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Original Article

Integrative analysis of bulk and single-cell RNA sequencing data reveals distinct subtypes of MAFLD based on N1-methyladenosine regulator expression☆

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ABSTRACT

Background: Metabolic dysfunction-associated fatty liver disease (MAFLD) is now the most prevalent chronic liver disease worldwide, with an increasing incidence rate. MAFLD is a heterogeneous disease that can have a low or high-risk profile for developing severe liver disease in its natural course. Recent evidence has highlighted the critical role of RNA methylation modification in the pathogenesis of various liver diseases. However, it remains unclear whether the RNA N1-methyladenosine (m^1A) modification of immune cells could potentially contribute to the pathogenesis and heterogeneity of MAFLD.

Materials and methods: To address this issue, we conducted an integrated bioinformatics analysis of MAFLD bulk and single-cell RNA sequencing (scRNA-seq) data to pinpoint m^1A regulators in the network. This was followed by a description of the immune landscape, pathway enrichment analysis, and molecular subtyping.

Results: The expression patterns of m^1A regulatory genes stratify MAFLD into two molecular subtypes, Cluster 1 and Cluster 2. These subtypes demonstrate different immune cell infiltration with distinct inflammation characteristics, which suggest different immune-inflammatory responses in the liver. Notably, Cluster 2 is associated with pro-inflammation and may be more likely to lead to progressive stages of MAFLD. Through intersection analysis of weighted gene co-expression network analysis (WGCNA) and m^1A regulatory genes, three true hub genes (*ALKBH1*, *YTHDC1*, and *YTHDF3*) were identified, all of which were strongly correlated with infiltrating immune cells. The specific signaling pathways involved in the three core genes were derived from genomic variation analysis. Furthermore, scRNA-seq data from 33,168 cells from six liver samples identified 26 cell clusters and eight cell types, with endothelial cells, macrophages, and monocytes showing the most significant differences between MAFLD and normal controls. The cell-cell communication network between immune cells and non-parenchymal cells was extremely sophisticated and changed significantly in MAFLD.

Conclusions: In summary, these findings demonstrate the involvement of m^1A in MAFLD heterogeneity and emphasize the crucial role of m^1A modulation of immune cells in regulating inflammation in MAFLD. These results may suggest potential therapeutic strategies for MAFLD.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a disorder associated with metabolic dysfunction that represents a broad range of conditions, from simple steatosis (NAFL) to non-alcoholic steatohepatitis (NASH), which can progress to liver fibrosis or cirrhosis, and

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even hepatocellular carcinoma.[1] Additionally, it has been suggested that NAFLD is a highly heterogeneous disease, and this heterogeneity is correlated with outcomes.[2] Due to these reasons, experts worldwide have reached a consensus that "metabolic dysfunction-associated fatty liver disease (MAFLD)" is a more appropriate overarching term.[3] Today, due to lifestyle changes, excess energy intake, and lack of exercise, MAFLD is becoming the most common chronic liver disease.[4,5] This condition is often accompanied by severe extrahepatic comorbidities, including dyslipidemia, diabetes mellitus, cardiovascular disease, and several cancers.[6] Despite numerous pharmacological candidates being tested in clinical trials every year, their efficacy, which has been confirmed in preclinical studies, is hampered by severe side effects, and almost all of them have failed in clinical trials.[7–9] One of the reasons behind this phenomenon is the lack of a clear classification and a comprehensive understanding of the MAFLD heterogeneity of different subtypes. Therefore, a classification based on an in-depth analysis of the molecular and cellular mechanisms of MAFLD is particularly important.

Decades ago, most efforts to elucidate the mechanism of MAFLD were focused on genomics at the transcriptional level.[10] However, in recent years, with the dramatic advances in sequencing technology, the dominance of gene-centric views has been challenged by the rapid development of RNA epigenetic approaches. This has brought RNA modification-based phenotypic variability into focus due to their essential function.[11,12] RNA modifications play a critical role in nearly every aspect of the biological process, ranging from early embryo development to disease pathogenesis.[13,14] Among over 140 distinct chemical modifications in RNA, methylation is the most prevalent chemical addition to RNA nucleotides. RNA methylation is dynamic and reversible at the post-transcriptional level,[15] and the reversibility of epigenetic modifications makes them more promising as potential drug targets. Apart from N6-methyladenosine (m^6A) modification, which is the most abundant internal messenger RNA (mRNA) modification in mammals, N1-methyladenosine (m^1A) is another prevalent RNA modification that involves the addition of a methyl group at the N1 position of adenosine in transfer RNA (tRNA), mRNA, and ribosomal RNA (rRNA). m^1A RNA methylation is orchestrated by methyltransferases and demethylases and recognized by m^1A-dependent RNA-binding proteins.[14] Although m^1A RNA modification has been found to be highly abundant in tRNAs and rRNAs, m^1A methylation modification in mRNAs cannot be underestimated. m^1A methylation plays a significant role in RNA metabolism, including RNA structure, stability, and mRNA translation, and is essential for gene expression and cellular processes. It has been shown to be involved in many physiological and pathophysiological conditions.[16–19] Studies have reported that m^1A RNA methylation plays an important role in glucose and lipid metabolism.[18,20] However, most previous studies have mainly focused on only one m^1A regulator in various cancers, and it remains to be fully elucidated whether m^1A modification has a potential role in the pathogenesis and heterogeneity of MAFLD. It is clear that the recognition of an epigenetic component in metabolic dysfunction or metaflammation could lead to new opportunities for early detection, classification, and prevention of MAFLD. Therefore, an all-round analysis of these m^1A regulators and their related enriched pathways will enhance our understanding of the molecular mechanisms of MAFLD.

MAFLD is a complex disorder that involves numerous influencing factors, including metabolism, endocrine function, immunity, and gut microbiota.[1,6] The transition from simple steatosis to steatohepatitis is a milestone in the natural course of MAFLD, which can be triggered by both intra- and extrahepatic events. In particular, studies have identified a fundamental role for the hepatic immune and inflammatory response during the development of NASH.[21–24] It has been reported that metabolic dysfunction and insulin resistance could fuel hepatic and systemic inflammation by directly activating innate and adaptive immune responses,[21,24] thus accelerating the development of MAFLD. Our published study demonstrated that an overactive innate immune response mediated metabolic inflammation, promoting the progression from NAFL to NASH.[25] Growing evidence suggests that the development of NASH is the consequence of aberrant recruitment of various immune cells to the liver, such as T-lymphocytes, macrophages, neutrophils, dendritic cells, and innate lymphoid cells in the liver, as well as in situ activation of liver resident non-parenchymal cells (NPCs) such as Kupffer cells (KCs), liver sinusoidal endothelial cells, and hepatic stellate cells (HSCs).[26,27] Although some animal studies have shown that inhibiting inflammatory cytokines and immune cells can attenuate MAFLD, this strategy only treats the symptoms and not the disease itself. Instead of simply suppressing these pro-inflammatory factors and cells, we need to understand the exact molecular and cellular mechanisms of liver inflammation, which is a major factor in determining the outcome of MAFLD. Therefore, it is crucial to determine the immune cell profile of MAFLD. Due to the complex and heterogeneous nature of MAFLD, a better understanding of the key triggers of disease progression could improve risk stratification of patients into categories such as inflammatory or non-inflammatory subgroups, as well as stable or progressive subgroups. Each patient may carry different risks based on different epigenetic and genetic backgrounds, and different molecular and cellular responses.[2] To identify patients at an elevated risk of progression to steatohepatitis or liver fibrosis, genetic analysis alone is insufficient, and epigenetic modifications must also be considered.[28,29] To provide a more comprehensive picture, we aim to unravel the pathogenesis and heterogeneity mechanisms of MAFLD from the perspective of RNA methylation and immune infiltration. In our study, we used bulk and single-cell RNA sequencing (scRNA-seq) data to reveal distinct subtypes of MAFLD based on m^1A regulator expression and found that they have different immune landscapes and inflammation characteristics. This provides a new direction for understanding the heterogeneity of MAFLD.

