Investigation of FOXO3a, S253 phosphorylation FoxO3a, and 14-3-3 Expression in Androgenetic alopecia VS Normal

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Abstract

The fundamental mechanism of androgenetic alopecia (AGA) is the depletion of the anagen phase causing clinical presentation of hair miniaturization. The imbalance of autophagy signaling led to a reduction of the anagen phase and an increase in senescence of dermal papilla (DP). FoxO3a, one of the upstream autophagy-regulated proteins, has been well studied in many cell types and relevant diseases. However, the function of FoxO3 and its relevant role in the autophagy mechanism becomes a mysterious phenomenon in hair regeneration and AGA. We randomly recruited multiple bulge areas of the outer root sheath from 10 tissue sections for each staining biopsied from 2 healthy volunteers and 3 early-onset androgenetic alopecia patients. Here, we aim to study FoxO3a expression by comparing early-onset AGA to normal. 5-µm formalin-fixed paraffin-embedded (FFPE) vertical sections of biopsied-scalp samples were used in immunohistochemical (IHC) assays and assessed scoring using Qu path software. The FoxO3a, S253 phosphorylated FoxO3a, and 14-3-3 were identified in the bulge area of the outer root sheet with significantly higher positive intensity and H-score in S253 phosphorylated FoxO3a IHC staining among the AGA group than the normal group. Interestingly, increased phosphorylated FoxO3a expression in the AGA group possibly demonstrated the loss of nuclear localization that was eventually lost to transcriptional factor activities encoding autophagy-related genes. These findings illustrated the clinicopathological significance of early-onset AGA and the imbalance in cell signaling of the protein involved in autophagy.

Keywords: *androgenetic alopecia, autophagy, FOXO3a, immunohistochemical study, Qu path*

1. Introduction

The most prevalent cause of progressive hair loss is androgenetic alopecia (AGA). Genetic predisposition, androgen induction, microinflammation, and oxidative stress are the main causes of AGA. The primary mechanism is signal dysregulation involving the shortening anagen phase with a gradually increasing telogen phase during hair cycling. AGA is distinguished by hair miniaturization when terminal hair becomes lighter and shorter with a marked decrease in hair follicle size known as "villus hair." (Jahoda 1998). This occurs between the late catagen and early anagen phases, affecting the dermal papilla and dermal sheath and eventually resulting in a smaller hair follicle (Whiting 2001).

Typically, hair follicle stem cells (HFSC) residing in the follicle bulge interact with mesenchymal stem cells (MSCs) and the dermal papilla (DP) cells, causing cell proliferation toward hair regeneration by inhibiting inhibitory effects of the cell signaling (Zhang, Kling, et al. 2014). Hair follicle stem cells are maintained in a quiescent state until they receive signals to proliferate and migrate either to the DP or to the epidermis leading to basal layer repopulation. Androgenetic alopecia could be associated with hair follicle stem or progenitor cells deprivation. Moreover, DP cells from the balding scalp of AGA patients exhibit signs of senescent characteristics, such as loss of replicative potential, changes in cell size and shape, decrease or loss of characteristic markers/molecular signatures (Bahta, Farjo, et al. 2008) and (Randall, Hibberts, et al. 1996). Similarly, DP cells from male AGA patients exhibit premature senescence in response to oxidative stress compared to occipital DP cells (Upton, Hannen, et al. 2015).

Autophagy is a stress-response mechanism that cells use to deal with stressors such as nutrient deficiency, oxidative stress, infection, and aging. Autophagy promotes anagen, inhibits catagen induction by removing senescent cells, and increases dermal papilla cell proliferation. For example, mTOR, an autophagy inhibitor, resulting from prolonged persistent Wnt signaling can potentially cause cell senescence. Moreover, rapamycin, an mTOR inhibitor, effectively prevents stem cell ablation (Castilho, Squarize, et al. 2009). Choi, Kang et al. (2021) demonstrated that Myristoleic Acid is required for autophagy induction, anagen signaling, cell cycle progression, and DPC proliferation by stimulating the ERK pathway. Furthermore, autophagy defects result in catagen induction and a lack of Atg7, an autophagy gene, leads to a hair growth disorder (Audesse, Dhakal, et al. 2019).

Forkhead box protein O3 (FoxO3a), which is highly expressed in a variety of tissues, including the brain, heart, kidney, spleen, and skin aging (Furuyama, Nakazawa, et al. 2000) is a critical regulator of cellular homeostasis, stress response, and longevity because it can adjust various of stressors such as nutrient deficiency, oxidative stress, hypoxia, heat shock, and DNA damage (Fasano, Disciglio, et al. 2019) by activating cell cycle inhibitors, pro-apoptotic genes, ROS scavengers, autophagy effectors, gluconeogenic enzymes, and other nuclear genes (Fasano, Disciglio, et al. 2019). FOXO proteins are important cell regulators in response to oxidative stress and cell fate decisions, specifically the autophagy pathway. As the critical transcription factor, FoxO3a recognizes as a credible upstream autophagy signaling. The FOXOs family primarily regulates autophagy as a transcriptional activator in multiple stages, including induction, nucleation, and fusion. In addition, the post-translational protein modification regulates the nuclear-cytoplasmic localization of FOXOs activity. Loss of FOXOs function involves the interaction of these proteins with 14-3-3 protein partners that modulate FOXOs protein's degradation by the ubiquitin-proteasome pathway (Obsil, Ghirlando, et al. 2003) and (Fasano, Disciglio, et al. 2019).

