

Transcriptional profiles of melanogenesis and genes related to enzymatic antioxidants in skins with periorbital hyperpigmentation

Perfis transcricionais da melanogênese e genes relacionados a antioxidantes enzimáticos em peles com hiperpigmentação periorbital

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ABSTRACT

Introduction: Identifying the causes of periorbital hyperpigmentation is crucial in selecting the best treatment. The identification of transcriptional profiles that may be related to hyperpigmentation around the eye area could contribute to a new approach in the treatment of periorbital hyperpigmentation – the gene therapy.

Objective: This study aims to assess the transcriptional profile of melanogenesis, and genes related to enzymatic antioxidants in skins with periorbital hyperpigmentation.

Methods: Based on clinical evaluation, 49 healthy volunteers were classified with or without periorbital hyperpigmentation. Genetic profiles of melanogenesis-related genes: microphthalmia-associated transcription factor (MITF), pro-opiomelanocortin (POMC), melanocortin 1 receptor (MC1R), tyrosinase (TYR), tyrosinase 1-related protein (TYRP1), and intracellular antioxidants – glutathione reductase (GR), glutathione peroxidase 1 (GPx-1), glutathione s-transferase (GST-1) – were determined by the polymerase chain reaction technique in real-time.

Results: MITF, TYR, and TYRP1 gene expressions were significantly higher in the periorbital hyperpigmentation group ($p < 0.01$). GR, GPx-1, and GST-1 gene expressions were comparable between the groups with and without periorbital hyperpigmentation.

Conclusions: The results of this study suggest that MITF is the primary regulator of melanin deposition in skins with periorbital hyperpigmentation. Up-regulated MITF is closely associated with increased TYR and TYRP1. These findings are essential in proposing a new therapeutic approach in the treatment of periorbital hyperpigmentation.

Keywords: Hyperpigmentation; Melanins; Microphthalmia-associated transcription factor

RESUMO

Introdução: A identificação das causas da hiperpigmentação periorbital é crucial na seleção do melhor tratamento. A identificação de perfis transcricionais que podem estar relacionados com a hiperpigmentação ao redor das áreas oculares poderia contribuir para uma nova abordagem no tratamento da hiperpigmentação periorbital, -a terapia genética-.

Objetivo: Este estudo tem como objetivo avaliar o perfil transcricional da melanogênese e genes relacionados a antioxidantes enzimáticos, em peles com hiperpigmentação periorbital.

Métodos: Com base na avaliação clínica, 49 voluntários saudáveis foram classificados em grupos com ou sem hiperpigmentação periorbital. Perfis genéticos de genes relacionados à melanogênese: -fator de transcrição associado à microftalmia (MITF), pró-opiomelanocortina (POMC), receptor da melanocortina 1, (MC1R), tirosinase (TYR), proteína relacionada à tirosinase 1 (TYRP1)-, e antioxidantes intracelulares: -glutathione reductase (GR), glutathione peroxidase 1 (GPx-1), glutathione s-transferase (GST-1)- foram determinadas pela técnica de reação em cadeia da polimerase em tempo real.

Resultados: as expressões dos genes MITF, TYR e TYRP1 foram significativamente maiores no grupo com hiperpigmentação periorbital. ($p < 0,01$). As expressões gênicas de GR, GPx-1 e GST-1 foram comparáveis entre os grupos com e sem hiperpigmentação periorbital.

Conclusões: Os resultados deste estudo sugerem que o MITF é o principal regulador da deposição de melanina em peles com hiperpigmentação periorbital. O MITF com regulação positiva está intimamente associado ao aumento da TYR e TYRP1. Esses achados são importantes na proposição de uma nova abordagem terapêutica no tratamento da hiperpigmentação periorbital.

Palavras-chave: Hiperpigmentação; Melaninas; Fator de transcrição associado à microftalmia

Original Article

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INTRODUCTION

Periorbital hyperpigmentation (POH), commonly known as dark circles under the eyes, features as bilateral, homogenous hyperchromic macules and patches primarily involving the lower and/or upper eyelids. In some severe cases, it extends to eyebrows, malar regions, temporal regions, and lateral nasal root.¹ POH is one of the most common cosmetic concerns, and it is notoriously resistant to treatment.

The data on the incidence and prevalence of POH is very scarce, and its pathogenesis remains elusive. It is believed that excessive pigmentation, thin and translucent lower eyelid skin, shadowing due to skin laxity and venous congestion are the main causative POH factors. Congenital and genetic factors are also the predisposing factors to POH development.^{2,3} Recently, Verschoree *et al.* assessed the melanin and hemoglobin distribution using a non-invasive method, i.e., spectrophotometric intracutaneous analysis (SIA)scopy, in the Indian population, suggesting that melanin deposits and blood stasis may play a role in POH.⁴ On the other hand, histology studies revealed that periorbital biopsy of Japanese and Brazilian patients showed dermal melanin deposit, which could be the main factor contributing to POH.^{5,6} Nevertheless, these authors did not mention the primary causes that may lead to melanin deposition surrounding eye areas. Other than limited histological studies on POH skin, to the best of our knowledge, the roles of pigmented-related gene expression in POH has not been elucidated yet.

