

SPP1 and AGER as potential prognostic biomarkers for lung adenocarcinoma

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Abstract. Overdue treatment and prognostic evaluation lead to low survival rates in patients with lung adenocarcinoma (LUAD). To date, effective biomarkers for prognosis are still required. The aim of the present study was to screen differentially expressed genes (DEGs) as biomarkers for prognostic evaluation of LUAD. DEGs in tumor and normal samples were identified and analyzed for Kyoto Encyclopedia of Genes and Genomes/Gene Ontology functional enrichments. The common genes that are up and downregulated were selected for prognostic analysis using RNAseq data in The Cancer Genome Atlas. Differential expression analysis was performed with 164 samples in GSE10072 and GSE7670 datasets. A total of 484 DEGs that were present in GSE10072 and GSE7670 datasets were screened, including secreted phosphoprotein 1 (SPP1) that was highly expressed and DEGs ficolin 3, advanced glycosylation end-product specific receptor (AGER), transmembrane protein 100 that were lowly expressed in tumor tissues. These four key genes were subsequently verified using an independent dataset, GSE19804. The gene expression model was consistent with GSE10072 and GSE7670 datasets. The dysregulation of highly expressed SPP1 and lowly expressed AGER significantly reduced the median survival time of patients with LUAD. These findings suggest that SPP1 and AGER are risk factors for LUAD, and these two genes may be utilized in the prognostic evaluation of patients with LUAD. Additionally, the key genes and functional enrichments may provide a reference for investigating the molecular expression mechanisms underlying LUAD.

Introduction

Lung adenocarcinoma (LUAD) is one of the three major histopathological subtypes along with squamous cell carcinoma (SqCLC) and large cell carcinoma (1). Currently, LUAD has become the most common lung cancer with increasing morbidity. This uptrend of incidence may be due to increasing smoking rate and air pollution (2). Furthermore, LUAD is diagnosed at late stages (stage III and IV), when the cancer has spread to nearby tissues and metastasis has occurred (3). As well as overdue diagnosis that leads to delayed treatment, the failure of prognostic evaluation also contributes to the low survival rate of LUAD (4,5). Therefore, there remains to be an urgent requirement for effective biomarkers for prognosis, where the outcome of patients with LUAD can be evaluated in time to provide adjuvant therapy.

Due to cancer largely being a genetic disease, the latest approaches to identify genes as biomarkers are based on microarray technology. Oncogenomic analysis has the ability to generate a wealth of data, which identifies the complex gene expression patterns in cancer (6,7). These gene expression patterns, including point mutations, structural variants at the DNA level and changes at the epigenetic level, are frequently studied to identify the association with the occurrence, development or the survival time of patients with cancer (8,9). A number of studies have identified specific genes that function as diagnostic or prognostic biomarkers for several types of cancer, including in LUAD (10-12). Additionally, gene expression profiling by microarray technology has been used to investigate the molecular mechanisms underlying a number of other diseases as well (13).

The reliability of biomarker identification relies on not only the analysis methods but also the database (14). The Cancer Genome Atlas (TCGA) provides comprehensive data on the molecular basis of various types of cancer (15). Significant gene patterns observed in one or several independent databases may also act in a similar genetic subtype of cancer (16). This may also be the case for different types of cancer. However, researchers must have the ability to evaluate and determine if this common pattern affects clinical phenotypes, including survival by using survival or clustering analysis with another independent dataset.

In the present study, the common differentially expressed genes (DEGs) were screened, including secreted phosphoprotein 1 (SPP1) with high expression and ficolin 3 (FCN3),

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advanced glycosylation end-product specific receptor (AGER; RAGE), transmembrane protein 100 (TMEM100) demonstrating low expression in tumor tissues. These four key genes (SPP1, FCN3, AGER and TMEM100) were verified using an additional dataset. The gene expression model was consistent with the two previous datasets that were analyzed. The dysregulation of highly expressed SPP1 and lowly expressed AGER significantly reduced the median survival time of patients with LUAD. These two genes were verified and indicated stable significance as risk factors for LUAD. This discovery enables these prognostic marker genes to be utilized in the evaluation of patients with LUAD. Additionally, the key genes and functional enrichments may provide reference to investigate the molecular expression mechanism underlying LUAD.