The impaired autophagy pathway has been demonstrated in the Androgenetic alopecia through the loss activity of the upstream autophagy-initiated protein, BECN-1, which FoxO3 regulates. Specifically, no shreds of evidence have been reported on the role of FoxO3a and relevant downstream autophagy mechanisms in the pathogenesis of androgenetic alopecia. More importantly, discovering new competent pathophysiology of AGA could pave a new promising way to understand a new effective treatment. Here the expressions of FoxO3a, S253 phosphorylation FoxO3a, and 14-3-3 have been studied in the bulge area of the outer root sheet (ORS) comparing between AGA and normal.

2. Objectives

This study aims to determine the clinicopathological significance of immunohistochemical expression of FoxO3a, S253 phosphorylation FoxO3a, and 14-3-3 expression in the outer root sheet (ORS) of bulge region by comparing between early-stage androgenetic alopecia and normal donor.

3. Materials and Methods

Population: We performed dermoscopy guided scalp biopsies to investigate the expression of certain proteins in each bulge region of the outer root sheath at the vertex area from 2 healthy volunteers and 3 early-onset androgenetic alopecia patients. The inclusion criteria for the AGA group are identified by males aged between 20 to 40 years old with androgenetic alopecia diagnosed by a clinical presentation showing Hamilton-Norwood stage II-III vertex and the presence of the following dermatoscopic features: hair miniaturization of more than 20% at the vertex but less than 20% at the occipital scalp, and brown peri pilar sign indicating an early stage of androgenetic alopecia. Whereas the normal group is defined by Healthy male volunteers aged 20-40 years with physical and dermoscopic examination showing normal hair and scalp with less than 20% of hair miniaturization. Patients taking other treatments for androgenetic alopecia before the study periods and having other hair loss-related diseases had been excluded.

Tissue section selection: 5-μm formalin-fixed paraffin-embedded (FFPE) vertical sections of biopsied-scalp samples were prepared for H&E staining to the identified hair follicle. Then, non-staining slides were carefully selected to stain for IHC-P and immunofluorescence assay referring to H&E staining slides. We identified each area of hair follicles from morphology including hair shaft, outer root sheath, inner

root sheath, hair matrix, dermal papillae, connective tissue sheath, sebaceous gland, and arrector pili muscle in 4 vertical areas namely infundibulum, bulge, supra bulb, and bulb region to localize and quantify protein expression in each bulge area of the outer root sheath. The bulge region is identified as the localization of label-retaining cells just above the insertion site of the arrector pili muscle and below the sebaceous glands (Ohyama 2007). We recruited multiple bulge areas acquired from 6 tissue sections of the AGA group and 4 tissue sections from the normal group for each staining.

Immunohistochemical study: Automated IHC assays were carried out in this study. IHC assays were performed on a Ventana Ultraview DAB detection kit in a Ventana Benchmark XT stainer (Ventana Medical Systems, Tucson, AZ) (Ying, Guo et al. 2013) and (Martinez 2017), using VENTANA reagents except as noted, according to the manufacturer's instructions. Slides were deparaffinized using EZ Prep solution (cat # 950–102) for 16 min at 72 °C. Epitope retrieval was accomplished with CC1 solution (cat # 950–224) at a high temperature (e.g., 95–100 °C) for a period (e.g., 32–92 min) that is suitable for a specific tissue type. An investigation of upstreaming autophagy regulators was achieved by applying the rabbit anti-human FoxO3a monoclonal antibody (1:200; cat. no. 12829S; Cell Signaling Technology, Inc.) (Feng, Jiang, et al. 2018), Anti-FoxO3A (phospho S253) antibody (ab47285), and pan 14-3-3 (sc-629; Santa Cruz Biotechnology, 1:4000) (Rizou, Frangou, et al. 2018) to detect FoxO3a, phosphorylated FoxO3a, and 14-3-3protein, respectively. Breast cancer tissues were used as a positive control for FoxO3a and phosphorylated FoxO3a IHC-P staining (Habashy, Rakha, et al. 2011). Whereas skin keratinocyte was used as a positive control in 14-3-3 IHC-P staining.

