Al-Azhar Med. J. *Vol. 45(4), October 2016, 921-930*
DOI: 10.12816/0034754

IMMUNOHISTOCHEMICAL EXPRESSION OF MELAN A AND ITS RELATION TO EXPRESSION OF CXCR3 IN VITILIGO LESIONS

By

Marwa A. El Kholy

Pathology Department, Faculty of Medicine for Girls, Al-Azhar University, Cairo, Egypt.

ABSTRACT

Background: Vitiligo is an acquired pigmentary disorder due to loss or destruction of melanocytes from the epidermis. The question about the presence of residual melanocytes in the depigmented skin remains and accurate methods of their identification are considered.

Objective: This study aimed to search about the presence of residual melanocytes in the skin of vitiligo patients and if there is a relation between destruction of melanocytes and presence of inflammatory cells mainly T-lymphocytes.

Patients and Methods: This study was conducted on 30 patients with vitiligo (localized and generalized) together with 10 healthy volunteers were investigated for Melan-A (A103 clone) expression by immunohistochemical analysis and for CXCR3 antibody.

Results: Melan-A+ cells were detected in depigmented skin as indication that the residual melanocytes are preserved in vitiligo lesions. Decrease of Melan-A+ melanocytes amount was revealed in perilesional normally pigmented skin of vitiligo patients ($P < 0.01$) compared with the skin of healthy volunteers. Also this study found significantly increased number of CXCR3-expressing cells in the dermis of vitiligo lesions (depimented skin) in comparison to healthy controls and those cells were mainly lymphocytes

Conclusion: Melan-A marker is useful not only for identifying melanocytes in vitiligo patients' skin but also for estimating their content in different zones of the vitiligo lesions. Clinically intact skin involvement in the pathological process should be taken into consideration if local treatment methods are recomended. CXCR3 and its ligands play role in the pathogenesis of vitiligo. So, blocking T-cell recruitment by blocking CXCR3 and its ligand chemotactic mechanism may present a new and effective therapy for vitiligo.

Keywords: **Immunohistochemistry, melanocytes, Melan-A marker, CXCR3 , vitiligo.**

INTRODUCTION

Vitiligo is an acquired pigmentary disorder characterized by the presence of well circumscribed depigmented milky white macules and patches ***(El Darouti et al., 2006)***. Its prevalence is 0.5–1%, occurring in the global population ***(Ezzedine et al., 2012).*** Depigmentation is due to loss or destruction of melanocytes from the epidermis ***(Wang et al., 2016).*** The generally accepted hypothesis of the cause of vitiligo is the autoimmune one ***(Oiso et al., 2011).***

The microscopic analysis of normal skin indicates that mature melanocytes are oval or fusiform, dendritic cells, smaller than keratinocytes. In the cytoplasm, there are special membrane-bound organelles producing melanin which are called melanosomes ***(Seiji and Fitzpatrick,***

2013). Melanocytes reside in the basal layer of epidermis where they form the epidermal melanin units as a result of the relationship between one melanocyte and 30-40 associated keratinocytes ***(Fitzpatrick and Breathnach, 2013).***

Studying Melan-A expression in vitiligo lesions is of significant interest because this melanosomal protein is one of the immunogenic determinants of melanocytes to which autoreactive circulating CD8+ T-cells were found in patients' blood ***(Teulings et al., 2014).***

A T-cell-dependent immune response contributes to the onset and evolution of the disease. Thus, the migration of T lymphocytes to areas of melanocyte destruction is a critical component in activating the immune system and inflammatory processes. This was one of the pioneering studies in investigating the involvement of chemokine pathways in vitiligo as CXCR3 and its ligands in the pathogenesis of vitiligo ***(Wang et al., 2016)***.

The aim of the present work was to detect the residual melanocytes in the skin of vitiligo patients, and the possible relation between the destruction of melanocytes and presence of inflammatory cells mainly T-lymphocytes.

MATERIAL AND METHODS

This study was conducted on 30 patients with vitiligo (localized and generalized) together with 10 healthy volunteers. Vitiligo cases were collected retrospectively by reviewing the surgical files of the Histopathology Departments of Al-Zahraa University Hospital (Al- Azhar University) during the period from April 2013 to August 2015. The clinical and dermatologic data were retrieved from the files of the patients after informed consent. In vitiligo patients, skin biopsy material was taken under local anesthesia from three zones, i.e. the zone of depigmented skin, marginal zone (bordering the vitiligo area), and zone of perilesional normally pigmented skin. Control biopsies were taken from healthy donors who were undergoing cosmetic surgery after informed consents.

