行政院國家科學委員會專題研究計畫 成果報告

黑色素生成相關基因及蛋白酶活化接受器 2(PAR-2)在肝斑病灶的表現情形，並評估與不同種類淡斑劑療效的相關性
研究成果報告(精簡版)

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計畫主持人：趙曉秋

計畫參與人員：住院醫師：魏楷哲

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行政院國家科學委員會補助專題研究計畫

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執行單位：國立成功大學醫學院皮膚學科

中華民國 96 年 10 月 30 日
Melasma is an acquired hypermelanosis, occurring symmetrically on sun-exposed areas of the body. Lesions are irregular light to dark brown macules and patches, usually involving the forehead, temples, upper lip, and cheeks. Although melasma can affect all people, Asian and Hispanic females are most commonly affected (Fitzpatrick, 1987; Kang et al. 2002).

Of the many etiologic factors associated with melasma, sunlight exposure appears to be the most significant (Sanchez et al., 1981). In a study by Sanchez et al. (1981), all patients resided in Puerto Rico and the onset of melasma occurred during the summer months in the majority, while patients felt their melasma to be less noticeable during the winter. These patients also stated that sun exposure would exacerbate their melasma. In addition, patients noted that melasma treated with topical depigmenting creams would recur with sun exposure (Sanchez et al., 1981). Other factors include genetic influences, pregnancy, oral contraceptives, certain cosmetics, endocrine factors, thyroid dysfunction, and medications, specifically anticonvulsants and photosensitizing agents (Sanchez et al., 1981; Fitzpatrick, 1987; Grimes PE, 1995; Pandya and Guevara, 2000; Kang et al., 2002). Some of these factors have been observational while others have been clinically studied. For example, in a study by Lufti et al. (1985), thyroid disorders were four times greater in patients with melasma than in matched controls. In a group of 212 patients taking oral contraceptives, 29% of individuals developed melasma (Resnick S, 1967).

In comparison to normal skin, melasma lesional skin was found to contain an increased amount of melanin in all epidermal layers and in the dermis, depending on the histological type of melasma (Table 1). In addition, the number of melanocytes was increased and the dendritic process of the melanocytes contained an increased number of Stage IV melanosomes in contrast to melanocytes of normal skin Stage IV melanosomes are the last stage in the maturation process of melanosomes (Sturm et al., 2003). Only Stage III and Stage IV melanosomes are capable of melanin synthesis (Sturm et al., 2003). The increase in melanosomes is accompanied by the finding of more organelles in melanocytes from melasma lesions. The latter contained more mitochondria, Golgi apparatus, rough endoplasmic reticulum, ribosomes, and dendrites, suggesting an increased production capability of these cells (Kang et al, 2002).

### Table 1. Classification of Melasma

<table>
<thead>
<tr>
<th>Type</th>
<th>Normal light</th>
<th>Wood’s light</th>
<th>Histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidermal</td>
<td>Light brown</td>
<td>Enhancement of color contrast</td>
<td>Melanin deposition in the basal and suprabasal layers of epidermis</td>
</tr>
<tr>
<td>Dermal</td>
<td>Ashen/Bluish gray</td>
<td>No enhancement of color contrast</td>
<td>Melanin-laden macrophages in a perivascular location found in superficial and middermis</td>
</tr>
</tbody>
</table>
An increased number of melanosomes were found in membrane-bound clusters in the keratinocytes of lesional skin as well as being more densely packed when compared to keratinocytes of normal skin. Thus, in addition to an increased synthesis of melanosomes in melanocytes, there also appears to be an increase in the transfer of melanosomes to keratinocytes (Kang et al, 2002).

Immunohistochemistry of lesional and nonlesional skin also revealed important differences. Mel-5 is a monoclonal antibody which is specific for detection of tyrosinase-related protein (TRP-1). TRP-1 is a 75-kDa glycoprotein involved in eumelanin synthesis (Sarangarajan and Boissey, 2001). Although its complete function is not yet known, TRP-1 is thought to help maintain the stability of tyrosinase and the ultrastructure of melanosomes, as well as modulate the enzymatic activity of tyrosinase (Sarangarajan and Boissey, 2001). It also affects both melanocyte proliferation and cell death. Increased intensity of staining was found in lesional skin when Mel-5 was applied to tissue sections (Kang et al, 2002), suggesting an increased expression of TRP-1.

In addition to the increase in TRP-1 in melasma lesional skin, an increased synthesis of tyrosinase also was found (Kang et al, 2002). In contrast to nonlesional skin, electron microscopy of lesional skin showed a large amount of reaction product in the cisternae and vesicles of the trans-Golgi network of melanocytes after the tissue had been incubated in DOPA solution, suggesting increased levels of tyrosinase.

