Identification of Hub Genes and Pathways With Immune Cell Infiltration in Cholangiocarcinoma by Weighted Gene Co-expression Network Analysis

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Research

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Abstract

Background: Cholangiocarcinoma (CCA) is the most frequent tumor in biliary tract and the second most common primary tumor of the liver. However, the molecular biomarkers in tumorigenesis of CCA remain unclear. Therefore, we aim to explore the underlying mechanisms of progression and screen for novel prognostic biomarkers and therapeutic targets.

Method: The genes expression sequencing of normal and CCA samples were selected from the Gene Expression Omnibus database (GEO) and the Cancer Genome Atlas (TCGA). The weighted gene co-expression network analysis (WGCNA) was used to build the co-expression network. Gene ontology (GO) as well as Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were applied for the selected genes. The protein-protein interactions of these modules are visualized using cytoscape. Furthermore, the significance of these genes was confirmed by survival analysis. The tumor immune estimation resource (TIMER) was presented to investigate assess the relationship between the hub genes and the immune cells infiltration.

Results: Ten hub genes with CCA development were identified in this study containing CDC20, CCNA2, TOP2A, AURKA, CCNB2, UBE2C, NUSAP1, PRC1, PTTG1 and MCM4. Key genes of CCNB2 and PTTG1 might be potential prognostic biomarkers for CCA. GO analysis indicated the enrichment terms of nuclear division, collagen-containing extracellular matrix and cell adhesion molecule binding. KEGG analysis demonstrated that the cell cycle pathway was the significantly altered pathway. There was a negative correlation between TOP2A, AURKA, CCNB2, PRC1 expression and the infiltration of CD4+T cell, while MCM4 expression was positively associated with the infiltration of neutrophil cells. No significant association between CDC20 levels and CD4+T cell, CD8+T cell, B cell, neutrophil, macrophage, or dendritic cell infiltration in CCA, the same as CCNA2, UBE2C, NUSAP1, PTTG1 respectively.

Conclusion: These candidate genes may involve in the development of CCA. Our results offer novel insights into the etiology, prognosis, and treatment of CCA.

Introduction

Cholangiocarcinoma (CCA), a group of heterogeneous carcinomas characterized by cholangiocyte differentiation, is the most common biliary tract tumor and the second frequent primary hepatic malignancy[1,2]. The incidence of CCA has risen to 10-25% of all primary liver malignancies during the past 40 years and accounted for approximately 2% of cancer-related deaths globally per year[3,4]. The diagnosis of CCA is challenging because of its atypical symptoms and anatomic location, and most patients are diagnosed with advanced CCA[5]. At present, the serum biomarkers routinely for CCA diagnosis, such as cancer antigen 125 and carbohydrate antigen 199, are not sensitive or accurate[6,7]. Overall, the prognosis of CCA is extremely poor, with an average 5-year OS rate of 5 to 10 %[8]. In non-operable CCA the 5-year OS is less than 5%[9]. Consequently, the use of novel biomarkers for early

detection and monitoring of CCA patients, as well as a better understanding of the molecular mechanisms associated with its development, are critical and may provide new insights for treatment.

In the last decade, gene expression analysis based on the next-generation sequencing (NGS) and high-throughput platforms has been widely used to detect significant expression changes in cancers [10,11]. Several studies indicated that different genomic alterations were involved in the development of CCA and might have potential value for its diagnosis and prognosis. Frequent mutations in oncogenes such as KRAS, as well as cancer suppressor genes of TP53 were identified in CCA[12,13].IDH1 mutation was also founded to be involved in downregulated genes with hypermethylation status in CCA patients[14,15]. Although several genes and mechanisms have been demonstrated to be closely implicated in the development of CCA, the comprehensive profile of the whole genes of CCA is still unclear. Weighted gene co-expression network analysis (WGCNA), a powerful biology method, has been successfully used to identify hub genes in many malignancies to describe the co-expression patterns between genes across microarray samples. In this study, to further investigate the molecular mechanisms of CCA development, we used WGCNA to explore the hub genes of CCA and analyze its relationship with immune infiltration.

Material And Methods

Data collection and screening

The gene expression profiles GSE26566 (GPL6104 Illumina humanRef-8 v2.0 expression beadchip) were collected from the Gene Expression Omnibus database (GEO, https://www.ncbi.nlm.nih.gov/geo/). This dataset included 6 normal samples and 104 CCA samples. To enhance the robustness, gene expression profile data and survival data of CCA were also downloaded from the Cancer Genome Atlas database (TCGA, https://www.cancer.gov/tcga), including 9 normal and 35 CCA samples. Differentially expressed genes (DEGs) between normal and CCA samples were identified using the R package “limma”.

Weighted Co-expression Network Construction and Module Detection

To further investigate the correlation of gene expression between the normal and CCA samples, we adopted WGCNA package in R software to build a co-expression network for GEO and TCGA databases respectively[16]. A hierarchical clustering tree was constructed and different branches of the tree were represented different gene modules. Then, the adjacency matrix was transformed into topological overlap matrix (TOM). Modules were identified using the dynamic tree cut algorithm based on the TOM-based dissimilarity measure. The pearson correlation examination was performed to explore the relation of module eigengenes (MEs).

Functional Annotation and Pathway Enrichment Analysis

The overlapping of DEGs and the selecting modules of WGCNA co-expression network in GEO and TCGA databases were portrayed using a Venn diagram. The biological function of the overlapping genes was elucidated through functional annotation and pathway enrichment analysis. The gene ontology (GO)

terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were annotated and visualized using the “ClusterProfiler package” in R software[17].

