

Endothelin-1 and Its A and B Receptors: Are They Possibly Involved in Vitiligo?

Dalia Gamal Aly¹, Samar Abdallah Mohamed Salem², Mahmoud Fawzy Abdel-Hamid¹, Nermeen Salah Youssef³, Marwa Abdel Moneim El Shaer⁴

¹Department of Dermatology and Venereology, National Research Center, Giza;

²Department of Dermatology and Venereology, ³Department of Pathology, Ain Shams University, Cairo; ⁴Department of Pathology, National Research Center, Giza, Egypt

Corresponding author:

Dalia Gamal Aly, MD
9 Moez El-Dawla St., off Makram Ebid St.
Nasr City, Cairo
Egypt
dalia.g.aly@gmail.com

Received: December 1, 2011

Accepted: December 10, 2012

SUMMARY Endothelin-1 (ET-1), expressed by keratinocytes, has paracrine effects on melanocytes. The endothelin 1-axis [ET-1, endothelin A receptor (ETAR) and endothelin B receptor (ETBR)] is thought to play a role in the depigmentation process occurring in vitiligo, with no studies on the cutaneous protein expression of this axis in the disease. The aim of the present study was to compare the expression of ET-1 axis in lesional and perilesional normal epidermis of vitiligo patients with healthy controls. Ten patients with non-segmental stable vitiligo and ten healthy controls were included. Skin biopsies from all subjects were studied immunohistochemically for ET-1, ETAR and ETBR expression. No significant difference was detected in the rate of expression and the degree of staining of ET-1 axis in controls compared with each of lesional vitiligo and perilesional normal epidermis ($P > 0.05$). There was no statistically significant difference between lesional vitiligo and perilesional normal epidermis regarding to the rates of ET-1, ETAR and ETBR expression ($P = 0.82$, $P = 0.5$ and $P = 0.99$, respectively). Semi-quantitative analysis of ETAR revealed higher staining grades in lesional compared with perilesional normal epidermis, with a statistically significant difference ($P = 0.04$). There was no statistically significant difference between the two groups regarding the staining grades of ET-1 and ETBR ($P > 0.05$ for both markers). A highly significant positive correlation was found between ET-1 and ETAR ($r = 0.99$, $P < 0.05$) and between ET-1 and ETBR ($r = 0.87$, $P < 0.05$). The study demonstrated unaltered expression of ET-1 axis in keratinocytes in lesional vitiligo and perilesional normal epidermis. Additional studies on the differential expression of this axis in keratinocytes and melanocytes are therefore required.

KEY WORDS: endothelin A receptor, endothelin B receptor, endothelin-1, melanocytes, vitiligo

INTRODUCTION

Vitiligo is a skin disease that occurs in approximately 0.5% of the world population. Hereditary factors define susceptibility to its development, and associated genes largely implement an autoimmune etiology (1). Multiple hypotheses have been proposed for its development as the cytotoxic metabolites, the neural, the hydrogen peroxide, the growth factor and

the melanocytorrhagy theory. Case reports and animal model also support the hypothesis that viral infections may play a role in the disease (1,2).

Nevertheless, melanocytes are considered central to the disease. They are considered not only as pigment-producing cells but also as important components of the skin immune system, as they have the

capacity to express HLA-DR, CD40 and adhesion molecules such as ICAM-1 and VCAM-1. In addition, they can produce various soluble mediators of inflammation such as IL-1, IL-6 and IL-8. Due to their strategic role in the skin, melanocytes can become targets of primary and secondary immune response as in vitiligo, which is characterized by their selective destruction and their absence in established lesions (2).

