Received: 17 August 2020 | Revised: 14 September 2020 | Accepted: 17 September 2020

DOI: 10.1002/mgg3.1519

Molecular Genetics & Genomic Medicine Open Access WILEY

ORIGINAL ARTICLE

TH17/IL23 cytokine gene polymorphisms in bullous pemphigoid

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Abstract

Background: TH17/IL-23 immune axis is considered to be involved in the pathogenesis of autoimmune and chronic inflammatory diseases. Bullous pemphigoid (BP) is the most frequent autoimmune blistering disease, characterized by the presence of autoantibodies against the components of the dermal-epidermal junction. Animal studies and characterization of patient samples point toward a contribution of TH17 cells in BP pathogenesis. However, genetic polymorphisms in the genes of TH17/IL-23 cytokines have not yet been well investigated in BP.

Methods: Detection of polymorphisms in *IL-17A* (rs2275913 and rs3819025), *IL-17F* (rs2397084 and rs763780), *IL-17RA* (rs2229151), and *IL-23R* (rs2201841, rs7530511, rs11209026, and rs10889677) genes were performed following the collection of blood samples and DNA extraction from BP patients and controls. Gene expression of *IL-23R* was determined by quantitative RT-PCR analysis.

Results: The prevalence of *IL-23R* rs7530511 genotypes and alleles, as well as IL-23R rs2201841 alleles, is significantly different between the BP patients and controls. While the minor C-allele of *IL-23R* rs7530511 is highly present in the patients, the G-allele distribution of *IL-23R* rs2201841 is significantly more prevalent in the control individuals compared to the BP patients. Genotypes and alleles of other SNPs in *IL-17A*, *IL-17F*, and *IL-17RA* were similarly distributed in patients and controls.

Conclusions: No alteration was found in the gene expression between wild and polymorphic genotypes of *IL-23R* (rs2201841 and rs7530511) variations, indicating they do not contribute to altering the levels of gene expression in blood. In summary, our data show that the alleles of two SNPs in *IL-23R* rs2201841 and rs7530511 are associated with BP.

KEYWORDS

autoimmune disease, bullous pemphigoid, gene expression, gene polymorphism, TH17/IL-23 cytokines

1 | INTRODUCTION

Bullous pemphigoid (BP) is the most common autoimmune subepidermal blistering disease which is characterized and caused by an autoimmune response against the proteins located at the dermal-epidermal junction (DEJ), namely BP180 and BP230 (Haeberle et al., 2018; Liu et al., 2017; Muramatsu et al., 2018; Schmidt & Zillikens, 2013). After binding of autoantibodies to their targets, a series of immunological responses and enzymatic processes are activated. These responses are mostly intervened by complement activation and inflammatory cell recruitment which leads to the destruction of basement membrane components and anchoring fibers and, ultimately to skin inflammation and subepidermal blister formation (Liu, 2003; Ludwig et al., 2017; Nakashima & Fujimoto, 2014). Cytokines play a major role in autoimmune skin diseases and dysregulation of cytokine production and alteration in their receptors are associated with the pathogenesis of inflammatory skin diseases (Bagci et al., 2017; Didona & Di Zenzo, 2018; Ludwig & Schmidt, 2009; Sauder, 1990; Tabatabaei-Panah et al., 2019).

TH17/IL-23 immune axis is implicated in the pathogenesis of several autoimmune and chronic inflammatory diseases such as psoriasis (Girolomoni et al., 2017). Although BP is considered to be mediated by TH1/TH2 cells associated with BP180/BP230 autoantibodies, immunohistochemical staining of patient skin showed that TH17 cells are present in BP (Arakawa et al., 2011; Chakievska et al., 2018; Fischer-Stabauer et al., 2012; Le Jan et al., 2014; Zebrowska et al., 2013). In a pre-clinical BP mouse model, IL-17A-deficient mice are completely protected from clinical disease induction, and pharmacological IL-17A blockade impairs disease induction. Hence, taken together, this points toward a contribution of IL17 to BP pathogenesis. IL-17A and -17F share a common receptor subunit (IL-17RA) for signaling and dysregulation of the IL-17A/IL-17RA axis contributes to the pathogenesis of inflammatory skin diseases (Papp et al., 2012). Herein, IL-23 is an upstream regulatory cytokine with a critical role in the maintenance of the TH17 cell phenotype (Stritesky et al., 2008) as well as the production of effector cytokines, such as IL-17A and IL-17F (Chan et al., 2006; Stritesky et al., 2008). IL-23 signals through IL-23R which can amplify Th17 cell response by inducing pro-inflammatory cytokines and dysregulated IL-23 production also promotes autoimmune inflammation (Langrish et al., 2005; Maddur et al., 2012). Furthermore, the pathogenicity of Th17 cells is associated with the hyper-activation of the IL23/IL23R signaling pathway (Di Meglio et al., 2011; McGeachy et al., 2009). Interestingly, the pharmacological blockade of the (IL12)/IL23 pathway using ustekinumab has shown diverse outcomes: In 2 cases, BP was newly diagnosed in psoriasis patients treated with ustekinumab, while in one case BP improved under ustekinumab treatment (Le Guern et al., 2015; Loget et al., 2017; Onsun et al., 2017).

