Mechanisms of Sex Differences in Ankylosing Spondylitis: Interleukin 2 Receptor Subunit Beta and CD3d Molecule Play an Important Role

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Research

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Abstract

Introduction:ankylosing spondylitis (AS) is characterized by sexual dimorphism in clinical manifestations. Our aim is to reveal the reason for gender difference in AS.

Material and Methods: we used bioinformatics to analyze gene expression data of GSE73754. And verified by PCR and ELISA.

Results: the pathogenic pathways leading to AS are Th1 and Th2 cell differentiation. The key gene involved in this pathway in male is interleukin 2 receptor subunit beta (IL2RB). The key genes involved in female are IL2RB and CD3d molecule (CD3D) . Both genes are immune-related genes.

Conclusion: differences in key genes contribute to gender differences in AS. Male AS may require only one gene, while female AS requires two genes.

1 Introduction

Ankylosing spondylitis (AS) is a chronic, inflammatory, systemic disease that primarily affects the axial joints, including the sacroiliac joints and the spine [1, 2, 3]. This disease may cause chronic pain in the lower back and hip, progressive damage to the hip joint, and other complications such as Cardiopulmonary disease, anterior uveitis, inflammatory bowel disease, etc [1, 2, 3, 4, 5, 6, 7, 8]. In the past, studies on the pathogenesis of AS have involved many fields, including genetics, environment, gut microbiota, hormone, etc [1, 2, 7, 8, 9]. Heredity is one of the factors of susceptibility in AS population and is considered as an endogenous promoter [2, 8, 11, 12]. Diversified clinical symptoms of AS may be due to the abnormal expression of multiple genes, but the key targeted genes have not been found. The pathogenesis of AS has not been fully elucidated, and there is no curative treatment.

Some researches have shown that AS is commonly seen in young males [1, 2, 13]. Qian Q et al. [14] found that the male-female ratio of patients with AS was 2.7:1, and the male was more likely to be HLA-B27 carriers than female, with a higher level of C-reactive protein (CRP). Other researches have suggested that the incidence of spinal cord changes is higher in male patients with AS, and their radiological progression is faster, while women may have more peripheral arthritis [15, 16, 17]. In addition, the effect of drugs on the treatment of AS is gender-dependent [17, 18, 19]. The different clinical characteristics and therapeutic effects of AS in both sexes have been confirmed [15, 16, 17, 17, 18, 19]. The pathogenesis of AS may be gender-dependent. Moreover, there is no obvious evidence for sex hormones as a trigger [20, 21, 22].

No researches have been conducted on the differentially expressed genes (DEGs) and pathways of AS in different genders. There may be differences in the expression of genes in male and female patients, which can reduce the accuracy of the results. Previous studies researches on pathogenesis of AS have ever not focused on gender. The investigation of key genes and related pathways will contribute to the search for the reasons of gender differences in AS. In order to fill the gap in the researches of gender differences in AS, we used multiple bioinformatics methods to analyze the data in GSE73754. The

purpose of the study is to reveal the key molecules and pathways in AS patients with different gender, which is conducive to a further comprehensive understanding of the biological characteristics of AS and to provide new molecular targets for personalized treatment.

2 Materials & Methods

2.1 Microarray Data

Data (GSE73754) were obtained from GPL10558 (Illumina humanht-12 V4.0 expression beadchip), including 52 AS samples (26 males, 26 females) and 20 healthy samples (10 males, 10 females). All samples were divided into 4 groups: male AS (M-AS) group, female AS (F-AS) group, male No-AS (M-NAS) group, female No-AS (F-NAS) group (Table 1). The data are analyzed and compared in the following order. This research is based on the data of open database. Ethics and patient consent are not applicable.

Table 1
grouping of samples in GSE73754

	AS		No-AS	
	Male	Female	Male	Female
Samples	26	26	10	10

2.2 Differentially Expressed Genes (DEGs)

GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/) is an online analysis tool, that is used to identify DEGs [23]. We used GEO2R to identify DEGs between the M-AS and M-NAS groups, and DEGs between F-AS and F-NAS groups, respectively. The analysis results were saved in the file format, and the truncation condition of DEGs was set as: adj $P < 0.05$, and $|Log\ fold\text{-}change\ (FC)| > 0.390$.

2.3 Enrichment Analysis of GO Function and KEGG Pathway

The DEGs were respectively analyzed by the plugin ClueGO [24] in the Cytoscape software(version: 3.6.1) [25] for Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment. Contents of GO Function analysis include Cellular component, Biological process and Molecular function. The standard setting with a statistically significant difference was $P < 0.05$ and Kappa score was 0.4. Benjamini-Hochberg corrected $P < 0.05$ was considered to be valid. Gene counts were $> = 3$ in the GO enrichment and $> = 4$ in the KEGG pathway.

2.4 PPI network construction

We integrated the DEGs into protein-protein interaction (PPI) network separately, and used String [26] (version 11.0) to evaluate the interaction between DEGs. The composite score of interaction was 0.4, which was considered to be statistically s1ignificant. The analysis results of PPI network were loaded into Cytoscape software for visual adjustment. Module-genes were screened by the plugin MCODE [27] with a default parameter setting that was degree cut-off = 2, node score cut-off = 0.2 and K-core value > 5.

CytoHubba [28] (version 0.1) is a plug-in in Cytoscape software to identify top 10 hub-genes from PPI networks by Degree method.

2.5 Identification of key genes and pathways

Hub-module-pathway genes were obtained by the intersection of hub genes, module genes and pathway genes. The networks of hub-module-pathway genes and KEGG pathways were constructed. Through the correlation between key pathways and immune genes, the key genes of men and women are found separately. The key genes and related pathways were analyzed.

2.6 Quantitative Real-time PCR(QPCR)

The blood were collected and divided into M-AS group (n = 3), M-NAS group (n = 3), F-AS group (n = 3), F-NAS group (n = 3) (Table 2). According to the manufacturer's instructions, Trizol reagent (Servicebio, Wuhan, China) was applied to extract total RNA from the samples. cDNA was synthesized using a cDNA reverse transcription Kit (Servicebio®RT First Strand cDNA Synthesis Kit, China). Then QPCR was performed using 2 × SYBR Green qPCR Master Mix (Low ROX) kit (Servicebio,Wuhan, China). The qPCR condition was set as follows: 95°C, 30 s, 94°C, 5 s, 58°C, 15 s, 72°C, 10 s, 40 cycles, followed by a 5-minute final extension at 40°C. Relative expression levels of mRNA were calculated using the 2-ΔΔCt method (Ct of target genes minus the Ct of GAPDH) [29]. The primer sequences are as follows: For interleukin 2 receptor subunit beta) (IL2RB), GTGGAACCAAACCTGTGAGCT (forward) and GTAGTGGGAGGCTTGGGAGAT (reverse); For CD3d molecule (CD3D), GGAACATAGCACGTTTCTCTCTG (forward) and AATGTCTGAGAGCAGTGTTCCC (reverse); For GADPH, GGAAGCTTGTCATCAATGGAAATC (forward) and TGATGACCCTTTTGGCTCCC (reverse).