2. Materials and methods

2.1. Study design and data acquisition

A schematic diagram of the study is shown in Fig. 1. Microarray data of liver biopsy samples from individuals with MAFLD (GSE164760) and scRNA-seq data of mouse MAFLD (GSE129516) were obtained from the publicly available Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). In addition, dataset GSE130970 was used for verification purposes. Nine m^1A regulators were selected based on previously published literature. The Corrplot package was utilized to assess the correlations between the nine m^1A regulators using Pearson correlation analysis.

2.2. Consensus clustering of MAFLD samples

Consensus K-means clustering was utilized to achieve consistent clustering of the 9 m^1A regulator expression profiles of 74 patients with MAFLD in GSE164760. The process involved 50 repetitions, each with 80% of the samples. The optimal number of clusters that distinguished the molecular subtypes of MAFLD was determined based on the cumulative distribution function (CDF) curves of the consensus score and consensus matrix heatmaps.

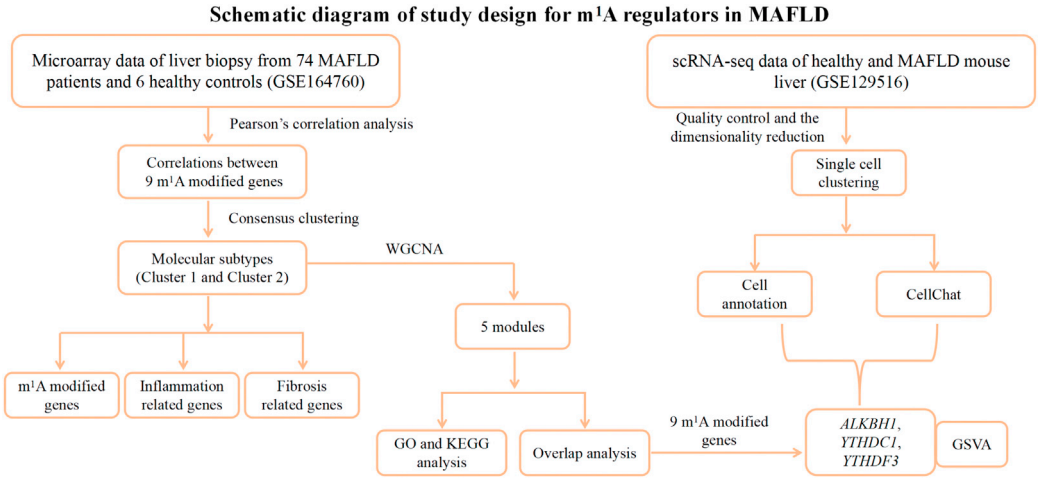

Fig. 1. Schematic diagram of the integrated analysis. We screened the Gene Expression Omnibus (GEO) database for differential N1-methyladenosine (m^1A) regulator genes in three datasets (GSE164760, GSE129516, and GSE130970), performed consensus clustering analysis, subtype annotation, WGCNA, immune infiltration analysis, external validation, and other specific analyses. Abbreviations: ALKBH1, AlkB homologue 1; GO, gene ontology; GSVA, gene set variation analysis; KEGG, Kyoto encyclopedia of genes and genomes; MAFLD, metabolic dysfunction-associated fatty liver disease; scRNA-seq, single-cell RNA sequencing; WGCNA, weighted gene co-expression network analysis; YTHDC1, YTH domain containing 1; YTHDF3, YTH domain family protein 3.

2.3. Weighted gene co-expression network analysis (WGCNA)

WGCNA was performed on MAFLD samples according to the differentially expressed genes (DEGs) in the two molecular subtypes. Unsigned co-expression networks were established using the WGCNA package in R, with the top 5000 genes based on expression variance. A soft thresholding power of 9 was selected based on the scale-free topology criterion for each set of genes. A pairwise Pearson correlation coefficient matrix was calculated, and an adjacency matrix was computed by raising the correlation matrix to the power of 9. The resulting weighted network was transformed into a network of topological overlap (TO), and genes were hierarchically clustered based on their TO. Modules were identified on the dendrogram using the dynamic tree cut algorithm. The singular value decomposition was used to summarize each module, with each module eigengene (ME) representing the first principal component of the module expression profiles. The ME with the highest variation in expression levels, denoted as I, was considered the most representative gene expression in the module.

2.4. Functional annotation of two molecular subtypes

The DEGs and the black module were performed using the clusterProfiler package in R. Subsequently, the DEGs and the black module were subjected to gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis. The GO analysis included molecular function, biological processes, and cell components. The results were found to be statistically significant with a *P*-value <0.05.

2.5. Single-cell RNA transcriptome landscape of cells in healthy and MAFLD mouse liver

2.5.1. Quality control and dimensionality reduction

All gene expression data were imported into the Seurat (v3.0) R toolkit using the read10 × function. Gene-cell matrices were filtered to exclude cells with nFeature RNA >50 & percent.mt < 5. Gene expression was represented as the fraction of the gene, converted into natural logarithm, and normalized. Principal component analysis (PCA) was performed using 20 highly variable genes. The t-distributed stochastic neighbor embedding (tSNE) method was then used for visualization of single-cell clustering.

2.5.2. Cell clustering and annotation

The location relationship between each cluster was determined through tSNE analysis. The mouse RNA-seq data (GSE129516) annotation file included in the celldex package was used to annotate the clusters. Some cells that are important for disease occurrence were also annotated. Finally, we set the logFC of FindAllMarkers to a threshold parameter of 1 in order to extract the marker gene of each cell subtype from the single-cell expression profile. We filtered for genes with |Log2FC| > 1 and *P*-value <0.05 to identify specific marker genes for each cell subtype.

2.6. Cell-cell interaction and immune cell infiltration in the liver tissue of MAFLD

To determine potential interactions among different cell types in liver tissues of MAFLD, we performed cell-cell interaction analysis using CellChat, an available repository of curated receptors, ligands, and their interactions. The "aggregateNet" function in CellChat was used to calculate the cell-cell communication network and visualize the signaling from each group. We evaluated the infiltration of immune cells in two molecular subtypes of MAFLD using the single-sample gene set enrichment analysis algorithm.

2.7. Functional analyses and mechanism exploration

We performed gene set variation analysis (GSVA) on ALKBH1, YTHDC1, and YTHDF3 using the GSVA package in R, with the hallmark gene sets as the reference gene set and a cut-off criteria of *P*-value <0.05 and *t*-value greater than 1. We identified the most significant changed pathways.

2.8. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed with HiScript II Q RT

SuperMix following the manufacturer's instructions. qRT-PCR assays were performed to analyze relative mRNA levels using AceQ® qPCR SYBR Green Master Mix (Vazyme, Nanjing, China). The mRNA amount for each gene was normalized to β-actin. The sequences of primers utilized in this study are listed in Supplementary Table 1.

2.9. Statistical analysis

The statistical analyses in this study were carried out with SPSS (version 25, IBM Corporation, Armonk, NY, USA) and R software (version 3.5.1). The results were presented as mean ± standard deviation (SD) from triplicate experiments. Student's *t*-test was utilized to estimate the difference between two groups. For comparisons of more than two groups, one-way analysis of variance was used. All *P* values were two-sided, and *P*-value <0.05 was considered significant.

