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Tumor necrosis factor-α -308 G>A and *interleukin-6* -174 G>C promoter polymorphisms and pemphigus

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ABSTRACT

The objective of this study was to analyze the possible involvement of the *tumor necrosis factor* (*TNF*)-α -308 G>A and *interleukin-6* (*IL-6*) -174 G>C polymorphisms in the susceptibility and/or disease profile of pemphigus in Egyptian patients. Detection of *TNF*-α -308 G>A by amplification refractory mutation system and *IL*-6 -174 G>C by restriction fragment length polymorphism was performed for 70 patients and 203 controls. No significant differences were observed in the distribution of *TNF*-α -308 in pemphigus patients and controls. However, GA+AA genotypes were more frequent in pemphigus vulgaris (PV) patients only versus controls ($p_c = 0.046$). The frequency of the C allele and CC/GC genotypes of *IL-6* -174 was significantly higher in pemphigus patients and those with the 2 major clinical forms (PV and pemphigus foliaceus [PF]) compared with controls ($p < 0.05$). Comparison of the distribution of *TNF*-α -308 and *IL-6* -174 variants in relation to clinical type of pemphigus (PV versus PF), activity score, recurrence, and demographic data of patients revealed no significant associations. The *IL-6* -174 CC genotype represents a marker of increased susceptibility to pemphigus in Egyptian patients and GG genotype can be considered a low-risk genotype; *TNF*-α -308 A– containing genotypes contribute to the susceptibility to PV only.

1. Introduction

Pemphigus is a group of autoimmune blistering diseases of the skin and mucous membranes that is characterized by tissue-bound and circulating immunoglobulin G autoantibodies directed against the cell surface of keratinocytes (cadherin-type adhesion molecule desmoglein [Dsg]1 and Dsg3) [1]. There are 2 major forms of pemphigus: pemphigus vulgaris (PV) and pemphigus foliaceus (PF) [2]. In PV, essentially all patients have mucosal membrane erosions and more than half also have skin blisters and erosions. The blisters of PV develop in the deeper part of the epidermis, just above the basal cell layer. In PF, patients have only cutaneous involvement without mucosal lesions and the splits occur in the superficial part of the epidermis, mostly at the granular layer [3].

Pemphigus is one of the most clearly defined autoimmune diseases mediated by autoantibodies and serves as a suitable model for investigation of the mechanisms of breakdown of self-tolerance [3]. PV is caused by autoantibodies against Dsg3, whereas PF is caused by autoantibodies against Dsg1 [4]. Autoantibodies against Dsg1 and -3 are capable of inducing the pathology of pemphigus by interfering with the function of desmosomes that mediate adhesion between epidermal keratinocytes, resulting in blister formation [5]. The etiology of pemphigus is complex, with interplay of genetic as well as environmental factors, most of which remain unknown [6].

The production or function of cytokines is at least partially regulated by polymorphisms in their gene sequences [7]. Numerous cytokine polymorphic variants are associated with the development of a broad spectrum of autoimmune diseases, including skin-affecting disorders such as psoriasis vulgaris and systemic lupus erythematosus [7,8]. Thus, it is reasonable to hypothesize that certain cytokine polymorphisms also participate in genetic susceptibility to PV [9].

Tumor necrosis factor-α (TNF-α) is a potent immunomediator and proinflammatory cytokine that has been implicated in the pathogenesis of a large number of human diseases [10]. Increased levels of TNF-α have been detected in the sera of PV patients [11]. Furthermore, Ragab et al. [12], in a study on Egyptian patients with pemphigus, reported significantly increased serum levels of TNF-α in PV compared with PF and a nonsignificant difference between PF patients and controls. However, the proinflammatory cytokines (interleukin [IL]-1, interferon-γ, and TNF-α) were detected in the inflammatory exudates of lesions in Brazilian patients with PF [13].

