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Expression of IL-17 and COX2 Gene in Peripheral Blood Leukocytes of Vitiligo Patients

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ABSTRACT

Vitiligo is a pigmentation disorder in which inflammatory mediators such as cytokines have a pivotal role in disease's pathogenesis. Interleukin 17 (IL-17A) is a proinflammatory cytokine which is involved in the induction of several proinflamatory mediators such as cyclooxygenase 2 (COX2). The aim of this study was to investigate the gene expression of IL-17 and COX2 in peripheral blood leukocytes of vitiligo's patients.

Peripheral blood leukocytes from 15 patients with vitiligo and 15 healthy controls were separated using a gradient density centrifugation method. After total RNA isolation and cDNA synthesis, IL-17 and COX2 gene expression were quantified by real-time polymerase chain reaction (PCR).

There were no significant differences in IL-17 and COX2 gene expression in lymphocytes of patients with vitiligo compared with control group ($p<0.05$). However there was an increased IL-17 and COX2 gene expression in neutrophils of patients compared to controls, but it did not reach statistical significance ($p=0.05$). We could not find any differences in IL-17 and Cox2 gene expression between clinical diseases subtypes, sex and age. There was a significant correlation between IL-17 and COX2 genes expression in the neutrophils of patients ($p=0.00$, $r=0.80$).

Our results showed an increased expression in IL-17 and Cox-2 genes in neurophils of patients with vitiligo. This suggested that these two factors are involved in the inflammatory process. Further studies with a larger sample size might help to establish the role of these factors in the pathogenesis of diseases.

Keywords: COX2; IL-17; Neutrophils, Real-time PCR; Vitiligo

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INTRODUCTION

Vitiligo is a depigmentation disorder resulting from the activity loss of melanocytes in the skin and affects 0.5-2 % of the world population.[1] It is associated with other autoimmune diseases such as autoimmune thyroid disease, rheumatoid arthritis (RA), psoriasis, adult-onset type 1 diabetes, pernicious anemia, systemic lupus erythematosus, and Addison's disease.[2] The immunopathogenesis of vitiligo has not been fully understood, however it is known as an inflammatory disorder which is associated with increased expression of inflammatory cytokines in skin[3] and blood.[4] Previous studies have demonstrated that the pathogenesis of other inflammatory diseases such as rheumatoid arthritis is associated with the presence of proinflammatory cytokines such as IL-17. IL-17, also referred to IL-17A, is a proinflammatory cytokine which has a pivotal role in autoimmune diseases[5] Although IL-17 is mainly produced by activated memory T cells,[6] other cells including $CD8^+$ memory T cells, γδ T cells, natural killer T (NKT) cells,[7] eosinophils,[8] neutrophils,[9] monocytes,[10] basophils and mast cells are able to produce IL-17 in certain circumstances. In contrast to relatively narrow expression of IL-17, its receptor has been found to be ubiquitously expressed in all cell types and tissues examined, suggesting most cells are potentially targets of IL-17.[11] The production of other inflammatory mediators such as COX2 is stimulated by IL-17 which leads to up regulation of gene expression and increase of local inflammation.[12]

Prostaglandin H2 synthase (cyclooxygenase or COX) is a membrane bound enzyme responsible for conversion of arachidonic acid (AA) to prostaglandins (PG).[13] Two different isoforms of COX has been identified, COX1 and COX2. COX-1 is constitutively expressed in most cells, but Cox2 is only expressed in specific cells including activated macrophage, monocytes, neutrophils and lymphocytes, in response to inflammatory stimuli such as mitogen, cytokines and growth factors.[14] Expression of COX2 leads to elevated protein production which in turn causes inflammation.[15] In several chronic and autoimmune diseases such as RA both IL-17 and COX2 are over expressed which indicates that these factors are involved in pathogenesis of these disorders. More Recently, Jandus et al., demonstrated that there is no change in the numbers of Th-17 cells, a subset of $CD4^+$ T cell which produce IL-17, in peripheral blood of patients with vitiligo.[16] Basak et al observed that serum IL-17 levels in these patients were positively correlated with the extent of body area involvement.[17] Furthermore, it has been shown an association between COX2 gene polymorphisms and the risk of vitiligo in the Chinese population.[18] There is no data to address the role of IL-17 and COX2 in the pathogenesis of vitiligo.

