Original Article

Assessment of Interleukin-8 in Bronchial Asthma in Iraq

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Abstract

It has been approved that neutrophils are responsible for many inflammatory lung diseases, such as acute respiratory distress syndrome, chronic obstructive pulmonary disease, and asthma. It is well documented that the CXC chemokine interleukin-8 (IL-8) plays a key role as a potent neutrophil recruiting and activating factor. Asthma is one of the most common major non-contagious diseases and has a substantial impact on the patient's quality of life. The current evidence suggests that asthma is a complex multifactorial disorder, and its etiology is increasingly attributed to interactions between genetic susceptibility, host factors, and environmental exposures. IL-8 plays an important role in respiratory diseases and is a known regulator of pulmonary inflammation and immunity, induced phagocytosis, and promoted angiogenesis. This study aimed to investigate the IL-8 gene expression in blood samples of bronchial asthma patients. Therefore, the blood samples were taken from two groups of participants, including the group of patients with asthma (n=100) in the age range of 20-61 years and the group of healthy individuals (n=50). The obtained results indicated that the expression of IL-8 mRNA in the group of asthma patients was three times higher than that in the group of healthy individuals. Therefore, it is suggested that the antagonism of IL-8 could be a potent therapeutic strategy in the treatment of asthma.

Keywords: Bronchial asthma, Gene expression, IL-8, qRT-PCR, RT-PCR

1. Introduction

Bronchial asthma is one of the most common non-communicable diseases and has a substantial impact on the patient's quality of life. Bronchial asthma is defined as a disorder in which the person's airways constrict and swell and cause him/her to cough up more mucus (1). This condition is characterized by variable and recurring symptoms, reversible airflow obstruction, and easily triggered bronchospasms. Symptoms include episodes of wheezing, coughing, chest tightness, and shortness of breath which may occur from a few times a day to a few times per week. Asthma symptoms may become worse at night or with exercise, depending on the person (2). Asthma is thought to be caused by a combination of genetic and environmental factors, such as exposure to air pollution and allergens (3). Other potential triggers include such medications as aspirin and beta-blockers (4-6).

Diagnosis is usually based on the pattern of symptoms, response to therapy over time, and spirometry testing of pulmonary function (7). Around 300 million people are suffering from asthma worldwide, and it is likely that a further 100 million may be affected by 2025 (8). This increase in asthma prevalence could be influenced by identification of milder asthma cases. Asthma is not a curable disease;

therefore, the ultimate goal of asthma management is to relieve symptoms, prevent the disease progression, and obtain a better quality of life (9).

Interleukin-8 (IL-8) or chemokine (CXC motif) ligand 8, (CXCL8) is a chemokine produced by macrophages and other cell types, such as the epithelial cells, airway smooth muscle cells, and endothelial cells (10). Endothelial cells store the IL-8 in their storage vesicles, the Weibel-Palade bodies (11). In humans, the IL-8 protein is encoded by the CXCL8 gene (12, 13). Many studies focused on the role of the IL-8 gene in association with the development of asthma (14). As an important upregulated cytokine for airway inflammation, IL-8 is mainly secreted during acute attacks of asthma and an inflammatory reaction in clinical remission (15). Moreover, IL-8 plays an important role in respiratory diseases and is a known regulator of pulmonary inflammation and immunity. With chemotaxis and activation effects on neutrophils, IL-8 leads to neutrophil accumulation in the airways of patients with BA (16-18).

This study aimed to detect the IL-8 gene expression in the blood sample of patients with bronchial asthma. The available methods for measuring IL-8levels are direct and quantitative real-time reverse transcription Polymerase Chain Reaction (qRT-PCR).

2. Material and Methods

This study was conducted from October 2020 to May 2021. All the study experiments were performed at the University of Technology and Iraqi Hereditary company (IHC), in Baghdad, Iraq.

2.1. Study Groups

The total number of participants in the study was 150 individuals who were assigned into two groups. Group 1 included 100 blood samples from Iraqi men and women in the age range of 20-61 years who were diagnosed with asthma. The blood samples were collected at the Specialist Centre for Allergy (Al-Russafa, Baghdad, Iraq), and the clinical information of participants was obtained from their hospital files and case-sheet records. Group 2 included50blood samples from apparently healthy individuals of both genders in the age range of 24-64 years that were considered as controls in this study (Table 1).

