Abstract. Lung adenocarcinoma (LUAD) is a common malignancy; however, the majority of its underlying molecular mechanisms remain unknown. In the present study, weighted gene co-expression network analysis was applied to construct gene co-expression networks for the GSE19804 dataset, in order to screen hub genes associated with the pathogenesis of LUAD. In addition, with the aid of the Database for Annotation, Visualization and Integrated Discovery, Gene Ontology, and Kyoto Encyclopedia of Genes and Genomes, pathway enrichment analyses were performed on the genes in the selected module. Using the GSE40791 dataset and The Cancer Genome Atlas database, the hub genes were identified. It was discovered that the turquoise module was the most significant module associated with the tumor stage of LUAD. After performing functional enrichment analyses, it was indicated that the turquoise module was mainly enriched in signal transduction. Additionally, at the transcriptional and translational level, nine hub genes were identified and validated: Carbonic anhydrase 4 (CA4), platelet and endothelial cell adhesion molecule 1 (PECAM1), DnaJ member B4 (DNAJB4), advanced glycosylation end-product specific receptor (AGER), GTPase, IMAP family member 6 (GIMAP6), chromosome 10 open reading frame 54 (C10orf54), dedicator of cytokinesis 4 (DOCK4), Golgi membrane protein 1 (GOLM1) and platelet activating factor acetylhydrolase 1b catalytic subunit 3 (PAFAH1B3). CA4, PECAM1, DNAJB4, AGER, GIMAP6, C10orf54 and DOCK4 were expressed at lower levels in the tumor samples, whereas GOLM1 and PAFAH1B3 were highly expressed in tumor samples. In addition, all hub genes were associated with prognosis. In conclusion, one module and nine genes were recognized to be associated with the tumor stage of LUAD. These findings may enhance the understanding of the progression and prognosis of LUAD.

Introduction

The incidence and mortality of lung cancer rank the highest among all types of cancer worldwide. In 2018, lung cancer was the most commonly diagnosed cancer (11.6% of all cancer cases) and the leading cause of cancer-associated mortality (18.4% of all cancer-associated mortality cases) across 20 world regions (1). Malignant epithelial tumors are the most frequently observed in lung cancer, and can be grouped into non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (2). NSCLC accounts for 85-90% of lung cancer cases, and lung adenocarcinoma (LUAD) is a common type of NSCLC (3). Although positive outcomes have been achieved following early diagnosis, the recurrence rate remains unacceptably high, and the 5-year overall survival rate of patients with LUAD remains low (4). Without sufficient early detection methods and effective therapeutic strategies during the early tumor stages, the mortality rate of patients with LUAD has not markedly decreased in recent years (5). Therefore, further insight into the mechanisms responsible for the development and progression of LUAD is urgently required (6).

Due to the development of high-throughput microarray technology, an increasing number of genes have been identified to serve an important role in tumor occurrence and in the progression of LUAD (7). Gene expression profiles were used to identify important genes associated with tumor progression (8). However, the majority of studies have focused on differentially expressed genes (DEGs) and not on the interconnection between genes (9-11). In order to obtain further information on the association between gene expression levels and important clinical features, scale-free gene co-expression...
networks were constructed using co-expression analysis. Previous studies have applied weighted gene co-expression network analysis (WGCNA) to analyze gene expression datasets and screen hub genes (12,13). Tumor stage is crucial to the clinical prognosis of patients with LUAD, and the survival status of patients at different tumor stages differs significantly (14). Therefore, tumor stage was selected as a main clinical feature. Subsequently, co-expression networks of the association between genes were constructed, and network-centric genes associated with the clinical features were identified. Finally, GSE40791 and UALCAN were applied to investigate the value of the candidate hub genes.

Materials and methods

Data sources and processing. The brief study flow is presented in Fig. 1. The gene expression profile GSE19804 dataset associated with LUAD was downloaded from the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/). GSE19804, which was based on the GPL96 platform (Affymetrix Human Genome U133A Array), contains 120 samples (60 normal and 60 LUAD samples) and 54,675 genes (15). The dataset was normalized with quantile normalization by the R package 'affy' (16). The top 25% most variable genes (13,669 genes) were then selected by analysis of variance for further study in R 3.5.1.