The aim of this study was to examine the expression of IL-17 and COX2 genes in peripheral blood leukocytes of patients with vitiligo to find out whether these factors are involved in the disease-associated inflammatory process.

MATERIALS AND METHODS

Subjects

This study included 15 vitiligo patients, who were referred to skin clinic at Ghaem Hospital, Mashhad University of Medical Sciences (MUMS), Iran. The criterion for different clinical forms of vitiligo has been explained elsewhere.[19] All subjects were interviewed and gave consent form to participate in the study. The study was approved by the Ethics Committee of MUMS. A complete history including age of onset disease, type of clinical feature of disease, family history of vitiligo and systemic or autoimmune disorders was recorded for each patient. Patients who had infectious or malignant diseases and a history of taking medications for vitiligo or anti-inflammatory medication were excluded from the study. Furthermore, all of the patients were examined for Hepatitis B surface antigen (HBsAg), anti-human immunodeficiency (HIV), anti-Hepatitis C virus (HCV) antibody and human T lymphotropic virus (HTLV-I) antibody by enzyme linked immunosorbent assay (ELSIA).

The control groups were 15 healthy individuals matched for age and sex and with no history of systemic or dermatological diseases.

Lymphocyte and Neutrophil Isolation

Fifteen milliliter blood samples were taken from each patient and healthy control. Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Hypaque 1077 (Sigma, UK) density gradient centrifugation. Neutrophils were separated by using gradient density centrifugation (Histopaque -1119,

Sigma, UK). The cell viability was examined by trypan blue dye staining.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from the lymphocytes using the RNeasy Mini kit (Roche, Germany) as described by the manufacture's instruction. Total RNA was isolated from neutrophils with TriPure Isolation Reagents (Roche Applied Science, Germany). The isolated RNA by TriPure was treated by DNase I (Fermentas, Germany), and the concentration of RNA was determined by an absorbance at 260 nm and 260 nm using Nanodrop ND-1000 instrument (Wilmington, USA).

We used a fixed volume of input RNA (1µL) for each cDNA reaction. Reverse transcription reaction was carried out with RevertAidTM H minus First Strand cDNA Synthesis Kit (Fermentas, Germany). Reverse transcription was performed at 42°C for 60 min followed by RT inactivation at 70°C for 5 min. cDNA was stored at - 20°C until use.

Real time PCR Analysis

Quantitative real time PCR was performed on the cDNA samples using SYBR Green I Master Mix (Invitrogen SYBR GreenER^{TM} qPCR SuperMix Universal, USA) in Rotor Gene system (Corbett Research, Mortlake, NSW, Australia). Primers were designed at exon-exon junctions (Beacon Designer http://WWW.premierbiosoft.com) to discriminate between genomic DNA and cDNA. All of the selected primers sequences were further analyzed with the program Oligo software (http://WWW.cambio.co.uk/index.php). The primers sequences are shown in table 1.

Beta 2 macroglobulin was used as a housekeeping gene to normalize the mRNA expression levels and control error between samples. Beta 2 microglobulin is a low molecular weight protein sub unit of MHC class1 molecules which are present on all nucleated cells and is suitable reference gene in gene expression studies of neutrophils[20] and is relatively constant in PBLs.[21] The PCRs were performed in glass capillaries (Rotor Gene Q, Qiagen, Germany), in a final volume of 20 µL containing 2 µL cDNA template, 0.4 µL of primer pairs (200 nM, β2M, COX2) or 1.2 µL (600 nM, IL-17) and 10 µL of the SYBR Green I Master Mix. The PCR conditions were as follow: one cycle at 95°C for 10 min, followed by 45 cycles at 95°C for 10 s, 60°C for 45 s (β2M, COX2) or 60°C for 60 s (IL-17). After each annealing and extension step, the SYBR Green fluorescence was measured at 60°C.

