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Evaluation of the Expression of Genes Associated with Inflammation and Apoptosis in Androgenetic Alopecia by Targeted RNA-Seq

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Keywords
Androgenetic alopecia · Targeted RNA-Seq · Apoptosis · Inflammation

Abstract
Androgenetic alopecia (AGA) or male pattern baldness is the most common form of hair loss in humans. Despite being a very frequent dermatological entity, molecular pathophysiology remains unclear. Several authors relate the presentation of AGA with a premature apoptotic process during the anagen phase and with an inflammatory microenvironment in the hair follicle. We evaluated a panel of 30 genes associated with inflammation and apoptosis in 5 AGA patients by targeted RNA-Seq. *WNT7A* gene was highly expressed in patients in stages 3V to 5 on the Hamilton-Norwood scale compared to patients with 5A stage. *CASP7* and *TNF* genes were overexpressed in stages 3V and 4 compared to stages 5 and 5A. Overexpression of these genes detected only at early stages of AGA proves the role of WNT pathway, apoptosis, and inflammation in the development of this disorder.

Introduction

Androgenetic alopecia (AGA), or male baldness pattern androgenetic alopecia, is the most common form of hair loss in humans and affects 80% of Caucasian men and 40–50% of Caucasian women [1]. In AGA, there is an alteration of the hair growth cycle [2]. AGA is characterized by miniaturization of the hair follicle, a phenomenon that occurs due to a rapid change from the anagen phase to the catagen/telogen phase, which probably reflects an ongoing follicle apoptotic process [3]. During the hair growth cycle, there is an important interaction between growth factors such as cytokines, hormones, and neurotransmitters and their receptors [4]. However, it is still not clear if AGA results from disruption of proliferation or an increase in apoptosis in the hair follicle [5]. Despite this, one of the main targets of topic and systemic antioxidant therapies for AGA is apoptosis inhibition [6]. In fact, studies of drugs such as finasteride have shown decreased levels of caspases after 6 months of treatment in patients with AGA compared with healthy subjects [7]. Most studies evaluating caspases in patients with AGA

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Table 1. Clinical data of the studied groups

Variables	Patients	Controls
Age range, years	22–52 (33.68±10.6)	21–22 (21.5±0.7)
Males, *n* (%)	10 (100)	2 (100)
Severity of disease (Hamilton-Norwood scale)		
1	–	–
2	–	–
3V	2	–
4	3	–
5	2	–
5A	3	–
Range of duration of disease, months	24–360 (108±93.6)	0

have been performed with immune-based techniques, such as immunohistochemistry and ELISA, to analyze gene expression. The purpose of this article was to evaluate the expression of a panel of genes associated with inflammation and apoptosis in AGA using targeted RNA-Seq.

Material and Methods

Tissue Samples and RNA Isolation

Ten patients with AGA (3V to 5A) and 2 control subjects were included in the study (Table 1). Clinical and dermatological evaluation was performed to confirm the diagnosis of AGA. Patients with diagnosis of seborrheic dermatitis, telogen effluvium, and other types of alopecias were excluded from this study. The Ethics and Research Committees of the School of Medicine and University Hospital of Universidad Autonoma de Nuevo Leon at Monterrey, Mexico, approved and registered the study protocol with the code BI14-001 to be conducted from January 2014 to August 2016. Two scalp biopsies of 4 mm were collected from each subject. Patients with AGA consisted of males aged from 18 to 40 years old, who had not been treated with finasteride or minoxidil. Biopsies were obtained from the affected area and the occipital region in these patients. Biopsies from control subjects were collected from the vertex and occipital regions. RNA*later*® solution (Thermo-Fisher Scientific, Waltham, MA, USA) for RNA stabilization and storage was used to preserve biopsies. RNA from scalp biopsies was isolated with the RNeasy Blood and Tissue kit™ (QIAGEN, Hilden, Germany) and was quantified with the Quant-iT™ RiboGreen® RNA Assay kit (Thermo-Fisher Scientific). Samples collected for this study were carefully preserved and stored at –80°C until use to maintain RNA integrity before the molecular analyses were performed. The RQI (RNA quality indicator) number was evaluated using the Experion™ automated electrophoresis system (Bio-Rad, Hercules, CA, USA). The cutoff for the analyzed RNA samples had values above 7.8. Values from 7 to 10 are considered of good quality for microarray analysis or sequencing [8].