Co-expression network construction. The R (R 3.5.1; https://www.r-project.org/) package ‘WGCNA’ (7) was used to construct gene co-expression networks for the filtered gene expression matrix. To construct a scale-free network, the power of β=12 (scale-free R²=0.89) was selected as the soft-thresholding parameter. After transforming the adjacency into a topological overlap measurement (TOM), the corresponding dissimilarity (1-TOM) was calculated and the dissimilarity of module eigengenes (MEs) was estimated. Using the DynamicTreeCut algorithm (17), the genes, which had similar expression profiles, were categorized into the same module.

Identification of clinically significant modules. The clinical trait of focus was the T stage of LUAD. The association between the clinical phenotype and MEs was determined to identify clinically significant modules. MEs were deemed to represent the expression levels of all genes in the associated module. In addition, the mediated P-value of each gene was calculated and the gene significance (GS=lg P) was identified. Finally, the most clinically significant module was selected according to module significance, which was the average GS of genes involved in the associated module.

Functional and pathway enrichment analysis. The Database for Annotation, Visualization and Integrated Discovery 5 (https://david-d.ncifcrf.gov/) (DAVID) is a database for several types of functional annotation. With the aid of DAVID, the biological relevance of the genes in a given module was identified according to false discovery rate (FDR) <0.05. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of the genes in the hub module were performed by DAVID.

Identification and validation of hub genes. The connectivity of the module can be measured by the absolute value of the Pearson's correlation. Additionally, the association between clinical traits and genes can be measured by the absolute value of the Pearson's correlation. The genes that had a high connectivity with the module and selected phenotype were regarded to be hub genes in hub module (cor.geneModuleMembership >0.8 and cor.geneTraitSignificance >0.2) (18). Survival analysis was performed to explore the association between the expression level of hub genes and overall survival rate in lung adenocarcinoma (based on The Cancer Genome Atlas data in Gene Expression Profiling Interactive Analysis, http://geopia.cancer-pku.cn/). Furthermore, other data of LUAD from GSE40791 (19) and UALCAN (http://ualcan.path.uab.edu/) were used for validation. GSE40791 was used to identify DEGs between normal tissues and LUAD tissues by using the ‘limma’ package in R (20). There are 100 normal samples and 94 tumor samples in the GSE40791 dataset. To overlap the genes in the turquoise module and DEGs, a Venn diagram was constructed using the online tool jvenn (http://jvenn.toulouse.inra.fr/app/example.html). UALCAN is a useful online tool for analyzing cancer transcriptome data, which is based on public cancer transcriptome data from The Cancer Genome Atlas (https://portal.gdc.cancer.gov) and MET500 (21) transcriptome sequencing (22). Independent-sample t-test was used to validate the hub genes in UALCAN. Validation of the genes that were selected from protein levels using The Human Protein Atlas (http://www.proteinatlas.org) was also performed.

Results

Weighted co-expression network construction and key module identification. Using the method of average linkage hierarchical clustering, 120 samples from the GSE19804 dataset were clustered (Fig. 2). Using the ‘WGCNA’ package, genes with similar expression levels were divided into modules to construct co-expression networks. The power of β=12 (scale free R²=0.89) was selected as the soft-thresholding parameter (Fig. 3). In total, 20 modules were identified and the turquoise module exhibited the highest association with the T stage of LUAD (Fig. 4). Therefore, the turquoise module was selected for further analysis as the clinically significant module.

GO and pathway enrichment analysis. To obtain further information on the function of candidate genes, the genes from the turquoise module were categorized into biological process (BP), cellular component (CC) and molecular function (MF) terms. The outcome of GO enrichment analysis is presented in Table I. The BP terms were generally enriched in the ‘positive regulation of transcription from RNA polymerase II promoter’, ‘signal transduction’, ‘negative regulation of transcription from RNA polymerase II promoter’, ‘cell adhesion’ and ‘positive regulation of GTPase activity’; the CC terms were mainly focused on ‘cytoplasm’, ‘plasma membrane’, ‘extracellular exosome’, ‘extracellular region’ and ‘integral component of plasma membrane’; and the MF terms were focused on ‘protein binding’, ‘calcium ion binding’, ‘actin binding’, ‘transcriptional activator activity’, RNA polymerase II core promoter proximal

\[ \text{Identification and validation of hub genes.} \]
region sequence-specific binding' and 'heparin binding'. In addition, KEGG analysis was performed to obtain the pathways in the turquoise module. The results presented in Table II indicated that these genes were included in 'focal adhesion', 'cGMP-PKG signaling pathway' and 'tight junction'. Overall, the genes in the turquoise module were primarily associated with signal transduction.

