The effects of green tea (*Camellia sinensis*) flower extract on melanin synthesis in B16-F10 melanoma cells

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Abstract: The present study observed the effects of a green tea (*Camellia sinensis*) flower extract (GTFE) on melanin synthesis in B16-F10 melanoma cells. GTFE exhibited antioxidant activity on 2,2-diphenyl-1-picrylhydrazyl and inhibited mushroom tyrosinase activity in a dose-dependent manner. Furthermore, GTFE significantly diminished α-melanocyte stimulating hormone (α-MSH) stimulated cellular melanin content and tyrosinase activity throughout the concentration range evaluated. Based on RNA sequencing analysis, differential gene expression patterns observed in α-MSH stimulated B16-F10 melanoma cells were normalized by the addition of GTFE. In particular, the expression levels of melanoregulin and tyrosinase genes which are key regulating genes in melanin synthesis were up-regulated by 3.5 and 3 fold respectively by α-MSH, and were normalized to control levels by the addition of GTFE. The results suggest that GTFE inhibits melanin synthesis in α-MSH stimulated B16-F10 melanoma cells by normalizing expression of genes that are essential for melanin synthesis. Overall, the results suggest that GTFE could be applied in the development of a whitening agent for the treatment of dermal hyperpigmentation.

Keywords: B16-F10 melanoma cells, RNA sequencing analysis, antioxidant effect, green tea flower, melanin synthesis inhibition, tyrosinase activity inhibition

Introduction

Melanin is a well-known pigment which is commonly distributed and found in animals, plants, fungi and bacteria [17]. Melanocytes produce and pack melanin within melanosomes and transfer it to neighbouring keratinocytes in the epidermis, resulting in visible skin pigmentation which provide photoprotection and thermoregulation [15, 20]. Melanogenesis is initiated with the oxidation of tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) which is further oxidized into dopaquinone which is catalyzed by tyrosinase [5]. Therefore tyrosinase inhibitors can be clinically useful for the treatment of some dermatological disorders associated with melanin hyper pigmentation [2, 23].

Ultraviolet (UV) is a major melanogenesis factor that causes skin aging such as melasma, blackness lentigine, and age spots in the epidermis [14]. Since regulation of melanin synthesis is highly influenced by reactive oxygen species (ROS), UV-induced melanogenesis could be mitigated by ROS scavengers and ROS generator inhibitors [11]. Stimulation of antioxidative levels suppresses melanogenesis and therefore antioxidants are important in protecting human skin from UV radiation [28].

Undesired increase of melanin might be an aesthetic problem and recently there has been a continued interest in protecting healthy skin from persistent environmental pollution [22, 31]. Hyperpigmentation has prompted the requirement of screening of a melanogenesis inhibitory agent. Skin lightening cosmetics made of plant-derived extracts has become a concern as commercially available skin whitening chemicals had adverse effects [3, 27].

The green tea is produced from the leaves of *Camellia sinensis* which contains high levels of flavonols, polyphenols and catechins [6, 21, 37]. Green tea is known to be one of the most active tyrosinase inhibitors with a major active constituents of epicatechin gallate (ECG), gallocatechin gallate (GCG), and epigallocatechin gallate (EGCG) which is a potential natural candidate for the hypopigmentation reagent [25]. Previous studies reported that EGCG in green tea (*Camellia sinensis*) flower extract (GTFE) attenuates microphthalmia-associated transcription factor (MITF) and tyrosinase production in melanocytes meanwhile significantly suppressing melanin synthesis [16, 37].

