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Mining of Gene Modules and Identification of Key Genes for Early Diagnosis of Gastric Cancer

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ABSTRACT

Gastric cancer (GC) is one of the most common malignant tumors with high incidence and mortality rates. Most patients with GC are not diagnosed until the advanced stage of cancer or during tumor screening, resulting in missing the best treatment time. This study identified key modules and hub genes associated with GC by weighted gene co-expression network analysis (WGCNA). The "limma" package in R was used to identify differentially expressed genes (DEGs) in GC samples from TCGA, and a total of 4892 DEGs were identified. GO enrichment and KEGG pathway enrichment analyses were conducted to detect the related pathways and functions of DEGs. These DEGs were primarily associated with extracellular matrix organization, DNA replication, cell cycle, and p53 signaling pathway. Gene modules associated with clinical characteristics were identified with WGCNA in tumor and normal samples. Six gene modules were obtained in the WGCNA network, of which two modules were significantly correlated with GC. Hub genes of key modules were identified using survival analysis and expression analysis. Finally, one-way ANOVA was used to explore the relationship between hub gene expression in normal tissues and different pathological stages of GC. Through survival and expression analysis, a total of 19 genes with good prognosis and significantly differential expressed were identified. The hub genes were significantly differential expressed in normal tissues and different pathological stages of GC, indicating that these genes have important diagnostic value for early GC and can be used as auxiliary indicators in the diagnosis of early GC.

Key words: *Gastric cancer, Bioinformatics, Differentially expressed genes, Weighted gene co-expression network analysis, Early diagnosis*

INTRODUCTION

Gastric cancer (GC) is a malignant tumor originating from the epithelial cells of gastric mucosa or the glands of the most superficial layer [1]. According to the global cancer statistics of GLOBOCAN 2020, GC has become the fifth leading cause of cancer-related morbidity and the fourth leading cause of cancer-related deaths worldwide [2]. GC is a multifactorial disease, which can be affected by many factors, such as environmental, dietary factors, *Helicobacter pylori* (*H. pylori*) infection incidence, obesity, and genetic factors during formation and development [3, 4]. Patients with GC have the characteristics of a high incidence rate, metastatic rate and mortality, low early diagnosis rate, radical resection rate, and 5-year survival rate [5]. The incidence of GC in young people is gradually increasing, and the mortality of GC patients is also increasing and gradually tends to be young people [6, 7]. Because patients with early GC are usually asymptomatic and have a low diagnosis rate, approximately 70% of

patients are first diagnosed with advanced GC [6]. Some patients with advanced GC even lose the chance to have the tumor surgically removed, so early detection, early diagnosis, and early treatment become very important. Therefore, mining new biomarkers of early GC is significant for the early diagnosis and treatment of GC, which will help in improving the overall prognosis of GC patients.

Weighted gene co-expression network analysis (WGCNA) is an analysis method for analyzing gene expression patterns of multiple samples. It can cluster genes with similar expression profiles to form gene modules, which can be combined with clinical information to identify hub genes in the modules [8-10]. WGCNA has been widely used to identify gene modules and hub genes related to clinical information in various cancers. Previous studies have identified some hub genes associated with the progression and prognosis of breast cancer, Colorectal cancer, and human clear cell renal cell carcinoma through WGCNA, providing a theoretical basis for the diagnosis and treatment of related cancers [11-13].

In this study, we combined WGCNA with other methods to analyze the clinical information and RNA sequencing data of GC patient samples downloaded from the TCGA database to identify hub genes associated with clinical traits (disease status (Tumor_Normal), gender, pathologic_T, pathologic_stage, vital_status, and initial_weight). These hub genes may be new biomarkers or therapeutic targets for early GC, providing a theoretical basis for the diagnosis and treatment of early GC.

MATERIALS AND METHODS

Data sources and pre-processing

We downloaded RNA sequencing data and clinical information data from TCGA (https://tcga.xenahubs.net) hosted at the Xena website of the University of California at Santa Cruz (http://xena.ucsc.edu/) [14]. The RNA sequencing data included 380 GC samples and 37 normal samples. The Principal Component Analysis (PCA) was used to detect the characteristics of the data set [15]. We excluded abnormal samples based on the results of the principal component analysis to ensure the reliability of the results **(Figure 1)**.