The protease-activated receptor-2 (PAR-2), expressed on keratinocytes but not on melanocytes, is involved in melanosome uptake via phagocytosis, and modulation of PAR-2 activation affects skin color (Sharlow et al., 2000; Seiberg et al., 2000(a, b); Paine et al., 2001). PAR-2 is UV-inducible (Scott et al., 2001), and inhibition of PAR-2 activation prevents UVB-induced tanning (Paine et al., 2001). Keratinocytes treated with SLIGRL or with UVB irradiation exhibited higher levels of secreted proteolytic activity relative to untreated controls (Sharlow et al., 2000; Scott et al., 2001). This proteolytic activity cleaves a peptide comprising the PAR-2 cleavage site in a dose-dependent manner (Scott et al., 2001). Trypsin, known as an essential food-digestive serine protease, is found in numerous tissues including epithelial cells of various tissues, leukocytes, and neurons (Koshikawa et al., 1997&1998). Treatment of pigmented epidermal equivalents with trypsin activates PAR-2 and induces melanogenesis to a similar level as obtained with UV irradiation (Seiberg et al., 2000(b)). Serine protease inhibitors inhibited PAR-2 activation,
resulting in reduced melanosome transfer and distribution. This inhibition led to a dose-dependent skin lightening in vivo (Seiberg et al., 2000; Paine et al., 2001). In this study, immunohistochemistry study is used to evaluate the levels and distribution pattern of the PAR-2 protein and trypsin at melasma lesion.

Hypopigmenting agents consist of phenolic and nonphenolic compounds. Phenolic agents include hydroquinone, which is one of the most effective of the available topical treatments. Hydroquinone is capable of inhibiting tyrosinase and, consequently, the conversion of dopa to melanin. Four percent hydroquinone is thought to be the gold standard for melasma therapy. The inhibition of the PAR-2 pathway by soymilk and the soybean-derived serine protease inhibitors are used as a natural alternative to skin lightening. In this study, we will compare the hypopigmented effect of hydroquinone and soybean-derived serine protease inhibitor and correlate with the messenger RNA levels of melanogenesis associated genes and expression of protease-activated receptor-2 (PAR-2) in melasma.

二、研究方法

1. **Human skin sample**
   Melasma skin samples were obtained from biopsy specimens with the patients’ informed consent following a protocol approved by the Institutional Review Board. Normal skin specimens are obtained from perilesional area. Specimens are fixed in buffered neutral 10% (v/v) formalin, processed by routine histological methods and embedded in paraffin.

2. **Probes**
   Probes that were used in this study were tyrosinase (374-2254, M38297), TYRP1 (63-2065, X51420) (parentheses show the cDNA fragments that were used as probes and the accession numbers of the sequences). Complementary DNA fragments of these genes were amplified from total RNA isolated from normal human melanocytes (tyrosinase, TYRP1) by reverse transcription polymerase chain reaction. Amplified fragments were inserted into PCR-II vector (Invitrogen, CA, USA). Digoxigenin-labeled cRNA probes for in situ hybridization were produced using a digoxigenin RNA labeling kit (Roche Diagnostics, Mannheim, Germany). Produced cRNA probes were shortened to about 130 bp by alkaline treatment.