In the present study, the inhibitory effects of GTFE on rad-
ical production, tyrosinase activity, and melanin synthesis were observed. Particularly, the relationship between the melanogenesis and the differential expression of genes related to melanogenesis and the differential expression of genes related to melano-}

Materials and Methods

Materials
α-Melanocyte stimulating hormone (α-MSH), 2,2-diphe-}

Preparation of GTFE
Dried green tea flowers were purchased from Research}

Cell culture and treatment
B16-F10 cells were grown in monolayers, adherent to the}

DPPH assay
The antioxidant activity of GTFE was determined by mea-

Cell viability assay (MTT assay)
Cell viability assay in the presence of GTFE was analyzed}

Mushroom tyrosinase assay
The inhibitory effect of GTFE on mushroom tyrosinase ac-

Cellular melanin synthesis inhibition assay
Melanin contents were measured as previously described}

Cellular tyrosinase inhibition assay
Cellular tyrosinase activity was measured as described previ-

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Dried green tea flowers were purchased from Research Institute of Plant Resource (Korea) and was ground to a powder. GTFE 100 g powder was soaked in 1 L of 80% ethanol (ethanol/water = 80/20, v/v) and was incubated in 60°C water bath for 1 h to facilitate extraction. The solvent was removed under reduced pressure in a rotary evaporator (HS-2005V, HanShin, Korea). The extract was filtered using Whatman No.1 filter paper, and the filtrate was diluted to final concentration of 50 mg/mL (w/v).

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Cell culture and treatment
B16-F10 cells were grown in monolayers, adherent to the bottom of culture flasks filled with DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in a 5% CO₂ atmosphere. Cells were harvested in the exponential growth phase at 80% confluence. Cells were washed twice with PBS, exposed to trypsin EDTA solution for 30 sec at 37°C, centrifuged at 800 × g for 5 min, and cells (5 × 10⁵ cells/well) were seeded in 96-well plates.

DPPH assay
The antioxidant activity of GTFE was determined by measuring the DPPH free radical scavenging activity as described previously [14]. GTFE was dissolved in DMSO to make a concentration of 50 mg/mL (w/v). Fifty microliters of each sample was mixed with 20 µL of mushroom tyrosinase (1250 U/mL) in medium was removed and cells were treated with 0, 25, 50, 100, 200, 400 and 1,000 µg/mL of GTFE for 24 h. Then 20 µL of EZ-Cytox solution was treated for 2 h and absorbance values were measured at a 540 nm using ELISA microplate reader (Tecan, Switzerland).

Mushroom tyrosinase assay
The inhibitory effect of GTFE on mushroom tyrosinase ac-

Cellular melanin synthesis inhibition assay
Melanin contents were measured as previously described [33]. B16-F10 cells were seeded with 1 × 10⁵ cells/well in 3 mL of medium in 6 well plates, and incubated overnight to allow cells to adhere. The cells were incubated with various concentrations (0, 5, 10, 20, and 40 µg/mL) of GTFE in the presence or absence of 20 nM α-MSH for 2 days. Microscopic images of α-MSH-stimulated B16-F10 melanoma cells incubated with various concentration of GTFE were captured using an inverted biological microscope connected HD LCD Tablet camera (Neosence, Korea). Then cells were washed twice with PBS and were harvested through trypsinization with 1 mL of 1× EDTA and 1 mL of media. Cells were harvested by centrifugation (800 × g for 5 min) and pellets were dried at 60°C oven. Cells were lysed with 500 µL of 1 M NaOH containing 10% DMSO for 30 min at 80°C in a heating block and was briefly centrifuged (800 × g for 1 min). Supernatant (300 µL) was added into wells of 96-well plates, and the absorbance at 490 nm was measured using an ELISA plate reader.

Cellular tyrosinase inhibition assay
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DPPH assay
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Cell viability assay (MTT assay)
Cell viability assay in the presence of GTFE was analyzed using B16-F10 melanoma cells as described previously [30]. B16-F10 melanoma cells (1 × 10⁵ cells/well) were cultured in 96-well plates. Following 24 h of cell incubation, DMEM medium was removed and cells were treated with 0, 25, 50, 100, 200, 400 and 1,000 µg/mL of GTFE for 24 h. Then 20 µL of EZ-Cytox solution was treated for 2 h and absorbance values were measured at a 540 nm using ELISA microplate reader (Tecan, Switzerland).

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MSH. Cells were washed twice with PBS and were lysed in 500 µL of PBS containing 1% Triton X-100 and 2 mM PMSF, and stored at −80°C for 30 min to facilitate cell rupturing. After thawing at room temperature, cell extracts were separated by centrifugation at 15,000 × g for 30 min at 4°C. The supernatant (150 µL) was mixed with 2 mM L-DOPA 150 µL in a 96-well plate, incubated 37°C for 10 min, and the absorbance at 490 nm was measured using an ELISA plate reader.