3. Results

3.1. Differential expression profile of m^1A regulators in healthy controls and MAFLD

m^1A modification is a reversible and dynamic post-transcriptional RNA modification that can be added by methyltransferases, including tRNA methyltransferase (TRMT) 6, TRMT61A, and TRMT61B, and removed by demethylases, consisting of AlkB homologue (ALKBH) 1 and ALKBH3. Subsequently, it is recognized by RNA-binding proteins, including YTH domain containing 1 (YTHDC1) and three YTH domain family proteins (YTHDF1, YTHDF2, and YTHDF3).[14,30] Recent studies have shown that m^1A RNA methylation may be associated with metabolic and immune disorders.[18,20,31] To confirm this possibility, we identified nine m^1A regulators (Supplementary Table 2); then, we evaluated their relationships in bulk RNA-seq data (GSE164760). The triangle heatmap showed significant differences in the correlations between the nine m^1A regulators in MAFLD compared to healthy controls (Fig. 2A and B), indicating that m^1A regulators may exhibit a biological function in MAFLD. To confirm the reliability of these results, we treated Hep3B and HepG2 cells with either bovine serum albumin (BSA) or BSA-conjugated palmitate (PA) to establish a MAFLD model *in vitro*. We conducted qRT-PCR analysis to assess the expression levels of the identified genes and found that most m^1A regulators were upregulated in PA-treated Hep3B and HepG2 cells, with YTHDC1 showing a significant increase (Fig. 2C). Overall, the expression profiles of the m^1A regulatory genes were in good agreement between bioinformatic analysis and experiments.

3.2. m^1A regulators-mediated modification patterns characterize MAFLD heterogeneity

Next, our aim was to determine whether the expression patterns of the nine m^1A regulators could demonstrate heterogeneity in patients with MAFLD. Initially, we applied K-means consensus clustering to 74 patients with MAFLD from $k = 2$ to $k = 9$ based on the similarity of the m^1A regulators' expression. Notably, we determined the optimal clustering number using the CDF, and $k = 2$ displayed the optimal selection (Fig. 3A and B), which we referred to as Cluster 1 and Cluster 2. Subsequently, we validated the difference of the nine m^1A regulators between Cluster 1 and Cluster 2. The comparison of results showed that ALKBH3, TRMT6, TRMT61A, and YTHDF1 were significantly increased in Cluster 2, while ALKBH1, TRMT61B, YTHDC1, YTHDF2, and YTHDF3 were decreased in Cluster 2 (Fig. 3C). Moreover, to better illustrate the characteristics of m^1A regulators, we analyzed the relationship between inflammation and m^1A-associated clusters. We first conducted a gene-set analysis in MAFLD and healthy patient specimens using GEO database. The results indicate that the gene sets with the highest increase in MAFLD were the “inflammatory response group” and the “acute inflammatory response group”. In addition, the analysis showed that the identified genes were significantly enriched for inflammatory response, alpha amino acid metabolic process, and negative regulation of protein metabolic process signaling (Supplementary Fig. 1A and B). This suggests that the inflammatory response was hyperactivated in MAFLD. Further, we observed a marked increase in markers of liver inflammation, including interleukin (IL)-6, IL-10, IL-17A, and nucleotide-binding oligomerization domain-like receptor family, pyrin domain containing 3 (NLRP3), in Cluster 2 (Fig. 3D). This indicates a more severe metaflammation in Cluster 2. Taken together, these results suggest that the m^1A regulator-mediated modification patterns can clearly characterize MAFLD heterogeneity and are closely related to liver inflammation.

3.3. WGCNA to demonstrate heterogeneity between two molecular subtypes

Based on the results mentioned above, we have discovered that the expression patterns of m^1A regulators can divide MAFLD into two molecular subtypes. To identify the hub genes that influence the progression of MAFLD, we conducted a WGCNA. The soft threshold β was determined using the “sft$powerEstimate” function, and the optimal soft threshold was found to be 9. Using a topological overlap matrix, the inter-connectivity of genes among modules was visualized (Fig. 4A). We created five different co-expression modules, namely black (2052), brown (450), green (235), gray (2167), and red (96) (Fig. 4B). Additionally, we analyzed the correlation between modules and trials, and the results indicated that the black module had the most significant correlation between the two clusters (correlation coefficient = 0.74, $P = 0.00005$). Therefore, the black module was selected for further analysis. Subsequently, GO enrichment analysis and KEGG were employed to elaborate on the biological function of genes in the black module. The GO analysis revealed that the upregulated genes were enriched in the regulation of protein catabolic process, lipid catabolic process, ribosome, mitochondrial respirasome, and structural constituent of ribosome (Fig. 4C). The KEGG analysis suggested that upregulated genes were associated with ribosome, oxidative phosphorylation, NAFLD, and fatty acid metabolism pathways (Fig. 4D). These findings imply that the progression of the two molecular subtypes is closely linked to metabolic and inflammatory disorders. Finally, we overlapped the 9 m^1A regulators with the black module of WGCNA and identified three core genes, including ALKBH1, YTHDC1, and YTHDF3 (Fig. 4E). This suggests that the three core genes may play a critical role in MAFLD.

3.4. Analysis of scRNA-seq of MAFLD uncovered the heterogeneity of the immune microenvironment

The pathogenesis of MAFLD is a complex process that involves disturbances in glucolipid metabolism, as well as innate and/or adaptive immune responses and inflammation.[6,21,24] Recent re-analysis of MAFLD liver bulk RNA-seq data has revealed upregulation of genes involved in lipid metabolism, inflammation, and fibrosis. Moreover, emerging evidence suggests that responsive activation of local KCs, HSCs, liver sinusoidal endothelial cells, and infiltration of immune cells also play a critical role in accelerating disease progression.[26,27] However, it is still unclear whether hepatocytes and NPCs respond differently to the heterogeneity of MAFLD. To further understand the cellular heterogeneity in MAFLD pathogenesis, we re-analyzed scRNA-seq data to explore

