Bioinformatics analysis of genes associated with the patchy-type alopecia areata: CD2 may be a new therapeutic target

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Background. Alopecia areata (AA) is mainly a T cell-medicated autoimmune disease with non-scarring hair loss and limited treatment options. Of these, the patchy-type alopecia areata (AAP) is the most common and relatively easy to treat due to smaller areas of the scalp affected. To understand the pathogenesis of AAP and explore the therapeutic target, we focus on the molecular signatures by comparing AAP and normal subjects.
Methods. The gene expression profile (GSE68801) was obtained from Gene Expression Omnibus (GEO) database. Differentially expressed genes (DEGs) were identified between AAP patients and normal controls using the GEO2R. Then the Gene ontology (GO) analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis and Protein-Protein interaction (PPI) network analysis were performed for DEGs.
Results. A total of 185 DEGs were identified, including 45 up-regulated genes and 140 down-regulated genes. The up-regulated DEGs were related to the immune response and chemokine signaling pathway. Meanwhile, down-regulated DEGs were enriched in keratin filament and intermediate filament. Subsequently, the top 10 hub genes were picked out in the PPI network, among them, CD2 showed the highest connectivity degree and central roles.
Conclusion. Our data suggest that the CD2 may be a new therapeutic target for AAP. Further study is needed to explore the value of CD2 in the treatment of alopecia areata.

Key words: patchy-type alopecia areata, differentially expressed genes, function enrichment, protein-protein interaction network, CD2.

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INTRODUCTION

Alopecia areata (AA) is a multifactorial disease with nonscarring hair loss resulting from disrupted immune privilege, autoimmune-mediated destruction, and up-regulated cytokine pathways of hair follicle, which was a great mental burden on patients[1]. The prevalence of alopecia varies by race and region, ranging from 0.1% to 0.2% of the general population[2]. Due to the difference of clinical manifestations, AA was classified into three major phenotypic variants patchy-type AA (AAP), alopecia totalis (AT) and alopecia universalis (AU) (ref.[3]).

Histological examination shows that hair follicles of AA are infiltrated by $CD4^+$ and $CD8^+$ T cells, natural killer (NK) cells and macrophages, etc[4], but no essential differences have been established between AAP and AT/AU samples. Besides this, the genome wide association study (GWAS) and subsequent Genome-wide meta-analysis found that *ULBP* are risk genes for activating ligands of the NK cell receptor NKG2D, which interacts with mainly $CD8^+NKG2D^+$ T cells, accelerating the pathogenesis of AA (ref.[5-7]). Other recent studies also showed that cytotoxic $CD8^+NKG2D^+$T cells were critical for AA in mouse models of disease and inhibition of Janus kinases (JAKs) has potential clinical utility, based on the AA signature connected with interferon-γ, IL-2, TH1, TH2, IL-23, and IL-9/TH9 cytokine activation[8-10].

Although much research on AA has been done, the precise pathogenesis and etiology of AA remains unknown, especially the specific subtype. Of three major phenotypic variants, AAP is limited to well-demarcated patches and relatively easy to treat early before progressing to the entire scalp or body hairs. Thus, it is necessary to gain insight into the pathogenesis of AAP, using gene expression analysis to explore therapeutic targets. In the present study, we analyzed the gene expression profile of AAP patients versus normal controls to screen for differentially expressed genes (DEGs). Next, Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed

for DEGs. In the end, we created a PPI network to explore the hub genes related to AAP.

METHODS

Data resource

The gene expression of GSE68801 was obtained from Gene Expression Omnibus database (GEO, http://www.ncbi.nlm.nih.gov/geo/) of NCBI, which was performed on Affymetrix Human Genome U133 Plus 2.0 Array platform. Our dataset included 22 AAP patients samples and 36 normal controls.

Screening of DEGs

The GEO2R tool was performed to identify differently expressed genes (DEGs) between AAP patients and normal controls. GEO2R employed GEO query of BioConductor and package limma of *R* to perform the statistical analysis[11]. Adjusted *P*-value (adj. *P* Val)<0.05 and a *fold-change* (FC)<1.0 were set as the thresholds for DEGs.