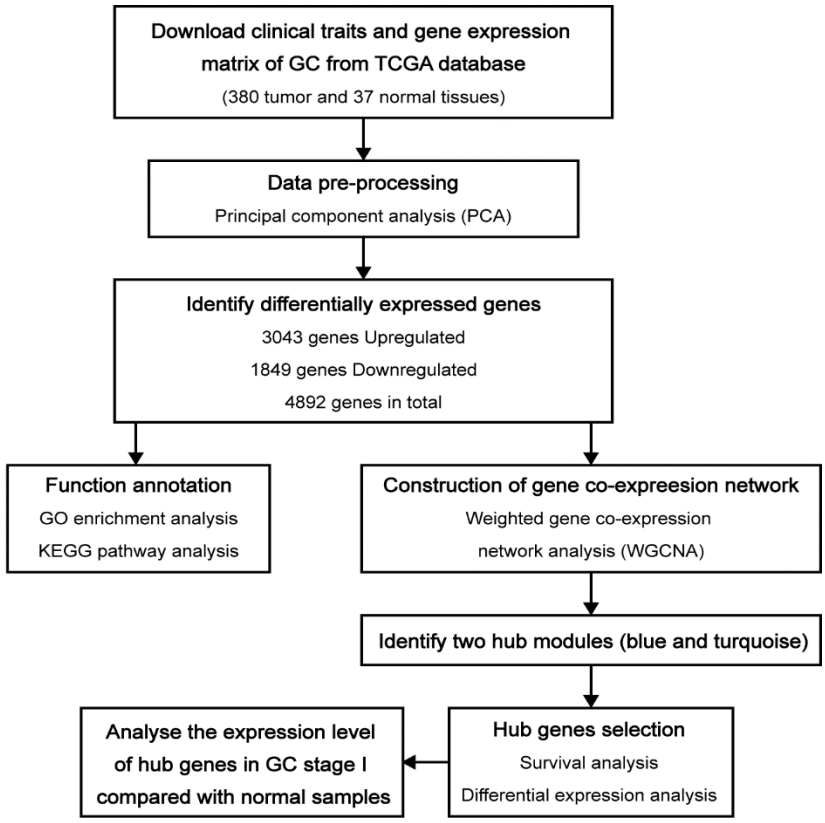


Figure 1. Workflow of searching hub genes in GC.

DEGs screening

The "limma" R package (version 3.44.3) [16] was used to perform differential expression analysis on the gene expression data of GC tumor samples and normal samples in TCGA. The cut-off criteria of DEGs screening were

$P < 0.05$.

KEGG pathway and GO enrichment analysis of DEGs

Enrichment analysis is a statistical analysis method that can speculate the functions of DEGs. Gene Ontology (GO) is a common bioinformatics tool that can be used to explore the potential functions of genes [17]. The Kyoto Encyclopedia of Genes and Genomes (KEGG) can be used to determine the main biochemical metabolic pathways and signal transduction pathways involved in genes [18]. We conducted GO enrichment analysis and KEGG pathway analysis on DEGs, respectively, in order to find the functions of DEGs. The "Clusterprofiler" package (version 3.16.1) [19] in R was used for GO enrichment analysis and KEGG pathway analyses of DEGs. GO terms with $P < 0.01$ and KEGG terms with $P < 0.05$ were used in the following analysis.

Identification of clinically significant modules

We used the "WGCNA" R package (version 1.70-3) to construct a co-expression network of the DEGs [8, 10]. Firstly, the samples were clustered by the flashClust tool in R to detect outlier samples. The Pearson correlation coefficient (PCC) between any two genes was calculated. Thirdly, selecting an appropriate soft threshold (β) made the constructed network more consistent with the characteristics of the scale-free network by using the pickSoftThreshold function (1). Fourth, the weighted adjacency matrix (AM) was created with the power function (1) where amn refers to the adjacency between gene m and gene n, cmn refers to the PCC between

$$amn = |cmn|\beta \quad (1)$$

those two genes and β refers to the soft threshold power. Then, the AM was transformed into the topological overlap matrix (TOM) using the adjacency function (2) where lmn represents the sum of the product of the adjacency coefficients that the nodes connected by genes m and gene n, km or kn represent the sum of the adjacency coefficients that gene m or gene n with all other genes in the weighted network. Based on TOM's

$$TOMmn = \frac{lmn + amn}{\min(km + kn) + 1 - amn} \quad (2)$$

dissimilarity measure (1-TOM), average linkage hierarchical clustering was performed to assign genes with similar expression profiles to the same gene module. The number of genes for the smallest gene module was set to 30, and the threshold for merging similar modules was set to 0.25. The Module Eigengene (ME) is the module's first principal component, representing the entire module's gene expression profile. The Pearson's correlation coefficient was calculated between the ME and the clinical phenotype to screen the modules with a higher correlation with the phenotype.

Hub gene selection

The Gene Significance (GS) represents the correlation between genes and clinical phenotypes, and the Module Membership (MM) represents the correlation between genes in the module and the other module. Genes were screened by setting the thresholds of GS and MM. In order to verify the biological significance of candidate genes, survival analysis and differential expression analysis in GEPIA (http://gepia.cancer-pku.cn) [20] were conducted to verify the further influence of these genes on the survival of GC patients and whether there were differences in their expression between early tumor tissues and normal tissues. The thresholds for survival analysis and differential expression analysis were set as $p<0.05$ and $p<0.01$, respectively.

Analysis of the diagnosis value of hub genes

For 241 samples provided by TCGA, clinical phenotypes and pathological stages of GC were selected to analyze the relationship between hub gene expression in normal tissues and different GC pathological stages, which was completed by one-way ANOVA in GraphPad Prism 8 software [21].

RESULTS AND DISCUSSION

Data pre-processing and screening of DEGs

After excluding 50 GC tumors and 26 normal samples that can not be classified clearly, the rest of the samples

were divided into GC tumor samples from normal samples according to PCA analysis, and PC1 and PC2 accounted for 8.8% and 5% of the observed differences respectively **(Figures 2a-2b)**. A total of 4892 DEGs were selected between normal and GC samples, including 3043 up-regulated genes and 1849 down-regulated genes **(Figures 2c-2d)**.

