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Study the Relationship Between *miR-let-7a* gene Expression and IL-13 Levels in Children with Asthma in Baghdad Governorate

Duaa fawzi Ahmed[1], Basima Qasim Hasan Alsaadi[2]

[1,2] Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad

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Abstract

Background: Asthma is a globally significant non-communicable disease with major public health consequences for both children and adults, including high morbidity, and mortality in severe cases. **Aims:** This study aimed to explore how molecular changes in the miR-let-7a gene may affect children with asthma and to determine interleukin-13 (IL-13) levels and their relationship with asthma**. Methods:** Blood samples were collected from 140 subjects (52 females and 88 males) aged 1–10 years with allergic asthma. The patients were separated into three groups: the first group consisted of those <1 year old, the second group included those aged 1–5 years, and the third group included those >5 years old. Patients were admitted to the Central Children's Hospital, Al-Kadhimiya Children's Hospital, Al-Alawiya Children's Hospital, and Al-Zahraa Center for Asthma and Allergy in Baghdad over the period from November 2023 to February 2024. **Results:** The study revealed that the second age group had the highest asthma prevalence at 51.25%, followed by the third age group at 38.75%, while the first group demonstrated the lowest percentage at 10.00%. The distribution of males and females among asthma patients was 65.00% and 35.00%, respectively, whereas among controls it was 58.33% and 41.67%, respectively. The results show significant differences in the expression levels of miR let-7a between the two groups. Specifically, patients exhibited significantly lower levels of Let-7a (0.14 ± 0.1094) compared to controls (1.25 ± 0.8710), with a p-value of 0.001. The IL-13 concentrations in patients were 63.72 ± 2.21, while the control group (60 individuals) had IL-13 concentrations of 50.12 ± 1.42. **Conclusion:** The results of the study indicate downregulation of the miR-let-7a gene, while high levels of interleukin-13 were observed in children who suffer from asthma."

Keywords: Asthma, *miR-let-7a* gene, Interleukin-13, Real Time PCR

Corresponding author: (Email: doaaff597@gmail.com)

Introduction

Asthma is a common condition among children worldwide, characterized by chronicrespiratory symptoms associated with bronchitis (1). Asthma in children significantly affects their behavior, academic performance, and engagement in learning, leading to frequent school absences and reduced classroom attendance. Poor physical health is one of the most prominent direct consequences of childhood asthma. (2, 3) In children, asthma is one of the most common chronic respiratory conditions. The 2018 Global Asthma Report estimates that 339.4 million people worldwide are anticipated to suffer from this illness, including 14% of children under the age of 15. Doctors face major challenges in diagnosing childhood asthma in resource constrained areas, such as sub-Saharan Africa, which makes it more difficult to provide appropriate care. According to (4), a thorough assessment conducted in three sub-Saharan African nations underscored the challenges of identifying childhood asthma and the necessity of workable solutions. In Iraq, a study showed a difference in the spread of asthma among children, as infection rates in Baghdad were higher compared to northern Iraq, specifically Erbil. This shows that geographical variation affects the spread of the disease (5-6). ***miR-let-7a*** **is** a member of the let-7 family, the second miRNA family to be discovered in humans, and is highly conserved across a wide range of species. Let-7 miRNAs have been shown to play important roles as regulators of many biological processes such as cell differentiation, proliferation, apoptosis, and metabolism. They are also involved in regulating the balance between cell proliferation and differentiation in both immune and non-immune systems (7). *miR-let-7a* has a central position in many biological pathways involved in the pathophysiology of asthma. Thus, miR-*let-7a* is known to regulate immune responses, particularly by increasing the differentiation of Th1 cells. (8). The role of let-7a-3p in asthma has been highlighted in several studies, particularly its function in immune regulation during allergic asthma in infants. One study shows that let-7d-3p exhibits seasonal variation in asthmatic children, with let-7d-3p potentially playing a protective role in reducing seasonal asthma symptoms (9). These findings suggest that let-7a-3p may influence asthma severity by modulating immune responses to environmental allergens. IL-13 is a multifunctional cytokine secreted primarily from activated Th2 cells, eosinophils, basophils, and mast cells. Its biological role has been extensively studied in various physiological and pathological conditions. IL-13 has been shown to contribute to Regulating immune responses aimed at neutralizing or killing pathogens, processes that may lead to tissue damage associated with these responses. IL-13 has multiple effects on immune system cells, making it an important target for studies to understand its precise roles in human health and disease (10). IL-13 plays a pivotal role in asthma and allergic diseases. IL-13 levels are elevated in the serum of patients with asthma. IL-13 is a key component of the Th2 inflammatory response characteristic of these conditions, contributing to many of the pathophysiological changes associated with asthma. IL-13 promotes airway hyperreactivity as a result of smooth muscle contraction, which becomes more sensitive to various stimuli with elevated IL-13 levels. Elevated IL-13 levels in asthma patients also lead to increased mucus secretion, which exacerbates asthma symptoms (11). The IL-13 gene is located on human chromosome 5q23-q31. Consists of four exons and three introns (12). Because the studies are little related to asthma in children and the impact of various environmental factors on this disease, the purpose of this study is to shed light on these factors and understand their impact on the symptoms and severity of the disease, which may contribute to improving methods of dealing with asthma and providing appropriate care for children with asthma