Given the influence of cytokine levels in the pathogenesis of the autoimmune diseases, single nucleotide polymorphisms (SNPs) in the genes may contribute to the altering of these levels, and thereby affect the susceptibility to autoimmune diseases. Although the polymorphisms of Th17/IL23 have been studied in autoimmune diseases, to our very best of knowledge, it has never been studied in BP patients. The present study aimed at testing the frequency of SNPs rs2275913 and rs3819025 (*IL-17A*, OMIM 603149), rs2397084 and rs763780 (*IL-17F*, OMIM 606496), rs2229151 (*IL-17RA*, OMIM 605461), and rs2201841, rs7530511, rs11209026, and rs10889677 (*IL-23R*, OMIM 607562) in patients suffering from BP compared with healthy controls. Since the polymorphism could affect the levels of gene expression, the respective cytokine gene expression was measured in the patients with allelic variation to compare with healthy individuals.

2 | MATERIALS AND METHODS

2.1 | Ethical compliance

Informed patient consent was collected and the Human Research Ethics Committee of Skin Research Center, Shahid Beheshti University of Medical Sciences has approved the study protocol. The study adhered to the principles outlined in the 1975 Declaration of Helsinki as revised in 2000.

2.2 | Subjects

Forty patients diagnosed with BP were recruited since 2013 at the Shohada Tajrish and Razi hospitals in Tehran. The diagnosis of BP was carried out according to investigational assessment guidelines (Feliciani et al., 2015). Briefly, it was based on positive direct immunofluorescence microscopy (DIF), clinical presentation, the biopsy subepidermal blisters, and compatible light microscopy findings. Following the diagnosis of BP, samples were collected and patient's data were analyzed. Samples with a lack of proper data were excluded from the study. Questionnaires were used to take relevant information from study participants. Forty age and sex-matched healthy individuals were included in the study.

2.3 | Analysis of single nucleotide polymorphism (SNP)

Genomic DNA was isolated from whole blood samples taken from the subjects using an isolation kit (DNGTM – Plus; SinaClon, Iran). Detection of polymorphisms in *IL-17A* (rs2275913 and rs3819025), *IL-17F* (rs2397084 and rs763780), *IL-17RA* (rs2229151), and *IL-23R* (rs2201841,

rs7530511, rs11209026, and rs10889677) genes were performed using PCR-based restricted fragment length polymorphism (PCR-RFLP) method with primers and respective restriction endonucleases as described previously (Chen et al., 2011; Csongei et al., 2010; Paradowska-Gorycka et al., 2010; Safrany et al., 2009; Shuang et al., 2015; Temel et al., 2016; Urabe et al., 2015).

2.4 | Gene expression analysis

Following RNA isolation and cDNA synthesis from blood samples of patients using RNX-Plus kit (SinaClon, Iran) according to the manufacturer's instructions, quantitative RT-PCR was performed to analyze the expression of the *IL-23R* gene as well as housekeeping glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene. The primer sequences and conditions of *IL-23R* (forward: 5′-TGG TGT CAT GGA GGA ATT ACA-3′; reverse: 5′-ATG AAG TTT CCT TGG TTG GC-3′ and *GAPDH* (forward: 5′-ATG GAG AAG GCT GGG GCT-3′; reverse: 5′-ATC TTG AGG CTG TTG TCA TAC TTC TC-3′) were designed by ABI PCR equipment (Applied Biosystems, USA). The *GAPDH* gene was used to measure the levels of gene expression by SYBR Green real-time RT-PCR.