For histopathological examination, 5-mm-thick sections were prepared from each tissue paraffin block and stained with Hematoxylin and Eosin for confirmation of the diagnosis. For immunohistochemical staining, two sections were cut from each case on positively charged slides and subjected to immunohistochemical staining using the streptavidin-biotin alkaline phosphate methods.

The primary antibodies used for immunohistochemical staining (with clone, manufacture, dilution, incubation period, and positive control) were mouse monoclonal Melan-A antibodies and A103 clone (Novocastra Laboratories Ltd., UK) for the phenotyping at a dilution of 1:25, and anti-CXCR3 antibody (R&D Systems, Minneapolis, MN, U.S.A.).

The sections were placed in an oven at 50°C for 30 min, de-paraffinized in xylene, rehydrated in graded alcohol dilution, washed in PBS, incubated with 0.3% hydrogen peroxide to block endogenous peroxidase activity, washed in PBS again, and boiled in citrate buffer

solution (pH 6.0) using a microwave for 10 min at 60°C for antigen retrieval. After cooling at room temperature, the sections were incubated with primary antibody overnight in a humidified chamber and rinsed with PBS. The sections were then incubated for 30 min at 37°C with biotinylated secondary antibody and streptavidin conjugated to horseradish peroxidase. After three rinses with PBS, the sections were incubated with diamino-benzidine substrate, and then rinsed with distilled water and counterstained with hematoxylin.

The amount of Melan-A+ cells was detected in the basal layer of the epidermis, and the calculation was made per 100 basal keratinocytes. Immuno-reactive cells were counted in five fields of vision.

The average value was calculated for each specimen (***Kubanov et al., 2016).*** CXCR3-expressing cells were counted as cells per x 400 magnifications and given as cells per high-power field.

Statistical analysis: The recorded results were statistically reviewed by computer using SPSS data editor software, version 22. The test used was Student's t-test for testing statistical significant difference. Data were considered significant when $p < 0.05$.

RESULTS

The vitiligo patients included 21 women (70%) and 9 men (30%). The patients were between 20 and 48 years old (median 34 years). The disease was localized in19 patients and generalized in 11 patients. All patients had a progressive (unstable) disease stage. The control group of 10 healthy volunteers included a woman (10%) and 9 men (90%) between 26 and 54 years old (median value 53 years).

Table (1): Clinical data of studied patients.

Count / Variables	No	%
Sex		
Male	**9**	**30**
Female	**21**	**70**
Distribution of vitiligo lesions		
Localized	**19**	**63.33**
Generalized	**11**	**36.67**

As regard the inflammatory infiltrate of skin biopsy of the studied patients 8 cases (26.67%) showed mild infiltrate while 10 cases (33.33%) and 12 cases (40%) exhibited moderate and excessive inflam-matory infiltrate respectively. Weak inflammatory infiltrate was noticed in the cases of control group.

In the vitiligo patients' skin, Melan-A expression was evident in the cells of basal layer of the epidermis: there were scattered individual cells in the zone of depigmented skin ranged from one to three (Fig. 1a), whereas in the marginal zone and in the zone of perilesional normally pigmented skin the amount varied from two to ten cells per 100 keratinocytes of the basal layer (Figs. 1b and 1c).

In the control group (healthy volunteers), Melan-A+ cells were revealed in the basal layer of the epidermis ranged from 8-10 cells per 100 keratinocytes of basal layer.