Materials and methods

The current study was carried out on a total of 140 subjects 60 control and 80 patients, (52 females and 88 males) aged 1–10 years with allergic asthma. The patients were separated into three groups: The first group consisted of those under 1 year, the Second group included those aged 1-5 years, and the Third group included those beyond 5 years old, which admitted at the Central Children's Hospital, Al- Kadhimiya Children's Hospital, Al-Alawiya Children's Hospital, and Al-Zahraa Center for Asthma and Allergy in Baghdad over the period from November 2023 to February 2024.

Blood Sample Collection

Five ml of venous blood was collecte using a sterile syringe. Divide this blood into 3 ml place it in a gel tube and leave it for 20 minutes to clot at room temperature (25-30°C). The tubes were then centrifuged at 3000 RPM for 15 minutes to separate the serum, and the serum was then stored in Eppendorf tubes at −20°C until used for immunohistochemical assays. The remaining 2 ml of blood was placed in Trizol tubes and kept frozen at −20°C for molecular analysis.

Ethical approval

The study was carried out by the ethical principles outlined in the Declaration of Helsinki. The study was performed following the acquisition of both verbal and written consent from the patients before collecting the samples, this case-control study was approved Committee of the Institute of Genetic Engineering and Biotechnology for past graduate study at the University of Baghdad, as well as the study was approved by the Ministry of Health and Environment of Iraq (73275 in 30-11- 2023).

Molecular study

Genomic RNA Extraction

The total RNA was extracted from the blood of asthma patients as well as from apparently healthy groups (control) by using the TransZol up Plus RNA Kit (Transgen). Then, RNA concentration and purity were measured by a nanodrop spectrophotometer (Thermo Fisher Scientific, USA) (13).

cDNA synthesis for mRNA

The reverse transcription of total RNA into complementary DNA (cDNA) was carried out using the EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix kit. Following the manufacturer's guidelines, the reaction volume was set at 20 µL, utilizing 20 µL of total RNA for the conversion process.as show in Table (1).

