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Cutaneous Forkhead Box P3 (FOXP3) Immune-Expression in Vitiligo Patients

A.D.El Hussiny , O.Y.Bassyoni, R.M.Zein, A.M.El Refaai and A.I.El Taweel
Dermatology Dept, Faculty of Medicine, Benha Univ,Benha, Egypt
E-Mail: R.Zein@gmail.com

Abstract

The exact etiology of vitiligo remains obscure. Studies have indicated a role for cellular immunity in the pathogenesis of vitiligo. The aim of this study is to assess tissue FOXP3+ natural regulatory T-cells (Tregs) in active vs. stable nonsegmental vitiligo. Immunohistochemical staining for expression of FoxP3 in lesional, marginal, and nonlesional skin of nonsegmental vitiligo was used to evaluate the abundance of Tregs in active and stable cases of vitiligo. Reduction in the number of FOXP3-expressing Tregs was detected in marginal skin in both stable and active vitiligo cases. The results revealed that FOXP3+ T-cells were lowest in marginal skin of active cases in comparison with lesional and nonlesional skin of the same group. Tregs were lower in marginal and nonlesional skin of stable vitiligo compared with lesional skin of the same group. Tregs were mostly present at the DEJ and upper dermis. The reduction in the number of FOXP3+ cells in the marginal skin suggests that this is the site where regulatory activity is needed to suppress the activity of helper and cytotoxic T-cells that are actively contributing to depigmentation.

Keywords Vitiligo, FOXP3, Immunohistochemistery, Regulatory T-cells.

1. Introduction

Vitiligo is a hypomelanotic autoimmune skin disease resulting from loss of functional melanocytes from the skin with a prevalence of 1–2%. The disease can affect individuals of any race or sex and manifests before the age of 20 years in approximately half of all cases [1].

Although vitiligo might be considered a minor disorder, the psychological effects of the disease are frequently considerable. The exact etiology and detailed pathogenesis of vitiligo are not fully understood, but autoimmunity has been strongly implicated in the development of the disease [2].

Several studies have identified a pivotal role for cytotoxic T cells in inducing melanocyte-specific destruction in vitiligo [3], including the findings that CD8+ T cells isolated from the vitiligo skin are cytotoxic to melanocytes, recognize melanocyte-specific autoantigens [3], and induce autologous melanocyte apoptosis through IL-6 and IL-13 [4] .

In addition, there is a global expansion and widespread activation of the CD8+ T cell population in vitiligo patients (5–7). So far, however, the exact mechanisms underlying the induction and activation of autoreactive CD8+ T cells and the loss of tolerance to melanocyte autoantigens in vitiligo are not clear, but as with other autoimmune disorders, it seems likely that this loss of tolerance to self must involve some defect in regulatory T cell (Tregs) function [5,6 and 8].

Regulatory T cells are a subset of CD4+ lymphocytes that play a key role in maintaining peripheral tolerance in vivo through the active suppression of self-reactive T cell activation and expansion Fig (1) [9].They maintain order in the immune system by enforcing a dominant negative regulation on other immune cells including actively suppressing the activation and expansion of autoreactive T cells that have escaped clonal deletion in the thymus.

Several studies have indicated perturbations in Treg cell numbers and/or function in vitiligo patients [5–22]. Such alterations might lead to the higher levels and activation of cytotoxic T cells which have been reported in individuals with the disease [5,6].

Assessment of circulating Tregs by flow cytometric analysis has revealed a decrease in their numbers in vitiligo patients compared to controls [5,6 and 8]. Decreased circulating Treg cell counts have been demonstrated in patients with active vitiligo as compared to those with stable disease [6]. Moreover, a striking reduction in the number of Tregs in the marginal and lesional skin of vitiligo patients has been observed [13,14]. Interestingly, some studies have demonstrated that the peripheral or lesional skin CD4 164 +CD25+FOXP3+ Treg cell numbers remain unaltered in vitiligo (20,21,23), and even that either may be increased [5,17].

Significant reductions in the function of peripheral Tregs have been demonstrated in progressive vitiligo using assays which measure how well these cells can inhibit the proliferation of and cytokine production from stimulated autologous CD8+ T cells [5,8]. The possible causes of Treg cell functional defects in vitiligo also have been investigated.

Forkhead box P3, the transcription factor Forkhead box P3 (FOXP3) is a specific marker for Tregs and serves as the dedicated mediator governing Treg cell development and function [23–25].