2.7 Enzyme-linked immunosorbent assay (ELISA)

Plasma samples were collected and stored in a frozen tube at -20℃, which were divided into M-AS group (n = 3), M-NAS group (n = 3), F-AS group (n = 3), F-NAS group (n = 3) (Table 2). Enzyme-linked immunosorbent assay (ELISA) kits were used to determine the protein expression levels of key genes in the sample, according to the manufacturer's instructions (Enzyme-linked Biotechnology, Shanghai, China). Optical density was measured at 450 nm absorbance. All procedures were carried out in compliance with the resolution of Helsinki. This study was approved by the Ethics Review Committee of the First Affiliated Hospital of Guangxi Medical University, and all patients provided written informed consent to participate in the study.

2.8 Statistical analysis

The data were expressed as the mean ± sd, and differences between 2 groups were analyzed with Student's t-test. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using the Statistical Program for Social Sciences (SPSS) software 19.0 (SPSS Inc., Chicago, IL, USA).

3 Results

3.1 Identify Differentially Expressed Genes (DEGs)

According to GEO2R screening analysis, there were 181 DEGs (61 up-regulated genes and 120 down-regulated genes) between the M-AS group and the M-NAS group, and 157 DEGs (26 up-regulated genes and 131 down-regulated genes) between the F-AS group and the F-NAS group. Volcano maps show significant genetic differences between the groups. (Fig. 1)

3.2 GO Functional Enrichment Analysis

Up-regulating or down-regulating DEGs of 2 groups were respectively subjected to GO enrichment analysis, and items were screened according to their P.adj.value (P.adj.value < 0.05). The results showed that there were differences in GO enrichment analysis between male and female DEGs. The up-regulated and down-regulated DEGs in male or female were all mainly concentrated in biological processes(Fig. 2). In the biological process, male up-regulated genes are involved in natural killer cell mediated immunity, negative regulation of DNA binding, regulation of lymphocyte apoptotic process, etc, etc (Fig. 2a). Male down-regulated genes are involved in regulation of heart growth, neural nucleus development, substantia nigra development, etc (Fig. 2b). Female up-regulated genes are involved in regulation of T cell receptor signaling pathway, regulation of CD4-positive, alpha-beta T cell activation (Fig. 2c). Female down-regulated genes participate in homeostasis of number of cells, erythrocyte differentiation, erythrocyte homeostasis, etc (Fig. 2d). In terms of Cellular component, Male down-regulated genes are involved in immunological synapse, cytosolic large ribosomal subunit, etc (Fig. 2a). Male down-regulated genes are involved in integrin complex, azurophil granule membrane, tertiary granule lumen, etc (Fig. 2b). Female down-regulated genes participate in lumenal side of membrane, MHC protein complex, umenal side of endoplasmic reticulum membrane, etc (Fig. 2d). In terms of Molecular function, Male down-regulated genes are involved in MHC protein complex binding, MHC protein binding, etc (Fig. 2a). Male down-regulated genes are involved in amyloid-beta binding, double-stranded RNA binding, aminopeptidase activity, etc (Fig. 2b). Female up-regulated genes are involved in MHC protein complex binding (Fig. 2c). Female down-regulated genes participate in amino acid binding, modified amino acid transmembrane transporter activity, promoter-specific chromatin binding (Fig. 2d).

3.3 KEGG Pathway Enrichment Analysis

KEGG analysis was performed respectively, and the most important KEGG pathways of DEGs are visually displayed (P. adj. value < 0.05). The main KEGG pathways for male DEGs to participate include: Th1 and Th2 cell differentiation, B cell receptor signaling pathway, Leukocyte transendothelial migration, etc (Fig. 3a). There are 36 pathway-genes in the male pathways. Female DEGs mainly participated in KEGG pathway including Th1 and Th2 cell differentiation, Antigen processing and presentation, Graft-versus-host disease, etc (Fig. 3b). There are 16 pathway-genes in the male pathways.

3.4 PPI Network Construction

The string database was used to evaluate the DEGs interaction and establish the PPI network structure. Data is entered into Cytoscape for visual adjustment. The male DEGs PPI network has 166 nodes and

308 edges (Fig. 4a). The female DEGs PPI network has 142 nodes and 168 edges (Fig. 4b). Module genes in male and female groups were screened separately. In the top modules of the PPI network, male and female not only share some same Module-genes, but also have unique Module-genes (Fig. 5a and 5b). The top hub 10 genes in male and female was respectively obtained through the degree method from the CytoHubba plugin (Fig. 6a and 6b).

3.5 Identification of Key Genes

There are four hub-module-pathway genes in male, including ITGAM (integrin subunit alpha M), ITGAX (integrin subunit alpha X), GZMB (granzyme B) and IL2RB (Fig. 7a). There are five hub-module-pathway genes in Female, including IL2RB, SPI1(Spi-1 proto-oncogene), KLRD1 (killer cell lectin like receptor D1), CD3D, and STAT5B (signal transducer and activator of transcription 5B) (Fig. 7b). The correlation networks between the hub-module-pathway genes and KEGG pathways were respectively constructed in male and female (Fig. 8a and Fig. 8b). Th1 and Th2 cell differentiation is a common pathway for male AS and female AS, which is the way to induce AS. In this pathway, the key genes involved in male AS are IL2RB, and the key genes involved in female AS are IL2RB and CD3D. Both genes are immune-related genes.

3.6 Quantitative Real-time PCR(QPCR)and Enzyme-linked immunosorbent assay (ELISA)

The QPCR analysis showed that the mRNA expression of IL2RB in the M-AS group was higher than that in the M-NAS group ($P<0.05$). The mRNA expression of IL2RB in the F-AS group was higher than that in the F-NAS group ($P<0.05$). The mRNA expression of CD3D in the F-AS group was higher than that in the F-NAS group ($P<0.05$). There was no significant difference in CD3D mRNA expression between M-AS and M-NAS groups ($P > 0.05$). (Fig. 9A and 9B). And ELISA analysis showed that the protein expression of IL2RB in the M-AS group was higher than that in the M-NAS group ($P<0.05$). The protein expression of IL2RB in the F-AS group was higher than that in the F-NAS group ($P<0.05$). The protein expression of CD3D in the F-AS group was higher than that in the F-NAS group ($P<0.05$). There was no significant difference in CD3D protein expression between M-AS and M-NAS groups ($P > 0.05$) (Fig. 9C and 9D).

