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Research Article

Network Cluster Analysis of PPI and Phenotype Ontology for Type 1 Diabetes Mellitus

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Background: Our knowledge of Type 1 Diabetes Mellitus (T1DM) etiology is incomplete; however, the pathogenesis of the disease includes T-cell-mediated destruction of β-cells.

Objective: The present study aimed to investigate the key gene pathways and co-expression networks in T1DM disease.

Material and Methods: TIDM-associated genes were identified from 13 databases, enrichment of pathways annotated with functional annotations, and analysis of protein-protein network interactions. Next, functional modules and transcription factor networks were constructed. The analysis of gene co-expression networks was conducted to discover associated pivotal modules.

Results: A total of 172 expressed genes and four variants (SNP) were filtered in the of T1DM disease; pathway enrichment analysis identified key pathways, such as inflammatory bowel disease, type I diabetes mellitus, cytokine-cytokine receptor interaction, Th17 cell differentiation, JAK-STAT signaling pathway, and graft-versus-host disease. A weighted correlation network analysis revealed one module that was strongly correlated with T1DM. Functional annotation revealed that the module was mainly enriched in pathways such as T cell activation, regulation of immune system process, and response to the organic substance. IRF2, IRF4, IRF8, and CDX2 were regulated in the module at a significant level.

Conclusion: The study identified IL-2 as a significant T1DM hotspot and highlighted the role of hub genes and transcription factors in the autoimmune disease, offering potentials for treatment and prevention.

Keywords: Autoimmune disease, Biomarker, PPI network, SNP, T1DM

1. Background

Type 1 Diabetes Mellitus (T1DM) is due to damage to β-cells that produce insulin in the pancreas. In terms of etiology, T1DM is categorized into two general subtypes, including idiopathic (type 1B) and autoimmune (Type 1A) (1). The pathogenesis and etiologic factors of T1DM are unclear; studies have shown that fulminant type 2 diabetes can be a part of this subtype (2) In addition, T1DM as a complication is multifactorial and induced by a complicated combination of environmental and genetic factors, so the genetic factors include many susceptibility genes.

In T1DM, the development of islet-specific auto-antibodies against cellular structures is accompanied by a heterogeneous process. The best-characterized autoantibodies that are signs of ongoing cell destruction

are islet cell antibodies (ICA), insulin autoantibodies (IAA), autoantibodies against IA-2 (IA-2A), Glutamic acid decarboxylase antibodies (GADA), and Zinc transporter 8 autoantibody (ZnT8A) (3). In childhood, IAA and GADA are the most frequently detected autoantibodies, while IA-2A and ZnT8A autoantibodies are found in relatively small numbers of patients (4). Autoantibodies are expected at the time of diagnosis; however, as the disease progresses, disturbances in the metabolism of glucose become more common (4).

There is a difference in the age of seroconversion for the various autoantibodies responsible for initiating autoimmunity. For instance, IAA peaks at the age of two, whereas GADA peaks at the age of 4-5 years and remains prominent throughout childhood (5). As positive autoantibodies increase, the risk of developing T1DM increases. When IA-2A levels are high, the risk of developing T1DM remains constant. However, the risk decreases when IAA and GADA levels are high (5). It is still necessary to perform a detailed analysis of this complex relationship, which includes the ZnT8 autoantibody. Children with islet autoantibodies and genetically susceptible to T1DM do not progress to clinical T1DM. Genetic susceptibilities are also associated with the rapid development of T1DM, as defined by HLA genotypes and non-HLA genes, the age at which autoantibodies appear, sex, and possibly yet unknown environmental factors (6). A variation in the duration of the asymptotic phase indicates that environmental factors, as well as genetic factors, influence the progression of the disease. IAA, IA-2A, and GADA perform a significant role in the progression of seroconversion to clinical T1DM and islet autoimmunity (7).