Protein-Protein Interaction Network Construction and Analysis

The search tool for the retrieval of interaction genes (STRING, https://string-db.org/) was used to construct the protein–protein interactions(PPI) networks[18]. The overlapping genes were mapped to STRING to evaluate the PPI information with a confidence score > 0.9 as the cut-off standard. Cytoscape is a practical open–source software tool for visually exploring biomolecule interaction networks consisting of proteins, genes and other types of interaction [19].Topological features of the network were calculated to filter the hub network. The maximal clique centrality (MCC) methods in cytohubba plug-in in cytoscape was used to select the key genes. Finally, the top 10 genes in MCC method were selected as the hub genes[20].

Survival Analysis

The prognostic role of hub genes was validated by survival analysis in TCGA database. R package “survival” was tested by log-rank tests and Kaplan-Meier survival curves were plotted [21].

Tumor-Infiltrating Immune Cells

The tumor immune estimation resource 2.0 (TIMER 2.0, http://timer.cistrome.org/) database was presented to comprehensively assess the relationship between the hub genes and the immune cells infiltration[22].

Statistical analysis

Statistical analyses were performed using IBM SPSS version 20.0 and the statistical software package R version 4.0.2. Absolute log2 fold-change >1 and *P*-value < 0.05 were considered statistically significant.

Results

Data processing

The whole work of this study is shown in Figure 1. The gene expression profile dataset GSE26566 was downloaded from the GEO database containing 104 CCA samples and 6 normal samples. Then, 168 significantly up–regulated DEGs and 595 significantly down–regulated DEGs were identified. The TCGA dataset contained of 35 normal samples and 9 CCA samples, and a total of 2808 up-regulated genes and 2467 down-regulated genes were identified. Figure 2A and Figure 2B show the volcano plot of DGEs in GEO and TCGA databases, respectively.

Construction of the weighted gene co-expression network and identification of key modules

Gene co-expression modules were established in the GEO and TCGA cohort by using WGCNA (Figure 3A,3C). In the GEO dataset, the black and green modules were the top two modules closely related to CCA with correlation coefficients at 0.43 and -0.41 respectively (Figure 3B). In the TCGA cohort, it was found that the correlation coefficients of the turquoise modules were greatest (Figure 3D). Consequently, the intersection of the MEgreen module in GEO cohort and MEturquoise in the TCGA cohort combined with DEGs in these two datasets is shown in Figure4A, including 89 genes. These genes were regarded as the essential genes connected with CCA development and were selected for further analysis.

Functional annotation and pathway enrichment analysis

All 89 intersection genes were analyzed by GO and KEGG pathway enrichment analyses. The results demonstrated that the top biological processes associated with these genes was nuclear division. The cellular components most related to these genes was collagen-containing extracellular matrix. Furthermore, the molecular function terms were indicated that these genes were enriched in cell adhesion molecule binding (Figure 4B). In KEGG analysis, cell cycle pathway was the top pathway with significant enrichment (Figure 4C).

PPI analysis and identification of hub genes

The PPI network was constructed by the STRING tool, consisting of 89 nodes and 246 edges (Figure 5A). The hub network extracted from PPI network based on a confidence score > 0.9 (Figure 5B). The result from STRING database was imported in cytoscape for subsequent analysis. The genes that scored in the top 10 by MCC method in cytohubba plus-in were selected as hub genes of CAA. These genes were cell division cycle 20(CDC20), cyclin A2(CCNA2), type IIA topoisomerases(TOP2A), aurora kinase A (AURKA), cyclin B1(CCNB2), ubiquitin-coupled enzyme 2C(UBE2C), nucleolar and spindle-associated protein 1(NUSAP1), polycomb repressor complex 1(PRC1), pituitary tumor transforming gene 1(PTTG1) and minichromosomal maintenance proteins 4 (MCM4), which may play an important role in CCA progression (Figure 5C).

Survival Analysis and Tumor-Infiltrating Immune Cells

The survival analysis results of all hub genes were also validated in TCGA databases. Overexpression of CCNB2 and PTTG1 had significantly predictive effect on the prognosis of CCA, while the other genes had no significant predictive effect (Figure 6). We further explore the correlation between the hub genes expression and immune infiltration using the TIMER database[22]. There was a negative correlation between TOP2A expression and the infiltration of CD4+T cell (Cor=-0.433, p = 9.43e-03), similarly between AURKA (Cor=-0.343, p = 4.35e-02), CCNB2 (Cor=-0.414, p = 1.34e-02), PRC1(Cor=-0.364, p = 3.14e-02) and CD4+T cell. MCM4 expression was positively associated with the infiltration of neutrophil cells (Cor=0.447, p = 7.13e-03). There was no significant association between CDC20 levels and CD4+T cell, CD8+T cell, B cell, neutrophil, macrophage, or dendritic cell infiltration in mesothelioma, the same in CCNA2, UBE2C, NUSAP1, PTTG1 respectively (Figure 7).

Discussion

CCA is the most common malignancy of bile duct and its incidence is increasing annually[23]. Due to its occult clinical characteristics, difficulties in early diagnosis as well as limited therapeutic approaches, the prognosis of CCA is still poor[24]. Although surgical resection was regard as a possible option for certain patients who suffer CCA, the 5-year OS is extremely low[25]. Therefore, it is urgent to investigate the pathogenesis of CCA, so as to develop new clinical treatment strategies.

