INTRODUCTION

Alopecia areata (AA) is an autoimmune skin disorder in which the immune system targets hair follicles, most commonly of the scalp, causing hair loss appearing as circular or oval patches. It may spontaneously recover, persist, or spread to other parts of the scalp or other body parts.\(^1\)

A peribulbar lymphocytic infiltration of CD4+ and CD8+ T-cells around anagen follicles is evident in the acute and subacute stages with a shift from the catagen hair growth stage to the telogen phase (the resting phase) and follicle miniaturization. Edema, microvesiculation, apoptosis, macrophages, and foreign body giant cells may be seen around the hair follicles. There is an increase in the catagen or telogen count during the chronic stage; however, the inflammation may or may not resolve.\(^2\)

It is hypothesized that there is a collapse of this immune privilege zone in AA from an unknown autoantigen. Interferon-gamma (IFN-\(\gamma\)) and interleukin (IL)-2 can then induce infiltration of CD8+, CD4+, natural killer cells (NKs), macrophages, and other inflammatory cells into the immune privilege zone. All of these alterations lead to inflammation of the hair follicle and may result in hair loss.\(^3\)

Tumor Necrosis Factor-like Weak Inducer of Apoptosis: A Novel Serum Marker in Patients with Severe Alopecia

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ABSTRACT

Background: Alopecia areata (AA) is a common form of nonscarring hair loss of scalp and/or body. Genetic predisposition, autoimmunity, and environmental factors play a major role in the etiopathogenesis of AA. Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) is a multifunctional cytokine expressed on various cell types and tissues and acts through binding to its sole receptor factor-inducible 14 (Fn14). TWEAK/Fn14 activation contributes to various pathological processes, including cell proliferation and death, angiogenesis, carcinogenesis, and inflammation

Aim: The aim of this current study was to measure serum levels of TWEAK in patients with AA and to assess the correlation between it and severity of the disease.

Subjects and Methods: This study included 50 patients who had patchy AA, in addition to 50 apparently healthy controls. Severity of AA was assessed using Severity of Alopecia Tool Score. Serum TWEAK levels in all participants were determined using ELISA technique and were correlated with the severity of the disease.

Results: Mean serum levels of TWEAK were significantly higher in AA patients, with a positive significant correlation between serum levels of TWEAK and severity of the disease.

Conclusion: TWEAK as a novel marker of many autoimmune inflammatory dermatological diseases, could be a promising marker in the diagnosis of AA, and also can be used as a prognostic marker for its severity.

Key words: Alopecia areata, Severity of Alopecia Tool Score, tumor necrosis factor-like weak inducer of apoptosis

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Tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK), an apoptosis inducer, belongs to the TNF ligand superfamily.\[^4\] TWEAK specifically binds to its sole receptor fibroblast growth factor-inducible 14 (Fn14).\[^3\]

It participates in many biological activities such as production of inflammatory cytokines, triggering vasculitis and angiogenesis, enhancing fibrogenic responses, and stimulation of cellular responses such as proliferation, migration, and apoptosis.\[^8\] Furthermore, skin inflammation activates TWEAK/Fn14 signaling pathway which modulates the keratinocytes inflammatory responses through activation of nuclear factor-κB signals and stimulation of cytokines production as ILs, monocyte chemotactic protein-1 (MCP-1), IFN-γ-induced protein, and ten regulated on activation, normal T-cell expressed, and secreted (RANTES).\[^7\]

It was noted that activation of TWEAK/Fn14 serves a major role in the pathogenesis of several skin disorders such as cutaneous vasculitis, atopic dermatitis, cutaneous autoimmune diseases, psoriasis and human papillomavirus infection and related skin tumors.\[^8\]

Our study has been conducted to analyze serum levels of TWEAK in AA patients and to evaluate its correlation with disease severity.

**SUBJECTS AND METHODS**

A case–control study carried on between January 2017 and May 2017, including 100 participants from the Outpatient Dermatology Clinic of Benha University Hospitals (50 patients suffering from any type of AA and 50 apparently healthy individuals as a control group in whom age and sex match the case group).

Approval of the Local Ethics Committee of Benha Faculty of Medicine on Research involving human participants was obtained, and each participant signed an informed written consent. Patients suffering from any inflammatory or infectious diseases in the scalp, concurrent significant medical conditions such as malignancy, diabetes, hepatic, renal or cardiovascular diseases, other autoimmune disease, or any dermatological disease were excluded from the study.

