Bioinformatics Approach to Investigate the Genes Manifesting Alopecia Areata

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Research

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Abstract

Background: Alopecia areata (AA) is a type of alopecia or hair loss, which is very common in human. It is classified as an autoimmune disorder, which has a variable course. It can be either relapsing or persistent type. The persistent type is seen in patients with extensive hair loss. AA affects young people most commonly with an age less than 20 years but can also concern adults. It makes up to 4% dermatology cases in China, around 2-3% in UK and USA and 0.7% in India. Patients with alopecia have social and economic suffering due to anxiety symptoms, avoidance behavior, and social anxiety disorder, making it a very important non lethal disease to study.

Methods: In the present study, microarray datasets GDS5274 and GDS5272 of AA have been re-analyzed from mouse as well as human respectively. The simultaneous analysis of model organism and patient data has provided two pronged validation approach to delineate potential biomarkers of the disease. Out of 45101 genes of model organism (*Mus musculus*), and 54675 genes of patient (*Homo sapiens*), top 100 up regulated and down regulated genes were selected and further analyzed by DAVID and Enrichr tools for KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways, GO (gene ontology) (cellular component, molecular function and biological process).

Results: Four genes viz. CXCL9, CXCL10, STAT1 and CCL5 were differentially regulated in both organisms, hence can be considered as plausibly contributing in triggering the AA. The network and pathway analysis by PathwayLinker2.0 revealed the partners of these crucial genes i.e. CCR1, CCR5, IGFBP7, VCAN, DPP4, CCR3, CXCR3 through which these genes might coordinate to manifest hair fall.

Conclusions: The dual analysis approach has helped to generate plausible novel biomarkers of the disease for diagnostic and therapeutic approach. Stimulation of any of these biomarkers by various triggers can damage hair follicle. These genes can be targeted therapeutically to halt the hair follicle damage by inhibiting their expression hence, providing novel future drug targets for AA.

Background

Alopecia areata (AA) is a type of alopecia or hair loss, which is very common in human. It is an autoimmune disorder with variable course, which can be either relapsing or persistent type with extensive hair loss(1). Non-scarring alopecia follows differential patterns in male and female and is the second most prevalent alopecia type(2). Peak incidence rate appears in the age of 15-29 years(3). According to the National Alopecia Areata Foundation (NAAF), it affects approximately 2% of the overall population(4). It has incidence rate of 4% in China, 2-3% in UK and USA, and around 0.7% in India (5) . [Alessandro Sette](#) and others used IEDB (The Immune Epitope Database) to study autoimmune epitope data related to AA(6). Bioinformatics approaches have been used extensively for identifying diagnostic and therapeutic biomarkers in various clinical conditions, however, in case of alopecia; no systematic bioinformatics analysis has ever been reported. Due to lack of data analytic approaches, progress in this field is very slow. In the present study an extensive bioinformatics based approach has been used to fill the gap in

alopecia data analysis. To make this approach holistic, two datasets of AA were taken from microarray data repository. Up and down regulated genes of both the datasets were selected. These were further analyzed by DAVID and Enrichr tools for cellular component, molecular function and biological process information. The dual analytic approach of model organism along with patient data analysis has given plausible pathways based novel biomarkers.

Methodology

- **Data Collection and Organization:** In GEO (Gene expression Omnibus), from (National Centre for Biotechnology Information) NCBI, AA was selected as disease name and 4 datasets were displayed. Two datasets **GDS5274** and **GDS5272** with similar experimental framework were selected from GEO database. The complete work flow for the present analysis has been detailed in figure 1. Datasets were assigned into various groups where GSM's **1105879-81** were taken as experimental and GSM's **1105882-84** were taken as control of series Similarly, GSM's **1105869-73** were taken as experimental and GSM'S **1105874-78** were taken as control of series **GSE45512**. Top 100 up and down regulated genes from *M.musculus* and *H.sapiens* were selected for the analysis.
- **Functional enrichment analysis and KEGG pathway analysis:** Retrieved genes were queried with two enrichment databases viz. Enrichr updated version 2018 and DAVID version 6.8 (The Database for Annotation, Visualization and Integrated Discovery) for evaluating the significant gene ontology (GO) terms. This includes evaluating the enrichment significance of gene ontology (GO) terms. P-value of <0.05 was selected as threshold. Data retrieved in common from both enrichment tools were selected for construction of networks among the queried genes and their neighbors by Pathwaylinker2.0 (<http://pathwaylinker.org/>).