2.5 | Statistical analysis

Statistical analysis was done using SPSS (IBM SPSS Statistics, IBM Corporation, Chicago, IL, USA) or GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). Data were shown as median and a p value less than 0.05 was considered statistically significant. A corrected p value (pc) was calculated based on the Bonferroni method for multiple analysis correction. Genotype and allele frequencies, as well as the agreement with the Hardy–Weinberg equilibrium, was detected by the Chi-square (χ^2) test. Haploview software version 4.2 and SNPStats online software was recruited for analysis of the delta coefficient (D′) of the population, haplotype analysis, and linkage disequilibrium (LD) analysis. The χ^2 test or t test was applied for detecting associations between polymorphisms and clinical-demographic factors. The probability incidence of BP was considered by logistic regression analysis. The normality distribution of the data was tested by Shapiro–Wilk or Mann–Whitney t test analysis.

3 | RESULTS

3.1 | Cohort description

In total, 40 BP patients and healthy individuals were recruited. All patients and controls were of Iranian heritage. The age and gender were observed to be similar between patients and controls (Table 1). The frequencies of genotypes in the patients in SNPs of *IL-17A* (rs2275913 and rs3819025) and *IL-23R* (rs7530511) deviated from the Hardy–Weinberg equilibrium.

3.2 | The C-allele of rs7530511 and G-allele of rs2201841 in *IL-23R* is associated with BP

To investigate if variations in the TH17/IL23 are associated with BP, nine polymorphisms were examined in BP patients and healthy controls. Of these, *IL-23R* rs7530511 SNP is significantly associated with susceptibility to BP. In contrast to healthy individuals with no CC genotype, patients carried 40% of the CC genotype (Table 2). More specifically, the C-allele of *IL-23R* rs7530511 is present at a higher frequency in BP patients (67.5%) compared to the controls (27.5%, $pc<0.001$, Table 3). Furthermore, logistic regression analysis confirms the C-allele as an independent risk factor in predisposing to BP, where the calculated odds ratio (OR) was 5.47 (95% confidence interval [CI]: 2.77–10.7). Besides, a trend toward significant differences between patients and controls was found for *IL-23R* rs2201841. However, in contrast to *IL-23R* rs7530511, the minor G-allele of *IL-23R* rs2201841 variation is more prevalent in healthy subjects (20%) than to that of BP patients (5%, $pc = 0.016$, Table 2).

No significant differences were observed in SNPs of *IL-17A* (rs2275913 and rs3819025), *IL-17F* (rs2397084 and rs763780), and *IL-17RA* (rs2229151), as well as two variations of *IL23R* (rs11209026 and rs10889677) between patients and controls in our cohort study and all genotypes (Table 2) and alleles (Table 3), distributed similarly. Of note, none of the patients and controls were carrying polymorphic

TABLE 1 Clinical and demographic characteristics in patients with BP and control subjects

Characteristics	Frequency (%)		
	Patients with BP (n = 40)	Controls (n = 40)	*p*-value
Age (mean, years)	69.9 ± 2.01	64.7 ± 1.72	0.053
Gender, male/female	12/28 (30/70)	20/20 (57.1/42.9)	0.069
Age of onset	67.5 ± 3.0	—	—
Disease duration (years)	20.2 ± 6.2	—	—

Note:: p values were performed based on the chi-square or *t* test and a value of <0.05 was considered statistically significant.

Abbreviations: BP, bullous pemphigoid; n, number.

TABLE 2 The *IL-17A*, *IL-17F*, *IL-17RA*, and *IL-23R* polymorphism genotype frequencies