In the present study, a total of 10 genes were identified as hub genes in the development of CCA, including CDC20, CCNA2, TOP2A, AURKA, CCNB2, UBE2C, NUSAP1, PRC1, PTTG1 and MCM4. The genes of CCNA2, CCNB2, CDC20 and UBE2C play a critical role in cell cycle progression. CCNA2 and CCNB2 are both the key members of the cyclin family [26,27]. Abnormal expression of CCNA2 or CCNB2 has been detected in various types of cancers. It was reported that CCNA2 was dysregulated in lung cancer, gastric cancer and colorectal carcinoma[28-30]. Several studies also pointed the dysregulate of CCNB2 in hepatocellular carcinoma, non-small cell lung cancer and bladder cancer[31-33]. However, the molecular mechanisms of these two genes in CCA remain unclear. CDC20 is an essential co-factor of the anaphase promoting complex (APC) as well as a downstream factor of the spindle assembly checkpoint[34,35]. Previous studies have indicated that CDC20 may represent a promising therapeutic target for human cancers[36,37]. Li et al indicated that CDC20 promoted the resistance of esophageal cancer chemotherapy by regulating E2F1 degradation and thymidylate synthase expression[38]. Diane et al demonstrated that CDC20 overexpression increased the ability of human glioblastoma stem-like cells to generate brain tumors in an orthotopic xenograft model and implicated the role of CDC20-APC-targeted strategies in glioblastoma therapy[39]. Shi M et al found that CDC20 played an important role in the development of hepatocellular carcinoma by governing prolyl hydroxylase 3 protein[40]. Thus, exploring the underlying mechanism of CDC20 in the pathogenesis of cholangiocarcinoma and the development of CDC20 inhibitors may contribute to improve the therapeutic efficacy of patients with CCA. UBE2C is a ubiquitin enzyme that is highly expressed in various human cancers, such as NSCLC, ovarian cancers and breast cancers[41,42]. Overexpression of UBE2C may cause the loss of mitotic spindle checkpoint activity and genomic stability[43], suggesting that UBE2C may associated with tumorigenesis of CCA.

DNA topoisomerases, especially TOP2A, demonstrate the ability to regulate the topological states of DNA during transcription. TOP2A has been indicated to be highly expressed in 33% patients with intrahepatic CCA[44]. Overexpression of TOP2A in CCA was also confirmed in this paper. TOP2A has also been confirmed as a therapeutic target with anticancer and antibacterial effects[45,46]. Therefore, we can speculate that TOP2A may also be a new therapeutic target for CCA. AURKA belongs to aurora family and plays key roles in regulating mitotic progression and cell proliferation[47]. Aberrant AURKA activity participates in oncogenic transformation through the development of chromosomal instability and tumor cell heterogeneity[48,49]. Ding et al has demonstrated that AURKA inhibitor can inhibit cell growth and promote apoptosis of CCA both in vitro and in vivo experiments. These results suggested that AURKA

may be an effective target for the treatment of CCA[50]. NUSAP1 is a microtubule-binding protein involving in chromosomal segregation[51,52]. NUSAP1 regulates metastasis of cervical carcinoma through Wnt/β-catenin signaling pathway[53]. The down-regulation of NUSAP1 can inhibit cell proliferation, migration and invasion in NSCLC, which is related to the regulation of BTG2/PI3K/Akt signaling pathway[54]. PRC1 is encoded by multiple homologous genes and shows the ability to promote ubiquitination of histone H2A[55]. Su et al revealed that PRC1 can coordinate stemness with immune suppression and point out the potential clinical application of targeting PRC1 in double-negative prostate cancer[56]. PTTG1, a ubiquitously expressed transcription factor, is involved in DNA damage repair, angiogenesis, cell differentiation and apoptosis[57]. It can promote the transcription of genes that are directly or indirectly involved in tumorigenesis[58]. Therefore, it is important to elucidate the mechanism of PTTG1 involvement in the development of CCA [59]. MCM4, a member of MCM family, is required to initiate genome replication in eukaryotes. It may be involved in the formation of replication forks and the recruitment of other DNA replication-related proteins[60].

In GO analysis, nuclear division, collagen-containing extracellular matrix and cell adhesion molecule binding were the main pathways of gene enrichment. Furthermore, the genes were closely related to the cell cycle process in the KEGG analysis. This result was consistent with the related functions of the hub genes including CCNA2, CCNB2, CDC20 and UBE2C.Cell cycle dysregulation has been confirmed to be associated with the development and progression of various types of cancers, including CCA[61,62]. Chen et al found that the inhibition of Kelch-like family member 21 (KLHL21) could significantly reduce proliferation, migration and invasion of CCA cells. In addition, KLHL21 knockdown alleviated the activation of the Erk signaling pathway via decreasing the expression of phospho-Erk1/2, suggesting that it might be a potential therapeutic target for CCA[63]. Several studies have shown that some drugs, such as telmisartan and cryptotanshinone, can induce cell cycle arrest and apoptosis in CCA and are potential therapeutic agents for CCA[64,65]. Obviously, more attention should be paied to the cell cycle-related pathways and genes to explore the mechanism of development and treatment of CCA.