Next-Generation Sequencing

RNA (100 ng) from 5 patients (biopsies from affected and occipital areas) and 2 control subjects was used to obtain cDNA using the ProtoScript Reverse Transcriptase kit (New England Biolabs, Ipswich, MA, USA) and for the library preparation using TruSeq® Targeted RNA (Illumina, San Diego, CA, USA). Sequencing of 30 selected transcripts plus an endogenous *GAPDH* transcript control (Table 2) was carried out with MiSeq® Reagent Kit v3 (Illumina) (150 cycles) and Truseq® Target RNA Custom Panel Libraries (Illumina). Libraries were quantified using Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo-Fisher Scientific). Targeted RNA-Seq data analysis was performed using TruSeq Targeted RNA v1.0 App in BaseSpace (Illumina). After demultiplexing and FASTQ file generation, readings of each sample were aligned against specified references in the manifest using a banded Smith-Waterman alignment, which produces target hits files that contain raw aligned replicate counts for each transcript. Differential expression analysis was performed in which depth of sequencing normalization, variance estimation, and *p* values were calculated. *p* values reaching statistical significance for the differential expression were adjusted for false discovery rate (FDR) using the Benjamini-Hochberg method. A heat map was generated using Graph Pad Prism 7 (Graph Pad Software, Inc., La Jolla, CA, USA), considering the gene expression value as $\log_{10}$ of the number of readings for each gene per patient.

Validation by Real-Time PCR

Differential expression of *CASP7*, *WNT7A*, and *TNF* genes identified by targeted RNA-Seq was confirmed using real-time PCR analysis. The altered expression of *WNT7A* (ID: Hs01114990_m1), *CASP7* (ID: Hs00169152_m1), and *TNF* (ID: Hs00174128_m1) genes in AGA were further confirmed in independent scalp biopsies, using *AK3* (ID: Hs00750254_s1) as endogenous gene, based on the low variability observed in all samples tested by a previous microarray analysis. First strand cDNA was then synthesized from 2 μg total RNA using Superscript First Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). Subsequently, real-time PCR reactions were performed using cDNA, TaqMan Universal PCR Master Mix, and Taqman Gene Expression Assays (Thermo-Fisher). The analysis was performed in a LightCycler 480 II Real-Time PCR System (Roche Applied Science, Pleasanton, CA, USA), all according to the manufacturers' instructions.

Statistical Analysis

Comparison of numerical variables between the study groups was made using Student *t* test for independent samples normally distributed. *p* values <0.05 were considered significant. An unpaired Student *t* test analysis was used to detect gene expression by real-time PCR analyses using GraphPad Prism 7 software.

Results

Next-Generation Sequencing

A panel of 30 genes associated with inflammation and apoptosis was performed for 5 patients with AGA and 2 controls by targeted RNA-Seq panel (Table 2). Higher expression of *CASP3* ($p = 0.0003$), *CASP7* ($p = 0.0001$),