Materials and methods

Data source. Gene expression profiling of three individual datasets for microarray analysis [GSE19804 (17), GSE10072 (18) and GSE7670 (19)] were downloaded from Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>). There were 120 samples in GSE19804, including 60 LUAD tissues and 60 normal tissues. GSE10072 consisted of 107 samples (49 tumor samples and 58 normal tissues). GSE7670 contained 54 samples in total (27 tumor tissues and 27 normal lung tissues). Datasets GSE10072 and GSE7670 were employed for the identification of DEGs on the Affymetrix Gene Chip™ Human Genome U133A arrayplatform (Affymetrix; Thermo Fisher Scientific, Inc., Waltham, MA, USA). GSE19804 was used to verify the significance of selected DEGs. This verification was based on Affymetrix Gene Chip Human Genome U133 Plus 2.0 (Affymetrix; Thermo Fisher Scientific, Inc.). Finally, the prognostic analysis of the selected genes was performed with the gene expression data and follow-ups of patients with LUAD by using RNAseq (Illumina HiSeq 2500; Illumina, Inc., San Diego CA, USA) in TCGA (<https://cancergenome.nih.gov/>).

Quality control. The quality assessment of the Affymetrix Gene Chip datasets was performed using the affyPLM package in R software (version 3.6; <https://www.r-project.org>) (20). In the present analysis, the linear modeling procedures were applied at a probe-level. The Relative Log Expression (RLE) and Normalized Unscaled Standard Errors (NUSE) were generated to test the consistency of the data trends. RLE and NUSE were displayed in box plots. Then AffyRNAdeg package in R software (version 3.6; <https://www.r-project.org>) was used to test the degradation of RNA. Finally, datasets with consistent trends as well as improved RNA quality were selected for the following analysis.

Data preprocessing. To ensure the integrity and comparability of data sets for analysis, the gcrma package (version 3.6) (<https://www.bioconductor.org/packages/release/bioc/html/gcrma.html>) (21) was used to standardize and adjust for background intensities in the Affymetrix array data, eliminating system errors between gene chips. The correlation between gene expression levels of the samples is an important indicator to evaluate the reliability of experiments and sample selection. Therefore, the overall correlation

of the selected samples was analyzed. A correlation chart of all the samples was obtained using Pearson's correlation coefficient (22).

Screening of DEGs. The DEGs of the samples between tumor and normal samples were identified using the limma package in R (<http://bioconductor.org/packages/release/bioc/html/limma.html>) (23). The threshold used is as follows: (fold change, log₂>1 and P<0.05). Then, the differential expression of these genes was visualized in a Venn diagram, 'Volcano plots' or heatmaps were generated using ggplot2 in R (24).

Functional analysis of DEGs. To further understand the potential pathways that these DEGs may participate in or regulate, functional analysis was performed in The Database for Annotation, Visualization and Integrated Discovery (DAVID) (25). All DEGs were firstly annotated using the functional annotation tool and then analyzed for enriched pathways in Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) terms. For every functional item, DAVID calculated the P-value of the enrichment and false discovery rate using Benjamini correction. The threshold for significantly enriched pathways was P<0.05.

Verification analysis. The common up- and down-regulated genes were selected from the top DEGs in two individual datasets (GSE7670 and GSE10072). To evaluate the expression of these candidate DEGs for the prognosis of patients with LUAD, an independent dataset GSE19804 containing 120 samples (60 tumor tissues and 60 normal tissues) was analyzed using Affymetrix Human Genome U133 Plus 2.0.

Prognostic and statistical analysis. Survival analysis was performed by using SPSS version 24 (26,27) to investigate the prognostic impact of selected DEGs on patients with LUAD. The data are presented as the mean ± standard deviation. Significance analysis was performed using Kaplan-Meier analysis with the log-rank test. Log-rank (Mantel-Cox) test was used with a significance level of P<0.05. All statistical analysis was performed with GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA). The P-value of the enrichment and false discovery rate was obtained using Benjamini correlation. P<0.05 was considered to indicate a statistically significant difference.

Results

Data source and quality control. Combining correlation analysis of the raw data was performed with linear regression in affyPLM. The RLE box plot indicated that the majority of the gene expression data from samples in GSE7670 and GSE10072 were consistent (Fig. 1). More sensitive quality analysis of the data was displayed in the NUSE box plot. It was not able that three sample chips (GSM185844, GSM185848, GSM185856, GSM185858 and GSM185862) in the dataset GSE7670 had deviations >1.05, and therefore these were removed. Furthermore, two heavily deviated chips (GSM254636, GSM254639, GSM254670, GSM254671, GSM254677, GSM254678, GSM254684, GSM254700, GSM254709 and GSM254712) were excluded.