2.2. Blood Sampling

The blood samples were collected were from October2020 to January 2021. In total, 3 ml of venous blood was obtained from each participant and was directly collected into an EDTA-containing tube. This procedure was performed under aseptic conditions. The blood samples were immediately frozen at −20°C and stored for further analysis.

2.3. Total RNA Extraction from Blood Samples

The RNA was extracted from whole blood samples of patients and healthy controls using the TransZolUp Plus RNA Kit (19).

2.4. cDNA Synthesis from mRNA

Total RNA was reversely transcribed to complementary DNA (cDNA) using EasyScript®One-Step gDNA Removal and cDNA Synthesis Super Mix Kit (TransGen, China) (20, 21). The procedure was carried out in a reaction volume of 20 μl, according to the manufacturer's instructions. The total RNA volume for reverse transcription was 20μl. Thermal cycler steps of cDNA reverse transcription conditions are presented in table 2. Primers and probes used in this study and their sequences are tabulated in table 3.

Table 1. Age distribution of study groups

Age Groups (year)	Control No. (%)	Patients No. (%)
>30	5 (10%)	28 (28%)
30-40	22 (44%)	30 (30%)
>40	23 (46%)	42 (42%)

Table 2. Thermal cycler steps

	Step 1	Step 2	Step 3
Temperature	$25\,^{\circ}$c	$42\,^{\circ}$c	$85\,^{\circ}$c
Time	10min	15min	5sec
Reaction	Random Primer (N9) binding	Anchored Oligo(dT)18 binding	Inactivate reverse transcriptase enzyme

Table 3.Primers

Primer/probe	Sequence (5′→3′ direction)
Forward	GAGAGTGATTGAGAGTGGACCAC
Reverse	CACAACCCTCTGCACCCAGTTT
GAPDH house-keeping gene	
Forward	TGAGAAGTATGACAACAGCC
Reverse	TCCTTCCACGATACCAAAG

2.5. qRT–PCR

The qRT-PCR test was performed using the Stratagene Real-time PCR System (Analytik Jena Technologies) with qPCR software (22). The gene expression levels and fold changes were quantified through the measurement of the threshold cycle (Ct) using the 2xqPCR Master Mix Kits (Trans Gen, China) (23, 24).The components are presented in table 4. Every reaction was performed twice and included a non-template control, non-amplification control, and non-primer control (NPC) as the negative controls.

2.6. The qRT-PCR Reaction Run

The cycling protocol was programmed according to the thermal profile presented in table 5.

2.7. Housekeeping Gene Amplification

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene was used as an internal control in the calculation of the ΔCT value. A qRT-PCR reaction for GAPDH amplification was conducted with the thermal profile shown in table 6.

2.8. qRT-PCR analysis of IL-8 gene expression
2.8.1. ΔCT

The expression ratio was calculated without a calibrator sample $2^{-\Delta Ct}$, according to the following equation:

ΔCT (test)= CT gene of interest (target, test) – CT internal control

Eventually, the expression ratio was calculated according to the following formula:

$2^{-\Delta Ct}$ = Normalized expression ratio

2.8.2. ΔΔCT

The $2^{-\Delta\Delta Ct}$ method was used to compare the transcript levels between different samples (25). The CT of the gene of interest was normalized to that of the internal control gene. The difference in Ct values between the housekeeping gene (internal control gene) and IL-8 gene (interest gene) was calculated, according to the following formula:

ΔCT (test) = CT gene of interest (target, test) – CT internal control

ΔCT (calibrator) = CT gene of interest (target, calibrator) – CT internal control

Table 4. Components of the qRT-PCR used in IL-8 gene expression experiment

Component	Volume
2× PerfectStart™ Green qPCR SuperMix	10 µl
Forward primer	2 µM
Reverse primer	2 µM
cDNA	3 µl
Nuclease free water (N.F.W)	3 µl
Total volume	20 µl