Immunohistochemical imaging analysis: Digital images of IHC-stained slides were obtained by slide scanner, MoticEasyScan, and uploaded to the Motic digital slide server. Immunohistochemical staining was assessed and scored by using Qu path software (Bankhead, Loughrey, et al. 2017) and (Morriss, Conley, et al. 2020). Full details of QuPath including source code, documentation, links to the software download, and illustrative video supplements are available at https://qupath.github.io. The scanned images were uploaded into QuPath and manually selected the interesting area within 300 x 300 mm including the bulge area of the outer root sheath and excluded areas represented or with artifacts (tissue folding, for example) precluding assessment. According to the positive controls, the specimens required manual calibration of negative (<0.05), weak (0.05), moderate (0.1), and strong immunostaining (0.15) thresholds based upon mean cytoplasm and nuclear DAB optical densities for FoxO3a and S253 phosphorylation FoxO3a, respectively. In 14-3-3 stained, the manual calibration of negative (<0.2), weak (0.2), moderate (0.4), and strong immunostaining (0.6) thresholds based upon mean cytoplasm DAB optical densities were used. The “H-score” method of scoring was applied. H-score = $\sum$ Pi (i+1), where ‘‘Pi’’ is the percentage of stained cells in each intensity category (0–100%) and ‘‘i’’ is the intensity indicating weak (i=1), moderate (i=2), or strong staining (i=3). The H-score was determined by adding the results of multiplication of the percentage of cells with staining intensity ordinal value (scored from 0 for “no signal” to 3 for “strong signal”) with 300 possible values. In this system, <1% of positive cells were considered to be a negative result (Yilmaz, Gul, et al. 2018).

Ethical approval: Ethical approval was granted from the Ethics Committee of Thammasat University Diagnosis was established (Project no. MTU-EC-OO_6-085/64). Following the informed consent, a 5- µm punch biopsy was performed at both clinically peripilar signs of vertex balding scalp of AGA patients and from the vertex non - balding scalp of normal volunteers.

Statistical analysis: The SPSS 25.0 program was used for the statistical analyses. The nonparametric test (Mann-Whitney U test) was used to analyze protein expression among bulge areas of the outer root sheath in both normal and AGA tissue sections. A two-sided $P < 0.05$ was defined as statistically significant, and all analyses were performed using SPSS software (SPSS Version 25, Chicago, IL). ,(Feng, Jiang, et al. 2018)

4. Results and Discussion

4.1. Results

Androgenetic alopecia was diagnosed by a clinical presentation showing Hamilton-Norwood stage II-III vertex and the dermoscopic examination presenting hair miniaturization of more than 20% at the vertex

but less than 20% at the occipital scalp, and brown peri pilar sign as shown in Figure 1b. The normal group showed normal hair and scalp as illustrated in Figure 1a with the dermoscopic features showing hair follicular units containing 2-4 terminal hairs with less than 20% of hair miniaturization.

An immunohistochemical examination of FoxO3a, S253 Phosphorylation FoxO3a, and 14-3-3 expression was performed on cells residing in the bulge area of the outer root sheath at the vertex area from two healthy volunteers and three early-onset androgenetic alopecia patients. The results demonstrated original images of the immunohistochemical assay at bulge areas of the outer root sheath in the AGA group stained with FoxO3a (Figure 2a), S253 phosphorylation FoxO3a (Figure3a), and 14-3-3 (Figure4a) and in the normal group stained with FoxO3a (Figure 2c), phosphorylation FoxO3a (Figure 3c), and 14-3-3 (Figure 4c). Mark up images based on mean DAB nuclear staining intensity in S253 phosphorylation FoxO3 and mean DAB cytoplasm staining intensity in FoxO3, and 14-3-3 as follows: blue (negative), yellow (weakly positive), orange (moderately positive), and red (strongly positive) showing cell detection and classification immunoscoring by QuPath among both groups. The markup images of the AGA group stained with FoxO3a, S253 phosphorylation FoxO3a, and 14-3-3 were illustrated in Figure2b, 3b, and 4b, respectively. In the normal group, the markup images stained with FoxO3a, S253 phosphorylation FoxO3a, and 14-3-3 were shown in Figure2d, 3d, and 4d, respectively.

Although, the H-score and the percentage of total positive of FoxO3a among the AGA group were much higher than the normal group at the median of 254.14 and 98.97%, respectively, these variables were not statistically different. The H-score and the percentage of total positive of S253 phosphorylated FoxO3a were significantly higher than the normal group with a P-value < 0.001 at the median value of 32.18 and 22.37%, respectively. The expression of 14-3-3 protein compared between both groups was not significantly different, see Table 1.