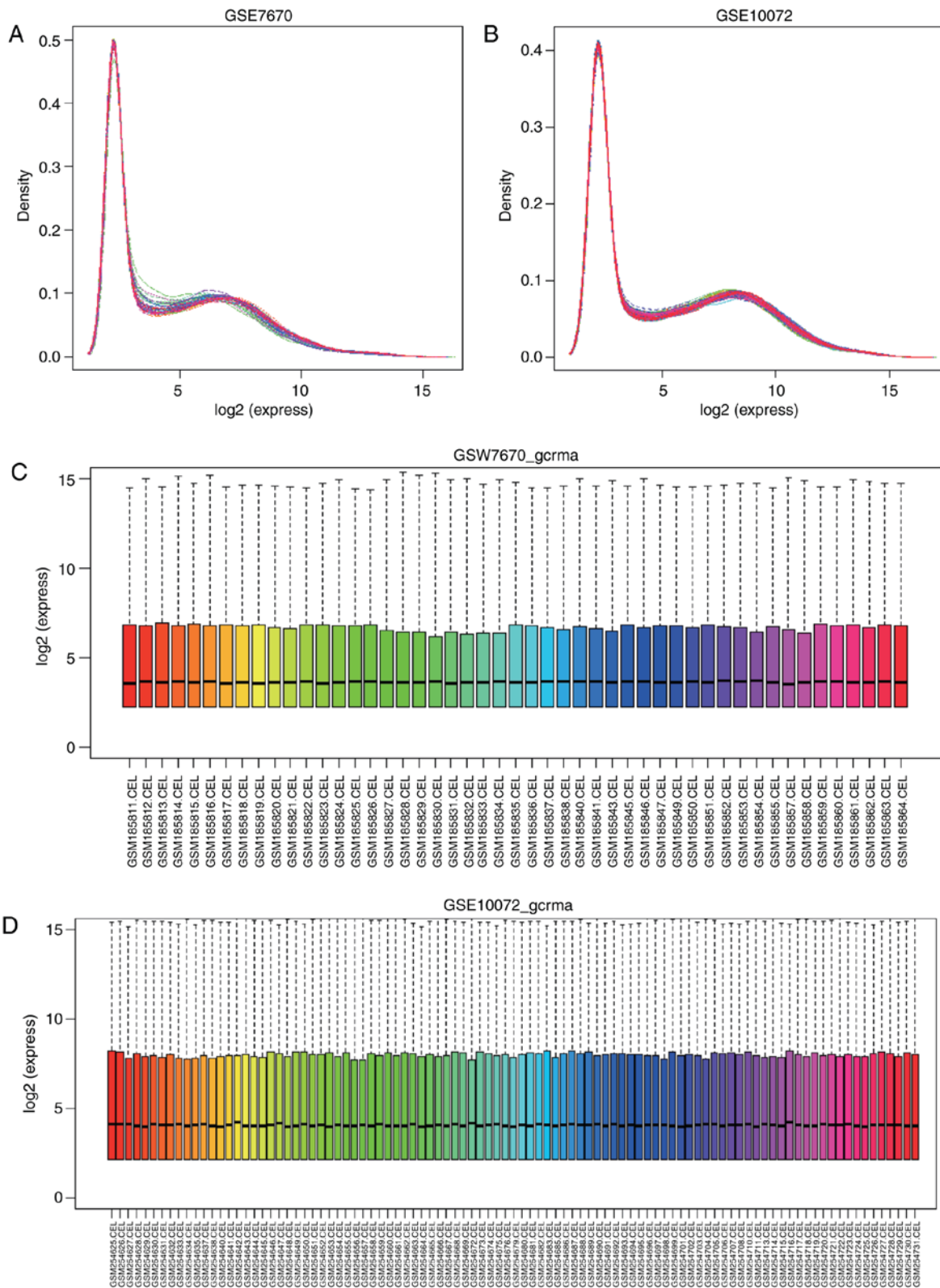


Figure 1. Expression density curve of the standardized samples. (A) Expression density curve of gene data in the GSE7670 dataset following standardization. (B) Expression density curve of gene data in GSE10072 following standardization. (C) Box plot of gene data in GSE7670 following standardization. (D) Box plot of gene data in GSE10072 following standardization.

Data preprocessing. Following quality assessment, all data were standardized with gcrma (Fig. 1). Expression density curves and box charts illustrated that the expression ranges of the 2 datasets were between 0 and 16. The expression value of gene data in GSE7670 and GSE10072 datasets were

concentrated around 4 following standardization, indicating a similar expression trend.