The immune system in CCA presents a complex landscape and most CCA is thought to origin from chronic inflammatory of the biliary tract[66]. In this study, TOP2A, AURKA, PRC1, MCM4 were confirmed to be associated with immune cell infiltration using the TIMER 2.0 algorithm. Understanding the interaction between infiltrating immune cells and tumor cells in CCA may open new opportunities for treatment. Patients with neutrophil distribution in CCA may indicate poor prognosis[67]. The chronic inflammatory status and the resulting immune state deserve further study and are meaningful for subsequent treatment of cancers. Tumor-associated macrophages (TAMs) play a pivotal role in promoting angiogenesis, tumor cell proliferation and suppression of adaptive immunity[68]. TAMs drive the occurrence of CCA by producing various soluble mediators, including cytokines, chemokine and reactive nitrogen intermediates[69]. It has been proven to be a prognostic marker and potential therapeutic intervention in CCA[70]. Moreover, depletion of the enrichment of CD163+TAMs, CD4+ T cell, as well as CD8+ T cell, are correlated with reducing relapse-free survival and inducing gemcitabine resistance[71].

Mature dendritic cell may enhance CD4+ and CD8+ T cells infiltration into cancers and improve prognosis in patients with CCA[72]. B cells have been identified in the tumor infiltrating lymphocytes of CCA, but are rarely observed in patient tissues[73,74]. Goeppert et al showed that the total number of tumor-infiltrating B cell is correlated with a longer OS probability in biliary tract cancer. Whether this observation just reflected an occasional phenomenon in the context of immune response activation, or indicated of the true effect of B cell on tumor control should be determined in future studies.

There are still several limitations in this study. First, although a relatively large number of CCA samples were analyzed, more studies with larger samples sizes are needed to verify the findings of this study. Second, although the immune cell infiltration results of the hub genes were evaluated in this study, they were based only on the information from an online database. Consequently, further experiments should be performed to validate the results in this study and to reveal the mechanisms of these hub genes in CCA progression and prognosis.

Conclusion

In summary, by using WGCNA and a series of other bioinformatics approaches, we identified 10 hub genes (CDC20, CCNA2, TOP2A, AURKA, CCNB2, UBE2C, NUSAP1, PRC1, PTTG1 and MCM4), which may contribute to the development and prognosis of CCA. Further studies of the hub genes identified in the present study are urgently required to determine the underlying mechanisms associated with their function in CCA.

Abbreviations

CCA, Cholangiocarcinoma; OS, overall survival; NGS, next generation sequencing; WGCNA, Weighted gene co-expression network analysis; GEO, The Gene Expression Omnibus database; TCGA, The Cancer Genome Atlas database; DEGs, differentially expressed genes; TOM, topological overlap matrix; MEs, module eigengenes; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; STRING, Search the Tool for The Retrieval of Interaction Genes; PPI, Protein–protein interaction; GEPIA, Gene Expression Profiling Interactive Analysis; TIMER, The Tumor Immune Estimation Resource; HPA, The Human Protein Atlas; CCNA2, Cyclin A2; CCNB2, cyclin B1; CDC20, Cell division cycle 20; APC, anaphase promoting complex; TOP2A, type IIA topoisomerases; AURKA, Aurora kinase; UBE2C, Ubiquitin-conjugating enzyme 2C; NUSAP1, Nucleolar and spindle-associated protein 1; PRC1, Polycomb Repressor Complex 1; PTTG1, Pituitary tumor-transforming gene 1. MCM4, Minichromosomal maintenance proteins 4.

Declarations

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Author contributions

YY and SJ conceived the concept and designed the study. SJ and ZL collected and analysised the data. HS and GL prepared the figures. YY and SJ wrote the paper. All authors reviewed and approved the manuscript.

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Competing Interests

The authors declare that they have no conflicts of interest.

