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Original Article

Dermatology Section

IL-8 mRNA and Serum Levels in Vitiligo Patients: A Case Control Study

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ABSTRACT

Introduction: Many theories are suggested to explain aetiology of vitiligo, such as autoimmune, genetic and biochemical. Interleukin-8 (IL-8) is a pro-inflammatory chemokine which is evaluated in the pathogenesis of some skin diseases, like psoriasis, however, in vitiligo, few studies were reported regarding this issue.

Aim: The aim of the present study was to investigate serum level of IL-8 and IL-8 mRNA in patients with different types of vitiligo to validate its role in aetiopathogenesis of vitiligo.

Materials and Methods: This case-control study was conducted on 39 vitiligo patients and 15 age and gender matched healthy controls. The participants were selected from Dermatology Outpatient Clinic, Menoufia University Hospitals from October 2014 to October 2015. They were subjected to clinical history, examination and full general examination. Detailed dermatological examination including VASI score was applied. IL-8 serum level was measured by ELISA and IL-8 mRNA was quantitated by Real Time-PCR. The statistical analysis was done using SPSS, version 20.0. Mann Whitney U test was used to compare two quantitative not normally distributed. Chi-square test (χ^2) was used to study association between two qualitative variables. Spearman correlation test was applied to assess correlation between two continuous quantitative variables. A $p \leq 0.05$ was considered significant.

Results: IL-8 serum level and mRNA concentration mean values were significantly elevated in vitiligo patients (26.25±43.28 pg/mL and 8.48±11.92 ng/mL) than controls (0.57±0.50 pg/mL and 0.60±0.32 ng/mL) (p=0.002, for both), and they showed significant positive correlation with each other (r=0.622; p≤0.001). Moreover, both of them revealed significant high values in localised vitiligo (62.20±74.39 pg/mL and 12.42±13.85 ng/mL) than generalised (18.39±29.49 pg/mL and 7.61±11.52 ng/mL) (p≤0.000 and p=0.004), respectively.

Conclusion: Serum IL-8 chemokine and its mRNA increased significantly in vitiligo patients indicating that it may have a dynamic role in vitiligo development and participate in its pathogenesis.

Keywords: ELISA, IL-8 mRNA, Pathogenesis, Real time PCR

INTRODUCTION

Vitiligo is an acquired skin disease characterised by having white and well demarcated macules and patches of different distribution and variable sizes [1], in addition to that the melanocytes in the skin lesion are destroyed [2,3]. The disease is estimated to affect about 0.5-1% of the population worldwide, affecting men and women equally [4].

Aetiology of vitiligo is complex and multifactorial. Many theories, such as genetic, autoimmune, biochemical and adhesion defect have been suggested trying to explain its pathogenesis; however none of them succeeded in describing the full spectrum of the disease [5]. The autoimmune theory hypothesises that the destruction of melanocytes is caused by many active immunological components like memory cytotoxic T-cells and autoantibodies specific to surface or cytoplasmic antigens on melanocytes, such as γ-enolase, α-enolase and heat-shock protein 90 [6,7].

Some inflammatory cytokines were proven to be involved in vitiligo pathogenesis [8], like TNF-α [9], IFN-γ, IL-10 and IL-1β [10], IL-6 [11] and IL-8 [11,12]. Oxidative stress has a well proven role in starting the destruction of melanocytes, thereof it could be a possible theory of vitiligo [13].

IL-8 (CXCL8) is a pro-inflammatory cytokine. It is a member of CXC chemokine family that working on CXCR1 and CXCR2 receptors [14,15]. It is generated by a wide type of cells, such as neutrophils, monocytes, endothelial cells, in addition to keratinocytes [15,16]. Also, CXCR1 and CXCR2, are expressed on the surface of many cells types, like monocytes, neutrophils, CD8+ cells, immature monocyte-derived dendritic cells, natural killer cells, myeloid derived suppressor cells, keratinocytes, and melanocytes [15].

A number of different stimuli mediate the expression of IL-8, such as inflammatory signals, exposure to chemotherapy agents, hypoxia, and male sex hormones [17]. IL-8 gene expression is highly sensitive to oxidants, and anti-oxidants reactive oxygen intermediates [18], also, it is induced by TNF–α, IFN–γ, other chemokines including IL–1, and lipopolysaccharide of the bacterial wall [19,20].