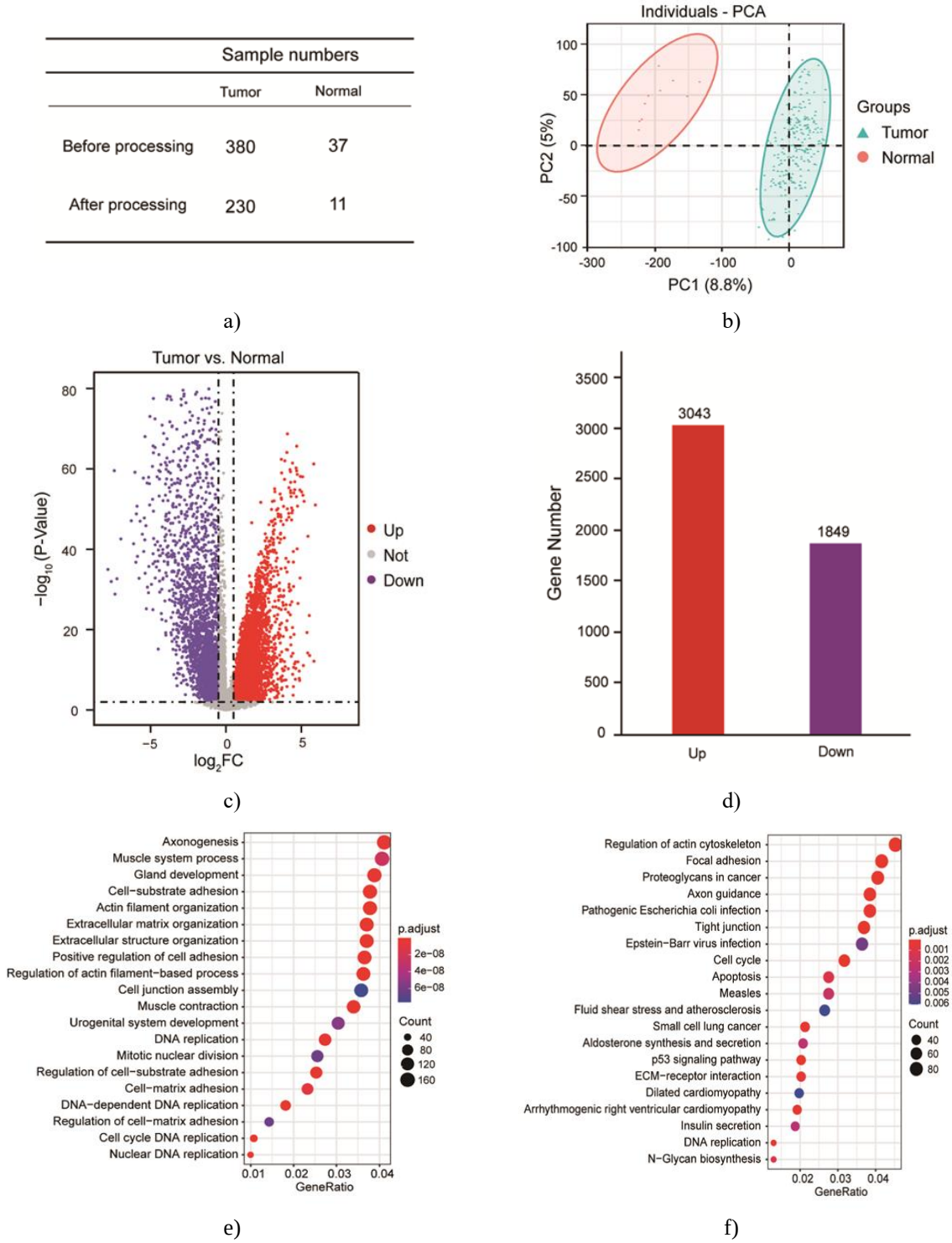


Figure 2. Identification and functional enrichment analysis of DEGs in GC. (a) Numbers of samples. (b) Principal component analysis. (c) The volcano plot. Red dots: up-regulated genes; gray dots: not significant genes; purple dots: down-regulated genes. (d) Numbers of DEGs. (e) GO enrichment analysis. (f) KEGG pathway analysis. The dots' color reflects the significance of enrichment, and the size of the dots indicates the number of genes enriched under the given term (top 20 enrichment results shown according to p-value).

