Chapter

A Novel P53/POMC/\(\alpha\)-MSH/SASH1 Autoregulatory Feedback Loop and Pathologic Hyperpigmentation

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Abstract

P53-regulated proteins in transcriptional level are associated with many signal transduction pathways and p53 plays a pivotal role in a number of positive and negative autoregulatory feedback loops. Although POMC/\(\alpha\)-MSH productions induced by ultraviolet (UV) are directly mediated by p53, p53 is related to UV-independent pathological pigmentation. In the process of identifying the causative gene of dyschromatosis universalis hereditaria (DUH), three mutations encoding amino acid substitutions were found in the gene SAM and SH3 domain containing 1 (SASH1). SASH1 was identified to interact with guanine nucleotide-binding protein subunit-alpha isoforms short (G\(\alpha\)s). However, for about 90 years, the pathological gene and the pathological mechanism of DUH are unclear. Our study indicates that SASH1 is physiologically medicated by p53 upon UV stimulation and a reciprocal SASH-p53 inducement is existed physiologically and pathophysiologically. A novel p53/POMC/\(\alpha\)-MSH/G\(\alpha\)s/SASH1 signal cascade regulates SASH1 to foster melanogenesis. SASH1 mutations control a novel p53/POMC/G\(\alpha\)s/SASH1 autoregulatory positive feedback loop to promote pathological hyperpigmentation phenotype in DUH-affected individuals. Our work illustrates a novel p53/POMC/G\(\alpha\)s/SASH1 autoregulatory positive feedback loop that is mediated by SASH1 mutations to foster pathological hyperpigmentation phenotype.

Keywords: SASH1 substitution mutations, p53, autoregulatory feedback loop, DUH, pathological hyperpigmentation phenotype

1. Introduction

The skin pigmentation is formed by the synthesis of melanin in the melanocytes. Melanocyte is a kind of epithelial cells mainly locating basal cell layers of epidermis, and a few number of melanocytes are located in mucosa. Pigment granules constituted with melanin can distribute and transport to neighboring keratinocytes [1]. Mutations in melanocortin-1-receptor (MC1R) are pivotal for human skin’s tanning and pigmentation. MC1R belongs to a G-protein-coupled receptor (GPCR) that is expressed in epidermal melanocytes in a preferential
α-Melanocyte-stimulating hormone (α-MSH), the GPCR's ligand, is a propigmentation hormone which is generated and secreted by both keratinocytes and melanocytes in the skin. After UV irradiation, α-MSH can activate GPCR. Pro-opiomelanocortin (POMC) is a multicomponent precursor for α-MSH (melanotropic), ACTH (adrenocorticotropic), and the opioid peptide β-endorphin, and α-MSH and other bioactive peptides are the cleavage products of POMC [2]. Normal synthesis of α-MSH and ACTH is extremely important to constitutive human pigmentation and the cutaneous response to UV [2].

In melanocytes, the amount and type of pigment production are regulated by MC1R. So MC1R is an important determiner of skin phototype, sensitivity to UV radiation-induced damage, and skin cancer risk [3]. The heterotrimeric G proteins consist of α, β, and γ subunits. Upon ligand binding, a signal is transmitted by GPCRs to heterotrimeric G proteins, which results in the separation of the α subunit from the Gβγ subunit of G proteins. ATP is catalyzed to be directly transformed to cAMP by the G proteins of the Gαs class and cAMP is in charge of melanogenesis including the sensitization of tyrosinase in melanin biosynthesis upon being activated by ligands such as α-MSH [4].

p53 is not only a transcriptional factor but also a tumor-suppressor protein, which is documented to directly sensitize the transcription of a lot of genes including those that control cell cycle, apoptosis, and others. POMC/MSH inducement by UV irradiation in skin is directly regulated by p53 and POMC promoter is stimulated in response to UV irradiation. p53 involves in UV-independent pathologic pigmentation and could imitate the tanning response [1]. Dipyrimidine C to T substitutions including CC to TT frameshift mutations in the p53 gene can be uniquely induced by UV in the skin of UV-irradiated mice months before tumor development [5]. In addition, p53 has been demonstrated to be necessary to the presentation of “sunburn cells,” which are a sign of sunburns [5].

DUH characterized by extensively mottled pigmentation is a heterogeneous disorder, which was first diagnosed by two Japanese researchers in two generations of two pedigrees for about 80 years [6, 7]. Similar Chinese DUH pedigrees with dyschromatosis symmetrica hereditaria (DSH) with autosomal dominant DUH had been reported by us in 2003 [8] and diagnosed as DUH rather than DSH afterward. Although novel mutations of SASH1 have been identified to be associated with dyschromatosis universalis hereditaria [9], less pathogenesis of DUH has not been investigated. The pathogenesis of DUH remain unclear and indefinite for 80 years [7].

SASH1, a previously described novel family of putative adapter and scaffold proteins transmitting signals from the ligand to the receptor, was first showed to be a candidate tumor-suppressor gene in breast cancer and colon carcinoma [10–12]. Our previous study demonstrates that SASH1 interacts with Gαs, the downstream player of α-MSH/MC1R signaling pathway [13]. Our previous report indicated that in the several affected DUH individuals, hyperpigmented macules became more obvious after strong UV irradiation particularly in summer [8], but no further investigations was performed to identify the reasons of photosensitivity [14].

The significance of expression of p53/POMC/α-MSH in UV-photopigmentation response and UV-independent hyperpigmentation has been explained [1]. However, few investigations were performed to reveal that the mutations in SASH1 gene are related to hyperpigmentation and how these mutations result in hyperpigmentation phenotype.