In recent years, evidence has suggested that paracrine cytokines produced by keratinocytes and their receptors expressed on melanocytes might play important roles in the maintenance and activation of melanocyte function in the skin, leading to normal or accentuated pigmentation (3,4). Among the known keratinocyte-derived cytokines is endothelin-1 (ET-1), originally known to be a potent vasoconstrictor peptide expressed in vascular endothelial cells and has been implicated in several diseases such as hypertension, atherosclerosis, malignant arrhythmia and chronic heart failure (5). Its expression is also increased in various human malignancies such as ovarian, colorectal and prostate cancer (6,7). Additionally, high levels of ET-1 have been found in the skin and serum of psoriatic patients (8). ET-1 has also been defined as a strong mitogen and melanogen for human melanocytes (4). It was found to influence their homeostasis, proliferation and pigmentation (5,9). To date, ET-1, ET-2, and ET-3 have been identified, where their biological effects are mediated by their binding to specific cell surface receptors known as endothelin A receptor (ETAR) and endothelin B receptor (ETBR) that belong to the family of G-protein coupled receptors. While ETAR is selective for ET-1 and ET-2 with high affinity and ET-3 with low affinity, ETBR binds all ET isopeptides with equal affinity. The endothelins and their receptors are referred to as the ET-axis (10).

During the past years, important findings started to shed light on ET-1 in the pathogenesis of depigmentation occurring in vitiligo (11). Altered levels of this melanogenic mediator have been described in the disease (12). Defective ET-1 function can lead to a failure to prevent apoptotic cell death and restore incidentally injured melanocytes in patients with defective ET-1 gene function (5). Also, point mutations of melanogenic ligand-specific receptors such as ETBR have been proposed as the cause for the loss of pigment seen in vitiligo (13).

To the best of our knowledge, no studies have investigated ET-1 together with its two receptors, A and B, in vitiligo. We therefore investigated immunohistochemically the expression of ET-1 axis in lesional and perilesional skin biopsies of vitiligo patients, aiming to clarify its potential involvement in the disease.

MATERIALS AND METHODS

Patients

This pilot study included 20 subjects, ten patients (8 female and 2 male) with non-segmental stable vitiligo and ten healthy controls (5 male and 5 female) undergoing cosmetic surgery for breast reduction or face lift. The clinical diagnosis was based on the presence of well-demarcated, depigmented patches, confirmed by Wood's lamp examination. We excluded patients receiving topical treatment for the last 2 weeks or systemic treatment for the last 2 months prior to the study, and those with any associated systemic disease. Subjects were recruited from the Dermatology Outpatient Clinic, National Research Center and Ain Shams University Hospitals and the Plastic Surgery Clinic of Ain Shams University Hospitals. All subjects gave an informed consent to participate in the study. The study was approved by the research ethics committee of the National Research Center, Giza, Egypt.

METHODS

Sampling

Five-mm punch biopsies were obtained from lesional vitiligo skin, perilesional normal skin and controls. Assessment of distinct border between the lesional and perilesional normal skin was done by Fontana-Masson staining. In vitiligo patients, skin biopsies were obtained from the back, abdomen and thigh. Biopsies were then fixed in formalin and embedded in paraffin. Four-micron thick sections from the paraffin embedded biopsies were immunohistochemically stained for ET-1, ETAR and ETBR.

Immunohistochemical staining

Immunohistochemical staining was performed using rabbit polyclonal antihuman ET-1 antibody, rabbit polyclonal ETAR antibody and rabbit polyclonal ETBR antibody (1:200 dilution; Abcam, San Francisco, USA, for all markers used). Avidin-biotin immunoperoxidase complex technique was used by applying the super sensitive detection kit (Biogenex, CA, USA). Prepared tissue sections were fixed on poly-L-lysine coated slides that were deparaffinized with graded concentrations of xylene and ethanol, and washed with 0.6% H₂O₂ in methanol for 30 minutes at room temperature to block endogenous peroxidase activity. Tissue was then incubated with 5% normal bovine serum for 10 minutes at room temperature to reduce nonspecific background staining and then with the primary antibody in humidified chambers for 2 hours at room temperature. All treated slides were then



incubated with biotinylated anti-mouse immunoglobulin (secondary antibody) at room temperature, followed by incubation with avidin and biotinylated horseradish peroxidase complex. Peroxidase activity was visualized by a diaminobenzidine substrate kit for 5 minutes. The sections were counterstained with hematoxylin. Appropriate positive and negative controls were included for the slides.