Total RNA isolation, library preparation, sequencing and data analysis

B16-F10 cells were cultured in 6 well plates (1 × 10^5 cells/well) and incubated at 37°C, for 24 h. Cells were washed with 1× PBS, and treated with α-MSH and different concentrations of GTFE. Total RNA was separated from melanoma cells after 48 h cultivation and cells were dissolved in 500 µL of Trizol reagent (Invitrogen, USA) according to manufacturer’s instructions, and stored at −80°C until use. RNA quality was assessed by Agilent 2100 Bioanalyzer using the RNA 6000 Nano Chip (Agilent Technologies, The Netherlands), and RNA quantification was carried out using ND-2000 Spectrophotometer (Thermo Fisher Scientific, USA). SENSE 3’ mRNA-Seq Library Prep Kit (Lexogen, Austria) was used to perform the construction of a library for control and test RNAs according to the manufacturer’s instructions. Total RNA 500 ng was prepared and an oligo-dT primer containing an Illumina-compatible sequence at its 5’ end was hybridized to the RNA and reverse transcription was performed. Following RNA degradation complimentary strand synthesis was started by random primer containing an Illumina-compatible linker sequence at its 5’ end. Magnetic beads were used to remove all reaction components. The library was amplified to add the complete adapter sequences required for cluster generation. The completed library was purified from PCR components. High-throughput sequencing was performed as single-end 75 sequencing using NextSeq 500 (Illumina, USA). SENSE 3’ mRNA-Seq reads were aligned using Bowtie2 ver. 2.1.0 [18]. Bowtie2 indices were either generated from genome assembly sequence or the representative transcript sequences for aligning to the genome and transcriptome. The alignment file was used for assembling transcripts, estimating their abundances and detecting differential expression of genes. Differentially expressed genes were determined based on counts from unique and multiple alignments using EdgeR within R ver. 3.2.2 (R development Core Team, 2011) using Bioconductor ver. 3.0 [12]. The read count data were processed based on Quantile normalization method using the Genowiz ver. 4.0.5.6 (Ocimum Biosolutions, India). Cytoscape (ver. 2.7), an open source bioinformatics platform developed by the Institute of Systems Biology, USA, was used to construct network diagrams and to illustrate clustering of the genes in our dataset within specific pathways. Gene classification was based on searches done by MEDLINE databases (National Centre for Biotechnology Information, USA).

Data analysis

All experiments were done in triplicates or more, and the results were expressed as mean ± standard error for each sample concentration. Data were statistically analyzed with SPSS Statistics (Ver. 17.0; IBM, USA). The statistical differences among groups were analyzed with one way analysis (ANOVA) followed by Turkey’s test.

Results

Radical scavenging activity

DPPH assay was performed to evaluate the antioxidant activity of GTFE. GTFE displayed DPPH free radical scavenging activity in a dose dependent manner. DPPH radical scavenging activities of 25, 50, 100, 200 and 400 µg/mL of GTFE were estimated to be 5.8%, 11.7%, 20.6%, 34.8% and 40.5% respectively (Fig. 1A). The EC_{50} of GTFE was estimated to be 0.266 mg/mL, which is corresponding to 2328.58 mg of AA/g based on the ascorbic AEAC calculation as described in Materials and Methods.
Cellular toxicity of GTFE on B16-F10 melanoma cells was measured using MTT assay. GTFE showed no cellular toxicity on B16-F10 melanoma cell at the concentration up to 200 µg/mL (Fig. 1B). However, cellular toxicity was increased significantly (25.6%) from the GTFE concentration of 400 µg/mL.

Inhibition of mushroom tyrosinase activity
Inhibitory effect of GTFE on mushroom tyrosinase activity was observed as described in Materials and Methods. As shown in Figure 2A, GTFE inhibited mushroom tyrosinase activity in a dose-dependent manner. Mushroom tyrosinase inhibition of 25, 50, 100, 200, 400 and 1,000 µg/mL of GTFE was estimated to be 5.3%, 6.6%, 19.6%, 31.1%, 44.4% and 58.4% respectively (Fig. 2A).