Studies have shown that the microphthalmia-associated transcription factor (MITF) modulation can alter skin pigmentation in dark-skinned Yucatan swine.⁷ On the other hand, MC1R has also been reported to be highly polymorphic, and its genetic variants are associated with various pigmentation phenotypes in the skin, hair, and eyes.⁸ Surprisingly, its gene variants are also associated with an increased risk for hyperpigmentation disorders, i.e., cutaneous melanoma, which is largely independent of skin type and hair color.⁹ Tyrosinase-related protein 1 (TRP-1) and tyrosinase-related protein 2 (TRP-2) present in the membrane of melanosomes are proteins similar to tyrosinase. Their precise role is nuclear, but it has been proposed that TRP-1 acts on the activation and stabilization of tyrosinase, melanosome synthesis, increased eumelanin/pheomelanin ration, and also against oxidative stress.¹⁰

This study assumes that deregulation of pigmentation-related gene expression might involve POH development. Furthermore, it raises the hypothesis that deregulation of intracellular enzymatic antioxidants might be associated with overregulated melanogenic gene expression.

METHODOLOGY

Ethical Approval of Studies

Medical Research & Ethics Committee (NMRR-13-1267-16770) and Sunway Medical Center Independent Research Ethics Committee (011/2013/ER) were obtained prior to the commencement of the study.

Subject recruitment

Healthy subjects requested for blepharoplasty in Lim Plastic and Surgery Clinic (Kuala Lumpur, Malaysia) and Sunway

Medical Centre (Selangor, Malaysia) were randomly invited to participate in this study. Subjects diagnosed with nevus of Ota, nevomelanocytic nevi, café-au-lait, Hori's nevus, ephelides, localized post-inflammatory hyperpigmentation due to a recognizable insult, cutaneous inflammatory diseases/ulceration, allergies/asthma, hyperpigmentation associated with systemic diseases, i.e., Addison's disease, were excluded from the study.

A total of forty-nine (n=49) subjects had been recruited. Written informed consent was obtained from each subject. Plastic surgeons evaluated the periorbital areas and classified them into pigmented (POH) or non-pigmented (Non-POH) eyelid skins. The excised eyelid skins following blepharoplasty were collected and kept in 10% formalin for histological analysis or RNeasy[®] RNA stabilization reagent solution for gene expression study. The specimens were processed within 3 days of upon sample collection.

Histological Analysis

Paraffin-embedded skin tissues were processed for the Fontana-Masson silver stain to observe the melanin deposition and pattern of distribution in eyelid specimens. The depth of melanin distribution, i.e., down to papillary dermis or down to reticular dermis, was reported.

Total RNA extraction and cDNA conversion

A small portion of biopsy specimens was preserved in RNeasy[®] RNA stabilization reagent (Qiagen, Germany). Specimens were homogenized using gentleMACS M tubes (Miltenyi Biotec, Germany). Total mRNA was extracted using RNeasy[®] Lipid Tissue Mini Kit (Qiagen, Germany). Reverse transcription-polymerase chain reaction (RT-PCR) was conducted with High Capacity RNA-to-cDNA Kit. Up to 2µg of total RNA was used in the conversion of RNA-to-cDNA in a 20µl reaction. The reaction consisted of 10 µl of 2× RT Buffer, 1µl of 20× Enzyme Mix, 2µg of RNA, and sufficient quantity of nuclease-free H₂O up to the final volume of 20µl.

Gene Expression Assay

A real-time PCR technique was utilized to determine the level of mRNA expression associated with melanogenesis as well as the antioxidant defense system. The genes which have been quantified were microphthalmia-associated transcription factor (MITF), proopiomelanocortin (POMC), and melanocortin 1 receptor (MC1R), tyrosinase (TYR) and tyrosinase-related protein 1 (TYRP1), glutathione reductase (GR), glutathione peroxidase-1 (GPx-1) and glutathione s-transferase-1 (GST-1). Beta-actin (ACTB) acts as the housekeeping gene. Taqman[®] Gene Expression Assays were purchased from Applied Biosystem with gene code Hs01117294_m1 (MITF), Hs01596743_m1 (POMC), Hs00267167_s1 (MC1R), Hs00165976_m1 (TYR), Hs00167051_m1 (TYRP1), Hs00167371_m1 (GR), Hs00829989_gH (GPx-1), Hs00220393_m1 (GST-1) and Hs99999903_m1 (ACTB).