T1DM may show up at any age, however, it most frequently does so in children. Over one million children and adolescents under twenty worldwide were estimated to have more cases of T1DM in 2021, representing 149.5 per 1000 people/annum (8). There has been a correlation between clinical T1DM and reduced mass of β-cells by 30-60 percent. However, there is a difference between these numbers among children and adults with early-stage and long-standing diabetes (9). The global incidence of T1DM has steadily increased during the past century (10). A recent report indicates that the incidence of the disease has leveled off in populations with the highest increase. It is essential to predict autoimmunity risks early to select appropriately matched case-control groups for prevention trials. Children with T1DM are at an increased risk of mortality, other complications, and cardiovascular problems (11). On the other hand, a timely diagnosis can reduce complications at the time of onset (12).

Gene expression profile analysis has been used extensively in pathological mechanism research, disease diagnosis, and treatment. Numerous investigations utilizing microarray analysis have been dedicated to examining the molecular genetics underlying the development of T1DM. The specific genes expressed in T1DM individuals, and the functional pathways and co-expression network remain to be determined. These findings provide fresh perspectives on pathogenesis, development, and drug discovery of T1DM.

2. Objective

In this study, we obtained datasets from 13 databases, filtered reported expressed genes for T1DM disease, annotated the enrichment of functional pathways, and analyzed the PPI network; transcription factors and modules analyzed performed for protein-protein interaction (PPI) networks of expressed genes. To identify the key gene modules related to the disease, the co-expression network of the dataset was also analyzed.

3. Material and Methods

3.1. Identification of GDAs and SNPs Sites

Thirteen databases were selected for searching for Autoimmune Diabetes and T1DM, namely ClinVar (13), UniProt (14), GWAS db (15), GWAS Catalog (15), ORPHANET (16), CTD (17), GENOMICS ENGLAND (18), CLINGEN (19), PSYGENET (20), CGI (21), LHGDN (22), HPO (23), and BeFree (24) to analyze the gene-disease association data of T1DM and the relative SNPs.

3.2. Go and KEGG Pathway Enrichment Analysis

The g:Profiler includes analytical tools and an integrated biological knowledge base to extract the systematic biological meaning of broad lists of proteins or genes (25). It was used to carry out the functional analysis of genes, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) (26) was employed to analyze pathway enrichment. A threshold of at least 95% of

matches above the threshold is considered statistically significant for the GO and pathway enrichment analysis using the g:SCS algorithm (25).

3.3. PPI and Module Analysis

The STRING database (27) analyzed the expressed genes PPI network with a confidence score >0.9 for significant results. Cytoscape software (version 3.10.0; The Cytoscape Consortium, New York City, NY, USA) (28) visualizes the PPI network. A plugin for Cytoscape called Molecular Complex Detection (MCODE) (29) was used to analyze the PPIs' network modules. MCODE detects densely connected modules of networks that might represent molecular complexes. The performed plugin criteria for significant modules score and rank set as follow; degree cut-off: 5; node score cut-off: 0.2; k-core: 2; and max. depth: 100. The obtained top significant modules were ranked and scored. The gene with the highest weighted vertex was designated as a seed by MCODE.

3.4. Transcription Factor Analysis

The analysis of transcription factors within the indicated modules was conducted using the iRegulon plugin of Cytoscape (30). The default criteria for the iRegulon plugin were set. Putative transcription factors were identified with a normalized enrichment score (NES) greater than 5. Each module's top three transcription factors with the highest NES were enumerated.

4. Results

To identify the gene-disease association data from human T1DM SNPs (four variants and the interfering genes (n=172; **Supplementary 1**), the g: Profiler web server was used. As indicated in **Table 1**, six genes performed four variants, including 3′ prime UTR, NMD transcript, Intron, Missenses, Non-coding transcript exon, non-coding transcript, and Synonymous.

