Dermatology Practical & Conceptual

Autophagy and Premature Graying of Hair: The Role of LC3 as a Biomarker in a Case-Control Study

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ABSTRACT

Introduction: Premature graying of hair (PGH) is a common disorder with a multifactorial etiology. Autophagy, which is self-cellular digestion, has been linked to melanin pigment formation; however, the role of autophagy in PGH has not been investigated well.

Objectives: The study aimed to evaluate the relationship between PGH and autophagy by measuring gene expression and serum microtubule-associated protein light chain 3 (LC3) concentration.

Methods: A case-control study was conducted on 39 PGH patients and 21 controls. Patients clinically diagnosed with PGH and aged <30 years were included in the study. Blood samples were taken to detect LC3B protein by ELISA in the serum of both groups. White hairs from both groups were collected to detect LC3B gene expression by PCR.

Results: There was a statistically significant difference between the two groups as regards expression levels of the LC3 gene by PCR (P<0.001), with the mean in the control group (0.71± 0.3) lower than in the PGH group (5.1 ± 1.4). Also, there was a positive significant correlation between LC3 concentration and LC3 gene expression in control (r=0.867, P< 0.001) and in PGH patients (r=0.954, P≤0.001). Multivariate logistic regression analysis for PGH predictors using age, sex (female), hemoglobin level, LC3 concentration, and LC3 gene expression revealed that the only predictor of PGH was LC3 gene expression.

Conclusions: Premature graying of hair may have a link with autophagy. LC3 gene expression was increased in PGH patients as compared to the control. LC3 gene expression may be an independent predictor of PGH development. Autophagy modulation may be a therapeutic target for PGH.

Introduction

Hair graying (canities) is a common age-related physiological change. On average, hair graying starts in Caucasians in their mid-30s, Asians in their late 30s, and Africans in their mid-40 [1]. The prevalence of hair graying varies among different races. A study has reported that half of the hair turns gray by the age of 50 in 6–23% of people [2]. Both sexes are equally affected [3]. The term premature graying of hair (PGH) is used when graying occurs before the age of 20 in Europeans, 25 in Asians, and 30 in Africans [4, 5]. Premature graying of hair has a multifactorial etiology, with an autosomal dominant pattern of inheritance [5], with various environmental factors, including psychological stress, smoking, drugs, ultraviolet light, and nutritional deficiency [4, 6]. It also may be associated with various autoimmune and genetic disorders [3]. The strongest associations have been detected between personal history and family history of PGH [7]. Several genes have been linked to the development of PGH, including the interferon regulatory factor 4 gene (IRF4), which has a role in melanin production and storage [8]. Also, the genes PAX3 and MITE play a vital role in melanocyte stem cell maintenance and differentiation, with their defective functions being linked to hair graying [9]. Numerous studies have demonstrated relationships between autophagy and the regulation of melanocytes and melanoma cell growth, senescence, and death [10]. Autophagy is the process by which cells digest themselves by sending cytoplasmic material to the lysosome for degradation [11]. Autophagy has vital roles in melanogenesis, melanosome transfer, and pigmentation, with the autophagic machinery proteins promoting the migration of developing melanosomes within melanocytes on microtubules and actin filaments [12]. The melanosomes are transported to keratinocytes, where autophagy restricts melanin accumulation [13]. Among many proteins known to be essential to the autophagic process, the microtubule-associated protein light chain 3 (LC3) plays a critical role in autophagy. Once synthesized as a soluble cytosolic form (LC3-I), LC3-I is then converted into a membrane-bound form (LC3-II) via an autophagy-specific conjugation process activated by autophagy-related gene (Atg7&3); LC3-II is then recruited to the autophagosomal membrane [14]. A study has attributed autophagic clearance of melanosomes in keratinocytes based on increased LC3 flux, which has been linked to the ethnic diversity of skin color [15]. Therefore, it is of interest to study the relationship between PGH, autophagy evaluation through LC3 gene expression by PCR, and serum LC3 concentration by ELISA.

Methods

A case-control study was conducted on 39 patients with PGH followed at the outpatient dermatology clinic and 21 controls (age- and sex-matched). The study was approved by the Medical Research Ethics Committee at the Faculty of Medicine (IRB; Soh-Med-22-11-16). Informed consent was obtained from all participants.