GO enrichment and KEGG pathway analysis of DEGs

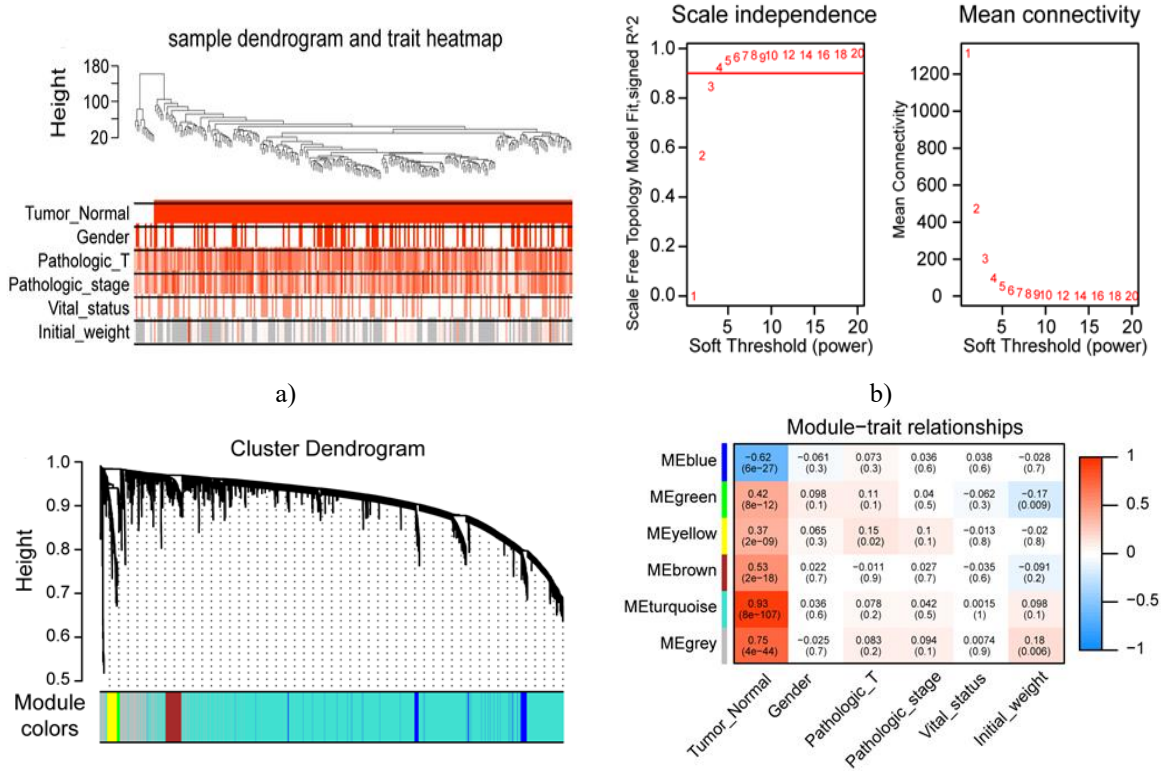
GO enrichment and KEGG pathway analysis were used to explore the potential biological functions of DEGs in GC. According to the GO enrichment analysis, extracellular matrix organization, DNA replication, cell cycle DNA replication, and nuclear DNA replication were the enriched terms that were always reported in previous studies **(Figure 2e)** [22, 23]. As for KEGG pathways, focal adhesion, proteoglycans in cancer, pathogenic Escherichia coli infection, cell cycle, apoptosis, small cell lung cancer, p53 signaling pathway, and ECM−receptor interaction most often enriched in DEGs **(Figure 2f)** [23, 24].

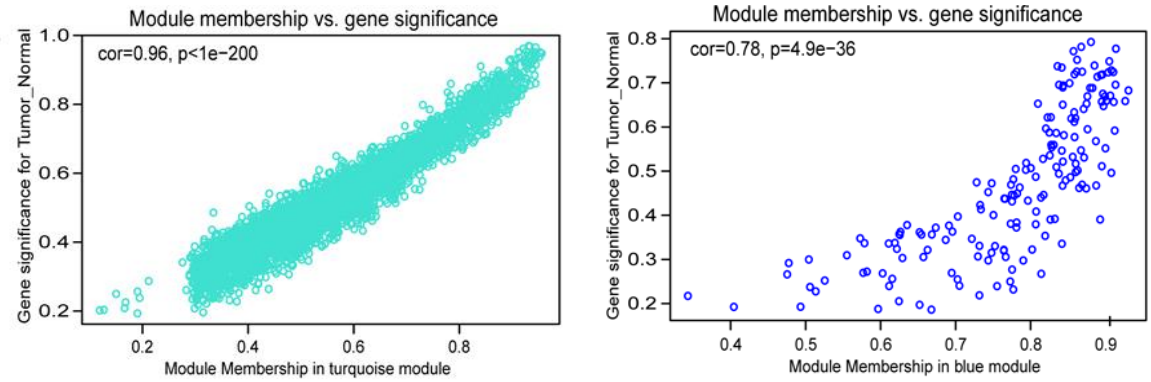
WGCNA and identification of key modules

The co-expression network was constructed through WGCNA, tumor samples, and normal samples was clustered into two branches, and there were no outliers to be deleted **(Figure 3a)**. The scale-free topology was set to 0.9, then the soft threshold β was 4 **(Figure 3b)**. Six gene modules (blue, green, yellow, brown, turquoise, and grey) were obtained finally, among which the grey module represented the gene set that could not be aggregated with other modules **(Figures 3c-3d)**. The correlation between these modules and the clinical phenotype showed that the turquoise module (cor=0.93, *P*=8e-107) was significantly positively correlated with GC, and the blue module (cor=-0.62, *P*=6e-27) was significantly negatively correlated with GC. These results indicate that the turquoise module may play an essential role in the tumorigenesis of GC, and the blue module may have anti-tumor effects. Therefore, the turquoise and blue modules are selected for further research and analysis.

