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Correlation of Gene's expression of IL4, IL21, and TNF-α in Pemphigus vulgaris Patients with Gender and Residence Area in Babylon Province

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Abstract

Introduction: Pemphigus vulgaris (PV) is a rare and possibly lethal autoimmune blistering disorder. PV induces flaccid blisters and erosions on the skin and mucous membranes as a result of keratinocyte dissociation inside the epidermis. The objective of this research was to examine the correlation between the gene expression levels of interleukin-4, interleukin-21, and tumor necrosis factor-alpha and the incidence of PV, while also assessing the effects of gender and geographical location.

Methods: A case-control study was conducted involving 26 clinically diagnosed PV patients and 52 healthy controls from Babylon Province, Iraq. Participants were aged 30 to 72 years. RNA was extracted from peripheral blood, and the gene expression of the target cytokines was quantified using one-step RT-qPCR with the $2^{(-\Delta\Delta Ct)}$ method.

Results: The study revealed that 19 patients (73.1%) were female and 9 (26.9%) were male, with ages ranging from 30 to 72 years. Most patients lived in urban areas 16(61.5%), while fewer resided in rural areas 10(38.5%). We found notably higher dysregulation in the gene expression of both IL-4 and IL-21 among PV patients. Although TNF-α expression levels showed no significant difference, the average for cases (2.10 ± 2.60) was still slightly higher than that of the control group (1.92 ± 1.83).

Conclusion: The significant downregulation of specific interleukins suggests a complex, stage-dependent immune dysregulation in PV that may be influenced by environmental factors such as urbanization. Further large-scale, multicenter studies are warranted to fully elucidate these cytokine dynamics.

Keywords: Pemphigus vulgaris, Gene expression, IL-4 and IL-21, TNF-α

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Introduction

Pemphigus vulgaris (PV) is an uncommon chronic autoimmune disorder affecting the skin and mucous membranes, distinguished by the formation of intraepithelial blisters and erosions due to pathogenic autoantibodies targeting several keratinocyte membrane proteins(1). PV is characterized pathophysiologically by the generation of pathogenic IgG autoantibodies. Despite substantial improvements in understanding the immunological mechanisms of PV, not all persons exposed to environmental triggers acquire the disease, indicating a strong hereditary component(2). Cytokine expression is known to cause both systemic and local inflammation(3). T cell activation leads to increased tissue inflammation, which in turn causes cytokine release. Additionally, T cells support B cell survival and development, enabling ongoing autoantibody synthesis(4). Some of the cytokines that have been examined and are likely to play pathogenic roles in PV include interleukin IL-4, IL-6, IL-17, and IL-21(5,6). TNF-α is a potent pro-inflammatory cytokine that has been shown to have elevated levels in serum and in situ expression in PV patients' lesional skin on multiple occasions(7). Compared to individuals with desirable disease outcomes, PV patients who experience emotional stress also had a higher level of TNF-α(8).

There are numerous indications that the pathophysiology of autoimmune illnesses is significantly influenced by heredity. Genetic variants could be utilized to predict treatment response in addition to influencing susceptibility to certain disorders(1). Typically, SNPs may affect messenger RNA stability, protein function, and promoter activity to affect gene expression (9). The goal of this study was to estimate gene expression of immune-related genes in PV patients against healthy individuals.

Methodology

Study subject

Between 2024 and 2025, patients with PV were recruited from those attending the dermatology clinic at Murjan Teaching Hospital in Babylon. The group included 26 individuals 7 males and 19 females with a mean age of 51.42 ± 10.92 years. Diagnosis was made clinically, and the Pemphigus Disease Area Index (PDAI) was used by specialist physicians to assess disease severity (10). A control group of 52 healthy individuals 14 males and 38 females had a mean age of 49.15 ± 9.23 years. All participants gave written informed consent to join the study, and consultations were carried out after obtaining

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verbal consent from those able to follow instructions and willing to participate.

RNA extracted and quantitative RT- PCR

RNA was extracted using RNA extraction kit (Geneaid/ Taiwan). cDNA synthesis and PCR were achieved byGoTaq® 1-Step RT-qPCR System (Promega / USA). Primers were obtained from Macrogene (Koria).

Estimation of RNA Concentration and Purity

Using the diode array scanning range of (200 to 320) Nm wavelength, RNA quantity and quality were assessed by nanodrop and adsorption profile processing leading to a calculation of RNA quantity and quality through measuring the 260/280 and 260/230 ratios. A ratio less than 1.8 for individual sample would lead to re-extraction (11).