The standardized and the logarithmically converted data were conveyed into cor function in R for Pearson's correlation coefficient. Hierarchical diagram of Pearson's correlation

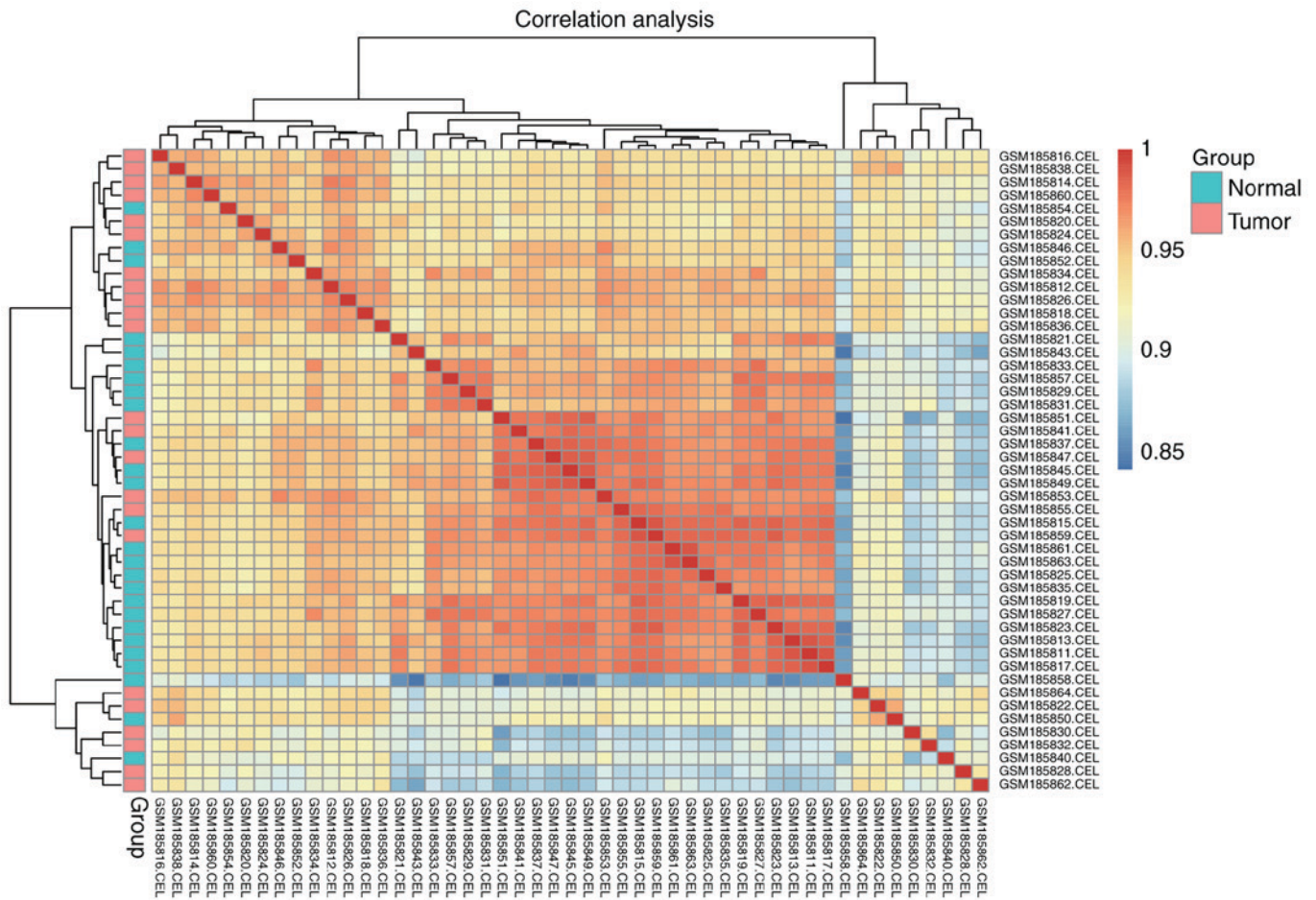


Figure 2. Hierarchical diagram of the correlations of gene expression between standardized samples. Hierarchical diagram of standardized gene samples in the GSE7670 dataset. Samples were analyzed with Pearson's correlation coefficient. Groups are on the left of the diagram, where red indicates tumor samples and blue indicates normal samples.

coefficient was obtained (Fig. 2). In Fig. 2, the minimum value of the correlation coefficient between samples in GSE7670 was 0.84. The majority of the normal samples were clustered together. The tumor samples could not be clustered together due to marked differences in expression. The minimum value in GSE10072 (0.84) was a little higher compared with the value in GSE7670 (0.87).