Identification and validation of hub genes

In the blue and turquoise modules, MM and GS showed a significant positive correlation **(Figures 3e-3f)**. The criteria for selecting hub genes were |MM| > 0.8 and |GS| > 0.6 in the blue module, and the thresholds for determining hub genes were |MM| > 0.8 and |GS| > 0.8 in the turquoise module. 89 and 216 genes were identified to satisfy these selection thresholds in the blue and turquoise modules. Following verification using the survival analysis and the expression level between GC tissues and normal tissues, 19 genes were selected as hub genes (*ASF1B*, *DPT*, *ZBTB16*, *WISP2*, *PRIMA1*, *EPCAM*, *PDZD4*, *ATP1A2*, *FAM83H*, *ABCA9*, *C8orf46*, *MAMDC2*, *TCEAL2*, *CEP55*, *LIMS2*, *LMOD1*, *PLP1*, *TMEM100*, *ADHFE1*). These hub genes were associated with the prognosis of GC, and there were significant differences in the expression of all hub genes between GC tissues and normal tissues **(Figures 4 and 5)**.





e) f)

Figure 3. WGCNA of DEGs in GC. (a) Clustering of GC samples and normal samples in clinical features. (b) The scale-free fit index and the mean connectivity for various soft threshold powers (β) are analyzed. (c) The cluster dendrogram of DEGs is based on the dissimilarity measure (1-TOM). (d) Heatmap of the correlation between the module eigengenes (ME) and different clinical traits of GC. (e, f) The scatter plots of the module membership (MM) and gene significance (GS) for genes in the turquoise(g) and blue(f) modules.

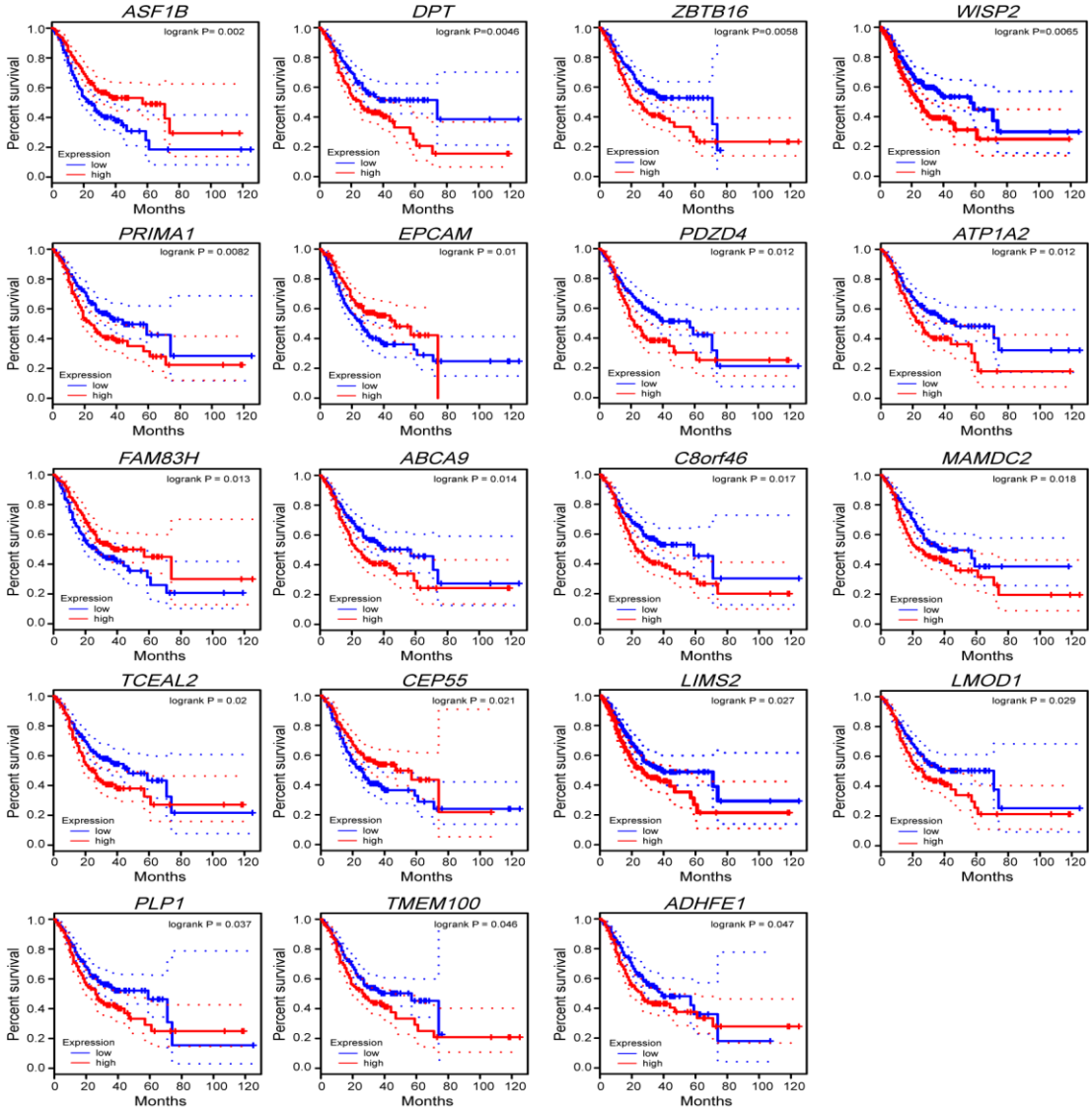


Figure 4. Survival analysis for hub genes in GC. These 19 genes are significantly related to survival, in which the red line indicates the high expression group of the gene, and the blue line indicates the low expression group of the gene. $P < 0.05$ was used as a significant criterion.