4 Discussion

AS a disease with a higher proportion of males, there are obvious differences in the clinical manifestations between male AS patients and female AS patients. Grubisi F et al. [30] indicated that the distribution of the clinical manifestations and specific radiological characteristics of AS in the sacroiliac joint and axial bone was gender-dependent, and that males were regarded as one of the risk factors associated with poor prognosis of AS. Jung YO et al. [31] found that males were younger at the onset of symptoms, had a higher positive rate of HLA-B27, and had a greater degree of involvement of the spinal cord on imaging. And females have less spinal involvement and better mobility, but a higher incidence of

plantar fasciitis. Jimenez-balderas FJ et al. [32] suggested that male patients were more common in uveitis, bamboo spine and hip arthroplasty. And Calin A et al. [33] found that female patients with AS had A longer delay in diagnosis, which may miss the optimal treatment time. As, rheumatoid arthritis and systemic lupus erythematosus are all considered as autoimmune diseases. However, Rheumatoid arthritis and systemic lupus erythematosus are more common in females [34, 35]. The latter two are considered to have distinct sexual dimorphism in the immune mechanism [34, 36, 37, 38]. Some researches suggested that estrogen plays an important role in the immune mechanisms of these diseases [39, 40, 41]. However, AS usually occurs in males [1, 2]. Some researches have shown that the role of sex hormones in AS is not prominent, they can not directly explain the dominant position of male, nor directly explain its sexual characteristics [20, 21, 22, 42].

Due to the gender differences in clinical manifestations of AS, as well as gender differences in the composition and content of blood, we divided the samples into males and females. The Bioinformatics analysis of DEGs (M-AS vs M-NAS group, F-AS vs F-NAS group) was carried out respectively, and it was found that there were differences in GO function and KEGG pathways between the two groups. In terms of GO function, both male and female DEGs were mainly concentrated in biological processes. The difference is that the male DEGs mainly concentrated in natural killer cell mediated immunity, regulation of heart growth, etc. And female DEGs mainly concentrated in regulation of T cell receptor signaling pathway, regulation of CD4-positive, homeostasis of number of cells, etc. On the KEGG pathway, the male DEGs mainly concentrated in the Th1 and Th2 cell differentiation, Complement and coagulation cascades, etc. Female DEGs mainly concentrated in Th1 and Th2 cell differentiation, Human T-cell leukemia virus 1 infection, etc. It was found from the correlation network between hub-module-pathway genes and KEGG pathways that the most likely key genes for males were IL2RB, and the pathway were Th1 and Th2 cell differentiation, while the most likely key genes for females were CD3D, IL2RB and STAT5B, and the pathway were Th1 and Th2 cell differentiation. The expression of these key genes was further verified by QPCR and ELISA.

IL2RB protein binds with interleukin-2 in an intermediate or high affinity manner and participates in T-cell-mediated immune response [43, 44, 45]. Abnormalities of IL2RB can induce immune dysregulation driven by T and NK cells, leading to autoimmune diseases and immunodeficiency diseases [46]. IL2RB is associated with a variety of immune diseases, such as inflammatory bowel disease, rheumatoid arthritis, multiple sclerosis, etc [47, 48, 49]. Polo Y La Borda J et al. [50] found that IL2RB was a participant in the development of peripheral arthritis of AS.

CD3D proteins are part of the T cell receptor/CD3 complex (TCR/CD3 complex) and participate in T cell development and signal transduction [51, 52]. TCR is responsible for identifying antigens associated with major histocompatibility complex (MHC) and initiating the cellular immune response [53, 54]. Doucey MA et al. [52] indicated that the coupling of CD3D and TCR is necessary for the effective activation and positive selection of CD8T cells. And activated CD8T cells were involved in the induction and maintenance of AS [55, 56].

Th1 and Th2 cell differentiation is the main pathogenesis of AS. There is only one key gene involved in this pathway in men, while there are two key genes involved in this pathway in women. This indicates that high expression of IL2RB may induce AS in men. The abnormalities of both IL2RB and CD3D may cause women to carry AS. Therefore, men are more likely to suffer from AS than women. This explains why the main population of AS patients is male. Both IL2RB and CD3D are immune-related genes, which further confirms that AS is an immune disease.

Conclusion

In the study, we found that the key genes of male AS are IL2RB, and the key genes of female AS are CD3D and IL2RB. Differences in key genes between the sexes may result in significant dimorphism in the clinical manifestations and therapeutic effects of AS. These findings indicate that gender differences in mechanisms should be considered when treating AS.

Future Perspective

AS is a disease that causes pathological changes in the spine and sacroiliac joints, which seriously affect the quality of life of patients. Numerous studies have shown that AS is characterized by sexual dimorphism in clinical manifestations. Its mechanism has not been fully elucidated. Our study revealed gender differences in the mechanism of AS, which provided new insights into the pathogenesis of AS. Therefore, sexual dimorphism should be considered in the research and treatment of AS. This finding will be conducive to a further comprehensive understanding of the biological characteristics of AS and to provide new molecular targets for personalized treatment.

Summary Points

• We used bioinformatics to analyze gene expression data of GSE73754 and identified key differentially expressed genes and related pathways for male and female AS.

• We found similarities and differences in the pathway of DEGs enrichment.

• We identified key genes and related pathways in males and females from the correlation network between the hub-module genes and the KEGG pathways.

• We compared the expression of key genes between male and female. And it was found that it is not the expression level of key genes, but the difference in key genes that leads to the gender difference in AS.

• Our study provides new insights into the pathogenesis of AS and provides new molecular targets for personalized treatment.

Declarations

Ethics approval and consent to participate

All procedures were performed in compliance with the resolution of Helsinki and approved by the Local Ethics Committee. All participants received written informed consent.

Consent for publication

All the authors agreed to publish the article.

Availability of data and material

The datasets supporting the conclusions of this article are available in the GEO repository, https://www.ncbi.nlm.nih.gov/geo/.

Competing interests

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Authors' contributions

Chaojie Yu designed the study. Xinli Zhan and Jinmin Zhao supervised the study. Chaojie Yu, Chong Liu, Tuo Liang, Shian Liao, Liyi Chen, Risheng Huang, Xunhua Sun, Zhen Ye, Hao Guo, and Yuanlin Yao analyze the data. Chaojie Yu and Chong Liu digital visualization. Chaojie Yu wrote and revised the manuscript. All authors read and approved the final manuscript.