Several observations indicate that there is a defect in FOXP3 expression in vitiligo patients that could impair the function of Treg cells. Firstly, FOXP3 expression is significantly decreased in CD4+CD25+ Tregs from vitiligo patients

compared to controls [6]. Secondly, the mean percentage area of FOXP3-positive immunostaining in the lesional skin and the levels of FoxP3-positive cells in the peripheral blood are significantly lower in vitiligo patients compared to controls [24]. Finally, FOXP3 mRNA levels in the lesional and perilesional skin are significantly reduced in vitiligo patients when compared with skin from healthy individuals [20]. In contrast, significantly higher levels of FOXP3 mRNA expression have been found to occur in the lesional vitiligo skin compared to the non-lesional vitiligo skin [8], suggesting the recruitment of Tregs into the affected site to balance the autoimmune response.

FOXP3 expression is relatively unique to regulatory T-cells. Most of the Tregs expressing FOXP3 were also expressing CD4 and CD25 antigens [9].

The aim of the present study was to assess tissue FOXP3+ Tregs in lesional, marginal, and non-lesional skin of active vs stable vitiligo patients.

2. Patients and methods

2.1Patients

This prospective study included 20 patients with nonsegmental vitiligo; 10 of them were suffering from active vitiligo, and the other 10 were stable cases of vitiligo with various degrees of disease extent. The activity of the disease was detected by asking the patient about the appearance of new vitiligo lesion(s), increase in size of existing lesions and/or reappearance of any healed lesion within the last six months. Clinical assessment of vitiligo lesions was performed to determine the distribution, clinical variant, and extent. Excluded from the study were patients taking any treatment in the last four weeks prior to the study. The study was conducted in Benha University Hospital, Egypt. Informed consent was taken from all subjects before participating in this work.

2.2 Methods

Four-millimeter punch biopsies were obtained under local anesthesia from lesional, marginal, and nonlesional skin of each patient with vitiligo included in this study. Lesional (L) and marginal (M) biopsies were taken from the most recent lesion. The marginal skin biopsy was taken spanning the border between depigmented and existing vitiligo lesion. Sections from each skin biopsy were immunohistochemically stained for the expression of FOXP3 antigen; positively stained cells appeared brown. For the immunohistochemical detection of FOXP3 antibody, 6.0 ml, prediluted mouse monoclonal antibody (**API 3164 AA**), was used according to the manufacturer's recommendations (Biocare Medical, 60 Berry, Dr Pacheco, CA 94553, USA).

2.3 Immunohistochemical staining analysis

FOXP3 was assessed via non-cross reactive staining in the epidermis; therefore, staining in the epidermis was not included in the statistical analysis. The tissue expression density in immunohistochemically stained sections were determined using the semiquantitative method and scored on a scale of 0–3 points using a pathological scoring system. The global pathological score was determined by average stained cell number in 10 microscopic fields in each section (9400 magnification); the score was 1 if there was staining in 1/3 of the total cell count, 2 if there was staining in 1/3–2/3 of the total cell count, 3 if there was staining in >2/3 of the total cell count, and 0 if there was no staining.

2.4 Statistical analysis

The collected data were summarized in terms of mean± Standard Deviation (SD), median and range for quantitative data and frequency and percentage for qualitative data. Comparisons between the different study groups were carried out using the Fisher Exact test (FET) to compare proportions. The test of proportion (Z-test) was used to compare two proportions. The Mann-Whitney test (MW) was used to compare two non-parametric variables. The Kruskal Wallis test was used to compare more than two groups regarding non-parametric data.

Spearman correlation coefficient (rho; ρ) was used to test for the correlation between FOXP3 and estimated parameters. Statistical significance was accepted at P value <0.05 (S). A P value <0.001 was considered highly significant (HS) while a P value >0.05 was considered non-significant. The statistical analysis was conducted using STATA/SE version 11.2 for Windows (STATA corporation, College Station, Texas).

3. Results

The age of selected cases ranged from 8-65years with a median age of 22 and a mean ± SD 26.5±15.73 years. There was no significant difference between active and stable vitiligo cases regarding sex, age (years), family history, VETI, type of vitiligo, Koebner phenomenon, associated chronic diseases, site of biopsy and duration of the disease Table (1).

FOXP3 expression

The FOXP3 expression scores of the groups are summarized in Table 2. The results revealed that the expression of FOXP3 in T-cells was reduced in the skin of vitiligo patients. The expression of FOXP3 in T-cells was lower in marginal skin of active cases in comparison with lesional and nonlesional skin of the same group. The expression of FOXP3 in T-cells was lower in marginal and nonlesional skin of stable vitiligo compared with lesional skin of the same group.