GO term and KEGG pathways analysis

GO annotation and KEGG pathway enrichment analysis of DEGs were performed using Database for Annotation, Visualization, and Integrated Discovery (DAVID, https://david.ncifcrf.gov/)[12] which is a bioinformatics library that integrates biological data and analysis tools to provide systematic functional annotations for large-scale gene or protein lists. *P*<0.05, FDR (false discovery rate) <0.05 and the count ≥ 5 were set as thresholds for the pathway enrichment analysis.

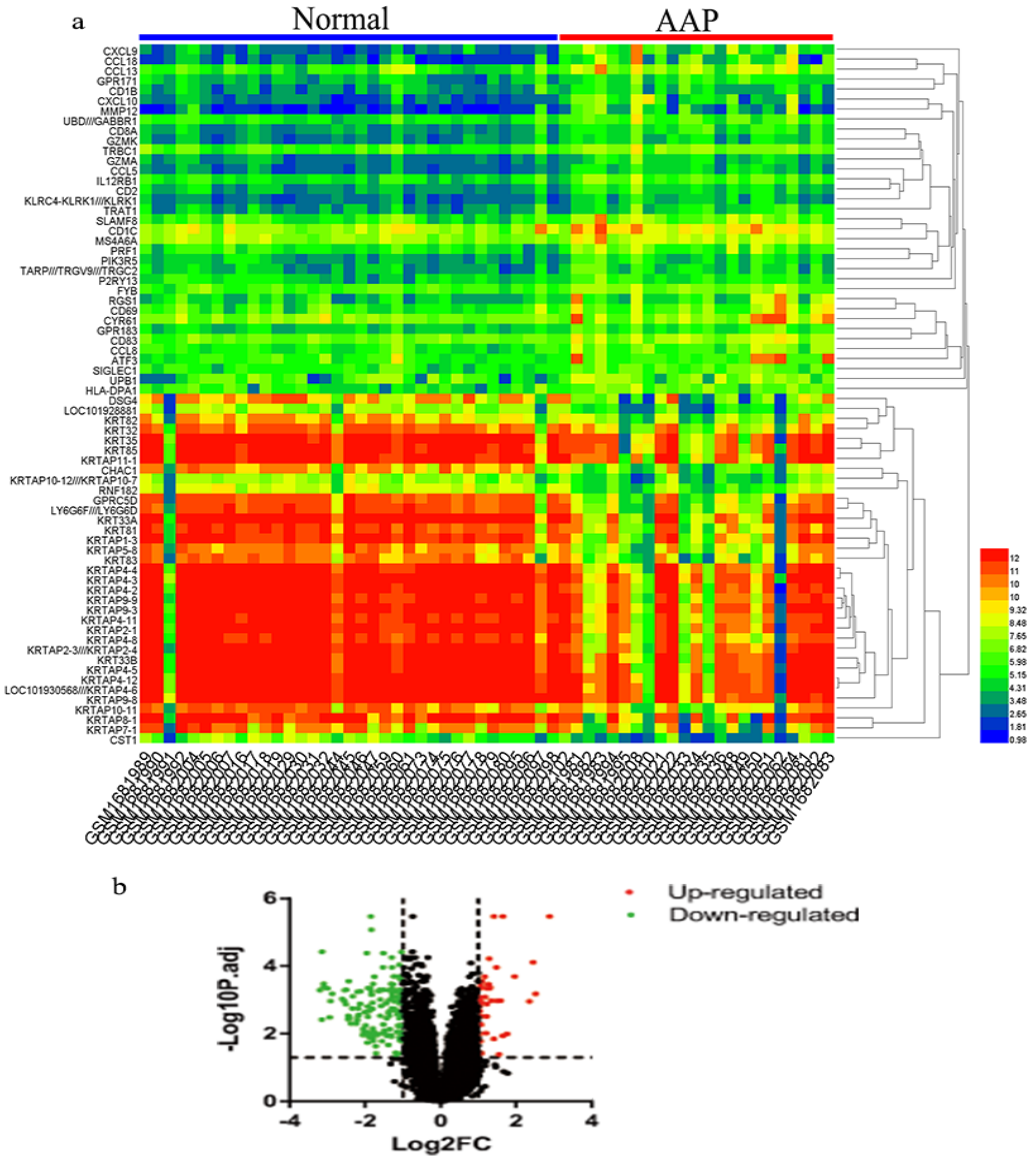

Fig. 1. The heatmap and volcano plot of DEGs. (a) In the heatmap, red color represents upregulated genes and the green color represents downregulated genes. (b)
In the volcano plot, X-axis is fold change (log2) and Y-axis is adjust *P* value (-log10). Red points (fold change>1) indicate upregulated genes, whereas blue points (fold change<-1) indicate downregulated genes. Moreover, the darker red indicates a stronger upregulation in expression and the darker green indicates a stronger downregulation.

Table 1. Top 10 up- and down-regulated DEGs between AAP patients and normal controls.

	Gene	logFC	*P*	FDR
UP-DEG	*CXCL9*	2.88676392	1.60E-10	0.00000339
	CCL18	2.51395408	1.69E-06	0.00065419
	CXCL10	2.44544086	2.98E-08	0.00007655
	MMP12	2.35115806	4.20E-06	0.00109837
	CCL13	1.96138773	1.71E-07	0.00020337
	RGS1	1.75863712	1.26E-04	0.01004194
	CD8A	1.65169431	2.76E-10	0.00000339
	ATF3	1.64663753	1.53E-04	0.01142259
	SLAMF8	1.58567182	3.82E-06	0.00104597
	UPB1	1.54259012	1.18E-03	0.04047649
DOWN-DEG	*DSG4*	-3.1948376	1.09E-06	0.00051453
	KRTAP8-1	-3.14079175	3.00E-05	0.00379357
	CHAC1	-3.13925852	5.54E-09	0.00003773
	GPRC5D	-3.09278597	4.27E-07	0.0003292