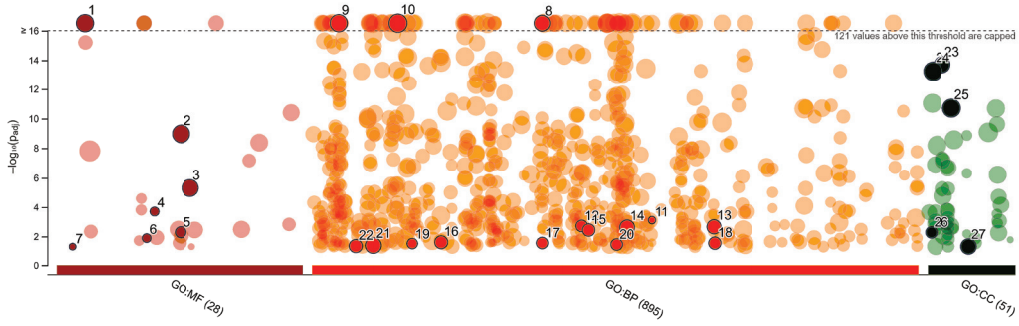
4.1. Function and Pathway Annotation

We uploaded all the genes to the g: Profiler online tool to conduct a pathway enrichment analysis. For KEGG pathway enrichment (**Fig. 1A**), the expressed gene mainly enriched in pathways in inflammatory bowel disease, type I diabetes mellitus, cytokine-cytokine receptor interaction, Th17 cell differentiation, JAK-STAT signaling pathway, rheumatoid arthritis, graft-versus-host disease, autoimmune thyroid disease, allograft rejection, and leishmaniasis (**Table 2**). For the GO term biological (GO: BP), molecular (GO:MF), and cellular (GO:CC) process analysis, the expressed gene enriched in positive regulation is also revealed in **Figure 1A**. Results are visualized for the GO term of biological process analysis to illustrate the experimental findings (**Fig. 1B**).

Table 1. **SNPs associated with T1DM**

Gene name	Chromosome	Gene ID	Alleles	Frequently reported variant						
				3′ prime UTR	NMD transcript	Intron	Missenses	Non-coding transcript exon	Non-coding transcript	Synonymous
SUMO4 TAB2	6	ENSG00000055208 ENSG00000177688	G/A		2	12	2		2	
AP4B1-AS1 PTPN22	1	ENSG00000134242 ENSG00000231128	A/G	2	2	4	10	2	2	
IFIH1	2	ENSG00000115267	C/T				4	2		
CTLA4	2	ENSG00000163599	C/T			1		2		4

A)



ID	Source	Term ID	Term Name	p_{adj} (query_1)
1	GO:MF	GO:0005102	signaling receptor binding	1.038×10^{-19}
2	GO:MF	GO:0042802	identical protein binding	1.084×10^{-9}
3	GO:MF	GO:0044877	protein-containing complex binding	4.868×10^{-6}
4	GO:MF	GO:0032395	MHC class II receptor activity	1.969×10^{-4}
5	GO:MF	GO:0042605	peptide antigen binding	5.065×10^{-3}
6	GO:MF	GO:0030881	beta-2-microglobulin binding	1.293×10^{-2}
7	GO:MF	GO:0004351	glutamate decarboxylase activity	4.923×10^{-2}
8	GO:BP	GO:0042110	T cell activation	3.920×10^{-45}
9	GO:BP	GO:0002682	regulation of immune system process	2.926×10^{-37}
10	GO:BP	GO:0010033	response to organic substance	4.215×10^{-37}
11	GO:BP	GO:0060559	positive regulation of calcidiol 1-monooxygenase ...	7.619×10^{-4}
12	GO:BP	GO:0045840	positive regulation of mitotic nuclear division	1.894×10^{-3}
13	GO:BP	GO:0072593	reactive oxygen species metabolic process	2.175×10^{-3}
14	GO:BP	GO:0051346	negative regulation of hydrolase activity	2.360×10^{-3}
15	GO:BP	GO:0046209	nitric oxide metabolic process	3.609×10^{-3}
16	GO:BP	GO:0019233	sensory perception of pain	2.467×10^{-2}
17	GO:BP	GO:0042119	neutrophil activation	2.777×10^{-2}
18	GO:BP	GO:0072678	T cell migration	2.837×10^{-2}
19	GO:BP	GO:0010888	negative regulation of lipid storage	3.032×10^{-2}
20	GO:BP	GO:0050691	regulation of defense response to virus by host	3.502×10^{-2}
21	GO:BP	GO:0007169	transmembrane receptor protein tyrosine kinase si...	4.103×10^{-2}
22	GO:BP	GO:0006090	pyruvate metabolic process	4.489×10^{-2}
23	GO:CC	GO:0009986	cell surface	1.960×10^{-14}
24	GO:CC	GO:0005615	extracellular space	5.604×10^{-14}
25	GO:CC	GO:0031410	cytoplasmic vesicle	1.964×10^{-11}
26	GO:CC	GO:0001772	immunological synapse	5.242×10^{-3}
27	GO:CC	GO:0043235	receptor complex	4.776×10^{-2}