**Identification and validation of hub genes.** Given the threshold of module membership (MM) > 0.8 and |GS| > 0.2, a total of
415 genes in the turquoise module were recognized as hub genes. Additionally, carbonic anhydrase 4 (CA4), platelet and endothelial cell adhesion molecule 1 (PECAM1), DnaJ heat shock protein family (Hsp40) member B4 (DNAJB4), advanced glycosylation end-product specific receptor (AGER), GTPase, IMAP family member 6 (GIMAP6), chromosome 10 open reading frame 54 (C10orf54), dedicator of cytokinesis 4 (DOCK4), Golgi membrane protein 1 (GOLM1) and platelet activating factor acetylhydrolase 1b catalytic subunit 3 (PAFAH1B3) were associated with overall survival and relapse-free survival (Figs. 5 and 6). After clarifying the hub genes, some methods were used for validation. Firstly, the 'limma' package in R was used to identify DEGs between normal tissues and LUAD tissues in the GSE40791 dataset. Defined by the threshold of $|\log_2\text{fold change}| \geq 2$ and FDR $\leq 0.05$, 3,295 DEGs were obtained. To overlap the genes in the turquoise module and DEGs, a Venn diagram was constructed using the online tool jvenn (Fig. 7). Secondly, the expression levels of these nine genes differed between the normal and LUAD samples in UALCAN (Fig. 8). CA4, PECAM1, DNAJB4, AGER, GIMAP6, C10orf54 and DOCK4 were expressed at lower levels in the tumor samples, whereas GOLM1 and PAFAH1B3 were highly expressed in the tumor samples. Finally, in the Human Protein Atlas database, the protein expression levels of six genes (PECAM1, DNAJB4, AGER, GIMAP6, GOLM1 and PAFAH1B3) in the LUAD samples were distinct from the normal samples (Fig. 9). There were no associated IHC samples of CA4, C10orf54 and DOCK4 in the database.

**Discussion**

The early diagnosis and recurrence prediction of LUAD are crucial for effective prevention and treatment. Therefore,
Figure 4. Identification of modules associated with the clinical traits of LUAD. (A) Dendrogram of all differentially expressed genes clustered based on a dissimilarity measure (1-TOM). (B) Heatmap of the association between MEs and clinical traits of lung adenocarcinoma. (C) Distribution of average gene significance and errors in the modules associated with tumor grades of LUAD. (D) Scatter plot of MEs in the turquoise module. LUAD, lung adenocarcinoma; MEs, module eigengenes; TOM, topological overlap measurement.

| Category | Term | Count, n | %   | FDR       |
|----------|------|----------|-----|-----------|
| BP       | GO:0045944-positive regulation of transcription from RNA polymerase II promoter | 157 | 8.26751 | 3.54x10^{-7} |
| BP       | GO:0007165-signal transduction | 157 | 8.26751 | 2.14x10^{-2} |
| BP       | GO:000122-negative regulation of transcription from RNA polymerase II promoter | 114 | 6.00316 | 2.66x10^{-4} |
| BP       | GO:0007155-cell adhesion | 98  | 5.16061 | 1.54x10^{-10} |
| BP       | GO:0043547-positive regulation of GTPase activity | 96  | 5.05529 | 1.08x10^{-4} |
| CC       | GO:0005737-cytosplasm | 577 | 30.38441 | 1.68x10^{-2} |
| CC       | GO:0005886-plasma membrane | 531 | 27.96209 | 1.33x10^{-12} |
| CC       | GO:0070062-extracellular exosome | 367 | 19.32596 | 5.00x10^{-4} |
| CC       | GO:0005576-extracellular region | 213 | 11.21643 | 6.62x10^{-4} |
| CC       | GO:0005887-Integral component of plasma membrane | 206 | 10.84781 | 5.04x10^{-7} |
| MF       | GO:0005515-protein binding | 953 | 50.18431 | 8.86x10^{-7} |
| MF       | GO:0005509-calcium ion binding | 112 | 5.89784 | 2.45x10^{-4} |
| MF       | GO:0003779-actin binding | 54  | 2.84360 | 1.10x10^{-3} |
| MF       | GO:0001077-transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding | 45  | 2.36967 | 1.72x10^{-2} |
| MF       | GO:0008201-heparin binding | 36  | 1.895735 | 3.57x10^{-3} |