Screening of DEGs. DEGs were identified using the limma package. In the GSE7670 dataset, a total of 626 DEGs were selected following comparison of 23 LUAD samples and 25 normal tissues. Following removing the duplicates 510 DEGs with annotation remained, including 264 upregulated and 362 downregulated genes (Fig. 3). Following the comparison of 51 lung tumor samples with the 46 normal samples in the GSE10072 dataset, 2,361 DEGs (2,340 were annotated) were obtained, including 1,030 DEGs that were significantly upregulated and downregulated. Following the removal of duplicates, 1,855 DEGs with annotation remained. When the DEGs in the GSE7670 and GSE10072 datasets were matched, there were 484 common genes. Of these, 196 genes were upregulated, and 288 genes were downregulated.

Functional analysis of DEGs. In GO terms, DEGs were significantly enriched in 250 items, including 167 items in

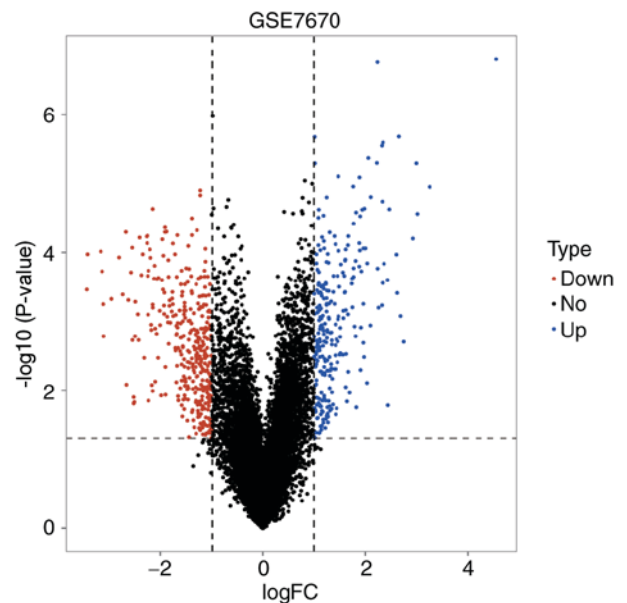


Figure 3. ‘Volcano plots’ for identification of differentially expressed genes in the GSE7670 dataset. Each point represents a gene from the individual dataset. Data points highlighted in blue represent upregulated genes and red points indicate downregulated genes. The black dots represent genes without significant difference in expression. The log₁₀ ratio of expression (normal/tumor) is presented on the y-axis, and logFC is depicted on the x-axis. logFC, fold change.

