCAGTTCTAGTTCTAT) (Tm 55.5 °C)	1
Reverse Primer (GAAAGAATCATTCAACCAGCG) (Tm 55.8 °C)	1
DNA Template	4
Distilled sterile water	6.5
Total	25

Determination of human TNF-α by enzyme-linked immunosorbent assay (ELISA)

TNF-α level were measured by using an ELISA kit from Wuhan USCN (China). According to the instruction's manual, the test was done. The microplate provided in this kit has been pre-coated with an antibody specific to TNF-α. By using (HumaReader HS, Germany), the optical density (OD) was measured at a wavelength of 450 nm. The OD of the samples was compared to the standard curve to determine the concentration of TNF-α in the tested samples.

Statistical analysis

Chi-square (χ2) and Hardy-Weinberg equation (HWE) were calculated to determine whether the study samples followed normal distribution or not. The influence of the polymorphism -308G/A on the response to Etanercept was determined according to the ODD ratio. Means and standard error of the mean (SEM) were calculated for continuous variables. The t-test was used to find the variation among study groups. P value lower than 0.05 was considered as statistically significant value.

Results

The age of patients ranged between 20-69 years, with an average of (48±1.35 years). The average of the 36 responders age was (47±2.08 years) with the highest percentage (28%) in the range (50-59 year), while the age average of 24 non-responders' patients was (50±1.37 years) with the highest percentage (54%) of age (50-59 year) as shown in the figure (1).

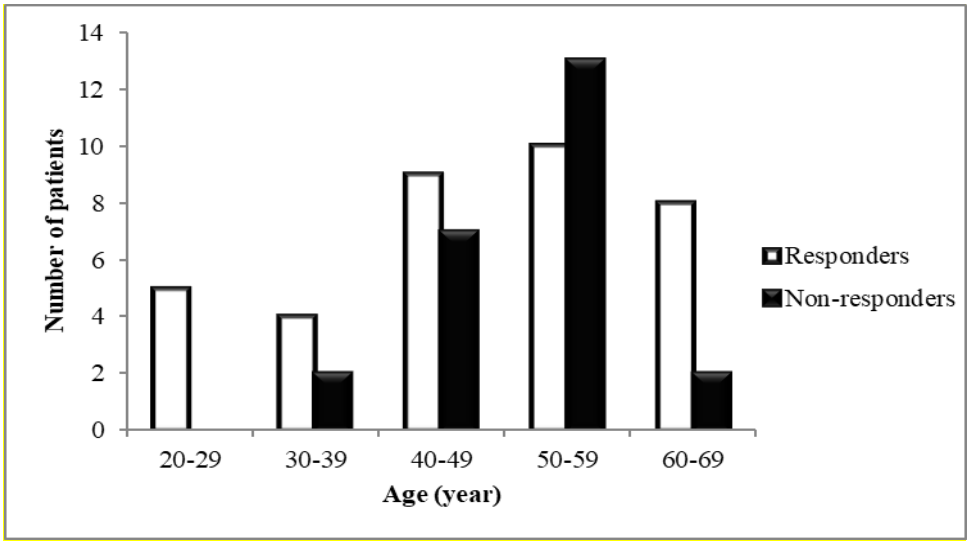

Figure 1. Age distribution of respondent vs. non-respondent rheumatoid arthritis patients

Molecular results

Genomic DNA extraction

The concentration of DNA obtained after extraction was range between 80-150 ng/µl with purity ranged from 1.8-2. This concentration and purity were matched the recommendations for PCR technique [10]. The DNA was electrophoresed on agarose gel (0.7%) as shown in figure (2).