In a word, we assume that a novel p53/POMC/α-MSH/Gαs that SASH1 involves in regulating UV-photopigmentation response and pathological hyperpigmentation phenotype.
2. Materials and methods

2.1 DUH pathologic gene sequencing and SASH1 mutation analysis

Two Chinese pedigrees recruited from the Henan and Yunnan provinces of China and one American pedigree with typical features of DUH were involved in this study. Three DUH pedigrees with an autosomal dominant inheritance pattern were diagnosed by skilled clinical dermatologists. The small American pedigree only offered peripheral blood samples of the affected individuals for investigations. This study was recognized by the ethical review committees from the appropriate institutions. Genotyping was implemented, and the two-point LOD score was counted as our previous description [8]. In total, 50 family members and 500 normal individuals (controls) involved in the research were provided with informed consent and specimens of peripheral blood DNA were acquired from all obtainable family members. PCR and sequencing were executed as our previous description [8]. ABI BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, CA) was used to perform the sequencing on an ABI PRISM 3130 DNA Analyzer (Applied Biosystems) and sequence analysis software, version 3.4.1 (Applied Biosystems) were used to analyze the data. Sequencher 4.10.1 (Gene Codes Corp.) was used to compare the sequence data with SASH1 reference sequence (GenBank NM_015278.30). Nucleotide numbering reflects complementary DNA (cDNA) numbering, with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence [8].

2.2 Constructing expression vectors of SASH1, \( \Gamma^\alpha \), POMC, and p53

Wild-type and mutant SASH1-PEGFP-C3 and wild-type and mutant SASH1-PBABE-Flag-puro were constructed according to the protocol of our previous study [13]. To generate the p53-HA-Pcna3.0, POMC-myc-PcDNA3.0 and \( \Gamma^\alpha \)-Pegfp-C3 vectors, high fidelity DNA polymerase (Phusion Hot Start High Fidelity Polymerase from New England Biolabs, Inc. or GXL Polymerase from Takara) and the primers

| Name of primers or probes | Sequences (5′-3′) |
|---------------------------|-------------------|
| \( \Gamma^\alpha \) forward primer (SalI site included) | ACGCGTGCAGATGGGCCCTGCCCTGGGAAC |
| \( \Gamma^\alpha \) reverse primer (XhoI site included) | CCGCTCGAGTTAGAGCAGCTCTGTAAGAGC |
| p53 forward primer (BamHI site included) | CGCGGATCCGCCACCACCATGGAGGAGCCGCAGTCAGATCCCT |
| p53 reverse primer (XhoI site included) | CCGCTCGAGTCAGTCTGAGTCAGGCCCTTCTGT |
| POMC forward primer (BamHI site included) | CGCGGATCCATGCCGAGATCGTGCTGC |
| POMC reverse primer (XhoI site included) | CCCAAGCTTTCACTCGCCCTTCTCTGAGGCTTCTTGAT |
| SASH1 probe 1# | GCCCAAGCTTTCACTCGCCCTTCTCCCTT |
| SASH1 probe 2# | CCAAGCTTTCACTCGCCCTTCTCCCTT |
| SASH1 probe 3# | CGTGCCACCTTAGACCCGGAGG |

Table 1. Sequences of primers or probes used in gene cloning and EMSA in this study.
indicated in Table 1 were used to amplify the bacteria (obtained from Han Jiahuai Lab, Xiamen University, Xiamen, China) containing the vector of full-length CDS sequences of and Gαs, p53 and POMC. Mammalian expression vector (Invitrogen) via the relative restriction sites and sequenced.

2.3 Culture of cell and vector transfection

SK-MEL-28, HEK-293T, and A375 cells were cultured according to our previous description [15]. Normal human epithelial melanocytes (NHEMs, C-12402, PromoCell, Germany) were maintained in M2 medium. Lipofectamine 2000 (11668-027, Invitrogen) as previously described [15, 16] or Entranster-D (18668-01, Engreen Biosystem Co., Ltd.) or polyethyleneimine (PEI) prepared by ourselves were, respectively, used for the transfection of SK-MEL-28, A375, B16, and HEK-293T cells. The transfected A375 and SK-MEL-28 cells were screened with 1.5 μg/ml puromycin or 2.0 μg/ml G418 to construct stable cell lines. Wild-type and mutant SASH1-pEGFP-C3 or co-transfected with wild-type SASH1-Pbabe-Flag-puro and Gαs-Pegfp-C3 vectors were transiently introduced into HEK-293T cells for immunoprecipitation experiments. p53-HA-Pcdn3.0, POMC-myc-Pcdn3.0, Gαs-Pegfp-C3, and wild-type SASH1-pEGFP-C3 according to pairwise combination were introduced into NHEMs and HEK-293 or HEK-293T cells to detect the expression of exogenous p53, POMC, Gαs, and SASH1 using PEI or PromoFectin (PK-CT-2000-MAC-1, PromoCell).