Expression of the markers

Immunostaining of ET-1, ETAR and ETBR was recorded according to the percentage of positive cells. Expression of proteins was graded semi-quantitatively from 0 to 3 in various samples examined using the scoring method by Zeiher *et al.* (14): grade 0 indicated absence of any staining; grade 1, positivity in <10% of the cells; grade 2, positivity in 10% to 30% of the cells; and grade 3, positivity in >30% of the cells. Grading was performed independently by two of the investigators without knowledge of the clinical diagnosis.

STATISTICAL ANALYSIS

Data were analyzed using the Statistical Program for Social Science (SPSS) 15.0.1 for Windows (SPSS

Inc., Chicago, IL, USA, 2001). Data were expressed as number (%) of cases. Comparison of proportions between lesional and perilesional epidermis was made by using the Wilcoxon Signed Ranks test (regarding scores) and Mc Nemar test (regarding binary data – or +). The relationship between ET-1 and ETAR and ETBR was tested using Spearman test. $P < 0.05$ was considered the cut-off value of significance.

RESULTS

The study included ten vitiligo patients, eight (80%) females and two (20%) males. Their age ranged from 22 to 42 years with a mean \pm SD of 31.80 ± 7.21 years. The control group included five (50%) females and five (50%) males. Their age ranged from 29 to 64 years with a mean of 47.9 ± 10.5 years. There was no statistically significant sex difference between patients and controls ($P > 0.05$). In lesional vitiligo epidermis, all 10 (100%) cases showed positive expression of ET-1 and ETAR, while only 8 (80%) cases showed positive expression of ETBR. On the other hand, perilesional normal epidermis showed that ET-1 was also positively expressed in all 10 (100%) cases, whereas ETAR was positively expressed in 8 (80%) and ETBR

Table 1. Comparison between lesional vitiligo, perilesional normal epidermis and controls as regards the rate of endothelin-1, endothelin A receptor and endothelin B receptor expression (McNemar test)

	Lesional vitiligo epidermis		Perilesional normal epidermis		Controls		P- value
	n	%	n	%	n	%	
Endothelin-1							
Negative	0	0.0	0	0.0	7	70.0	Controls vs. lesional vitiligo=0.25 Controls vs. perilesional normal epidermis=0.29 Lesional vs. perilesional normal epidermis=0.82
Positive	10	100.0	10	100.0	3	30.0	
Endothelin A receptor							Controls vs. lesional vitiligo=0.06 Controls vs. perilesional normal epidermis=0.12 Lesional vs. perilesional normal epidermis=0.5
Negative	0	0.0	2	20.0	5	50.0	
Positive	10	100.0	8	80.0	5	50.0	
Endothelin B receptor							Controls vs. lesional vitiligo=0.1 Controls vs. perilesional normal epidermis=0.48 Lesional vs. perilesional normal epidermis >0.99
Negative	2	20.0	3	30.0	2	20.0	
Positive	8	80.0	7	70.0	8	80.0	

Table 2. Comparison between lesional vitiligo, perilesional normal epidermis and controls as regards of endothelin-1, endothelin A receptor and endothelin B receptor staining grades (Wilcoxon Signed Ranks test)