Table (1): strand cDNA synthesis reaction component

Component	volume reaction
mRNA/miRNA	4 µl
Anchored Oligo(dT)18 Primer (0.5 µg / µl)	1 µl
Random Primer (0.1 µg / µl)	1 µl
GSP	1 µl/10 pmol
2xES Reaction Mix	10 µl
EasyScript ® RT/RI Enzyme Mix	1µl
gDNA Remover	1µl
RNase-free Water	1µl
Total volume	20µl

Gene expression of *miR-let-7a* by Quantitative Real Time PCR (qRT–PCR)

The total RNA underwent reverse transcription into complementary DNA (cDNA) using the EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix Kit, as provided by TransGen Biotech Co in China, within a reaction volume of 20 µl, following the manufacturer's guidelines. The Quantitative Real-Time PCR (qRT–PCR) was carried out using the QIAGEN Rotor- gene-Q Real-time PCR System (Germany). Each qRT-PCR reaction involved 2 µl of cDNA, 1 µl for both the forward and reverse primers (with a concentration of 10 µM) They were synthesized and lyophilized by Alpha DNA Ltd. (Canada) (14). as listed in Table 2, and 10 µl of the Perfect Start TM Green qPCR SuperMix kit from TransGen Biotech Co., China. The thermal profile consisted of an initial step at 94 °C for 5 minutes (one cycle), followed by 40 cycles involving denaturation at 94 °C for 5minutes, annealing at 58 °C for MiR- let7a and housekeeping gene (*miRU6*) for 15 seconds, and extension at 72 °C for 20 seconds. The final dissociation stage spanned from 55 to 95 °C, with each degree lasting 5 seconds. The specificity of the amplified product was confirmed through melting curve analyses. To evaluate the relative expression of the miR-*let-7a* gene in the samples from the study groups, the expressions were normalized to the reference gene miRU*6*. using the 2-Ct technique $2^{-(\Delta\Delta\square\square\square\square)}$ method (15). Compared to the healthy controls, the data were presented as the fold change in *miR-let 7a* gene expression within the study groups. This allowed for a normalization of the expression levels against the reference gene (*miRU6*). The median fold expression of *miR-let-7a* in the study groups were then utilized to assess whether there were statistically significant differences in *miR-let-7a* gene mRNA expression levels. Significant differences in *miR-let7a* gene mRNA expression

Table (2): primers sequences utilized in this study's assays.

Primer	Sequence (5′→3′ direction)	Primer size (bp)	Ta (°C)	
miRNA				
miR-let-7a	CTATACAATCTACTGTCTTTC	21	58	**Design in current study**
miRNA-universe R.P.	GCGAGCACAGAATTAATACGAC	22		
Universe R.transcription p	CAGGTCCAGTTTTTTTTTTTTTTTVN	26		
miRU6 F.P.	AGAGAAGATTAGCATGGCCCCT	22	58	

*** Ta: Annealing Temperature**

miR-let-7a genes expression Calculation

The fold variations of the quantitative expression of the mature RNAs were determined using the relative cycle threshold ($2^{-\Delta\Delta Ct}$) approach, which was described by Livak and Schmittgen in 2001. It is the ratio of the relative gene expression between control group and the test group. Double delta Ct (threshold cycle) analysis was used to assess the expression of *miR-let-7a-1-* genes, in which the housekeeping reference genes. The calculations were as the following: By using the real-time cycler software, the threshold cycle (CT) was calculated for each sample. The samples were duplicated and the average results were computed. The Ct values for the target genes MiR- let7a-1-, which were being evaluated in both patients and controls, were reported. The ΔCt, or difference in CT values, which is also referred to as the "normalized raw data," was determined by subtracting the specified normalization factor from the Ct value of each target gene and the housekeeping gene.

Assessment IL-13 by Enzyme-linked immunosorbent assay (ELISA)

Serum was obtained from vacutainer tubes using a serum separator. Following a 30-minute of incubation at room temperature, then, samples were centrifuged at 3,000 revolutions per minute for 10 minutes after being allowed to be incubated at room temperature for thirty minutes. The serum was kept in storage. The quantities of IL-13 were determined in serum through the utilization of sandwich immunoassays, in accordance with the guidelines provided by the manufacturer's protocol (Sunlog China). Optical densities were measured at 450 nm using a plate reader that was employed. Statistical analysis: The results of this study were analyzed according to the study objectives and presented based on the overall characteristics of the sample. Microsoft Excel 2010 and SPSS (version 25) software were used for statistics analysis. Microsoft package (Excel and Word). The data are expressed as mean ± SD, One-way ANOVA and T-test were used to significantly compare between means. The chi-square test was used to significantly compare between percentages (0.05 and 0.01 probability). As shown in the figure (1).