	LY6G6F///LY6G6D	-3.08911462	6.07E-07	0.000415
	KRTAP1-3	-2.9955714	6.96E-07	0.00045319
	KRTAP7-1	-2.93512285	2.35E-05	0.00324861
	KRT83	-2.91776267	4.09E-06	0.00107937
	KRT33A	-2.87255385	1.77E-06	0.00065875
	KRT81	-2.60421786	3.15E-06	0.00093709

DEG – differentially expressed genes; FDR – false discovery rate; log FC – log fold change

Table 2. Functional and pathway enrichment analysis of up-regulated and down-regulated genes in the AAP patients versus normal controls.

Category	Term	Description	Count	*P*	FDR
Up-regulated DEGs					
BP	GO:0006955	immune response	14	2.65E-12	3.49E-09
BP	GO:0071346	cellular response to interferon-gamma	6	1.51E-07	1.99E-04
BP	GO:0006935	chemotaxis	7	2.51E-07	3.30E-04
BP	GO:0070098	chemokine-mediatedsignaling pathway	6	4.60E-07	6.04E-04
BP	GO:0060326	cell chemotaxis	5	1.23E-05	0.016118368
BP	GO:0030593	neutrophil chemotaxis	5	1.30E-05	0.017131228
BP	GO:0007186	G-protein coupled receptor signaling pathway	11	1.71E-05	0.022427246
BP	GO:0050852	T cell receptor signaling pathway	6	1.73E-05	0.022677588
BP	GO:0007267	cell-cell signaling	7	1.77E-05	0.023274812
BP	GO:0007165	signal transduction	12	2.64E-05	0.034672508
CC	GO:0009897	external side of plasma membrane	8	3.02E-07	2.95E-04
CC	GO:0005887	integral component of plasma membrane	13	2.48E-05	0.024228567
MF	GO:0008009	chemokine activity	6	5.08E-08	5.49E-05
KEGGPATHWAY	hsa04062	Chemokine signaling pathway	7	1.87E-05	0.020847485
Down-regulated DEGs					
BP	GO:0042633	hair cycle	7	6.80E-11	9.70E-08
BP	GO:0007568	aging	8	2.00E-05	2.90E-02
CC	GO:0045095	keratin filament	32	1.60E-44	1.80E-41
CC	GO:0005882	intermediate filament	17	2.80E-17	3.10E-14
MF	GO:0005198	structural molecule activity	16	7.70E-12	9.10E-09