	Lesional vitiligo Žepidermis		Perilesional normal epidermis		Controls		P-value
	n	%	n	%	n	%	
Endothelin 1							
Grade 0	0	0.0	0	0.0	7	70.0	Controls vs. lesional vitiligo=0.1 Controls vs. perilesional normal epidermis=0.1 Lesional vs. perilesional normal epidermis=0.25
Grade 1	1	10.0	0	0.0	0	0.0	
Grade 2	1	10.0	7	70.0	3	30.0	
Grade 3	8	80.0	3	30.0	0	0.0	
Endothelin A receptor							
Grade 0	0	0.0	2	20.0	5	50.0	Controls vs. lesional vitiligo=0.5 Controls vs. perilesional normal epidermis=0.7 Lesional vs. perilesional normal epidermis=0.04
Grade 1	1	10.0	1	10.0	2	20.0	
Grade 2	1	10.0	2	20.0	3	30.0	
Grade 3	8	80.0	5	50.0	0	0.0	
Endothelin B receptor							
Grade 0	2	20.0	3	30.0	2	20.0	Controls vs. lesional vitiligo=0.3 Controls vs. perilesional normal epidermis=0.1 Lesional vs. perilesional normal epidermis=0.52
Grade 1	0	0.0	1	10.0	8	80.0	
Grade 2	2	20.0	1	10.0	0	0.0	
Grade 3	6	60.0	5	50.0	0	0.0	

in 7 (70%) cases. In the control group, both ET-1 and ETAR were expressed at a lower rate [3 (30%) and 5 (50%) subjects, respectively] than in either lesional vitiligo or perilesional normal epidermis, while ETBR was expressed at a rate comparable [8 (80%) subjects] with both lesional vitiligo and perilesional normal epidermis (Table 1).

Comparison between lesional vitiligo, perilesional normal epidermis and controls according to the rate of ET-1, ETAR and ETBR expression

Although ET-1 and ETAR were detected at a lower rate in controls compared with either lesional vitiligo or perilesional normal epidermis, the difference was statistically nonsignificant ($P>0.05$). Additionally, comparison between them and controls according

to the rate of ETBR expression showed comparable results with no statistically significant difference ($P>0.05$).

ETAR was detected at a higher rate in lesional epidermis compared with perilesional normal epidermis, but the difference did not reach statistical significance ($P>0.05$). Furthermore, no statistically significant difference was found between the two groups according to ET-1 and ETBR expression rate ($P>0.05$ and $P>0.99$, respectively) (Table 1).

Comparison between lesional vitiligo, perilesional normal epidermis and controls according to ET-1, ETAR and ETBR staining grades (Table 2)

Overall, comparison of the grades of staining in each lesional vitiligo and perilesional normal



epidermis with controls yielded no statistically significant difference ($P>0.05$). However, higher staining grades (grade 3) were detected only in lesional vitiligo and perilesional normal epidermis, but none of the control group.

Lesional vitiligo epidermis showed significantly higher grades of ETAR staining than perilesional normal epidermis ($P<0.05$) (Fig. 1). There was no significant difference between lesional and perilesional normal epidermis according to staining grades of either ET-1 (Fig. 2) or ETBR (Fig. 3) ($P>0.05$ for both markers).

Correlation between ET-1 and each of ETAR and ETBR in lesional vitiligo and perilesional normal epidermis

In lesional vitiligo epidermis, a highly significant positive correlation was noted between ET-1 and ETAR ($r^2=0.99$, $P<0.05$) and between ET-1 and ETBR ($r=0.87$, $P<0.05$). On the other hand, in perilesional normal epidermis, the correlation between ET-1 and each of ETAR and ETBR was nonsignificant ($r=-0.44$, $P>0.05$ and $r=-0.20$, $P>0.05$, respectively).

DISCUSSION

The complete knowledge of the etiology of vitiligo has been elusive for decades of intense research. Melanocytes are believed to be targeted by multiple aggressions leading to marked reduction of pigment cells and eventually to their complete destruction (11). It has been argued if melanocytes remain but have lost their ability to synthesize melanin in melanosomes (15).