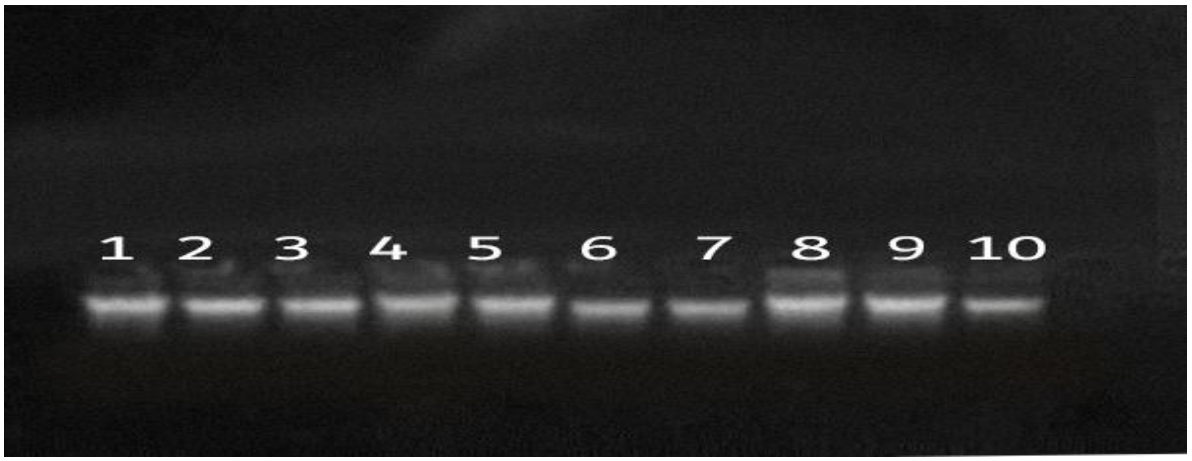

Figure 2. Gel electrophoresis on agarose gel (0.7%) of genomic DNA of the control and rheumatoid arthritis patents for 1 hour at 5 v/cm^2

Amplification of SNP region

This region was amplified by using specific primers and optimum conditions. The illustrated results in figure (3) showed a clear band with (369 base pairs) after electrophoresis of PCR products on agarose gel (2%). These fragments represent the target region in the promoter of the TNF-α gene, which contains rs1800629 SNP.

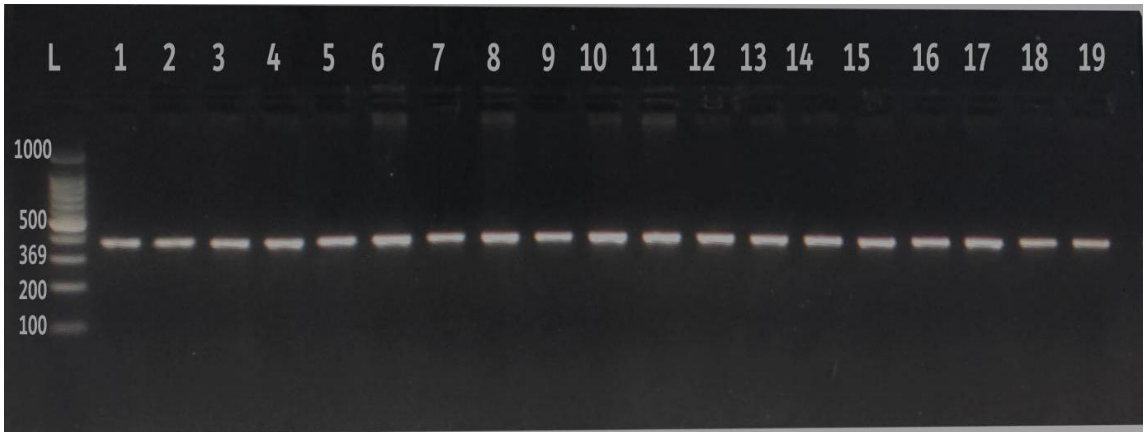

Figure 3. Gel electrophoresis of PCR products of the specific TNF-α promoter region that contain rs1800629 SNP on agarose gel (2%) for 1 hour at 5v/cm^2 volt in the presence of 1 kb DNA Ladder marker

Sequence results

Sanger sequencing method was used to achieve the sequence of amplified PCR products. The sequencing of the amplification products of the flanked regions of rs1800629 were achieved by Macrogen/USA. Then, the sequences of these products were compared with the reference data in the NCBI's GenBank for the TNF-α gene.

Hardy-Weinberg equation was used to evaluate normal distribution following by control and RA patients as shown in table (2).

Table 2. Results of the HWE for the TNF-α polymorphism rs1800629 G/A between rheumatoid arthritis patients and controls

Groups	NO.	GG (%)	GA (%)	AA (%)	χ^2	P value
RA patients	Observed	46 (76.7%)	14 (23.3%)	0	5.02	0.025
	Expected	(85%)	(15%)	0		
Control	Observed	20 (80%)	5 (20%)	0	0.127	0.72
	Expected	(77%)	(23%)	0		
Total Observed		66	19	0		

The results showed that the RA patients' group had a P value <0.05, which indicated that RA patients were out of normal distribution and inconsistent with HWE. While the controls group had a p value>0.05, which indicated that controls group are within the HWE. The genotyping and allele frequency for the TNF-α polymorphism rs1800629 G/A among RA patients and controls is illustrated in table (3).

It revealed the comparison between RA patients and a healthy control group regarding genotyping and allele frequency for the TNF-α polymorphism rs1800629 G/A. The heterozygous GA in RA patients had OR = 1.28 (0.3862 to 3.8378), p value > 0.05), which indicated no significant risk (1.28 folds) of the GA genotype compared to wild type GG. While in the AA homozygous was not appeared in both RA patients and controls group, which revealed that the AA genotype at rs1800629 may be not found in Iraqi population. Allele frequency of allele A in RA patients was slightly higher than controls group, but mutant allele A did not show a significant result (p-value >0.05).