DEGs – differently expressed genes; Counts – number of DEGs; BP – biological process; CC – cellular compartment; MF – molecular function

Construction of the protein-protein interaction (PPI) network

PPI network of DEGs was constructed using STRING database (http://string-db.org/), which maps a set of genes to be studied into network of relevant protein interactions to analyze hub proteins and screen out key genes associated with the specific disease. Interactions with Combined Score>0.4 were retained and then visualized using Cytoscape software (www.cytoscape.org/).

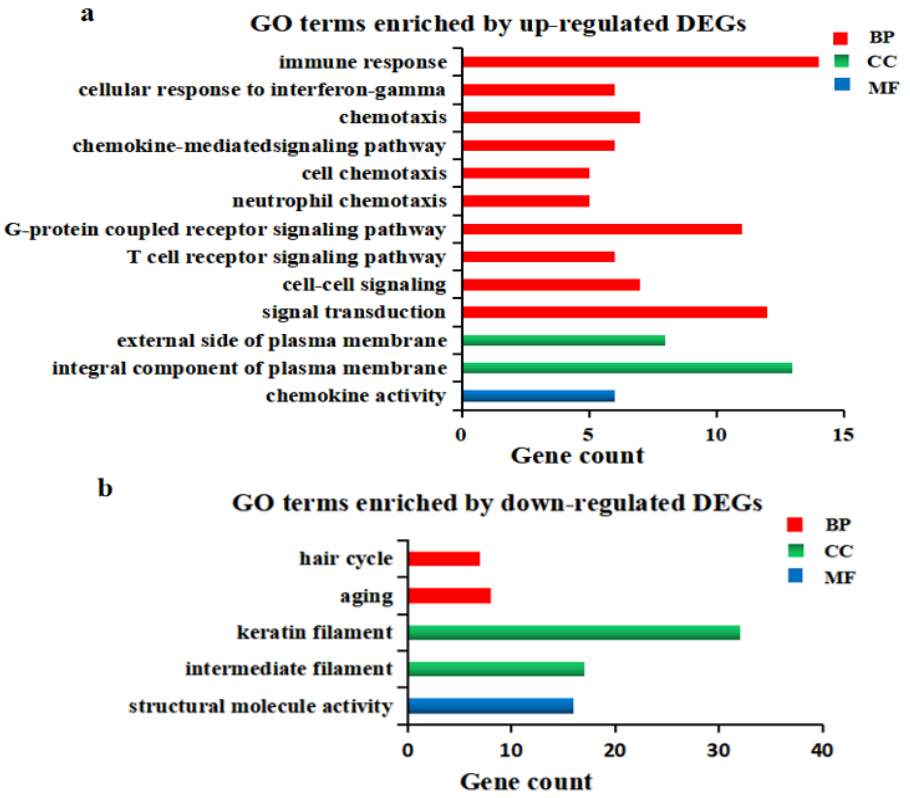

Fig. 2. The enriched (count≥5) gene ontology terms of up-regulated DEGs (a) and down-regulated DEGs (b).
BP – biological process; CC – cellular component; MF – molecular function.

RESULTS

DEGs Identification

According to the predetermined threshold (adj. *P* Val<0.05 and $|\log_2 FC| > 1.0$), a total of 185 DEGs, including 45 up-regulated genes and 140 down-regulated genes, were screened out. The top 10 up- and down-regulated DEGs are listed in Table 1. Subsequently, a heatmap of the top 35 up-regulated and down-regulated DEGs and volcano plot of all DEGs was created to visualize expression variation between AAP patients and normal controls (Fig. 1a, b).