IL-8 has many functions; it initiates migration of neutrophils to the site of inflammation in a process known as chemotaxis, mediates neutrophil adhesion to endothelial cells, and enhances activation of neutrophils [21].

In the field of dermatology, IL-8 is involved in the pathogenesis of many common skin diseases, such as bullous pemphigoid [22], psoriasis [23] and atopic dermatitis [24], however its role in vitiligo still needs to be elucidated.

The aim of the present study was to measure serum level of IL-8 and IL-8 mRNA in patients with different types and severity of vitiligo to shed light on its possible role in aetiopathogenesis of vitiligo.

MATERIALS AND METHODS

This case-control study was applied on 39 patients with different varieties of vitiligo, in addition 15 age and gender matched healthy volunteers were chosen controls. They were selected from Dermatology Outpatient Clinic, Faculty of Medicine, Menoufia University Hospitals during the period from October 2014 to

October 2015. Each individual in the study signed a written consent form approved by The Committee of Human Rights in Research of Menoufia University.

This study included newly diagnosed cases of vitiligo and old diagnosed cases stopped topical treatment for two weeks and systemic treatment for 1 month before joining the study.

Subjects having auto-immmune/inflammatory diseases, current infection or immunosuppression, pregnant or lactating women were excluded from our study.

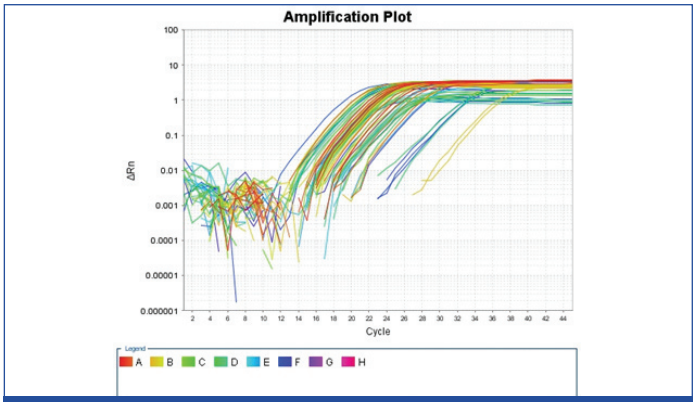
All participants in the study were subjected to thorough history taking, stressing on onset, duration and family history of vitiligo. General clinical examination, to detect any excluding factor and dermatological examination, to evaluate the vitiligo and to assess its severity based on VASI score were also performed [25].

Measurement of the serum levels of IL-8 protein by ELISA: From each participant, 5 mL of venous blood were withdrawn under complete aseptic condition, and 2 mL of them were put in a plain tube, left for 15 minutes at room temperature to clot, centrifuged for 10 minutes at 4000 rpm and the sera were kept frozen at -80°C for measurement of IL-8 by ELISA.
Serum IL-8 was measured using Human IL-8/CXCL8 ELISA Kit (Boster Biological Technology, USA) according to the manufacturer's instructions. This kit has sensitivity of less than 1 pg/mL with no detectable cross-reactivity with other relevant proteins.