At the end of the PCR, a melting curve analysis was performed by gradually increasing the temperature from 65 to 95°C.

Table 1. Primers sequence of β2M, COX2 and IL-17A

Genes	Sense	Anti Sense	Size (bp)
B2M	5'- CTTGTCTTTCAGCAAGGACTGG-3'	5'- CCACTTAACTATCTTGGGCTGTG-3'	127
COX2	5'- CCGAGGTGTATGTATGAGTGTG-3'	5'- TGAAGTGGGTAAGTATGTAGTGC-3'	161
IL-17A	5'- GTCAACCTGAACATCCATAACCG-3'	5'- ACTTTGCCTCCCAGATCACAG-3'	142

Table 2. Demographics of the subjects included in the study

Variables	Number of individuals	Mean ± SD	Range
Vitiligo patients	15		
Age (years)		25.40 ± 14	8-60
Males	6	28.5 ± 21.26	
Females	9	23.33 ± 8.8	
Healthy controls	15		
Age (years)		25.47 ± 12	10-52
Males	6	28.66 ± 18.5	
Females	9	23.33 ± 8.1	

Table 3. Clinical data of patients

Types of disease	Frequency	Percent
Generalized	2	13.3
Acrofacial	2	13.3
Universal	1	6.7
Segmental	1	6.7
Vulgaris	5	33.3
Focal	4	26.7
Total	15	100

Moreover, at the end of some experiments, 5 µL of PCR products (alongside with the Gene Ruler 100 bp DNA ladder) were UV-visualized following electrophoresis in ethidium bromide on 2% agarose gel, which confirmed the presence of the correct size of PCR products. All measurements were performed in duplicate. To establish the amplification efficiencies for the genes, relative standard curves were prepared using a ten-fold dilution series of a pooled cDNA for COX2 and β2M and two-fold dilution series of pooled cDNA for the IL-17 gene. Relative gene expression levels of COX2 and IL-17 were determined by using standard curves. The relative standard curves for both reference and target genes generated by plotting the threshold cycle value vs. the log of the dilution of the cDNA and PCR efficiencies ($10^{-1/slope}$ -1) was automatically calculated by the Rotor Gene Q system Software.

Statistical Analysis

Results are presented as mean± SD. Depending on the mode of distribution, by use of the one-sample Kolmogorov-Smirnov Test, statistical procedures were performed by the Mann-Whithny U Test or by the Independent-sample T Test.

Pearson or Spearman correlation tests were used for the relation between parameters and graphically described by scatter plots and linear regression lines. For investigating gene expression between different types of vitiligo, the Kruskal-Wallis Test was used. *p* value less than 0.05 was considered to be significant.

RESULTS

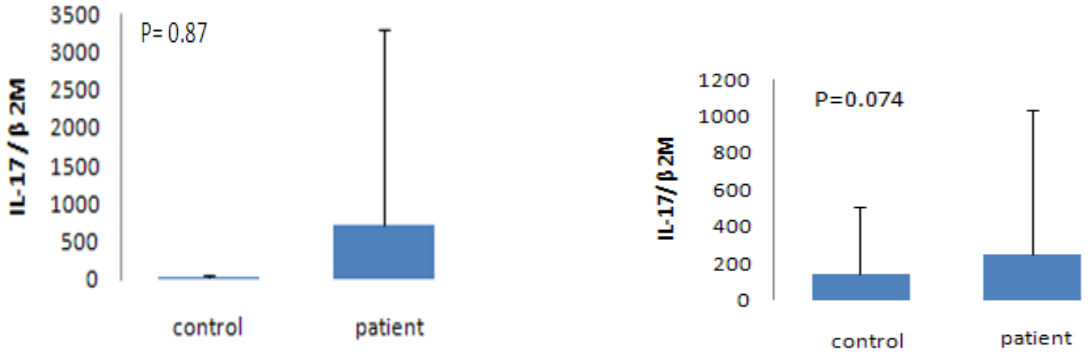
Family history was present in 5 patients (33.3%), 2 patients (13.3%) had other autoimmune disorders and in 5 cases (33.3%), There was report of autoimmune disorders in other family members or first-generation family members. The demographic and clinical data of patients are shown in Table 2 and 3. The percentage of viable cells was >99%. The neutrophil purity exceeded 99% when stained by Wright-Giemsa.