GO, Gene Ontology; FDR, false discovery rate; BP, biological process; CC, cellular component; MF, molecular function.
a deeper understanding of the molecular mechanisms associated with the development of these tumors is of utmost importance. In the present study, free-scale gene co-expression networks were constructed to identify genes with a high connectivity with the T stage of LUAD. WGCNA is a method of analyzing the association between
the expression levels of genes and important clinical features (12). Although co-expression does not mean causality, the module and the genes that are closely associated with a certain clinical phenotype in co-expression networks could be identified. Additionally, survival analysis was performed to screen hub genes, which were associated with overall survival and relapse-free survival rate. The hub genes (CA4, PECAM1, DNAJB4, AGER, GIMAP6, C10orf54, DOCK4, GOLM1 and PAFAH1B3) were differentially expressed between the normal and LUAD samples at the transcriptional and protein level, and were significantly associated with prognosis. Therefore, the hub genes serving an important role in tumor progression have the potential to be prognostic biomarkers for LUAD.

In the present study, the tumor stage of the patients with LUAD was of prime concern. The tumor samples of the GSE19804 dataset included different stages, and the findings of WGCNA for GSE19804 would be more convincing and more significant compared with other datasets in Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo). Although the GSE19804 dataset refers to lung cancer in female non-smokers, the result of WGCNA, in which the stage of the tumor was selected as a main clinical feature, could not be significantly influenced. Furthermore, other
Figure 7. Validation of hub genes in GSE40791. (A) Volcano plot visualizing DEGs in GSE40791 (100 normal samples and 94 lung adenocarcinoma samples). The vertical lines demarcate the fold change values. The right vertical line corresponds to ≥2-fold change (upregulation) and the left vertical line to ≥2-fold change (downregulation), whereas the horizontal line marks a -log_{10} adjusted P-value of 0.01. (B) Identification of common genes between DEGs and the turquoise module by overlapping them. The nine hub genes in the turquoise module were also DEGs in the GSE40791 dataset. DEGs, differentially expressed genes; CA4, carbonic anhydrase 4; PECAM1, platelet and endothelial cell adhesion molecule 1; DNAJB4, DnaJ member B4; AGER, advanced glycosylation end-product specific receptor; GIMAP6, GTPase, IMAP family member 6; C10orf54, chromosome 10 open reading frame 54; DOCK4, dedicator of cytokinesis 4; GOLM1, Golgi membrane protein 1; PAFAH1B3, platelet activating factor acetylhydrolase 1b catalytic subunit 3.

Figure 8. Gene expression levels in normal lung and tumor samples (based on The Cancer Genome Atlas data in UALCAN). mRNA levels of (A) CA4, (B) PECAM1, (C) DNAJB4, (D) AGER, (E) GIMAP6, (F) C10orf54, (G) DOCK4, (H) GOLM1 and (I) PAFAH1B3. (A-I) P<0.0001. TCGA, The Cancer Genome Atlas; CA4, carbonic anhydrase 4; PECAM1, platelet and endothelial cell adhesion molecule 1; DNAJB4, DnaJ member B4; AGER, advanced glycosylation end-product specific receptor; GIMAP6, GTPase, IMAP family member 6; C10orf54, chromosome 10 open reading frame 54; DOCK4, dedicator of cytokinesis 4; GOLM1, Golgi membrane protein 1; PAFAH1B3, platelet activating factor acetylhydrolase 1b catalytic subunit 3.
data of LUAD from the GSE40791 dataset and TCGA (in UALCAN), which exhibited no significant differences in sex and smoking status, were used for the validation of the hub genes. Following adjustment for other predictors, the results demonstrated the important role of the hub genes in the progression and prognosis of LUAD. Validation of the hub genes based on protein levels in the Human Protein Atlas was also performed.

