Estimation of Matrix Metalloproteinase-9 and Interleukin-17 Tissue Levels in Stable, Non-Segmental Vitiligo

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Abstract

**Background:** Patients with vitiligo have disturbed melanocytic milieu either by rise or decline in levels of certain mediators.

**Objectives:** The study aimed at measuring Matrix Metalloproteinase-9 (MMP-9) and Interleukin-17 (IL-17) tissue levels in patients with stable, Non-Segmental Vitiligo (NSV) and determining if there is any relation between their levels and patients' age and sex, and disease activity, extent and duration.

**Patients and Methods:** Thirty patients with stable NSV were recruited. Punch skin biopsies were taken and examined by ELISA to detect the levels of MMP-9 and IL-17.

**Results:** All cases with stable NSV had low MMP-9 and elevated IL-17 levels. None of them was related to the age, sex, disease activity and duration, and extent of vitiligo.

**Conclusion:** Imbalance in MMP-9 and IL-17 levels is directly related to vitiligo pathogenesis and targeting these mediators could be a promising line of treatment.

**Key Words:** Vitiligo – NSV – MMP-9 – IL-17.

Introduction

VITILIGO is a chronic pigmented disorder which manifests as milky white macules and patches. Although its pathogenesis is still obscure, the convergence theory (in which there is a complex interaction between genetic, environmental, immunological, biochemical and neural events) could be considered the pillar to understand it [1].

Melanocytes are a prototype of a migratory cell, migrating from the neural crest to the epidermis during fetal life [2], and from the hair follicle infundibulum to the depigmented epidermis during adult life [3], but such migratory cells must penetrate ECM (Extra cellular matrix). Therefore, timely degradation of ECM is essential through activation of MMPs e.g. MMP-9 which is regulated by E-twenty-six-1 (Ets-1) [4,5]. MMPs are generally expressed in very low amounts and their transcription is tightly regulated either positively or negatively by cytokines and growth factors such as; interleukins (IL-1, IL-6), Epidermal Growth Factor (EGF), and TNF-a. Some of these regulatory molecules can be proteolytically activated or inactivated by feedback effect of MMPs [6,7]. MMP-9 plays a major role in the degradation of ECM and non-ECM in tissue remodelling, affects the immune cells function and is upregulated during inflammatory processes such as arthritis and diabetes [8].

Hence, MMP-9 plays an important role through paving a pathway in front of migratory melanocytes and represents a pro-pigmenting factor.

On the other hand, in vitiligo, there is skewing of responses toward Th1 and Th17 [9]. Th17 cells produce IL-17, –21 and –22, as well as chemokines. Various Th17-like cells exist and produce a similar array of cytokines; these include yS-T cells, Natural Killer (NK) cells and NKT cells [10]. Thus, IL-17 is produced by cells of both innate and adaptive immunity. IL-17 acts on a broad range of cells (fibroblasts, endothelial cells, keratinocytes and macrophages) inducing the expression of cytokines (TNF, IL-1 and IL-6), chemokines, and metalloproteinases [11]. Swope et al., [12] showed that TNF-a, IL-1 and IL-6 inhibit melanocyte proliferation and Mussette et al., [13] found that they may induce melanocyte apoptosis through activation of macrophages and cytotoxic lymphocytes. Moreover, IL-17 itself synergizes with these local inflammatory mediators causing further inhibition of melanocyte proliferation [14].
Accordingly, IL-17 has a pathogenic effect against melanocytes and could be considered as an anti-pigmenting factor.

**Aim of the study:**

To measure the tissue levels of two contradictory substances, MMP-9 and IL-17 in cases with stable NSV using Enzyme-Linked Immunosorbent Assay (ELISA), as well as, to evaluate the relation between MMP-9 and IL-17 tissue levels and patients’ age, sex, disease activity and duration, and the extent of vitiligo.

**Patients and Methods**

After approval of the Dermatology Research Ethical Committee (DermREC) of Faculty of Medicine, Cairo University, this study was performed at the Dermatology outpatient clinic of, Kasr-Al-Ainy Teaching Hospital. The study extended from March 2014 till March 2015.

Thirty patients fulfilling the following criteria were enrolled in the study and informed written consents were taken from patients or guardians of patients under eighteen years.

**Inclusion criteria:**

- Patients of both sexes.
- Patients older than twelve years.
- Patients with NSV.
- Patients with stable vitiligo for six months or more.