All participants were subjected to a thorough medical history taking and a complete general clinical assessment including the body mass index (BMI) determined through dividing the body mass by the square of the body height. The type and severity of AA were determined through a complete cutaneous examination and the application of the Severity of Alopecia Tool (SALT) score in which four areas of the scalp are considered: 40% (0.4) of the scalp surface area referred to as the vertex, 18% (0.18) of the scalp surface area referred to as the right profile of the scalp, 18% (0.18) of the scalp surface area referred to as the left profile of the scalp, and 24% (0.24) of the scalp surface area known as the posterior aspect of the scalp. The hair loss percentage of an area was calculated as the hair loss percentage multiplied by the percent surface area of the scalp in that area. SALT score was then identified as the sum of hair loss percentages of all aforementioned areas.\[^9\]

**Serum tumor necrosis factor-like weak inducer of apoptosis measurement by ELISA**

**Blood sample**

Each participant was subjected to aseptic withdrawal of 3 ml of venous blood in an empty test tube which was then kept at room temperature for 30 min to allow blood clotting and then centrifuged for 15 min at 1000 \( \times g \). The serum was then removed, aliquoted, and stored at ≤−20°C for a later assessment of serum TWEAK level.

**Test procedure**

Serum TWEAK concentration was determined by ELISA using a well-defined quantitative double-antibody sandwich technique. The commercial kits were obtained from the SunRed biotechnology company, Shanghai, China (Cat. No. 201-12-1821) with assay range – 15–4200 ng/ml.

Serial dilutions of the original standard were prepared by adding 120 μl of the original standard to 120 μl of standard diluent to obtain standard No. 5 with concentration = 2400 ng/ml, then the standard No. 4 with concentration = 1200 ng/ml was prepared by adding 120 μl of standard No. 5 to 120 μl of standard diluent. The operation was repeated consequently for preparation of the standard No. 3 with concentration = 600 ng/ml and then for standard No. 2 with concentration = 300 ng/ml and lastly for standard No. 1 with concentration = 150 ng/ml. The 30 × washing concentrate was diluted 30 times by adding 580 ml of distilled water.

The microtiter plate used was coated with anti-TWEAK monoclonal antibodies to bind TWEAK in the samples. The first well was left as a blank while the standard wells were identified and injected with 50 μl of the standard solutions from standard No. 1 to standard No. 5. Forty microliter
of the sample followed by 10 μl of Biotin-TWEAK antibodies were injected into the test wells. Consequently, 50 μl of streptavidin-horseradish peroxidase was added in all wells except the blank, followed by sealing with the sealing membrane and then incubated at 37°C for 60 min. After incubation, the membrane was carefully removed, the liquid was drained, and the remaining water was shaken away using the previously prepared washing solution. Into each well, including the blank, 50 μl chromogen solution A, then 50 μl chromogen solution B were added, gently mixed, and incubated for 10 min at 37°C away from light. The reaction was stopped by the addition of 50 μl of stop solution, then the blue color was changed into a yellow one immediately.

The amount of TWEAK was assayed by a colorimetric reaction, the blank was taken as zero, and the optical density was measured under 450 nm wavelength within 15 min after adding the stop solution in a Stat Fax 2100 Microplate Reader (Awareness Technology Inc., Palm City, FL, USA).

Statistical methods

Quantitative data are expressed as mean ± standard deviation and range whereas qualitative data are presented as frequency and percentage. Chi-square test and Fisher’s exact test were used to compare the different study groups. Differences in parametric and nonparametric data between two groups were assessed using the Student’s t-test (t) and the Mann–Whitney test, respectively. Correlations between serum level of TWEAK and studied parameters were detected by the Spearman’s correlation coefficient. The receiver operating characteristic (ROC) curve was used to determine the diagnostic ability of serum TWEAK levels for AA. The best cutoff levels, the corresponding sensitivity, specificity, positive predictive value, and negative predictive value, and the area under the curve (AUC) were estimated. All statistical analyses were performed using the STATA/SE version 11.2 of Windows (STATA Corporation, College Station, Texas, USA).

RESULTS

The study included 100 participants allocated into two groups: Group I of 50 AA cases with a mean age of 28.2 ± 6.6 years, 41 (82%) were males and 9 (18%) were females and Group II (control group) of 50 apparently healthy individuals with a mean age of 27.9 ± 4.09 years, 41 (82%) were males and 9 (18%) were females. The mean BMI of cases was 21.96 ± 2.88 in comparison to controls which was 21.62 ± 2.09. No differences of a statistical significance were detected between cases and controls regarding age and sex as well as BMI with P values (P = 0.783, 0.1060, and 0.501), respectively.

Regarding history and clinical presentations of cases, 24 (48%) patients were smokers, 11 (22%) patients reported a positive family history of AA, and 10 (20%) patients gave a history of positive consanguinity. Only three patients (6%) had a family history of other autoimmune diseases. As regards clinical data and the history of current disease, 38 (76%) patients presented with one lesion and 12 (24%) patients presented with multiple lesions, with a disease duration ranged between 2 weeks and 1 year. About 40% of the cases had a history of a previous attack and 36% had tried different types of treatment.