Table 5. Thermal profile of IL-8 gene expression

		Temperature	Time	cycle
Stage 1	Denaturation	94ºC	30 Sec	1
Stage 2	Denaturation	94ºC	5 Sec	
	Annealing	64ºC	15 Sec	40-45
	Extension	72ºC	10 Sec	
Stage 3	Dissociation	72ºC	1 m	1

Table 6. Thermal profile of GAPDH gene expression

Step	Temperature	Duration	Cycles
Enzyme activation	95ºC	30 Sec	Hold
Denature	95ºC	5 Sec	40
Anneal/extend	60ºC	40 Sec	
Dissociation	95 ºC 55 ºC 95 ºC	1min 30 sec 30sec	

3. Results and Discussion

The distribution of patients' age range showed that 42 patients (42%) were in the age group >40 years (Table 7). However, age range distribution reflects a generalized increase of asthma in older patients. Individuals in all study groups were randomly selected; therefore, the differences in the age distribution are not significant (P=0.0074).

The results of the current study were in line with those of previously published studies which demonstrated that asthma prevalence increased in old individuals due to airway responsiveness and lower levels of lung function (28).

Table 7. Age distribution of patients' group

Age groups (year)	Patients No. (%)	P-value
>30 yr.	28 (28.00%)	0.0074 **
30-40 yr.	30 (30.00%)	
>40 yr.	42 (42.00%)	
Mean ± SE	38.45 ±1.17	0.184 NS

** (P≤0.01), NS: Non-Significant

The Ct value of GAPDH, the housekeeping gene used in the present study is shown in table 8. The mean±SD of Ct value for GAPDH in the healthy group and the asthma patients group was determined at 24.676±2.85and 24.679±3.08, respectively. A significant difference was found between these groups regarding the mean Ct value of GAPDH, (P=0.844;P<0.05) and an LSD value (5.219).

The inalienable supposition in the utilization of housekeeping genes in atomic examinations is that their appearance stays steady in the cells or tissue being scrutinized (29).

Barber, Harmer (30) studied the expression of 1,718 genes using qRT-PCR, and they applied the GAPDH as a reference gene in 72 kinds of normal human tissue (30). They found that the use of the GAPDH gene results in a reliable normalization of gene expression data using qRT-PCR when applied in clinical studies.

To improve this and despite the fact that there was a critical contrast in the mean Ct esteem between bunches in the current examination, the variety of complete change in articulation of GAPDH was concentrated in various investigation bunches using the 2^{-Ct} value and the ratio of 2^{-Ct} (Table 9).The 2^{-Ct} value was obtained at3.731E-08and 3.723E-08 in the groups of healthy individuals and patients, respectively. The computed ratio for gene fold expression was estimated at 1.000 and 0.997 for the healthy individuals and patients' groups, respectively. These little varieties in gene overlap articulation between the study groups render the GAPDH gene as a valuable control gene.

Table 8. Comparison of different groups in terms of the mean±SD of Ct value of GAPDH

Group	No.	Mean±SD of Ct value	Range
Group 1 Patients	100	24.679±3.08	23.18-25.82
Group 2 Healthy	50	24.676±2.85	23.58-25.93
LSD		5.219 NS	
P-value		0.844	

NS: Non-Significant

Table 9. Comparison of GAPDH Fold expression between the study groups

Group	Means Ct of *GAPDH*	2^{-Ct}	Experimental group/ Control group	Fold of gene expression
Group 1 Patients	24.679	3.723E-08	3.723E-08/3.731E-08	0.997±0.216
Group 2 Healthy	24.676	3.731E-08	3.731E-08/3.731E-08	1.00± 0.00
T-test				0458 NS
(P-value)				0.794