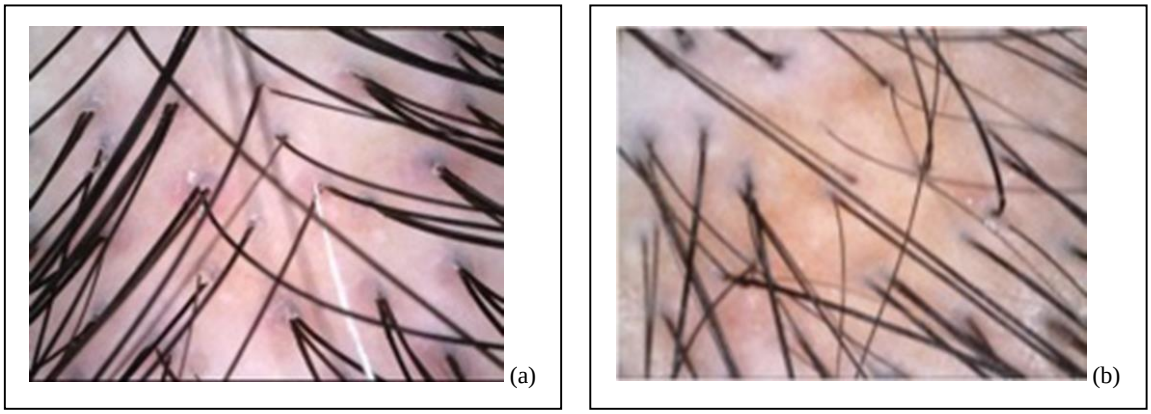

Figure 1 The pictures of dermoscopic examination of the normal group(2a) and the AGA group(2b)

Table 1 Results of the nonparametric test (Mann-Whitney U test) for H-score and percentage of total positive comparing between the AGA group and the normal group

		Normal			AGA				
Staining	**intensity**	**N**	**Mean (SD)**	**Med (Min/Max)**	**n**	**Mean (SD)**	**Med (Min/Max)**	**Z*.**	**2 tailed P-value**
FoxO3	H-score	10	205.50 (85.53)	239.67 (56/292.07)	11	243.06 (34.32)	254.14 (187.60/297.67)	-0.49	0.654
	Total positive	10	83.64 (20.80)	95.66 (43.00/100.00)	11	98.02 (2.32)	98.97 (93.00/100.00)	-1.83	0.070

		Normal			AGA				
Staining	**intensity**	**N**	**Mean (SD)**	**Med (Min/Max)**	**n**	**Mean (SD)**	**Med (Min/Max)**	**Z*.**	**2 tailed P-value**
S253 Phospho -rylation FoxO3a	H-score	11	5.34 (7.37)	2.87 (0.00/22.27)	10	32.18 (19.29)	27.16 (9.09/75.44)	-3.45	<0.001**
	Total positive	11	4.11 (5.22)	2.09 (0.00/17.00)	10	22.37 (11.75)	19.83 (6.00/48.00)	-3.59	<0.001**
14-3-3	H-score	10	299.73 (0.56)	300.00 (298.21/300)	11	299.42 (0.92)	299.60 (296.86/300)	-1.07	0.311
	Total positive	10	99.96 (0.08)	100.00 (100/100.00)	11	99.96 (0.06)	100.00 (100/100.00)	-0.69	0.462

*Z-Mann-Whitney U-test was used, **P-value <0.05, Med: median, N = bulge areas from the normal group, n = bulge areas from the AGA group, SD: Standard deviation.

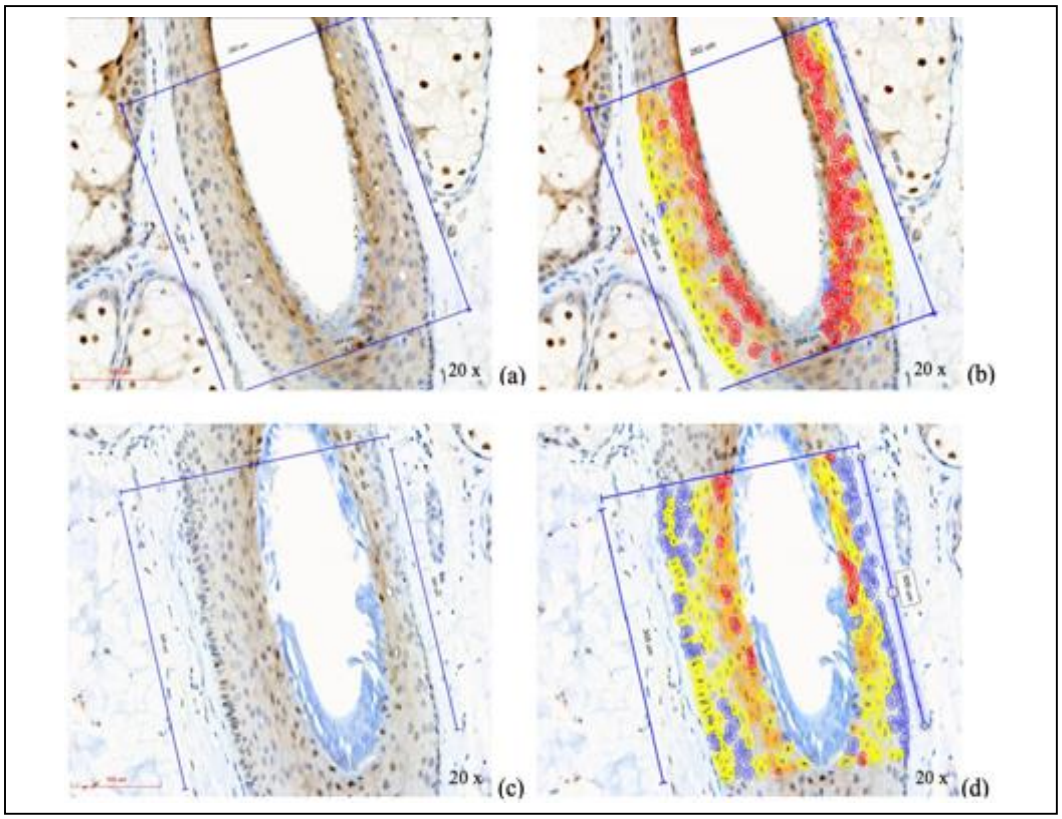

Figure 2 The Original images of the outer root sheath bulge stained with FoxO3a in AGA (2a) and normal (2c) and the markup images showing cell detection and classification using QuPath in AGA (2b) and normal (2d)