Patients clinically diagnosed with PGH and aged <30 years were included in the study. Exclusion criteria included patients with graying of hair as a part of other conditions such as vitiligo, cutaneous disease involving the scalp, and pregnant or lactating women. Iron deficiency anemia was also excluded.

Participant Assessment

All participants were subjected to the following:

1. Medical history: A detailed history was taken regarding age at onset, origin, pattern, progression, family history, and personal histories such as smoking and alcohol intake.
2. General examination: To exclude systemic diseases (anemia, thyroid disorders).
3. Dermatological examination: General dermatological and hair examinations were done. A local examination of the scalp was done to detect any abnormality. Examination of the hair was done to evaluate the site and severity of hair graying. Diagnosis of PGH was made clinically and was graded into three groups as follows, mild: <10; moderate: 10–100; severe: >100 gray hairs.
4. Laboratory investigations, including:

Blood Sample

Blood samples (5cm) were taken on EDTA tubes for examination in the central laboratory of Sohag University, Egypt.

a. Complete blood count and serum ferritin were done to exclude iron deficiency anemia.
b. Immunohistochemistry evaluation to detect LC3B protein by ELISA in the serum of both groups.

The serum was left to coagulate at room temperature for 10-20 minutes, then centrifugation 20-min at the speed of 3000 r.p.m was done. The specimen was kept at -20° C to be preserved until evaluation. Human Microtubule-Associated Protein 1 Light Chain 3 beta (MAP1LC3B) ELISA (enzyme-linked immunosorbent assay) kit (*201-12-5566, Sunred biotechnology, China*) was used.

First, the MAP1LC3B was added to the monoclonal antibody enzyme, which was pre-coated with human MAP1LC3B monoclonal antibody, incubation for 60 minutes at 37° C, then MAP1LC3B antibodies labeled with biotin was added and combined with Streptavidin-HRP (Streptavidin Horseradish peroxidase) to form an immune complex. Then, incubation and washing were done again to

remove the uncombined enzyme. Then, chromogen solution A & B were added and the color of the liquid changed to blue.Under the effect of acid, the color became yellow. The chroma of the color and the concentration of the human substance MAP1LC3B of samples were positively correlated.

The sensitivity of this assay, defined as the lowest protein concentration that could be differentiated from zero, was determined by subtracting two standard deviations from the mean optical density value of twenty-zero standard replicates and calculating the corresponding concentration. The assay range was 0.5-150ng/ml.

Hair Samples

At least six white hairs from patients with PGH and six normal hairs from controls were collected by hair plucking to detect LC3B gene expression by polymerase chain reaction (PCR) in the National Research Centre (NRC), Cairo, Egypt. Hair samples were processed with the following four steps: extraction of RNA from hair samples; reverse transcription into cDNA; real-time PCR quantification; interpretation of the result.

Extraction of RNA from Whole Hair Samples

Total cellular RNA was extracted from all hair samples with the QIAamp®RNA Mini Kit (*52904, QIAGEN, Germany*). All preparation and handling steps of RNA took place in a laminar flow hood under RNase-free conditions.

Reverse Transcription into cDNA

Total RNA was reversely transcripted to cDNA using a High-Capacity cDNA reverse transcription Kit (*4368814, Applied Biosystems™, USA*).

Real-time PCR Quantification

A real-time quantitative RT-PCR (RQ-PCR) based on TaqMan fluorescence methodology (*4333458, Applied Biosystems™, USA*) was used for LC3B quantitation. Amplification of cDNA using TaqMan master mix (*16495, Applied Biosystems™, USA*) and primer assay of the gene using real-time PCR.

Procedure:

1. Total PCR volume was 25 µl, including 5 µl of RT reaction, 10 µl TaqMan universal PCR master mix (10 µM), 1 µl primer, and probe assay mix of each gene (LC3B) (10 µM) was completed for a total volume by adding 9 µl distilled water.
2. GAPDH was used as a reference gene or internal control: Sense, 5-CCTCAAGATCATCAGCAAT-3;
   Antisense, 5'-CCATCCACAGTCTTCTGGGT-3';
   Probe, 5'-FAM-ACCACAGTCCATGCCATCAC-TAMRA-3'
3. PCR cycling conditions were as follows: denaturing at 94° C for 20 s, followed by annealing at 56° C for 20 s, and extension at 72° C for 30 s, 80° C for 20 s.
4. Quantification of PCR product after normalization to GAPDH gene expression was done and calculated relative to the untreated control group.