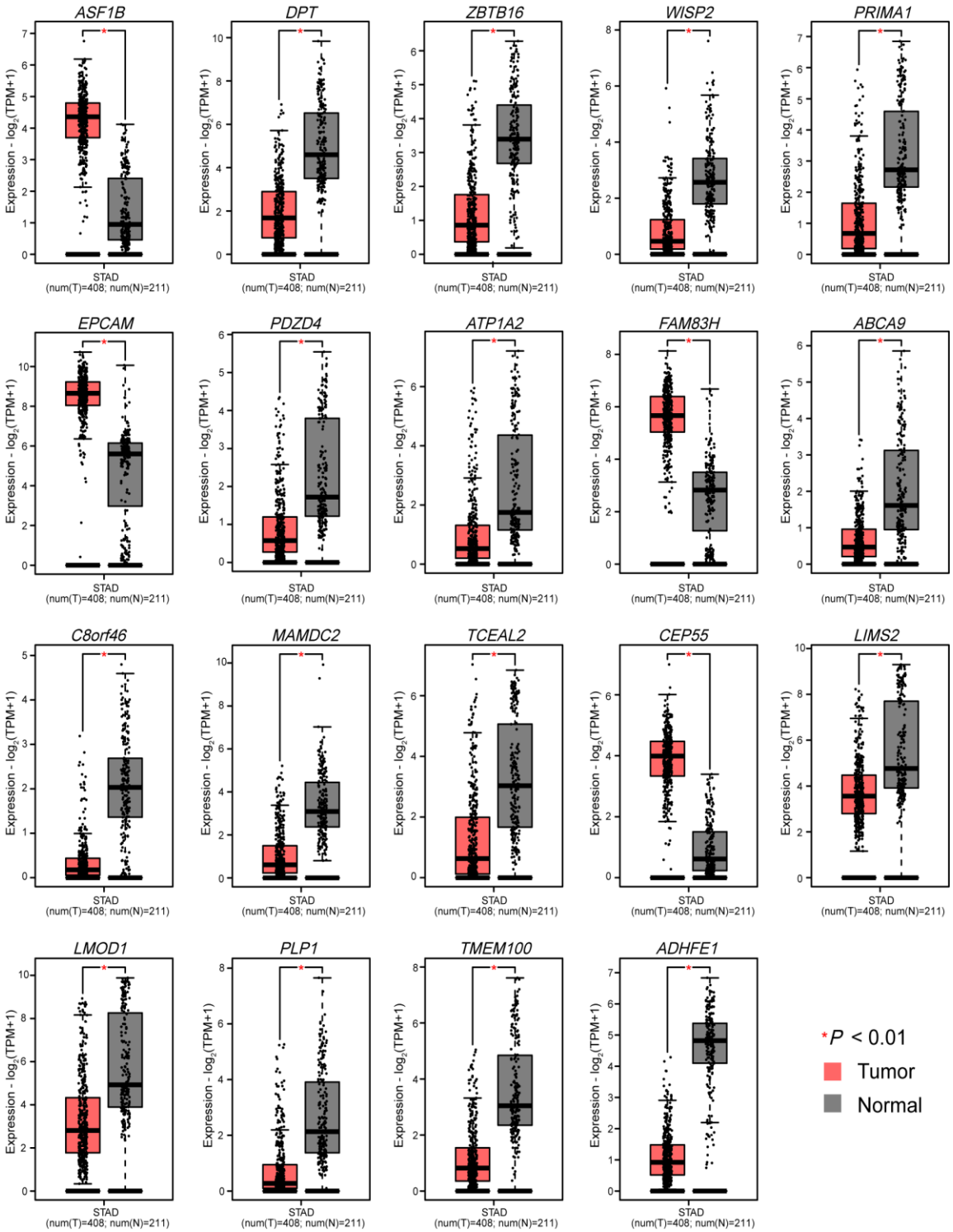


Figure 5. The expression level of hub genes in GC tissues and normal tissues. Hub genes were analyzed using the GEPIA online database to further verify their expression levels between GC tissues and normal tissues. * $P < 0.01$ was considered statistically significant.

Early diagnostic biomarkers for GC

In verifying the correlation of these hub genes at the pathologic stage of GC, we found that the expression levels of 19 hub genes in normal GC tissues were significantly different from those in early GC tissues, among which *ASF1B*, *EPCAM*, *FAM83H* and *CEP55* were higher expressed in early GC than normal gastric, while other genes showed lower expression levels in early cancer, indicating that these hub genes may have important clinical value in the early diagnosis of GC **(Figure 6)** [25]. The expression level of *ASF1B* and *CEP55*, which was reported to be related to the development of multiple cancers and mitotic exit and cytokinesis, was close to 0 in normal gastric while was high in tumor tissues, including the early stage. Contrarily, the expression level of *PDZD4*, *ATP1A2*,

ABCA9, *C8orf46,* and *TCEAL2* was a high-level expression in normal tissues while close to 0 in the early stage of GC **(Table 1, Figure 6)**. The combination of the 19 hub genes, especially for *ASF1B*, *CEP55*, *PDZD4*, *ATP1A2*, *ABCA9*, *C8orf46,* and *TCEAL2*, can be effective biomarkers utilized in early GC detection.