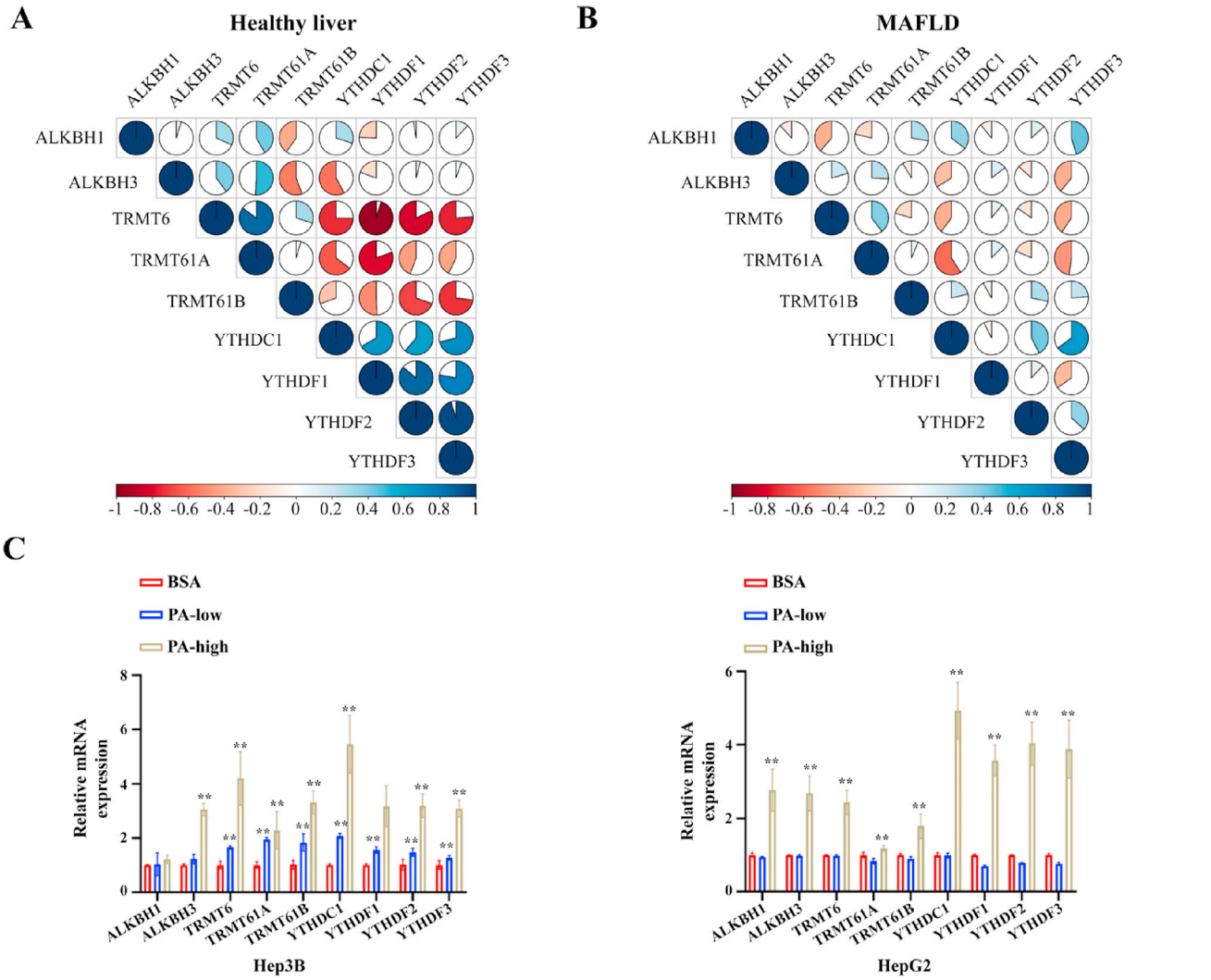

Fig. 2. Expression pattern of nine m^{1}A regulators in MAFLD. (A) Pearson correlation of nine m^{1}A regulators in healthy liver. **(B)** Pearson correlation of nine m^{1}A regulators in MAFLD liver. **(C)** Hep3B or HepG2 cells were treated with bovine serum albumin (BSA) or BSA-conjugated palmitate (PA) for 24 h, and m^{1}A regulator expression was detected using qRT-PCR. $^{**}P < 0.01$ *vs*. BSA group. Abbreviations: ALKBH, AlkB homologue; MAFLD, metabolic dysfunction-associated fatty liver disease; m^{1}A, N1-methyladenosine; qRT-PCR, quantitative real-time polymerase chain reaction; TRMT, transfer RNA methyltransferase; YTHDC1, YTH domain containing 1; YTHDF, YTH domain family protein.

hepatocyte and NPC transcripts in normal and MAFLD mouse livers. In this study, we profiled 33,168 cells for additional analysis (normal = 3, MAFLD = 3) after quality control and standardization. After gene expression normalization, PCA revealed no noticeable batch effect among the samples (Fig. 5A). Subsequently, unbiased clustering based on tSNE analysis was used to visualize the cells, and 26 cell clusters were identified simultaneously (Fig. 5B). Based on the expression of markers, these 26 cell clusters were annotated as endothelial cells, natural killer (NK) cells, B cells, macrophages, monocytes, hepatocytes, T cells, and fibroblasts (Fig. 5C). The most significant differences between MAFLD and normal controls were observed in endothelial cells, macrophages, and monocytes (Fig. 5D). These findings suggest that the heterogeneous pathogenesis of MAFLD may be associated with cellular heterogeneity.

As previously mentioned, the association of m^{1}A regulators with MAFLD heterogeneity leads us to speculate that the landscape of m^{1}A regulators may explain the cellular heterogeneity observed in MAFLD. Our results suggest that reader proteins (Ythdc1, Ythdf1, Ythdf2, and Ythdf3) and eraser protein Alkbh1 are significantly expressed in T cells, macrophages, monocytes, and endothelial cells. In contrast, Alkbh3, Trmt6, and Trmt61a are only slightly expressed in various liver cells (Fig. 5E and F). These findings support the idea that different expressions of m^{1}A regulators contribute to cellular heterogeneity, which may promote immune cell infiltration in MAFLD.

3.5. Construction of the cell-cell interaction and immune cell infiltration network in MAFLD

To further investigate the function of m^{1}A regulators in immune cell infiltration, we used CellChat to decipher ligand-receptor interactions in multicellular systems. We predicted the number and strength of ligand-receptor interactions and found that they displayed extremely distinct cell communication networks in healthy livers and MAFLD (Fig. 6A and Supplementary Fig. 2A). In addition, we identified specific ligand-receptor interactions among different cells from healthy livers and MAFLD, including strong interactions among macrophages and B cells, T cells and B cells, fibroblasts and B cells, macrophages and NK cells through ligand–receptors, such as Mif–(Cd74-Cxcr4) and Cxcl16–Cxcr6, which might induce an inflammatory response in MAFLD (Fig. 6B). Furthermore, macrophages and fibroblasts closely interacted with other cells in MAFLD