IL-6 is a major proinflammatory cytokine that plays a role in immune response. Increased levels of IL-6 have been detected in serum from patients with PV, and its level has been correlated with disease severity [11]. Furthermore, Tron et al. [14] speculated that the IL-6 gene is the only gene that contributes to PF pathogenesis. IL-6

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not only supports autoantibody production through its stimulatory effects on B and T cells [15] and participates in blister formation, but also favors processing and presentation of autoantigen-derived peptides by antigen-presenting cells to Dsg-specific autoreactive T cells by altering antigen processing and presentation [16].

Hence, in pemphigus, TNF-α and IL-6 seem to play a role as mediators of acantholysis because *in situ* transcription of these cytokines was significantly increased in epidermal lesion cells in different forms of pemphigus and higher expression was observed in PF [17].

Few studies investigating the association of cytokine gene polymorphism and pemphigus have been previously published [9,18–21]. To the best of our knowledge, there is no such report for Egyptians and this study may be the first. Considering the role of TNF-α and IL-6 in pemphigus pathogenesis, we planned this case–control study to analyze the possible involvement of *TNF*-α -308 G>A and *IL*-6 -174 G>C promoter polymorphisms in the susceptibility and/or disease profile of pemphigus in Egyptian patients.

2. Subjects and methods

2.1. Patients and healthy controls

The study included a total of 70 patients with pemphigus (51 PV and 19 PF). The patients included 18 males and 52 females with a mean age of 46.6 ± 15.3 years and minimum–maximum of 9–90 years. Patients were consecutively recruited into the study during a 1-year period from the outpatient clinic of the dermatology department of Mansoura University Hospital, Egypt. The diagnosis was based on clinical, histological, and immunofluorescence criteria of pemphigus. Lesional skin biopsy for routine histopathology revealed the classic features of pemphigus (suprabasal cleft and acantholysis of keratinocytes with intact epidermis at the base of bulla in PV and intragranular and upper epidermal cleft with acantholysis in PF). Also, direct immunofluorescence on normal-appearing perilesional skin revealed immunoglobulin G deposition on the surface of keratinocytes. The control group included 203 unrelated healthy age- (mean ± SD, 45.1 ± 14.9) and sex- (26.1% were males and 73.9% were females) matched subjects with neither symptoms nor family history of pemphigus and/or other autoimmune disease. They were living in the same geographical area and had the same ethnic origin as the patients. Written informed consent was obtained from the patients and controls after the study protocol was approved by the local ethical committee.

2.2. Methods

2.2.1. Clinical evaluation

A thorough skin and oral examination was performed for patients and the subtype of pemphigus was assigned as either PV or PF. The disease activity was determined by assessment of the pemphigus area and severity score according to Daneshpazhooh et al. [22]. Skin severity was graded as follows: grade 0, quiescent; grade 1, up to 5 blisters and/or erosions; grade 2, 5 to 20 blisters and/or erosions; grade 3, >20 discrete blisters and/or erosions; and grade 4, extensive and confluent blisters and/or erosions. The oral involvement was graded as follows: grade 0, quiescent; grade 1, up to 2 erosions; grade 2, 3 to 10 erosions; grade 3, >10 erosions; and grade 4, extensive confluent erosion and/or generalized desquamative gingivitis. All studied patients had active disease with flaccid bullae and/or erosions in the skin. PV patients also had oral erosions.

2.2.2. Typing of TNF-α -308 G>A gene polymorphism using the amplification refractory mutation system technique

DNA was extracted from whole venous blood using the EZNA blood DNA extraction kit (Omega bio-tek, Norcross, GA, Lot No. D 3392-01). Typing of the *TNF*-α (6p21.3) -308 G>A (rs1800629, in gene promoter) gene polymorphism was performed with an amplification refractory mutation system reaction similar to that first described by Perrey et al. [23]. The sequences of primers (Biolegia, BV Nijmegen, The Netherlands) used were as follows: G primer (*TNF* 1 allele), 5′-ATA GGT TTT GAG GGG CAT GG-3′; A primer (*TNF* 2 allele), 5′-AATA GGT TTT GAG GGG CAT GA-3′; and generic primer, 5′-TCT CGG TTT CTT CTC CAT CG-3′. Internal control primers of human growth hormone were used to check for successful polymerase chain reaction (PCR): forward primer, 5′-GCC TTC CCA ACC ATT CCC TTA-3′; and reverse primer, 5′-TCA CGG ATT TCT GTT GTG TTT C-3′.