Interpretation of the Result:

The relative of both LC3B expressions in a sample was determined by subtracting the cycle threshold of the reference gene (GAPDH) from that of the target gene (LC3B) expression getting the normalized amount of the mRNA, then the value was compared to that of the calibrators.

Statistical Analysis

Data were verified, coded by the researcher, and analyzed using SPSS version 24. Descriptive statistics: Means, standard deviations, median, range, and percentages were calculated. Test of significances: chi-squared test was used to compare the difference in the distribution of frequencies among different groups. A test of normality for the main variables was carried out using the Shapiro-Wilk test. Independent t-test/Mann-Whitney U test analysis was carried out to compare the means of normally/not normally distributed binary data, respectively. For non-parametric variables with more than two categories, the Kruskal Wallis test was used to compare the difference in medians, and the post-hoc test with Bonferroni correction was used for pairwise comparisons. The clinical and demographic factors with proven statistical significance from the univariate analyses were further included in the multivariate logistic regression models (odds ratio (OR), 95% confidence (CI)). Spearman's rank correlation analysis was used to test the association between variables. A p-value of ≤ 0.05 was considered statistically significant.

Results

The study was conducted on 60 cases: 21 control and 39 PGH patients. The mean age was 27.03± 3.7 years in the control group and 27.1± 1.7 years in PGH patients. Females constituted 66.7% and 82.1% in the control and PGH groups, respectively. There was no statistical significance as regards age, sex, or hemoglobin level, as shown in Table 1. The history of age at onset in the PGH group was 19.77± 3.5 years old, with the mean disease duration of 7.28± 2.8 years. Family history of PGH was positive in 89.7% (35/39). About 3/4 of PGH patients (74.3%, 29/39) had severe disease, with the majority of patients (84.6%, 33/39) having ≥ four affected sites with PGH. Other clinical characteristics are shown in Table 2. There was a statistically significant difference between the two groups as regards expression levels of the LC3 gene by PCR (P<0.001). However, there was no

Table 1. Basic Clinical and Laboratory Characteristics of PGH patients (N=39) and control (N=21) group.

Parameter	Group 1 control (N = 21)	Group 2 cases (N = 39)	P-value
Age (years)	27.10 ± 1.7	27.03 ± 3.7	0.922*
Sex • Male • Female	 7 (33.3%) 14 (66.7%)	 7 (17.9%) 32 (82.1%)	0.179**
Hemoglobin (g/dl)	12.76 ± 0.8	12.41 ± 0.8	0.119*

*Student t-test was used to compare the mean difference between groups. **Chi-squared test was used to compare proportions between groups.

Table 2. Clinical Characteristics of PGH Patients (N=39).

Parameter	PGH Cases (N=39)
Age at Onset/years (Mean ± SD) 19.77 ± 3.5	
Categories of Age at Onset	
• 5 - 10 years • 10 - 15 years • 15 - 20 years • 20 - 25 years	1 (2.6%) 2 (5.1%) 19 (48.7%) 17 (43.6%)
Categories of Age at Onset	
• ≤ 20 years • > 20 years	22 (56.4%) 17 (43.6%)
Disease Duration (years) (Mean ± SD)	7.28 ± 2.8
Categories of Disease Duration	
• 1 - 5 years • 5 - 10 years • 10 - 15 years	6 (15.4%) 25 (64.1%) 8 (20.5%)
Categories of Disease Duration	
• ≤ 7 years	20 (51.3%)
Cosmetic Procedure	
• Yes	12 (30.8%)
Family History	
• Yes	35 (89.7%)
Disease Severity	
• Mild 1 • Moderate • Severe 29	(2.6%) 9 (23.1%) (74.3%)
Number of Affected Areas	
• Two • Three • ≥Four	3 (7.7%) 3 (7.7%) 33 (84.6%)