Complementary DNA Synthesis

GoTaq® 1-Step RT-qPCR Master Mix is specifically designed for high sensitivity, high efficiency copy synthesis and amplification in one-step RT-qPCR. Though the first-strand cDNA is synthesized using RNA as templates and reverse gene-specific primers, and then the qPCR is performed with both forward and reverse gene-specific primers in sequence with the synthesized cDNA used as templates to achieve one step from reverse transcription to qPCR within a single tube. The sequences of the designated primers are detailed in Table 1.

Reaction steps

For quantifying gene expression of IL-4, IL-21, TNF-α and housekeeping (β-actin), quantitative real time PCR was based on the fluorescent power of Syber Green. It was the component with their quantity as per kit.

RT- qPCR Program

The program for RT-qPCR was set up with indicated thermocycling protocol as shown in Table 2.

Analysis Gene Expression

In order to evaluate the expression of IL-4 and IL-21, the housekeeping gene (β-actin) served as the internal control gene, and the double delta Ct (threshold cycle) analysis was used. A 2-ΔΔCt, which stands for the Relative Fold Change, was measured in order to ascertain the expression fold change for the IL-4, IL-21, and TNF-α genes. Hence, the findings were presented as a fold shift in the target gene's expression level, relative to the calibrator, and normalized to a housekeeping gene (β-actin). Gene expression data was generated by calculating relative quantification using the Pfaffl Method(9).

Table 1. List of primers sequences

Gene	Primer sequence (5' -3')	Reference
IL-4	F: CGGCAACTTTGTCCACGGA R: TCTGTTACGGTCAACTCGGTG	(12)
IL-21	F: GAGGAAACCACCTTCCACAA R: CAGGAATCTTCACTTCCGTGT	(13)
TNF-α	F: CTGGGGCCTACAGCTTTGAT R:GGCTCCGTGTCTCAAGGAAG	(14)
Housekeeping β-actin (ACTB)	F: GGA CTT CGA GCA AGA GAT GG R: TGT GTT GGG GTA CAG GTCTTG	(15)

Statistical analysis

Data Analysis the data was analyzed using (version 20). The outcomes were presented as simple statistic scores including mean and standard deviation. Quantitative data percentages were analyzed and compared across different groups, including the control group using the chi-square test (χ^2-test). Additionally, Mann-Whitney U test was conducted to compare between two independent groups of cases and controls. Statistical significance was defined as p-value ≤ 0.05.

Results

A dermatologist diagnosed 26 cases of PV, all classified as the mucocutaneous type. More women were affected than men. The study involved twenty-six patients with a mean age of 51.42 ± 10.93 years and fifty-two apparently healthy controls with a mean age of 49.15 ± 9.24 years. Of the patients, 19 were female (73.1%) and 7 were male (26.9%), giving a male-to-female ratio of 1:3.6. In terms of residential areas, there was no difference between the case and control groups. However, most patients lived in urban areas 16(61.5%), while a smaller proportion resided in rural areas 10(38.5%), Table 3.

The provided Table 4 contains quantitative RT-qPCR data comparing gene expression levels of IL-4, IL-21, and *TNF-α* between PV patient and control groups.

The RT-qPCR analysis showed higher significant differences in IL-4 and IL-21 expression between the control and patient groups, while TNF-α differences were not significant. Even though TNF-α expression levels didn't differ significantly, the mean value for cases (2.10 ± 2.60) was still higher than that of the control group (1.92 ± 1.83), as presented in Table 5.

The data in Table 6 show notable differences in TNF-α fold and IL4_fold for the urban study group, with values

Table 2. RT-qPCR of IL-4, IL-21, TNF-α and β-actin amplification program

Step	Temperature	Time	Cycles No.
Hold temper1	37ºC	15 min	1
Hold temper2	94 ºC	10 min	
Denature	94ºC	10 sec	40
Annealing	60 ºC	30 sec	
Extension	72	30 sec	
Melting temper	60-95 ºC	2-5 sec/step	

Table 3. Baseline area of residence for pemphigus vulgaris groups and control

Groups	Area of residence		Total	*P* value
	Rural No. (%)	Urban No. (%)		
Control	17(32.7%)	35(67.3%)	52(100.0%)	
Cases	10(38.5%)	16(61.5%)	26(100.0%)	0.81
Total	27(34.6%)	51(65.4%)	78(100.0%)	

Not significant (*P*>0.05).