Real time PCR Validation

Specificity of the β2M, COX2 and IL-17 products was checked by melting curve analysis and agarose gel electrophoresis. The PCR products size of β2M, COX2 and IL-17 was 127bp, 161bp and 142bp, respectively with melting temperatures of 79°C, 78.5°C and 81°C. The melt curve analysis and sequencing of COX2 and IL-17 PCR products was performed to further confirm that the correct gene sequence had been amplified (data not shown).

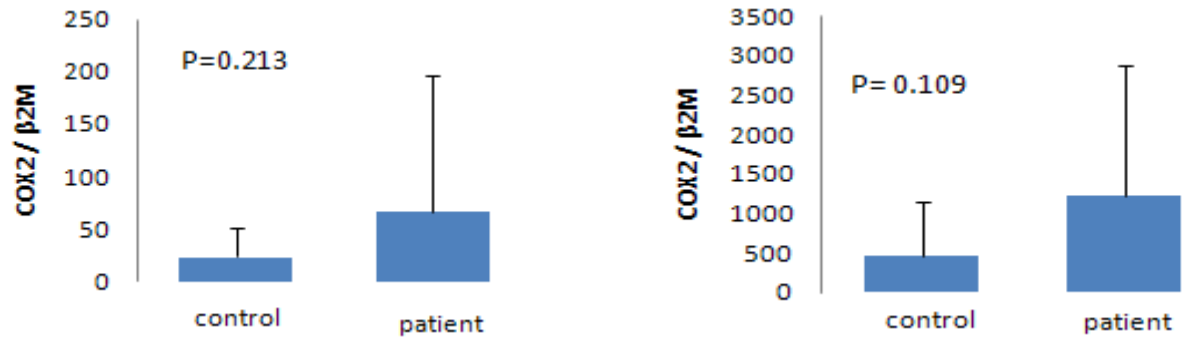
IL-17 mRNA Expression

The expression of IL-17 mRNA was analyzed in lymphocytes of patients with vitiligo and healthy controls. The mean of IL-17 mRNA expression levels in lymphocytes of cases (2570±718) was higher than control group (30±30), however no significant differences was observed between two groups (*p*=0.87). Furthermore, we examined the expression of IL-17 mRNA levels in the neutrophils of patients and controls.



(A) **(B)**

Figure 1. Expression of IL-17 gene in patients with vitiligo and healthy controls: lymphocytes and neutrophils were separated by gradient density centrifugation and IL-17 mRNA expressive levels were examined in lymphocytes (A) and neutrophils (B). No significant differences in IL-17 expression were observed in lymphocytes and neutrophils between the two groups. P value was calculated by the Mann-Whitney U test.



(A) **(B)**

Figure 2. COX2 mRNA expression in patients with vitiligo and healthy controls: Lymphocytes and neutrophils were separated by gradient density centrifugation and COX2 mRNA expression levels were examined in lymphocytes (A) and neutrophils (B). No significant differences in COX2 mRNA expression were found in lymphocytes and neutrophils between two groups. P value was calculated by the Mann-Whitney U test.

Although the mean mRNA expression levels was higher in patients (790±245) when compared with controls (336±138), statistical analysis did not reveal any significance (p=0.074) (Figure 1).