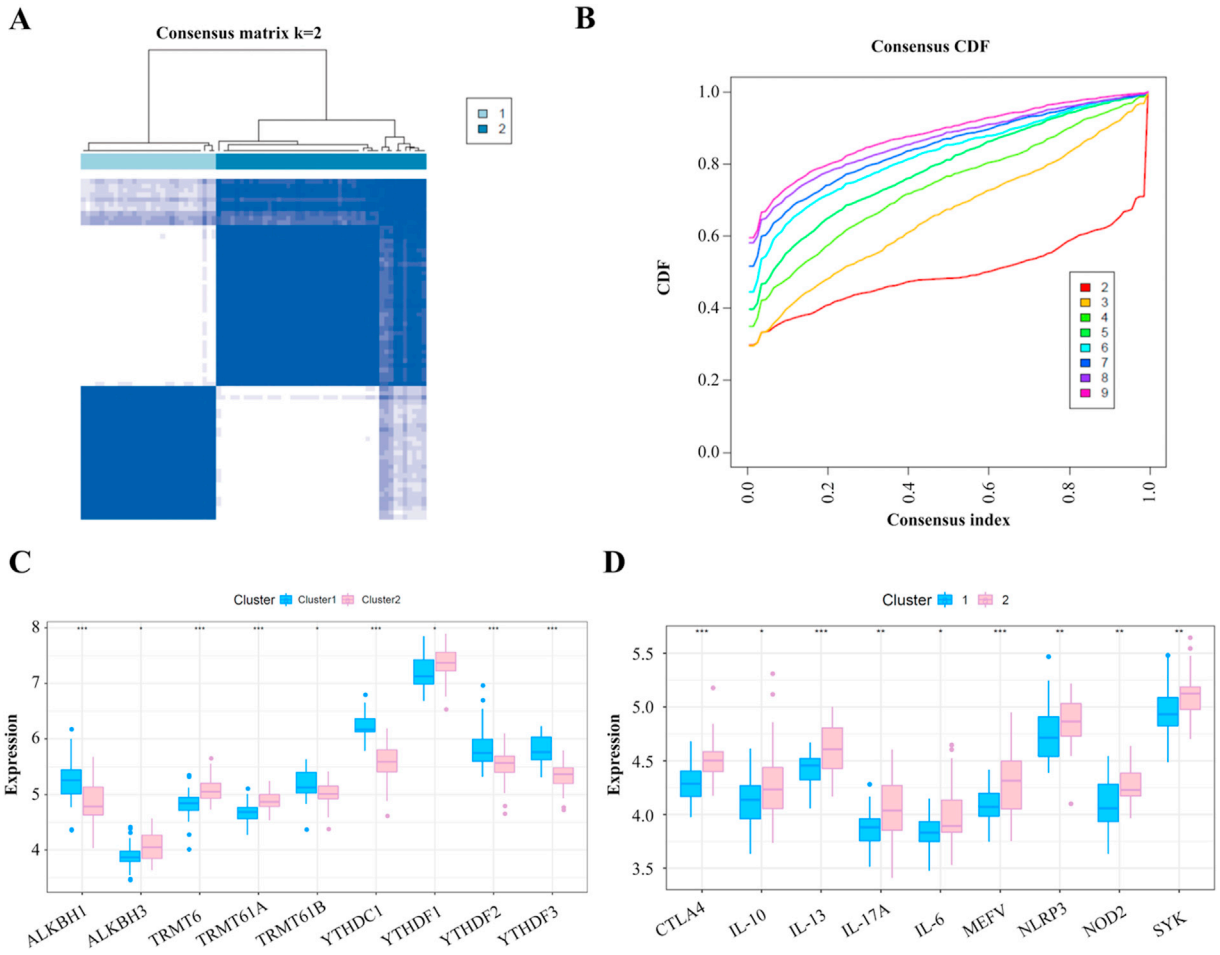

Fig. 3. Characterization of optimal MAFLD subtypes based on m^{1}A regulators. (A) Consensus matrix. The values of the consensus matrix are presented in shades of white to dark blue, and the consensus matrix was ranked according to consensus clustering. The bars between the dendrogram and the heatmap represent the molecular subtypes. **(B)** Cumulative distribution function (CDF) curves representing the CDF of each cluster based on different subtype numbers (k = 2, 3, 4, 5, 6, 7, 8, and 9) are exhibited in corresponding colors. **(C)** Differential expression of nine m^{1}A regulators in two molecular subtypes of MAFLD. **(D)** Differential expression of inflammatory genes in two subtypes of MAFLD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Abbreviations: ALKBH, AlkB homologue; CTLA4, cytotoxic T lymphocyte antigen 4; IL, interleukin; MAFLD, metabolic dysfunction-associated fatty liver disease; m^{1}A, N1-methyladenosine; NLRP3, nucleotide-binding oligomerization domain-like receptor family, pyrin domain containing 3; NOD2, nucleotide-binding oligomerization domain containing 2; SYK, spleen tyrosine kinase; TRMT, transfer RNA methyltransferase; YTHDC1, YTH domain containing 1; YTHDF, YTH domain family protein.

(Fig. 6C). Overall, the data showed that sophisticated cell-cell interactions existed between immune cells and NPCs in the liver, and more importantly, the intercellular communication network changed in MAFLD. These results suggest that NPCs may play an essential role in MAFLD by mediating immune infiltration.

The immune microenvironment is mainly composed of various immune cells, extracellular matrix, growth factors, inflammatory factors, and unique physical and chemical features. These factors significantly determine the sensitivity of clinical treatment and diagnosis of MAFLD. Therefore, we investigated the infiltration of immune cells in two molecular subtypes. The results revealed that the contents of inflammation-promoting cells, mast cells, plasmacytoid dendritic cells (pDCs), T cell co-inhibition, T cell co-stimulation, and T helper (Th) cells were significantly higher in Cluster 2 than in Cluster 1, while CD8$^+$ T cells and parainflammation cells were significantly lower in Cluster 2 than in Cluster 1 (Fig. 6D), indicating that molecular subtype Cluster 2 had the most advanced stage of inflammation. The RNA-seq data from GSE130970 were also divided into two molecular subtypes based on 9 m^{1}A regulators (Supplementary Fig. 3A and B), and further analysis showed enhanced B cells and T follicular helper (Tfh) cells in Cluster 2, which was consistent with the GSE164760 data (Supplementary Fig. 3C). Subsequently, we evaluated the correlation between three core genes (*ALKBH1*, *YTHDC1*, and *YTHDF3*) and immune infiltration. As shown in Fig. 6E, all three core genes were strongly correlated with immune cell infiltration. Taken together, these results suggest that ALKBH1, YTHDC1, and YTHDF3 may play a crucial role in intercellular crosstalk that promotes immune cell infiltration.

3.6. GSVA for ALKBH1, YTHDC1, and YTHDF3

To identify the specific signaling pathways involved in immune infiltration mediated by the three core genes, we conducted GSVA of ALKBH1, YTHDC1, and YTHDF3 in the molecular signatures database. The results showed strikingly positive enrichment of oxidative phosphorylation, fatty acid metabolism, adipogenesis, and PI3K/AKT/mTOR signaling, while Kras signaling, myogenesis, and Wnt/β-catenin signaling were negatively enriched in ALKBH1, YTHDC1, and YTHDF3 (Fig. 7A, B, and C). This suggests that the three core genes may regulate these signaling pathways and affect the progression of MAFLD.

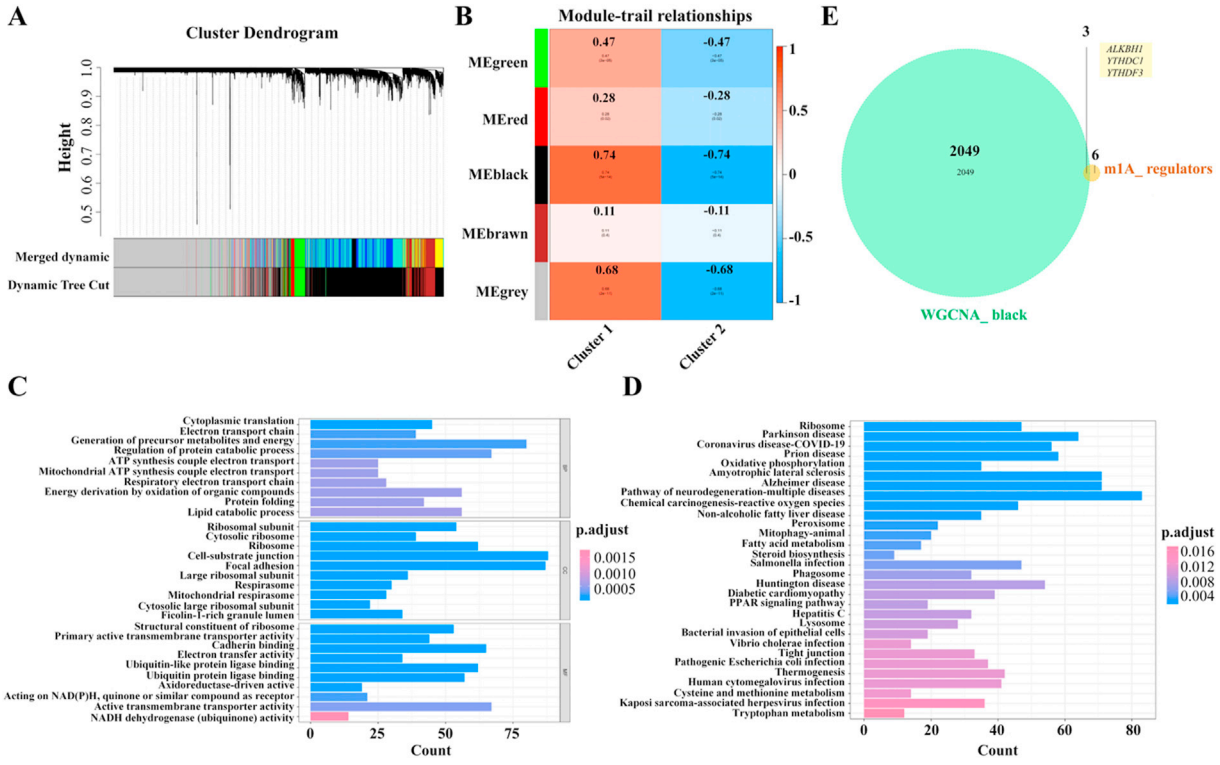

Fig. 4. Weighted gene co-expression network analysis (WGCNA) demonstrating the heterogeneity of two molecular subtypes. (A) Hierarchical cluster analysis of co-expression clusters using WGCNA. **(B)** The five modules were assigned the following colors: black, brown, green, gray, and red. The heatmap displays the correlations between the five different modules. **(C)** Gene ontology (GO) enrichment analysis of differentially expressed genes (DEGs) in the black module. **(D)** KEGG pathway analysis of DEGs in the black module. **(E)** The Venn diagram shows significant overlap between m^{1}A regulators and the black module. Abbreviations: ALKBH1, AlkB homologue 1; KEGG, Kyoto encyclopedia of genes and genomes; m^{1}A, N1-methyladenosine; ME, module eigengene; YTHDC1, YTH domain containing 1; YTHDF3, YTH domain family protein 3.