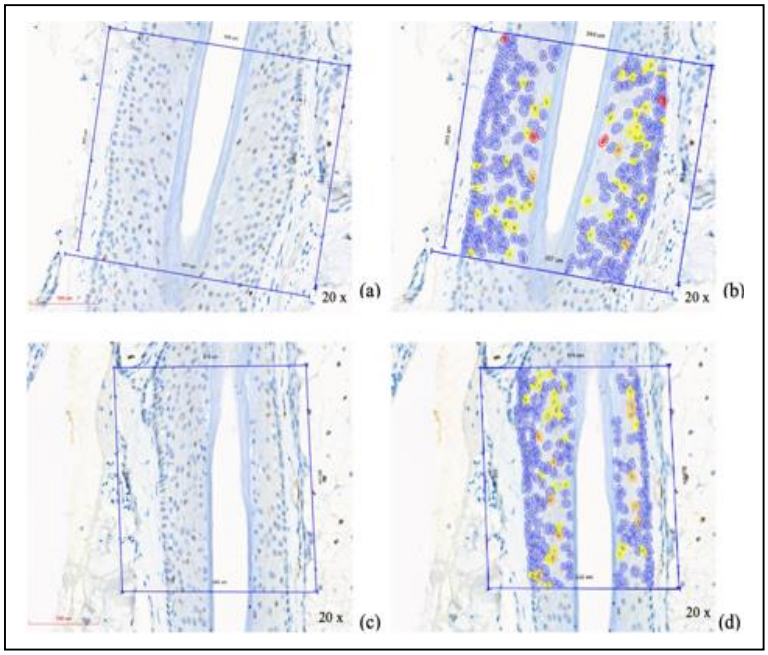

Figure 3 The Original images of the outer root sheath bulge stained with S253 Phosphorylation FoxO3a in AGA (3a) and normal (3c) and the markup images showing cell detection and classification using QuPath in AGA (3b) and normal (3d)

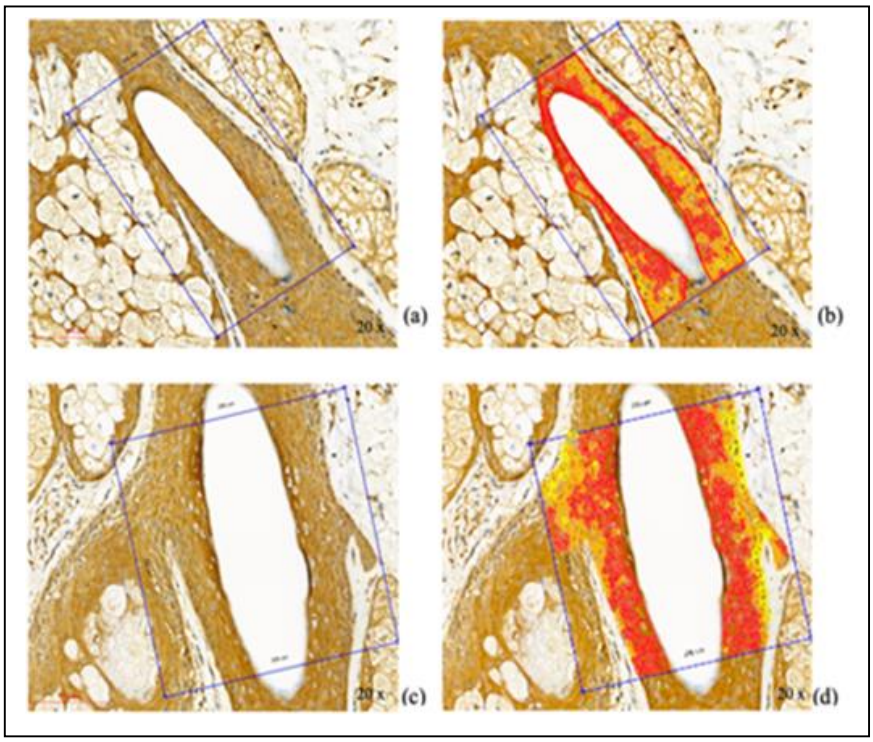

Figure 4 The Original images of the outer root sheath bulge stained with 14-3-3 AGA (4a) and normal (4c) and the markup images showing cell detection and classification using QuPath in AGA (4b) and normal (4d)

