Evaluation of the Role of Impaired Cell to Cell Adhesion in Vitiligo: Immunohistochemical Expression of MART1 and E-Cadherin

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Abstract

Background: Vitiligo is an acquired, hypomelanotic skin disorder characterized by circumscribed depigmented macules. Most studies on vitiligo have concentrated on the abnormality of melanocytes rather than the abnormality of keratinocytes. Some studies supported the hypothesis that keratinocytes impaired intercellular adhesion may play a role in the pathogenesis of vitiligo.

Objectives: To assess the role of impaired intercellular adhesion in the pathogenesis of vitiligo through the studying of immunohistochemical presence and distribution of MART1 and E-cadherin in the epidermis.

Patients and Methods: Thirty five patients having non-segmental vitiligo were recruited from the outpatient clinic (all active therapies were stopped at least 3 weeks prior to inclusion in the study) as well as twenty (age and sex matched) volunteers (vitiligo free) were included in the study as control group. All patients were subjected to complete history taking with special emphasis on the duration of the disease. Patients underwent skin biopsies (lesional, perilesional and non lesional) and 1 skin biopsy was taken from every control volunteer. H & E staining was performed for histopathological examination and Immunohistochemical staining with MART1 was done for lesional biopsies to confirm the clinical diagnosis. E-cadherin immunostaining was done for all biopsies and statistical analysis was done to compare the results in patients and controls.

Results: Regarding to immunostaining of E-cadherin there was a highly significant difference between lesional biopsies of cases and control groups. Also there was a highly significant difference between lesional biopsies of cases and perilesional and non lesional biopsies of the same cases. No significant difference was noted regarding the disease stability.

Conclusion: Vitiligo is not a disease confined to melanocytes. Keratinocytes impaired cell to cell adhesion may play prominent role in the pathogenesis of the disease.

Key Words: Vitiligo – Cell adhesion – E-cadherin.

Introduction

VITILIGO is an acquired, hypomelanotic skin disorder characterized by circumscribed depigmented macules resulting from the loss of functional melanocytes. Various factors which may be responsible for precipitating this disorder in susceptible patients are oxidative stress, auto-immunity and neurochemicals [1].

Numerous hypotheses about the etiology of vitiligo had been found however, none of them was totally proven. One way to understand the etiology was to determine the mechanism by which melanocytes were destroyed [2].

Most studies on vitiligo have concentrated on the abnormality of melanocytes rather than the abnormality of keratinocytes; however, epidermal melanocytes form a functional and structural unit with neighboring keratinocytes. It has been demonstrated that non-functioning melanocytes may still be present in the white epidermis of vitiligo patients [3].

By electron microscope vitiligo lesional skin samples showed the absence of melanocytes. Keratinocytes were of distorted architecture with no melanosomes and some could only be identified by the presence of desmosomes. Increased intercellular spaces were noted compared with perilesional skin. Irregular nuclei and prominent rough endoplasmic reticulum were seen in keratinocytes with peripheral margination of tonofilaments. Perilesional skin of vitiligo patients showed melanocytes with prominent rough endoplasmic reticulum and vacuolization. Keratinocytes were of normal architecture but had irregular nuclei and contained some vacuoles. There was no increase in intercellular spaces. Melanosomes in both melanocytes...
and keratinocytes appeared electron dense and scattered within the cytoplasm [3].

Cross-talk between keratinocytes and melanocytes has been observed. Actually, direct cell-to-cell contact stimulates in vitro proliferation of melanocytes, and growth factors produced by adjacent keratinocytes regulate the proliferation and differentiation of melanocytes [4].

Keratinocytes produce and release numerous synergistic mitogens in culture, such as basic Fibroblast Growth Factor (bFGF), endothelins, Stem Cell Factor (SCF), hepatocyte growth factor, nerve growth factor, granulocyte macrophage colony stimulating factor, leukemia inhibitory factor, α-melanocyte stimulating hormone, and adrenocorticotropic hormone61. In particular, SCF is essential for melanocyte survival. Melanocytes express c-kit, a receptor for SCF [8].