COX2 mRNA Expression in Lymphocytes and Neutrophils

We analyzed the expression of COX2 mRNA in lymphocytes and neutrophils of patients with vitiligo and healthy controls. The expression of COX2 mRNA in the lymphocytes of cases was (130±66) and in controls was (28±23). No significant difference was found between the two groups (p=0.213). We also examined the expression of COX2 mRNA in neutrophils of the two groups. The mean of COX2 mRNA expression levels in patients with vitiligo was 1650±1225) and in control group (691±459). No significant differences in COX2 mRNA expression were found at baseline between cases and controls (p =0.109) (Figure 2).

Correlation between IL-17 and COX2 mRNA Expression in Lymphocytes and Neutrophils of Vitiligo Patients

We examined the correlation of IL-17 with COX2 mRNA levels in lymphocytes and neutrophils of patients and controls by Pierson test. As shown in figure 4, there was no significant correlation of IL-17 mRNA levels with COX2 expression in both patients' (r=0.116, p =0.68) and control's lymphocytes (r=0.273, p =0.38).

There was also no significant correlation of IL-17 mRNA with COX2 in neutrophils of control group (r=0.343, p=0.38), while there was a significant and positive correlation of IL-17 mRNA with COX2 in vitiligo patients (r=0.80; p=0.01) (Figure 3).

No Correlation between IL-17 and COX2 mRNA Expression with Sex and Age in Vitiligo Patients

The correlation between IL-17 and COX2 mRNA levels with age in neutrophils and lymphocytes of patients was examined. No significant correlation of IL-17 mRNA expression was observed with neither lymphocytes (p=0.97) or neutrophils (p=0.45) in vitiligo patients. No significant correlation was also between mRNA COX2 with age in lymphocytes (p=0.19) and neutrophils (p = 0.93) of patients with vitiligo.

We further analyzed the correlation of mRNA IL-17 and COX2 with sex in lymphocytes and neutrophils of patients with vitiligo and the results were not statistically different (p>0.05) (data not shown).

Comparison of IL-17 and COX2 Gene Expression in Different Clinical Types of Vitiligo Disease

To examine the mRNA levels of IL-17 and COX2 in lymphocyte and neutrophils of patient with different clinical types of disease, the mean mRNA of IL-17 and COX2 was compared.

No significant differences in IL-17 and COX2 mRNA levels was found among clinical types of disease (p>0.05).