Inhibition of cellular tyrosinase activity
Inhibitory effect of GTFE on cellular tyrosinase activity was evaluated in B16-F10 melanoma cells after stimulating melanin synthesis in cells using α-MSH. Cellular tyrosinase activity was significantly increased in the presence of α-MSH, whereas the activities were decreased in a concentration dependent manner of GTFE. GTFE 5, 10, 20 and 40 µg/mL inhibited cellular tyrosinase activity by 23.2%, 44.4%, 49.3% and 89.0% respectively (Fig. 2B).

Inhibition of melanin synthesis
Inhibitory effect of GTFE on melanin synthesis was observed in B16-F10 melanoma cells after stimulating melanin synthesis in cells using α-MSH. Melanin synthesis was significantly increased in the presence of α-MSH, whereas declining trend of melanin was observed throughout the concentration range evaluated (Fig. 3A and B). GTFE 5, 10, 20 and 40 µg/mL suppressed melanin synthesis by 15.5%, 18.4%, 22.9% and 41.3% respectively (Fig. 3B).

Differential gene expression
The differential gene expression of B16-F10 cells treated with α-MSH and GTFE were observed using RNA sequencing analysis. Based on RNA sequencing results, among 23,282 of total genes in B16-F10 cells, 2,420 genes were up-regulated (higher than 2.0) and 3,236 genes were down-regu-
The effects of GTFE on melanin synthesis in B16-F10 melanoma cells

Table 1. Differential expression of functional genes in B16-F10 melanoma cell treated with α-MSH only

| Gene number | Total | A | Ang | AP | CC | CD | CDi | CM | CP | DR | EM | IR | InR | N | RS | S |
|-------------|-------|---|-----|----|----|----|-----|----|----|----|----|----|----|----|---|---|---|
| % of total  | 100.0 | 1.1 | 1.3 | 3.3 | 4.5 | 3.6 | 14.1 | 3.0 | 2.4 | 1.6 | 1.7 | 3.8 | 1.8 | 6.4 | 1.2 | 2.0 |
| Up significant | 2420.0 | 22.0 | 31.0 | 89.0 | 87.0 | 96.0 | 260.0 | 67.0 | 51.0 | 40.0 | 26.0 | 71.0 | 28.0 | 120.0 | 20.0 | 46.0 |
| % of up significant | 10.4 | 8.3 | 10.2 | 11.5 | 8.3 | 11.4 | 7.9 | 9.6 | 9.3 | 10.6 | 6.4 | 8.1 | 6.7 | 8.1 | 6.9 | 9.8 |
| Down significant | 3236.0 | 38.0 | 41.0 | 115.0 | 225.0 | 122.0 | 436.0 | 93.0 | 77.0 | 92.0 | 51.0 | 95.0 | 52.0 | 210.0 | 48.0 | 57.0 |
| % of down significant | 13.9 | 14.3 | 13.5 | 14.8 | 21.6 | 14.5 | 13.3 | 13.4 | 14.0 | 24.4 | 12.6 | 10.8 | 12.5 | 14.1 | 16.7 | 12.1 |

Table 2. Differential expression of functional genes in B16-F10 melanoma cell treated with α-MSH and GTFE (20 µg/mL)