Gαs-GFP, HA-p53, myc-POMC, and GFP-SASH1 recombined vector were introduced into HEK-293T cells. 24 h later, Entranster™-R transfection reagent (18668-06, Engreen Biosystem Co., Ltd) was used to transfect Gαs- and POMC-specific siRNAs synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China) to silence the expression of exogenous Gαs, p53, and SASH1 in the transfected HEK-293T cells. The sense/antisense sequences of each siRNA for Gαs, POMC are documented in Table 2.

| Gene name   | Forward primer (5′–3′)                  | Reverse primer (5′–3′)                  |
|-------------|-----------------------------------------|-----------------------------------------|
| SASH1       | CGGAAACGTCAGTCAATCGGA                  | ATCTCCTTTCTTGAGCTAG                     |
| TYRP1       | CACAGGCAAGGTACGTCCTC                   | CTGAACTCCATGGTGCTTCTGTG               |
| Pmel17      | AAGTTTGACAGTGGAGCTGGCA                 | TTTCACGGCTCTGCTAG                     |
| Rab 27A     | AACTAGTGCTGCAATGGGACA                 | TTTGATCGACACCACCTGTC                   |
| Gαs         | GTCTTCTGGGAAATCG                      | CGGAGGTAATAGGGTAG                     |
| p53         | CCACTCCTACTACAATCAT                   | TCCAGCACAGGCAAC                          |
| POMC        | AGTCTAAGAGGGAGCTGCTG                  | CATGAACCGCGGTAGG                      |
| GAPDH       | CACCCACTCCCTCAGGTCTCGG                 | ACCACCTGCTCGCTGTG                     |
| Gαs siRNA 1 | GAGGACUACUUUCCAGAAUTT                | AUUCUGGAAGAUAGUAGUCCUTT               |
| Gαs siRNA 2 | GAGGACUACUUUCCAGAAUAGUAGG            | AUGUAGGAAGUAGUAGGAGUAGUAGG           |
| POMC siRNA1 | ACCUCACCAGGAAAGCAATT                  | UUGCUUUCGUGUGUGAGGUTT                 |
| POMC siRNA2 | AGUAGCUAGGAGGCGCCCACUUATT           | AUGGAGCCAGGAGUAGUAGUAGG              |
| GAPDH       | GUAUGACACAGCCAUACAAAG                 | CUUGAGGCUUGUGUGUAGUAGUAGG            |
| Negative control | UUCUUCGAAGUGUGACAGUAGG       | ACUGAGCAAGUAGGAGAATT                   |

Table 2.
Primers used for site directed mutagenesis, real time RT-PCR and RNAi.
2.4 Pull-down experiments and nanoflow LC-MS/MS and bioinformatic assays

Procedure of the pull-down assay, LC-MS/MS analyses, database search, and bioinformatic analyses for functional classification are mainly as performed as our previous description [13].

2.5 Immunoprecipitation and western blotting

Transfected HEK-293T cells or HEK-293 cells or NHEMs with ectopic exogenous genes were washed in a gentle way for three times with PBS and lysed in IP-western blot lysis buffer (P0013, Beyond Time BioScience and Technology Company) in the presence of a complete protease inhibitor cocktail, 1 μM sodium orthovanadate, and 1 mM sodium fluoride per 10 cm dish on ice for 20 min to acquire lysisprotein. Cell lysates were centrifuged for 10 min at 12,000 rpm at 4°C. 600 μl of supernatants of cell lysates were pre-cleaned with Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Inc.) for 1 h. GFP-Tag (7G9) mouse mAb (Shanghai Abmart, Inc.) or DYKDDDDK-Flag-Tag mouse mAb (Shanghai Abmart) or HA-Tag mouse mAb (Shanghai Genomics) was used to immunoprecipitate the corresponding proteins at 4°C for 2 h. Then, the cell lysates were mixed with 20 μl of Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Inc.) at 4°C for 10 h for co-immunoprecipitation or immunoprecipitation analyses. Finally, immunoprecipitates were washed for three times with PBS and subjected to western blotting. GFP-Tag mouse Ab, Flag-tag mouse mAb, DYKDDDDK-Flag mouse mAb, GAPDH mouse mAb and anti-β-tubulin mouse mAb (Shanghai Abmart, Inc.), anti-Gas rabbit polyclonal Ab (Gene Tex, Inc.), myc-tag mAb and HA-tag mouse mAb (Shanghai Genomics), SASH1 Rabbit mAb (Bethyl Laboratories, Inc.), TYRP1 (TA99) mouse mAb and melanoma gp100 Rabbit mAb (Abcam), Rab 27a mouse mAb (Abnova) were used for immunoblotting assay as previously described [17, 18].

2.6 Immunohistochemical analyses and immunofluorescence staining and melanin staining

2.6.1 Immunohistochemistry

All participating patients in this study were given the written informed consent regarding tissue and data use for scientific purposes. Epithelial tissues from affected individuals with the Y551D SASH1 mutation from pedigree I were fixed and embedded in paraffin. Paraffin sections (5 μm) were baked at 56°C for 3 h, and then deparaffinized and rehydrated using xylene and an ethanol gradient. SASH1 monoclonal antibody, rabbit anti-ACTH antibody, MiTF polyclonal antibody, the antibodies of melanogenesis related molecules including HMB45, TYRP1, and Rab 27a and p53 monoclonal antibody was used to bind the corresponding proteins on paraffin sections, respectively. Finally, counterstaining of hematoxylin was performed and the sections were photographed under the positive position microscope BX51.

2.6.2 Immunofluorescence (IF) and confocal microscopy detection

A375 stable cells with ectopic wild-type or mutant SASH1 in 6-well chamber slides were analyzed with indirect immunofluorescence analysis. SASH1 rabbit
mAb (Betheyl Laboratories, Inc.) and DYKDDDDK-Flag mouse mAb (Shanghai Genomics, China) were used to assess SASH1 localization and expression, as described previously [13].

2.6.3 Melanin staining

The melanin staining of paraffin sections obtained from epithelial tissues were performed as our previous descriptions and observed under a light microscope [18].