NS: Non-Significant

Each quantification PCR reaction was run in triplicate for each sample. Samples from the healthy and asthma groups were used in each run. Plots of each run (i.e., amplification plots and dissociation curves) were recorded as well. Figures 1, 2, 3, and 4 in the appendix present the amplification plots and dissociation curves for GAPDH andIL-8, respectively.
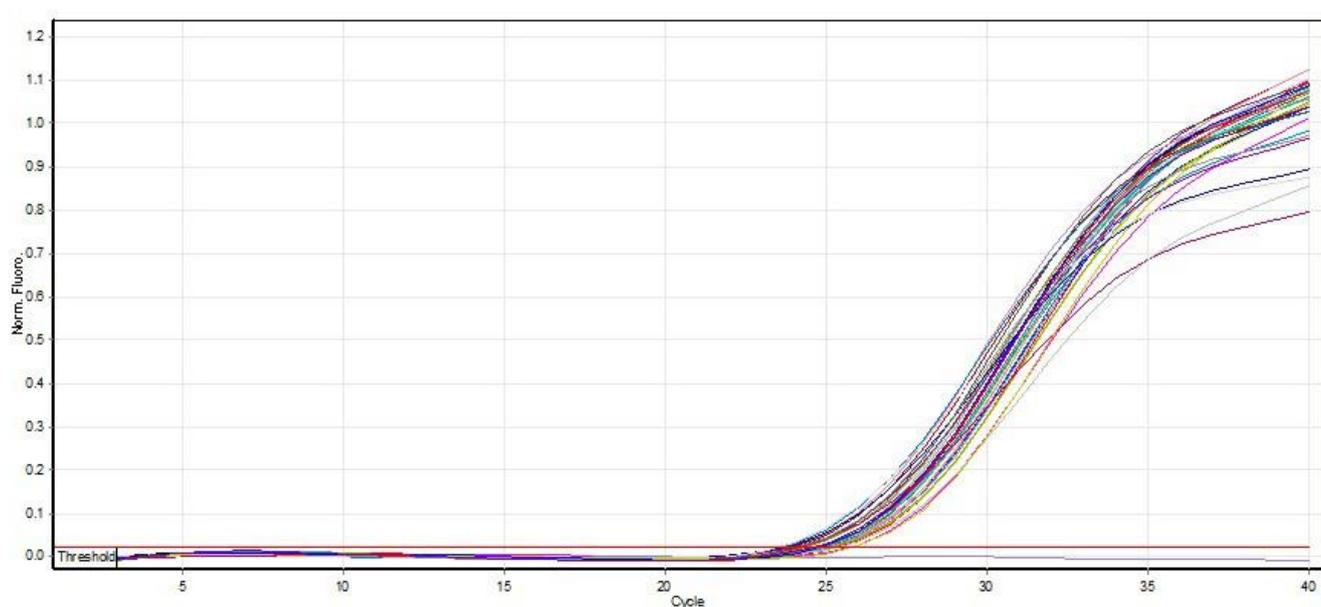