Results And Discussion

Bioinformatics tools have panned a way greatly in exploring the new vistas for finding biomarkers and targets for various diseases(7–9). In the present study, GEO the genomic data repository was used for data sourcing. Two GEO datasets: GDS5272 (*H.sapiens*) and GDS5274 (*M.musculus*) were extracted. Out of 45101 genes of model organism (*M.musculus*), and 54675 genes of AA patient (*H.sapiens*) investigated, top 100 up and downregulated genes were selected in each datasets and further analyzed as shown in Venn diagrams depicted in the workflow (Figure1).

In order to find the commonalities and corroboration of patient data with experimental model organism data, both the datasets were analyzed in a combinatorial approach(10). Gene enrichment analysis was done to interpret the functional annotation of distinctively expressed genes(11). In order to find out whether the functionally expressed genes were related to a particular biological process or molecular function(12), the calculation of enrichment p-value was done by comparing the observed frequency of an annotation term with frequency expected by chance. Only the genes with p-value less than 0.05 (p-value<0.05) were deemed enriched. Figure S₁.1 (a) mentioned in supplementary data shows KEGG

pathways for up regulated genes of *M.musculus* from Enrichr and S₁.1 (b) shows that from DAVID. Five common pathways of up regulated genes from both the tools were selected as shown in Table 1. The genes associated with these pathways were further screened. Involvement of Chemokine signaling pathway was seen in both the tools hence it was chosen along with CXCL10, 1TK, CXCL11, CXCL9, STAT1, STAT2, CCL5, CCL2, CXCR6 and CCR5 genes, which were validated by both the tools. Figure S₁.2 give details of GO cellular component domain of *M.musculus* providing functional location of the genes. CXCL10, CXCL9, CXCL11, IFNG, CCL2, CCL5, LTB, SPP1, and TNFSF10 were shown to be present in extracellular space. In the detailed result of GO biological processes in figure S₁.3, both Enrichr and DAVID result ratifies that the genes CXCL10, CXCL9, CXCL11, CCL5 seem to be involved in positive regulation of T cell chemotaxis. GO Molecular function domain is shown in figure S₁.4 which shows that the genes CCL2, CCL5, CXCL10, CXCL11, CXCL9 perform Chemokine activity.

KEGG pathway	<p>Chemokine signaling pathway CXCL10,1TK,CXCL11,CXCL9,STAT1,STAT2,CCL5,CCL2,CXCR6,CCR5</p> <p>Cytokine-cytokine receptor interaction CXCL10,CXCL11,CXCL9,CXCR6,CCR5,IFNG,TNFSF10,LTB</p> <p>Influenza A CXCL10,IFNG,STAT1,STAT2,CCL5,TNFSF10,CCL2</p> <p>Toll-like receptor signaling pathway CXCL10,CXCL11,CXCL9,STATA1,CCL5,SPP1</p> <p>Herpes simplex infection IFNG,STAT1,STAT2,CCL5,CCL2,IFIT1</p>
GO Cellular component	<p>Extracellular space CXCL10, CXCL9,CXCL11, IFNG, CCL2, CCL5,LTB, SPP1, TNFSF10</p> <p>Cytoplasm CCL2,CCL5, CCR5, IFNG,IFIT1,SPP1, STAT1, STAT2</p> <p>Extracellular region CCL2,CCL5,CXCL10, CXCL9,IFNG,SPP1</p> <p>External side of plasma membrane CCR5, CXCL10,CXCL9, IFNG</p>
GO Molecular function	<p>Chemokine receptor binding CXCL10,CXCL11,CXCL9</p> <p>Tumor necrosis factor receptor binding LTB, STAT1, TNFSF10CXCR3</p> <p>Heparin binding CXCL11, CXCL10, CCL5, CCL2</p> <p>Chemokine activity CCL2, CCL5, CXCL10, CXCL11, CXCL9</p> <p>Cytokine activity CXCL10, CXCL9, IFNG, SPP1, TNFSF10, CCL1, CCL2, CCL5,LTB</p>
GO Biological process	<p>Inflammatory response CXCL10,CXCL9,CXCL11,CCL5,CCL2</p>