Function enrichment analysis of DEGs related to AAP

GO terms and KEGG pathways enrichment analysis was performed using DAVID to demonstrate the function of screened DEGs (Table 2). GO enrichment analysis indicated that the up-regulated genes were mainly enriched in biological processes associated with the immune response, signal transduction and inflammatory response (Table 2 and Fig. 2a). Down-regulated genes were mainly enriched in the hair cycle and aging (Table 2 and Fig. 2b). In terms of the cellular component, up-regulated genes were mainly enriched in the plasma membrane, including the integral component of plasma membrane and external side of plasma membrane, while down-regulated DEGs were primarily enriched in keratin filament and intermediate filament. In terms of molecular function, up-regulated genes were significantly enriched in chemokine activity, whereas down-regulated genes were mainly enriched in structural molecule activity. Subsequently, the results of KEGG pathway analysis indicated that the up-regulated DEGs were primarily enriched in Chemokine signaling pathway. However, there is no pathway for down-regulated genes to be enriched in.

Table 3. Degree of the DEGs (differential expressed genes) in the protein-protein network.

Gene	Degree
CD2	17
CCL5	16
CXCL9	16
CD3D	15
CXCL10	14
KRTAP17-1	13
CD69	13
GZMA	11
BMP2	10
CD163	10

PPI network construction and hub genes identification

By mapping separately the up- and down-regulated DEGs into STRING database, the PPI networks of DEGs were visualized using Cytoscape (Combined Score ≥ 0.4). A total of 93 nodes and 184 edges were contained in the PPI network (Fig. 3). Degrees ≥10 is used as a cutoff criterion for judging of hub protein. The top 10 hub genes were screened out, including *CD2, CCL5, CXCL9, CD3D,*