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Figures

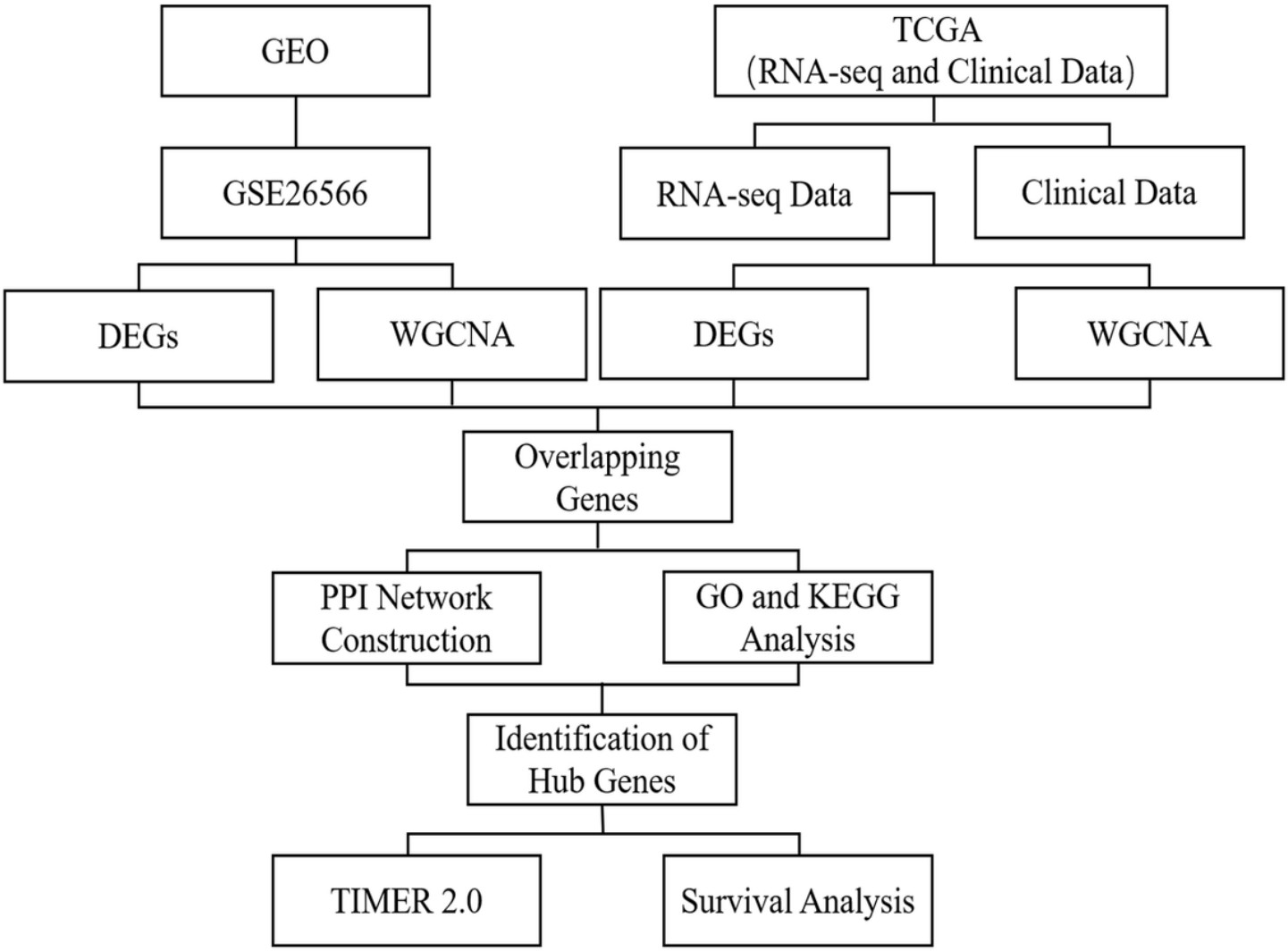

Figure 1

Flow chart of data collection, processing and analysis.

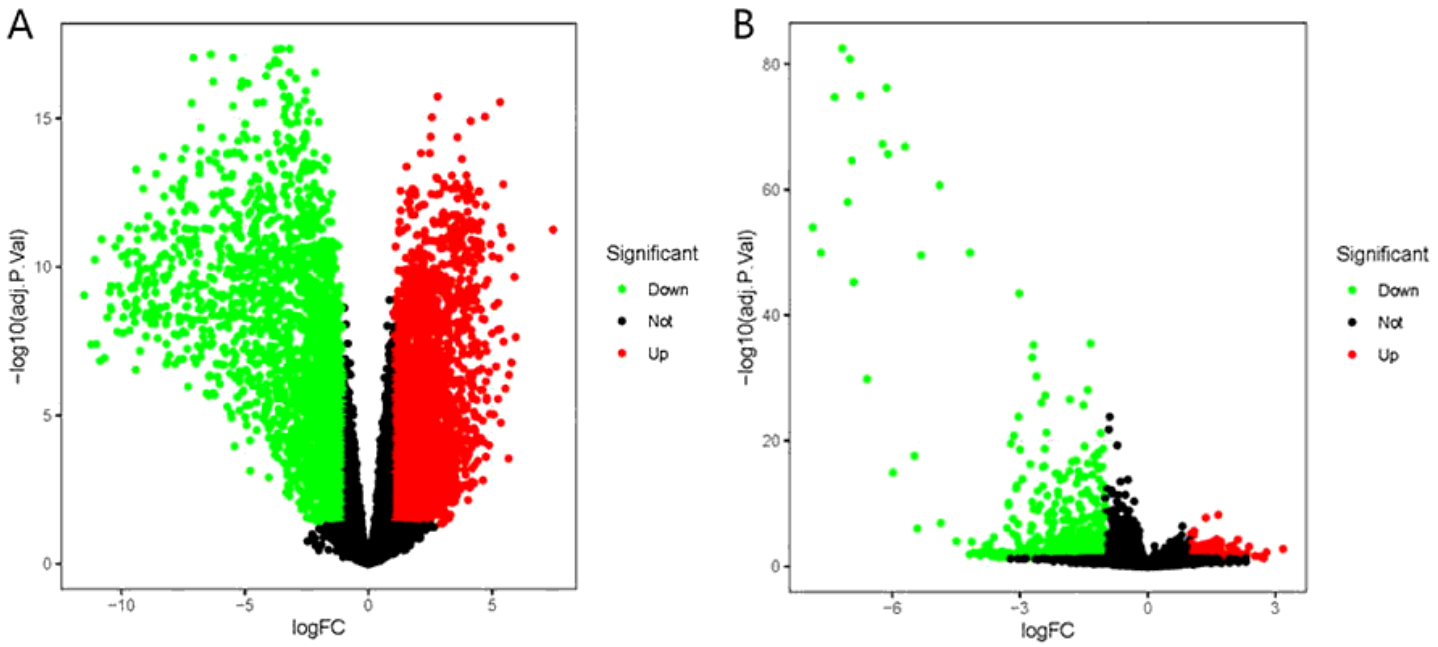

Figure 2

A volcano plot revealing the expression of all genes in GEO (A)and TCGA(B) databases, respectively. The red dots represent all upregulated DEGs, and green dots represent all downregulated DEGs.