T+type I interferon signaling pathway

STAT1,STAT2,CCL2,IFIT1

Positive regulation of T cell chemotaxis

CXCL10,CXCL9,CXCL11,CCL5

Table 1

KEGG, GO analysis of up regulated genes of *M. musculus* (p-value<0.05)

Results for down regulated genes of *M.musculus* were obtained and validated as shown in supplementary data (Fig.S₂), similarly results for up and down regulated genes of *H.sapiens* were obtained and validated using both the tools Enrichr and DAVID. (Fig S₃-S₄)

The up regulated genes of *M.musculus* mostly included chemokines. CXCL10 (C-X-C Motif Chemokine Ligand 10) is an antimicrobial gene encoding a Chemokine of the CXC subfamily and ligand for the receptor CXCR3. The present data also suggests that in AA lesions the infiltration of CXCR3+ Th1 cells around the hair bulbs might be induced by the increased activity of CXCL10 which is actually a Th1 Chemokine as shown in Table 1(13) .

The function of innate immune response is based on distinctive receptors called PRRs (pattern-recognition receptors) which have an ability to recognize conserved microbial structures called PAMPs (pathogen associated molecular patterns). Innate immune response discriminates between self and non-self antigens because of PRRs. Toll-like receptors (TLRs) are a group of PRRs which play an active role in identification of danger and initiation of immune response(14). In the present study, as depicted in Table 1, CXCL10, CXCL11, CXCL9, STATA1, CCL5, SPP1 genes might be involved in Toll- like receptor signaling pathway. Alzolibani and others confirmed that as compared to healthy individuals, the gene expression of intracellular TLRs (TLR-3, 7, 8, 9) have been found to be higher in AA patients. Dysregulated expression of TLR-3, TLR-7, TLR-8 and TLR-9 in peripheral blood cells of AA patients involved in their signaling cascade leads to dysregulation of Th-1, Th-17 and regulatory T-cell cytokines(15). Therefore they deduced that in the pathogenesis of AA, the poorly regulated expression of TLRs and cytokines play an important role due to the improper activation of TLRs.

The present study also shows IFNG as up regulated gene component, which is an interferon gamma gene encoding a soluble cytokine, a member of type II interferon class. It is involved in pathways like Herpes simplex infection, Cytokine-cytokine receptor interaction at extracellular space and external side of plasma membrane as shown in Table 1. It is produced by lymphocytes and plays an important role in immunoregulatory functions. Duncan and others stated that feeding high levels of dietary Vitamin A to mice accelerated the onset of AA which is further associated with decreased IFNG level(16). Results from few other studies have also suggested that although IFNG is important for AA outset but the level of IFNG involved in skin drops as AA advances.

Down regulated genes of *M.musculus* were investigated by KEGG pathway analysis as shown in Table 2. Circadian entrainment, Gastric acid secretion, Insulin secretion, Glutamatergic synapse, salivary secretion, GABAergic synapse pathways have been shown to be indirectly related to AA(17,18). In another study it was found that in AA skin, the expression of cytokines (CXCL10, CX3CL1, CCL5, CXCL1) gene controlling complicated immune responses was over expressed(19). CX3CL1 is instigated by the action of IFN-gamma, and is responsible for the amplification of one way response of polarized T-helper 1, indicating a Th1 type of response in AA skin. Increased expression of CCL5 due to the action of gene IL-1 beta and TNF-alpha have attributes to high Th1 pathway (20). Bellavista and others presented that during Herpes zoster (HZ) infection, the pain could be considered as a stress factor, which potentially triggers recurrent AA. One of the possibilities is that HZ manifests cutaneous inflammatory reactions such as Koebner phenomenon which in turn induces AA (21). Koebner phenomenon is also known as koebnerization or isomorphic response which is described as the formation of skin lesions on parts of the body that are not typically involved, meaning the lesions appear in the areas other than the usual spots which are affected by cutaneous disease like psoriasis(22–28).