The real-time PCR was conducted in 20 µl reaction mixture consisting of 2µl of template cDNA, 1µl of Taqman[®] Gene Expression Assay and Taqman[®] Fast Advanced Master Mix. The sample was first denatured at 95 °C for 20 s, followed by 40

cycles of denaturing step at 95 °C for 1 s and annealing step at 60 °C for 20 s. The data was collected at the end of each annealing step. Relative expression of mRNA was determined by the comparative 2- $\Delta\Delta$ CT method.

Statistical analysis

Quantitative data were analyzed using statistical software, SPSS 18.0. Normally distributed continuous data were analyzed using parametric statistical test and expressed as mean and standard deviation. Categorical data were analyzed via chi-square test.

RESULTS

A. Demographic Data and association with POH

Forty-nine (10 men and 39 women) with a mean age of 52.9 ± 9.2 years old met the inclusion criteria and agreed to participate in the study. A total of 47% (n=23) of subjects were evaluated with POH, and 53% (n=26) were categorized into the non-POH group. A total of 67.3% of the subjects were Fitzpatrick skin phototype III, while 22.3% were phototype IV, 8% were phototype I, and 2% were phototype V. Pearson Chi-square showed that Fitzpatrick scale was not associated with the POH group ($\chi^2 = 2.675, p=0.445$). However, the Pearson Chi-square showed that the invagination of melanin deposit into the dermal layer was more prominent in the POH group compared to the non-POH group ($\chi^2 = 8.349, p < 0.05$, Table 1).

B. Gene Expression Study

MITF, TYR, and TYRP1 gene expressions were significantly higher in the POH group ($p < 0.01$, graph 1). Gene expressions of GR, GPx-1, and GST-1 were comparable between POH and non-POH groups (Table 2).

DISCUSSION

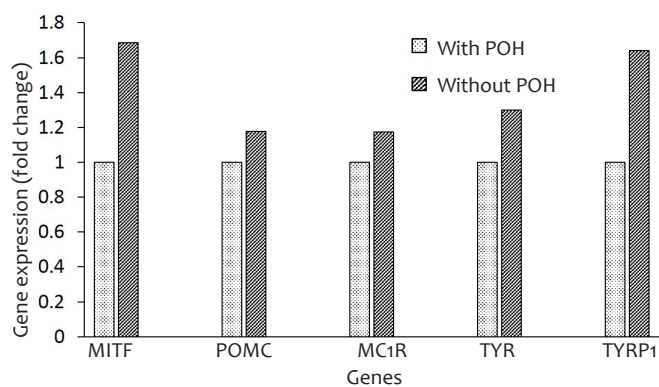
The findings of this study suggest that (i) dermal hyperpigmentation could be the predominant underlying cause of periorbital hyperpigmentation, which may be associated with (ii) the overexpression of MITF, TYR, and TYRP1, which in turn triggers the melanogenesis in periorbital skins.

In agreement with the findings of this study, growing evidence showed that pigmentation surrounding the eyelids is not restricted in the epidermal layer but also deep in the dermal layer, and it is resistant to treatments.^{2, 6, 11-14} Macrophages normally phagocyte melanin that falls into the reticular dermis to form melanophages, causing a bluish appearance. Dermal hyperpigmentation is less responsive to common depigmenting agents. Partially, it occurs because most of the depigmentation therapies focus on epidermal hyperpigmentation, and they are

not effective in eliminating dermal melanophages. The findings of this study suggest that incorporating topical depigmenting agents to transdermal drug delivery may be beneficial in reducing dermal hyperpigmentation. For instance, using a transdermal vehicle like synthetic peptide ACSSSPSKHCG (TD1) and oligoarginine (R8) could be beneficial in treating periorbital hyperpigmentation. However, which therapeutic molecule should be delivered to the dermal layer to achieve the maximal therapeutic effect? Extensive studies focus on the depigmenting roles of TYR inhibitors, but scarce have considered MITF as a drug target for treating hyperpigmentation disorders, especially periorbital hyperpigmentation.

Topical eye creams, mainly tyrosinase-inhibiting agents, i.e., hydroquinone, azelaic acid, tretinoin, and kojic acid, are widely used as depigmenting agents.^{15, 16} Nevertheless, their therapeutic effects are inconsistent and unsatisfying in treating periorbital hyperpigmentation. Laser and intense pulsed light treatments are newer approaches to remove the pigments under the eyes.¹⁷ Still, these therapies are relatively more expensive and require high skilled professions to conduct the treatment procedure. Therefore, innovative strategies are required to identify new drug targets. This study proposes that targeted suppression of MITF rather than its downstream melanogenic genes such as TYR and TRP1 would be a better candidate for a new generation of depigmenting agents.