The reaction volume was 25 μL: 5 μL DNA at 100 ng/μL, 15.0 μL DreamTaq Green mater mix (Fermentas, St. Leon-Rot, Germany, Lot No. 116050-96), 1.0 μL of specific primer mix (0.5 μL of 1 of the 2 allele-specific primers and 0.5 μL of generic primer), 1 μL of internal control primers mix, and 3.0 μL H_2O. Reaction conditions were carried out in a thermocycler PTC-100 (Bio-Rad, Hercules, CA) at 95°C for 1 minute followed by 10 cycles of 95°C for 15 seconds, 65°C for 50 seconds, and 72°C for 40 seconds and then 20 cycles of 95°C for 20 seconds, 56°C for 50 seconds, and 72°C for 50 seconds. The amplified PCR products were analyzed by 2% agarose gel and ethidium bromide staining followed by ultraviolet visualization. The PCR product for *TNF*-α (-308) was detected at 184 bp and at 429 bp for internal control primers.

2.2.3. IL-6 -174 G>C gene polymorphism typing by restriction fragment length polymorphism (RFLP)

The *IL-6* -174 G>C single nucleotide polymorphism (SNP; rs1800795) in the promoter region of the *IL-6* gene (7p21) was genotyped using the PCR–RFLP method previously reported by Mazzatti et al. [24]. Genomic DNA was amplified using PCR with different primers (forward and reverse). The sequences of primers (Biolegia) used were as follows: forward primer, 5′-TGA CTT CAG CTT TAC TCT TTG T-3′; and reverse primer, 5′-CTG ATT GGA AAC CTT ATT AAG-3′. The reaction volume was 25 μL: 5 μL DNA at 100 ng/μL, 15.0 μL DreamTaq Green mater mix (Fermentas, Lot No. 39428), 0.5 μL of each primer (25 pmoL/μL), and 4.0 μL H_2O. Reaction conditions were carried out in a thermocycler PTC-100 (Bio-Rad) at 94°C for 10 minutes followed by 35 cycles of 94°C for 60 seconds, 55°C for 90 seconds, and 72°C for 60 seconds and a final extension step at 72 C for 10 minutes. A total of 10 μL of PCR products was resolved in 2% agarose gel to check the PCR products at the 198-bp fragment.

RFLP analysis was performed using FastDigest *Nla*III (Fermentas, Lot No. 51153) in 30 μL total volume by mixing 10 μL of PCR products + 1.0 μL of *Nla*III restriction enzyme + 2.0 μL 10× FastDigest green buffer + 17 μL nuclease-free water. The mixture was incubated at 37°C for 10 minutes followed by heating at 65°C for 10 minutes. DNA fragments were resolved in 2.5% agarose gels. Digestion of PCR products yielded 153 + 45 bp fragments (CC), a single 198-bp fragment (GG), and 198 + 153 + 45 bp fragments (GC).

2.3. Statistical analysis

The statistical analysis of data was performed using the SPSS statistical package for social science version 16 (SPSS, Inc, Chicago, IL). The description of the data was given in the form of means ± SD for quantitative data and frequency and percentage for qualitative data. For quantitative data, the Student *t* test was used to compare between 2 groups. For qualitative data the χ^2 test or Fisher's exact test was used, as appropriate. *p* value significant at ≤0.05, odds ratios (OR), and 95% confidence intervals (95% CI) were calculated. Significant probability values obtained were corrected for multiple testing using Bonferroni's formula by multiplying numbers of markers studied in the *p* value (p_c = number of comparisons × *p* value), and the p_c value was significant at ≤0.05.