**Exclusion criteria:**

- Patients who received vitiligo treatment either topically or systemically over the past month.
- Patients who gave history of bleeding tendency or positive koebnerization.

**Methodology:**

Patients were subjected for:

- **Detailed history taking as regards:**
  - Age and sex.
  - Disease duration and course [progressive, regressive or stationary] and accordingly, VIDA scoring (vitiligo disease activity) (Table 1) was done.
  - Treatment history.
  - History of koebnerization or bleeding tendency.

- **Clinical examination:** To detect the type and extent of vitiligo.
- One vitiliginous lesion/patient was chosen.

**Work up:** 2mm Punch biopsies were taken from vitiligo lesions for which Enzyme-Linked Immunosorbent Assay (ELISA) for MMP-9 and IL-17 was done as follows: Skin biopsies were weighted and rinsed with 1X Phosphate-Buffered Saline (PBS), homogenized in 300uL of 1X PBS and stored overnight at 20ºC. After two freeze-thaw cycles, skin homogenates were centrifuged for 5 minutes at 5000g. The supernate was removed for quantitative determination of MMP-9 and IL-17 by Enzyme-Linked Immunosorbent Assay (ELISA) method using [MMP-9 Platinum ELISA BMS2016/2/BMS2016/2TEN, and IL-17 Platinum ELISA BMS2017/BMS2017TEN supplied from eBioscience, affymetrix (Vienna, Austria)] and concentrations were expressed as ng/gm and pg/gm tissue homogenates respectively.

<table>
<thead>
<tr>
<th>VIDA score</th>
<th>Description</th>
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<tr>
<td>+4</td>
<td>Stable for 6 weeks or less duration</td>
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<tr>
<td>+3</td>
<td>Stable for 6 weeks to 3 months</td>
</tr>
<tr>
<td>+2</td>
<td>Stable for 3-6 months</td>
</tr>
<tr>
<td>+1</td>
<td>Stable for 6-12 months</td>
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<tr>
<td>0</td>
<td>Stable for 1 year or more</td>
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<tr>
<td>–1</td>
<td>Stable with spontaneous repigmentation since 1 year or more</td>
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ELISA tests were performed according to manufacturer’s instructions. Microwell plate was coated with anti-Human MMP-9 antibody to which human MMP-9 binded. Then, a 50µl of biotin-conjugated anti-human MMP-9 antibody was added and got adsorbed to human MMP-9 captured by the first antibody. The plate was covered with an adhesive film and incubated at room temperature (18 to 25ºC) for 2 hours on a microplate shaker set at 400rpm. The microwell strips were washed 4 times to remove unbound biotin-conjugated anti-human MMP-9 antibody. 100µl of diluted Streptavidin-HRP was added and binded to the biotin-conjugated anti-human MMP-9 antibody. The plate was reincubated for 1 hour on a microplate shaker at 400rpm. The unbound Streptavidin-HRP was removed by washing the plate 4 times followed by adding 100µl of tetramethyl-benzidine, a substrate solution reactive with HRP, to the well. Incubation was done for about 10min with protection from direct exposure to intense light.

The enzyme reaction was stopped by quick pipetting of 100µl stop solution (1M Phosphoric acid) and absorbance was read on a spectrophotometer using 450nm as the primary wave length. A coloured product was formed in propor-
tion to the amount of human MMP-9. A standard curve was prepared by plotting the mean absorbance for 7 human MMP-9 standard dilutions on the ordinate against the human MMP-9 concentration on the abscissa. The best fit curve through the points of the graph was drawn and human MMP-9 sample concentrations were determined.

In the same way, ELISA was done for measuring IL-17 levels.

**Statistical methods:**

Data were coded and entered using the statistical package SPSS (Statistical Package for the Social Sciences) version 23. Data was summarized using mean, standard deviation, median, minimum and maximum in quantitative data and using frequency (count) and relative frequency (percentage) for categorical data. Comparisons between quantitative variables were done using the non-parametric Kruskal-Wallis and Mann-Whitney tests. *p*-values less than 0.05 were considered as statistically significant.

**Results**

**Patients’ data:**

The current study included thirty patients, seven males (23.3%) and twenty three females (76.7%) with stable, non-segmental and non-acral vitiligo. Ages ranged from 13 to 60 years with a mean of 27.40 years ±12.458.

Clinical data of the patients were summarized in (Tables 2-4).