2.7 Quantitative RT-PCR

TRIZol reagent (Invitrogen) was used to isolate the total RNA from the different groups of SK-MEL-28 cells. Reverse transcription was performed according to the manufacturer’s protocol for the PrimeScript™ RT Reagent Kit (DRR037A, TaKaRa) for qRT-PCR. The sense and antisense primer sequences for SASH1, TYRP1, Pmel17, Rab27a, Gus, POMC, and GAPDH are showed in Table 2. The PCR products were identified by agarose gel electrophoresis. The Applied Biosystems 7500 System was applied to detect the expression of corresponding genes with SYBR Premix Ex Taq™ (DRR041A, TaKaRa). The quantity of each mRNA was normalized to that of GAPDH mRNA.

2.8 UV irradiation

Human foreskin tissues from a 14 year-old boy were irradiated for enough time under a ultraviolet phototherapy instrument (NBUVB SS-05, Sigma) to reach the expected UV intensity. The irradiated tissues were fixed in 10% formalin and embedded in paraffin for immunohistochemistry analyses. We conformed to the guidelines of the World Medical Assembly (Declaration of Helsinki) to acquire the human foreskin tissues. In vitro UV experiments were mainly referred to the protocol of our institute [19]. HEK-293T cells and NHEMs transiently with ectopic myc-POMC were subcultured to approximately 70–80% confluence and were irradiated with 100 mJ/cm² UVC delivered via a HL-2000 Hybrilinker with a 254 nm wavelength and followed by the indicated recovery time. Finally, immunobloting was performed to identify the corresponding proteins’ levels.

2.9 Electrophoretic mobility shift assay

Three probes binding with/without biotin targeting the promoter sequence of SASH1 gene were synthesized. The sequences of probes were as indicated in Table 1. Electrophoretic mobility shift assay was performed as described as the protocol provided with LightShift® Chemiluminescent EMSA (20148, Thermo Scientific, Pierce Biotechnology) to detect the bindings of SASH1 with p53 [18].

2.10 Statistical analyses

The data are indicated as mean ± standard error of the mean (SEM)s. The homogeneity of variance test was first used to analyze the variance homogeneity of data and the data were analyzed the change of variable test. Statistical significance was determined by a one-factor analysis of variance (ANOVA) using LSD on SPSS version 16.0 to produce the required p-values. Cartograms were plotted with GraphPad Prism 5.
3. Results

3.1 Mutations in SASH1 gene in the DUH-affected individuals result in up-regulated SASH1 in vitro and in vivo

The gene that is responsible for DUH had been localized to chromosome 6q24.2-q25.2. The 10.2 Mb region on chromosome 6 (6q24.2-q25.2) containing more than 50 candidate genes is flanked by the markers D6S1703 and D6S1708 [8]. Direct sequencing of the PCR products of exons amplified from genomic DNA of affected, unaffected, and control individuals was performed to screen the selected genes in this region for possible pathological mutations. 50 candidate genes were sequenced. Finally, in the probands in each of the two nonconsanguineous Chinese DUH-affected pedigrees (families I and II) and in one nonconsanguineous American DUH-affected pedigree (family III), three heterozygous mutations encoding amino acid substitutions in SAM and SH3 domain containing I (SASH1) were found in the three pedigrees. The substitution mutations in SASH1 gene were as follows: a TAC→GAC substitution at nucleotide 2126 in exon 14, causing a Y551D (p.Tyr 551 Asp) mutation at codon 551 in family I, a CTC→CCC substitution at nucleotide 2019 in exon 13, causing a L515P (p.Leu to Pro) mutation at codon 515 in family II, and a GAA→AAA substitution at nucleotide 2000 in exon 13, resulting in a E509K (p.Glu to Lys) mutation in family III. These sequence alterations were identified in all of the affected pedigree members, but were not observed in unaffected pedigree members, correlating the presence of the mutations with the presence of the phenotype. In any of the 500 normal controls or in any of the current databases, including the HapMap database, these three SASH1 mutations were not found [18]. So, these three mutations are impossible to be common single nucleotide polymorphisms (SNPs) [8].

In A375 stable cells with ectopic SASH1 gene mutants, mutated SASH1 were found to be significantly up-regulated (Figure 1B). Western blot showed that up-regulation of SASH1 was found in A375 cells stably expressing either wild-type (WT-A375 cells) or mutant SASH1, when compared to the expression of endogenous SASH1 in A375 cells with expression of pBABE-puro empty vector (VECTOR-A375 cells) or BLANK-A375 cells (Figure 1B) [18].

To identify the stability of SASH1 proteins, 20 µg/ml of the protein synthesis inhibitor cycloheximide (CHX) was used to treat HEK-293T stable cells with ectopic wild-type or mutant SASH1 expressing for the indicated times to assess the half-life of SASH1. SASH1 protein levels were induced to decrease by CHX treatment in a time-course-dependent manner. Wild-type SASH1 levels was decreased with a half-life of approximately 4 h, however, mutant SASH1 proteins began to degrade with CHX treatment for 6 h or longer. Therefore, it is deduced that the three mutant SASH1 proteins were more steady than the wild-type, confirming the conclusion offered above that expression levels of mutated SASH1(s) are higher levels than that of wild-type (Figure 2A and B). Endogenous SASH1 was not a stable protein with an half-life of approximately 3 h as identified by western blot (Figure 2C) [18].

The subcellular localization of SASH1 in A375 cells and skin epithelial layers was further characterized. A homogenous pattern of expression of SASH1 protein was observed in VECTOR-A375 cells and the skin epithelial layers from normal controls (Figures 1C and 3-a4). However, heterogeneity expression of SASH1 protein was showed in WT-A375 cells and mutant-A375 cells (Figure 3-b4–e4) as well as in the epithelial tissues of affected individuals (Figure 1C). In addition, as identified by Mitf, a melanocyte indicator, most of the SASH1-positive cells were Mitf-nucleic positive-melanocytes in the epithelial tissues of DUH-affected individuals. These Mitf-nucleic positive-melanocytes in the affected epithelial layer showed a heterogeneous...
distribution compared to those of unaffected individuals (Figure 1C). Some Mitf-tenuiigenin-positive staining is of false positivity (Figure 1C). Melanocytes or SASH1-positive epithelial cells not only localized at the basal layers, but also the suprabasal layers of the affected epidermal tissue, the phenomenon of which coincides with our previous descriptions that SASH1 mutations promotes melanocyte movement from the affected basal layers to the superficial ones [13].