The genotyping and allele frequency for the TNF-α polymorphism rs1800629 G/A among responders and non-responders' group is illustrated in Table (4).

The results shown in Table (4) revealed the comparison between responders and non-responders' group regarding genotyping and allele frequency for the TNF-α polymorphism rs1800629 G/A. The heterozygous GA in the non-responders' group had OR= 0.78 (0.2283 to 2.7304), P value >0.05), which indicated no significant influence (0.78 folds) of the GA genotype on the response to etanercept. Allele frequency of allele A in RA patients was slightly higher in the responders than non-responders' group, but mutant allele A did not show a significant effect on the response to therapy (P value >0.05).

Table 3. Genotypes and allele frequency results for the TNF-α polymorphism rs1800629 G/A between rheumatoid arthritis patients and controls

rs1800629	Frequencies (%) Control (*n* = 25)	Frequencies (%) RA patients (*n* = 60)	P value	Odd ratio (95% CI)
GG (Wild)	20 (80%)	46 (76.6 %)	---	1.00 (Reference)
GA	5(20%)	14 (23.3%)	0.7370	1.28 (0.3862 to 3.8378)
AA	0	0	0	0
Allele frequency				
G	45 (90%)	106 (88.3%)	---	1.00 (Reference)
A	5 (10%)	14 (11.6%)	0.7536	1.89 (0.4041 to 3.4969)

Table 4. Genotypes and allele frequency results for the TNF-α polymorphism rs1800629 G/A between responders and non-responders' group

rs1800629	Frequencies (%) Responders (*n* = 36)	Frequencies (%) Non-responders (*n* = 24)	P value	Odd ratio (95% CI)
GG (Wild)	27 (75%)	19 (79 %)	---	1.00 (Reference)
GA	9(25%)	5 (21%)	0.7	0.78 (0.2283 to 2.7304)
AA	0	0	0	0
Allele frequency				
G	63 (88%)	43 (89.5%)	---	1.00 (Reference)
A	9 (12%)	5 (10.5%)	0.72	0.8 (0.2552 to 2.5963)

Immunological biomarkers

Level of human TNF-α at baseline tested by ELISA

The level of TNF-α in the serum at baseline was measured for each RA patients and healthy control group using ELISA. The results revealed that the level of TNF-α was significantly difference (P <0.05) between RA patients (190.82±12.23 pg/ml) and the control group (106.62±4.15 pg/ml) as shown in figure (4). While there was no significant difference of the level of TNF-α between responders (188.53±15.81 pg/ml) and non-responders (194.25±19.45 pg/ml), as shown in figure (5).