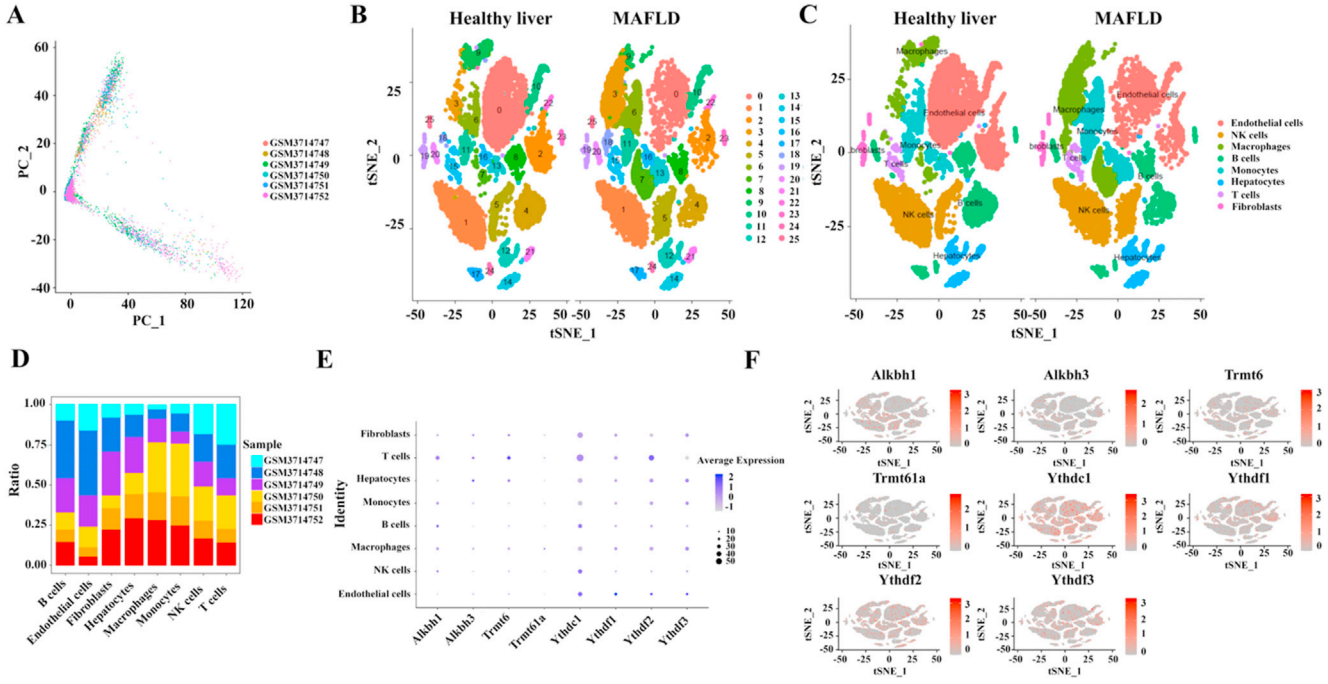

Fig. 5. Single-cell RNA sequencing (scRNA-seq) analysis of MAFLD livers in mice. (A) principal component analysis (PCA) of 33,168 single cells. **(B)** t-Distributed stochastic neighbor embedding (tSNE) visualization of liver cell clusters based on 33,168 single-cell transcriptomes. **(C)** tSNE plot of the eight main cell types identified in liver cell clusters based on 33,168 single-cell transcriptomes. **(D)** Relative proportion of healthy and MAFLD mouse liver cells across each cluster. **(E)** Circle plot of m^{1}A-modified gene expression in different cell clusters. **(F)** Feature plot for m^{1}A-modified genes in different cell clusters. Abbreviations: Alkbh, AlkB homologue; MAFLD, metabolic dysfunction-associated fatty liver disease; m^{1}A, N1-methyladenosine; NK, natural killer; Trmt, transfer RNA methyltransferase; Ythdc1, YTH domain containing 1; Ythdf, YTH domain family protein.

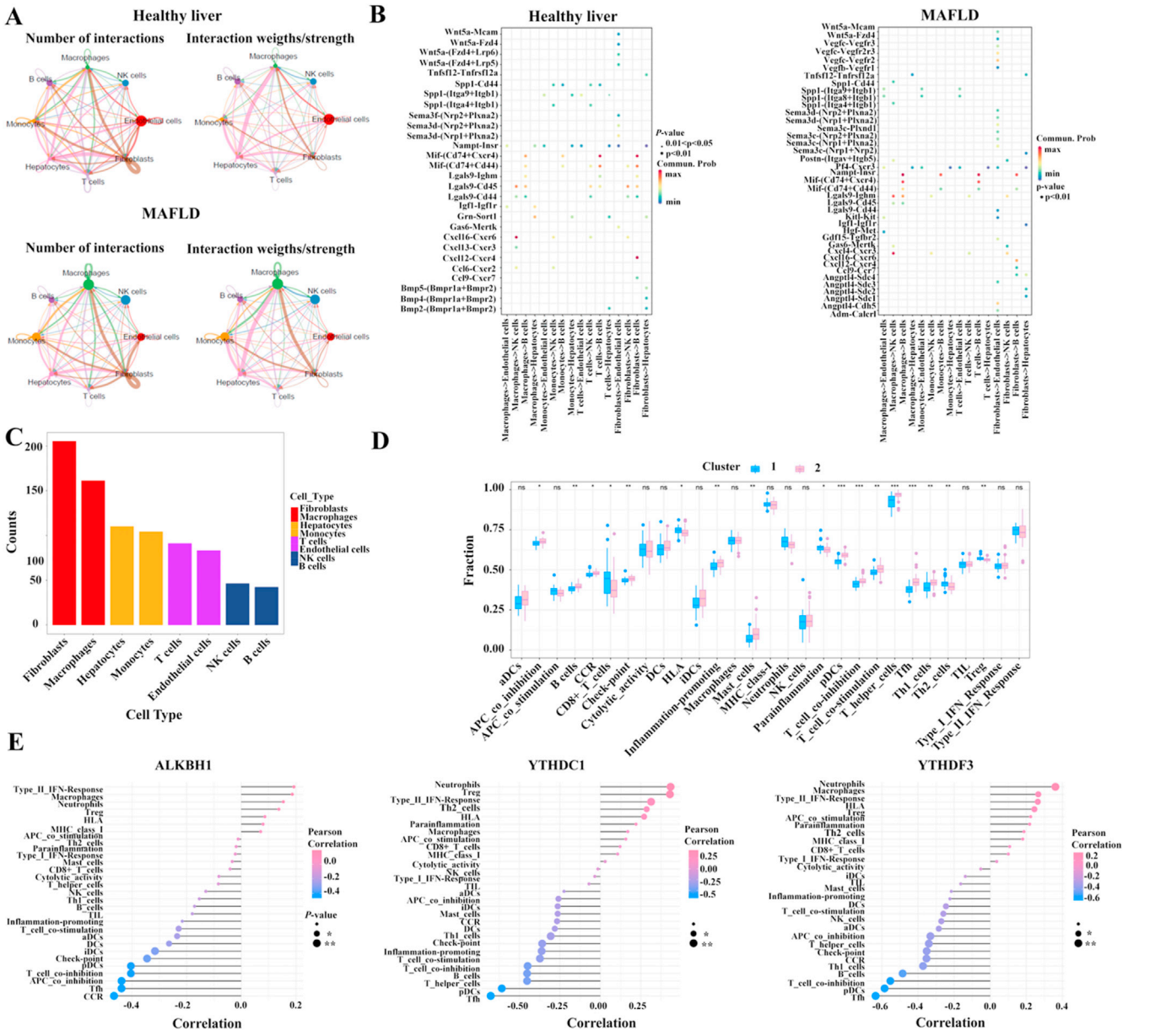

Fig. 6. Dense network and multiple immune responses in MAFLD. (A) Intercellular communication between different cell clusters in the healthy liver and MAFLD. **(B)** Summary of ligand-receptor interactions between different cell clusters from the healthy liver and MAFLD. **(C)** Relative percentage of cell-cell interactions. **(D)** Comparisons of various immune cells between two molecular subtypes. **(E)** Associations of ALKBH1, YTHDC1, and YTHDF3 with immune infiltration cells in MAFLD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. ns, not significant. Abbreviations: ALKBH1, AlkB homologue 1; APC, anaphase-promoting complex; Bmp, bone morphogenetic protein; Bmpr, bone morphogenetic protein receptor; Cxcl, C-X-C motif ligand; Cxcr, C-X-C motif receptor; HLA, human leukocyte antigen; IFN, interferon; MAFLD, metabolic dysfunction-associated fatty liver disease; MHC, major histocompatibility complex; NK, natural killer; pDCs, plasmacytoid dendritic cells; Tfh, T follicular helper; Treg, regulatory T; YTHDC1, YTH domain containing 1; YTHDF3, YTH domain family protein 3.