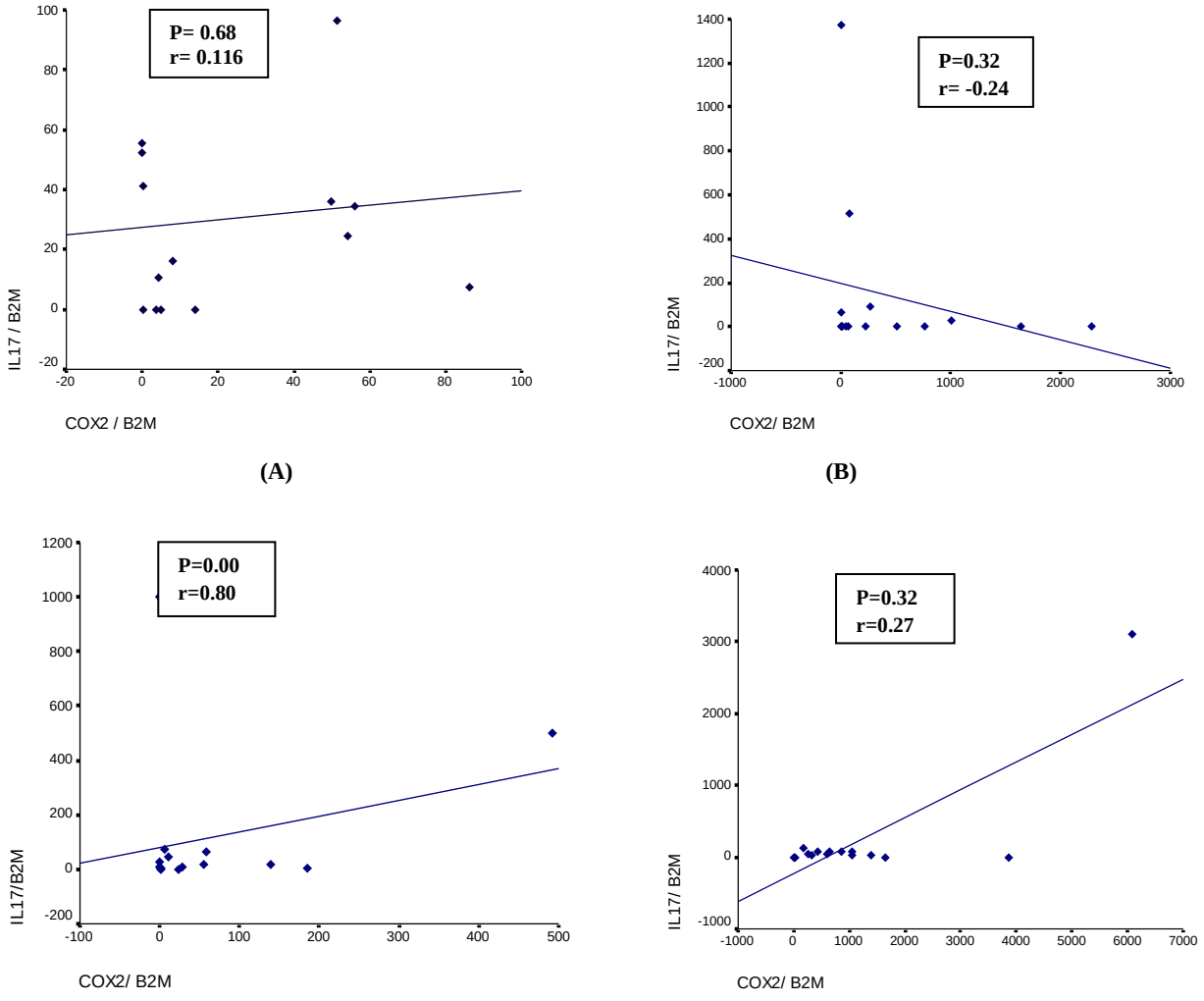


Figure 3. Correlation between mRNA IL-17 levels with mRNA COX2 levels in lymphocytes and neutophils of patients with vitilio and controls: (A) lymphocytes from controls, (B) neutrophils from controls, (C) lymphocytes from patients, and (D) neutrophils from patients. Pearson correlation test was used to assess the correlation of mRNA IL-17 with COX2.

DISCUSSION

Although the pathogenesis of vitiligo has not been fully understood, however an autoimmune theory has been proposed to explain the cause of disease.[22] It has been shown that cytokines, as the inflammatory mediators which play a pivotal role in autoimmune diseases, may involved in depigmentation.[23,24]

To our knowledge this is the first study which examines the expression of COX2 and IL-17 genes in both lymphocytes and neutrophils in vitiligo patients. The results showed that the mRNA levels of IL-17 and COX2 in lymphocytes of patients were not significantly higher than control group, but the mRNA levels of IL-17 in patients' neutrophils were higher than control group and a considerable difference in the COX2 gene expression in neutrophils between two groups was observed, although it was not statistically significant.

The studies on cytokines in vitiligo patients are limited and inconsistent.[17] IL-17 has been classified as a pro-inflammatory mediator, based on its ability to induce a wide range of inflammatory effectors in target cells.[11] Locally IL-17 stimulates production of PGE2/COX2, nitric oxide, and IL-6.[25] IL-17 mediates chemotaxis of monocytes and neutrophils to sites of inflammation through the chemoattractant mediators such as IL-8, MCP-1, (Gro, α), CXCL1, CXCL10,

CCL2, CCL7, and CCL20.[26] Therefore, IL-17 is considered as one of the main mediators of tissue inflammation. The relation between IL-17 and several autoimmune disorders includes: RA,[27] psoriasis,[28] have been studied.