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Figures

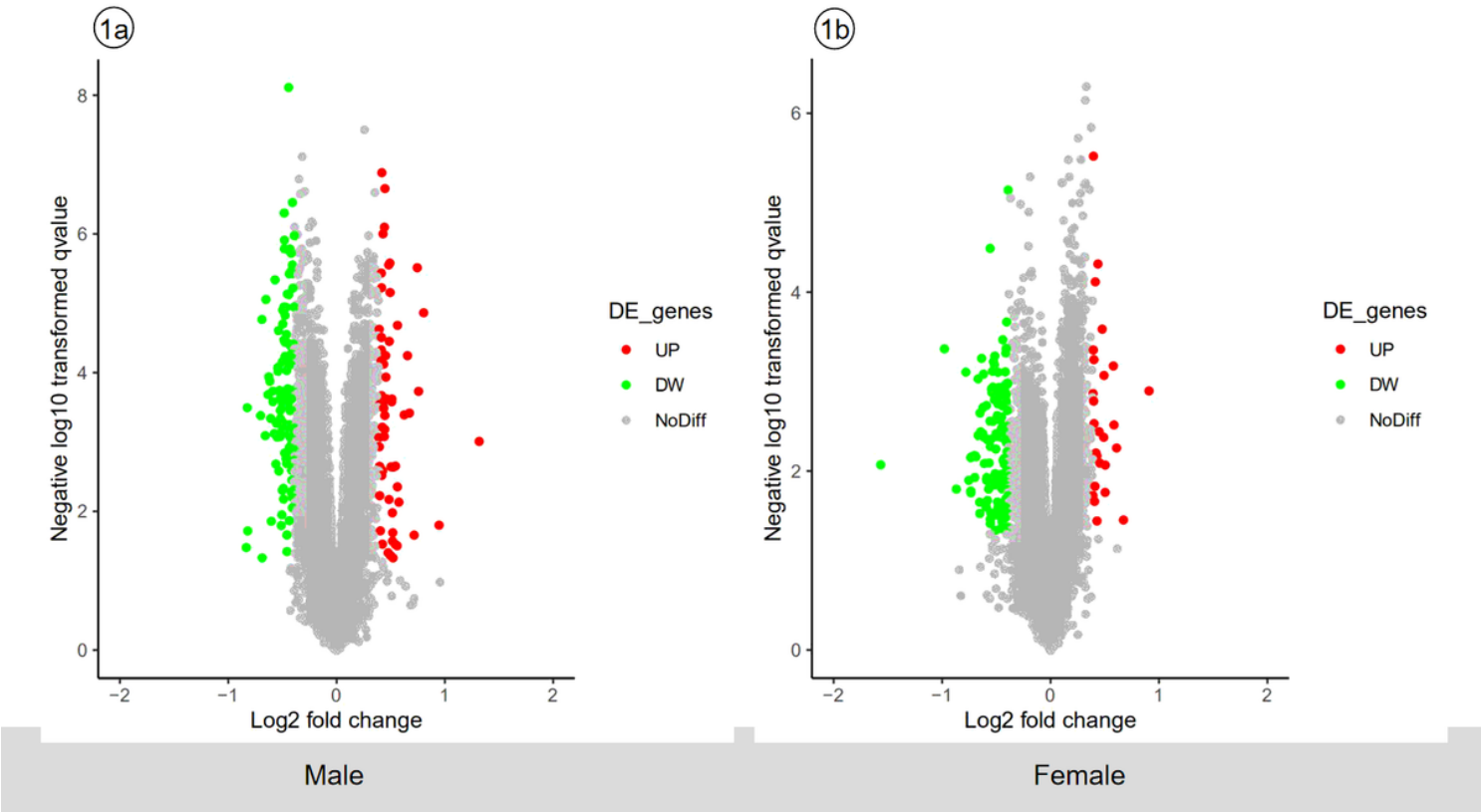

Figure 1

active volcano map of DEGs, screening criteria: P<0.05 and |log2(FC)|>0.390. The red dots represent up-regulated genes and the green dots represent down-regulated genes. a a volcano map of DEGs between M-AS and M-NAS groups; b a volcano map of DEGs between F-AS and F-NAS groups.

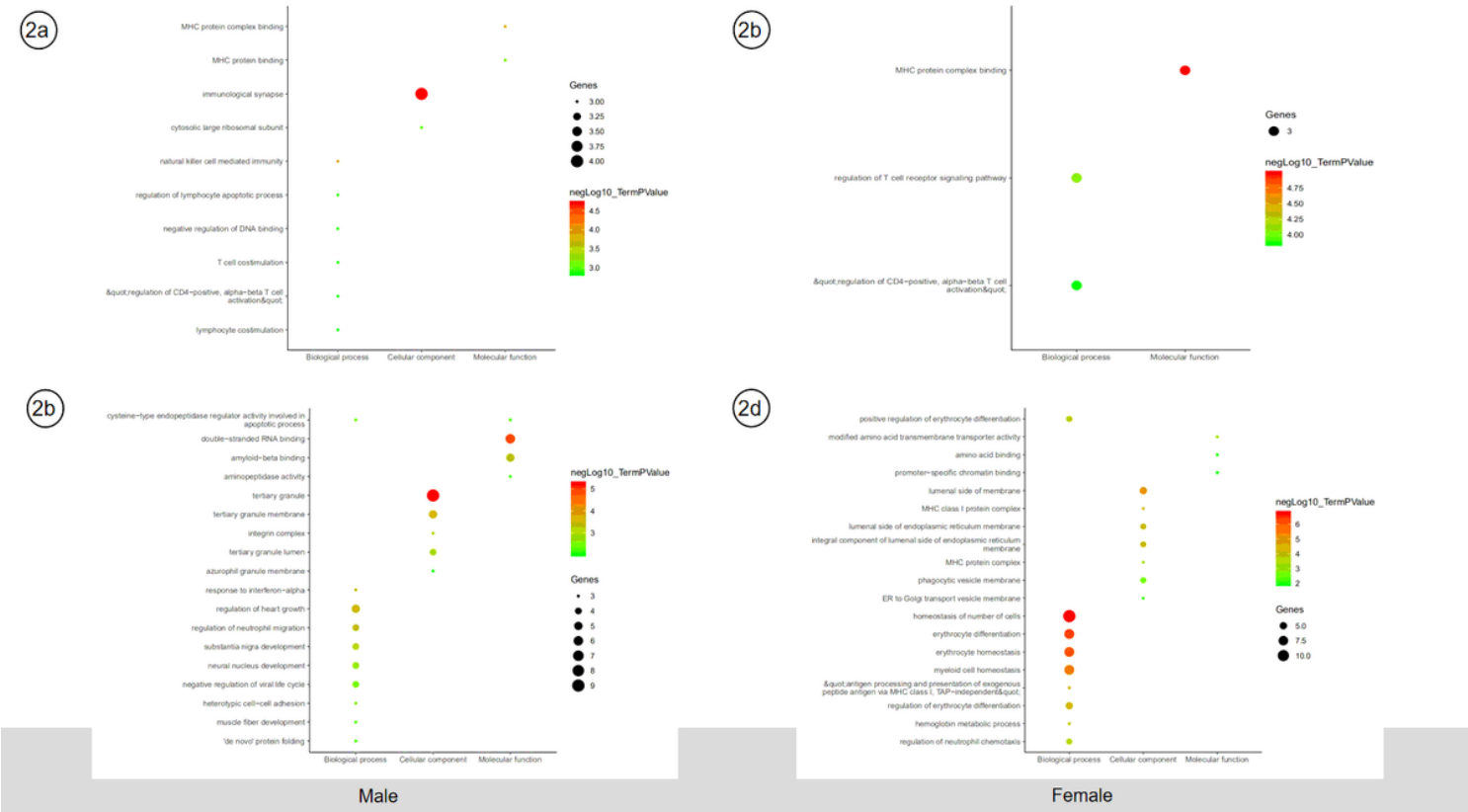

Figure 2

top 10 GO enrichment analysis of DEGs. The function of DEGs in tissues is described according to its GO characteristics (Biological process, Molecular function, Cell components). -Log10 (p. adj.value) < 0.05 and count of gene is > =3. a up-regulated genes in male ; b down-regulated genes in male; c up-regulated genes in female ; d down-regulated genes in female.