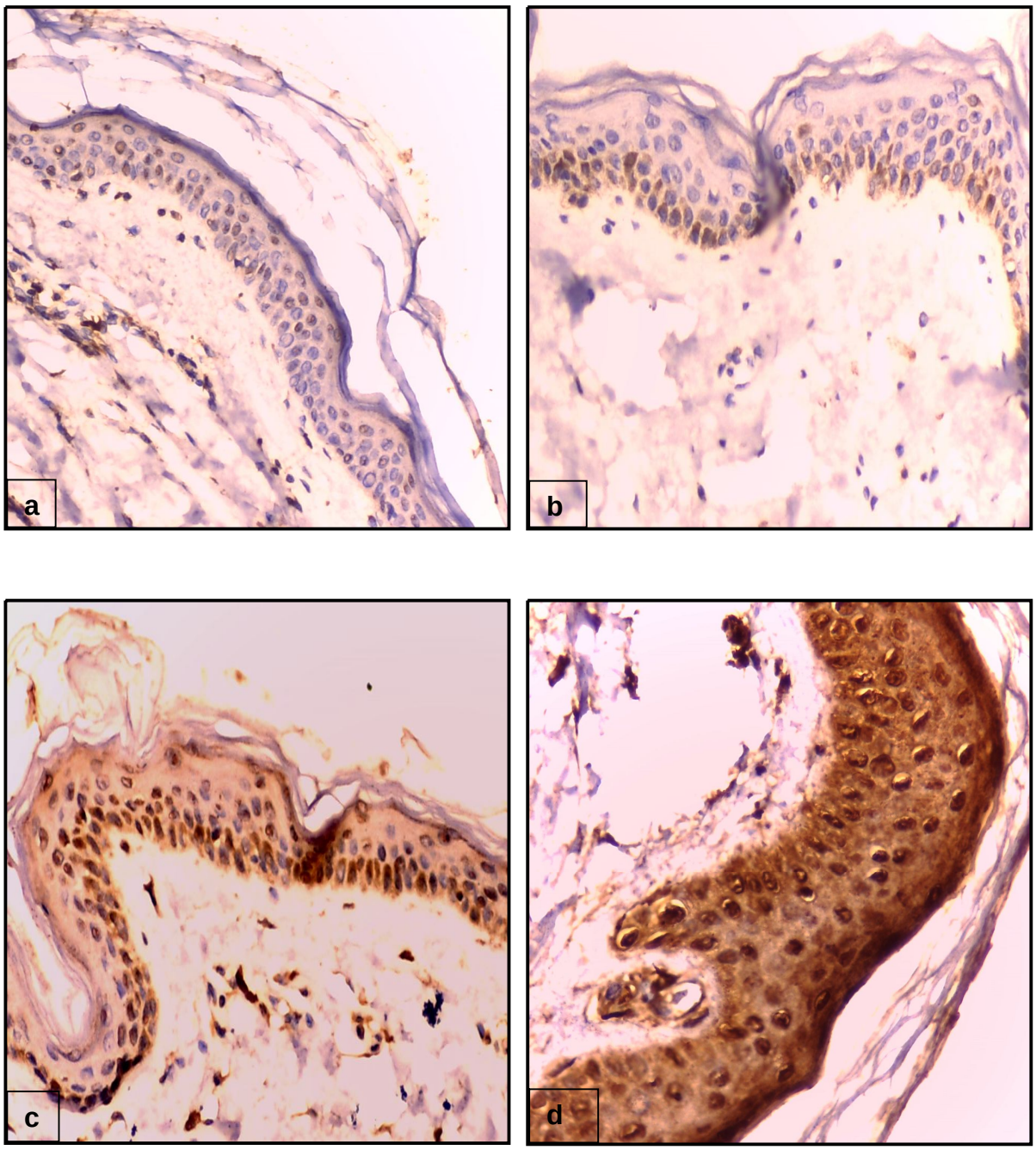

Figure 1 (a): Expression of Melan-A in zone of depigmented skin of a vitiligo patient. Immunohistochemical reaction with monoclonal antibodies A103, ×200. **(b).** Expression of Melan-A in marginal zone of the skin of a vitiligo patient. Immunohistochemical reaction with monoclonal antibodies A103, ×200. **(C).** Expression of Melan-A in zone of perilesional normally pigmented skin of a vitiligo patient. Immunohistochemical reaction with monoclonal antibodies A103, (×200).**(d).** Expression of Melan-A in zone of normal skin of control group. Immunohistochemical reaction with monoclonal antibodies A103, (×200).

Statistical analysis showed a significant decrease in the amount of Melan-A+ cells in all three areas of vitiligo lesions compared with the skin of healthy volunteers ($P < 0.01$-Table 2).

Table (2): Content of Melan-A+ melanocytes in the epidermis of patients with vitiligo and healthy volunteers (number of cells per 100 basal keratinocytes, average values).