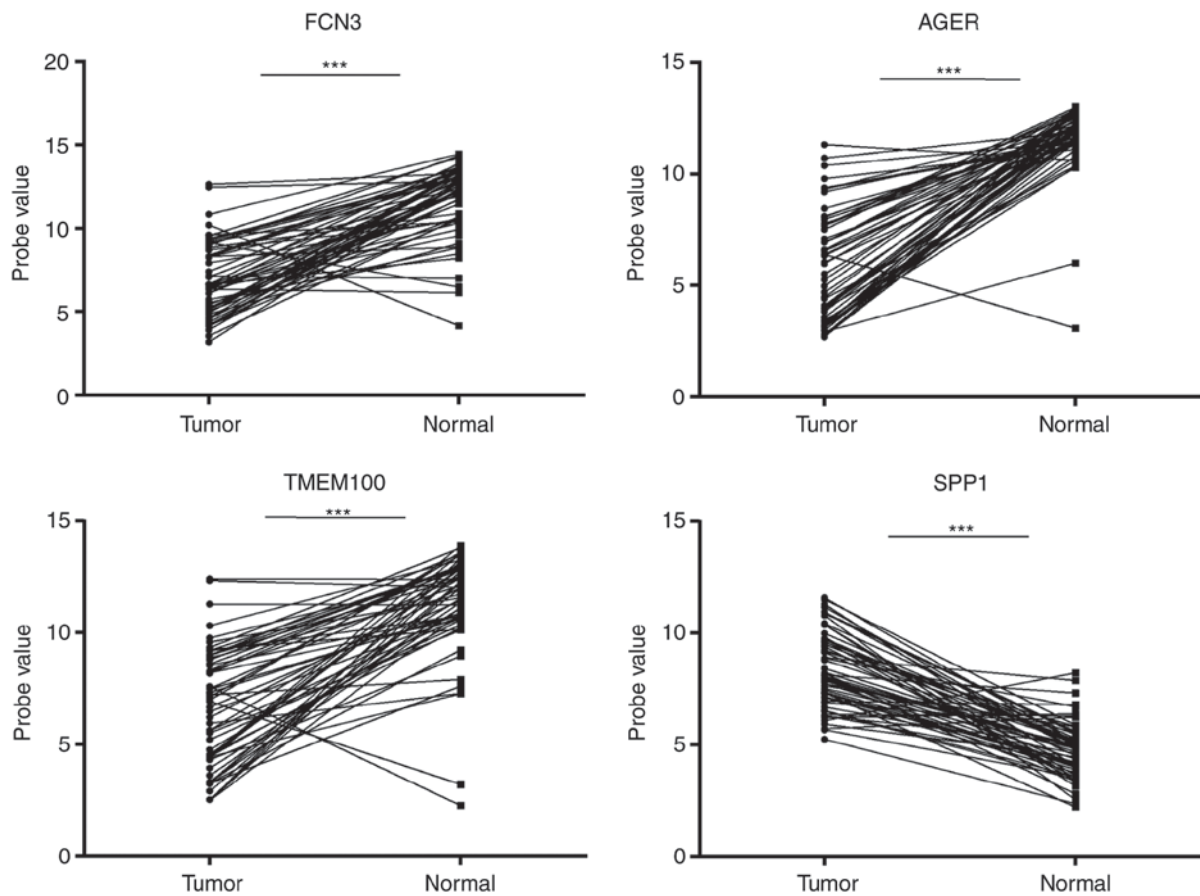


Figure 4. Expression of four candidate genes in the GSE19804 dataset. Data for LUAD tissue samples are indicated as 'tumor', and 'normal' represents normal tissues. *** $P < 0.001$. SPP1, secreted phosphoprotein 1; FCN3, ficolin 3; AGER, advanced glycosylation end-product specific receptor; TMEM100, transmembrane protein 100.

biological process (BP) and 40 in molecular function (MF). For functions in BP, more DEGs were enriched in nitric oxide transport, negative regulation of hepatocyte proliferation, caveola assembly and elastic fiber assembly. In MF, the top three functions were transforming growth factor β -activated receptor activity, small molecule binding and retinoid binding. For functions in cellular component (CC), collagen type I trimer, lamellar body and clathrin-coated endocytic vesicle were the top three popular enrichments. The pathway analysis of KEGG indicated that ECM-receptor interaction was highly enriched among the eight signaling pathways.

Verification analysis. The common up and downregulated genes were selected from the top five DEGs in two individual datasets (GSE7670 and GSE10072), respectively. There were one upregulated DEG (SPP1) and three downregulated DEGs (FCN3, AGER and TMEM100), which were obtained as candidate biomarkers. To evaluate the expression of these candidate DEGs for the prognosis of patients with LUAD, an independent dataset GSE19804, which contains 120 samples (60 tumor tissues and 60 normal tissues), was analyzed on Affymetrix Human Genome U133 Plus 2.0.

In the independent validation analysis, the expression of genes FCN3, AGER and TMEM100 all significantly decreased in tumor tissues from patients with LUAD compared with normal tissues. By contrast, the expression of SPP1 was increased in tumor tissues compared with normal

tissues (Fig. 4). These up and downregulated genes were indicated to participate in the development and/or prognosis of LUAD.

Prognostic analysis and statistical analysis. To further analyze the prognostic impact of the DEGs, survival analysis on the expression data of patients with LUAD was performed using the TCGA database. There were 543 samples available, including 249 males and 294 females (>60 years, $n=369$; ≤ 60 , $n=174$) as listed in Table I. In statistical analysis, the lowly expressed genes FCN3 and TMEM100 did not affect the survival of the patients in the samples tested (Fig. 5). However, the upregulated SPP1 (high expression) and downregulated AGER (low expression) significantly affected the survival of patients with LUAD. The median survival time of patients with downregulated SPP1 was 1,454 days, whilst that of patients with upregulated SPP1 expression was 1,258 days [log-rank (Mantel-Cox) test $P=0.0463$; hazard ratio (HR), 1.366; 95% confidence interval (CI), 1.006-1.865]. The median survival time for patients with downregulated AGER (1,229 days) was 150 days less compared with patients with upregulated AGER (1,379 days). A low expression of AGER indicated poor effects on the prognosis of patients with LUAD [log-rank (Mantel-Cox) test $P=0.0238$; HR, 1.426; 95% CI, 1.050-1.945]. This data indicated that the genes SPP1 and AGER were risk factors that affect the survival time of patients with LUAD.