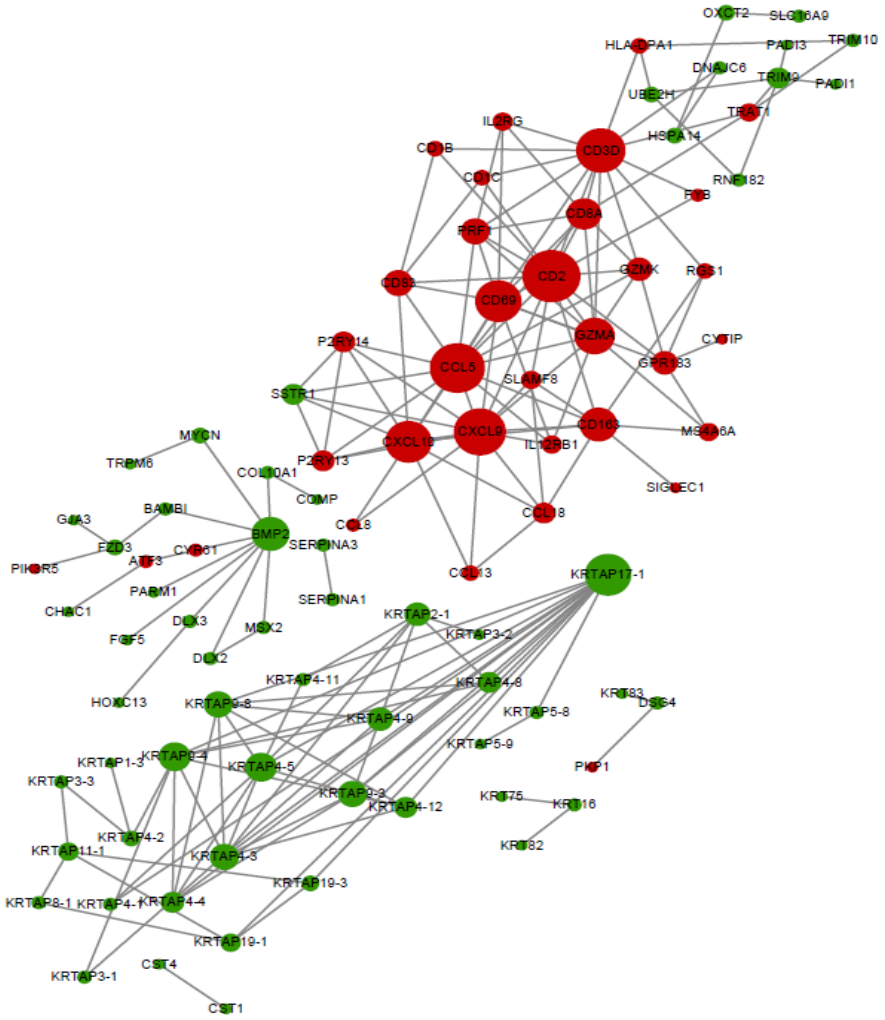

Fig. 3. Protein-protein interaction network constituted by up- and down- regulated DEGs and visualized using Cytoscape (Combined Score>0.4). Red nodes stand for up-regulated genes, while green nodes stand for down-regulated genes. The lines represent interaction relationship between nodes. Besides, the size of nodes represents the connectivity degree and the larger the size, the larger the degree.

CXCL10, CD69, KRTAP17-1, GZMA, BMP2, CD163 (Table 3). Among them, *CD2* showed the highest connectivity degree, and all the genes except *KRTAP17-1* and *BMP2* were up-regulated.

DISCUSSION

Alopecia areata (AA) is a multifatorial, non-scarring hair loss disease in which common clinical manifestations is a clear round or oval patchy hair loss on the scalp. Among three major phenotypic variants, AAP is the most common and relatively easy to treat due to smaller areas of the scalp area or beard area. Although the key role of autoimmunity in the pathogenesis of AA has been proved, the core genes and therapeutic targets of AAP still need further identification.

Hence, in our study, GSE68801 was utilized to screen the DEGs between 22 AAP patients and 36 normal controls from the GEO database. We identified a total of 185 DEGs, in which 45 genes were up-regulated and 140 genes were down-regulated. Based on the results of GO and KEGG pathway enrichment analysis, the up-regulated DEGs were mainly enriched in immune response, which are consistent with previous reports[9]. The down-regulated DEGs were mainly involved in cell component associated with keratin filament and intermediate filament. Further, it was reported that only these four functional keratins genes (*KRT81, KRT83, KRT85 and KRT86)* are related to human genetic disorders of the hair and/or nails[13-15]. Besides this, *KRT85* plays a more critically important role in the structure of hair and nail than other keratins, therefore, mutations in *KRT85* may cause alopecia universalis and exceptionally abnormal nails, due to recessive ectodermal dysplasia of hair and nails[14]. According to our research, although the keratins DEGs of *KRT81, KRT83,* and *KRT85* were all significantly down-regulated, these patients still suffer from AAP, implying the close connection between the genes encoding four keratins and pathogenesis of AA. Further, the PPI analysis showed

that desmoglein 4 (*Dsg4*) and plakophilins 1 (*PKP1*) were connected with the *KRT83,* which was in accordance with relevant researches on the importance of *PKP1* and *Dsg4* for the hair shaft to maintain integrity[15-18]. Therefore, down-regulated *Dsg4* and up-regulated *PKP1* may play critical roles in the pathogenesis of AAP.

Furthermore, we analyzed the PPI network and the top 10 core genes were identified, including *CD2, CCL5, CXCL9, CD3D, CXCL10, KRTAP17-1, CD69, GZMA, BMP2, and CD163*.

CD2, also known as LFA-2, acts as an adhesion molecule expressed on T cells and natural killer cells that binds CD58 on APCs, facilitating TCR binding and signal transduction. Meanwhile, various studies have demonstrated the important role of CD2 in T-cell activation, the production of cytokines by T-cells and T-or NK-mediated effector responses[19-21]. Besides this, the protein encoded by *CD3D* is part of the T-cell receptor/CD3 complex (TCR/CD3 complex) and is involved in T-cell development and signal transduction. More importantly, an increasing number of studies show that anti-CD2 treatment that block the CD2-CD58 co-stimulatory signals could be the therapeutic strategies for dampening autoimmune diseases, since CD2-CD58 interactions promote the synthesis of IL-2 and interferon-γ (IFN-γ), as well as T and NK cells-mediated cytotoxicity[20,22,23]. Hence, although there is no direct research on anti-CD2 treatment to reverse the alopecia areata, the prospect is still cheerful.