Average values of Melan-A+ melanocytes / **Site of biopsy**	**0-2**	**2-4**	**4-6**	**> 6**	**P value**
Healthy volunteers (n = 10)	0	0	0	10	P_1= 0.033
• Lesional skin	19	11	0	0	P_2= 0.025
• Marginal zone	0	10	12	8	P_3= 0.018
• Perilesional normally skin	0	7	9	14	

P1: levels of statistical significance during comparison of average values of Melan-A+ melanocytes in of healthy volunteers and lesional skin in patients group **P2:** levels of statistical significance during comparison of average values of Melan-A+ melanocytes in healthy volunteers and marginal zone in patients group **P3:** levels of statistical significance during comparison of average values of Melan-A+ melanocytes in healthy volunteers and perilesional normally pigmented skin in patients group.

There was a significantly increased number of CXCR3-expressing cells in the dermis of vitiligo lesions (depigmented skin) in comparison to healthy controls (Fig. 2 a,b), and those cells were mainly lymphocytes (Table 3).

Table (3): CXCR3 expression in studied groups.

Groups / **No. of CXCR3 +ve cells /HPF**	**Cases (30)**		**Control (10)**		**P value**
	No	**%**	**No**	**%**	
0-10	0	0	8	80	0.027
11-20	2	6.67	2	20	0.004
21-30	11	36.67	0	0	0.012
31-40	17	56.67	0	0	0.019

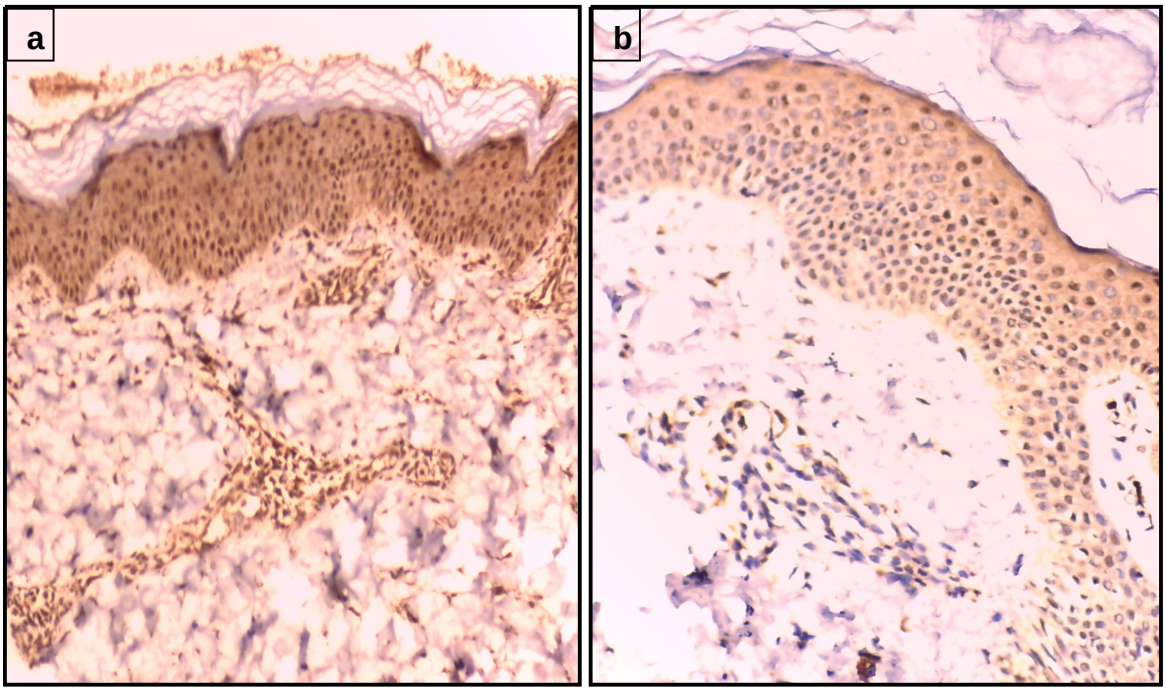

Figure 2: (a) Overexpression of CXCR3 in the dermis of lesional skin of vitiligo patients. **(b)** Few CXCR3-producing cells in the dermis of the healthy controls (×200)**.**

DISCUSSION

Vitiligo is an acquired cutaneous disorder of pigmentation, with an incidence of 0.5% to 2% worldwide. There are three major hypotheses for the pathogenesis of vitiligo that are not exclusive of each other: biochemical/ cytotoxic, neural and autoimmune. Some data provide strong evidence supporting an autoimmune pathogenesis of vitiligo ***(Ghafouriani et al., 2014).***