4. Discussion

A hallmark of MAFLD is its heterogeneity, which refers to significant variation in disease progression among patients.[2,3,32] For a long time, MAFLD has been classified into two subtypes based on inflammation and fibrosis, which rely mainly on tedious and expensive clinical examination. However, as more detailed molecular mechanisms underlying MAFLD have been identified, experts have begun to question the accuracy and plausibility of this classification. This is especially true given that most of the clinical trials testing new drugs have failed,[7–9,33] with a reasonable explanation being a lack of understanding of disease heterogeneity. Underestimating MAFLD heterogeneity can lead to serious deviations in treatment, which is unfavorable for new drug research. Therefore, it is of great significance to explore a regulatory analysis that affects heterogeneity and gain a comprehensive understanding of MAFLD pathogenesis. To address these issues, we utilized the GEO database to identify DEGs of m^1A regulators between MAFLD patients and healthy controls. We then analyzed the correlation between these regulators, immune landscape, and inflammatory responses. Based on the expression levels of m^1A regulators, we determined two different regulation patterns (Cluster 1 and Cluster 2) using the unsupervised clustering method. The two clusters exhibited distinct immune cell infiltration, and Cluster 2 showed

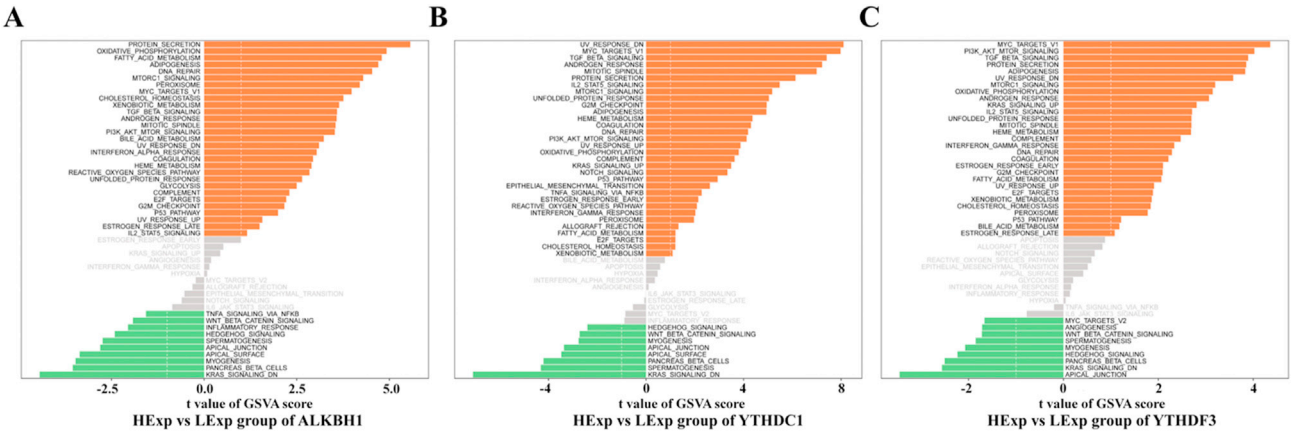

Fig. 7. Gene set variation analysis (GSVA) of three m^{1}A core genes. GSVA of **(A)** ALKBH1, **(B)** YTHDC1, and **(C)** YTHDF3 from the molecular signatures database. Abbreviations: ALKBH1, AlkB homologue 1; YTHDC1, YTH domain containing 1; YTHDF3, YTH domain family protein 3.

higher inflammation, suggesting that it is more likely to be a progressive subtype of MAFLD. Using various bioinformatics analysis, we identified three m^{1}A regulatory hub genes (*ALKBH1*, *YTHDC1*, and *YTHDF3*) and described their related signaling pathways and immune cell infiltration. This study sheds new light on the mechanism of MAFLD in terms of RNA m^{1}A methylation modification (Fig. 8). It emphasizes the interpatient heterogeneity of MAFLD and may provide a novel theoretical basis for the primary prevention and treatment of MAFLD.

In recent years, epigenetic mechanisms have gained new perspectives in MAFLD, in addition to genetic regulation.[28,34] The epigenome is more susceptible to modification by environmental changes and other effectors compared to the genome sequence, contributing significantly to phenotypic diversity in many aspects.[35,36] Therefore, epigenetic modification is essential for the heterogeneity of MAFLD. RNA methylation is a type of epigenetic modification that has been attracting more attention due to its crucial roles in gene expression and molecular phenotype. Compared to m^{6}A, m^{1}A modification is relatively new, and the detection and analysis approaches are not fully equipped. However, our perception of m^{1}A methylation modification is constantly improving with studies. In liver cancer, scientists found that the methyltransferase complex TRMT6/TRMT61A promoted m^{1}A methylation at position 58 (m^{1}A58) of tRNA, increasing peroxisome proliferator-activated receptor delta (PPARδ) translation. This, in turn, triggered cholesterol biosynthesis, eventually driving the self-renewal of liver cancer stem cells and tumorigenesis via activating hedgehog signaling.[18] More recently, Wu *et al.*[20] reported that m^{1}A can regulate the translation and transcription of ATP5D and then regulate glycolysis in cancer cells. This study was the first to reveal a crosstalk between mRNA m^{1}A modification and cell metabolism. Currently, research on m^{1}A methylation modification primarily focuses on cancer development, leading to an undervaluation of its role in other chronic benign diseases. The two aforementioned studies suggest that m^{1}A RNA modification is closely related to cell metabolism, motivating further investigation into the correlation between m^{1}A methylation and metabolic diseases. In the present study, we found that all recognized regulators of m^{1}A methylation were differentially expressed in healthy individuals and those with fatty liver disease. Additionally, the m^{1}A RNA methylation landscape stratified MAFLD into two subtypes with distinct metaflammation characteristics. To our knowledge, this is the first report to highlight the biological significance of m^{1}A methylation modification in MAFLD and emphasizes the relationship between m^{1}A modification patterns and MAFLD heterogeneity. However, these preliminary studies contribute to a field of epitranscriptomics in MAFLD that is still in its embryonic stage, and further work will be necessary to determine how m^{1}A regulators can influence liver function and the pathogenesis of MAFLD.

Studies have identified the fundamental role of hepatic immune and inflammatory responses in NASH development.[21,22,24,37,38] Therefore, we further investigated the relationship between the two m^{1}A subtypes and immune cell infiltration. Cell-cell ligand-receptor interaction analysis revealed that the relationship among various immune cells in fatty livers is extremely complex, and different immune cells play different roles. NK cells and DCs are reported to contribute to MAFLD pathogenesis through cytotoxic effects and cytokine production in the innate immune system.[24,39] For example, cytokines IL-1β and tumor necrosis factor-alpha (TNF-α) are common pro-inflammatory cytokines that drive liver inflammation and fibrosis. T cells, as the main cellular component of the adaptive immune system, consist of multiple differentially active subsets, including Th cells, regulatory T (Treg) cells, and CD8$^+$ cytotoxic T (Tc) cells, among others.[21,39–42] These adaptive immune cells specifically produce pro-inflammatory cytokines, such as interferon-gamma (IFN-γ), IL-2, TNF-α, IL-33, and others, all of which may accelerate the progression of MAFLD.[43,44] Both the aforementioned studies and our present research indicate that the complex immune microenvironment consists of many immune cells that participate in the development of MAFLD and each perform their duties. It is important to note that, despite the complexity of immune cell infiltration, the expression of pro-inflammatory cytokines is consistently upregulated in Cluster 2 in our study, which may suggest a more severe metaflammation in this Cluster. Overall, many immune cell subsets have not been thoroughly investigated in the context of MAFLD and need further definition. Due to a lack of sufficient MAFLD liver samples, we did not perform more validation experiments. Our findings could be the beginning of new research in this field, intending to offer valuable suggestions.