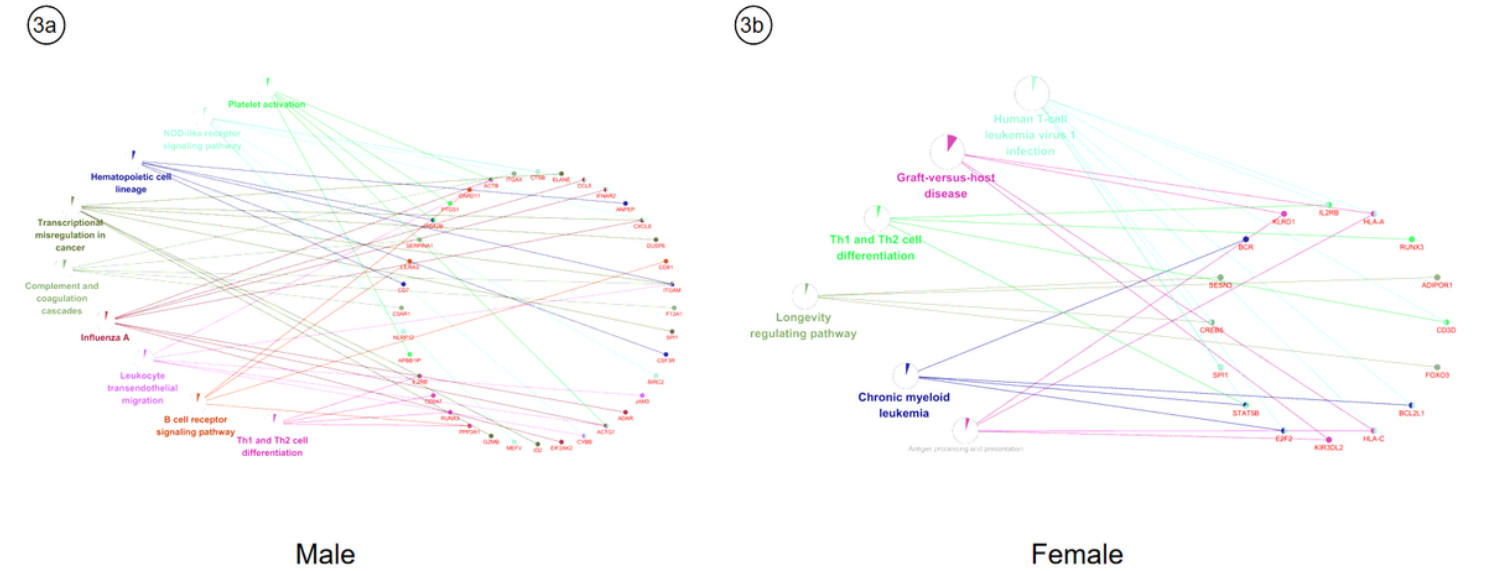

Figure 3

KEGG pathway analysis of DEGs. -Log10 (p. adj.value) < 0.05 and count of gene is > =4. a male; b female.

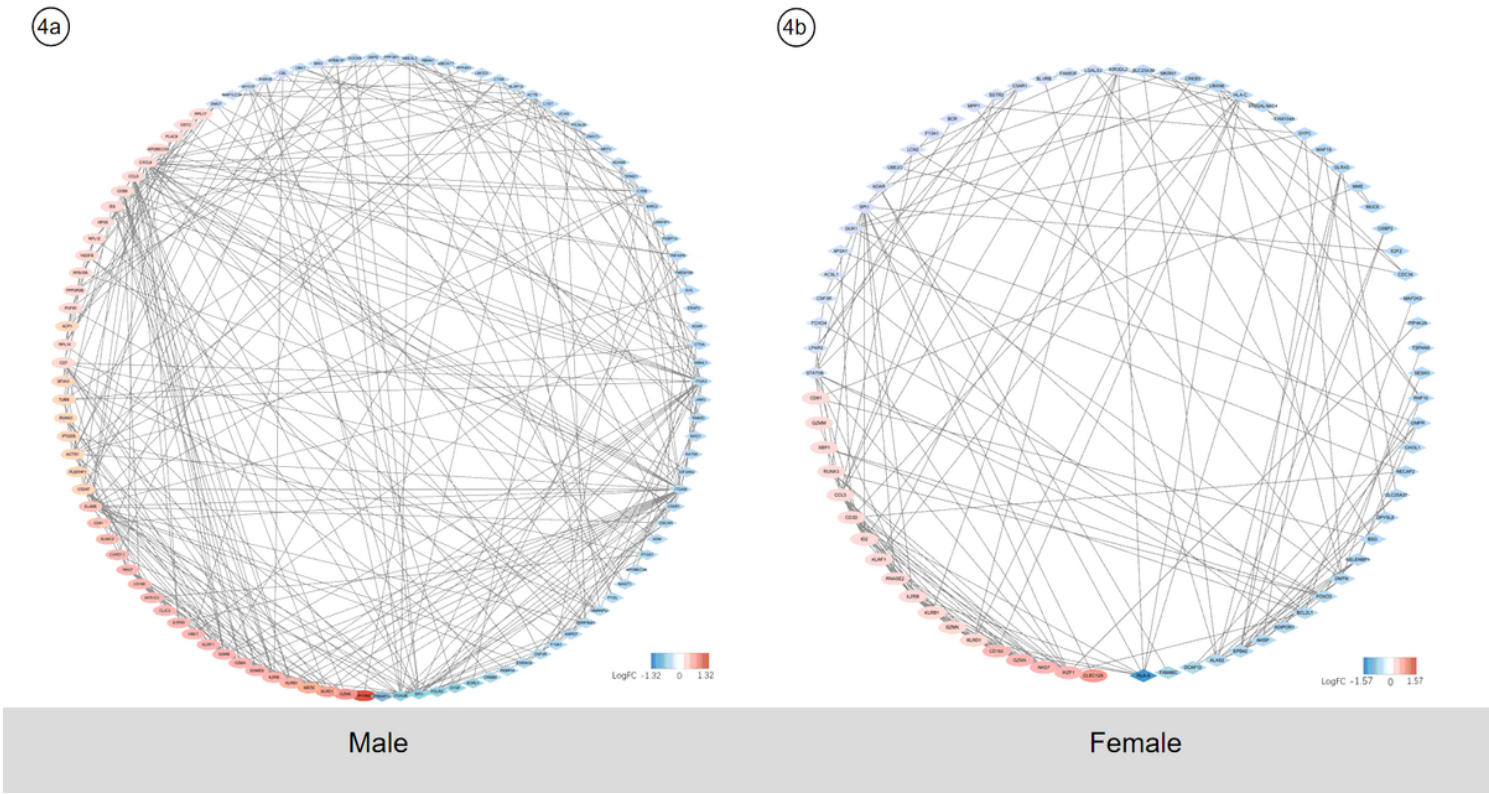

Figure 4

all DEGs PPI networks are visualized in Cytoscape. Red balls represent the up-regulated DEGs, and blue diamonds represent the down-regulated DEGs. a male; b female.