statistically significant difference between the two groups as regards LC3 concentration by ELISA (*P*=0.229), as shown in Table 3. Classification of PGH patients according to age at onset (≤20 vs >20 years old) revealed a statistically significant difference in LC3 concentration by ELISA (*P*=0.042), as shown in Table 3. In addition, the classification of PGH patients according to disease duration (≤7 vs >7 years) showed a statistically significant difference in both LC3 gene level by PCR (*P*=0.016) and LC3 concentration by ELISA (*P*=0.024), as shown in Table 3. However, the classification of PGH patients according to disease severity showed no statistically significant difference between both LC3 gene level by PCR and LC3 concentration by ELISA, as shown in Table 3. There was a positive significant correlation between LC3 concentration and LC3 gene expression in control (r=0.867, *P*<0.001), as shown in Figure 1, and in PGH patients (r=0.954, *P*=< 0.001), as shown in Figure 2. There was a positive correlation between LC3 gene expression and age at onset of PGH (r=0.321, *P*=0.023), as shown in Figure 3. Also, there was a positive significant correlation between LC3 concentration and age at onset of PGH (r=0.364, *P*=0.011), as shown in Figure 4. In addition, there was a negative correlation between LC3 gene expression and disease duration (r=-0.393, *P*=0.007), as shown in Figure 5, and a negative correlation between LC3 concentration and disease duration (r=-0.382, *P*=0.008), as shown in Figure 6. A multivariate logistic regression analysis for PGH predictors using age, sex (female), hemoglobin level, LC3 concentration, and LC3 gene expression was done; after adjusting for all factors, the only predictor of PGH was LC3 gene expression. In other words, with a one-point increase in the LC3 expression, there was a six-time increase in the risk of having PGH, as shown in Table 4.

Discussion

Premature graying of hair is a very common disorder. The prevalence of PGH varies among different clinical studies, from 27.3% [16] up to 69% [17]. The etiology of PGH is multifactorial, with the genetically determined, age-related exhaustion of hair follicle pigmentary capacity as the main cause. This may be mediated through increased reactive oxygen species and dysregulation of the anti-oxidant capacity, which lead to melanocyte DNA damage and accumulation of gene mutations with aging [5]. Autophagy plays an important role in melanogenesis and melanosome transfer [12]

Table 3. Comparison of LC3 Concentration by ELISA and LC3 Expression by PCR between Controls and PGH patients according to the Age at Onset, Disease Duration, and Disease Severity.

	LC3 Concentration by ELISA	LC3 Expression by PCR
Comparison between controls and PGH patients:		
Group 1: controls (N=21)	28.50 ± 12.8 27 (15.5 – 79)	0.71 ± 0.3 0.7 (0.4 – 1.3)
Group 2: PGH cases (N=39)	44.16 ± 43.3 29 (3 – 172)	5.10 ± 1.4 5 (2 – 11)
P-value	= 0.229*	**< 0.001****
According to age at onset (≤ 20 vs > 20):		
≤ 20 years (N=22)	36.87 ± 31.5 26 (3 – 172)	4.89 ± 2.1 4.6 (2.1 – 11)
> 20 years (N=17)	4.89 ± 2.1 4.6 (2.1 – 11)	5.38 ± 1.6 5.2 (2.5 – 8)
P-value	**0.042***	0.081*
According to disease duration (≤ 7 vs > 7):		
≤ 7 years (N=20)	48.59 ± 41.4 32 (20.5 – 171)	5.52 ± 1.2 5.2 (3.5 – 8)
> 7 years (N=19)	39.51 ± 35.9 25 (3 – 172.5)	4.66 ± 2.3 4.5 (2.1 – 11)
P-value	**0.024***	**0.016***
According to disease severity:		
Mild/Moderate (N=10)	37.06 ± 28.3 28.9 (11.5 – 143)	4.51 ± 1.4 4.9 (2.1 – 6.2)
Severe (N=29)	46.61 ± 45.3 29.3 (3 – 172.5)	5.31 ± 2.1 5 (2.1 – 11)
P-value	0.579*	0.418*

*Mann-Whitney U test was used to compare the median difference between groups; **Student t-test was used to compare the mean difference between groups