Gene	rs number	Genotype	Patients with BP (n = 40)	Control subjects (n = 40)	OR (95% CI)	*p*	*pc*
IL-17A	rs2275913	GG	0.15	0.15	1.6 (0.72-3.53)	0.243	—
		GA	0.60	0.75			
		AA	0.25	0.10			
	rs3819025	GG	1.00	0.50	—	0.998	—
		GA	0	0.50			
		AA	0	0			
IL-17F	rs2397084	AA	0.85	0.85	1.29 (0.47-3.55)	0.614	—
		AG	0.10	0.15			
		GG	0.05	0			
	rs763780	AA	0.85	0.90	1.81 (0.58-5.59)	0.301	—
		AG	0.10	0.10			
		GG	0.05	0			
	rs2229151	GG	0.85	0.90	1.58 (0.41-6.12)	0.502	—
		GA	0.15	0.10			
IL-17RA		AA	0	0			
IL−23R	rs2201841	AA	0.95	0.80	0.45 (0.20-1.03)	0.059	—
		AG	0	0			
		GG	0.05	0.20			
	rs7530511	TT	0.05	0.45	17.4 (4.1-72.9)	**<0.001**	**<0.001**
		TC	0.55	0.55			
		CC	0.40	0			
	rs11209026	GG	0.95	0.85	0.58 (0.23-1.42)	0.233	—
		GA	0	0.05			
		AA	0.05	0.10			
	*rs*10889677	AA	0.15	0.30	0.47 (0.19-1.14)	0.096	—
		AC	0.75	0.65			
		CC	0.10	0.05			

Note:: p values (*p*) were performed based on the logistic regression test and a value of <0.05 was considered statistically significant. Significant *p* values are shown in bold.

Abbreviations: BP, bullous pemphigoid; CI, confidence interval; n, number; OR, odds ratio.

AA genotypes in *IL-17A* rs3819025. Moreover, the patients were all homogenous for GG genotypes for this variation.

Furthermore, clinical and demographical factors were considered verifying an association between the polymorphic and wild-type genotypes in patients. No significant difference was found in our further investigation between wild and polymorphic genotypes of all investigated SNPs and clinical and demographical features (data not shown).

3.3 | Linkage disequilibrium (LD) analysis

To uncover the association between polymorphisms in *IL-17A*, *IL-17F*, and *IL-23R*, the pairwise LD was performed for all related SNPs (Figure 1). LD analysis indicates a strong and a moderate LD between two SNPs (rs2397084 and rs763780) of *IL-17F* and two SNPs of *IL-23R* (rs2201841 and rs7530511), respectively, while SNPs in *IL-17A* (rs2275913 and rs3819025) as well as in *IL-23R* (rs11209026 and rs10889677) seems to be almost independent of one another. Haplotype frequencies were analyzed between *IL-17A*, *IL-17F*, and *IL-23R* SNPs to compare their association with BP. No significant differences were noticed in haplotype frequencies between patients and controls based on the global and individual haplotype score analysis ($p > 0.05$).

3.4 | *IL-23R* variations do not alter the levels of gene expression in blood cells

Given an association between *IL-23R* (rs2201841 and rs7530511) SNPs and susceptibility to BP, a quantitative

TABLE 3 The distribution of alleles in *IL-17A*, *IL-17F*, *IL-17RA*, and *IL-23R* polymorphisms

Gene	rs number	Allele	Patients with BP (n = 40)	Control subjects (n = 40)	OR (95% CI)	*p*	*pc*
IL-17A	rs2275913	G	0.450	0.525	1.35 (0.72-2.51)	0.343	—
		A	0.550	0.475			
	rs3819025	G	1.000	0.750	—	0.998	—
		A	0	0.250			
IL-17F	rs2397084	A	0.900	0.925	1.37 (0.45-4.14)	0.577	—
		G	0.100	0.075			
	rs763780	A	0.900	0.950	2.11 (0.60-7.31)	0.239	—
		G	0.100	0.050			
IL-17RA	rs2229151	G	0.925	0.950	1.54 (0.41-5.68)	0.516	—
		A	0.075	0.050			
IL-23R	rs2201841	A	0.950	0.800	0.21 (0.06-0.66)	**0.008**	**0.016**
		G	0.050	0.200			
	rs7530511	T	0.325	0.725	5.47 (2.77-10.7)	**<0.001**	**<0.001**
		C	0.675	0.275			
	rs11209026	G	0.950	0.875	0.36 (0.11-1.22)	0.104	—
		A	0.050	0.125			
	*rs*10889677	A	0.525	0.625	1.50 (0.80-2.83)	0.202	—
		C	0.475	0.375			

Note:: p values (*p*) were performed based on the logistic regression test and a value of <0.05 was considered statistically significant. Significant *p* values are shown in bold.

Abbreviations: BP, bullous pemphigoid; CI, confidence interval; n, number; OR, odds ratio.