Table (1) Comparisons between Active & stable cases of vitiligo

Variable		Active Vitiligo (no=10)		Stable vitiligo (no.=10)		Total (no.=20)		Test	P
		No.	%	No.	%	No.	%		
Sex	Females	3	30.0	5	50.0	8	40.0	FET	0.65
	Males	7	70.0	5	50.0	12	60.0		
Age (years)	8-20	5	50.0	3	30.0	8	40.0	Z= 0.91	0.36
	20-40	3	30.0	5	50.0	8	40.0	Z= 0.91	0.36
	40-65	2	20.0	2	20.0	4	20.0	Z= 0.00	1.00
	Mean ±SD median (range)	23.1±12.25 19.5 (8-48)		29.9±18.62 24.5 (12-65)		26.5±15.73 22 (8-65)		MW= 0.87	0.38
Family history	Negative	10	100.0	8	80.0	18	90.0	FET	0.47
	Positive	0	0.0	2	20.0	2	10.0		
VETI	Mean ±SD median (range)	7.17±5.67 6.25 (0.6-17)		3.77±2.14 (3.52 (0.8-7.6)		5.47±4.52 3.81 (0.6-17)		MW= 1.13	0.26
Type of vitiligo	Acrofacial	4	40.0	2	20.0	6	30.0	Z= 0.97	0.33
	Focal	0	0.0	2	20.0	2	10.0	Z= 1.49	0.14
	Generalized	5	50.0	6	60.0	11	55.0	Z= 0.45	0.65
	Universalis	1	10.0	0	0.0	1	5.0	Z= 1.03	0.30
KP	Negative	7	70.0	7	70.0	14	70.0	FET	1.00
	Positive	3	30.0	3	30.0	6	30.0		
Associations	Diabetes mellitus	1	10.0	1	10.0	2	10.0	Z= 0.00	1.00
	Thyroid disease	1	10.0	0	0.0	1	5.0	Z= 1.03	0.30
	No	8	80.0	9	90.0	17	85.0	Z= 0.63	0.53
Duration (years)	Mean ±SD median (range)	3.8±3.15 2 (1-9)		5.7±4.35 4.5 (1-15)		4.75±3.82 3.5 (1-15)		MW= 1.14	0.25
Site of biopsy	Acral	7	70.0	7	70.0	14	70.0	FET	1.00
	Non acral	3	30.0	3	30.0	6	30.0		

P: Probability; SD: Standard Deviation; FET: Fisher Exact Test; MW: Mann-Whitney test

Table (2) The statistical comparison of the immunohistochemical assessment results for FOXP3 in lesional, marginal, and non-lesional skin specimens between the groups

Variable	FOXP3 Dermis Pathology	Active vitiligo (no.=10)		Stable vitiligo (no.=10)		Total (no.=20)		Test	P
	expression score	No.	%	No.	%	No.	%		
Lesional	0	3	30.0	2	20.0	5	25.0	Z= 0.52	0.60
	1	5	50.0	6	60.0	11	55.0	Z= 0.45	0.65
	2	2	20.0	2	20.0	4	20.0	Z= 0.00	1.00
	Mean ±SD median (range)	0.9±0.74 1 (0-2)		1±0.67 1 (0-2)		0.95±0.69 1 (0-2)		MW= 0.34	0.74
Marginal	0	6	60.0	4	40.0	10	50.0	Z=0.89	0.37
	1	0	0.0	1	10.0	1	5.0	Z=1.03	0.30
	2	4	40.0	4	40.0	8	40.0	Z=0.00	1.00
	3	0	0.0	1	10.0	1	5.0	Z=1.03	0.30
	Mean ±SD median (range)	0.8±1.03 0 (0-2)		1.2±1.13 1.5 (0-3)		1±1.08 0.5 (0-3)		MW=0.84	0.40
Nonlesional	0	4	40.0	4	40.0	8	40.0	Z=0.00	1.00
	2	5	50.0	4	40.0	9	45.0	Z=0.45	0.65
	3	1	10.0	2	20.0	3	15.0	Z=0.63	0.53
	Mean ±SD median (range)	1.3±1.16 2 (0-3)		1.4±1.26 2 (0-3)		1.35±1.18 2 (0-3)		MW=0.25	0.80

Pathology score: 0, no staining; **1**, staining less than 1/3 of mononuclear cell infiltrate (inflammatory cells); **2**, staining between 1/3 and 2/3 of mononuclear cell infiltrate (inflammatory cells); **3**, staining more than 2/3 of mononuclear cell infiltrate (inflammatory cells). n, number of cases.