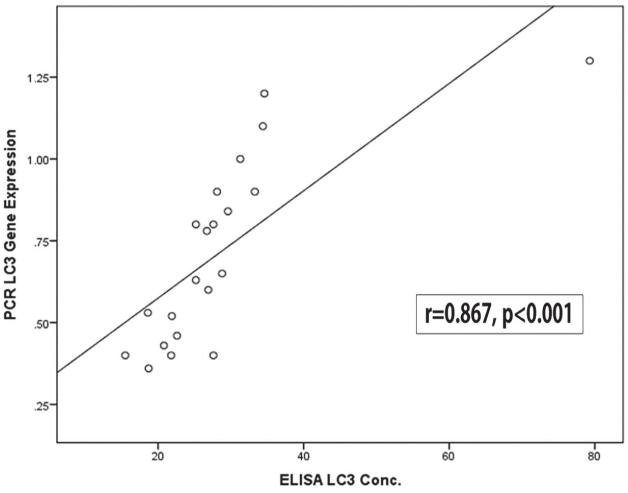

Figure 1. Correlation between LC3 concentration and LC3 gene expression in controls (N=21).

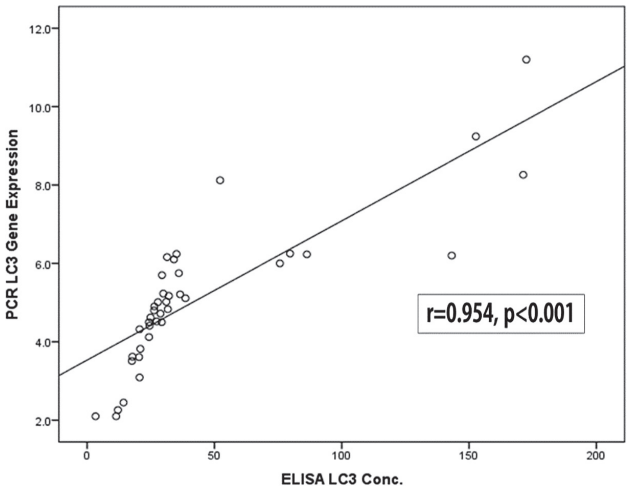

Figure 2. Correlation between LC3 concentration and LC3 gene expression in PGH patients (N=39).

PGH cases compared to the controls, but this increase was not significant. More interestingly, the expression levels of the LC3 gene were not significantly higher in PGH cases with an age at onset of >20 years old and significantly higher in and might contribute to ethnic skin color diversity by regulating melanosome degradation in keratinocytes [15]. One of the autophagic proteins is the microtubule-associated protein light chain 3 (LC31A/1B), with its cytosolic form

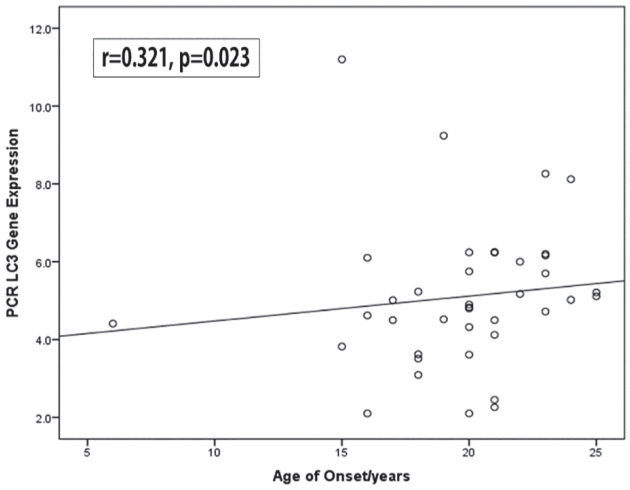

Figure 3. Correlation between LC3 gene expression and age at onset in patients (N=39).

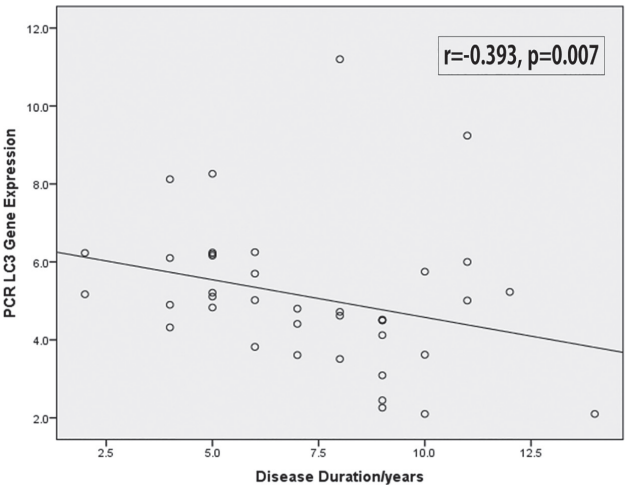

Figure 5. Correlation between LC3 gene expression and disease duration in patients (N=39).