Table 2. Genes included in the RNA-Seq panel

Gene	Function
TNF	This cytokine is involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation
CASP3	Is a member of the cysteine-aspartic acid protease (caspase) family; plays a central role in the execution phase of cell apoptosis
CASP7	Is a member of the cysteine-aspartic acid protease (caspase) family; plays a central role in the execution phase of cell apoptosis/inflammation
CASP8	Is a member of the cysteine-aspartic acid protease (caspase) family; plays a central role in the execution phase of cell apoptosis
CASP10	Is a member of the cysteine-aspartic acid protease (caspase) family; plays a central role in the execution phase of cell apoptosis
BAX	BCL2-associated X protein, act as anti- or pro-apoptotic regulator that is involved in a wide variety of cellular activities
TNFRSF1A	Is one of the major receptors for the tumor necrosis factor-α; can activate NF-κB, mediate apoptosis, and function as a regulator of inflammation
TXN	Thioredoxin; inhibits oxidative stress and caspase 3 activity
BCL2	Encodes an integral outer mitochondrial membrane protein that blocks the apoptotic death
POMC	One peptide produced from the POMC protein is α-MSH involved in regulating the pigment-producing cells of the skin and hair (melanocytes), where it binds to melanocortin 1 receptor (MC1R)/inflammation
MC1R	Controls melanogenesis/inflammation inhibitor
MC4R	Encoded protein interacts with adrenocorticotropic and MSH hormones and is mediated by G proteins/inflammation inhibitor
TPT1	Microtubule stabilization/inflammation
FASLG	Fas ligand (TNF superfamily, member 6); induction of apoptosis triggered by binding to FAS
BCL3	Involved in regulation of cell proliferation and contributes to transcriptional regulation of NFKB. Pro-survival and pro-inflammatory
NFKB1	Inflammation/cell damage. Nuclear Factor Kappa B Subunit 1; is a transcription regulator which stimulates the expression of genes involved in a wide variety of biological function
TYRP1	Encodes a melanosomal enzyme that belongs to the tyrosinase family and plays an important role in the melanin biosynthetic pathway. Inflammation
DCT	Regulating eumelanin and phaeomelanin levels/inflammation
MLANA	Involved in melanosome biogenesis/oxidative stress
WNT7A	Wnt Family Member 7A, implicated in oncogenesis and in several developmental processes. Hair growth/inflammation inhibitor
MITF	Involved in melanocyte survival, controls the expression of genes related to melanin synthesis. Cell differentiation, proliferation, and survival/inflammation
MAPK1	Mitogen-activated protein kinase 1, involved in cell proliferation and differentiation/ inflammation
CAPN3	Intracellular protease/activated in apoptotic cells
CFLAR	Is a regulator of apoptosis and is structurally similar to caspase-8/apoptosis inhibitor
CCBL2	Cysteine conjugate-beta lyase 2, involved in the regulation of oxidative stress/inflammation

Table 2 (continued)

Gene	Function
CDC5L	Cell cycle control/inflammation
CSNK1G3	WNT signaling/inflammation
PHACTR2	Phosphatase and actin regulator 2/inflammatory cytokine in dermal cells
GGT1	Involved in maintenance of intracellular GSH level. It is part of the cellular antioxidant defense mechanism/inflammation
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase. Endogenous control/constitutive expression.
GPD1	Glycerol-3-phosphate dehydrogenase 1, involved in the redox metabolism
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase. Endogenous control/constitutive expression

The function of the genes was extracted from: GeneCards Human Gene Database and UCSC Genome-Browser.