KEGG pathway	Circadian entrainment PER2, RASD1, ADCY1, GNG13
	Glutamatergic synapse GRIK1, ADCY1, SLC38A3, GNG13
	Gastric acid secretion ATP1B4, SLC26A7, ADCY1
	Insulin secretion KCNC1, ATP1B4, ADCY1
	GABAergic synapse ADCY1, SLC38A3, GNG13
	Salivary secretion KCNMA1, ATP1B4, ADCY1
GO Cellular component	Terminal bouton GRIK1, KCNMA1
	Dendrite GRIK1, KCNMA1, KCNC1
	Neuronal cell body KCNC1, KCNMA1, GRIK1
GO Molecular function	Structural molecular activity KCNMA1
GO Biological process	Potassium ion transport KCNMA1, KCNC1
	Circadian rhythm PER2, ADCY1, KCNMA1

Table 2

KEGG, GO analysis of down regulated genes of *M.musculus* (p-value<0.05)

Another up regulated gene Granzyme B (GZMB) encodes a member of the granzyme subfamily of proteins, a part of the peptidase S1 family of serine proteases and is involved in allograft rejection and type I diabetes mellitus (Table 3). It participates in inducing apoptosis of target cells for NK (natural killer) cells and cytotoxic CD8+ lymphocytes which are part of the innate immune system. Boivin and others

have revealed the role of GZMB in severing ECM (extracellular matrix) proteins, auto antigens, and receptors NOTCH1 and FGFR1(29). This impacts the hair follicle in AA skin type and changes the structure of the connective tissue layer and signaling within the hair follicle stem cells and dermal papilla. If the extracellular matrix is damaged, its loss may lead to cell death, vacating the place for immune cells to infiltrate the follicular space and breakdown its immune privilege. Thus, remodeled immunolocalization of GZMB by vitamin A can also cause a lot of cellular damage at different follicular sites.

KEGG pathway	<p>Cytokine-cytokine receptor interaction</p> <p>XCL1, XCL2, CXCL9, CXCL10, CCL13, CCL18,CCL5,TSLP,IL7</p> <p>Herpes simplex infection</p> <p>HLA-DRB5, HLA-DRB4, HLA-DRB3, HLA-DRB1, HLA-DQA1,HLA-DQB1, CCL5,STAT1</p> <p>Allograft rejection</p> <p>HLA-DRB5, HLA-DRB4,GZMB, HLA-DRB3, HLA-DRB1, HLA-DQA1,HLA-DQB1</p> <p>Type I diabetes mellitus</p> <p>HLA-DRB5, HLA-DRB4,GZMB, HLA-DRB3, HLA-DRB1, HLA-DQA1,HLA-DQB1</p> <p>Influenza A</p> <p>CCL5,CXCL10,HLADRB1,RSAD2,STAT1, HLA-DQA1,HLA-DQB1</p> <p>Rheumatoid arthritis</p> <p>HLA-DRB5, HLA-DRB4, HLA-DRB3, HLA-DRB1, HLA-DQA1,HLA-DQB1,CCL5</p> <p>Toxoplasmosis</p> <p>HLA-DRB5, HLA-DRB4, HLA-DRB3, HLA-DRB1, HLA-DQA1,HLA-DQB1, STAT1</p>
GO Cellular component	<p>Extracellular space</p> <p>CCL13, CCL18, CCL5, CXCL10, CXCL9, XCL1, IL7, TSLP</p> <p>Extracellular region</p> <p>CCL13, CCL5, CXCL10, CXCL9, XCL1,IL7</p> <p>External side of plasma membrane</p> <p>CXCL10,CXCL9,HLA-DRB1</p> <p>MHC class II protein complex</p> <p>HLA-DRB1, HLA-DQA1, HLA-DQB1</p> <p>Integral component of lumenal side of endoplasmic reticulum membrane</p> <p>HLA-DRB1, HLA-DQA1, HLA-DQB1</p> <p>Transport vesicle membrane</p> <p>HLA-DRB1, HLA-DQA1, HLA-DQB1</p> <p>Clathrin-coated endocytic vesicle membrane</p> <p>HLA-DRB1, HLA-DQA1, HLA-DQB1</p> <p>ER to Golgi transport vesicle membrane</p>