Assessment of IL-8 mRNA by using Real Time -PCR for mRNA expression of IL-8 [12]: The other 3 mL of the collected venous blood were put in EDTA tube for extraction of total RNA from whole blood by GeneJET Whole Blood RNA Purification Mini Kit (Thermo scientific), according to the manufacturer's protocol. The extracted RNA samples were stored at -80°C until analysis. The concentration of RNA was determined by measuring its absorbance at 260nm (A260). Absorbance readings should be greater than 0.15 to ensure significance. The ratio between the absorbance value at 260 and 280nm (A260/A280) gave an estimate of RNA purity. A260/A280 ratio greater than 1.6 was accepted. Two steps RT–PCR was done as follows: for reverse transcription step; a reverse transcriptase kit (SensiFAST cDNA synthesis kit, Bioline Reagents Ltd., UK) was used for complementary DNA (cDNA) synthesis on 2720 thermal cycler (Singapore). For cDNA synthesis, RNA (10 µL) was reverse transcribed in a final volume of 20 µL containing 1 µL of reverse transcriptase enzyme, 4 µL of 5x TransAmp buffer and 5 µL of DNase/RNase free water. The samples were incubated at 25°C for 10 minutes (primer annealing), and 42°C for 15 minutes (reverse transcription). Reverse transcriptase was then inactivated by heating at 85°C for 5 minutes. All products were stored at -20°C till the next step. For cDNA amplification: a relative quantitation of IL-8 mRNA expression normalised to the endogenous β-actin reference gene was performed by real time reverse transcription PCR (RT-PCR), using the 2x SensiFAST MSYBR® No-ROX Kit (Bioline Reagents Ltd.), on Applied Biosystems 7500 Real Time PCR System. Forward primer of IL-8: ACTGAGAGTGATTGAGAGTGGAC and reverse primer of IL-8: AACCCTCTGCACCCAGTTTTC [26]. Forward primer of β-actin primer: 5'AGTTGCGTTACACCCTTTCTTG3' and reverse primer: 5'TCACCTTCACCGTTCCAGTTT3'. The PCR reaction mixture (final volume, 25 µL) contained 12.5 µL of 2x SensiFAST MSYBR® No-ROX Master Mix, 1 µL of each primer (Sigma), 5.5 µL of DNase/RNase free water and 5 µL of cDNA. Thermo-cycling conditions were 10 minutes at 95°C, followed by 45 cycles at 95°C for 15 second, and 60°C for 1 minute. For relative quantification; The comparative Cycle threshold (Ct) method was used. Analysis was performed using Applied Biosystems 7500, software version 2.0.1. The amplification plot of IL-8 was shown in [Table/Fig-1].

STATISTICAL ANALYSIS

The data were collected, entered and processed on IBM PC compatible computer using SPSS software (version 20.0) (SPSS Inc., Chicago, U.S). Two types of statistics were done; the descriptive methods (for example, mean and standard deviations for normal continuous variables, and range for non-normal continuous variables and frequencies and percentages for categorical variables) and analytic statistics: e.g., Mann-Whitney U test, a nonparametric test used to compare two quantitative not normally distributed. Chi-square test (χ^2) was used to study association between two qualitative variables. Spearman correlation test (rho) to assess correlation between two continuous quantitative variables not normally distributed. A $p \leq 0.05$ was considered statistically significant.



[Table/Fig-1]: Amplification Plot of IL-8 gene.

RESULTS

Demographic and Clinical Data

This study was conducted on 39 patients with vitiligo, they were 15 males (38.5%) and 24 females (61.5%). Their age ranged between 5 to 70 years with 40.31±18.70 years as mean. Our control group included 15 healthy volunteers, their age ranged from 7.0 to 65.0 years, with a mean value of 36.20±18.20 years. They were 5 males (33.3%) and 10 females (66.7%). They were age and sex matched with the vitiligo cases (p=0.471 and p=0.727) respectively [Table/Fig-2].

Parameter	Vitiligo group (n=39)		Control group (n=15)		Test of significance	p-value
Age (years) Mean±SD Range	5.0-70.0 40.31±18.70		7.0–65.0 36.20±18.20		Mann-Whitney = 0.73	0.471
Gender	No	%	No	%	χ^2=0.12	0.727
Males	15	38.5	5	33.3		
Females	24	61.5	10	66.7		

[Table/Fig-2]: Comparison between demographic data of the studied groups.

Thirty two cases presented with generalised vitiligo (82.1%), while 7 cases with localised vitiligo (17.9%). The duration of the disease ranged between 1 month to 40 years with 12.30±10.88 years as mean value. The age of onset of the disease ranged from 4 to 59 years with 28.80±15.48 years as the mean. Family history of vitiligo was positive in 11 cases (28.2%). The severity of vitiligo ranged from 0.09 to 100% with 35.13±19.21% as the mean value [Table/Fig-3].