E-cadherin is a cell adhesion transmembrane molecule which is a member of a family of functionally related transmembrane glycoproteins that mediate Ca2+-dependent intercellular cellular adhesion [6,7].

E-cadherin is important for epidermal intercellular adherence because it is required for the adhesive properties of keratinocytes and skin differentiation and loss of E-cadherin-mediated cell adhesion is one rate-limiting step in the progression from adenaoma to carcinoma. Furthermore, it determines the morphogenesis and hair follicle cycling. The intracellular domain of E-cadherin binds directly to β-catenin and γ-catenin. The N-terminal portions of both β- and γ-catenin interact with α-catenin, which links E-cadherin to the underlying actin cytoskeleton. Some studies showed that treatment with the PI3K inhibitor, wortmannin, inhibited E-cadherin-catenin expression and PI3K phosphorylation in cultured keratinocytes [8].

Cadherin is involved in the calcium-dependent cell-to-cell adhesion of molecules. Increases in intracellular calcium could recruit PI3K to the E-cadherin-catenin complex at the plasma membrane, resulting in PI3K activation. Because activation of the PI3K/AKT pathway is required for keratinocyte differentiation [9,10].

Some studies showed that phosphorylated PI3K decreased in the depigmented epidermis of patients with vitiligo compared to that in normally pigmented epidermis. Also it was identified that AKT1 phosphorylation and E-cadherin, β-catenin, and γ-catenin expression decreased significantly in depigmented epidermis [11].

Reduced E-cadherin gene (CDH1) activity in mice and melanocytes of human reconstructed epidermis, in the presence of mechanical or oxidative stress, decreases pigmentation, number of basal melanocytes. Comparison between lesional and non-lesional skin of vitiligo patients shows reduced E-cadherin expression [12,13].

It was found that E-cadherin is absent from or discontinuously distributed across melanocyte membranes of vitiligo patients long before clinical lesions appear. This abnormality is associated with the detachment of the melanocytes from the basal to the suprabasal layers in the epidermis [7].

Interestingly, E-cadherin also anchors Langerhans cells to keratinocytes, regulating its tolerogenic differentiation. A dysfunction of this mechanism could induce autoimmune/inflammatory disease, this can explain the association among patients presenting autoimmune co-morbidities [13].

**Aim of the work:**

We aimed by this work to evaluate the role impaired cell to cell adhesion as possible hypotheses for vitiligo. Immunohistochemical expression of MART 1 (as a melanocytes specific marker) and E-cadherin (as intercellular adhesion molecule) was evaluated in lesional, perilesional, non lesional skin biopsies taken from affected patients and skin biopsies from normal control cases.

**Patients and Methods**

Thirty five patients having non segmental vitiligo were recruited from the outpatient clinic, Dermatology Department. Fayoum General Hospital and private clinics along a period of one year from August 2012 to August 2013 (all active therapies were stopped at least 3 weeks prior to inclusion in the study).

Twenty (age and sex matched) volunteers (vitiligo free) with healthy skin appearance (attended the dermatology or plastic surgery clinics for cosmetic problems) were included in the study as control group.

*After taking a written consent from patients and controls, every patient was subjected to the following:*

- Complete history taking with special emphasis on the duration of the disease. According to Ezzedine et al., 2012 a 12-month period of lesion stability-without clinical progression-is deemed appropriate to consider a lesion 'stable' for the purpose of surgical treatment [14].
- Dermatological examination.
Methods:

Three mm. punch biopsies were taken from the patients (lesional, perilesional and non lesional) and one 3mm punch biopsy was taken from the controls.

All specimens were fixed in formaline, routinely processed and embedded in paraffin. Serial 5-micron thick sections were obtained from the paraffin blocks and stained with H & E for routine histopathological evaluation.

Three sections were mounted on positively charged slides and stained for immunohistochemical evaluation.