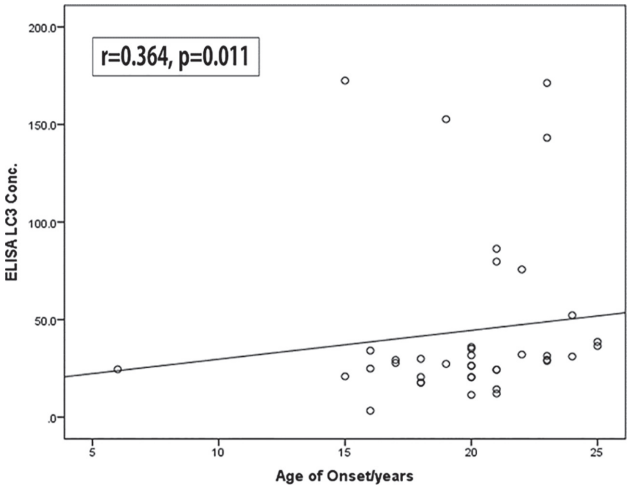

Figure 4. Correlation between LC3 concentration and age at onset in patients (N=39).

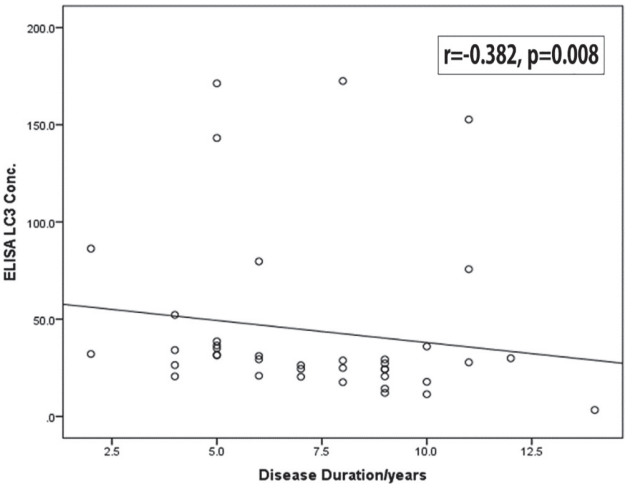

Figure 6. Correlation between LC3 concentration and disease duration in patients (N=39).

Table 4. Multivariable Logistic Regression Analysis for PGH Predictors.

Variable	AOR	95% CI	P-value
Age/years	0.993	0.837 – 1.178	= 0.934
Sex (Female)	2.286	0.674 – 7.754	= 0.185
HGB Leve (g/dl)	0.571	0.286 – 1.143	= 0.113
LC3 Conc. By ELISA	1.019	0.993 – 1.047	= 0.159
LC3 Gene Expression By PCR	6.031	2.256 – 9.162	**< 0.001**

Abbreviations: AOR, adjusted odds ratio, CI, confidence interval

(LC3-I) converted to a conjugated form (LC3-II) during the activated autophagic process [14]. Therefore, LC3 detection by immunoblotting or immunofluorescence is considered a valuable tool for monitoring autophagy [18]. It must be noted that the role of LC3 in regulating pigmentation is complex and still controversial. Increased LC3 has been linked to both increased and decreased pigmentation. The current study evaluated both the expression levels of the LC3 gene by PCR and serum LC3 concentration by ELISA in 39 cases and 21 healthy controls. The expression levels of the LC3 gene were significantly higher in PGH cases compared to the controls. Also, the serum LC3 concentration was higher in