3. **In situ hybridization**
   In situ hybridization was carried out according to a protocol provided by WAKO (Tokyo, Japan). Cross-sectional samples (thickness of 4 µm) were cut from paraffin-embedded skin samples. The samples were washed three times in xylene for 5 min, and three times with 100% ethanol for 5 min, then washed in 90, 80, 70, and 50% ethanol (5 min each), and left in phosphate-buffered saline for 10 min. They were treated with proteinase K (5 mg per ml in phosphate-buffered saline) for 20-30 min at 37°C, and washed in glycine
solution (2 mg per ml glycine in phosphate-buffered saline, WAKO, Osaka, Japan) for 10 min, then twice in phosphate-buffered saline for 3 min. Next they were each placed in 200 ml of acetylation buffer, and 500 ml of acetic anhydride was slowly added. The mixture was incubated for 15 min with continuous agitation. They were then washed twice in 4 X sodium citrate/chloride buffer for 10 min, and incubated in prewarmed prehybridization solution (2 X sodium citrate/chloride buffer, 50% deionized formamide, at 43°C) for more than 30 min. Excess prehybridization solution was wiped off, hybridization solution containing labeled cRNA probes (1 ng per ml) was placed on the samples, and incubation was conducted overnight at 43°C. The samples were washed in prewarmed prehybridization solution three times for 20 min at 43°C. After the incubation in prewarmed NTE buffer for 5 min at 37°C, the samples were treated with prewarmed RNase A solution (20 mg per ml in NTE buffer) for 30 min at 37°C, then returned to NTE buffer, and incubated for 3 min at 37°C. They were washed in prewarmed 0.1 X sodium citrate/chloride buffer three times for 20 min at 43°C, and then washed once in Buffer 1 (0.1 M Tris-HCl pH 7.5, 0.15 M NaCl) for 1 min. Blocking solution [5% blocking agent in Buffer 1] was placed on the samples, and incubation was continued for about 30 min. The samples were washed with Buffer 1 for 1 min, the samples were treated with anti-digoxigenin-alkaline phosphatase conjugate (diluted to 1/500 in Buffer 1 with 1% blocking agent and 0.075% Brij 35) for 1 h. They were washed with Buffer 1 with 0.075% Brij35 three times for 10 min and incubated in Buffer 1 for 5 min. Then they were washed with Buffer 3 (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 0.05 M MgCl2) for 3 min, substrate solution [1/20 diluted solution of NBT/BCIP stock solution in Buffer 3] was placed on the samples and incubation was conducted overnight at room temperature. They were then washed with TE buffer (10 mM Tris-HCl pH 8.0, 1 mM ethylenediamine tetraacetic acid) to stop the reaction. For less abundant mRNA (P-protein and MITF) the GenPoint System (K0620, DAKO, CA, USA) was used for the detection step according to the manufacturer's instructions. In both methods (WAKO, Osaka, Japan and DAKO, CA, USA), RNA stained violet and could be easily distinguished from brownish melanin.

4. Antibodies and Immunohistochemistry

Paraffin sections (6 µm) were placed on slides coated with poly-L-lysine (Sigma, St Louis, MO, USA). Immunohistochemistry was performed with the ABC Elite Kit from Vector Laboratories (Burlingame, CA, USA), using heat retrieval in antigen unmasking solution (Vector Laboratories). Rabbit polyclonal PAR-2 antibodies were raised against the human PAR-2 peptide fragment (31 RSSKGRSLIGKVD41) by Research Genetics (Huntsville, AL, USA). This sequence enables recognition of both the cleaved and uncleaved receptors and therefore recognizes both the active and inactive forms of PAR-2. Results were confirmed with a
monoclonal PAR-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), which
was raised against the peptide (SLGKVDGTSHVETGK). PAR-2 antibodies were used
at a 1:1000 dilution. Polyclonal rabbit anti-human trypsin antibodies were purchased from
BioTrend (Cologne, Germany). Mouse monoclonal b-actin antibodies were purchased
from Sigma. Secondary antibodies were used according to the manufacturer’s instructions
(Vector Laboratories).

美白產品的使用及效果評估

本研究將收集臉頰有黑斑的志願者，兩側臉頰塗抹不同類型的美白產品（抑制
tyrosinase 活性（hydroquinone）、inhibition of the PAR-2 pathway 活性的產品
(soybean-derived serine protease inhibitors)及三合一美白產品）。
a. 使用前先用 chromometer (Minolta CR 200) 檢測其黑斑指數，並照相記錄。
b. 分別在使用後 4 週、8 週及 12 週測定其黑斑指數及照相紀錄。
c. 計算使用前後黑斑指數的改變，評估其治療效果。
d. 問卷調查受試者的滿意度。

6. 根據 In situ hybridization 及 PAR-2 activity 的評估與臨床使用美白產品的療效作一
correlation，期望能將 melasma 分類並找到最好的治療方式。

三、結果、討論與自評（含結論與建議）

1. 美白產品的使用及效果評估

收集臉頰有黑斑的志願者，分別塗抹不同類型的美白產品，比較使用前後之黑斑指
數，發現三合一美白產品優於 soybean-derived serine protease inhibitors 及
hydroquinone。受試者滿意度的問卷調查有相同的結果。
使用 soybean-derived serine protease inhibitor

使用 hydroquinone
2. **In situ** hybridization 及 PAR-2 activity 的評估

檢體收集困難，幾乎參加臨床試驗的受試者都不願接受切片檢查，雖解釋切片檢
查，對其找出適合治療方式可能有幫忙，但只有三位受試者接受切片，因檢體數目
太少，無法與臨床治療效果作一 correlation。後續仍會努力收集檢體。

3. Hydroquinone 之前一直是治療 melasma 的主要藥物，經由上述研究發現使用
soybean-derived serine protease inhibitor 的效果優於 hydroquinone，但仍低於三合一
美白產品。soybean-derived serine protease inhibitor 可提供敏感性肌膚或害怕類固醇
副作用的黑斑患者另一選擇。

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