Immunohistochemical staining:

**Antibodies used in this study:**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>MART-1 monoclonal antibody (Clone A103 mouse Mo Ab)</td>
<td>Melanocytes</td>
<td>Thermo Scientific, UK</td>
<td>1:100</td>
</tr>
<tr>
<td>E-cadherin monoclonal antibody (Clone 36B5 mouse Mo Ab)</td>
<td>E-cadherin</td>
<td>Thermo Scientific, UK</td>
<td>1:25</td>
</tr>
</tbody>
</table>

**Interpretation of immunostaining:**

Positive staining is indicated by the presence of a brown cell membrane and cytoplasmic color in case of E-cadherin Ab, brown cytoplasmic staining in case of MART-1 Ab.

For each immunostaining marker, in every staining session a section of the control tissue previously known to be positive for the specific marker was used.

Semiquantitative assessment of E-cadherin expression was performed using a modified H-score in which both intensity and proportion of staining was categorized. Category A indicated the proportion of positive cell staining throughout the section and was assigned a scale from 0 to 3 (0=0-4%; 1=5-24%; 2=25-49%; 3=50-100%). Category B represented the average intensity; the presence of negative, weak, intermediate and strong staining was given a score from 0 to 3. Category A was multiplied by category B to form a multiplicative score. The cases were sorted into three subgroups; H-score 0 referred to negative expression; H-score 1-2 to weak expression; H-score 3-9 to moderate/strong expression [15].

**Statistical analysis:**

Data were collected and statistically analyzed using Statistical Package for Social Sciences SPSS Version 17.

A- Descriptive statistics:

- Arithmetic mean for central tendency.
- Standard Deviation (SD) for measuring dispersion.
- Median and range for non-normally distributed data.
- Percentage (%).

B- Analytic statistics:

- Non-parametric t-test (Mann Whitney test) was used for comparison of means of two independent groups.
- Spearman rank coefficient was used as a measure of association of quantitative variables.
- A p-value of less than 0.05 was considered to be statistically significant.

Results

The present study included 35 non segmental vitiligo patients recruited from the outpatient clinic of the Dermatology Department in Fayoum General Hospital and private clinics through a period of one year (from August 2012 to August 2013), the diagnosis was based on clinical examination and confirmed with immunohistochemical staining of lesional biopsies with MART-1/Melan-A mouse monoclonal antibody. Twenty (age and sex matched) volunteers (vitiligo free) with healthy skin appearance (attended the dermatology or plastic surgery clinics for cosmetic problems) were included in the study as control group.

This study included 25 cases of clinically stable vitiligo representing (71.5%) and 10 clinically non stable vitiligo representing (28.5%). A 12-month period of lesion stability-without clinical progression-is deemed appropriate to consider a lesion 'stable' for the purpose of surgical treatment [11].

Table (1): Comparison of E cadherin lesional. Perilesional, and non-lesional among cases and in comparison with control values.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cases</th>
<th>Controls</th>
<th>p-value (cases vs. controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-cad lesional</td>
<td>1.0 (0-3)</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>E-cad perilesional</td>
<td>6 (4-9)</td>
<td>9 (6-9)</td>
<td>0.002</td>
</tr>
<tr>
<td>E-cad nonlesional</td>
<td>9 (6-9)</td>
<td></td>
<td>0.26</td>
</tr>
</tbody>
</table>
Table (1) shows that there was an obvious difference between cases (both lesional and perilesional biopsies) and control group values regarding the sum of intensity and proportion of staining. This difference was significant for both lesional biopsies (\(p\)-value <0.001) and perilesional biopsies (\(p\)-value=0.002). On the other hand there was no significant difference between non lesional biopsies (\(p\)-value=0.26) if compared with control cases.

A comparison was done between lesional to perilesional, and to non-lesional biopsies among cases regarding to E-cadherin immunohistochemical staining as shown in Table (1) and Graph (1).

Both the Table (1) and Graph (1) show significant difference in the sum of intensity and proportion of E-cadherin immunohistochemical staining in lesional biopsies when compared with perilesional biopsies (\(p\)-value <0.001) and non lesional biopsies (\(p\)-value <0.001) of the same cases.