PGH cases with disease duration ≤7 years. In addition, the serum LC3 concentration was significantly higher in PGH cases with an age at onset of >20 years old and significantly higher in PGH cases with disease duration ≤7 years. In the current study, PGH patients showed significantly higher LC3 gene expression (although the non-significant high serum LC3) as compared to controls. This may be explained by the usual poor correlation between mRNA and protein concentrations [19]. Furthermore, it is unknown how much of the relative concentration of the mRNA level is translated to the protein level [20]. Moreover, there is great variability in the rate of production and degradation of proteins irrespective of the corresponding mRNA levels [10]. A clinical study [21] compared the hypopigmented macules of tuberous sclerosis complex (TSC) with normal skin as regards the possible role of autophagy. The melanocytes of the TSC- hypopigmented macules showed dysregulation of autophagy with increased LC3 expression and p62 accumulation, which is one of the autophagic substrates [21]. A recent in vitro study [22] evaluated the effect of a synthetic autophagy inducer PTPD-12 on human melanocytes and keratinocytes containing melanosomes. The induction of the autophagic flux led to increased degradation of melanosomes, with visible lightening of melanocyte and keratinocytes cell pellets. However, the autophagic flux inhibition by chloroquine led to a decreased degradation of melanosomes leading to return of the normal melanogenesis pathway. This suggests the complex role of autophagy in melanocyte biology and melanogenesis[22]. ATG7 gene deletion in melanocytes suppressed LC3B expression and induced mild hypopigmentation in mice skin [23]. The melanin content of the hair of ATG7 deficient mice was reduced by 10-15% as compared with the control. The ATG7 deficient melanocytes showed oxidative stress-induced damage with premature growth arrest. More interestingly, in vitro cultures of melanocytes from ATG7 deficient mice and control demonstrate an equal amount of melanin per cell. Therefore, ATG7-dependent autophagy is important for both melanocyte function and melanogenesis [23]. Moreover, suppression of ATG7-dependent autophagy in natural human epidermal melanocytes (NHEM) leads to inhibition of melanocyte proliferation, with an increase in their oxidative stress-mediated apoptosis, which leads to premature melanocyte senescence. This reflects the important role of ATG7 in controlling oxidative stress homeostasis and consequently melanocyte functions, including melanogenesis. Also, this ATG7 suppression has been associated with a decrease in the conversion of LC3-I to LC3-II, with an increase in the autophagy adapter protein p62, reflecting the stoppage of the autophagic flux process in NHEM [24]. Several studies [10, 12, 15, 21-23] support the important role of autophagy in melanogenesis in the literature. Melanosome trafficking is mediated by the autophagic proteins LC3B and ATG4B. This LC3B associated with melanosomes does not only affect the steady state level of melanosomes but also enables their intracellular movement on specific microtubules and actin tracks. Therefore, the transfer of the melanosomes to keratinocytes is affected by LC3B knockdown [12]. The role of autophagy in hair physiology has been studied through the use of the organ culture of human scalp hair follicles (HFs). In anagen, the keratinocytes of organ-cultured HFs showed an active autophagic flux as evidenced by the elevation of LC3B expression and autophagosome visualization. This autophagic flux changes during catagen. Therefore, the anti-hair loss products may prolong the anagen phase by enhancing HFs autophagy [25]. The current study showed a significant positive correlation between LC3 expression and concentration by ELISA among controls as well as PGH cases. After adjusting for all factors, the only predictor of PGH was LC3 gene expression. In other words, with a one-point increase in the LC3 expression, there was a six-time increase in the risk of having PGH. Family history and age at onset of PGH are important risk factors associated with PGH development [26]. In a recent meta-analysis, several risk factors were associated with PGH, including smoking, mineral deficiency (low serum iron and calcium), and vitamin deficiency (B7, B12, and folic acid) [27]. The current study has several limitations. The first is the small sample size of the included PGH cases and the healthy controls. Investigations of patients and controls for nutritional deficiencies such as mineral deficiency and vitamin deficiency are needed to decrease bias. The second is that this autophagic evaluation does not reflect the autophagic flux. The transmission electron microscopic visualization of autophagosomes is considered the gold standard for autophagy diagnosis. Also, the relationship between autophagy and oxidative stress was not evaluated in PGH, especially since oxidative stress has an important role in PGH [28]. Finally, the current study could not establish either the cause or effect relationship between hair graying and elevated levels for the studied LC3 gene product, but only showed an association. A multicenter case-control study with a larger sample size is needed to evaluate serum and gene expression of several autophagic markers including LC3, p62, and beclin together with autophagosome visualization with the transmission electron microscope. Also, autophagic manipulation might be a potential therapeutic modality for the treatment of PGH.

Study Approval Statement: This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Medical Research Ethics Committee at the Faculty of Medicine (IRB; Soh-Med-22-11-16).

Consent to Participate Statement: Informed consent was obtained from all participants included in the study.

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