<table>
<thead>
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<th>Table (2): Disease duration.</th>
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<tr>
<td>Data</td>
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<td>Disease duration (years)</td>
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<th>Table (3): Extent of vitiligo.</th>
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<td>Extent of vitiligo (%)</td>
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<td>&lt;25</td>
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<td>25-50</td>
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<td>50-75</td>
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<td>&gt;75</td>
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<th>Table (4): VIDA scoring for the patients.</th>
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<td>VIDA scoring (stability duration)</td>
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<td>0 = Stable for 1 year or more</td>
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<td>+1 = Stable for 6-12 months</td>
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**Tissue baseline levels for MMP-9:**

Baseline levels for MMP-9 (measured by ELISA) in the vitiligo lesions were from 80 to 1,286 ng/gm tissue with a mean of 239.6±258.299.

It has been found that MMP-9 tissue levels were not statistically significant when related to the age (*p*-value=0.830), sex (*p*-value=0.162), disease activity (*p*-value=0.288), extent of vitiligo (*p*-value=0.180) and VIDA score (*p*-value=0.464).

**Tissue baseline levels for IL-17:**

IL-17 tissue levels (measured by ELISA) ranged from 896 to 2,588 pg/gm tissue with a mean of 1,514±395.615.

It is noteworthy that IL-17 basal levels showed no statistical significance when related to the age (*p*-value=0.979), sex (*p*-value=0.148), disease activity (*p*-value=0.329), extent of vitiligo (*p*-value=0.083) and VIDA score (*p*-value=0.477).

**Discussion**

Vitiligo pathogenesis is multifactorial and no single mechanism could explain it. Moreover, numerous tissue mediators attribute to the pathogenesis through several sequential steps [1].

Thirty vitiligo patients with stable, non-segmental and non-acral vitiligo were enrolled in this study. A single vitiligo lesion per patient was biopsied to detect the baseline levels of MMP-9 and IL-17.

The current study revealed low MMP-9 levels (80-1286ng/gm tissue) which were in agreement with Kumar and colleagues, [18] who documented absence of Ets-1 expression in vitiligo melanocytes which could possibly decrease MMP-2 and MMP-9 expression. Therefore, activity of MMP-2 and MMP-9 was absent in the vitiligo melanocytes compared with the control melanocytes.

Our study is the first to detect that tissue MMP-9 levels were not statistically significant when related to the patients’ age and sex. Furthermore, relation to the duration of the disease, extent of vitiligo and VIDA score were of no statistical significance.

In this study, basal tissue IL-17 levels were high (896-2588pg/mg tissue) similar to Bassiouny and Shaker, [19] Esmaeili et al., [20] and Habeb et al., [21]. It has not been of statistical significance when related to the extent of vitiligo and VIDA score which was concording with Habeb et al., [21] but against Basak et al., [14] and Bassiouny...
and Shaker (19) who found that serum levels of IL-17 in vitiligo patients correlated positively with percentage BSA (body surface area) involvement and disease duration. These findings could be due to the multifactorial pathogenic nature of the disease and denote the presence of many other factors controlling the disease activity.

IL-17 levels were not statistically significant when related to patients’ age and sex, and the disease duration.

In conclusion, both low MMP-9 and high IL-17 tissue levels contribute to the poor melanocytic function in vitiligo and this augments the multifactorial nature of vitiligo pathogenesis.

Recommendations:
Further studies on therapeutic modalities which can increase MMP-9 levels (e.g fractional CO₂ laser) or decrease IL-17 levels (e.g Ustekinumab) should be done.

References
الملخص العربي

يعد السبب الرئيسي لمرض البهقاغ غير معروف لذا فإن العديد من الدراسات تهدف لكشف أسبابه.

وقد أجري هذا البحث على ثلاثين من مرضى البهقاغ الذين نشطوا باللهجة، الفيظان والفضول، وتم قياس مستوى مادة (أنتروپيون-17) و(أم ام ب-9) في العينات الجلدية المأخوذة من الأماكن المصابة بالبهقاغ. أوضح النتائج ارتفاع نسبة (أنتروپيون-17) وانخفاض نسبة (أم ام ب-9) مما يؤثر سلبياً على نشاط وحركة الخلايا الصبغية.

كما أثبت عدم وجود علاقة إحصائية بين نسب المادتين التي تم قياسهما وسن وجنسي المريض ومدة المرض ونشاطه ومساحة المناطق المصابة.

هذا الاختلاف في مستوى المادتين يحفز الاتجاه إلى الطرق العلاجية التي تساعد على استعادة النسب المثلى، أما بزيادة (أم ام ب-9) أو بانخفاض (أنتروپيون-17).