The Melan-A marker has been increasingly used in diagnosing various pigment neoplasms and diseases of the skin ***(Prieto and Shea, 2011).*** It is well expressed in melanocytes of healthy skin ***(Busam and Jungbluth, 2009)*** and transformed melanocytes of benign and malignant neoplasms of melanocytic genesis ***(Fernandes et al., 2007).***

This study used the Melan-A marker to demonstrate melanocytes in various zones of vitiligo lesions and normal skin of volunteers. The Melan-A marker is presented both in the skin of healthy volunteers and in the skin of vitiligo patients with a decreased amount of Melan-A+ cells in all three areas of vitiligo lesions compared with the skin of healthy volunteers. The decrease in Melan-A+ melanocyte number in perilesional normally pigmented skin of vitiligo patients, as compared to the skin of healthy volunteers indicates that the pathological process may involve not only depigmented skin but also normally pigmented, clinically free skin.

Nearly, the same result was reported by ***Kubanov et al. (2016)*** who found Melan-A+ melanocytes and melanin granules using the Fontana-Masson method, in all

the zones of vitiligo lesions, with a greater than three-fold decrease in Melan-A+ melanocyte amount was revealed in perilesional normally pigmented skin of vitiligo patients, as compared to the skin of healthy volunteers. In another ultra-structural analysis by ***Kubanov et al. (2016)*** of depigmented skin, mature melanin granules were found in the basal and suprabasal layers of the epidermis in 10 of 12 patients examined. These melanin granules were synthesized by residual melanocytes. ***Anbar et al. (2012)*** used electron microscopy to detect de-generative changes in perilesional melanocytes of non-segmental vitiligo patients in the form of vacuolization of the melanocytic cytoplasm, pyknosis of nuclei, and peripheral margination of chromatin.

The current work was consistent with the results of studies of ***Wańkowicz-Kalińska et al. (2003)*** which revealed the association of microscopic signs of melanocyte destruction in clinically intact skin of patients with generalized vitiligo with skin infiltration with T-cells.

This may be explained by the results of ***Wood et al. (2009)*** who found that, in vitiligo, H_2O_2 mediated oxidation affected many proteins and peptides, yielding altered or even complete loss of functionality.

CXCR3 is a chemokine receptor that is highly expressed on effector T cells and plays an important role in T cell trafficking and function. CXCR3 is rapidly induced on naive cells following activation and preferentially remains highly expressed on Th1-type CD4+ T cells and effector CD8+ T cells. CXCR3 is activated by three interferon-inducible ligands CXCL9 (MIG), CXCL10 (IP-10) and CXCL11 (I-TAC) ***(Joanna and Andrew, 2011).***

The role of CXCR3 and its ligands in vitiligo might be more than just recruiting T cells. CXCR3 expression on T cells requires activation by triggering a non-persistent T-cell receptor (TCR) in the presence of IFN-c ***(Lacotte et al., 2009).*** Interestingly, all three CXCR3-binding chemokines, are induced by IFN-c. At the same time, the role of IFN-c in vitiligo has also been proposed recently ***(Rashighi et al., 2014).*** Overexpressed IFN-c was detected in both the skin and blood of patients with vitiligo and was able to induce direct melanocyte apoptosis in vitro ***(Yang et al., 2015).***

This study hypothesized a significantly increased number of CXCR3-expressing cells in the dermis of vitiligo lesions' depigmented skin in comparison to healthy controls. These findings suggested that the CXCR3 and its ligands may be involved in recruiting T lymphocytes into these areas.

In agreement with our results, ***Wang et al. (2016)*** found that IFN-c regulate the accumulation of T cells into tissue through the up regulation of CXCR3 and its ligands. The abundant secretions of CXCL9 and CXCL10 promote the recruitment of CXCR3+ effector T cells. Subsequently, these cells produce more IFN-c, which amplifies the infiltration of effector cells, thus forming an ongoing inflammatory response. This IFN-c–chemokine–CXCR3 loop allows for the recruitment of CXCR3+ T cells, which

may induce and sustain the autoimmune process against melanocytes.

CONCLUSION

Melan-A marker was useful for identifying melanocytes in vitiligo patients' skin and estimating their content in different zones of the vitiligo lesions. We have to consider the involvement of clinically intact skin by the pathological process if local treatment methods are prescribed.