| Gene number | Total | A | Ang | AP | CC | CD | CDi | CM | CP | DR | EM | IR | InR | N | RS | S |
|-------------|-------|---|-----|----|----|----|-----|----|----|----|----|----|----|----|---|---|---|
| % of total  | 100.0 | 1.1 | 1.3 | 3.3 | 4.5 | 3.6 | 14.1 | 3.0 | 2.4 | 1.6 | 1.7 | 3.8 | 1.8 | 6.4 | 1.2 | 2.0 |
| Up significant | 2876.0 | 21.0 | 38.0 | 77.0 | 89.0 | 87.0 | 264.0 | 74.0 | 50.0 | 32.0 | 52.0 | 80.0 | 26.0 | 135.0 | 28.0 | 50.0 |
| % of up significant | 12.4 | 7.9 | 12.5 | 9.9 | 8.5 | 10.3 | 8.0 | 10.6 | 9.1 | 8.5 | 12.8 | 9.1 | 6.2 | 9.1 | 9.7 | 10.6 |
| Down significant | 2697.0 | 28.0 | 28.0 | 97.0 | 177.0 | 103.0 | 364.0 | 93.0 | 64.0 | 71.0 | 37.0 | 85.0 | 37.0 | 178.0 | 28.0 | 56.0 |
| % of down significant | 11.6 | 10.3 | 9.2 | 12.5 | 17.0 | 12.2 | 11.1 | 13.4 | 11.7 | 18.8 | 9.1 | 9.6 | 8.9 | 12.0 | 9.7 | 11.9 |

Table 3. Differential expression of functional genes in B16-F10 melanoma cell treated with α-MSH and GTFE (40 µg/mL)

| Gene number | Total | A | Ang | AP | CC | CD | CDi | CM | CP | DR | EM | IR | InR | N | RS | S |
|-------------|-------|---|-----|----|----|----|-----|----|----|----|----|----|----|----|---|---|---|
| % of total  | 100.0 | 1.1 | 1.3 | 3.3 | 4.5 | 3.6 | 14.1 | 3.0 | 2.4 | 1.6 | 1.7 | 3.8 | 1.8 | 6.4 | 1.2 | 2.0 |
| Up significant | 2454.0 | 20.0 | 26.0 | 76.0 | 73.0 | 82.0 | 265.0 | 70.0 | 47.0 | 36.0 | 72.0 | 72.0 | 22.0 | 129.0 | 20.0 | 48.0 |
| % of up significant | 10.5 | 7.5 | 8.6 | 9.8 | 7.0 | 9.7 | 8.1 | 10.1 | 8.6 | 9.5 | 8.2 | 8.2 | 5.3 | 8.7 | 6.9 | 10.2 |
| Down significant | 2624.0 | 31.0 | 40.0 | 93.0 | 227.0 | 103.0 | 359.0 | 91.0 | 73.0 | 85.0 | 86.0 | 86.0 | 51.0 | 174.0 | 39.0 | 41.0 |
| % of down significant | 11.3 | 11.7 | 13.2 | 12.0 | 21.8 | 12.2 | 10.9 | 13.1 | 13.3 | 22.5 | 9.8 | 9.8 | 12.2 | 11.7 | 13.5 | 8.7 |

A, aging; Ang, angiogenesis; AP, apoptosis process; CC, cell cycle; CD, cell death; CDi, cell difference; CM, cell migration; CP, cell proliferation; DR, DNA repair; EM, extracellular matrix; IR, immune response; InR, inflammation response; N, neurogenesis; RS, RNA splicing; S, secretion.

Analysis of RNA sequencing data showed that 8 genes related to melanogenesis including Atp6v1g2, Try, Slc1a4, Mreg, Cited1, Hspa5, Slc3a2 and Pdia4 genes were up-regulated by 44.9, 3.5, 3.0, 3.2, 2.6, 2.2 and 2.0 folds compared to the control group by addition of α-MSH. However the levels were normalized to control level by the addition of GTFE (Fig. 4A). In particular, the expression levels of Mreg and Tyr genes which are key regulating genes in melanin synthesis were up-regulated by 3.5 and 3 fold compared to control by α-MSH and were normalized to control level by the addition of GTFE (Fig. 4A).

Seven genes related to melanin synthesis including Stom, Rab2a and Rab29 genes were down-regulated by 0.5, 0.4 and 0.4 fold compared to control group by α-MSH and were normalized to control level by the addition of GTFE (Fig. 4B).
Green tea is one of the most popular beverages in the world, and considered to have many scientifically proven beneficial effects on human health [6]. It has been reported that *Camellia sinensis* flowers have similar chemical compositions with *Camellia sinensis* leaves and GTFE contains a high amount of flavonoids, polyphenols, catechins, ECG and EGCG which directly scavenge free radicals and ROS [37]. However, despite many published results, the ameliorative effect of GTFE on melanogenesis has not been reported. In the present study, we investigated the effects of GTFE on melanin synthesis, and on the expression levels of genes related to melanin synthesis in B16-F10 melanoma cells.