4.2. Discussion

The pathophysiology of Androgenetic alopecia (AGA) is caused by signal dysregulation in the hair cycle, consequently leading to the diminished duration of the anagen phase. The autophagy pathway regulates tissue homeostasis during skin and hair cycle development. The transition from anagen to catagen phase reveals a significant decrease in the number of LC3B-positive fluorescent dots and sequestosome 1 (SQSTM1/p62) proteins for autophagosome formation in the human anagen hair matrix keratinocytes (Parodi, Hardman, et al. 2018). Autophagy contributes to new anagen formation in mouse skin by using the specific autophagy-induced small molecule treated telogen hairs (Chai, Jiang, et al. 2019). Interestingly, a reduction in autophagic flux-related protein among the AGA group was illustrated by (Liu, Li et al. 2021) which showed that inhibition of autophagy especially from the initiation step by an interaction between BECN-1 and BCL2, led to hair miniaturization by enhancing premature catagen and cell apoptosis. Nonetheless, autophagy and its modulation mechanism in hair diseases like AGA have never been investigated. One of the upstream autophagy-controlled proteins, FoxO3a (forkhead box O3a), has been extensively researched in a variety of cell types and disorders, however, the function of FoxO3 in the hair follicle remains unknown.

The recent studies showed the relationships between the FOXOs (forkhead box O) superfamily acting as a transcription factor in various skin conditions. In skin injury, an up-regulation of FoxOs appears to aid in the healing process by influencing the proliferation, migration, and apoptosis of keratinocytes, fibroblasts, and other cells accumulating at the injured site (Tsitsipatis, Klotz, et al. 2017). FoxO1 and FoxO3 have been addressed as the key signaling axis in the regulation of epidermal morphogenesis and melanin biosynthesis, respectively (Tsitsipatis, Klotz, et al. 2017).

Since the FOXOs family primarily regulates autophagy as a transcriptional activator at multiple stages, FoxO3a is regarded as a reliable upstream autophagy signaling. In primary mouse skeletal muscle, AMPK directly phosphorylates FoxO3a, activating the FoxO3a transcription factor by promoting the production of autophagy-related proteins such as LC3B-II, Gabarapl1, and Beclin1(BECN-1) (Sanchez, Csibi, et al. 2012). Furthermore, knocking out FoxO1 and FoxO1+3 caused a significant reduction in light chain 3 (LC3), Beclin1, and sirtuin 1 (SIRT-1) protein levels after the treatment with the oxidant tert-butyl hydroperoxide (tBHP) (Akasaki, Alvarez-Garcia, et al. 2014). However, the function of FOXOs transcription factors as well as their autophagy regulation has never been elucidated in the hair follicle and hair regeneration mechanism.

Our findings showed a significant elevation of S253 Phosphorylation FoxO3a among the AGA group at the bulge area of the outer root sheath. This is the first study of FoxO3a and its related proteins in hair disease. From the previous knowledge, active AKT translocate into the nucleus and phosphorylates FoxO3a at three conserved residues (Thr 32, Ser 253, and Ser 315) generating a binding site for 14-3-3 causing FoxO3a nucleocytoplasmic translocation and eventually cytoplasmic FoxO3a degradation (Brunet, Bonni, et al. 1999). The effect of 14-3-3 on human FOXOs was later validated that 14-3-3 binding to the aminoterminal locations prevents FoxO-DNA interactions (Obsil, Ghirlando, et al. 2003) and (Silhan, Vacha, et al. 2009).

Herein, the significant expression of S253 Phosphorylation FoxO3a at the bulge area of the outer root sheath in the AGA presumably represented the binding of these molecules with 14-3-3 protein resulting in FoxO3a being excluded from the nucleus, sustained in the cytosol, and eventually degraded. Therefore, a reduction of an active form of FoxO3a in the nucleus led to poor transcriptional activities regarding the autophagy-related genes. However, an overall FoxO3a protein expression was not statistically significantly higher in the AGA group than in the normal group. This might be caused by an accumulation of cytoplasmic FoxO3a, an inactive form. Hence, the immunofluorescence study for cytoplasmic and nuclear accumulation of FoxO3a could be demonstrated to confirm the preferential subcellular localization of this protein in the disease and normal conditions.

In conclusion, we firstly demonstrated the clinicopathological relationship between FoxO3a and its related proteins in hair disease. We hypothesized that increased expression of S253 Phosphorylation FoxO3a in the AGA group could indicate that an imbalanced of this molecule is involved in the impairment of cellular

homeostasis, particularly autophagy, in stem cells located in the bulge of the outer root sheath area by decreasing anagen induction (Choi, Kang, et al. 2021), catagen inhibition (Castilho, Squarize, et al. 2009) ; (Audesse, Dhakal, et al. 2019); (Parodi, Hardman, et al. 2018), and dermal papilla cell proliferation (Choi, Kang, et al. 2021), leading to clinical manifestations of hair miniaturization. Moreover, if we can correct the depletion of S253 Phosphorylation FoxO3a, we might have a new way to treat androgenetic alopecia driven by dysregulated autophagy.