Figure 1. GAPDH amplification plots by qPCR Samples included all study groups. Cycle threshold values ranged from 23 to 25. The photograph was taken directly from the Qiagen rotor Gene qPCR system.
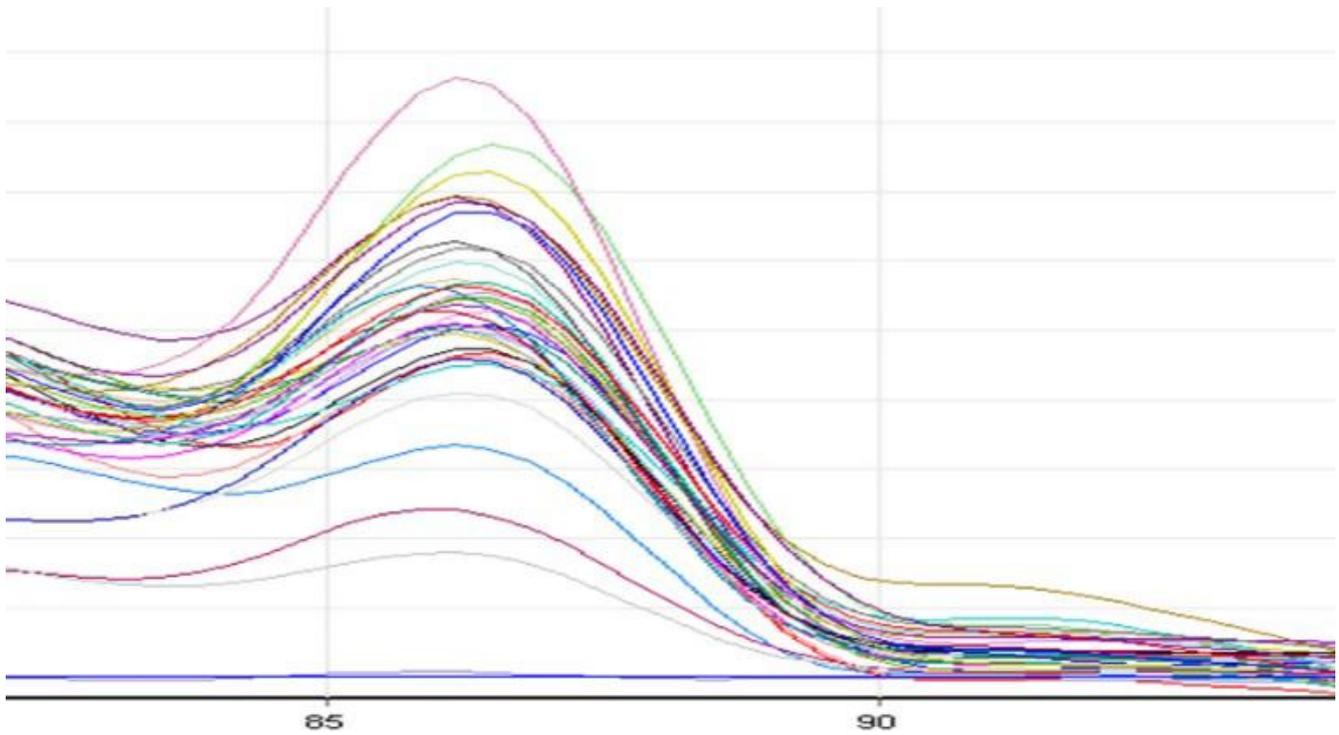
Figure 2. GAPDH dissociation curves by qPCR Samples included all the study groups. Melting temperature ranged from 86°C to 87°C. The photograph was taken directly from the Qiagen Rotor-Gene qPCR system.
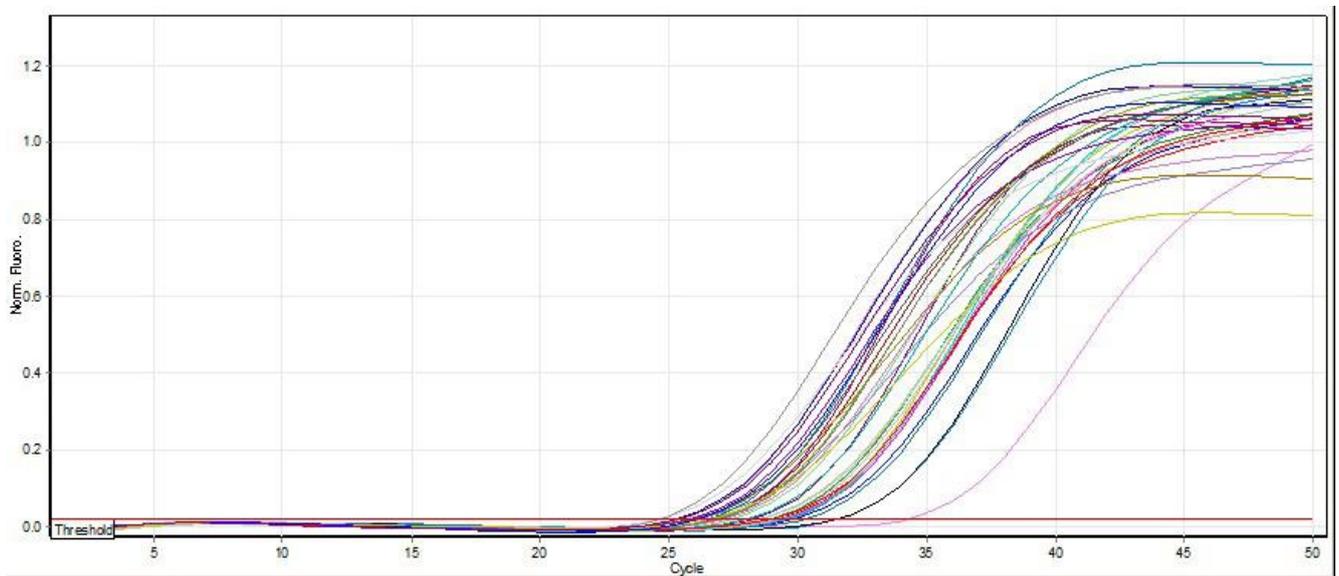