3.2 SASH1 binds to Gαs and is induced by the canonical p53/POMC/Gαs cascade

SASH1 contains two functional domains, SAM and SH3 domain, which indicates SASH1 may plays an important role in a signaling pathway acting as a signaling molecule adapter or an associated scaffolding protein [8, 9]. Therefore, we performed a pull-down assay and a mass spectrometry analysis to investigate which signaling
pathways are regulated by SASH1. Pull-down experiments and nanoflow LC-MS/MS analysis demonstrated that SASH1 interacts with \( \text{G}\alpha_s \) and CALM, both of which are important in melanogenesis process (Tables 3 and 4) in WT-A375 cells. \( \text{G}\alpha_s \) connects receptor-ligand associations with the activation of adenylyl cyclase and many cellular responses, serving as a pivotal player in the conventional signal cascades [20]. To investigate the associations between SASH1 and \( \text{G}\alpha_s \), HEK-293T cells were co-transfected with Flag-SASH1 and GFP-\( \text{G}\alpha_s \). Exogenous SASH1 was immunoprecipitated with both exogenous \( \text{G}\alpha_s \) (GFP-\( \text{G}\alpha_s \)) and endogenous \( \text{G}\alpha_s \). Immunoprecipitates of exogenous SASH1 had different observed band sizes of \( \text{G}\alpha_s \) (Figure 4B and C) [18].

\( \text{G}\alpha_s \) mediates cAMP production in melanocytes which is stimulated by \( \alpha\)-MSH and melanocortins [21] and our study here shows that \( \text{G}\alpha_s \) is associated with SASH1. Hence, we examine whether \( \text{G}\alpha_s \) is required for the induction of SASH1 and how \( \text{G}\alpha_s \) mediates SASH1 expression, we introduced exogenous \( p53 \), POMC, \( \text{G}\alpha_s \), and SASH1 gene into HEK-293T and NHEMs (normal human epithelial...
melanocytes) to assess the effects of p53 and POMC on Gαs. Exogenous Gαs was induced in the co-existence of exogenous p53 and POMC (Figure 4C lane 5 and Figure 4D lane 5) and both inducements of exogenous Gαs and exogenous SASH1 were observed in the co-existence of exogenous p53 and POMC in two types of normal cells (Figure 4C lane 6 and Figure 4D lane 6). Meanwhile, in the presence of GFP-SASH1, GFP-Gαs was also induced (Figure 4C lane 4 and Figure 4D lane 4).

Figure 3.
Subcellular localization of SASH1. The fluorescence signals that were detected by confocal microscopy indicate that the overexpression or mutation of SASH1 results in the heterogeneous expression of SASH1 in vitro in A375 stable cells. The green fluorescence represents the Flag-tag label. Both exogenous and endogenous SASH1s are labeled with a red fluorescent tag. The nuclei are labeled with DAPI (in blue). The yellow fluorescence indicates the overlap of the green and red fluorescent staining. The red arrowheads indicate the activated SASH1-Flag fusion proteins that were expressed in the cytoplasm of WT-A375 cells or mutant-A375 cells. The blue arrowheads indicate the regions that do not express the activated SASH1-Flag fusion protein in the cytoplasm of WT-A375 or mutant-A375 cells. The endogenous SASH1 presents a uniform pattern of expression in all of the VECTOR-A375 cells (Figure 3A-a4). Scale bar = 5 μm.
A Novel P53/POMC/Gα/SASH1 Autoregulatory Feedback Loop and Pathologic...

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...lane 4), which indicated that SASH1 is necessary for activation of GFP-Gα. And immunoblot showed that Gα was identified to be induced by exogenous p53 and SASH1 (Figure 4E and F). Our results also demonstrated that POMC was mediated by p53 in HEK-293T and melanocytes, which were consistent with previous conclusions [1] (Figure 4G and H).

To confirm the fact that POMC, p53, and Gα are needed for the induction of SASH1 and exogenous POMC, p53, Gα, and SASH1 were transfected into HEK-293T cells and followed by silence of Gα and POMC by two specific pairs of siRNA, respectively. As identified in HEK-293 cells, deletion of Gα gene directly induced significant reduction of SASH1 (Figure 5C and D). Deletion of POMC by siRNA resulted in the downregulation of Gα and SASH1 (Figure 5E and F). Taken above,
it is believed that Gαs serves as a pivotal downstream of p53/POMC cascade and SASH1 is regulated by a novel p53/POMC/Gαs cascade.