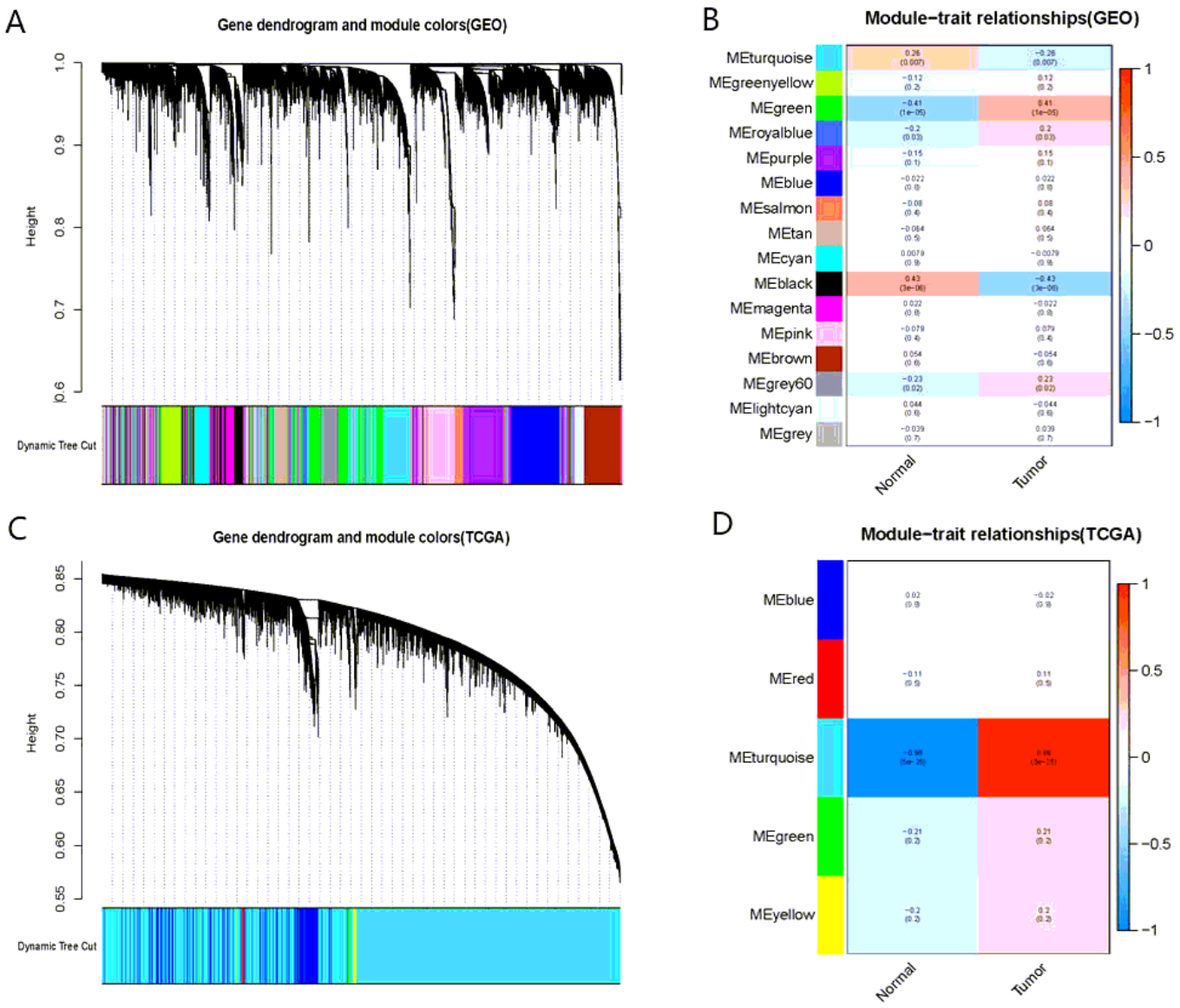

Figure 3

The construction of WGCNA. (A), (C)Dendrogram of all expressed genes clustered based on a dissimilarity measure in GEO and TCGA dataset, respectively. (B), (D) Module relationships and P-values between normal and CCA samples in GEO and TCGA dataset, respectively. Each row corresponds to a module eigengene, and each column corresponds to normal or tumor sample.