Whitening efficacy of *Camellia sinensis* flower extract in B16-F10 melanoma cells was verified in the current study by radical scavenging assay, tyrosinase inhibition assay, and melanogenesis inhibition assay followed by the differential gene expression patterns. Significant radical scavenging ability, mushroom tyrosinase inhibition activity, α-MSH stimulated B16-F10 melanoma cellular melanin content, and cellular tyrosinase activity inhibition were expressed by GTFE concentration dependently.

The present study observed that GTFE scavenged DPPH radicals in a dose dependent manner. As observed previously, the skin exposure to UV radiation generates both melanin and harmful amount of ROS [4]. As ROS are essential in melanocyte proliferation and melanogenesis, ROS scavengers will reduce hyperpigmentation and UV-induced melanogenesis [36]. Overall results suggest that the radical scavenging effect of GTFE might contribute to the inhibition of melanogenesis in cells.

Previous studies revealed that ECG, GCG, and EGCG, the major components of green tea, are the most effective tyrosinase inhibitors [17, 25]. In the present study GTFE showed inhibitory effect on both mushroom tyrosinase activity and cellular tyrosinase activity. GTFE inhibited mushroom tyrosinase activity in a cell-free system revealing the direct inhibitory effects of GTFE on tyrosinase activity. The results suggest that the components of GTFE directly inhibit cellular tyrosinase activity, and mainly contribute to the reduction of pigments in melanoma cells.

In α-MSH stimulated B16-F10 melanoma cells, the expression levels of Mreg and Tyr genes which are key regulating genes in melanin synthesis were up-regulated by 3.5 and 3 fold respectively by α-MSH, and were normalized to control level by addition of GTFE, which normalized the melanin synthesis and its transport.

Tyr gene is a key regulating gene in the initial steps of melanin synthesis by catalyzing the oxidation of tyrosine to DOPA quinone [32]. Mreg gene is known to be essential for the lysosome maturation, intracellular trafficking and vesicular transporting of pigments in epithelial cells [10]. In addition, the gene expression levels of other genes related to melanogenesis including Slc1a4, Hspa5 and Cited1 were upregulated by α-MSH and were restored to control level by GTFE.

Slc1a4 is a target gene of MITF which is involved in amino acid and lipid metabolism in melanocytes [13]. Hspa5 is known to protect tyrosinase as a molecular chaperone [32]. Cited1 plays a vital role in melanogenesis by regulating tyrosinase, dopachrome tautomerase and more enzymes in pigmented system [24]. In the present study, GTFE was found to restore α-MSH induced up-regulated gene expression levels of the genes related to melanogenesis including Atp6v1g2, Tyr, Slc1a4, Mreg, Cited1, Hspa5, Slc3a2 and Pdia4 in B16-F10 melanoma cells to the control level upholding the antimelanogenesis effect of GTFE.

Microarray studies further showed that the expression levels of 3 genes including Stom, Rab2a and Rab29 which are related to melanogenesis were down-regulated by α-MSH and were normalized by GTFE. A lipid raft-associated protein
Stom gene which involves in melanosome degradation [7, 8, 19] and golgi complex regulating genes Rab29 and Rab2a which contribute to the proteolysis of tyrosinase were downregulated by α-MSH and were restored by GTFE hindering skin pigmentation [1, 2, 34, 35]. Therefore, the results indicate that components of GTFE normalized the expression of genes related to melanogenesis which were disturbed by α-MSH.

In conclusion, this study explored that GTFE inhibits the cellular tyrosinase activity and melanin synthesis in α-MSH stimulated B16-F10 cells by regulating genes related to melanogenesis. The overall results suggest that GTFE can be applied for the development of whitening agent for the treatment of dermal hyperpigmentation.

Acknowledgments

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