Regarding the SALT score of AA, the mean SALT score was 11.33 with a range of 1.80 up to 33. Most patients (86%) were S1 grade while only 7 (14%) patients were S2 grade.

As regards to serum levels of TWEAK, a higher serum TWEAK was observed in cases compared to controls with a significant P value (P = 0.001). Moreover, cases with multiple lesions had a significantly higher serum TWEAK than cases with a single lesion (P = 0.001) as shown in Table 1.

Levels of serum TWEAK significantly correlated with the BMI and SALT score in AA patients but neither correlated with patients’ age nor disease duration [Table 2].

The ROC curve revealed that TWEAK had an excellent ability to diagnose AA as it showed an AUC of 0.983 with 95% confidence interval ranged from 0.959 to 1.0. The best cutoff point for the serum TWEAK level was 257 ng/mL which gave a sensitivity and specificity of 100% and 94%, respectively [Figure 1].

Table 1: Comparison between serum levels of tumor necrosis factor-like weak inducer of apoptosis in different studied groups

|                  | TWEAK (mean±SD) | MW* | P     |
|------------------|-----------------|-----|-------|
| Patients (n=50)  | 1095.7±1089.7   | 6.05| 0.001*|
| Controls (n=50)  | 116.4±90.6      |     |       |
| Patients with single lesion (n=38) | 556.3±320.9 | 13.5 | 0.001* |
| Patients with multiple lesions (n=12) | 2801.8±873.1 |     |       |

*Mann-Whitney test of nonparametric data. *P*≤0.05 is significant. TWEAK – TNF-like weak inducer of apoptosis; MW – Mann–Whitney; SD – Standard deviation.
DISCUSSION

Several types of leukocytes produce intracellular TWEAK-like human resting and activated monocytes, NK cells, and dendritic cells (DCs). Many human peripheral blood innate immune cells such as macrophages, polymorph nuclear leukocytes, DCs, and NK cells express TWEAK mRNA.\[10\]

Previous studies demonstrated that disturbed TWEAK protein level was linked to various autoimmune diseases such as psoriasis\[11\] and bullous pemphigoid.\[12\] As far as we know, this is the first study to measure the serum TWEAK in cases of AA.

According to the current study, the serum TWEAK was significantly higher in AA cases than controls. Furthermore, patients with multiple lesions showed significantly higher levels of serum TWEAK than patients with a single lesion.

As AA is an autoimmune inflammatory disorder, many inflammatory cells may be implicated in AA pathogenesis as they accumulate in and around the affected hair bulbs including CD4+ T-cells (60%–80%), CD8+ T-cells (20%–40%), mast cells, DCs, in addition to macrophages, foreign body giant cells, and NK cells.\[13\] Consistent with this idea, it is not surprising that this study detected high serum levels of TWEAK in AA, which may be produced by these excessive inflammatory cells which are considered the factory of TWEAK.

Another explanation of the relationship between AA and TWEAK is through IFN-γ, the main cytokine produced in AA through a CD4+ Th1-mediated response by the perifollicular or follicular antigen-presenting cells, which has been shown to upregulate the TWEAK protein in cultured human peripheral blood monocytes, DCs, and NK cells.\[14,15\]

Since AA is an autoimmune disorder, the results presented by the current study agree with similar studies involving other autoimmune disorders that confirm this concept. For instance, Bilgiç et al\[11\] reported that psoriasis cases demonstrated a significantly higher serum TWEAK than their healthy controls. Furthermore, Liu et al\[12\] reported a higher serum TWEAK in bullous pemphigoid cases which positively correlated with the anti-BP180 IgG.

Regarding severity of AA, serum TWEAK positively correlated with a statistical significance with the SALT score. This finding is supported by the findings that the serum TWEAK level is closely correlated with RANTES and IL-8 which were secreted from keratinocytes in response to TWEAK/Fn14 activation,\[16\] and it was noted that IL-8 and RANTES levels were elevated in the extensive form of AA.\[17\]

According to our study, serum TWEAK positively correlated with a statistical significance with the BMI of the cases' group. This could be attributed to the production of Fn14 in mature adipocytes and preadipocytes;\[18\] so, the increased serum level of TWEAK acts on its receptors in adipose tissue exerting a pro-inflammatory effect on adipocyte cells which in turn trigger the production of the pro-inflammatory cytokines – MCP-1 and IL-6.\[19\]

Sato et al\[15\] also showed a significantly positive correlation between TWEAK and weight gain since elevated serum TWEAK causes metabolic dysfunctions, visceral obesity, and insulin resistance. Such finding suggests that inhibition of TWEAK could be used for the prevention of weight gain and Type II diabetes.