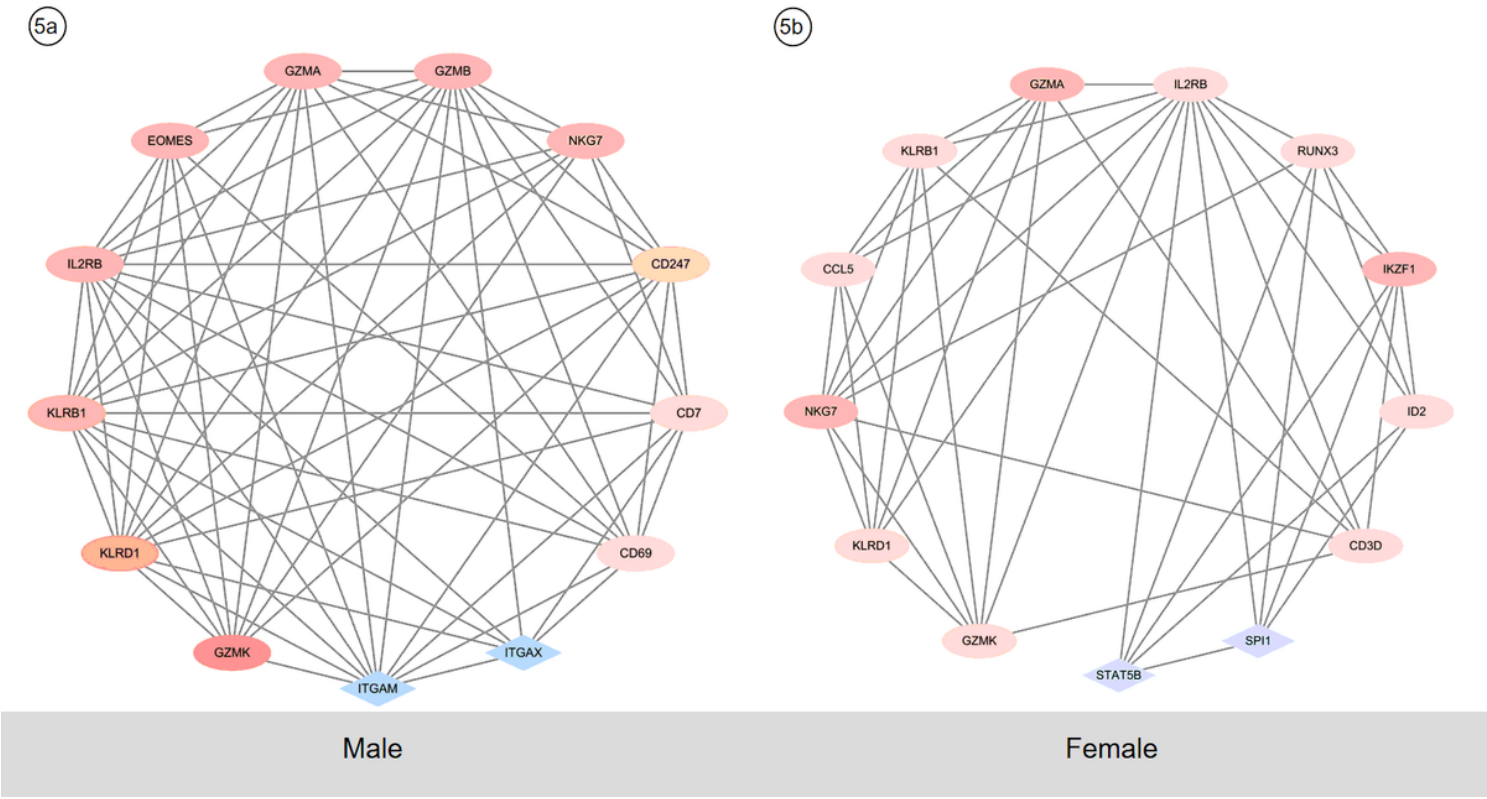

Figure 5

top module from the PPI network. Red balls represent the up-regulated DEGs, and blue diamonds represent the down-regulated DEGs. a male; b female.