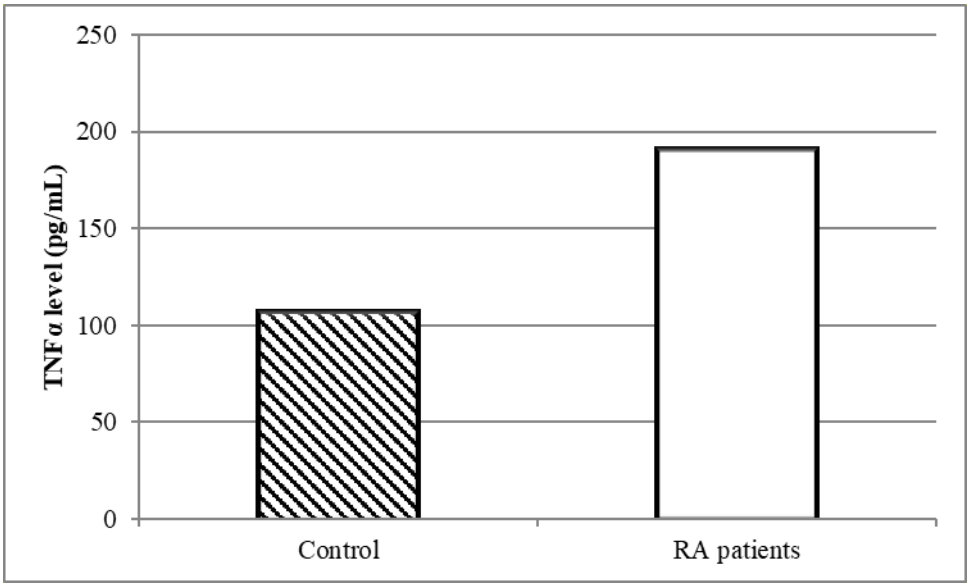

Figure 4. Level of TNF-α of control vs. rheumatoid arthritis patients

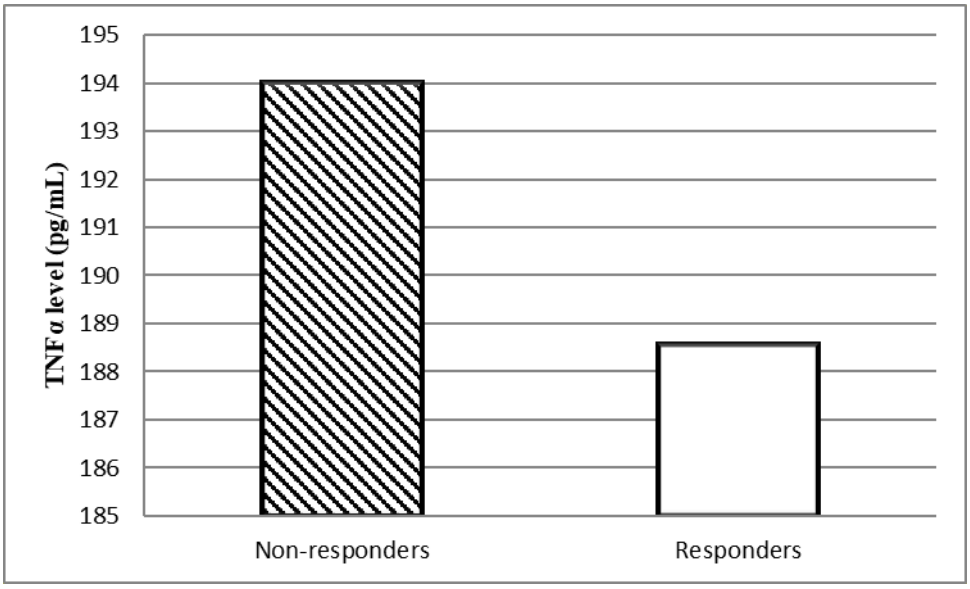

Figure 5. Level of TNFα of respondents vs. non-respondents' rheumatoid arthritis patients

Discussion

Polymorphisms have been associated with numerous diseases in genetic research [11]. The ethnicity and country of a certain population affect the prevalence of each polymorphism. Studying the distribution of polymorphism may give an early biomarker of susceptibility and/or progression of diseases [12]. This study indicated that the GG genotype at rs1800629 was most prevalent in Iraqi control and RA patients (about 80%). While the GA genotype compromised 20% and the AA genotype was absent in control and RA patients. The genotype distribution frequency in this study was similar to other studies [13,14]. Current results indicate that the G/A polymorphism does not correlates with RA risk factor in Iraqi individuals, which is agree with other studies that showed that the TNF-α rs1800629 G/A polymorphism could possibly represent an

important RA risk factor in Latin Americans but not in Arab, European or Asian populations [14,15]. Some studies have found that no association of TNF-α -308G/A polymorphisms with susceptibility to RA [16-18]. The genotypes GG and GA have no significant effect on response to etanercept in our study. While other study reported that the response to TNF-α inhibitors can be predicted by the presence of TNF-α -308 G/A [19,20], and the response failure was more often in RA patients having the GA genotype instead of GG genotype. The difference in obtained results in our study and other studies may be influenced by population ethnic diversity and the number and sex of the patients.

This study indicates that the level of TNF-α elevated in RA patients in compare with control as it was reported by many researchers [21-23]. TNF-α plays a crucial role in the regulation of RA development. The expression of TNF-α is heightened in patients with RA, and the excessive expression of TNF-α leads to the development of autoimmune arthritis in transgenic animals [24,25]. The production of the TNF-α precursor molecule occurs mostly in the form of a transmembrane protein known as mem TNF-α. Subsequently, this protein is cleaved by a metalloproteinase, namely TNF-α converting enzyme, resulting in the liberation of soluble TNF-α (sTNF-α) [26]. TNF-α signaling has multiple roles in the development of RA. It causes the release of inflammatory cytokines like interleukin (IL)-6, IL-1, and TNF-α by activating endothelial cells and drawing in synovial fibroblasts and macrophages which lead to overexpression of TNFα in the RA patients [25,26]. In addition, mass cytometry and single-cell transcriptomics studies have shown that T cells and B cells are the chief sources of TNF-α in RA-affected synovium, which contributes to raising the TNF-α level [26,27].

The level of TNF-α in the responders' group was slightly less than in the non-responders group. Although there is no significant difference between the level of TNF-α between the two groups. So it may be that the level of TNF-α at baseline have no influence on the response to Etanercept.

In conclusions, there is no correlation between TNF-α gene polymorphism -308 G/A with the response to Etanercept. The serum level of TNF-α at baseline have no influence to response to etanercept.

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Author contribution
Abbas: Methodology, investigation, formal analysis and writing draft. Dr. Mohammad and Dr. Naif: Project idea and planning, supervision, writing reviewing and editing.

Conflict of interest
The authors have no conflict of interest.

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Correspondence to Basman F. Abbas
E-mail: basman_fadhel@yahoo.com
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