REFERENCES

1. ***Anbar TS, El-Sawy AE, Attia SK, Barakat MT, Moftah NH, El-Ammawy TS, Abdel-Rahman AT and El-Tonsy MH. (2012):*** Effect of PUVA therapy on melanocytes and keratinocytes in non-segmental vitiligo: histopathological, immuno-histochemical and ultrastructural study. Photodermatol Photoimmunol Photomed., 28 (1):17-25.

2. ***Busam KJ and Jungbluth AA (2009):*** Melan-A, a new melanocytic differentiation marker. Adv Anat Pathol., 6:12-18.

3. ***El Darouti MA, Marzouk SA, Azzam O, Fawzi MM, Abdel Halim MR, Zayed AA and Leheta TM. (2006):*** Vitiligo vs hypopigmented mycosis fungoides. Eur J Dermatol., 16 (1):17-22.

4. ***Ezzedine K, Lim HW, Suzuki T , Katayama I, Hamzavi I, Lan CC, Goh BK, Anbar T, Silva de Castro C, Lee AY, Parsad D, van Geel N, Le Poole IC, Oiso N, Benzekri L, Spritz R, Gauthier Y, Hann SK, Picardo M and Taieb A (2012):*** Revised classification/ nomenclature of vitiligo and related issues. The Vitiligo Global Issues Consensus Conference. Pigment Cell Melanoma Res., 25(3): 1–13.

5. ***Fernandes BF, Odashiro AN, Saraiva VS, Logan P, Antecka E and Burnier MN Jr (2007)***: Immunohistochemical expression of melan-A and tyrosinase in uveal melanoma. J Carcinogenesis, 6: 1-6.

6. ***Fitzpatrick TB and Breathnach AS (2013):*** The epidermal melanin unit system. Dermatol Wochenschr., 147: 481–489.

7. ***Ghafouriani E. S., Mohebi R., Shokooittni Y. S. and Hamat R.A. (2014):*** Vitiligo: symptoms pathogenesis and treatment. International Journal of Immunopathology and Pharmacology, 27(4): 485-489.

8. ***Joanna R. G. and Andrew D. L. (2011):*** CXCR3 in T cell function. Exp Cell Res., 317(5): 620–631.

9. ***Kubanov A., Proshutinskaia D., Volnukhin V., Katunina O. and Abramova T. (2016):*** Immunohistochemical analysis of melanocyte content in different zones of vitiligo lesions using the Melan-A marker. Acta Dermato-venerologica Alpina, actaapa, 25:5-9.

10. ***Lacotte S, Brun S, Muller S and Dumortier H (2009):*** CXCR3, inflammation, and autoimmune diseases. Ann N Y Acad Sci., 1173:310–317.

11. ***Oiso N, Suzuki T, Fukai K, Katayama I and Kawada A (2011):*** Nonsegmental vitiligo and autoimmune mechanism. Dermatology Research and Practice, 2011: 1-7.

12. ***Prieto VG and Shea CR (2011):*** Immunohistochemistry of melanocytic proliferations. Arch Pathol Lab Med., 135:853-859.

13. ***Rashighi M, Agarwal P, Richmond JM, Harris TH, Dresser K, Su MW, Zhou Y, Deng A, Hunter CA, Luster AD and Harris JE. (2014):*** CXCL10 is critical for the progression and maintenance of depigmentation in a mouse model of vitiligo. Sci Transl Med., 6(223):223.

14. ***Seiji H and Fitzpatrick TB (2013):*** The reciprocal relationship between melanization and tyrosinase activity in melanosomes (melanin granules). J Biochem., 49: 700–706.

15. ***Teulings HE, Willemsen KJ, Glykofridis I, Krebbers G, Komen L, Kroon MW, Kemp EH, Wolkerstorfer A, van der Veen JP, Luiten RM and Tjin EP. (2014):*** The antibody response against MART-1 differs in patients with melanoma-associated leucoderma and vitiligo. Pigment Cell Melanoma Res., 27: 1086-1096.

16. ***Wang X. X., Wang Q. Q., Wu J. Q., Jiang M., Chen L., Zhang C. F. and Xiang L. H. (2016):*** Increased expression of CXCR3 and its ligands in patients with vitiligo and CXCL10 as a potential clinical marker for vitiligo. British Journal of Dermatology, 174:1318–1326.