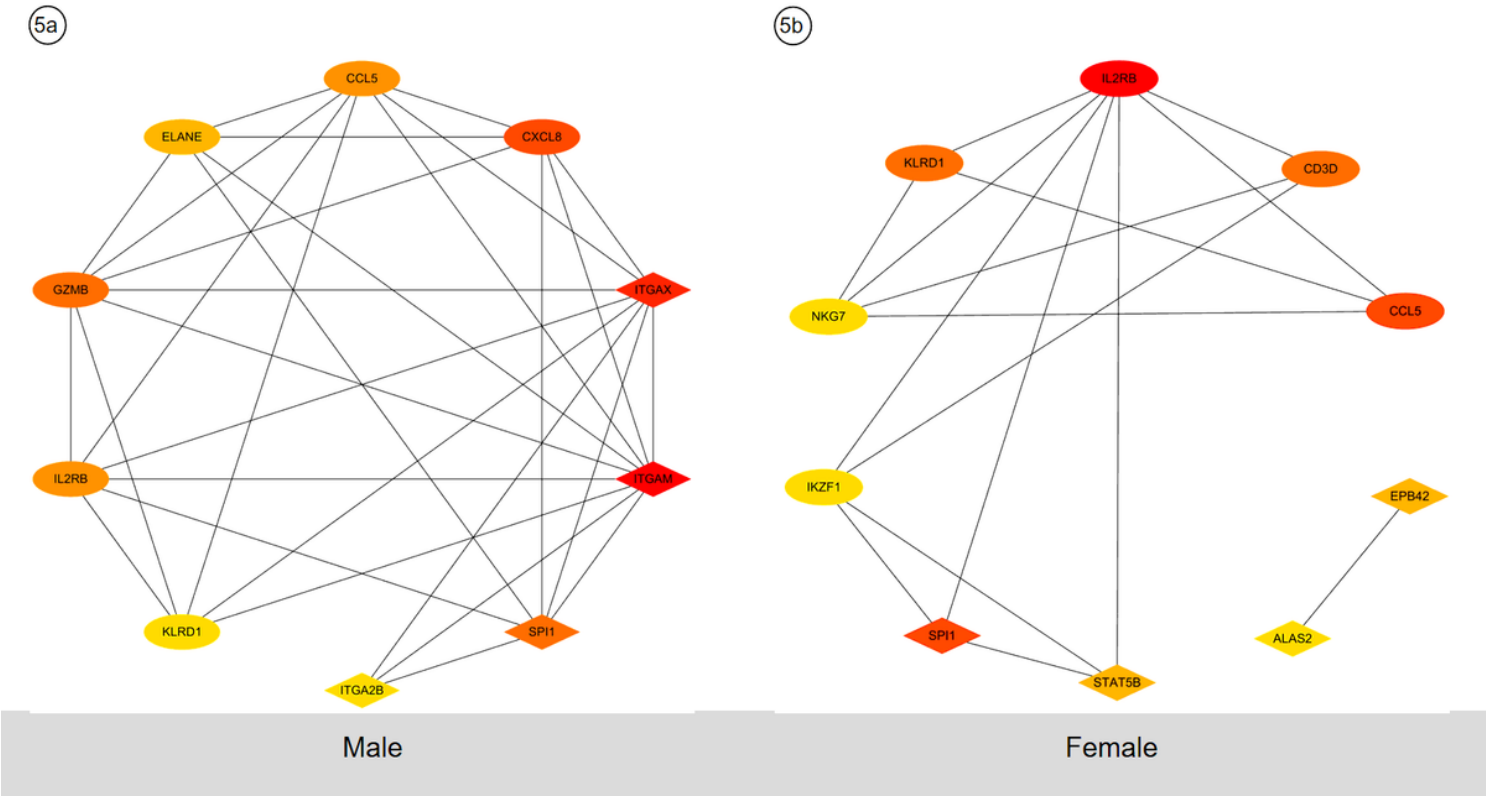

Figure 6

the top hub 10 genes was obtained through the degree method from the CytoHubba plugin. Balls represent the up-regulated DEGs, and diamonds represent the down-regulated DEGs. a male; b female.

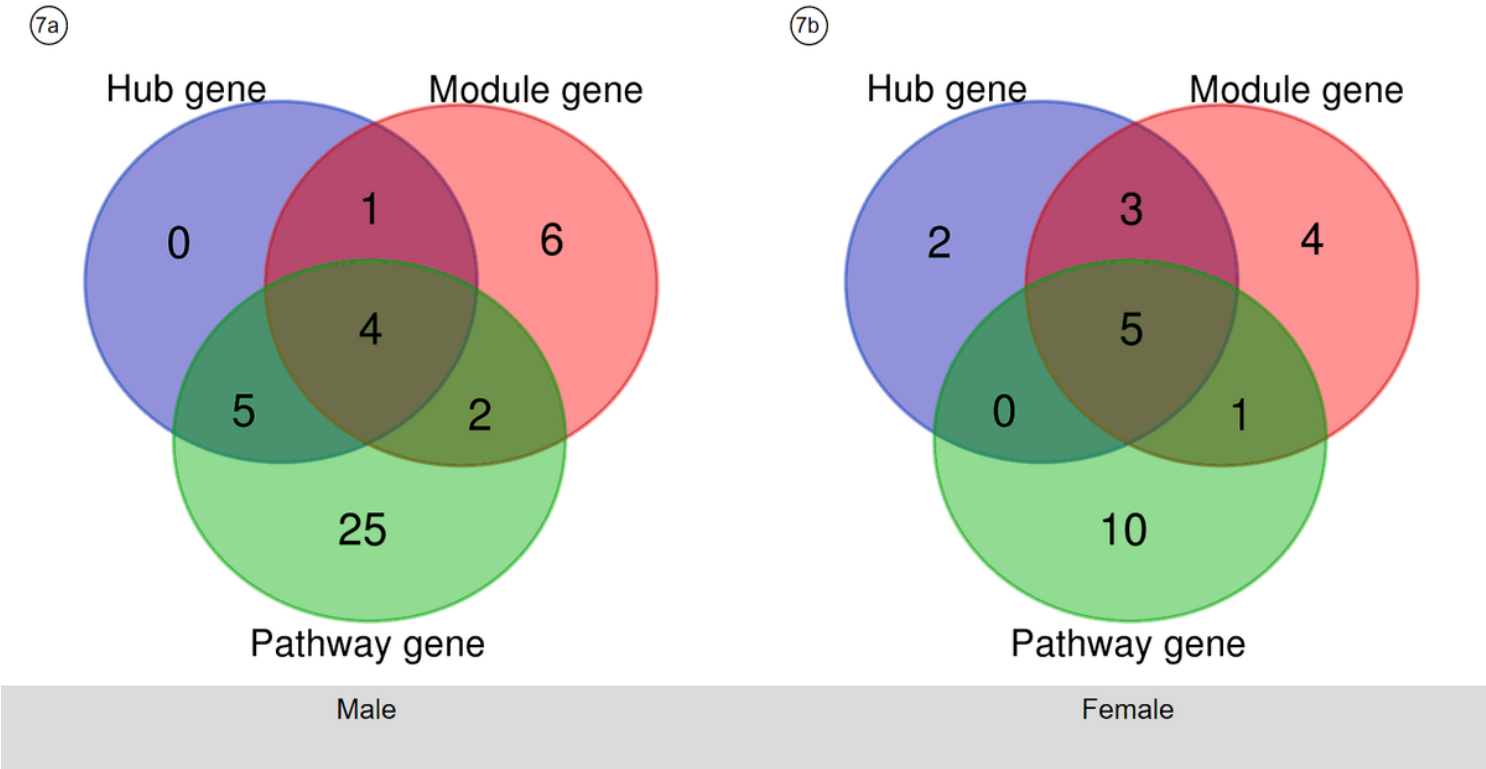

Figure 7

intersection diagram of hub genes, module genes and pathway genes. a male; b female.