	<p>HLA-DQA1,HLA-DQB1, HLA-DRB1</p> <p>Endocytic vesicle membrane</p> <p>HLA-DQA1,HLA-DQB1, HLA-DRB1</p> <p>Trans-Golgi network membrane</p> <p>HLA-DQA1,HLA-DQB1, HLA-DRB1</p> <p>Integral component of membrane</p> <p>HLA-DQA1,HLA-DQB1, HLA-DRB1</p> <p>Lysosomal membrane</p> <p>HLA-DQA1,HLA-DQB1, HLA-DRB1</p>
GO Molecular function	<p>Chemokine activity</p> <p>CCL13,CCL18,CCL5,CXCL10,CXCL9,XCL1</p> <p>MHC class II receptor activity</p> <p>HLA-DQA1,HLA-DQB1,HLA-DRB1</p> <p>CCR chemokine receptor binding</p> <p>CCL13,CCL18,XCL1</p> <p>Peptide antigen binding</p> <p>HLA-DQA1,HLA-DQB1,HLA-DRB1</p> <p>Cytokine activity</p> <p>CXCL9,IL7,TSLP</p>
GO Biological process	<p>Immune response</p> <p>CCL13, CCL18, CCL5, CXCL10, CXCL9, IL7, HLA-DQA1, HLA-DQB1, HLA-DRB1</p> <p>Cell-cell signaling</p> <p>CCL13, CCL18, CCL5, CXCL10, CXCL9,XCL1,IL7</p> <p>Chemokine-mediated signaling pathway</p> <p>CCL13, CCL18, CCL5, CXCL10, CXCL9, XCL1</p> <p>G-protein coupled receptor signaling pathway</p> <p>CCL13, CCL18, CCL5, CXCL10, CXCL9,XCL1</p> <p>Inflammatory response</p> <p>CCL13, CCL18, CCL5, CXCL10, CXCL9</p> <p>Chemotaxis</p>

CCL13, CCL18, CCL5, CXCL10, CXCL9

Signal transduction

CCL13, CCL18, XCL1, CXCL10, CXCL9

Table 3

KEGG, GO analysis of up regulated genes of *H.sapiens* (p-value<0.05)

In GO Cellular Component, very important and significant finding of current investigation was genes HLA-DRB1 and HLA-DQB1. Both of these genes are involved in notable pathways like type 1 diabetes mellitus, Influenza A, toxoplasmosis etc. and clathrin-coated endocytic vesicle and MHC (major histocompatibility complex) class II receptor activity as shown in Table 3. Human Leukocyte antigen (HLA) system is a gene complex, responsible for the production of MHC protein, acting at the surface of cells and is responsible for all immune actions of the cell. In the present study, HLA is involved in all three GO components and pathways as shown in Table 3. HLA-DRB1*11:04 allele has been strongly associated with alopecia in Iraqi Arab Muslims patients and is highly involved with early onset and severe patchy AA (30). HLA-DRB1*04 allele group poses a risk factor for the development of AA whereas the allele DRB1*0401 predominates in the population of Belgium and Germany(31). This shows that genetic effects of the HLA system play a crucial role in familial cases of AA (32), similar findings have been corroborated by the present study. Many more studies were done in UK and North America, conferring the risk of DRB1*04 in AA (32,33). 80% of AA patients were affected by HLADQB1*03 allele, and this allele also covers 92% of patients with total or universal AA(34)

KEGG pathway analysis of down regulated genes of *H.sapiens* indicated that HLA-DRB4, FGG, CNTNAP2, HLA-DRB4, COMP, FGF18 genes are involved in the most significant enriched pathways, as shown in Table 4. GO cellular component analysis indicates that COMP, FGG, FGF18 genes are present in extracellular space. FGF18 is the predominant gene in the hair follicles; it is responsible for inducing anagen from telogen stage hair follicles. It has been shown as one of the main target and well-known marker of the Wnt signaling pathway which is down-regulated in AA lesional skin. Coda and others proved that Wnt/ β -catenin signaling pathway are down regulated in the blood of AA patient by up-regulated Wnt suppressors, which claims that an over-expression of Wnt/ β -catenin signaling inhibitors may govern to develop AA(35). Similar pathways such as Chemokine signaling pathway, Cytokine-cytokine receptor interaction, Influenza A, Toll-like receptor signaling pathway, Herpes simplex infection were obtained in data analytic approach from *H.sapiens* as well as *M.musculus*, which further validates the use of this species as experimental model organism in studies related to AA.