Result

separated into three groups is: the first the study cohort consisted of individuals aged between 1 and 10 years. The patient samples were group consisted of those under 1 year old, the second group included those aged 1-5 years, while the third group included those beyond 5 years old. The study revealed that; the second group had the highest asthma prevalence at 51.25%, followed by the third group at 38.75%. The first group demonstrated the lowest percentage at 10.00% As for the distribution of males and females among asthma patients, it was 65.00% and 35.008%, respectively, whereas among controls it was 58.33% and 41.67%, respectively. The frequencies of these two groups did not exhibit any significant difference (p-value = 0.0286), as recorded. As for the season, patients were categorized into four groups: the first group with a percentage of 78.75% during winter, the second group with a percentage of 21.25% during fall, and the third group with a percentage of 12.50% during spring. Lastly, the fourth season July observed a complete absence of patients (0.00%). The study revealed that the winter group had the highest asthma prevalence, with 53 patients, followed by the autumn group with 17 patients. The spring group had the lowest number of participants, with 10 patients. Table (3).

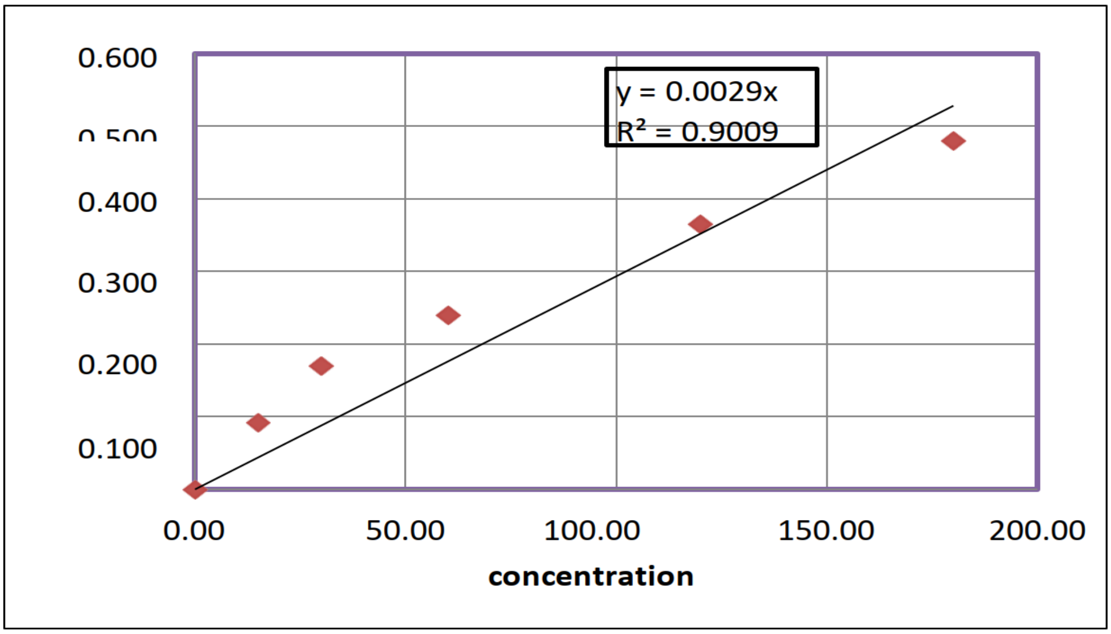

Figure (1): Standard Curve of IL-13

Table (3): Distribution of sample study according difference factors in patients and control groups