Table 4. Expression Levels of *IL4, IL21 and TNF-α* in controls and pemphigus vulgaris

Group		Ct Target Gene	Ct HKG Gene	Δ Ct	ΔΔCt	2^-ΔΔCt	Fold change
				Mean ± S. D			
Patients	*IL-4*	21.60 ± 2.60	17.8 ± 0.43	3.79 ± 2.29	1.49 ± 2.31	0.77 ± 1.45	0.43
Control		20.52 ± 2.16	18.26 ± 0.72	2.23 ± 1.99	0.03 ± 1.98	1.78 ± 2.00	2.31
Patients	*IL-21*	22.6 ± 2.47	17.8 ± 0.43	4.81 ± 2.33	1.65 ± 2.31	0.65 ± 0.68	0.26
Control		21.70 ± 3.46	18.26 ± 0.72	3.23 ± 2.65	0.03 ± 2.65	2.44 ± 2.60	3.75
Patients	*TNF-α*	21.04 ± 1.99	17.8 ± 0.43	3.1 ± 1.7	-0.10 ± 1.72	2.10 ± 2.6	1.07
Control		21.59 ± 2.11	18.26 ± 0.72	3.25 ± 1.95	0.01 ± 1.95	1.96 ± 1.83	0.93

Table 5. Mean statistic of expression levels of *IL4, IL21 and TNF-α* in controls and pemphigus vulgaris

	Group Statistics					U test *P* value
	Groups	No.	Mean	Std. Deviation	Std. Error Mean	
IL4	control	52	1.7840	2.00446	.27797	0.000**
	cases	26	0.7254	1.29065	.25312	
IL21	control	52	2.4414	2.60025	.36059	0.001**
	cases	26	0.6522	.68355	.13405	
TNF-α	control	52	1.9258	1.83879	.25499	0.733
	cases	26	2.1002	2.60493	.51087	

* Refer to significant difference at $P<0.01$.

Table 6. Relationship between gene expression folds of *IL4, IL21 and TNF-α* in controls and pemphigus vulgaris with residence area and sex.

Group Statistics						
	Area of residence	NO. 78	Mean	SD	Std. Error Mean	*P* value
TNF- α _fold	Rural	27	2.0798	2.07474	.39928	0.81
	Urban	51	1.9706	2.13407	.29883	
IL21_fold	Rural	27	1.2023	1.24766	.24011	**0.03***
	Urban	51	**2.1853**	2.66257	.37283	
Il4_fold	Rural	27	.8553	.76456	.14714	**0.01***
	Urban	51	**1.7358**	2.17765	.30493	
Sex						
TNF- α _fold	Male	21	2.5093	2.77610	.60579	0.53
	Female	57	1.8239	1.78534	.23647	
IL21_fold	Male	21	1.6494	2.82357	.61615	0.59
	Female	57	1.9171	2.11898	.28067	
Il4_fold	Male	21	1.9205	2.66118	.58072	0.47
	Female	57	1.2507	1.44834	.19184	

* Refer to significant difference at $P\leq0.05$.

of 2.18 ± 2.66 compared to 1.20 ± 1.24, and 1.73 ± 2.17 compared to 0.85 ± 0.76, respectively.

Discussion

This research found that the mean age of PV patients was 51.42 years, consistent with worldwide epidemiological statistics suggesting that PV start often occurs between 40 and 60 years, with the majority of diagnoses made between 45 and 65 years of age(10,11). A recent systematic study by Zhao et al. calculated the worldwide pooled incidence rate of PV at 2.83 per million person-years, affirming that PV is a rare but clinically relevant autoimmune disease(11). The average age of presentation in polycythemia vera (PV) significantly differs across populations, spanning from 36.5 years in Kuwait to 72.4 years in Bulgaria(10), these discrepancies may be ascribed to disparities in sample sizes, ethnic compositions, and environmental exposures of the research cohorts.