Basak et al.[17] measured the serum level of IL-17 by ELISA in vitiligo patients and reported that the serum levels of IL-17 in patients with vitiligo significantly decreased when age of disease onset increased. Furthermore, serum IL-17 level was associated with body area. In another study, Jandas et al.[16] investigated the role of Th-17 cells in the pathogenesis of several autoimmune disorders including five vitiligo patients. They could not find any increase in the number of these cells in peripheral blood of vitiligo patients in compare with control group. In both study the role of main inflammatory cells of immune system, neutrophils, was not considered. Van den Broom et al., reported the IL-17-producing $CD8^+$ T cells in vitiligo patient who only one experiencing halo nevi, and poliosis in nearly all lesions, suggesting these cells might be involved in diseases pathogenesis.[29] In the present study we expected to observe high levels of IL-17 expression in neutrophils of patients, however no significant difference was seen between patients and controls. A larger sample would allow for greater statistical analyses between the patients and controls.

Cox2, inducible form of cyclooxygenase enzyme, is the key factor of inflammatory development and express in certain cells including lymphocytes[30] and neutrophils[31] following extracellular stimuli such as mitogens, lipopolysaccharide (LPS) and inflammatory cytokines such as: IL-1, TNF-α and growth factors.[15] The expression of Cox2 in neutrophils and lymphocytes increases by pro-inflammatory stimuli[31] and T cell receptor triggering with OKT3 antibodies, respectively.[30] COX2 is up-regulated in angiogenesis-related disorders such as RA, psoriasis, lupus,[32] and many cancer cells.[33] The products of COX2, prostaglandin E2, which is also produced in response to ultraviolet radiation (UVR), plays an important role in melanocyte proliferation and melanogenesis[34] and loss of menalocytes is the main defects in vitiligo.[35]

Recently Miao et al.[18] suggested the association between three functional Cox2 polymorphisms and the risk of vitiligo. In our studty, the increased of Cox2 expression in patient's neutrophils might suggest that this gene have a pivotal role in innate immune response on melanogenesis.

Studies have suggested that IL-17 and Cox2 show reciprocal effect on each other. Our result demonstrated a positive correlation of IL-17 mRNA with COX2 in neutophils of patients with vitiligo. In vitro, stimulation of stroma cells by IL-17 induces secretion of certain pro inflammatory mediators, especially Cox2.[36]

IL-17 is one of the effective cytokines in destruction and inflammation of cartilage. This effect is mediated through the increase of other genes such as Cox2.[37] Lemus et al.[38] demonstrated that locally produced COX2-derived prostaglandins are essential for IL-17 production in RA patients.

In the present study we couldn't find any relation between IL-17 and COX2 genes expression and gender, age or types of vitiligo.

It has been suggested that different pathogenesis mechanisms could account for different clinical types of vitiligo. Neural theory is usually related to segmental whereas the auto immune hypothesis is thought to be involved in the generalized (non-segmental) form of disorder.[1] Locally immune response is observed in generalized type while it has not been reported in segmental type.[39] Therefore it would be valuable to study the immune response locally in each clinical type of disease.

The gene expression of IL-17 and Cox2 has not been studied in the skin. Previous studies demonstrated the presence of epidermal cytokines in the site of vitiligo lesions[40,41] and other inflammatory and autoimmune disease such as psoriasis,[42] suggesting that these changes can occur without any relation with peripheral blood.[17] Thus, investigation of IL-17 and COX2 genes expression in site of vitiligo lesions might help to understand the mechanisms of pathogenesis in vitiligo.

CONCLUSION

In conclusion, our results showed a positive correlation of IL-17 mRNA with COX2 in neutophils of patients with vitiligo . Furthermore the expression of Cox2 was higher in neutrophils of patients compared with control group. Testing of IL-17 and COX2 genes expression in more cases in lymphocytes, neutrophils, and investigations of these mediators in heterogeneous clinical forms diseases and skin of vitiligo patients might help to clarify the disease pathogenesis.

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