Factors		**Patients No (%)**	**Control No (%)**	**P-value**
Sex	Male	52 (65.00%)	35 (58.33%)	0.0286 *
	Female	28 (35.008%)	25 (41.67%)	
Age	<1 yr.	8 (10.00%)	3 (5.00%)	0.0001 **
	1-5 yr.	41 (51.25%)	24 (40.00%)	
	>5 yr.	31 (38.75%)	32 (53.33%)	
Season	Autumn	17 (21.25%)	0 (0.00%)	0.0001 **
	Winter	53 (78.75%)	0 (0.00%)	
	Spring	10 (12.50%)	32 (53.33%)	
	Summer	0 (0.00%)	28 (46.67%)	

*** (P≤0.05), ** (P≤0.01).**

Distribution of IL-13 Serum level of Studied Groups

Statistical analysis of the results using t-test with a value of (**5.650) revealed a highly significant increase in the level of IL13 in the serum of asthma patients when compared to the control group. (63.72 ng/mL. 50.12 ng/mL), with significant differences (p= 0.0001), (Table 4), The shape showed comparison of IL-13 concentrations between a group exhibited a mean IL 13 concentration of 63.72 ng/ml, with a standard error of the mean (SE) of 2.21 ng/ml. In contrast, the control group, comprising 60 individuals, had a mean IL-13 concentration of 50.12 ng/ml, with an SE of 1.42 ng/ml presents it. as Figure 2.

Table (4): Distribution of IL-13 serum level in asthma patients compared to control group.

Group	No	Mean ±SE of IL-13 conc. (ng/ml)
Patients	80	63.72 ±2.21
Control	60	50.12 ±1.42
T-test	---	5.650 **
P-value	---	0.0001