The results indicate a clear tendency toward females, with a female-to-male ratio of 3.6:1 (73.1% female). This female predominance is consistent with earlier global epidemiological studies and recent prevalence analyses. Wertenteil et al. reported that 61% of PV patients in a United States population-based study were women(12), Kridin and Schmidt (2021) identified a female predominance in virtually all research cohorts with non-endemic pemphigus, with female-to-male ratios mostly ranging from 1:1 to 2:1.(10). The notably higher ratio in

our study (3.6:1) may reflect regional and ethnic variations, as Middle Eastern populations have been reported to have distinct epidemiological profiles(13). Estrogen is thought to play a key role in the higher incidence of PV among females, as it modulates immune system responses and enhances autoimmune susceptibility(14). Lavaee et al. (2021) further supported this notion by demonstrating a significant reduction in estrogen serum levels among patients with PV, suggesting that hormonal dysregulation may contribute to disease pathogenesis in postmenopausal women(15). Interestingly, in the two groups of PV patients analyzed in our study, although gender may impact illness risk, no statistically significant differences were found in gene expression between male and female patients, it does not drastically alter the expression of IL-4, IL-21, or TNF-α in this cohort.

Regarding the area of residence, most patients lived in urban areas (61.5%). While our study found no significant difference in overall prevalence between urban and rural groups, a notable finding was that IL-4 and IL-21 gene expression fold changes were significantly higher in urban patients compared to rural patients. The relationship between urbanization and autoimmune diseases is complex and multifactorial. Voskarides demonstrated that there is a considerable correlation between urbanization and the prevalence of autoimmune illnesses, with urban environments contributing to altered immune profiles through factors such as pollution, dietary changes, and reduced microbial diversity(16). Conversely, Serwin et al. reported that the incidence of pemphigus in rural areas of North-East Poland (4.96 per 100,000) was significantly higher than in urban areas (2.70 per 100,000)(17), while Barcelos et al. found that most pemphigus patients in Brazil were from peri-urban (53.1%)(18). These conflicting findings highlight the need for further investigation into geo-epidemiological factors and their influence on cytokine gene expression in PV.

Given the limited information on the levels and relationships of interleukin and cytokine gene expression in pemphigus, we compared the values of gene expressions of these interleukins and cytokines with their values in sera reported in recent literature. Pemphigus was once considered a Th2-dominant disease, but growing evidence now points to an important role for other T helper cell subsets, including Th17 and Tfh cells(19,20). Holstein et al. conducted a thorough immunophenotyping study, revealing that TH17/TFH17 cells play a crucial role in the pathogenesis of pemphigus and facilitate the production of desmoglein 1/3-specific autoantibodies(20). In their recent review of PV pathogenesis, Yang and Zuo highlighted that the principal processes underlying the development of PV include elements that facilitate B cell differentiation and autoantibody synthesis, alongside pathogenic consequences mediated by signaling pathways., including the JAK/STAT pathway, along with other contributing factors such as genetic and epigenetic elements(19).

In the current study, PV patients showed significantly lower levels of IL-4 and IL-21 gene expression compared to the control groups. These results contrast with a recent study by Arnold et al, on IL-4 gene expression in Bullous Pemphigoid, which reported an upregulation of IL-4 expression(21), The comprehensive analysis by Geng et al. established broad biomarker patterns, including higher levels of Th2 cytokines, including IL-4, IL-6, IL-10, and IL-13, in patients with PV across 66 studies involving 2,463 individuals (22). However, the findings of this research are highly consistent with those of Shahbazian et al, who measurements of IL-4 and IL-21 in blood revealed that the serum levels of both cytokines are dramatically decreased in newly diagnosed PV patients compared to healthy individuals (23). The observed downregulation of these cytokines in specific patient cohorts may indicate the intricate and evolving characteristics of the immune response in PV, which can differ according to disease stage, previous treatments, and individual variability. Schwartz et al. conducted an extensive cytokine profiling study involving 116 patients with PV. Their findings indicate that, in comparison to control subjects, PV patients typically exhibit an activated immune response characterized by cytokines and chemokines. Nonetheless, substantial interindividual diversity in cytokine levels occurs, coupled with limited activation of several T helper cell pathways across individuals (24). Furthermore, even healthy individuals carrying PV susceptibility HLA alleles showed upregulation of certain cytokine levels comparable to those seen in PV patients(24), suggesting that genetic background significantly modulates cytokine expression.