Referring to the paracrine linkage between keratinocytes and melanocytes within the epidermis secreting and responding to cytokines and to published data in the literature (3,13), the importance of the ET-1 axis in vitiligo was proposed. In particular, its release together with the coordinated expression of its respective receptors on melanocytes and the level of functioning keratinocytes may affect the melanocyte dysfunction observed in vitiligo epidermis (13,16). Accordingly, we investigated ET-1 and its receptors (ETAR and ETBR) expression in lesional and perilesional normal skin biopsies from 10 vitiligo patients in comparison to those from 10 healthy subjects.

Interestingly, in the present work, we were able to demonstrate that ET-1 was positively expressed in all specimens of both lesional vitiligo and perilesional normal epidermis. The positive expression of ET-1 in lesional vitiligo epidermis was actually unexpected. Knowing that ET-1 is the only cytokine reported to date that can stimulate both proliferation and melanization of human melanocytes at concentrations as low as 1 nM (13), we expected a deficient

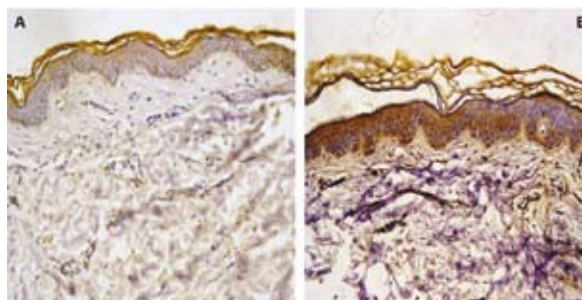


Figure 1. Immunohistochemical staining for endothelin-A receptor: (A) negative expression in perilesional normal epidermis of grade 0 immunostaining (IHC, X200); (B) positive expression in the epidermis of lesional vitiligo of grade 3 immunostaining (IHC, X200).

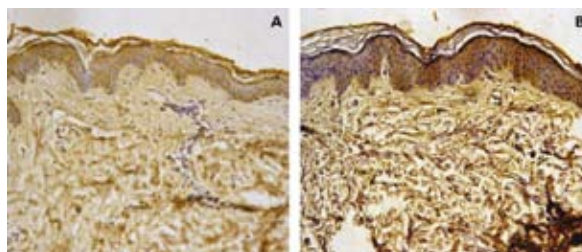


Figure 2. Immunohistochemical staining for endothelin-1: (A) positive expression in perilesional normal epidermis of grade 2 immunostaining (IHC, X200); (B) positive expression in the epidermis of lesional vitiligo of grade 3 immunostaining (IHC, X200).

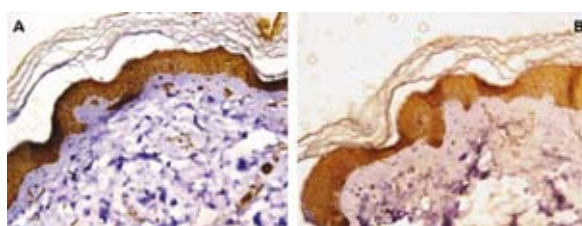


Figure 3. Immunohistochemical staining for endothelin-B receptor: (A) positive expression in perilesional normal epidermis of grade 3 immunostaining (IHC, X200); (B) positive expression in epidermis of lesional vitiligo of grade 3 immunostaining (IHC, X200).

expression of ET-1, which could contribute to the hypopigmentary status of this disease. In contrast, our finding indicated normal production of ET-1 from keratinocytes without any diminished ability in vitiligo epidermis in a fashion similar to that of perilesional normal epidermis and normal controls. Non conclu-

sive and sometimes opposing results to ours at the level of keratinocyte derived ET-1 have been reported in vitiligo epidermis (3,17,18), most likely because of differences in the methods used and in patient selection (12). Further large-scale studies would be beneficial to confirm or refute our findings.