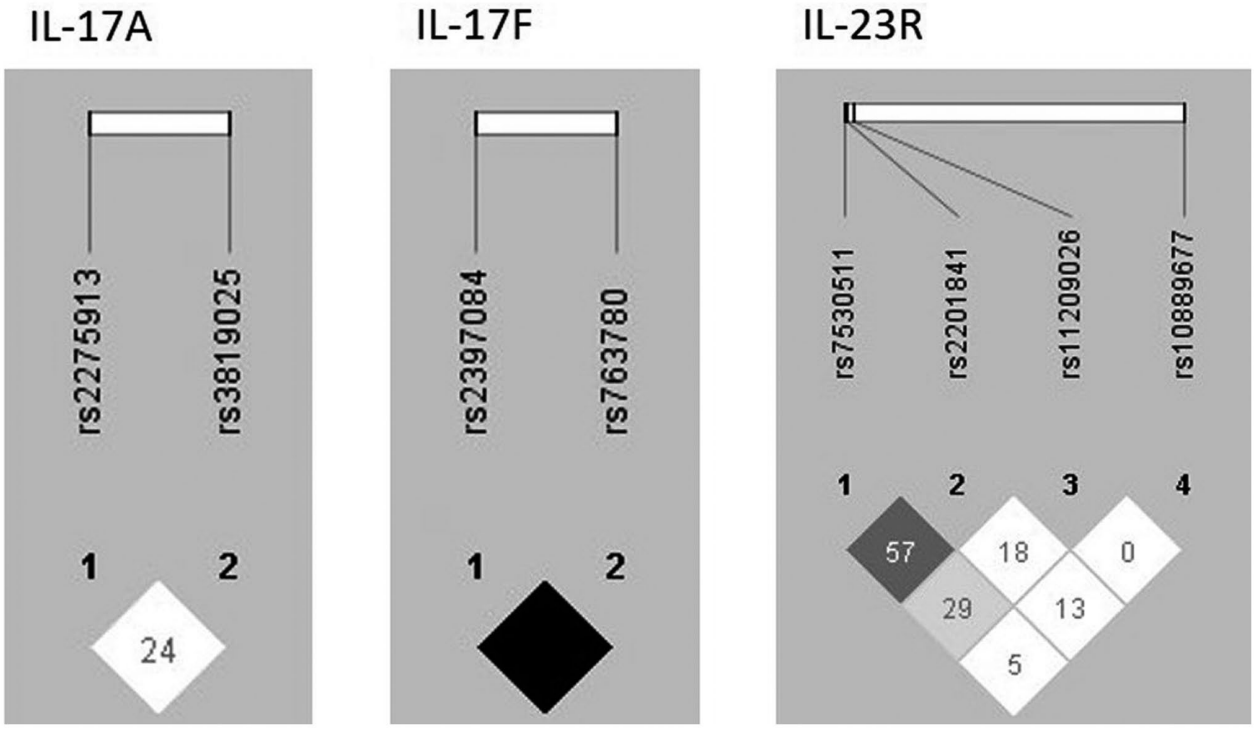

FIGURE 1 Linkage disequilibrium pattern of *IL-17A*, *IL-17F*, and *IL-23R*

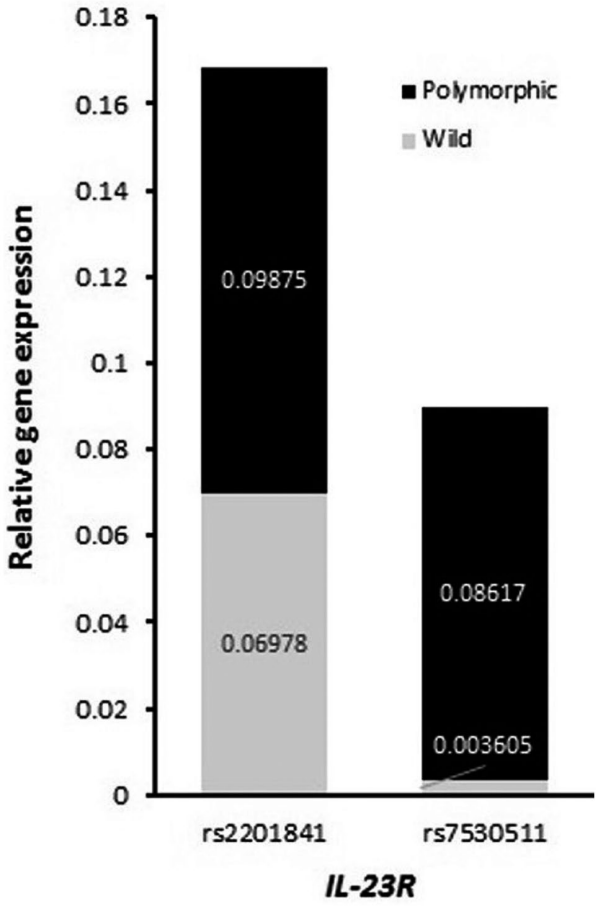

FIGURE 2 The relative gene expression analysis of two *IL-23R* SNP genotypes in the patient group is shown. Data are expressed as median with interquartile range and were compared based on the Mann–Whitney *t* test

analysis was performed to uncover any impact of these polymorphisms on gene expression (Figure 2). Our data revealed no alteration in the gene expression of polymorphic and wild genotypes, indicating they are not involved in modifying the gene expression levels in blood cells.

4 | DISCUSSION

In the current study, our data indicate an association between *IL-23R* rs7530511 and rs2201841 SNPs and BP susceptibility. The C-allele of *IL-23R* rs7530511 was found to be more frequent in the patients, while the G-allele distribution of *IL-23R* rs2201841 is significantly recognized in the control individuals.

Autoantibody-induced infiltration of inflammatory cells in BP is a prerequisite for initiating the inflammatory processes involving in blister formation. Cytokines play a significant role in the pathogenesis of autoimmune diseases and therefore, their alteration may influence the predisposition to BP disease. Since the Th17/IL23 pathway plays a significant role in tissue immunosurveillance and autoimmunity mechanisms, it has recently been under intensive research. Gene variations in the Th17/IL23 axis may influence its function, and therefore may cause dysregulation of this pathway in BP. Given the influence of single nucleotide substitution in cytokine genes on their functional importance, it is the first study to our knowledge that investigates an eventual contribution of the most important Th17/IL23 axis gene variations in the pathogenesis of BP.

Although altered levels of cytokines have been shown in skin lesions, serum, or blister fluid of BP patients (Bagci et al., 2017; Didona & Di Zenzo, 2018; Sauder, 1990), contradictory observations have so far been reported. Increased levels of IL-17 in blister fluids of BP patients could be in line with findings indicating the contribution of IL-17-producing cells in BP disease, which may confirm that BP regulation is beyond the Th1/Th2 paradigm (Arakawa et