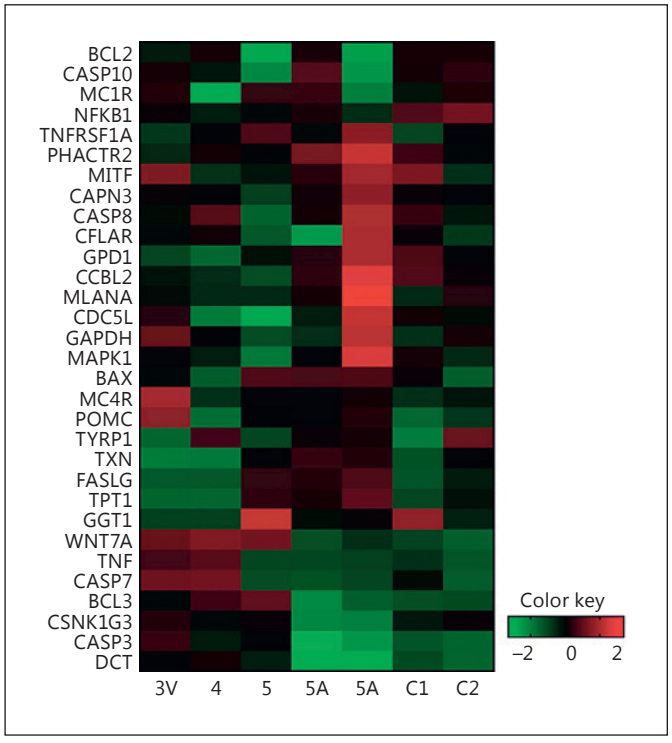

Fig. 1. Heat map of the profile of expression differences between the balding regions of patients with types 3V to 5A of androgenetic alopecia (Hamilton-Norwood scale). Genes with high expression are observed in red (upregulated), while those with low expression are represented in green (downregulated) ($p < 0.05$). Data was processed using the Graph Pad Prism 7 program. Each column represents a type of androgenetic alopecia according to the Hamilton-Norwood scale and each row represents a gene. The gene expression value corresponds to $\log_{10}$ of the number of readings for each gene per patient.

BCL3 ($p = 0.0166$), *WNT7A* ($p = 0.0005$), and *TNF* ($p = 0.0004$) was found in the affected area of patients with 3V, 4, and 5; and underexpressed in 5A patients (Fig. 1).

Validation by Real-Time PCR

Targeted RNA-Seq analysis was confirmed using real-time PCR analyses for selected genes. Overexpression of *CASP7* ($p = 0.0001$) and *TNF* ($p = 0.0005$) in patients with types 3V and 4 was confirmed. Underexpression of these same genes was also confirmed in patients with types 5 and 5A. *WNT7A* ($p = 0.0001$) was overexpressed in patients with types 3V to 5, whereas it was underexpressed in type 5A patients. Differences were confirmed using unpaired Student *t* test between two groups (3V–4 vs. 5–5A and 3V–5 vs. 5A) (Fig. 2). Expression analyses of *CASP3* and *BCL3* were not evaluated due to limitations in sample availability.

Discussion

The role of the Wnt pathway in AGA is well established, as it is involved in the hair growth cycle and the morphogenesis of the hair follicle. Therefore, overexpression of *WNT7A* in moderate degrees (3V to 5) and its underexpression in the 5A severe phenotype prove that in late stages of AGA, the hair growth cycle is suppressed, causing follicles to remain in the catagen/telogen phase. In this study, *CASP7* and *TNF* showed overexpression in stages 3V and 4, but not in stages 5 and 5A (Fig. 1). *CASP7* belongs to the subgroup of executioner caspases, as does

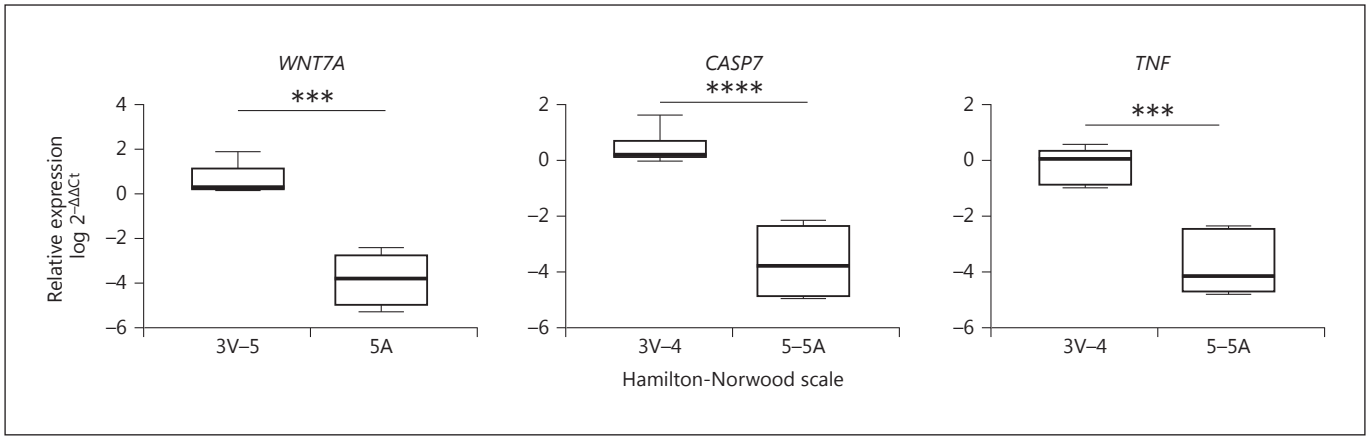

Fig. 2. Validation of the expression of *WNT7*, *CASP7*, and *TNF* genes by real-time PCR. Unpaired *t* test was used to detect differences in gene expression between 2 groups (3V–4 and 5–5A types) using the Graph Pad Prism 7 software. Significance: *** $p < 0.001$ and **** $p < 0.0001$.