CONCLUSION

TWEAK appears to serve a major role in the pathogenesis of autoimmune skin disorders including AA which...
correlates with disease severity. Hence, it could be considered a promising therapeutic target for autoimmune diseases.

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**Conflicts of interest**

There are no conflicts of interest.

**REFERENCES**

1. Gilhar A, Etzioni A, Paus R. Alopecia areata. N Engl J Med 2012;366:1515-25.
2. Yoon TY, Lee DY, Kim YJ, Lee JY, Kim MK. Diagnostic usefulness of a peribulbar eosinophilic infiltrate in alopecia areata. JAMA Dermatol 2014;150:952-6.
3. Pratt CH, King LE Jr., Messenger AG, Christiano AM, Sundberg JP. Alopecia areata. Nat Rev Dis Primers 2017;3:17011.
4. Wen J, Doerner J, Weidenheim K, Xia Y, Stock A, Michaelson JS, et al. TNF-like weak inducer of apoptosis promotes blood brain barrier disruption and increases neuronal cell death in MRL/lpr mice. J Autoimmun 2015;60:40-50.
5. Armstrong CL, Galisteo R, Brown SA, Winkles JA. TWEAK activation of the non-canonical NF-κB signaling pathway differentially regulates melanoma and prostate cancer cell invasion. Oncotarget 2016;7:81474-92.
6. Chen T, Guo ZP, Fu LX, Cao N, Qiu S. Anti-TWEAK monoclonal antibodies reduce vascular damage and leucocyte infiltration in a mouse model of cutaneous reverse passive arthus reaction. Clin Exp Dermatol 2016;41:871-7.
7. Cheng H, Xu M, Liu X, Zou X, Zhan N, Xia Y, et al. TWEAK/Fn14 activation induces keratinocyte proliferation under psoriatic inflammation.
8. Xu WD, Zhao Y, Liu Y. Role of the TWEAK/Fn14 pathway in autoimmune diseases. Immunol Res 2016;64:44-50.
9. Olsen EA, Hordinsky MK, Price VH, Roberts JL, Shapiro JI, Canfield D, et al. Alopecia areata investigation assessment guidelines – Part II. National alopecia areata foundation. J Am Acad Dermatol 2004;51:440-7.
10. Sharif MN, Campanholle G, Nagiec EE, Wang J, Syed J, O’Neil SP, et al. Soluble fn14 is detected and elevated in mouse and human kidney disease. PLoS One 2016;11:e0155368.
11. Bülge O, Sivrikaya A, Toker A, Ulu A, Altnyaziz C. Serum levels of TWEAK in patients with psoriasis vulgaris. Cytokine 2016;77:10-3.
12. Liu Y, Peng J, Li J, Liu C, Hu X, Xiao S, et al. TWEAK/Fn14 activation contributes to the pathogenesis of bullous pemphigoid. J Invest Dermatol 2017;137:1512-22.
13. Castellana D, Paun R, Perez-Moreno M. Macrophages contribute to the cyclic activation of adult hair follicle stem cells. PLoS Biol 2014;12:e1002002.
14. Zimmermann M, Koreck A, Meyer N, Basinski T, Meiler F, Simone B, et al. TNF-like weak inducer of apoptosis (TWEAK) and TNF-α cooperate in the induction of keratinocyte apoptosis. J Allergy Clin Immunol 2011;127:200-7, 207.e1-10.
15. Sato S, Ogura Y, Kumar A. TWEAK/Fn14 signaling axis mediates skeletal muscle atrophy and metabolic dysfunction. Front Immunol 2014;5:18.
16. Jin L, Nakao A, Nakayama M, Yamaguchi N, Koijima Y, Nakano N, et al. Induction of RANTES by TWEAK/Fn14 interaction in human keratinocytes. J Invest Dermatol 2004;122:1175-9.
17. Kurwano Y, Fujimoto M, Watanabe R, Ishiura N, Nakashima H, Ohno Y, et al. Serum chemokine profiles in patients with alopecia areata. Br J Dermatol 2007;157:466-73.
18. Alexaki VI, Notas G, Pelekanou V, Kampa M, Valkanou M, Theodoropoulos P, et al. Adipocytes as immune cells: Differential expression of TWEAK, BAFF, and APRIL and their receptors (Fn14, BAFF-R, TACI, and BCMA) at different stages of normal and pathological adipose tissue development. J Immunol 2009;183:5948-56.
19. Tiller G, Fischer-Posrovzsky P, Laumen H, Finck A, Skurz T, Keuper M, et al. Effects of TWEAK (TNF superfamily member 12) on differentiation, metabolism, and secretory function of human primary preadipocytes and adipocytes. Endocrinology 2009;150:373-83.