IL-8 (serum and mRNA) levels

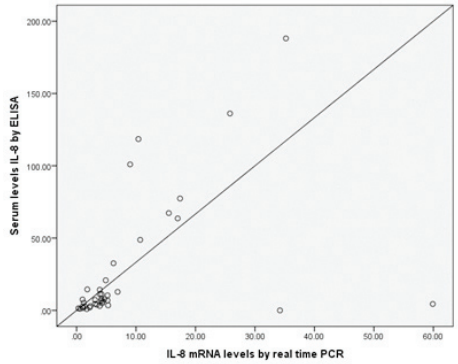
Serum IL-8 level of vitiligo group (26.25±43.28 pg/mL) was significantly higher than that of the controls (0.57±0.50 pg/mL) (p=0.002). Also, mean of IL-8 mRNA level was higher in the vitiligo group revealing 8.48±11.92 ng/mL, whereas it was 0.60±0.32 ng/mL in the control group with statistically high significant value (p=0.002) [Table/Fig-4]. Moreover, there was statistically significant positive correlation between the measured serum IL-8 level with that of IL-8 mRNA (r=0.622 and p<0.001) in vitiligo patients group [Table/Fig-5].

Parameter	Vitiligo patients (n=39)	No. %
Type of vitiligo		
Generalised	32	82.1
Localised	7	17.9
Previous treatment for vitiligo		
Receive ttt	20	51.3
Not receive ttt	19	48.7
Family history of vitiligo		
Positive	11	28.2
Negative	28	71.8
Age of onset of vitiligo (years) Mean±SD Range	4.0-59.0 28.80±15.48	
Duration of illness with vitiligo (years) Mean± SD Range	1 month-40 year 12.30±10.88	
VASI score of patients with vitiligo Mean± SD Range	35.13±19.21 0.09-100	

[Table/Fig-3]: Characteristics of vitiligo patients.

Parameter	Vitiligo group (n=39)	Control group (n=15)	Mann-Whitney test	p-value
Serum IL-8 (pg/mL) Mean± SD	26.25±43.28	0.57±0.50	3.03	0.002
IL-8 mRNA (ng/mL) Mean±SD	8.48 ±11.92	0.60±0.32	3.05	0.002

[Table/Fig-4]: Comparison of serum IL-8 and IL-8 mRNA levels between the studied groups.

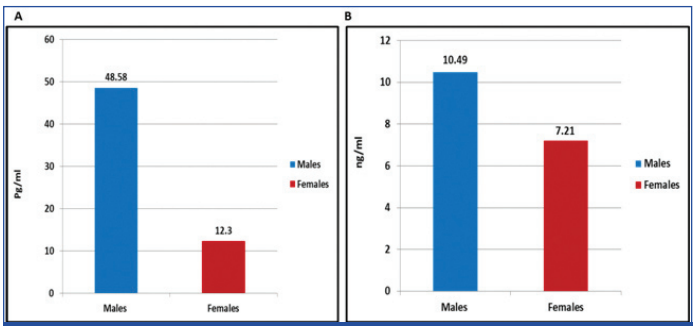


[Table/Fig-5]: Correlation between IL-8 mRNA levels and serum levels of IL-8 in vitiligo cases.

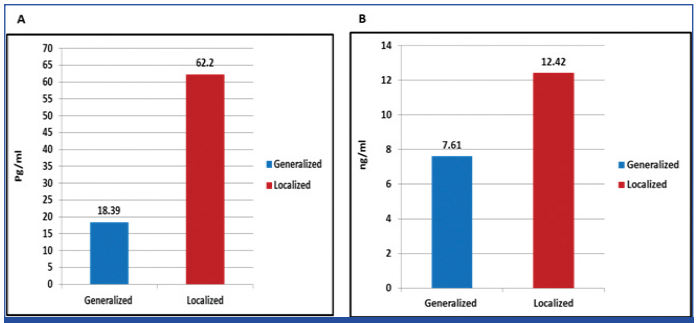
Relationship between IL-8 serum and IL-8 mRNA levels with demographic and clinical criteria

IL-8 serum and IL-8 mRNA concentration increased significantly (p=0.005 and p=0.003, respectively) in males vitiligo patients (48.58±57.98 pg/mL and 10.49±9.35 ng/mL, respectively) than females patients (12.30±22.81 pg/mL and 7.21±13.31 ng/mL, respectively) [Table/Fig-6].