Figure 3. IL-8 amplification plots by qPCR. Samples included those taken from the healthy study group. Cycle threshold values ranged from 21.97 to 23.75. The photograph was taken directly from the Qiagen Rotor-Gene qPCR system.
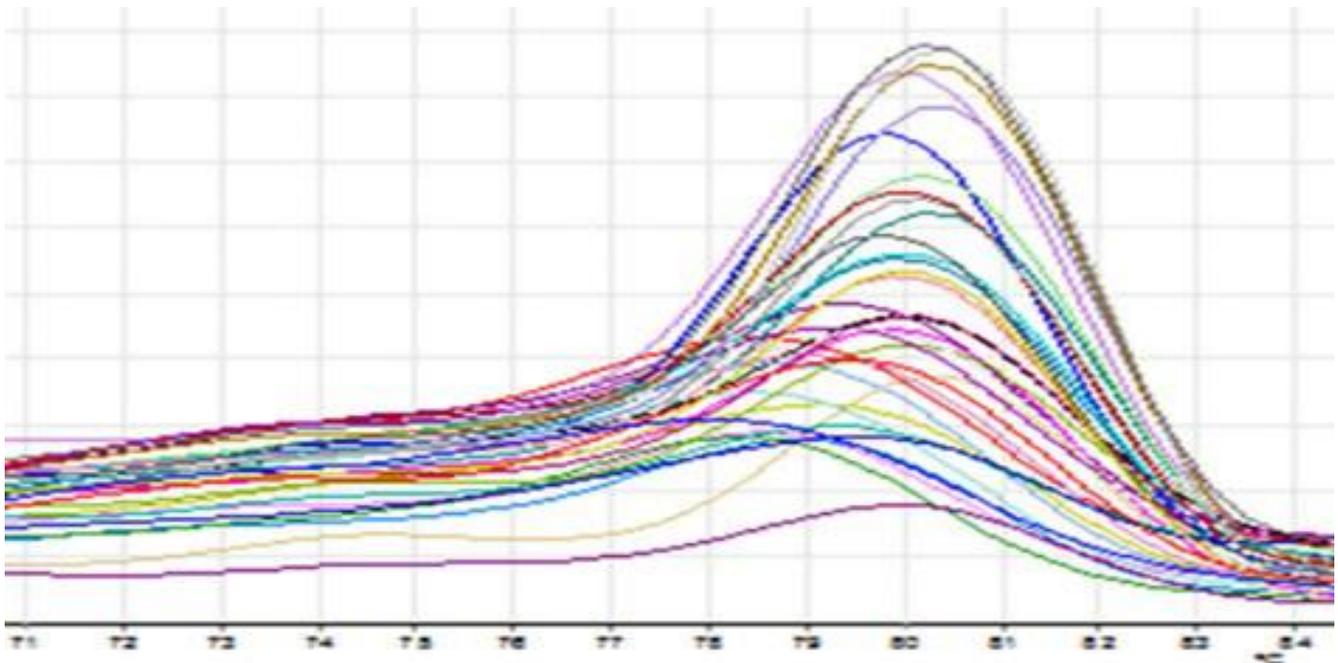
Figure 4. IL-8 dissociation curves by qPCR Samples included a healthy study group. Melting temperature ranged from 80.0°C to 81.0°C. The photograph was taken directly from the Qiagen Rotor gene qPCR machine.

The Ct values of IL-8 cDNA amplification ranged from 21.97 to 23.75 in the healthy group and from 19.42 to 20.62 in the patients' group. The mean Ct values in the asthma group were higher than those in the healthy group. This is important in reflecting the original mRNAs which are present in the samples. It is evident from these results that the patients' group is associated with the highest copy number of mRNAs that is indicative of its higher expression. This study aimed to investigate the potential effect of IL-8 polymorphism on genetic damage and asthma risk. These results are consistent with those reported in previously published studies (31, 32).

In the current study, the mRNA expression of IL-8 was analyzed using qRT-PCR assay, and the results were compared between the groups of healthy controls and asthma patients.