Table 1. Functional roles of the 19 hub genes

Gene	Function	Source PMIDs#
ASF1B	Related to the development of multiple cancers	35362843; 21179005
DPT	A non-collagenous extracellular matrix component that can regulate tumor cell proliferation and invasiveness	30391671; 25149533; 21796630
ZBTB16	Acts as a transcriptional repressor to inhibit the proliferation and metastasis of tumor cells	10688654; 24359566; 29358655; 24339862; 32517789; 30431129
WISP2	Bidirectional regulation effect on tumor cells	34385183; 30808397; 32711570
PRIMA1	Interacts with AChE and anchors AChE onto the neural cell membranes	11804574
EPCAM	Influences on cell cycle and proliferation directly and up-regulate proto-oncogene c-myc and cyclin A/E	15195135
PDZD4	A new human gene with the PDZ domain plays a role in tumor cell proliferation	15077175
ATP1A2	The catalytic component of the active enzyme, which catalyzes the hydrolysis of ATP coupled with the exchange of sodium and potassium ions across the plasma membrane	33880529
FAM83H	Regulates epithelial cell migration	23902688
ABCA9	Transporter that may play a role in monocyte differentiation and lipid transport and homeostasis	12150964
C8orf46	Involved in neurogenesis	32558188
MAMDC2	Inhibiting tumor activity	32707597
TCEAL2	Tumor suppressor	33061644
CEP55	Plays a role in mitotic exit and cytokinesis	16198290; 17853893
LIMS2	Modulating tumor cell spreading and migration	16959213
LMOD1	Promote tumor cell migration	35488236
PLP1	Formation and maintenance of myelin multilayer structure	30094605
TMEM100	Inhibition of tumor cell metastasis	34687431; 31188741
ADHFE1	Associated with tumor cell proliferation and embryonic development	16959974; 23517143; 29202474; 24886599