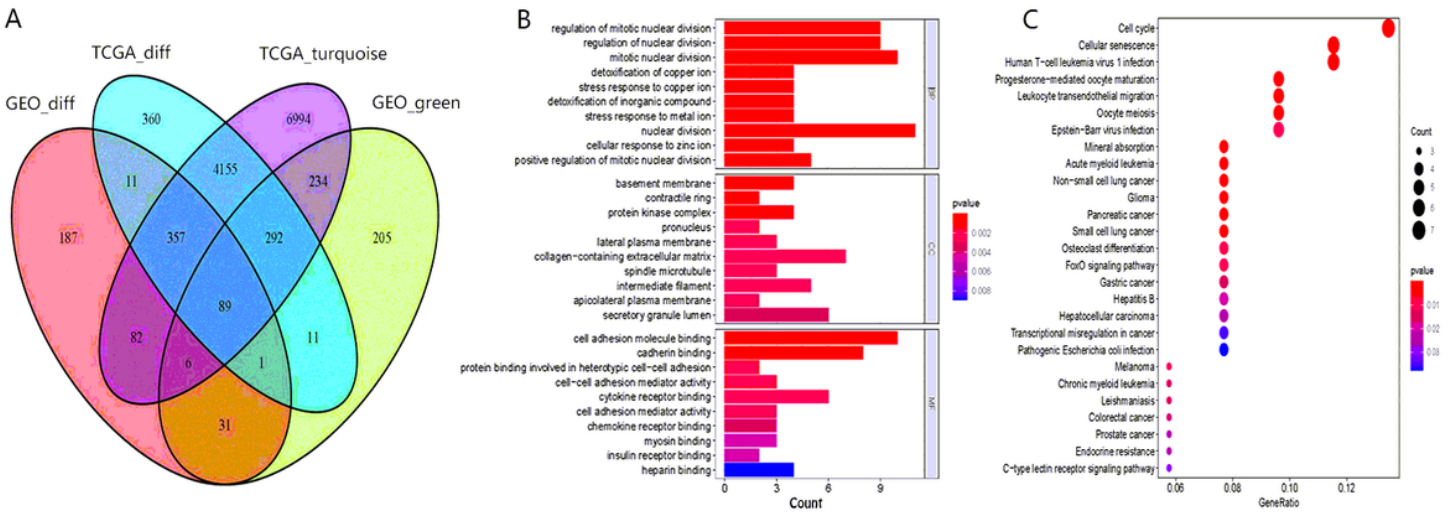

Figure 4

Functional annotation and pathway enrichment analysis. (A)Venn diagram to identify intersectional module core genes combined with DEGs. (B) GO enrichment analysis. (C) KEGG pathway enrichment analysis.

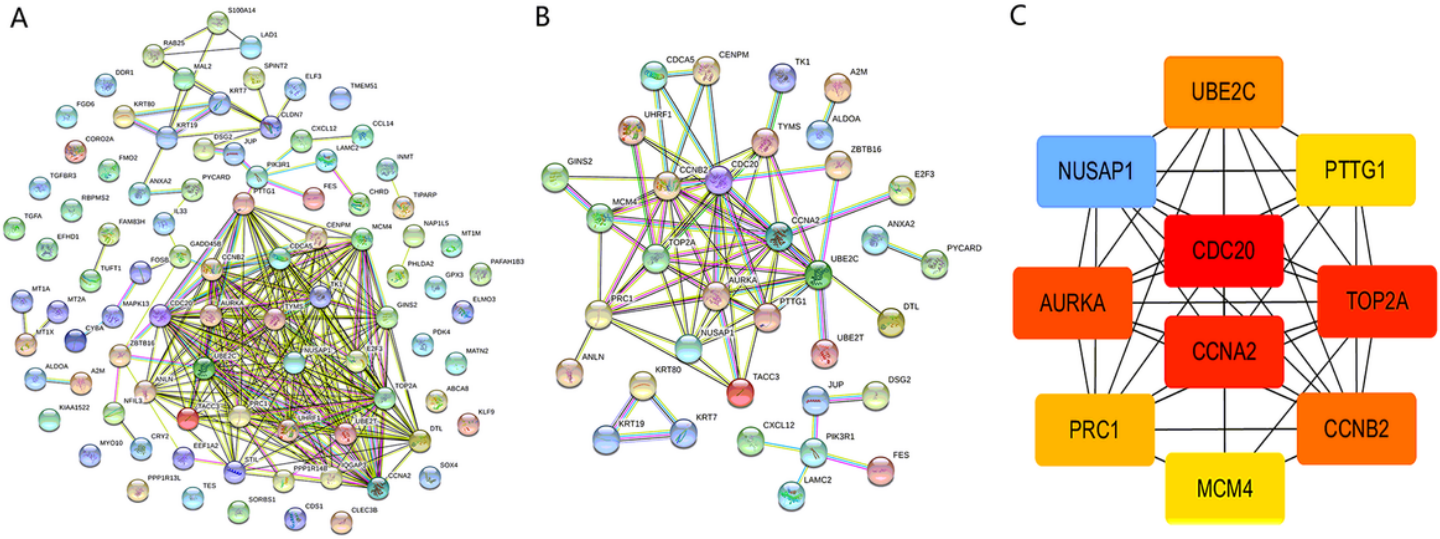

Figure 5

Construction of PPI and selection of hub genes. (A) PPI network of all 89 intersection genes. (B) Hub network extracted from (A) based on a confidence score > 0.9. (C) Top significant sub-module cluster was identified by using by MCC method in cytohubba plus-in in cytoscape.