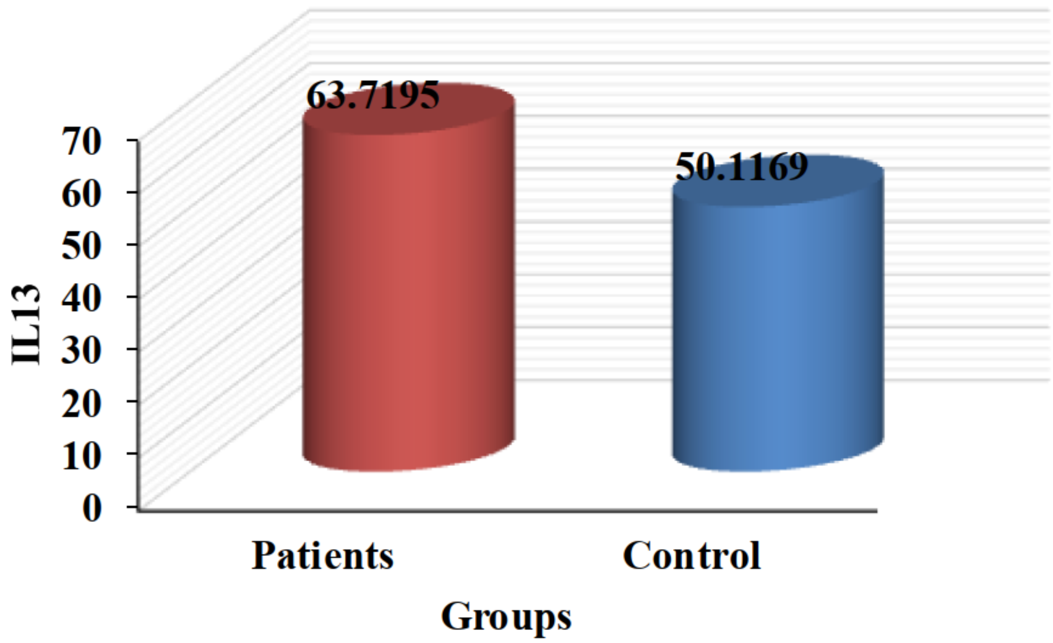

Figure (2): IL-13 concentration in patients' control groups

Discussion

different trend, showing that females are This study is consistent with the study conducted by Harvey and McElvaney (16) where it was found that boys before puberty are more susceptible to asthma compared to girls, a trend that is reversed after puberty where the prevalence of the condition is higher among women. A study conducted by Boulet et. al (17). This difference is attributed to biological factors including the influence of sex hormones, in addition to social, cultural, and behavioural factors, that affect the incidence of asthma in both sexes differently. This is consistent with a study confirmed by Al-Iede et. al (18). They confirmed that the interaction between biological, environmental and social factors greatly affects the prevalence of asthma among children, with noticeable differences between males and females while previous studies have shown that male children are more likely to develop asthma than females, a study conducted by Similar (15) in Saudi Arabia revealed a more likely to develop

In contrast, Abdulkareem and Al Saadi (22) refuted these findings, as their study determined that younger children are more susceptible to asthma. However, this result aligns with the findings of the current study. This result agreed with those of the study where the proportion of patients with symptoms is consistently greater in winter (23). About 75 percent of patients say that cold air aggravates their asthma symptoms, A study of children in Ireland found that infants living in cold homes were 41% more likely to develop asthma and 47% more likely to wheeze (24). These results are consistent with the findings of this study, which shows that cold air is a factor that increases asthma symptoms in children with asthma. Cold temperature trigger childhood asthma, as airway cooling in cold days may exacerbate inflammation, which causes a narrowing of airways (25). This is consistent with the current study.

asthma than males. This discrepancy is not consistent with the results of the current study, suggesting that there may be different environmental or social factors that may influence the gender of those affected, whether male or female. The results of the current study in terms of the age group most susceptible to infection are consistent with the results of (19) as they showed the highest results of asthma infection from the age of (1-4) years. This similarity enhances the reliability of the results, as mentioned in (20) that confirmed the important role of age, as this study provided a comprehensive analysis of asthma in childhood, indicating that the highest rates are found in very young children aged (1-4) years. Bloom et. al. (19) demonstrated that asthma rates decrease as children get older.

“Furthermore, Hamdan et al. (21) demonstrated that the age group with the highest proportion of asthma patients were between 18 and 25 years old, accounting for 47.9%.

The results show a significant difference in IL-13 concentrations between the patient group and the control group. This indicates that IL-13 levels were significantly higher in patients compared with controls, suggesting a strong association between elevated IL-13 and the presence of allergic asthma. These findings are consistent with previous studies, such as a study in Iraq-Baghdad that investigated serum levels of total IgE and IL-13 in a sample of allergic asthma patients by Jebur and Saud (25), which also reported higher IL-13 concentrations in patients with allergic asthma compared with the control group. The results of the study also agree with what was indicated by Al Qadi and Al-Saadi (26), as their study found a significant increase in IL-13 levels in asthma patients, which supports the study that IL-13 plays a pivotal role in enhancing the inflammatory response and increasing symptoms in these patients, and this indicates the existence of a strong relationship between high levels of interleukin 13 and exacerbation of asthma symptoms. However, some studies, like those of (27), did not find a statistically significant difference in IL 13 levels between asthmatic patients and controls.

Conclusion

The findings of the current study demonstrated a significant downregulation in the expression levels of *miR-let-7a*, accompanied by an increase in IL-13 concentrations in the blood of children with asthma compared to the control group. These results suggest a potential role for miR-*let-7a* as a negative regulator of IL-13 levels, where reduced *miR-let 7a* expression may lead to elevated IL-13 production, contributing to the inflammatory response and exacerbation of asthma symptoms in patients. This study highlights the importance of targeting *miR-let-7a* as a potential therapeutic pathway to reduce IL-13 levels and control asthma-associated inflammation.

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