The gene expression fold changes were calculated through relative measurement. This depends on the normalization of the Ct values and calculation of the ΔCt which is the difference between the mean Ct

values of a replica of IL-8 cDNA amplification for every single case and that of the GAPDH. Table 10 shows the mean of ΔCt (normalization Ct values) in each study group. Moreover, the ΔCt means in the groups of healthy individuals and asthma patients were obtained at 2.941 and 4.634, respectively. A significant difference was noticed between the study groups (P=0.0001). The mean of the 2^{-Ct} value of the patients' group (1.130) was significantly higher, compared to the 2^{-Ct} value of the healthy group (0.040).

The gene expression folds were calculated in relation to the housekeeping genes. Furthermore, the result of $2^{-\Delta Ct}$ of each group was measured in relation to that of controls. The results are tabulated in table 11. The fold of gene expression in the patients' group was 3 times higher than that in the healthy group. These results indicated a significant increase in the expression of the IL-8 gene in the patients' group.

The relative expression of the IL-8 gene in all study groups was calculated using the $2^{-\Delta\Delta Ct}$ method. The mean of $2^{-\Delta\Delta Ct}$ values of the healthy group was 59.54,

while it was 192.53in the asthma patients group (Table 9). Therefore, there was a significant difference between these two groups in terms of the mean $2^{-\Delta\Delta Ct}$ value (P=0.0001).

The study groups were divided into two subgroups of "high expression", in which the fold change of gene expression was above 1, and "low expression" in which the fold change was lower than 1. It is well documented here that the high expression was evident in the asthma group, in comparison with the healthy group. The induction of IL-8gene expression is apparently due to asthma itself. There was a statistically significant difference between the study groups (P=0.0001). This suggests the importance of the obtained results in showing high expression of the IL-8 gene in the asthma group which confirms the working mechanism of the IL-8 gene. The researchers believed that IL-8wasinvolved in the asthma attack progression. Therefore, it is possible to consider the high expression of the IL-8gene as a marker for severe asthma. The IL-8 in the serum sample can be used as a marker that assists in diagnosing bronchial asthma based on the results of the interleukin concentration obtained through the ELISA test. High IL-8 gene expression is present in the bronchial asthma group. This reflects a high DNA damage and warrants follow-up.

Table 10. Fold of IL-8 expression depending on the $2^{-\Delta\Delta Ct}$ method

Groups	Means Ct of IL8	Means Ct of GAPDH	ΔCt (Means Ctof IL8 - Means Ct of GAPH)	Mean ΔCt Calibrator (ctIL8-ctGAPDH)	ΔΔCt	$2^{-\Delta\Delta Ct}$	Experimental group/ Control group	Fold of gene expression
Group 1 Patients	27.62	24.679	2.941	10.53	-7.589	192.53	192.53/59.54	3.233±0.38
Group 2 Healthy	29.31	24.676	4.634	10.53	-5.896	59.54	59.54/59.4	1±0.00
T-test (P-value)								0.431 ** (0.0001)

** (P≤0.01)

Table 11. Fold ofIL-8 expression depending on $2^{-\Delta Ct}$ Method

Groups	Means Ct of IL8	Means Ct of GAPDH	ΔCt (Means Ct of IL8 - Means Ct of GAPDH)	$2^{-\Delta Ct}$	Experimental group/ Control group	Fold of gene expression
Group 1 Patients	27.62	24.679	2.941	0.130	0.130/0.040	3.23±0.37
Group 2 Healthy	29.31	24.676	4.634	0.040	0.040/0.040	1±0.00
T-test (P-value)						0.431 ** (0.0001)

** (P≤0.01)

Authors' Contribution

Study concept and design: M. F. J.

Acquisition of data: H. M. A.

Analysis and interpretation of data: B. H. A.

Drafting of the manuscript: M. K. A. A.

Critical revision of the manuscript for important intellectual content: M. F. J., H. M. A. and B. H. A.

Statistical analysis: M. K. A. A.

Administrative, technical, and material support: M. F. J.

Ethics

All procedures performed in this study involving human participants were in accordance with the ethical standards of the University of Technology, Baghdad, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgment

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