Table I. Follow-ups and candidate common differentially expressed genes of patients with lung adenocarcinoma.

Factor	Total	SPP1		FCN3		AGER		TMEM100	
		Low	High	Low	High	Low	High	Low	High
Sex									
Male	249	124	125	125	124	125	124	123	126
Female	294	147	147	145	149	146	148	148	146
Age, years									
>60	369	184	185	174	195	180	189	179	190
≤60	174	87	87	96	78	91	83	92	82

SPP1, secreted phosphoprotein 1; FCN3, ficolin 3; AGER, advanced glycosylation end-product specific receptor; TMEM100, transmembrane protein 100.

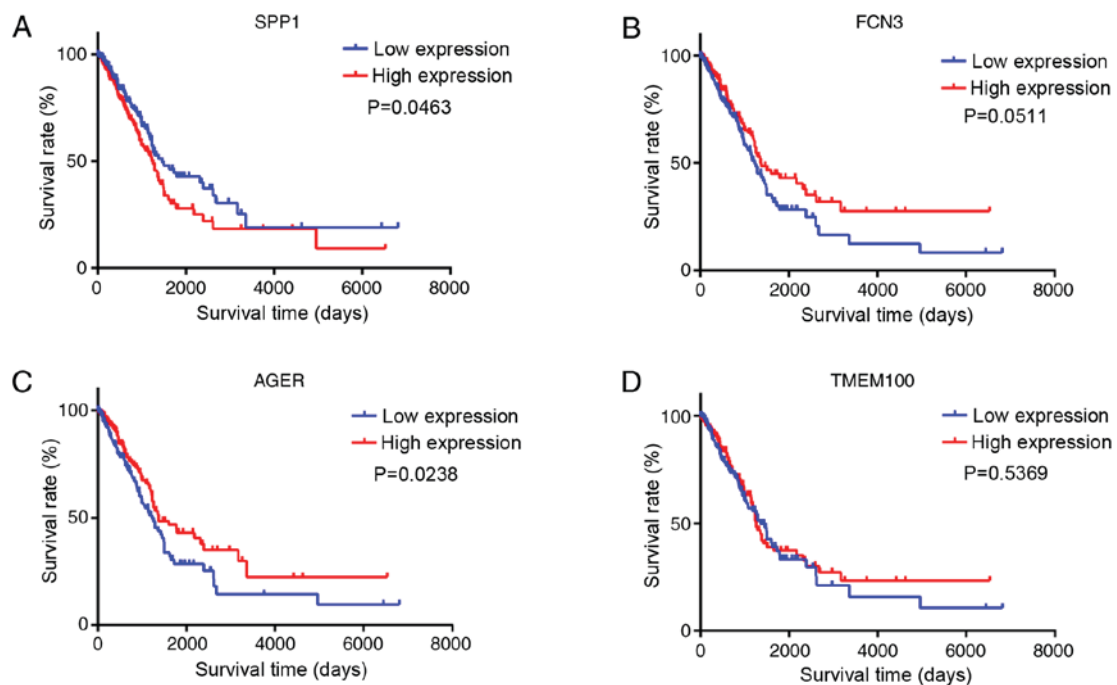


Figure 5. Survival analysis of differentially expressed genes. Survival analysis of (A) SPP1, (B) FCN3, (C) AGER and (D) TMEM100. Blue, genes with low expression; red, genes with high expression. $P < 0.05$; log-rank (Mantel-Cox) test. SPP1, secreted phosphoprotein 1; FCN3, ficolin 3; AGER, advanced glycosylation end-product specific receptor; TMEM100, transmembrane protein 100.

Discussion

Lung cancer is the most common malignant tumor globally with high occurrence and mortality (28-31). It is indicated that lung cancer is not only a genetic disease; it is also affected by environmental factors. For example, it was determined that ~85% of smokers were at a higher risk of lung cancer compared with non-smokers (32). Additionally, the occurrence and mortality of lung cancer were also indicated to be associated with air pollution (33,34).