In view of the lack of previous literature reports on the studies investigating both ET-1 receptors in vitiligo, we consequently investigated whether ETAR or ETBR expression was altered in our patients. ETBR was expressed in both lesional vitiligo and perilesional normal epidermis, with a nonsignificant difference between them or between them and controls. However, ETAR was found to be expressed at a higher rate (although statistically nonsignificant) and at a significantly higher intensity in lesional compared with perilesional normal epidermis, and in both at a nonsignificant level compared with controls. This together with a significant positive correlation between ET-1 and ETAR in lesional compared with perilesional normal epidermis suggest an important role of this receptor in vitiligo.

Collectively, the evidence available in the literature suggests an important and pivotal role of cytokine/receptor linkages in up-regulating or down-regulating melanocyte function. ET-1 produced by keratinocytes plays an important role during the induction of pigmentation both as a mitogen and melanogen for normal human melanocytes through acting on the ETB and A receptors present on their cell surface. Action on B receptor results in the activation of various intracellular signal transduction pathways in melanocytes resulting in mobilization of intracellular calcium, and activation of mitogen-activated protein kinase (19,20) while action on A receptor results in the activation of tyrosinase enzyme (19).

The pigmentation or depigmentation in the skin is associated directly with the induction or suppression of tyrosinase in melanocytes (16). In their study conducted in 2004, Akio *et al.* (21) found suppression of tyrosinase activity by endothelin and eventual decrease in the specific activity of tyrosinase despite the increase in tyrosinase protein expression of melanocytes. This suppression of tyrosinase activity was regulated by ETAR antagonist. From this aspect and on the basis of our findings, it would be valuable to assess the quantitative and functional characteristics of tyrosinase in relation to ET-1 axis, as vitiligo could result from a tyrosinase dysfunction rather than defect in its quantitative expression.

ET-1 is an autocrine growth factor for keratinocytes through its action on ET B and A receptors (4), which might result in further ET-1 secretion and hence

human melanocyte proliferation. Consequently, our findings of comparable expression of ET-1 axis in lesional and perilesional normal epidermis as well as in controls indicated that the hypopigmentation process in vitiligo could be the result of either decreased expression of endothelin receptors on melanocytes or a blockage in the intracellular signal transduction pathway or in the ligand receptor interaction. Assessment of the effect of expression of ET-1 axis markers on proliferation and melanogenesis in co-culture system of keratinocytes and melanocytes is thus required to investigate this speculation.

CONCLUSION

This pilot study shows unaltered expression of ET-1 axis in keratinocytes in lesional vitiligo and perilesional normal epidermis. Additional studies on the differential expression of this axis in keratinocytes and melanocytes are therefore required. Better understanding of the role of ET-1 axis in the depigmentation process of vitiligo may help in finding novel therapeutic modalities for this common disease.

References

1. Ellassiuty YE, Klarquist J, Speiser J, Yousef RM, El Refaee AA, Hunter NS, *et al.* Heme oxygenase-1 expression protects melanocytes from stress-induced cell death: implications for vitiligo. *Exp Dermatol* 2011;20:496-501.
2. Yu N, Zhang S, Sun T, Kang K, Guan M, Xiang L. Double-stranded RNA induces melanocyte death *via* activation of toll-like receptor 3. *Exp Dermatol* 2011;20:134-9.
3. Kitamura R, Tsukamoto K, Harada K, Shimizu A, Shimada S, Kobayashi T, *et al.* Mechanisms underlying the dysfunction of melanocytes in vitiligo epidermis: role of SCF/KIT protein interactions and the downstream effector, MITF-M. *J Pathol* 2004;202:463-75.
4. Kadono S, Manaka I, Kawashima M, Kobayashi T, Imokawa G. The role of the epidermal endothelin cascade in the hyperpigmentation mechanism of lentigo senilis. *J Invest Dermatol* 2001;116:571-7.
5. Kim HJ, Choi CP, Uhm YK, Kim YI, Lee JW, Yoon SH, *et al.* The association between endothelin-1 gene polymorphisms and susceptibility to vitiligo in a Korean population. *Exp Dermatol* 2007;16:561-6.
6. Bagnato A, Salani D, Di Castro V, Wu-Wong JR, Tectec R, Nicotra MR, *et al.* Expression of endothelin-1 and endothelin-A receptor in ovarian carcinoma: evidence for an autocrine role in tumor growth. *Cancer Res* 1999;59:720-7.