No previous research has focused on the relevance of m^{1}A in the pathogenesis and immune microenvironment of MAFLD. Therefore, we aim to clarify the effect of m^{1}A on changes in the immune microenvironment and inflammatory responses in MAFLD. In this study, we utilized scRNA-seq data to analyze immune cell subtypes and contents in fatty livers. The results of PCA showed natural heterogeneity not only in the distribution of immune cells between MAFLD and normal liver tissue but also in different clusters based on the distinct expression of m^{1}A regulators. On one hand, we observed higher levels of monocytes and macrophages in MAFLD

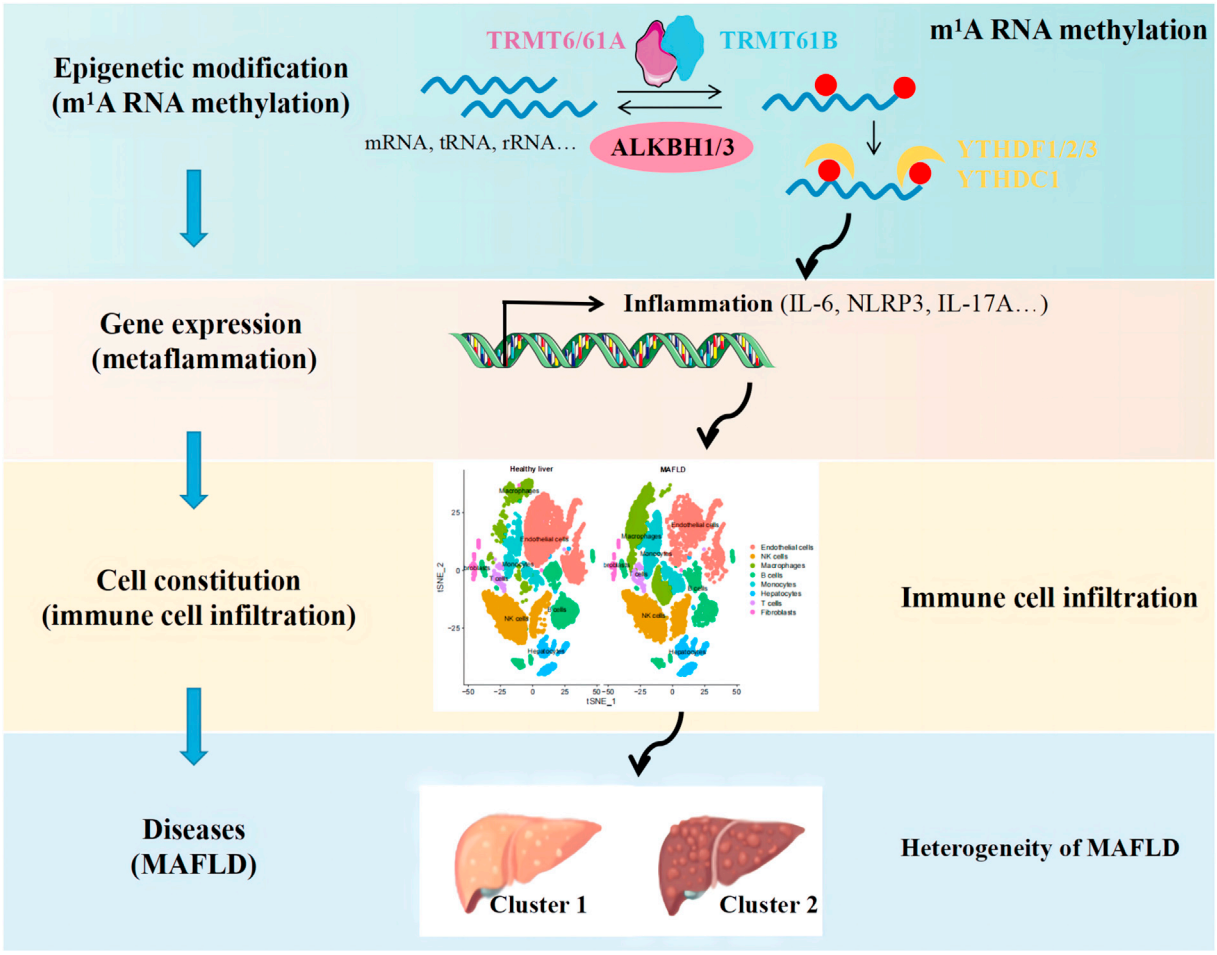

Fig. 8. Schematic representation of the different molecular subtypes in the pathogenesis of MAFLD mediated by m^{1}A RNA methylation. m^{1}A methylation plays a significant role in various RNA structures, stability, and mRNA translation, making it essential for gene expression and cellular processes. The expression patterns of m^{1}A regulatory genes stratify MAFLD into two subtypes (Cluster 1 and Cluster 2) with distinct immune inflammation characteristics. This indicates that m^{1}A RNA modification may affect MAFLD progression by mediating the differentiation of immune cells to modulate the metaflammatory microenvironment. Abbreviations: ALKBH, AlkB homologue; IL, interleukin; MAFLD, metabolic dysfunction-associated fatty liver disease; m^{1}A, N1-methyladenosine; mRNA, messenger RNA; NK, natural killer; NLRP3, nucleotide-binding oligomerization domain-like receptor family, pyrin domain containing 3; rRNA, ribosomal RNA; TRMT, transfer RNA (tRNA) methyltransferase; tSNE, t-distributed stochastic neighbor embedding; YTHDC1, YTH domain containing 1; YTHDF, YTH domain family protein.

tissues. On the other hand, further analysis revealed that Cluster 2 was accompanied by higher levels of inflammation-promoting cells, mast cells, pDCs, T cell co-inhibition, T cell co-stimulation, and Th cells, as well as higher expression of inflammatory factors. More importantly, we found that three essential m^{1}A regulators (*ALKBH1*, *YTHDC1*, and *YTHDF3*) strongly correlated with distinct immune cell contents. All these data indicate that m^{1}A RNA modification may affect MAFLD progression by mediating the differentiation of immune cells to modulate the immune-inflammatory microenvironment. However, these preliminary data require further validation in MAFLD animal models and clinical specimens. Moreover, we understand that the best phenotyping approaches in clinical practice should consider patient variables such as race, age, gender, body mass index, microbiota composition, and comorbidities, and integrate with genetic and epigenetic factors to better predict "high-risk" factors for MAFLD. Thus, this study still needs to be investigated in human models.

5. Conclusions

In summary, to the best of our knowledge, this study is the first to demonstrate that m^{1}A methylation modification plays a crucial role in the development of MAFLD. It may lead to the reprogramming of the immune microenvironment and trigger metaflammation in the liver. These findings will enhance our understanding of the mechanisms underlying MAFLD and may provide a novel theoretical basis for its primary prevention and treatment.

Authors' contributions

J. He, C. Xiao, and C. Li contributed equally to this work. C. Du designed the study and was responsible for the data integrity and accuracy of the analysis. F. Yang reviewed and edited the manuscript. J. He, C. Xiao, and C. Li wrote the manuscript and contributed to the collection and analysis of the data. All authors reviewed and approved the final version of this manuscript.

Declaration of competing interest

The authors declare that there is no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.livres.2023.06.001.

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