We propose additional immunofluorescence studies to localize S253 Phosphorylation FoxO3a in the nucleus as well as co-localize S253 Phosphorylation FoxO3a with other autophagy-related proteins such as BECN-1, lipidated Light Chain 3B (LC3B), and sequestosome 1 (SQSTM1/p62) in the outer root sheath bulge region to determine the interaction of FoxO3a and autophagic flux proteins in both the AGA and the normal group.

5. Conclusion

This is, to our concern, the first study demonstrating the clinicopathological significance of early-onset AGA and the imbalance in cell signaling of the protein involved in autophagy including FoxO3a, S253 Phosphorylation FoxO3a, and 14-3-3. S253 Phosphorylation FoxO3a was found to be significantly increased among the AGA group in the bulge area of the outer root sheath region, which could be the potential key molecule to correct dysregulation of autophagy in androgenetic alopecia. However, further investigation should be conducted to determine the interaction between FoxO3a and autophagy-related proteins by localizing activated nucleus FoxO3a as well as co-localizing FoxO3a with other autophagy-related proteins.

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7. References

Akasaki, Y., Alvarez-Garcia, O., Saito, M., Caramés, B., Iwamoto Y., and Lotz, M. K. (2014). FoxO transcription factors support oxidative stress resistance in human chondrocytes. *Arthritis Rheumatol*, *66*(12), 3349-3358.

Audesse, A. J., Dhakal, S., Hassell, L. A., Gardell, Z., Nemtsova, Y., and Webb, A. E. (2019). FOXO3 directly regulates an autophagy network to functionally regulate proteostasis in adult neural stem cells. *PLoS genetics*, *15*(4), e1008097.

Bahta, A. W., Farjo, N., Farjo, B., and Philpott, M. P. (2008). Premature senescence of balding dermal papilla cells in vitro is associated with p16(INK4a) expression. *J Invest Dermatol*, 128(5), 1088-1094.

Bankhead, P., Loughrey, M. B., Fernández, J. A., Dombrowski, Y., McArt, D. G., Dunne, P. D., McQuaid, S. R., Gray, T., Murray, L. J., and Coleman, H. G. (2017). QuPath: Open source software for digital pathology image analysis. *Scientific reports*, *7*(1), 1-7.

Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *cell*, *96*(6), 857-868.

Castilho, R. M., Squarize, C. H., Chodosh, L. A., Williams, B. O., and Gutkind, J. S. (2009). mTOR mediates Wnt-induced epidermal stem cell exhaustion and aging. *Cell Stem Cell*, *5*(3), 279-289.

Chacón-Martínez, C. A., Klose, M., Niemann, C., Glauche, I., and Wickström, S. A. (2017). Hair follicle stem cell cultures reveal self-organizing plasticity of stem cells and their progeny. *Embo j*, *36*(2), 151-164.

Chai, M., Jiang, M., Vergnes, L., Fu, X., de Barros, S. C., Doan, N. B., Huang, W., Chu, J., Jiao, J., and Herschman, H. (2019). Stimulation of hair growth by small molecules that activate autophagy. *Cell reports*, *27*(12), 3413-3421. e3413.

Choi, Y. K., Kang, J. I., Hyun, J. W., Koh, Y. S., Kang, J. H., Hyun, C. G., Yoon, K. S., Lee, K. S., Lee, C. M., and Kim, T. Y. (2021). Myristoleic Acid Promotes Anagen Signaling by Autophagy through Activating Wnt/β-Catenin and ERK Pathways in Dermal Papilla Cells. *Biomolecules & Therapeutics*, *29*(2), 211-219.

Fasano, C., Disciglio, V., Bertora, S., Signorile, M. L., and Simone, C. (2019). FOXO3a from the nucleus to the mitochondria: a round trip in cellular stress response. *Cells*, *8*, Article No. 1110, 1-28.

Feng, S., Jiang, Z. J., Yu, D., Li, J., Liu, G., and Sun, J. J. (2018). FOXO3a expression and its diagnostic value in pancreatic ductal adenocarcinoma. *International journal of clinical and experimental pathology*, *11*(11), 5422-5429.

Furuyama, T., Nakazawa, T., Nakano, I., and Mori, N. (2000). Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. *Biochem J* ***349****(Pt 2)*, 629-634.

Habashy, H. O., Rakha, E. A., Aleskandarany, M., Ahmed, M. A., Green, A. R., Ellis, I. O., and Powe, D. G. (2011). FOXO3a nuclear localisation is associated with good prognosis in luminal-like breast cancer. *Breast cancer research and treatment*, *129*(1), 11-21.

Jahoda, C. (1998). Cellular and developmental aspects of androgenetic alopecia. *Experimental dermatology*, *7*(5), 235-248.

Lay, K., Kume, T., and Fuchs, E. (2016). FOXC1 maintains the hair follicle stem cell niche and governs stem cell quiescence to preserve long-term tissue-regenerating potential. *Proc Natl Acad Sci U S A*, *113*(11), E1506-1515.