B)

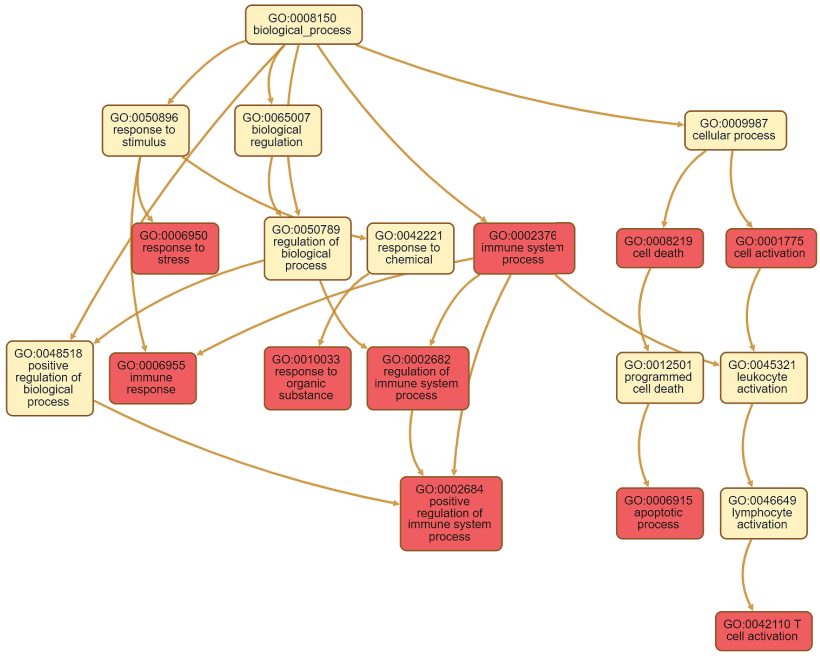


Figure 1. Enrichment analyses for: A) Kyoto Encyclopedia of Genes and Genomes pathways and **B**) GO terms of biological process analysis from expressed genes in type 1 diabetes disease.