7. Asham E, Shankar A, Loizidou M, Fredericks S, Miller K, Boulos PB, *et al.* Increased endothelin-1 in colorectal cancer and reduction of tumour growth by ET(A) receptor antagonism. *Br J Cancer* 2001;85:1759-63.
8. Simeone P, Teson M, Latini A, Carducci M, Venuti A. Endothelin-1 could be one of the targets of psoriasis therapy. *Br J Dermatol* 2004;151:1273-5.
9. Hachiya A, Kobayashi A, Yoshida Y, Kitahara T, Takema Y, Imokawa G. Biphasic expression of two paracrine melanogenic cytokines, stem cell factor and endothelin-1, in ultraviolet B-induced human melanogenesis. *Am J Pathol* 2004;165:2099-109.
10. Eltze E, Bertolin M, Korsching E, Wülfing P, Maggino T, Lellé R. Expression and prognostic relevance of endothelin-B receptor in vulvar cancer. *Oncol Rep* 2007;18:305-11.
11. Falabella R. Vitiligo and the melanocyte reservoir. *Indian J Dermatol* 2009;54:313-8.
12. Moretti S, Fabbri P, Baroni G, Berti S, Bani D, Berti E, *et al.* Keratinocyte dysfunction in vitiligo epidermis: cytokine microenvironment and correlation to keratinocyte apoptosis. *Histol Histopathol* 2009;24:849-57.
13. Imokawa G. Autocrine and paracrine regulation of melanocytes in human skin and in pigmentary disorders. *Pigment Cell Res* 2004;17:96-110.
14. Zeiher AM, Goebel H, Schächinger V, Ihling C. Tissue endothelin-1 immunoreactivity in the active coronary atherosclerotic plaque. A clue to the mechanism of increased vasoreactivity of the culprit lesion in unstable angina. *Circulation* 1995;91:941-7.
15. Gauthier Y, Cario Andre M, Taïeb A. A critical appraisal of vitiligo etiologic theories. Is melanocyte loss a melanocytorrhagy? *Pigment Cell Res* 2003;16:322-32.
16. Baharav E, Merimski O, Shoenfeld Y, Zigelman R, Gilbrud B, Yechezkel G, *et al.* Tyrosinase as an autoantigen in patients with vitiligo. *Clin Exp Immunol* 1996;105:84-8.
17. Moretti S, Spallanzani A, Amato L, Hautmann G, Gallerani I, Fabiani M, *et al.* New insights into the pathogenesis of vitiligo: imbalance of epidermal cytokines at sites of lesions. *Pigment Cell Res* 2002;15:87-92.
18. Lee AY, Kim NH, Choi WI, Youm YH. Less keratinocyte-derived factors related to more keratinocyte apoptosis in depigmented than normally pigmented suction-blistered epidermis may cause passive melanocyte death in vitiligo. *J Invest Dermatol* 2005;124:976-83.
19. Kobayashi A, Hachiya A, Ohuchi A, Kitahara T, Takema Y. Inhibitory mechanism of an extract of *Althaea officinalis* L. on endothelin-1 induced melanocyte activation. *Biol Pharm Bull* 2002;25:229-34.
20. Ma HJ, Zhu WY, Wang DG, Yue XZ, Li CR. Endothelin-1 combined with extracellular matrix proteins promotes the adhesion and chemotaxis of amelanotic melanocytes from human hair follicles *in vitro*. *Cell Biol Int* 2006;30:999-1006.
21. Akio M, Hajime I, Hideo O, Norio K. Tyrosinase induction in normal human cultured melanocytes by endothelin-1. *J Cardiovasc Pharmacol* 2004;44 Suppl 1:S439-S442.