17. ***Wańkowicz-Kalińska*** A, ***van den Wijngaard RM, Tigges BJ, Westerhof W, Ogg GS and Cerundolo V (2003):*** Immunopolarization of CD4+ and CD8+ T cells to Type-1-like is associated with melanocyte loss in human vitiligo. Lab Invest., 83: 683-695.

18. ***Wood JM, Decker H, Hartmann H, Chavan B, Rokos H, Spencer JD, Hasse S, Thornton MJ, Shalbaf M, Paus R and Schallreuter KU (2009):*** Senile hair graying: H2O2-mediated oxidative stress affects human hair color by blunting methionine sulfoxide repair. FASEB J., 23:2065-2075.

19. ***Yang L, Wei Y, Sun Y, Shi W, Yang J, Zhu L and Li M (2015):*** Interferon-gamma inhibits melanogenesis and induces apoptosis in melanocytes: a pivotal role of CD8+cytotoxic T lymphocytes in vitiligo. Acta Derm Venereol., 95:664–670.

دراسة هيستوكيميائية لصبغة ميلان أ فى بشرة مرضى البهاق وعلاقته بصبغة CXRC3 فى أدمة الجلد

مروة عبد المنعم الخولى

قسم الباثولوجيا العامة- كلية طب بنات - جامعة الأزهر- القاهرة

خلفية البحث :مرض البهاق هو مرض فقدان صبغة الجلد المكتسب ، والسبب إما بسبب فقد أو تكسير الخلايا الصبغية فى جلد هؤلاء المرضى. ويبقى السؤال عن وجود بعض الخلايا الصبغية فى مرضى البهاق والطرق المستعملة فى الكشف عنها.

الهدف من البحث: هو البحث عن وجود خلايا صبغية فى جلد مرضى البهاق فى المناطق المصابة والمناطق المجاورة لها؛ وهل هناك علاقة بين تكسير الخلايا الصبغية وزيادة عدد الخلايا اللمفاوية- ت.

المرضي وطرق البحث: تم اجراء هذا البحث على 30 من مرضى البهاق و10 من المتطوعين.وقد تم إدر اج المرضى في الدر اسة بعد أخذ الموافقة علي إدر اجهم في البحث، وتم صبغ العينات بصبغة **ميلان أ** وصبغة **CXCR3**

النتائج: صبغة **ميلان أ** أعطت نتائج إيجابية فى مناطق الجلد المصابة فى مرضى البهاق مما يعنى أنه لا يزال هناك خلايا صبغية متبقية فى هذه المناطق. ومن ناحية أخرى؛ كانت صبغة **ميلان أ** ضعيفة فى الأماكن المحيطة بمناطق الإصابة بالمقارنة بمثلها فى جلد المتطوعين، وكانت النتائج ذات درجة من الدلالة الإحصائية. كذلك وجدت زيادة فى عدد الخلايا المصبغة بصبغة **CXCR3** فى نسيج أدمة جلد مرضى البهاق بالمقارنة بعدد قليل من نفس الخلايا فى المتطوعين؛ وهذه الخلايا معظمها من الخلايا اللمفاوية- ت.

الاستنتاج : صبغة **ميلان أ** يمكن أن تستعمل ليس فقط لمعرفة وجود خلايا صبغية فى مرضى البهاق؛ ولكن لتحديد عدد هذه الخلايا فى الأماكن المصابة؛ وكذلك الأماكن المحيطة بها. ونظرا لقلة عدد الخلايا الصبغية فى الجلد المحيط بمناطق الإصابة والذى يبدو ظاهريا أنه طبيعى فإنه لا بد أن يؤخذ ذلك فى الاعتبار عند استعمال علاج موضعى، ولا يقتصر العلاج فقط على أماكن الاصابة فقط. مستقبلات صبغة **CXCR3** والجزيئات الملتحمة بها لها دور فى إحداث هذا المرض عن طريق جذب الخلايا اللمفاوية- ت الى مكان الإصابة والتى تقوم بتكسير الخلايا الصبغية. لذلك، فان ايقاف جذب هذه الخلايا اللمفاوية- ت باستعمال أدوية توقف عمل هذه المستقبلات والجزيئات الملتحمة بها؛ وبالتالى توقف تكسير الخلايا الصبغية فى هؤلاء المرضى ويكون إكتشاف علاج جديد وفعال لمرضى البهاق.