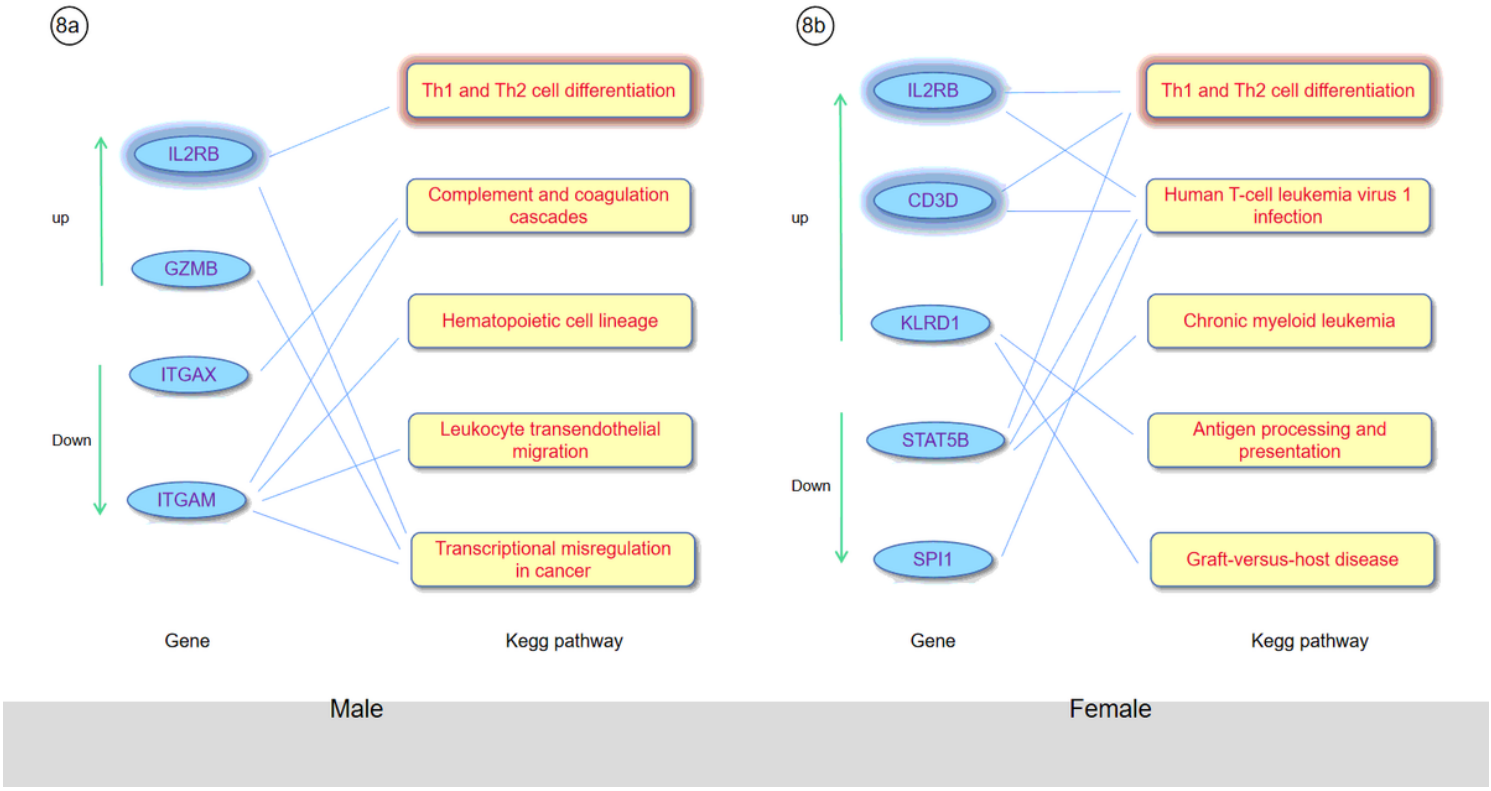

Figure 8

key genes were from correlation network between hub-module-pathway genes and KEGG pathways. a male; b female.

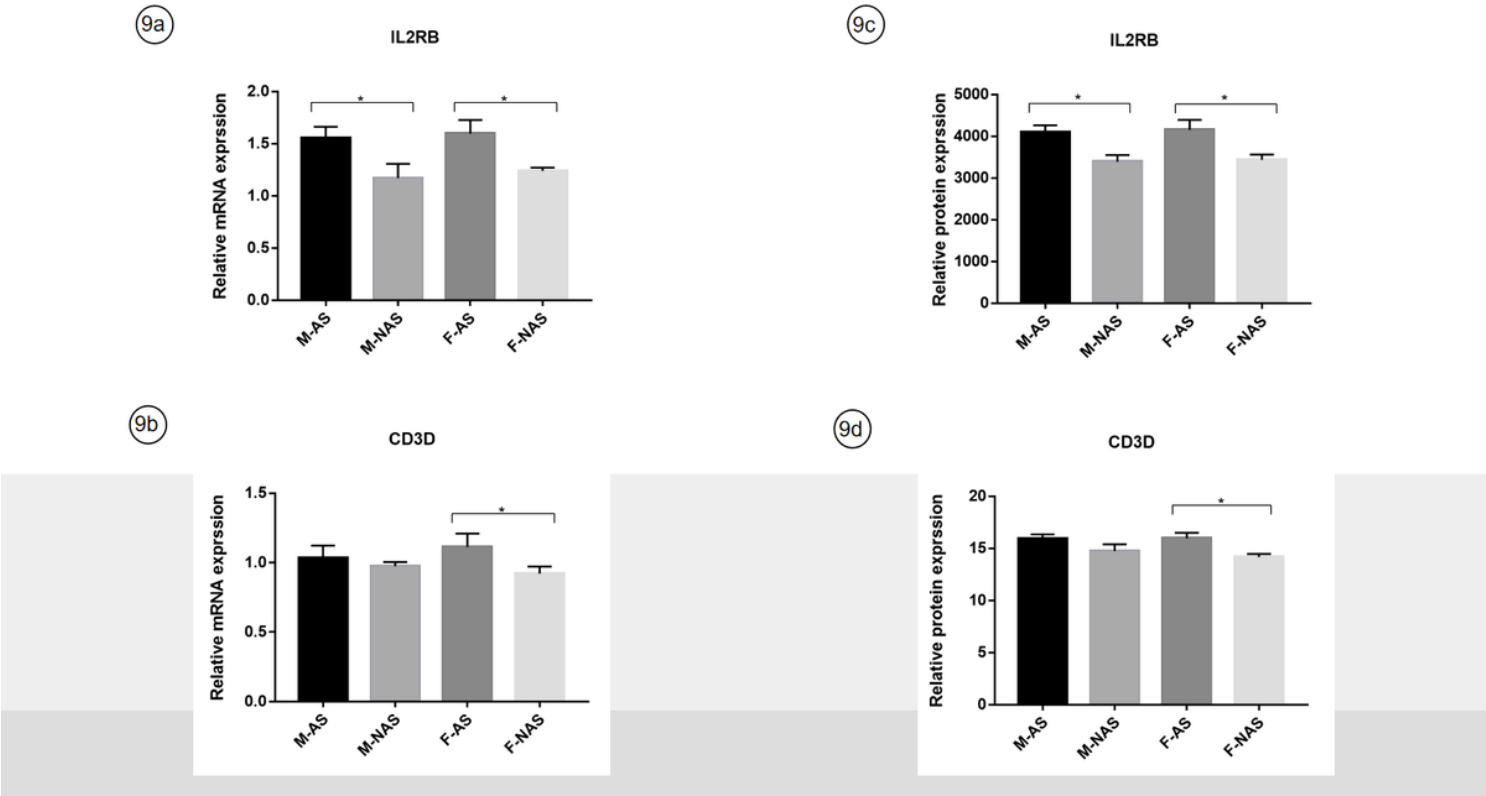

Figure 9

a relative mRNA expression of IL2RB in AS verified by QPCR. b relative mRNA expression of CD3D in AS was verified by QPCR. c relative protein expression of IL2RB in AS was verified by ELISA. d relative protein expression of CD3D in AS was verified by ELISA.