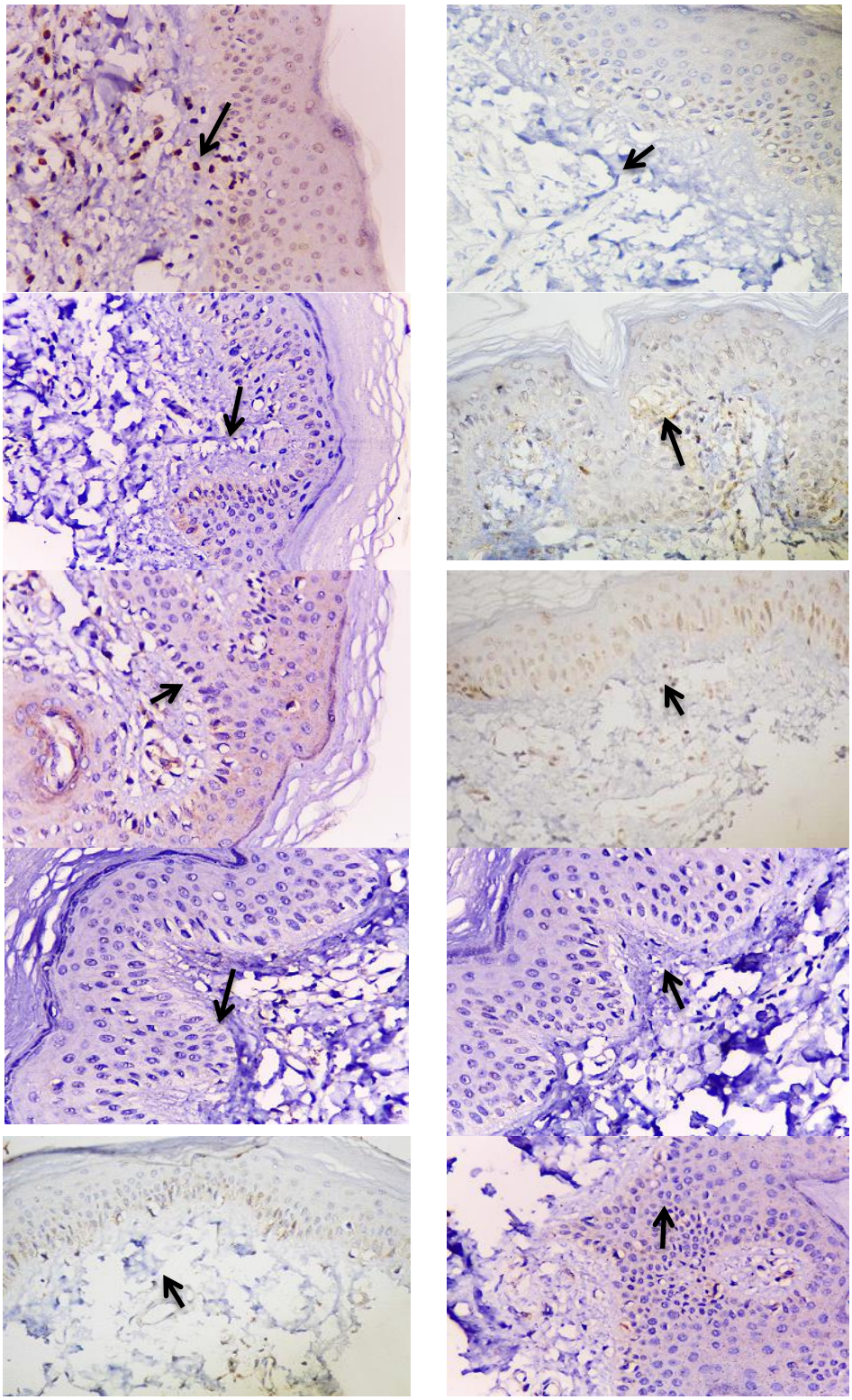

Fig (1) Immunopathological image examples of pathological scoring considered the density of FOXP3-expressed cells (brown cells) in the upper dermis. (a) pathological score 3, (lesional area, active case), (b) pathological score 0, (marginal area, active case NO.16), (c) pathological score 0, (lesional area, active case), (d) pathological score 1, (marginal area, active case), (e) pathological score 2, (marginal area, active case) ,(f) pathological score 3, (non-lesional area, active case NO.16),(g) 0, (lesional area, active case NO.16), (h) pathological score 0, (marginal area, stable case NO.10), (i) pathological score 2, (non-lesional area, stable case NO.10) and (j) pathological score 1, (lesional area, stable case NO.10).