Leiros, G. J., Attorresi, A. I., and Balana, M. E. (2012). Hair follicle stem cell differentiation is inhibited through cross-talk between Wnt/beta-catenin and androgen signalling in dermal papilla cells from patients with androgenetic alopecia. *Br J Dermatol*, *166*(5), 1035-1042.

Liu, W., Li, G., Wang, K., Yang, L., Qu, Q., Fan, Z., Sun, Y., Huang, J., Miao, Y., and Hu, Z. (2021). Impairment of autophagy may be associated with follicular miniaturization in androgenetic alopecia by inducing premature catagen. *The Journal of Dermatology*, *48*(3), 289-300.

Martinez, L. (2017). The Optimization of the Nuclear Protein in Testis (NUT) Antibody and its Importance and Impact in the Pathology Lab. Thesis for Master Student, University of South Florida, US.

Morriss, N. J., Conley, G. M., Ospina, S. M., Meehan III, W. P., Qiu, J., and Mannix, R. (2020). Automated Quantification of Immunohistochemical Staining of Large Animal Brain Tissue Using QuPath Software. *Neuroscience*, *429*, 235-244.

Obsil, T., Ghirlando, R., Anderson, D. E., Hickman, A. B., and Dyda, F. (2003). Two 14-3-3 binding motifs are required for stable association of Forkhead transcription factor FOXO4 with 14-3-3 proteins and inhibition of DNA binding. *Biochemistry*, *42*(51), 15264-15272.

Ohyama, M. (2007). William J. Cunliffe Scientific Awards. Advances in the study of stem-cell-enriched hair follicle bulge cells: a review featuring characterization and isolation of human bulge cells. *Dermatology*, *214*(4), 342-351.

Parodi, C., Hardman, J. A., Allavena, G., Marotta, R., Catelani, T., Bertolini, M., Paus, R., and Grimaldi, B. (2018). Autophagy is essential for maintaining the growth of a human (mini-) organ: Evidence from scalp hair follicle organ culture. *PLoS biology*, *16*(3), e2002864.

Randall, V. A., Hibberts, N. A., and Hamada, K. (1996). A comparison of the culture and growth of dermal papilla cells from hair follicles from non-balding and balding (androgenetic alopecia) scalp. *Br J Dermatol*, *134*(3), 437-444.

Rizou, M., Frangou, E. A., Marineli, F., Prakoura, N., Zoidakis, J., Gakiopoulou, H., Liapis, G., Kavvadas, P., Chatziantoniou, C., and Makridakis, M. (2018). The family of 14-3-3 proteins and specifically 14-3-3σ are up-regulated during the development of renal pathologies. *Journal of cellular and molecular medicine*, 22(9), 4139-4149.

Sanchez, A. M., Csibi, A., Raibon, A., Cornille, K., Gay, S., Bernardi, H., and Candau, R. (2012). AMPK promotes skeletal muscle autophagy through activation of forkhead FoxO3a and interaction with Ulk1. *Journal of cellular biochemistry*, *113*(2), 695-710.

Silhan, J., Vacha, P., Strnadova, P., Vecer, J., Herman, P., Sulc, M., Teisinger, J., Obsilova, V., and Obsil, T. (2009). 14-3-3 protein masks the DNA binding interface of forkhead transcription factor FOXO4. *Journal of Biological Chemistry*, *284*(29), 19349-19360.

Tsitsipatis, D., Klotz, L. O., and Steinbrenner, H. (2017). Multifaceted functions of the forkhead box transcription factors FoxO1 and FoxO3 in skin. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *1861*(5), 1057-1064.

Upton, J. H., Hannen, R. F., Bahta, A. W., Farjo, N., Farjo, B., and Philpott, M. P. (2015). Oxidative stress–associated senescence in dermal papilla cells of men with androgenetic alopecia. *Journal of Investigative Dermatology*, *135*(5), 1244-1252.

Whiting, D. A. (2001). Possible mechanisms of miniaturization during androgenetic alopecia or pattern hair loss. *Journal of the American Academy of Dermatology*, *45*(3), S81-S86.

Yilmaz, E., Gul, M., Melekoglu, R., and Koleli, I. (2018). Immunhistochemical analysis of nuclear factor kappa Beta expression in etiopathogenesis of ovarian tumors. *Acta cirurgica brasileira*, *33*(7), 641-650.

Ying, J., Guo, L., Qiu, T., Shan, L., Ling, Y., Liu, X., and Lu, N. (2013). Diagnostic value of a novel fully automated immunochemistry assay for detection of ALK rearrangement in primary lung adenocarcinoma. *Annals of oncology*, *24*(10), 2589-2593.

Zhang, P., Kling, R. E., Ravuri, S. K., Kokai, L. E., Rubin, J. P., Chai, J. K., and Marra, K. G. (2014). A review of adipocyte lineage cells and dermal papilla cells in hair follicle regeneration. *J Tissue Eng*, *5*, doi.org/10.1177/2041731414556850