The evaluated mean value of serum IL-8 in cases with localised vitiligo



[Table/Fig-6]: a) IL-8 serum; and b) IL-8 mRNA levels in vitiligo patients in relation to gender.



[Table/Fig-7]: a) IL-8 serum; and b) IL-8 mRNA levels in vitiligo patients in relation to clinical types of vitiligo.

was 62.20±74.39 pg/mL and in generalised vitiligo was 18.39±29.49 pg/mL, this difference was statistically significant (p<0.001). Also, there was a statistically significant (p=0.004) difference in IL-8 mRNA level between patients of localised vitiligo (12.42±13.85 ng/mL) and those with generalised (7.61±11.52 ng/mL) vitiligo [Table/Fig-7].

However, the correlation between both IL-8 serum and IL-8 mRNA expression levels in the studied vitiligo cases with other studied demographic and clinical data could not reach the level of significance (data were not shown).

DISCUSSION

To best of our knowledge, this study may be the first one that investigates both IL-8 serum level and its mRNA concentration at the same time in vitiligo patients, aiming to throw light on the possible hypothesised role of IL 8 in vitiligo development.

In the current study, we reported significantly increase in serum IL-8 level mean value in vitiligo patients than their controls (p=0.002). In agreement with our findings, Yu HS et al., revealed a significant elevation in IL-8 serum level in their studied vitiligo patients than control group [11]. Additionally Li YL et al., observed that melanocytes produce IL-8 following stimulation by anti-melanocyte IgG antibodies [27], that are present in most vitiligo patients [28].

However, Miniati A et al., reported no significant differences between vitiligo patients and controls regarding serum level of IL-8 measured by the microbead array [29]. This difference could be attributed either to small sample size in their study (15 vitiligo cases), or different laboratory method used for analysis.

In the same context, we evaluated IL-8 mRNA total amount using real time PCR. Parallel to IL-8 serum level, our result showed significantly increase in IL-8 mRNA mean value in vililigo patients than their peers (p=0.002). Likewise, Miniati A and his colleagues reported that the lesional skin of patients with new-onset and active non-segmental vitiligo showed increased IL-8 gene expression evaluated by quantitative reverse transcriptase PCR [29]. The authors suggested that in the initial stage of the disease, where melanocytes are still found in epidermis, IL-1β and TNF produced by many skin cell types have the ability to stimulate high IL-8 skin expression by melanocytes.

It was suggested that IL-8 may attract T-cells to vitiligo lesions leading to enhancement of the inflammatory reaction and melanocyte destruction [12]. Also, IL-8 is a powerful chemokine that can induce oxidative stress causing indirectly apoptosis of keratinocyte and these apoptotic cells could release increasing amounts of pro-inflammatory cytokines, such as IL-1 and TNF enhancing skin inflammation melanocyte [30]. Added to this, IL-8 may directly inhibit the growth and mediate expression of antigens on or in the cytoplasm of melanocytes [31].

Within the vitiligo patient group, evaluated serum IL-8 showed significant positive correlation with the measured IL-8 mRNA. This correlation was not reported till now, besides, it may reflect the source of serum IL-8 in vitiligo patients.

Moreover, both serum IL-8 and IL-8 mRNA were correlated significantly with male gender that could be attributable to the male

sex hormones which have stimulatory effect on the IL-8 expression, resulting in high IL-8 levels in males than females [17].

Additionally, both serum IL-8 and its mRNA were higher in the localised type of vitiligo than generalised form, that could be partially explained by increased IL-8 in the early stages of the disease because of exposure to oxidative stress, initiating its occurrence, then IL-8 level was consumed and declined in the generalised vitiligo.

LIMITATION

Small number of cases; finally, we recommend more studies on a large scale of vitiligo patients to confirm our results. Elegant studies for evaluation of the possible use of anti-IL-8 agents as therapeutic means in vitiligo treatment program, also suggested.

CONCLUSION

IL-8 serum level and its mRNA expression increased significantly in vitiligo patients than their matched peers, indicating that IL-8 may have an active role in vitiligo development and participate in its pathogenesis. Moreover, from this piece of work, IL-8 may be a novel candidate that represents, in our opinion, a future therapeutic target in the pathogenesis of vitiligo.

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