3.3 P53 physiologically triggers SASH1 upon UV irradiation

To reveal the phenomenon that P53 physiologically triggers SASH1, discarded normal human foreskin samples were irradiated to gradually increased dose of UV and stained for the histological analyses of p53, ACTH/POMC, and SASH1.
Immunohistochemical (IHC) analyses demonstrated p53 is quickly induced in basal layers at the 0.5 J/cm² dose of UV exposure. The quick inducement of SASH1 and POMC/ACTH at UV irradiation 1.0 J/cm² dose in melanocytes is followed closely...
by p53 up-regulation (Figure 6A). Previous study had indicated that up-regulated POMC gene is induced at both protein and mRNA levels following UV exposure of skin [22, 23]. Followed the previous reports [1], a 100 J/m² UVB dose was administered in this study. The 100 J/m² UVB dose equates to the standard erythema dose (SED), which is commonly used as a measure of sunlight [24]. Therefore, both endogenous p53 and SASH1 protein levels in HEK-293T cells and NHEMs with ectopic exogenous POMC after UV irradiation were assessed by immunoblot. Expression of exogenous POMC and endogenous SASH1 was markedly induced by 6 h after UV irradiation, which accords with its known stabilization by UV in NHEMs. At 24 h, in NHEMs, UV irradiation maximally promoted the expression of POMC, p53, and SASH1 protein (Figure 6B). Similar induction of exogenous POMC and endogenous p53 and SASH1 was detected in HEK-293T cells after UV irradiation (Figure 6C). Hence, it is believed that both POMC and SASH1 serve as novel downstream players which respond to p53 inducement by UV irradiation.

Figure 6.
SASH1 is induced physiologically by p53 after UV irradiation. (A) Upon UV irradiation or without UV irradiation, immunohistochemistry analyses of p53, POMC, and SASH1 in human foreskin indicated that p53 is activated by UV-induced-increase of POMC and SASH1. The human foreskin tissues obtained from a 14-year-old boy were irradiated at different doses of UV intensity, then fixed in 10% formalin and embedded in paraffin for immunohistochemical analyses. Scale bar: 20 μm. The representative positive cells of p53, ACTH, and SASH1 were donated by red arrows. (B) and (C) NHEMs and HEK-293T cells with ectopic exogenous POMC (myc-POMC) expression were irradiated with UV irradiation (100 ml/cm²) and recovered for the indicated times. Transfected cells were lysed and at different time-points after irradiation as indicated. Western blot was used to detect the protein levels of endogenous p53, endogenous SASH1, and exogenous POMC along with GAPDH or beta-tubulin as a loading control.
3.4 p53-SASH1 reciprocal inducement in normal cells

To assess whether p53 is required for the inducement of SASH1, exogenous p53, and POMC gene were transfected into HEK-293T and NHEMs to test the induction of p53 and POMC to SASH1. In NHEMs and HEK-293T cells with ectopic of POMC (myc-POMC) in NHEMs and HEK-293T cells, exogenous SASH1 were induced to up-regulate by p53 (Figure 7). Increased protein levels of exogenous SASH1 was induced by increasing amounts of exogenous p53 in two normal cells (Figure 8A and B). On the contrary, exogenous p53 (HA-p53) was also triggered by increasing amounts of exogenous SASH1 (Figure 8C and D). The induction of exogenous SASH1 to endogenous p53 was also identified. It has been documented that, in two types of normal cells, increased endogenous p53 was induced by increasing doses of exogenous SASH1 (Figure 8E and F).

Since SASH1 is mediated by p53, we want to investigate whether there is a direct relationship between SASH1 and p53. As indicated in Figure 9A and B, HA-p53 did not bind to GFP-SASH. So, we tested the proximal 1 kb promoter region of the SASH1 gene to find the consensus transcription-factor-binding elements that are conserved between human, rat, and mouse. Among the various consensus elements searched for, p53 gene was remarkable. A most possible p53-binding site, sequence of which is “tgcccaagctttcacacttgttt” was identified in the SASH1 5’ flanking region about 550 bp upstream of the transcription initiation site in humans (Figure 9C). So, three synthesized probes were used to investigate the associations of p53 protein with SASH1 gene promoter. However, analyses of electrophoretic mobility shift assay (EMSA) revealed that there was no p53 protein bind the promoter region of the SASH1 gene (Figure 9D).

In summary, it is believed that SASH is regulated by the p53/POMC/α-MSH/Gas signal cascade and p53/POMC/α-MSH/Gas cascade and SASH1 constitute a novel autoregulatory loop. The p53/POMC/α-MSH/Gas/SASH1 regulatory loop acts as an auto-feedback circuit to regulate the p53-SASH1 reciprocal inducement (Figure 8G).

![Figure 7](image_url)

Figure 7. Exogenous p53 triggers expression of SASH1. (A) Exogenous p53 caused up-regulation of exogenous SASH1 in HEK-293T cells. HA-TP53, GFP-SASH1, and myc-POMC were transfected into HEK-293T cells for transient expression. Cells were lysed in 0.5% NP40 buffer containing protease inhibitors and subjected to western blot along with GAPDH as loading control. (B) Exogenous p53 caused up-regulation of exogenous SASH1 in NHEMs.
Figure 8.
There is a reciprocal induction between p53 and SASH1 in normal cells. (A) and (B) Exogenous SASH1 was triggered by exogenous p53 (HA-p53) in a dose-dependent manner. Different amounts of HA-TP53 plasmid were introduced into HEK-293T cells and NHEMs as indicated. After 48-h post-transfection or transfection, total RNA of HEK-293T cells and NHEMs was extracted and exogenous SASH1 RNA levels were assessed by quantitative RT-PCR and normalized to GAPDH. Expression of exogenous p53 protein and SASH1 were analyzed by western blot along with GAPDH as a loading control. (C) and (D) Protein and RNA levels of exogenous p53 were promoted by exogenous SASH1 promotes expression. Different amounts of GFP-SASH1 plasmid and a certain amount of exogenous p53 were transfected to HEK-293T cells and NHEMs cells. As revealed by QRT-PCR and western blot, enhanced expression of exogenous TP53 was induced by increasing amounts of GFP-SASH1. (E) and (F) Increased endogenous p53 was induced by exogenous SASH1. Different amounts of GFP-SASH1 were transfected in to HEK-293T cells and NHEMs. At 36 h after transfection, cells were lysed and subjected to western blot to analyze the expression of GFP-SASH1 with GAPDH as loading control. Results are the representative of three independent experiments. (G) A novel reciprocal induction of p53 and SASH1 is mediated by an autoregulatory p53/POMC/Gas/SASH1 loop. p53 is activated by different types of stress, which fosters POMC, Gas, and SASH1 successively. The inducement of SASH1 by p53/POMC/Gas cascade promotes the up-regulation p53 in nucleus, then induced nucleic p53 conversely activates POMC/Gas/SASH1 cascade, which consists an autoregulatory p53/POMC/Gas/SASH1 loop.