Prior studies have shown that blood IL-4 levels in individuals with PV may be elevated (25), or remain unchanged (26), when comparison to control groups. IL-4 is mostly produced by Th2 cells and aids in Th2 cell development via an autocrine mechanism; also, it encourages isotype shift towards IgG4, a recognized class of IgG antibodies linked to pemphigus(23,27). The variation in IL-4 findings among studies could be attributed to several factors, including the level of disease activity during sampling, the treatment status of participants, and the measurement techniques employed, such as serum protein levels compared to mRNA gene expression analysis.

Mesas-Fernández et al. provided a comprehensive review of IL-21 in inflammatory skin diseases, emphasizing its central role in driving pathogenic immune responses through multiple signaling pathways(28). A prior study using RT-PCR by Holstein et al. found that IL-21 expression is elevated in pemphigus patients, particularly in TFH17 cell subsets(20). Additionally, Tavakolpour proposed IL-21 as a novel potential player in pemphigus pathogenesis, highlighting its involvement via different signaling pathways associated with B cell differentiation and autoantibody production(29). Our finding of decreased IL-21 gene expression aligns with the serum-level findings of Shahbazian et al. and may suggest a compensatory downregulation or a specific phase of immune dysregulation in our patient cohort(23). Notably, Shahbazian et al. observed that while IL-21 levels were significantly lower in newly diagnosed patients, they

were not different from controls in patients with PV in remission, suggesting that IL-21 dynamics are closely linked to disease activity(23).

While TNF-α expression levels did not differ significantly in the study, the mean value for cases was still slightly higher than that of the control group. Lee et al. reported that serum TNF-α and IL-6 levels were significantly elevated in PV patients compared to healthy controls(26), and Khozeimeh et al. similarly found notably higher TNF-α levels in PV patient sera(30). The systematic review by Geng et al. classified TNF-α as a characteristic Th1 biomarker that was elevated in PV patients, with genetic polymorphisms in TNF-α found to be associated with PV and TNF-α levels correlating with disease severity(22). It has been suggested that PV IgG-induced TNF-α might trigger acantholysis, with the cytokine potentially playing a direct role in the blistering process.

Recent advances have underscored the significance of the JAK-STAT signaling pathway in the mediation of cytokine effects within the context of autoimmune bullous diseases. Lin et al. conducted a review on the involvement of cytokines and the JAK-STAT pathway in PV and bullous pemphigoid, highlighting that JAK inhibitors have demonstrated potential in altering pathogenic cytokine signaling(31). Juczynska et al. provided evidence of altered expression of JAK3, STAT2, STAT4, in PV skin lesions, thereby reinforcing the role of this pathway in the pathogenesis of the disease (32). The collective findings highlight the intricate nature of cytokine networks in PV and suggest the possibility of targeted therapeutic interventions.

Cytokine production or function is partly influenced by genetic variations in their sequences(1), making it reasonable to suspect that certain cytokine polymorphisms may contribute to a genetic predisposition to PV. Vodo et al. emphasized that genetic variants could be utilized not only to predict disease susceptibility but also to anticipate treatment response(1). However, studies exploring this link have been limited, likely due to the disease's rarity in most populations. The Fang et al. review on the role of T cells in PV and bullous pemphigoid highlighted that T cell activation leads to increased tissue inflammation, cytokine release, and support for B cell survival and development, enabling ongoing autoantibody synthesis(4). Kim et al. further demonstrated that targeting ICOS expressed on CXCR5+PD-1+ T helper cells could suppress the progression of PV, offering novel therapeutic perspectives(33-36).

Conclusion

The study demonstrates a significant downregulation in the gene expression of IL-4 and IL-21 among patients with PV compared to healthy controls, highlighting a complex and potentially stage-dependent immune dysregulation in the pathogenesis of the disease. These findings show the differences in cytokine gene expression based on the area of residence, with significantly higher IL-4 and IL-21 fold changes in urban compared to rural patients, underscore the potential impact of environmental factors such as urbanization, pollution, and altered microbial diversity on the immune profiles of affected individuals.

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Artificial Intelligence Use Disclosure

AI techniques were used just for language editing and grammatical enhancement. All scientific content, data analyses, and conclusions are solely the authors' responsibility.

Authors' Contribution

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Competing Interests

None.

Data Availability Statement

The datasets used and/or analyzed during the present research are not publically accessible due to the privacy and confidentiality of participants but are accessible from the author responsible on reasonable request.

Ethical Approval

The Declaration of Helsinki was the basis for the study's ethical clearance. The university's ethics committee evaluated and accepted the written and verbal permission that the patients supplied (document number 342B, October 2024).

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