As one of the main subtypes of lung cancer, LUAD presents similar characteristics in occurrence and mortality. Although LUAD and SqCLC are types of non-small cell lung cancer, LUAD is more likely to occur in non-smoking females, particularly young females (age 20-35) (35,36). The rate of morbidity of LUAD is lower compared with squamous and

undifferentiated carcinoma (37-39). However, the outcomes and survival time of LUAD are poor with an overall five-year survival rate of only 15% (40).

Current studies focusing on the identification of biomarkers are becoming more frequent. However, present research remains insufficient for clinical application. Key genes that are associated with the outcomes and prognosis of patients with LUAD may have a considerable effect in preventing the delay of treatments by predicting survival time (3,36).

In the present study, two independent databases GSE10072 (containing 58 normal tissues and 49 tumor samples) and GSE7670 (containing 27 normal and tumor samples) were employed. With these comprehensive 164 samples, the DEGs were screened and the expression patterns and functional enrichments of the DEGs were identified. A total of 484 common DEGs were identified, including SPP1 with a

high expression and FCN3, AGER and TMEM100 with a low expression in tumor tissues. To confirm this identification, these four DEGs were verified in an additional database, GSE19804. It was indicated that the expression patterns of these four genes in the verification database are in accordance with those in GSE10072 and GSE7670. The differences in expression patterns were all significant. Furthermore, the dysregulation of these candidate genes was associated with the survival rate of the patients. The upregulation of SPP1 and downregulation of AGER suggested that these were risk factors for LUAD.

SPP1 was reported to promote the survival of cancer cells and regulate tumor-associated angiogenesis and inflammation (41). Additionally, SPP1 also served an important role in the development and metastasis of lung cancer (42) by activating the nuclear factor- κ B signaling pathway in LUAD (43). In the study by Wesselkamper *et al* (44), it was discovered that the expression of SPP1 was notably increased in mice with acute lung injury.

AGER is a protein that is associated with diabetic angiopathy and thymic hyperplasia by functioning in toll-like receptor 4 and advanced glycation end product (AGE)-RAGE signaling pathways (45). Under normal conditions, AGER mediates macrophages (46). However, under pathological conditions, the cross-linking reaction between the AGE and extracellular matrix is promoted, resulting in increased thickness and permeability of the endovascular membrane (47). It was also identified that the RAGE gene was suppressed in tumor tissues (48). The expression and mutation of RAGE were discovered to be highly increased in esophageal cancer but reduced in lung cancer (49). Additionally, a high expression of RAGE was indicated as a signal for lung inflammation and aggravation of other lung diseases (50). In combination with the results in the present study, SPP1 and AGER a close association with LUAD.

FCN3 is a member of the FCN/opsonin p35 lectin family. It was identified that activating the complement pathway in association with mannan-binding lectin serine peptidases and small MBL-associated proteins (51). Additionally, in ovarian cancer (OC), the differential expression of FCN3 has been discovered predominantly in serum molecules (52). In addition, FCN3 was also identified in a previous study in SqCLC (53). Although the differentially expressed TMEM100 has not been identified in lung cancer, the overexpression of TMEM100 inhibited the invasion, migration and proliferation of lung cancer cells (54). As the expression of TMEM100 is notably decreased in tumor tissues compared with normal tissues, it could be indicated that it was reliable as a prognostic biomarker for patients with LUAD. In survival analysis, SPP1, FCN3, AGER and TMEM100 worked well to cluster the patient samples in an independent database. These up and downregulated genes were indicated to participate in the development and or prognosis of LUAD.

In conclusion, among the 484 common DEGs, a high expression of SPP1 and a low expression of FCN3, AGER and TMEM100 were identified in tumor tissues from patients with LUAD. The four key genes (SPP1, FCN3, AGER and TMEM100) were verified using an independent dataset GSE19804. The present study indicated that these two genes (SPP1 and AGER) were risk factors for LUAD. Additionally, these four genes were able to cluster the patient samples in survival analysis, thereby indicating that these gene candidates

may serve as prognostic markers to evaluate the prognosis of patients with LUAD. However, in order to investigate the exact functions and the mechanisms that underly LUAD, further research on functional analysis is required. Furthermore, a validation of the associations of the identified DEGs with LUAD is required in future studies. The application of these four candidate genes in clinical treatment and survival evaluation may be promising.

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Availability of data and materials

The datasets generated and analyzed in the present study are included in this published article.

Authors' contributions

WZ was responsible for the majority of the analysis and manuscript writing. JF QC, CL, BQ, and QL contributed to data collection and selection.

Ethics and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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