al., 2011; Chakievska et al., 2018; Fischer-Stabauer et al., 2012; Le Jan et al., 2014; Zebrowska et al., 2013). In our cohort study, nine polymorphisms in *IL-17A*, *IL-17F*, *IL-17RA*, and *IL-23R* genes were studied to find out whether variations in the TH17/IL23 axis contribute to susceptibility to BP disease. No significant difference was found between *IL-17A*, *IL-17F*, and *IL-17RA* between patients and controls. However, among four studied SNPs in the *IL-23R* gene (rs2201841, rs7530511, rs11209026, and rs10889677), the rs2201841 and rs7530511 were found to be significantly associated with susceptibility to BP disease. Furthermore, there was only a moderate LD between two SNPs of *IL-23R* (rs2201841 and rs7530511).

IL23/IL23R signaling pathway plays a crucial role in the development and pathogenicity of Th17 cells (McGeachy et al., 2009) which implicated in the development of autoimmune inflammatory diseases (Aggarwal et al., 2003; Bettelli et al., 2006). It is also shown that mice deficient for IL-23 or IL-23 receptor are partially resistant to develop experimental models of auto-inflammatory diseases, suggesting a significant influence of IL-23 in the inflammatory process (Cua et al., 2003; Kikly et al., 2006; Langowski et al., 2006; Murphy et al., 2003). Moreover, clinical data in psoriasis demonstrate that blocking the p40 subunit in IL-23 and IL-12 may be used in the therapy of the disease (Leonardi et al., 2008). Upon analyzing patients versus controls in our cohort study, the minor G-allele for IL23R (rs2201841), showed higher prevalence in controls, indicating a protective role in the development of BP disease, whereas for *IL23R* (rs7530511) the minor C-allele revealed a higher frequency in BP patients, suggesting an independent risk factor in predisposing to BP disease. In an investigation with Tunisian patients with pemphigus foliaceus, genetic associations between *IL-23R* (rs11209026) as well as *IL-17F* (rs763780) and the susceptibility to pemphigus foliaceus were reported (Ben Jmaa et al., 2018). Xavier et al. reported data of meta-analyses that confirmed the role of some SNPs of *IL-23R* in the risk of Behçet's disease in Iranian patients, indicating the involvement of candidate SNPs in susceptibility of inflammatory diseases (Xavier et al., 2012). In