Figure 9.
p53 is not associated with SASH1 and SASH1 is not transcriptionally regulated by p53. (A) and (B) HEK-293T cells were co-transfected with the pEGFP-C3-SASH1 and Pcdna 3.0-HA-p53 vectors. At 24-h post-transfection, GFP-SASH1 was immunoprecipitated and the associated HA-p53 was detected by western blot analysis using an anti-HA antibody. Similarly, HA-p53 was immunoprecipitated and the associated GFP-SASH1 was detected by western blot analysis using an anti-GFP antibody. (C) Showed a schematic representation of the SASH1 locus, which indicates location of a p53-binding consensus sequence. (D) EMSA analyses demonstrated that there was none of among three probes of SASH1 gene promoter to bind p53 recombined protein.
3.5 Enhance expression of p53 and POMC is induced by SASH1 mutations

SASH1 up-regulation is mediated by SASH1 mutations, which is unfathomable enigma to us for lone time. Therefore, HEK-293T cells and NHEMs were transfected with wild-type or mutant SASH1 (wt SASH1 or mut SASH1), exogenous p53 and exogenous POMC to assess the effects of SASH1 mutations on p53 and POMC. As demonstrated in Figure 10A and B, increased expression of p53 and POMC was induced by SASH1 mutations. The effects of SASH1 mutations on endogenous p53 at protein level were also assessed. Increased endogenous p53 was also induced by mutated SASH1 (Figure 10C and D). In order to identify that p53 is induced by SASH1 mutations in vivo, immunostaining of p53 in the affected epithelial tissues with SASH1 Y551D mutation was performed. IHC analyses indicated that more nucleic expression of p53 in epithelial tissues and more p53-positive cells in affected epithelia layers were induced by SASH1 Y551D mutation (Figure 10E).

All of these indicate that not only SASH1 is positively regulated by the p53/POMC/α-MSH/Gas/SASH1 autoregulatory loop, but also SASH1 mutations serve more as molecular rheostats rather than an on-off switch to control this regulatory loop.

3.6 Expression of melanosomes matrix molecules was triggered by SASH1 mutations

Since there is a SASH1-p53 autoregulatory loop, the changes of downstream partners of SASH1 need to be tested. Therefore, we further identified the effects

![Figure 10](image-url)
of mutated SASH1 on the protein levels of matrix proteins and transport proteins. Enhanced expression of TYRP1, Rab 27a, Pmel17, and tyrosinase in SK-MEL-28 cells, a pigmented melanoma cell line and NHEMs was significantly induced by SASH1 mutations (Figure 11A and B). QRT-PCR also indicated that Pmel17, TYRP1, and Rab 27a were up-regulated by mutations of SASH1 in SK-MEL-28 stable cells (Figure 11C). Pmel17, TYRP1, and Rab 27a was heterogeneously distributed in the epithelial cells of tissues from the DUH-affected individuals (Figure 11D). Increased levels of melanogenesis molecules were observed in some hyperpigmentation areas in the affected epithelial layers. In the hyperpigmentation plaques, the superfluous production and secretion of melanin was clearly presented in the basal layers and in the suprabasal layers of the affected epidermal as (Figure 11D).

4. Conclusion

Our study reveals that a novel p53-SASH1 reciprocal induction triggers pigmentation of skin through an autoregulatory p53/POMC/α-MSH/αs/MSH/SASH1 loop. SASH1 mutations enhance SASH1-mediated induction of p53 and POMC. POMC is induced by p53 overexpression and resulted in UV-dependent hyperpigmentation UV-independent pathological hyperpigmentation [1]. Our work indicates that POMC up-regulation is induced by SASH1 mutations, which ultimately results
in the pathological hyperpigmentations of affected DUH individuals. These data indicate that SASH1 activation induced by mutations in melanocytes acts as a "UV sensor/effector" for skin pigmentation or SASH1 mutations-mediated up-regulation is the "chief criminal" of pathological hyperpigmentation of DUH, and its underlying mechanistic role is SASH1-p53 reciprocal inducement. Our data indicate that the definitions of the positive feedback p53/POMC/α-MSH/Gαs/SASH1 loop help us to recognize an important linkage between the p53 pathway and MC1R pathway by SASH1.

Recently, a c.1067T>C (p.Leu356Pro) mutation in exon 3 of ABCB6 (ATP-binding cassette subfamily B, member 6) was found in a large five-generation Chinese family with DUH family. Two additional missense mutations, c.508A>G (p.Ser170Gly) in exon 1 and c.1736G>A (p.Gly579Glu) in exon 12 of ABCB6 were found in two out of six patients using sporadic DUH patients [25]. Ac.1663C>A, (p.Gln555Lys) missense mutation in ABCB6 was identified in a Chinese family with typical features of autosomal dominant DUH. Two deletion mutations (g.776delC, c.459delC) in ABCB6 were found in an unrelated sporadic affected individual [26]. In addition, missense mutations in ABCB6 were also found in the sporadic affected DUH individuals [27, 28]. Silence of ABCB6 by siRNA destroyed PMEL amyloidogenesis in early melanosomes and resulted in aberrant increase of multilamellar aggregates in pigmented melanosomes. In the retinal pigment epithelium of ABCB6 knockout mice, morphological analysis indicated an obvious decrease of melanosome numbers [29]. All of these sequencing results and functional analyses of causing genes responsible for DUH indicate there exist novel pathogenicity genes and novel gene variations which is responsible for pathogenesis of DUH or there exists novel subtype of DUH.