3.1 Clinical assessment

When the vitiligo group was divided into subgroups of vitiligo cases and compared to each other as:

1- The vitiligo cases with a positive Koebner phenomenon (n = 6, 30%) vs. the patients with negative Koebner phenomenon.
2- The vitiligo cases with a positive family history of vitiligo (n = 10 %) vs. the vitiligo cases with no family history of vitiligo (n = 18, 90 %).
3- The vitiligo cases with concomitant disease (n = 3, 15 %) vs. the vitiligo cases without concomitant disease (n = 17, 85 %),

There were no statistically significant differences in terms of FOXP3 expressions in lesional, marginal or nonlesional skin specimens between the subgroups ($P > 0.05$).

4.Discussion

In the study, FOXP3 immunohistochemical staining revealed lower FOXP3 + Tregs expression in marginal skin than lesional skin in both active and stable vitiligo groups. Similar results were found by [22], [13].

[22].study revealed decreased percentage of peripheral CD4+CD25+ Tregs and decreased expression of FOXP3+. they proposed that impairment of the suppressive activity of Treg cells on cell proliferation with breakage of tolerance to melanocyte self-antigens could contribute to the pathogenesis of vitiligo.

This also support the theory proposed by [13] that Tregs regulatory activity is needed at the marginal skin to suppress the activity of helper and cytotoxic T-cells that are actively contributing to depigmentation. Therefore, their deficiency plays a role in progression of the disease process.

Regulatory cytokines produced by Treg cells, such as IL- 10 and TGF-β, were also suggested to be related to the stability of vitiligo [26].

[27] found that Foxp3 expression, and thus Treg density, was lower in the skin sections of vitiligo compared to controls and he suggested that the role of insufficient Treg-cell function in the pathogenesis of vitiligo is probably due to an IL-10 reduction.

In contrast to these studies, [18] and [28] reported a discrepancy between the relative abundance of Treg cells present in the circulation of vitiligo patients as compared to their skin. The authors suggested that this may be explained by a failure of Tregs to migrate into the skin.

Current results may also suggest that even in stable cases, progression of the disease is ongoing at tissue level in a subclinical manner. This is very important when surgical treatment is considered for such cases. It may determine the prognosis in individualized cases.

However, these findings could be related to the duration of stability in our cases. There is a debate about considering one year duration of clinical stability enough to label these cases as stable vitiligo. [29], proposed that stability of vitiligo should be considered after at least 2 years of clinical stability.

In the current study, FOXP3+ Tregs expression levels showed no difference between marginal and nonlesional skin of stable vitiligo group, while levels were reduced in marginal than nonlesional skin in active vitiligo group. This further supports the cardinal role of T regs in disease activity.

This is opposite to the study done by [13]. It showed that FOXP3 expression in nonlesional stable specimens was lower than the expression in the marginal specimens of the same group.

In the present study, there was no significant difference between lesional, marginal, and nonlesional skin Foxp3+ Tregs in relation to the type of vitiligo, duration of the disease, VETI score or presence of kobner phenomenon.

This is in contradiction to the proposal of [30] (The Double Strike Hypothesis). Michelsen suggested that vitiligo is caused by at least two different major pathomechanisms: an antibody-based pathomechanism and a T-cell-based pathomechanism. The antibody-based pathomechanism is dominant in diffuse (generalized) vitiligo, while the T-cell based pathomechanism is dominant in localized vitiligo.

The current study indicates that the same pathogenic factors take place in different types of vitiligo and with different other variables and further supports the homeostatic role of Tregs in both humoral and cellular immune response.

Clinical and exprimental studies have suggested that vitiligo is a result of systemic autoimmune disease. However, this will be accepted when the mechanisms involved in its pathogensis are revealed.

In this study, tissue sections consisting of only lesional, marginal and nonlesional skin specimens of vitiligo patients were assessed for ethical reasons. Skin specimens from controls were not done, representing a limitation of the study.

In conclusion, this study supports that FOXP3 is an important marker for Tregs in tissues. It shows decreased activity of Tregs in vitiligo and its role in disease progression. The reduction in the expression of FOXP3 in the marginal skin suggests that this is the site where regulatory activity is needed to suppress the activity of both helper and cytotoxic T-cells that are actively contributing to depigmentation. The study of different aspects of both active and stable vitiligo helps in understanding some of the mechanisms for vitiligo pathogenesis.

5.Acknowledgments

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