another study with a case-case study comparing severe and mild psoriasis phenotypes, an association between SNPs (rs7530511 and rs2201841) within the *IL23R* genes was indicated in the severe phenotype of psoriasis (Nikamo et al., 2015). Nevertheless, our findings are in agreement with the later study, suggesting the rs2201841 and rs7530511 as a risk factor with susceptibility to autoimmune skin disease.

Given the importance of IL-17/IL-23 in BP, inconsistent data have been so far reported. It has been shown that IL-17 is highly expressed in biopsies of perilesional and lesional skin as well as in the serum of BP patients (Zebrowska et al., 2013). In another study, increased levels of IL-17/IL-23 in blister fluid but not in the serum, mainly produced by innate immune cells, especially neutrophils, and treatment by corticosteroids decrease the IL-17 expression and clinical symptoms of BP disease (Le Jan et al., 2014). Plée et al. showed that in contrast to IL-17, serum concentrations of IL-23 are significantly more elevated in the serum from BP patients than in controls (Plee et al., 2015). A recent publication confirmed an association between BP and mutations in IL-17A as well as mRNA upregulation of IL-17A and related mediators in the perilesional skin of BP patients (Chakievska et al., 2018). Furthermore, they could also show a correlation between serum levels of IL-17A and disease severity in the antibody-transfer mouse model of BP (Chakievska et al., 2018). Since an association was found in this study between *IL-23R* (rs2201841 and rs7530511) variations and susceptibility to BP, a possible influence of these SNPs on *IL-23R* gene expression in blood cells was analyzed. Our data revealed that the polymorphic genotypes of respective SNPs had no impact on the gene expression in the cells extracted from blood in our further investigation. However, these data should be interpreted by the differences between dermal cell phenotypes and systemic, where *IL23R* might be more involved in the skin, but lesser in the blood. Likewise, it should be noted that the polymorphism could be more likely to affect the receptor-ligand binding than receptor expression itself.

Given an association between *IL-23R* SNPs and predisposition to BP disease, it should be noted that these data have to be interpreted concerning the study limitations. First, since we had a power of 85%, missing the significant associations between SNPs and disease must be taken into account, because of the low power of the study. Nevertheless, this is the first study to investigate these relationships with a significant association, and therefore, our data could offer an advantage to calculate an exact sample size for future investigations. Second, clinical presentation and DIF were the basis of BP diagnosis. According to the BLISTER study (Williams et al., 2017) with a subsequent serological analysis (Holtsche et al., 2018), they could diagnose BP disease in almost 90% of the patients. Therefore, a different diagnosis, such as mucous membrane pemphigoid, p200 pemphigoid, or epidermolysis bullosa acquisita in a maximum of 10% of our patients is not excluded. Finally, no functional understanding of the investigated associations is expected from genetic association studies and these findings could be helpful for future researches, pointing out the functional influence of associated gene polymorphisms that have recently been noticed.

In conclusion, we could show that the alleles of two SNPs in *IL-23R* are associated with BP disease. Finding possible susceptible and protective impacts of *IL-23R* polymorphisms on autoimmune disease could provide additional information and supports in the direction of developing potential therapeutic approaches in patients with BP.

ACKNOWLEDGMENT

We appreciate the staff of the Skin Research Center, Razi, and Shohada Tajrish hospitals at Tehran, Iran for kind collaboration.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

P-ST-P and RA contributed to the conception and design of the study. HM provided the samples and performed the diagnosis of the disease. All authors were involved in the analysis and interpretation of the data and drafting the article. RA wrote the manuscript and RL critically revised the manuscript.

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How to cite this article: Tabatabaei-Panah PS, Moravvej H, Aghaei S, et al. TH17/IL23 cytokine gene polymorphisms in bullous pemphigoid. *Mol Genet Genomic Med*. 2020;8:e1519. https://doi.org/10.1002/mgg3.1519