The transcriptional network of p53-responsive genes produces proteins that interact with a large number of other signal transduction pathways in the cell and a number of positive and negative autoregulatory feedback loops act upon the p53 response [30]. Feedback loops of p53 and p53-responsive genes provide a means to connect the p53 pathway with other signal transduction pathways and coordinate the cellular signals for growth and division [30]. Our findings suggest that SASH1 serves as a "Hinge" to connect p53/POMC/α-MSH pathway with MC1R/Gαs/cAMP/PKA cascade to form an autoregulatory p53/POMC/Gαs/SASH1 circuit to mediate the melanogenesis process [18, 31].

Most recently, SASH1 is showed to involve in autosomal-dominant lentiginous [32] and autosomal-recessive SASH1 variants (c.1849G>A; p.Glu617Lys), which are associated with a new genodermatosis with a pigmentation defects, palmoplantar keratoderma and skin carcinoma and SASH1 is first reported to be predisposed to skin cancer [33]. Dyschromatosis universalis hereditaria (DUH) is a clinically heterogeneous disorder that presents as generalized mottled pigmentation and was first reported by Ichigawa and Hiraga in 1933 [7]. Stuhrmann and colleagues identified the first locus responsible for autosomal-recessive DUH, and this findings is consistent with recent evidence demonstrating that DSH and DUH are genetically distinct disorders [34]. Zhang et al. mapped the causative gene of DSH to 1q11-1q21 and found that a novel mutation of a heterozygous nucleotide A → G at position 2879 in exon 10 of the DSRAD gene is involved in DSH [35]. Subsequent research on dyspigmentation has demonstrated that the pathogenic genetic variant that causes DSH is localized to the DSRAD gene on chromosome 1q [15, 36–41]. Expanding Stuhrmann and Nuber’s findings and our own previous work providing photographic evidence of dyschromatosis presenting as large hyperpigmented bodies on DUH-affected individuals [6, 8, 34], we believe that we have discovered the first locus associated with autosomal dominant DUH, identifying SASH1 as the causative gene of autosomal dominant DUH.
Our findings first identify the pathological gene of DUH and reveal the pathological mechanism of hyperpigmentation patches of DUH-affected individuals. In addition, our work will enrich the crosstalk of p53 pathway with other transduction pathways in cells and give a new definition of the p53-responsive genes and their associations, which will perfect the p53 programmed responses to stress and pathologic conditions.

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**Conflict of interest**

No conflict between the authors.

**Notes/thanks/other declarations**

The chapter text was mainly referred to our article entitled as “A Novel P53/POMC/\(\alpha\)-MSH/SASH1 Auto-regulatory Feedback Loop Activates Mutated SASH1 to Cause Pathologic Hyper-pigmentation” (Journal of Cellular and Molecular Medicine 2017, 21(4):802-815) which we published in journal of cellular and molecular medicine in April, 2017. In this chapter, we rewrite the chapter text according to the suggestions of reviewers.

The chapter figures were taken from the figures and supplementary figures of our article entitled as “A Novel P53/POMC/\(\alpha\)-MSH/SASH1 Auto-regulatory Feedback Loop Activates Mutated SASH1 to Cause Pathologic Hyper-pigmentation”. The chapter tables were taken from the supplementary tables of our published article.

**Declarations**

Our article entitled as “A Novel P53/POMC/\(\alpha\)-MSH/SASH1 Auto-regulatory Feedback Loop Activates Mutated SASH1 to Cause Pathologic Hyper-pigmentation” is an Open Access article published under the terms of the Creative Commons Attribution License (CC BY). We are allowed to reuse the material without having to obtain permission provided that the original source of publication.

**Appendices and nomenclature**

| Abbreviation | Description                        |
|--------------|------------------------------------|
| ABCB6        | ATP-binding cassette subfamily B, member 6 |
| ACTH         | adrenocorticotropic                |
| \(\alpha\)-MSH | \(\alpha\)-melanocyte stimulating hormone |
cAMP cyclic adenosine monophosphate
CDS coding sequence
CHX cycloheximide
DSRAD double-stranded RNA-specific adenosine deaminase
DSH dyschromatosis symmetrica hereditaria
DUH dyschromatosis universalis hereditaria
EMSA electrophoretic mobility shift assay
GAPDH glyceraldehyde phosphate dehydrogenase
Gαs guanine nucleotide-binding protein subunit-alpha isoforms short
GPCR G-protein-coupled receptor
GS Griscelli syndrome
IF immunofluorescence
IHC immunohistochemical
LC-MS/MS liquid chromatograph-mass spectrometer
LOD log odds
LSD least-significant-difference
MC1R melanocortin-1-receptor
Mitf microphthalmia-associated transcription factor
NHEMs normal human epithelial melanocytes
PEI polyethyleneimine
POMC pro-opiomelanocortin
QRT-PCR quantitative reverse transcriptase polymerase chain reaction
RT-PCR reverse transcriptase polymerase chain reaction
SASH1 SAM and SH3 domain containing 1
SED standard erythema dose
TYRP1 tyrosinase protein1
UV ultraviolet

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