In addition to *CD2* and *CD3D,* another T cells surface antigen, *CD69*, an early T cells activation marker, has been reported to distinguish tissue-resident memory T cells from circulating subsets, and thus, the up-regulation of CD69 may trigger retention of memory $CD4^+$ and $CD8^+$ T cells in relevant tissues, resulting in autoimmune diseases such as rheumatoid arthritis, psoriasis as well as alopecia areata[24-27]. Therefore, our findings are consistent with the report by Suárez-Fariñas et al. (ref.[8]). and Krueger et al.(ref.[28]).

Also, it is interesting that *CXCL9/CXCL10*, known as the monokine and interferon γ-induced protein 10 (IP-10), respectively, are strongly induced by IFN-γ, leading to migration of immune cells to focal sites, such as scalp and hair follicles, through the CXCL9, CXCL10, CXCL11/CXCR3 axis[29,30]. Besides this, Xing et al. (ref.[7]) pointed out the potential clinical application of JAK inhibition in human AA by downregulating effectors of a series of cells surface cytokine receptors, including mainly IFN-γ and IL-2, which are the two most abundant cytokines produced by human $CD4^+$ and $CD8^+$ T cells. *CCL5*, one of the subfamilies of chemokines (C), plays a similar function to *CXCL9* and *CXCL10* in the recruitment and retention of T cells or other lymphocytes, contributing to this mainly T cell-mediated autoimmune hair loss disease. Collectively, our results support the view that *CXCL9, CXCL10* and *CCL5* are associated with up-regulation of inflammation of alopecia areata, rheumatoid arthritis and psoriasis, which was also involved with the JAK/STAT signaling pathway[31-34].

Combined with the roles that the above 6 hub genes play, we verified that the CD2/CD3D/CD69 promote T cell activation, and subsequently $CD4^+$ and $CD8^+$ T cells produce cytokines IFN-γ and IL-2 to induce up-regulation of CXCL9, CXCL10 and CCL5. Our research confirms further the effect of T cells-mediated autoimmune and the upregulated cytokine pathways on hair follicle damage in alopecia areata.

In addition, the Keratin-associated protein 17-1 (*KRTAP17-1*) is a member of the ultrahigh sulfur keratin-associated protein (KAP) family[35]. The KAP proteins are involved in forming a matrix of keratin intermediate filaments that support the structure of hair fibers, in turn, the down-regulation of *KRTAP17-1* may accelerate the pathogenesis of AAP. Granzyme A (*GZMA*) is an abundant serine protease in the cytolytic granules of cytolytic T cells and NK cells, inducing caspase-independent cell death when delivered into target cells by perforin[36]. Bone morphogenetic protein 2 (*BMP2*), an extra-follicular macro-environment modulator, inhibited the transition of hair cycle from telogen to anagen and kept the hair stem cells relatively dormant[37]. It means that the down-regulated *BMP2* could promote the hair regeneration. *CD163*, one member of the scavenger receptors, is exclusively expressed in monocyte/macrophage lineage, and increased soluble CD163 was significantly involved with the pathogenesis of diverse autoimmune diseases, including rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus[38].

CONCLUSION

In summary, the present study identified DEG differences between AAP and normal healthy control with bioinformatics data mining and found the potential key genes and pathway of AAP. Of ten hub genes, CD2 may be selected as a new therapeutic target for AAP. In addition, the role of keratins in the pathogenesis of AAP should not be ignored. However, further validation of these related genes and pathways is needed to investigate the pathogenic mechanism of AAP.

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Author contributions: JS, PP, WL: manuscript writing and literature search; PM: data analysis; CX: language correction; GN, SF: final revision.

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