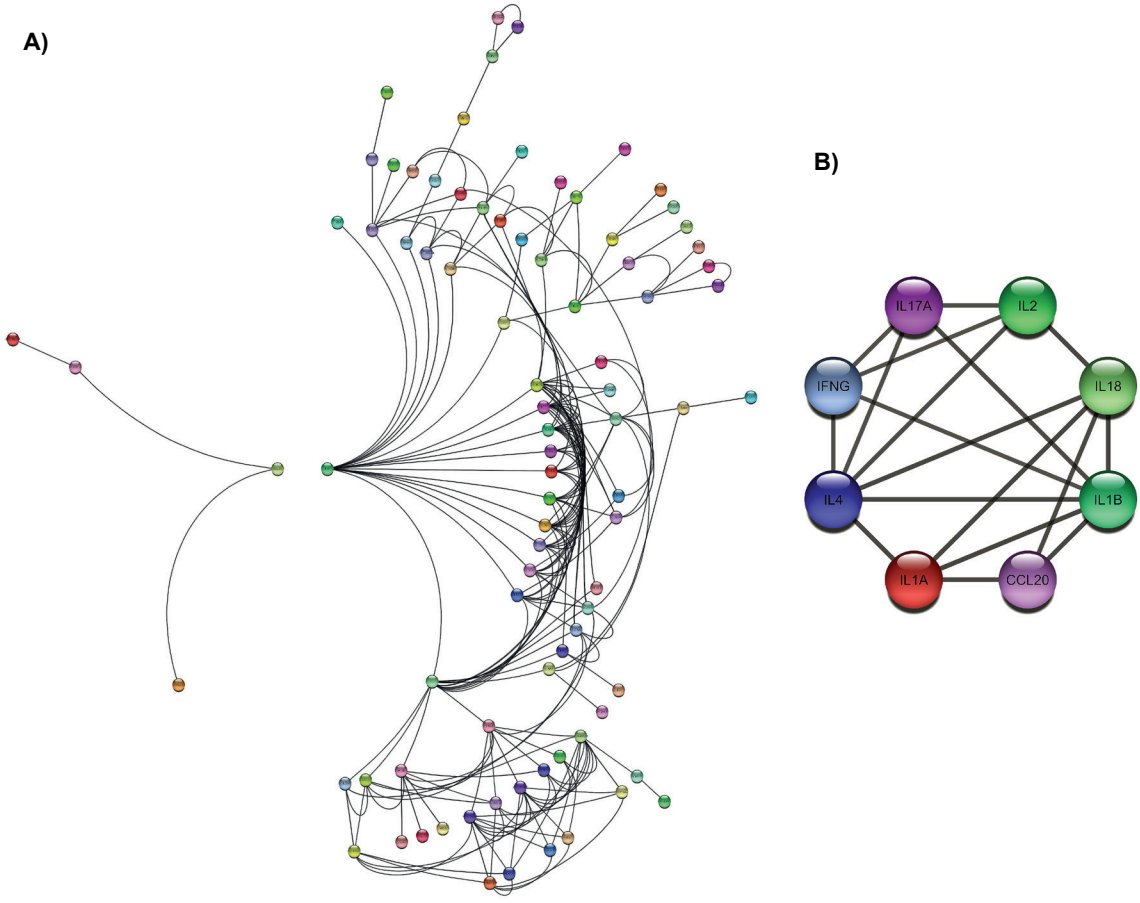


Figure 2. Protein–protein interaction network and module analysis of expressed genes in T1DM. **A**) Protein–protein interaction network based on 158 constructed by Cytoscape. **B**) The significant module identified from the protein–protein interaction network.

Table 2. Gene classification in the functional modules based on Genomes Terms and Kyoto Encyclopedia of Gene with a False Discovery Rate of <0.05 (Top 10)

Term ID	Count	P-value
has05321:Inflammatory bowel disease	19	2.40E-18
hsa04940:Type I diabetes mellitus	16	1.81E-17
hsa04060:Cytokine-cytokine receptor interaction	31	5.03E-16
hsa04659:Th17 cell differentiation	20	6.91E-15
hsa04630:JAK-STAT signaling pathway	22	5.23E-13
hsa05323:Rheumatoid arthritis	17	1.31E-12
hsa05332:Graft-versus-host disease	12	1.39E-11
hsa05320:Autoimmune thyroid disease	13	2.41E-11
hsa05330:Allograft rejection	11	1.55E-10
hsa05140:Leishmaniasis	14	2.88E-10

Table 3. Predicted transcription factor

Module	Transcription factor	P-value
1	IRF8	2.854E-2
2	CDX2	3.269E-2
3	IRF2	3.976E-2
4	IRF4	4.631E-2

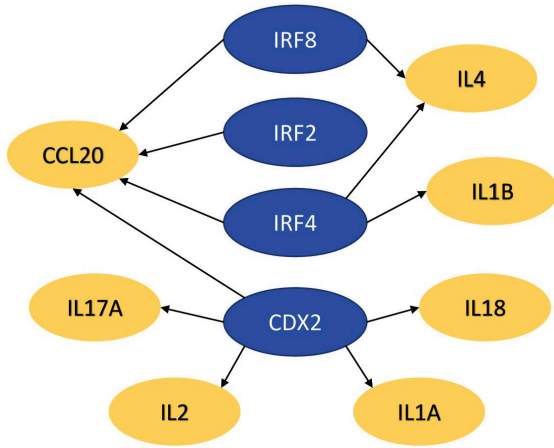


Figure 3. Transcription factor target networks of the four modules. Blue oval nodes represent the predicted transcription factor. Yellow oval nodes represent transcription factor-regulated genes.