Also correlation was done between the E-cadherin immunohistochemical staining in lesional, perilesional, and non-lesional biopsies and the clinical stability of the disease as in Table (2).

This table showed no significant difference in E-cadherin immunohistochemical staining in lesional, perilesional, and non-lesional biopsies regarding the clinical stability of the disease.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Non stable n=10</th>
<th>Stable n=25</th>
<th>(p)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-cad lesional</td>
<td>1.0 (0-2)</td>
<td>1 (0-3)</td>
<td>0.82</td>
</tr>
<tr>
<td>E-cad perilesional</td>
<td>6 (4-9)</td>
<td>6 (4-9)</td>
<td>0.26</td>
</tr>
<tr>
<td>E-cad nonlesional</td>
<td>9 (6-9)</td>
<td>9 (6-9)</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Fig. (1): Control case with MART1 immunohistochemical stain show positive basal cytoplasmic stain (black arrows) (H & E X200).

Fig. (2): A lesional biopsy from patient stained with MART1 immuno-staining show no basal stain (absent melanocytes) (H & E X400).

Fig. (3): Membranous and cytoplasmic E-cadherin expression in normal skin; showing mostly equal diffuse staining in all epidermal skin layers and doesn’t staining the horny layer, modified H score=9 (H & E X1000).
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Fig. (4): Lesional biopsy from vitiligo case stained with E-cadherin monoclonal Ab. Showing no expression of E-cadherin in all layers, modified H score=0 (H & E X1000).

Fig. (5): A case of vitiligo stained with E-cad, the non lesional biopsy showed strong E-cadherin expression, score=9 (A) (X200), peri-lesional intermediate expression, score=6 (B) (X200), non-lesional no expression, score=0 (C) (X200).

Discussion

Vitiligo is an acquired, hypomelanotic skin disorder characterized by circumscribed depigmented macules resulting from the loss of functional melanocytes [1].

Most studies on vitiligo have concentrated on the abnormality of melanocytes rather than the abnormality of keratinocytes; however, epidermal melanocytes form a functional and structural unit with neighboring keratinocytes. It has been demonstrated that non-functioning melanocytes may still be present in the white epidermis of vitiligo patients [3].

In fact, direct cell-to-cell contact stimulates in vitro proliferation of melanocytes, and growth factors produced by adjacent keratinocytes regulate the proliferation and differentiation of melanocytes. The potential role of keratinocyte-derived cytokines has also been presented [16].

E-cadherin, a transmembrane anchor, is expressed by epidermal melanocytes and functionally mediating its adhesion to keratinocytes in vitro. Also E-cadherin plays a pivotal role in keratinocyte differentiation. Some studies showed that E-cadherin, β-catenin, and γ-catenin expression decreased significantly in depigmented epidermis [7,13].

The present study included 35 non segmental vitiligo patients and 20 (age and sex matched) volunteers (vitiligo free) with healthy skin appearance.

In this work we stained all the lesional biopsies and some control cases with MART 1 (a melanocytic marker) to confirm the clinical diagnosis of vitiligo. In contrast to controls, all the lesional biopsies showed absent basal cytoplasmic staining, which is confirmatory to the clinical diagnosis of vitiligo.

This study included 25 cases of clinically stable vitiligo representing (71.5%) and 10 clinically non stable vitiligo representing (28.5%). In this work, we tried to find correlation between the disease stability and immunohistochemical expression of both P53 and E-cadherin.

This study showed no significant difference in E-cadherin immunohistochemical staining in lesional, perilesional, and non-lesional biopsies regarding the clinical stability of the disease.

Conclusion and Recommendations:

From our results we can conclude that vitiligo is not a disease confined to melanocytes only, keratinocytes also showed certain pathological changes in vitiligenous lesions.

As functional and structural units with melanocytes, keratinocytes in depigmented epidermis may constitute a different microenvironment compared to those in normally pigmented epidermis. These differences include obvious loss of cell to cell
adhesion between keratinocytes and melanocytes and between keratinocytes and each other, which in turn may affect the pigmentary system of the skin.