KEGG pathway	<i>Staphylococcus aureus</i> infection HLA-DRB4,FGG Cell adhesion molecules (CAMs) CNTNAP2, HLA-DRB4 PI3K-Akt signaling pathway COMP, FGF18
GO Cellular component	Extracellular space COMP, FGG,FGF18
GO Molecular function	Structural molecular activity FGG
GO Biological process	Vocalization behavior CNTNAP2

Table 4

KEGG, GO analysis of down regulated genes of *H. sapiens* (p-value<0.05)

After critically observing all the data, four genes came out to be common in both organisms which were executing their function in triggering the AA. These genes were further analyzed using Pathway Linker tool to assess their inter-relationship. The network and pathway analysis revealed the partners of these crucial genes which are CXCL9, CXCL10, STAT1 and CCL5. All four genes were found to be up regulated (Figure 2).

The suggested model for disesse progression has been constructed by taking common nodes among all the four genes as given in inset (Figure 3). The anagen hair follicle of AA expresses STAT1, CXCL9, CCL5 and CXCL10 genes along with IFNG in every part of follicular epithelium,which also includes the area adjacent to the dermal papilla of the hair follicle (Figure 3).

The hair follicle is itself a complex mini organ with specific immune and hormonal microenvironment. Immune privilege is the most interesting aspect of hair follicle integrity. AA occurs on disintegration of MHC class I based immune privileges of anagen hair follicles, which can be futher prompted predominately by these genes. Hair follicle enters into the anagen phase of the hair cycle, in which active melanogenesis i.e. the formation of melanin occurs. Consequently hair follicle autoantigens are recognised by intrafollicular infiltrate CD8+ T cells. Finally, an attack by CD8+ Tcell on the anagen hair follicular epithelium due to the presence of perifollicular infiltrate of CD4+ T cells results in hair loss. The pathway generated from the analysis using Enrichr, DAVID and PathwayLinker tools is shown in figure 3. The pathway depicts the regulatory phenomenon of anagen which could play a critical and crucial role in hair follicle damage in alopecia.

Conclusion

After all the critical analysis of data, a new pathway strategy for manifestation of AA has been suggested as depicted in figure 4 which explains how all the genes i.e. STAT1, CCR1, CCR5, CCL5, IGFBP7, VCAN, CXCL10, DPP4, CCR3, CXCL9, CXCR3 mentioned above in datasets analysed coordinate to manifest hair fall.

Future perspective

The genes viz CCL5, CXCL10, CXCL9, STAT1 are involved in manipulation of hair follicle structural integrity. Stimulation of any of these genes by various triggers can damage hair follicle. These genes can be targeted to halt the hair follicle damage by inhibiting their expression. Hence these genes can be future drug targets for AA.

List Of Abbreviations

AA Alopecia Areata

IEDB Immune Epitope Database and Analysis Resource

GEO Gene Expression Omnibus

NCBI National Centre for Biotechnology Information

DAVID The Database for annotation, visualization and integrated discovery

GO Gene Ontology

KEGG Kyoto Encyclopedia of Genes and Genomes

NAAF National Alopecia Areata Foundation

PRR Pattern-recognition receptors

PAMP Pathogen associated molecular patterns

TLR Toll-like receptor

HZ Herpes zoster

ECM Extracellular matrix

HLA Human Leukocyte antigen

MHC Major histocompatibility complex

NK Natural killer

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets analyzed during the current study are available in GEO (Gene Expression Omnibus) of NCBI (National Centre for Biotechnology Information) repository.

<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE45513>

<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE45512>

Competing interests

The authors declare that they have no competing interests.

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Author's Contributions

VP selected the Microarray datasets related to alopecia areata. Bioinformatics study strategy was designed by VP. SK performed the research, interpreted the results and wrote the manuscript. VP edited the manuscript.

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Figures

Figure 1

Flow chart of data processing and analysis. A) & B) Venn diagrams representing gene selection of *M. musculus* and *H.sapiens* respectively.

Figure 3

Diagrammatic representation of Alopecia areata anagen hair follicle. Inset: Linked pathway.

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