4.2. Construction and Analysis of the PPI Network

Employing the STRING database, we built the PPI network to investigate the functional relationships between all expressed genes. Cytoscape was used to evaluate and then visualize 172 different genes. Protein-functional interaction network (PPI), included 158 nodes and 218 edges (**Fig. 2A**). Furthermore, the MCODE plugin verified functional modules from the PPI network. The plugin detected one significant module that met the cut-off criteria. Module 1 (score: 5.143) comprised 8 nodes and 18 edges; the seed gene was IL-2A (**Fig. 2B**).

4.3. Transcription Factor Networks Construction of Modules

As transcription factors bind to specific DNA sequences, they regulate gene expression and function. Using the iRegulon plugin, we predicted the transcription factors in the module. **Table 3** displays predicted transcription factors with NES >5 values. In these modules, we predicted that IRF-8 would regulate IL4 and CCL20; CDX2 would regulate IL17A, IL2, IL18, CCL20, and IL1A; IRF-2 would regulate CCL20; and IRF-4 would regulate IL4, IL1B, and CCL20 (**Fig. 3**).

5. Discussion

We attempted to identify molecular functional pathways and co-expression networks in T1DM by analyzing gene profiles of patients with T1DM, which have been submitted to public NCBI GEO databases. Analyzing the associated genes would help us to reveal the relationships between genes and clinical features. Here, 178 genes and four variants were analyzed which were associated with T1DM (**Table 1**). Among the four variants, SUMO4 (31, 32) and IFIH21 (33) were limited to environmental factors like region and viral infection. The immune regulatory mechanisms function within tolerance ranges, and when they are not appropriately regulated, they may improve autoimmune reactions. Thus, it is notable that T1DM-associated genetic variants have a role in a distinct overlapping regulatory network potentially altering the autoimmune phenotypes development. Notably, there is a direct and indirect relationship between the CTLA-4 and PTPN2, which means that the variant can affect immune-response gene expression in a cell-type and tissue-specific manner.

KEGG pathway enrichment and GO analysis were performed for functional annotation to elucidate the underlying pathways (**Table 2**). We found that the expressed genes mainly enriched in inflammatory bowel disease, type I diabetes mellitus, cytokine-cytokine receptor interaction, Th17 cell differentiation, JAK-STAT signaling pathway, graft-versus-host disease, T cell activation, regulation of immune system process, and response to an organic substance (**Fig. 1A ,1B**). Type 1 diabetes is characterized by an imbalance in cytokine-cytokine receptor interactions, leading to chronic inflammation and immune dysregulation (34). In individuals with T1DM and confirmed

diabetic sensorimotor polyneuropathy (DSPN), there is an increase in both pro-inflammatory and anti-inflammatory cytokines, including IL-1α, IL-4, IL-12p70, IL-13, IL-17A, and TNF-α. These cytokines play a role in the immune response and may be part of compensatory mechanisms to balance immune activity (34). The JAK/STAT signaling pathway is crucial in various diseases, including T1DM. This pathway involves many critical biological processes, such as cell proliferation, differentiation, apoptosis, and immune regulation (35). Dysregulation of the JAK/STAT pathway has been associated with autoimmune diseases, including T1DM (36). In T1DM, the JAK/STAT pathway has been implicated in the viability and apoptosis of pancreatic β-cells. IL-6, a proinflammatory cytokine, activates the JAK/STAT signaling pathway, inhibiting pancreatic β-cell viability and increasing apoptosis. Additionally, high glucose levels can induce the activity of the JAK/STAT pathway, resulting in the activation of TGF-β1 and an increase in cardiac fibroblasts (35). In the T-cell activation process, $CD4^+$ T cells help autoreactive B cells, producing high-affinity autoantibodies; conversely, $CD8^+$ T cells directly contribute to the destruction of β-cells (37). In addition to T cells, other immune cell subsets such as macrophages, B cells, monocytes, and natural killer (NK) cells have been reported to contribute to insulitis and β-cell destruction in T1DM by infiltrating pancreatic islets and producing pro-inflammatory cytokines and nitric oxide (NO) (38). The role of the remaining enriched pathways in T1DM disease still requires further exploration.