More studies are needed to confirm and clarify the role of keratinocytes in vitiligo, including gene expression studies and studies on larger scale of patients.

It is hoped that an improved understanding of the pathogenesis of vitiligo will aid in providing novel treatment options directed to improving or enhancing the defect in adherence junction and to minimize cellular apoptosis, thereby leading to an amelioration of the tissue pathology and in turn the clinical presentation.

References

تقييم دور إضطرابات اتصال الخلايا ببعضها في البهاق؛
(E-cadherin) و (MART-1) التعبيرات المعنوية لكل من

البهاق هو مرض جلدي مكتسي يتميز بوجود بقع نافقة الصبغة بيانة عن فقدان وظيفة الخلايا الصباغية أو غياب الخلايا ذاتها. وقد
ركزت الدراسات على البهاق على الخلل في الخلايا الصباغية بدلاً من الخلل في الخلايا الكرياتية.

ويهدف هذا الهدف إلى تقييم دور ضعف إتصال الخلايا ببعضها البعض كحداً فرضيات ممكناً البهاق وذلك من خلال دراسة التعبير
المناعي ل (E-cadherin) في طبقات ما فوق الأدمة.

ولدراسة هذا الهدف تم اختيار خمسة وثلاثين من مرضى البهاق غير القطبي من العيادات الخارجية لقسم الأمراض الجلدية في مستشفى
الفيوم العام والعيادات الخاصة من خلال مدة سنة واحدة (من أغسطس 2012 إلى أغسطس 2013) وعشرين من المتطوعين (الاختيارات ظاهريًا
من البهاق) وأدرجوا في الدراسة كمجموعة ضابطة.

وقد أخذ تاريخ مرضى كامل لجميع المصابين بالبهاق مع التركيز على مدة المرض، بالإضافة إلى فحصهم إكلينيكياً. كما تم أخذ عينات
من الجلد من كل المرضى حيث تم أخذ عينة من كل مريض بالبهاق (عينة من المنطقة المصابية وعينة من المنطقة الملاصقة لها
وعينة من الجلد السليم ظاهريًا) ومثل أخذ عينة واحدة من متطوعي المجموعة الضابطة. وقد تم الصبغ بالهيماتوكسيلين والأيروسين من أجل الفحص
الميكتروسكوبى والتاج من سلالة العينة. بالإضافة إلى استخدام التعبير المناعي الهيستويميائي لتعيين إظهار وتوزيع (E-cadherin) في
كل المجموعات.

وقد أشارت النتائج إلى وجود اختلاف ذو دلالة إحصائية بين نتيجة العينات المأخوذة من منطقة الإصابة من المنطقة الملاصقة، ونسبة التعبير
في المجموعة المرضية وبين عينات المجموعة الضابطة بالنسبة للتعبيرات المناعية الهيستويميائية ل (E-cadherin). بينما لم يكن هناك اختلاف نو
دلالة إحصائية بين نتيجة العينات المأخوذة من المنطقة السليمة وعينات المجموعة الضابطة. كذلك كان هناك وجود اختلاف ذو
دلالة إحصائية عند مقارنة العينات المأخوذة من المناطق بكل من نتيجة العينات المأخوذة من المنطقة الملاصقة للإصابة ومن المنطقة السليمة
ظاهريًا في الحالات.

في حين أنه لم توجد علاقة ذات دلالة إحصائية بين إستقرار الحالة الإكلينيكية لمرض التعبير المناعي الهيستويميائي ل (E-cadherin)
في طبقات الجلد.

ومن هذه الدراسة نخلص إلى أنه في مرض البهاق غير القطبي يحدث تغييرات في التعبير المناعي الهيستويميائي ل (E-cadherin)
في طبقات الجلد. هذه التغييرات تؤثر في التعبير عن ضعف إتصال الخلايا ببعضها يمكن أن يلعب دوراً في إحداث الخلل الوظيفي لطبقات النسيج الطلائي
الجلد وتحفيز نشاط الخلايا فيه.