MITF is a key regulator involves in melanocytic development, and pigmentation. It is a critical regulator of survival, proliferation and differentiation of pigment cells, melanocytes.¹⁰ The three most commonly known signal pathways, which are cAMP-dependent (via MC1R), Wnt, and ERK (via c-Kit receptor) signaling pathways, control the MITF activity. Binding of α -MSH (also known as proopiomelanocortin, POMC) to its plasma membrane receptor, MC1R activates the cAMP-de-



** $p < 0.01$ compared to the group without POH

GRAPH 1: Fold change of gene expression in groups with and without POH

TABLE 1: DEPTH OF MELANIN DISTRIBUTION AND ITS ASSOCIATION WITH POH GROUP

POH Classification/ Depth of melanin distribution	POH (n = 26)	Non- POH (n = 23)	Total (n)	Pearson Chi-square distribution
Papillary dermis	11	19	30	$(\chi^2 = 8.349, p < 0.05)$
Reticular dermis	15	4	19	
Total (n)	26	23	49	

Pearson chi-square test showed that the depth of melanin distribution was associated with the POH group ($p < 0.05$).

TABLE 2: PEARSON CHI-SQUARE TEST SHOWED THAT THE DEPTH OF MELANIN DISTRIBUTION WAS ASSOCIATED WITH THE POH GROUP ($P < 0.05$).

Gene/Group	Gene Expression (Fold-change)	
	Non-POH	HPO
GR	1 ± 0.51	0.90 ± 0.41
GPx-1	1 ± 0.93	0.86 ± 0.68
GST-1	1 ± 0.48	1.06 ± 0.49

pendent pathway, and eventually increases MITF activity, and therefore stimulates melanogenesis.¹⁰ The findings of this study suggest that overexpression of MITF is unlikely via the activation of α -MSH-MC1R signaling pathway. It has been demonstrated that regardless of the high or low level of MC1R, elevated intracellular cAMP triggered by forskolin leads to induction of the TYR, and TYRP1 promoters.¹⁸ Further investigations on the association of MITF with increased intracellular cAMP, Wnt and ERK signaling pathways might provide more insights into the underlying mechanism of MITF overexpression in hyperpigmented eyelids.

This study has shown that MITF is highly expressed in periorbital hyperpigmented skins and closely associated with the degree of melanin production. Therefore, this study proposed that MITF is a potential therapeutic target molecule in treating periorbital hyperpigmentation. Earlier studies showed that the silencing of MITF gene expression via small interfering RNA (siRNA) technique, MITF-siRNA (a negative modulator of MITF), significantly reduced the TYR, TYRP1, and MC1R levels, and, therefore inhibit melanogenesis in melanoma cell culture.¹⁹ Furthermore, in a clinical study, the topical application of MITF-siRNA cream with transdermal vehicles significantly improved facial hyperpigmentation lesions.¹⁹ As the underlying cause of dark circles under the eyes is similar to melasma, which is mainly due to overexpression of MITF and melanin deposition in the dermal layer, this study suggests that MITF-siRNA probably exerts the similar therapeutic effect in skins with periorbital hyperpigmentation.

In addition to MITF-siRNA strategies, compounds, or drug molecules able to interfere with MITF, post-transcriptional regulation can be utilized to modulate the pigmentation process. DKK1 (dickkopf-1) is found predominantly in hypopigmented

palmar and plantar areas and exhibits suppression activities on MITF, therefore inhibiting melanocyte growth and pigment production.²⁰ This study postulates that targeted DKK1 stimulation could be a viable approach to modulate the overexpressed MITF in periorbital hyperpigmented skins. Therefore, there is two crucial conditions in designing a new therapeutic agent in treating periorbital hyperpigmentation, i.e., (i) it should be a small molecule and able to be delivered to transdermal layer, (ii) it should be able to enter the cell and suppress the MITF expression.

Exposure to ultraviolet (UV) irradiation and oxidative stress has been associated with the pathogenesis of periorbital hyperpigmentation.^{1,21,22} UV has been shown to trigger the expression of MITF, tyrosinase, and MSH, which eventually leads to the production of melanin pigments.¹⁰ UV irradiation is also known to induce the production of reactive oxygen species (ROS) in human skin, resulting in oxidative stress and photo-damage to the skin. Antioxidants have been widely used for depigmentation in skin products, including eye creams, to reduce skin damage and melanin deposition.^{2,23} Nevertheless, this study showed that there is no significant difference in gene expressions of the major intracellular enzymatic antioxidants like glutathione reductase (GR), glutathione peroxidase (GPx), and glutathione-s-transferase (GST) in hyperpigmented and non-hyperpigmented eyelid skins. These findings suggest that periorbital hyperpigmentation is unlikely due to inadequate intracellular antioxidants.

In conclusion, the findings of this present study suggest that MITF is the master regulator for melanin deposition in POH skins, upregulated MITF is closely associated with increased TYR and TYR1. These findings are essential in proposing a new therapeutic approach in POH treatment. ●

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