#PMID: PubMed Unique Identifier

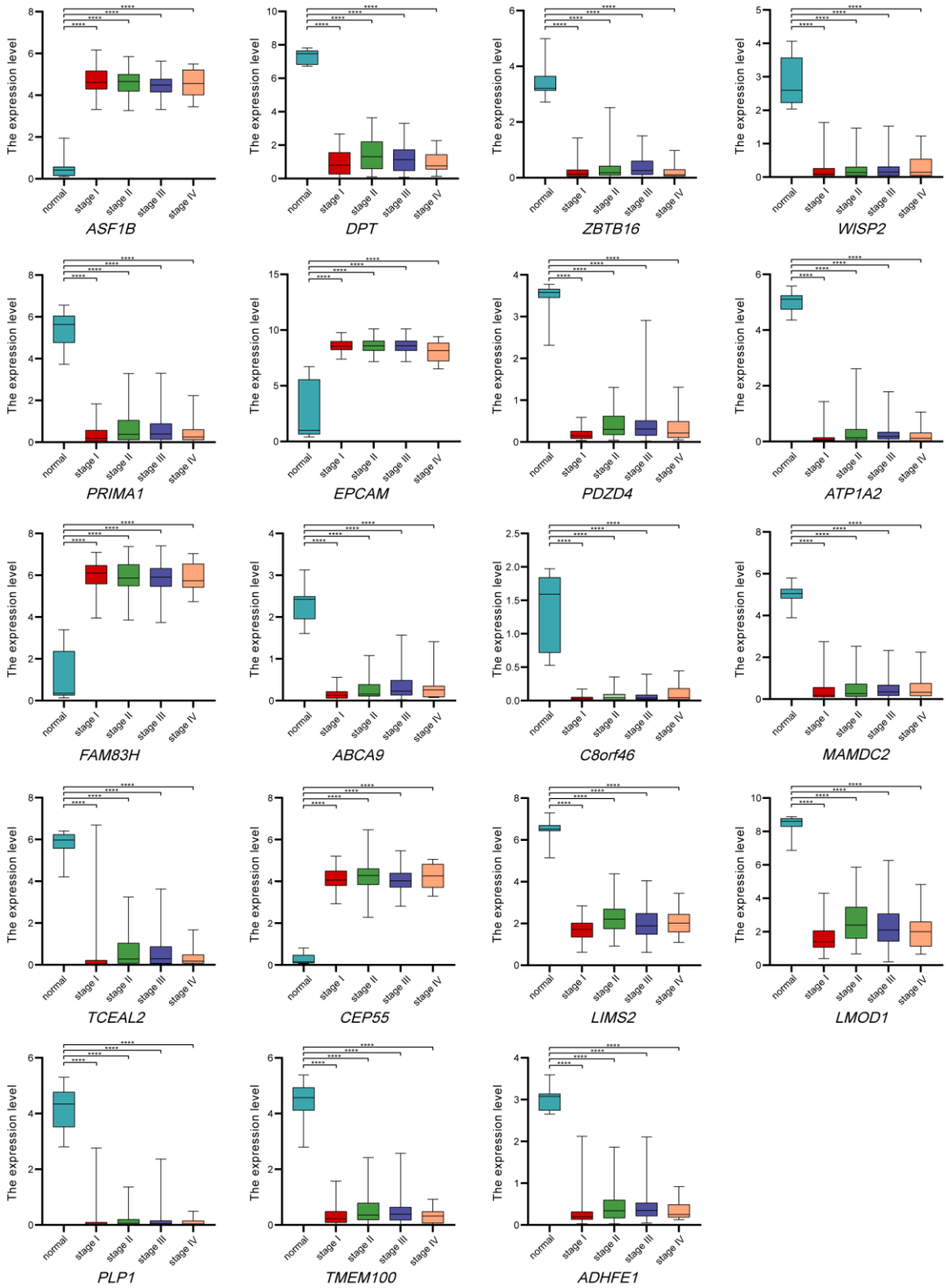


Figure 6. Association between hub genes expression and tumor stage. **** was considered as $P < 0.0001$.

As one of the most common malignant tumors threatening human health, GC has significant limitations in the treatment of advanced GC, and many problems have not been solved [26]. Therefore, it is critical to screen for new biomarkers or the hub genes for therapeutic targets of early GC. In this study, 4892 DEGs were screened. The results of GO and KEGG analyses showed that these DEGs were significantly associated with extracellular matrix organization, DNA replication, cell cycle, and p53 signaling pathway. The cell cycle is often dysregulated during the formation of many tumors, and targeted regulation of cancer cell cycle therapy is a potential treatment for cancer [27, 28]. Thus, studying the cell cycle pathway may improve the understanding of GC carcinogenesis

mechanisms and treatment options. DNA replication is closely related to the cell cycle. DNA replication, including incomplete, erroneous, or untimely replication, may cause mutations, chromosomal poly- or aneuploidy along with gene copy number variation, and these variations may lead to cancer [29]. Extracellular matrix (ECM) is involved in the regulation of cancer development and plays an important role in tumor metastasis [30]. The p53 signaling pathway is a typical cancer signaling pathway; the presence of p53 is required for DNA-damaged cells to arrest, repair, and re-enter the cell cycle [31]. Therefore, we may think that these DEGs may play a role in the progression of GC and may affect prognosis by regulating the p53 signaling pathway, resulting in a poor prognosis of GC.

WGCNA identified gene co-expression modules associated with GC progression. A total of 6 modules were identified, among which the genes in blue and turquoise modules were significantly related to GC (Tumor_Normal). Then, the genes in blue and turquoise modules were screened and verified, and 19 hub genes were screened by survival analysis and differential expression analysis. These results indicate that the verified hub genes may have a promising future in the diagnosis and treatment of GC.

At present, the early detection of cancer has important clinical significance and value, which challenges the frontier of early detection of cancer. Therefore, we investigated the differential expression of these 19 hub genes between different pathological stages of GC and normal tissues. Analysis showed that the expression levels of *ASF1B*, *EPCAM*, *FAM83H,* and *CEP55* in early GC were significantly higher than those in normal tissues, and the expression levels of the other 14 hub genes were lower in early GC, suggesting that the hub genes may have important clinical significance in the early diagnosis of GC.

Many of the 19 hub genes we identified have previously been reported to be associated with cancer. A study reports that high expression of *ASF1B* is associated with an increase in disease progression and metastasis rate of breast cancer [32]. Researchers have demonstrated that *DPT* is related to cell adhesion and invasiveness and plays an important role in regulating the invasion and metastasis of oral squamous cell carcinoma [33]. As for *ZBTB16*, *MAMDC2*, *TCEAL2,* and *TMEM100*, four studies have reported that they can inhibit the activity, proliferation, or metastasis of tumor cells, including gallbladder cancer, breast cancer, renal cell carcinoma and non-small cell lung cancer [34-37]. *PDZD4* and *ADHFE1* have been proven to be associated with synovial sarcoma and colorectal cancer cell proliferation, respectively [38, 39]. Two studies have reported that *LIMS2* and *LMOD1* can promote the migration of GC cells [40, 41]. As for *WISP2*, researchers have demonstrated that it has a bidirectional regulatory effect on tumor cells, with *WISP2* overexpression inhibiting the growth of esophageal cancer cells and its absence inhibiting the proliferation of tumor cells in ovarian cancer [42, 43]. In particular, *LIMS2*, *LMOD1*, *TCEAL2*, *TMEM100,* and *ZBTB16* have been reported to play important roles in the development of GC [40, 41, 44-46]. These results suggest that the hub gene we screened may have an important potential role in GC and may act as a biomarker for the early screening of GC.

Early detection, diagnosis, and treatment of cancer can not only significantly improve the survival rate but also have a higher quality of life. This study advances our understanding of the early diagnosis of GC. These hub genes may be used as biomarkers and potential therapeutic targets for early GC, and their discovery has important clinical significance for the early diagnosis, treatment, and survival prognosis of GC patients. However, our study still has some shortcomings and lacks further molecular biology experiments to confirm the function of these hub genes in GC.

CONCLUSION

Based on WGCNA, we obtained six co-expression network modules and then screened the genes in the key modules. Combined with the GEPIA database, a total of 19 hub genes were verified. The 19 identified hub genes can be used as biomarkers of early GC, which is conducive to the detection and treatment of early GC and the reduction of the mortality of GC patients and provides a theoretical basis for the prediction, diagnosis, and treatment of early GC.

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