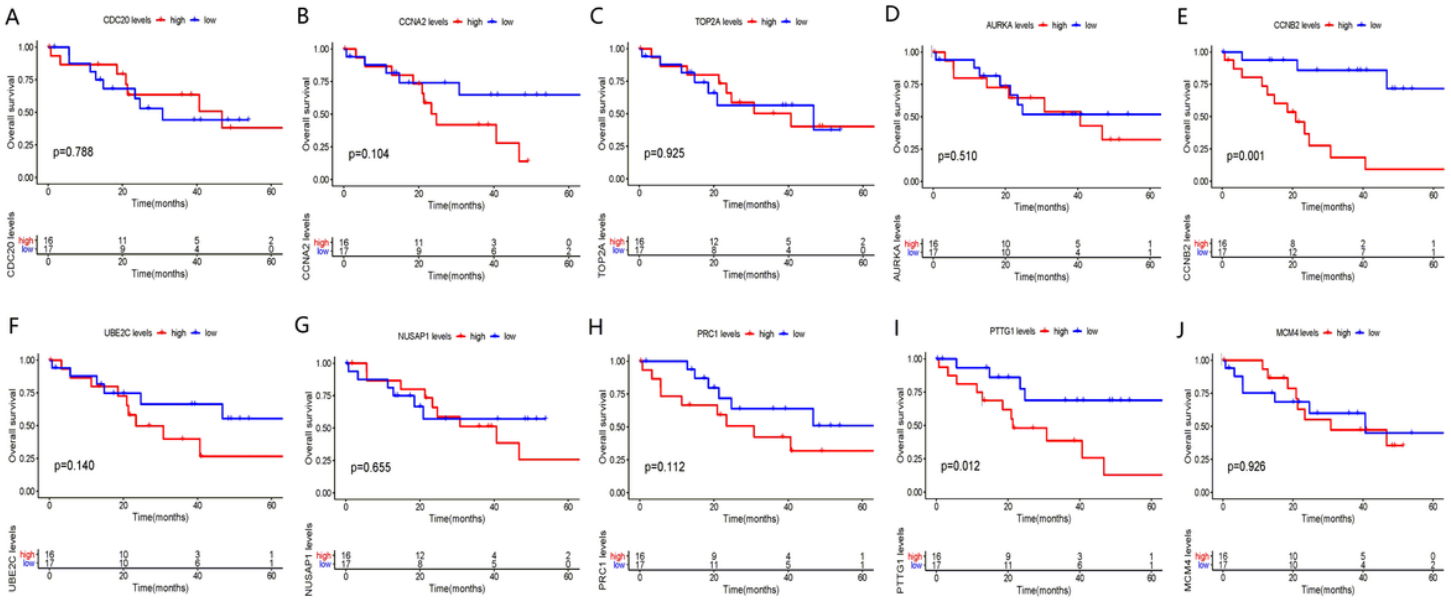

Figure 6

The prognostic value of hub genes. (A) CDC20; (B) CCNA2;(C) TOP2A; (D) AURKA; (E) CCNB2; (F) UBE2C; (G) NUSAP1; (H) PRC1;(I) PTTG1;(J) MCM4.

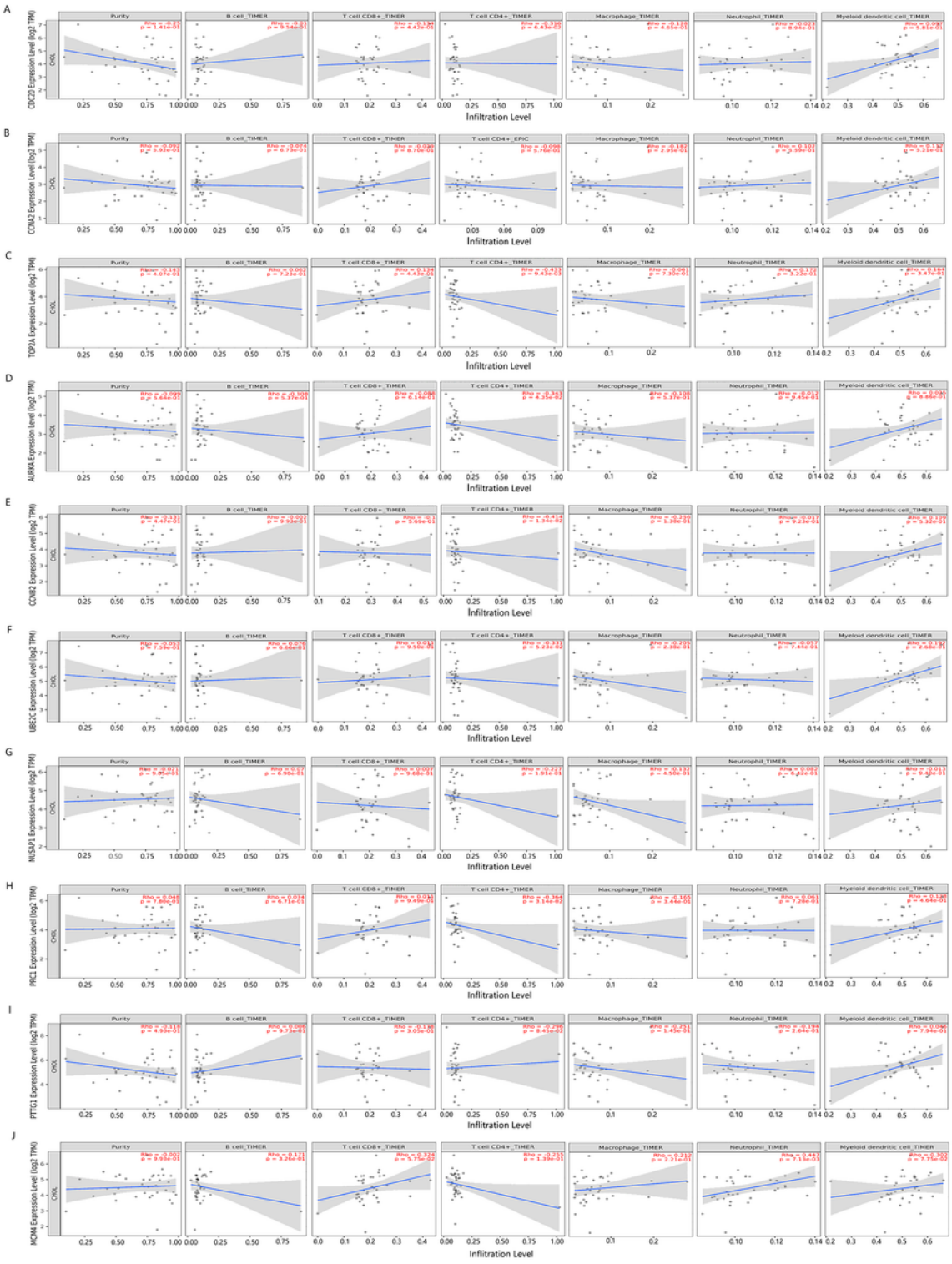

Figure 7

Correlation between hub genes and immune cell infiltration. (A) CDC20; (B) CCNA2;(C) TOP2A; (D) AURKA; (E) CCNB2; (F) UBE2C; (G) NUSAP1; (H) PRC1;(I) PTTG1;(J) MCM4.