Furthermore, we constructed the PPI network by all genes for functional interactions (**Fig. 2A**). The most significant three functional modules were filtered (**Fig. 2B**). We found that the seed gene of the module was IL2. IL-2, a cytokine that affects T cells mainly through the JAK-STAT, Erk, and PI3K pathways, has been studied primarily in T cells. However, IL-2 can also influence other cell types if they express the IL-2 receptor (39). IL-2 is also involved in the activation and proliferation of T cells, particularly CD8+ cytotoxic T cells, which are critical for antitumor immune responses (40). The upregulation of CTLA-4 and Treg cells through low production of IL2 is one of the associated risk factors with anti-diabetic immune tolerance (41). Lack of negative costimulation induced by the PD-1/PD-L1 pathway can lead to increased T cell stimulation and activation, contributing to the development of T1DM. Additionally, the attendance or lack of GAD antibodies (GADA) has been linked to the period between the start of immune checkpoint inhibitors (ICIs) therapy and the onset of T1DM (41). The balance between pathogenic and regulatory cells, including Treg cells, contributes to developing autoimmunity in T1DM (39). Several T1DM susceptibility loci, including IL2, CTLA4, IL10, PTPN2, and IL2RA, could influence both effector T cells and Treg cells FOXP3, a transcription factor, is used as a marker of CD4+ Treg cells (39). CCL20 got the same score as IL-2 in the module (**Fig. 2B**). CCL20, also known as macrophage inflammatory protein (MIP)-3α, is a chemokine that plays a role in leukocyte migration during inflammatory reactions (42). The CCL20-CCR6 axis has been extensively studied in various diseases, including diabetes. CCL20 is highly expressed by M2-type macrophages activated by IL4 in pancreatic cancer, which promotes the epithelium-mesenchymal transition (EMT) and invasion of cancer cells. Additionally, the CCL20-CCR6 axis has been shown to promote growth of pancreatic cancer (42). The present results showed that IL-2 is associated with T1DM T cell activation, and CCL20 might have a potential role in the development of T1DM. The interactions between this seed gene and T1DM still require further investigation.

The T1DM is likely caused by several transcription factors identified by our study (**Fig. 3 and Table 3**). Several transcription factors have been implicated in the pathogenesis of T1DM, including IRF2, IRF4, IRF8, and CDX2. It has been reported that IRF2 can inhibit the transcriptional activation of IRF1, another member of the IRF family. However, more study is required to comprehend IRF2's involvement in T1DM properly (43).

There is limited direct evidence linking IRF4 to the disease. However, IRF4 has been shown to play a role in other autoimmune disorders, suggesting its potential involvement in T1DM. For example, a study demonstrated that IRF4 is abnormally expressed in various mature lymphoid neoplasms and acts as an oncogene (44). This suggests that dysregulation of IRF4 could contribute to developing autoimmune diseases, including T1DM. Additionally, IRF4 expression has been observed in dermatopathic lymphadenopathy (DL), reported the expression of MUM1/IRF4, a variant of IRF4, in the case of DL (45). While DL is not directly related to T1DM, this finding suggests that IRF4 may

be involved in the immune response associated with chronic inflammatory conditions.

IRF8 was found to promote M1-type polarization of macrophages, which is associated with pro-inflammatory responses. In the context of diabetes, IRF8 activation was shown to promote macrophage autophagy and M1-type polarization, contributing to inflammation (46). However, there is limited research on the role of IRF8 in T1DM, and more studies are needed to elucidate its involvement in the disease.

While CDX2 is primarily associated with intestinal development and homeostasis, recent studies have suggested its potential involvement in the pathogenesis of T1DM. The TCF7L2 polymorphism, associated with T2DM, is associated with the clinical signs and autoimmune characteristics of T1DM (47). Meanwhile, the association between other transcription factors and T1DM must still be fully elucidated.

6. Conclusion

Aside from IL-2 being a determined significant candidate of the T1DM network hotspot, our results contribute to our understanding of the complicated autoimmune disease by determining the hub genes and their transcription factors in the modulating functional network that can be an opportunity to treat and prevent the human T1DM disease.

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Supporting information

S1. T1DM-associated genes list.

Author Contributions

Mahnaz Azarnia contributed to the study's conception and design. Davood Zaeifi prepared data collection, analysis, and the first draft of the manuscript. All authors read and approved the final manuscript.

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