CASP3 and *CASP6*. Interestingly, *CASP7* proteolytic maturation is involved in apoptosis and inflammation, in contrast to *CASP3*, which participates in the apoptosis process only [9]. *CASP7* expression is observed in cultured keratinocytes and in the anagen phase of the hair follicle in vivo [10, 11]. Nevertheless, the role of *CASP7* in the hair follicle is still controversial, since the gene remains active during the proliferation and differentiation processes in absence of evident apoptosis, suggesting additional roles for this enzyme [12].

Conversely, *TNF* is an inflammatory cytokine that plays a well-established role and acts on several different signaling pathways through two cell surface receptors, being probably the most potent inducer of apoptosis [13]. TNF has been recently added to the list of growth factors regulating hair follicle morphogenesis and has been traditionally associated with host defense, immunity, inflammation, and cancer [14]. TNF appears to protect against hair loss. The blockade of TNF by monoclonal antibodies resulted in alopecia areata in a patient treated with infliximab [15]. *TNF* overexpression in early phases of AGA and its downregulation in advanced stages of the disease suggest a protective effect for this protein.

Caspase-3 plays an important role in apoptosis, as it is activated in the apoptotic cell by extrinsic (death ligand) and intrinsic (mitochondrial) pathways [16]. Overexpression of *CASP3* was only found in stages 3V to 5, indicating that this gene is involved in early stages of AGA. In contrast, *CASP3* is less expressed in advanced stages, probably reflecting the profuse destruction of hair follicles and the resulting perifollicular fibrosis. These results coincide with the report by Fawzi et al. [17], which stated that *DKK1* levels, a WNT inhibitor, only influenced early stages of AGA. Overexpression of *BCL3* in the clinical presentations 3V to 5 of AGA may reflect a response of the affected hair follicles to *CASP3* expression, since elevated *BCL3* activity points towards increased cell proliferation and survival [18].

There are several limitations in our study. The sample size for each type of the Hamilton-Norwood scale needs to be increased to clarify *CASP3* and *WNT7A* overexpression at early stages, like in types 1–3, where minimal bitemporal hair recession is evident. In addition, the number of transcripts analyzed in this study may result limited to study additional pathways involved in AGA progression. Due to the small number of samples, it was not possible to evaluate the expression of *CASP3* and *BCL3* genes by real-time PCR, which were also overexpressed in types 3V to 5 AGA patients. However, in this pilot study we proved the efficiency of molecular techniques as RNA-Seq and real-time PCR for the analysis of androgenetic alopecia, since to date, most studies evaluating caspases in patients with AGA have used immunohistochemistry and ELISA techniques to analyze the gene expression. It is also important to mention that the number of samples has been the limitation of other breakthrough studies in the AGA research field, since the diagnosis of this entity does not required a biopsy. Also, techniques such as microarray, sequencing, and real-time PCR generate a higher cost, compared with techniques of immunohistochemistry and ELISA.

In conclusion, this study proves that *WNT7A*, *CASP7*, and *TNF* are overexpressed in alopecia areas in patients with AGA at early stages of the disease. This result confirms the role of WNT pathway, apoptosis, and inflammation on early stages of this disorder. Targeted RNA-Seq is a useful technique for the evaluation of gene expression in AGA samples, as it only requires a small amount of input material for evaluating large numbers of genes.

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Statement of Ethics

The Ethics and Research Committees of the School of Medicine and University Hospital of Universidad Autonoma de Nuevo Leon at Monterrey, Mexico, approved and registered the study protocol with the code BI14-001 to be conducted from January 2014 to August 2016.

Disclosure Statement

The authors declare that there is no conflict of interest that could be perceived as prejudicial to the impartiality of the reported research.

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