There were 100 normal samples and 94 tumor samples in the GSE40791 dataset. For validation, the GSE40791 dataset was used to identify DEGs between normal and LUAD tissues, and the dataset had a sufficient sample number. Additionally, the tumor tissues in the GSE40791 dataset included 69, 12 and 13 stage I, II and III LUAD frozen tissues, respectively. The tumor samples with various stages of LUAD provided more reliable results.

To obtain further information on the role of the hub module (turquoise module) in tumor progression, GO and KEGG pathway enrichment analyses were performed based on DAVID. The genes were generally enriched for ‘positive regulation of transcription from RNA polymerase II promoter’, ‘signal transduction’, ‘negative regulation of transcription from RNA polymerase II promoter’, ‘cell adhesion’ and ‘positive regulation of GTPase activity’. Currently, inhibitors of RNA polymerase II have the potential to be effective anticancer drugs (23). The hub genes, which are associated with regulating RNA polymerase II, may serve as therapeutic targets for drug design. Signaling by small GTPase, Ras-related protein

Figure 9. Validation of six hub genes in the turquoise module using The Human Protein Atlas database. There were no associated immunohistochemistry samples of carbonic anhydrase 4, chromosome 10 open reading frame 54 and dedicator of cytokinesis 4 in the database. Expression of (A) PECAM1, (B) DNAJB4, (C) AGER, (D) GIMAP6, (E) GOLM1, (F) PAFAH1B3. (A-F) Translational expression levels of six hub genes were positively associated with the disease status, as they were upregulated in the LUAD samples. PECAM1, platelet and endothelial cell adhesion molecule 1; DNAJB4, DnaJ member B4; AGER, advanced glycosylation end-product specific receptor; GIMAP6, GTPase, IMAP family member 6; GOLM1, Golgi membrane protein 1; PAFAH1B3, platelet activating factor acetylhydrolase 1b catalytic subunit 3; LUAD, lung adenocarcinoma.
expression (25). E-cadherin functions as a suppressor of invasion, whereas N-cadherin and vimentin promote cell motility and the inhibition of N-cadherin and vimentin expression and the inhibition of N-cadherin and vimentin promote cell motility and invasion in cancer (25-27).

The protein encoded by CA4 is one of 12 active human isozymes. It is also one of four existing on the extracellular surfaces of certain epithelial and endothelial cells (28). By interacting with Wilms' tumor 1-associating protein (WTAP), CA4 influences WTAP protein degradation through polyubiquitination (25). Furthermore, it has been demonstrated that a low expression of carbonic anhydrase IV can promote the proliferation of cancer cells (29). PECAM1 can code for CD31, which belongs to the adhesion molecule in the immunoglobulin superfamily (30). Via the wingless-related integration site signaling pathway, PECAM1 can maintain and restore vascular integrity (30). This indicates that PECAM1 is involved in the tumorigenesis of LUAD by regulating the expression of vascular endothelial growth factor. Additionally, CD31 is a member of I transmembrane glycoprotein that is enriched in platelets, monocytes, endothelial cells and discrete circulating lymphocytes (31). DNAJB4 belongs to the heat shock protein family (Hsp40/DnaJ), and serves an important role in suppressing cancer metastasis (32). AGER is a member of the immunoglobulin superfamily of cell surface molecules. AGER engagement activates multiple intracellular signaling mechanisms to fuel chronic inflammatory conditions, which may lead to the evasion of apoptosis caused by high intracellular PAF concentrations (47,48). In addition, by regulating an optimal landscape of signaling lipids, PAFAH1B3 can weaken the aggressiveness of cancer and regulate cancer cell pathogenicity (49).

In conclusion, the present study identified the turquoise module and nine hub genes (CA4, PECAM1, DNAJB4, AGER, GIMAP6, C10orf54, DOCK4, GOLM1 and PAFAH1B3), which are of importance to the development of LUAD. Through the turquoise module, further information on the mechanisms of tumorigenesis in LUAD was obtained. In the future, these nine hub genes that serve a vital role in LUAD tumorigenesis may also contribute to early diagnosis and treatment.

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Availability of data and materials
The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions
WH and SL conceived and designed the study. DY, JH and XL performed the analysis procedures. DY, JH, XR, CC and XL analyzed the results. XR, XL, WH and SL contributed to the analysis tools. DY and JH contributed to the writing of the manuscript. All authors reviewed the manuscript.

Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.
Competing interests

The authors declare that they have no competing interests.

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