

Identification of lung adenocarcinoma biomarkers based on bioinformatic analysis and human samples

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Abstract. Lung adenocarcinoma is one of the most common malignant tumors worldwide. Although efforts have been made to clarify its pathology, the underlying molecular mechanisms of lung adenocarcinoma are still not clear. The microarray datasets GSE75037, GSE63459 and GSE32863 were downloaded from the Gene Expression Omnibus (GEO) database to identify biomarkers for effective lung adenocarcinoma diagnosis and therapy. The differentially expressed genes (DEGs) were identified by GEO2R, and function enrichment analyses were conducted using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO). The STRING database and Cytoscape software were used to construct and analyze the protein-protein interaction network (PPI). We identified 376 DEGs, consisting of 83 upregulated genes and 293 downregulated genes. Functional and pathway enrichment showed that the DEGs were mainly focused on regulation of cell proliferation, the transforming growth factor β receptor signaling pathway, cell adhesion, biological adhesion, and responses to hormone stimulus. Sixteen hub genes were identified and biological process analysis showed that these 16 hub genes were mainly involved in the M phase, cell cycle phases, the mitotic cell cycle, and nuclear division. We further confirmed the two genes with the highest node degree, DNA topoisomerase II α (*TOP2A*) and aurora kinase A (*AURKA*), in lung adenocarcinoma cell lines and human samples. Both these genes were upregulated and associated with larger tumor size. Upregulation of *AURKA* in particular, was associated with lymphatic metastasis. In summary, identification of the DEGs and hub genes in our research enables us to elaborate the molecular mechanisms underlying the genesis and progression of lung adenocarcinoma and identify potential targets for the diagnosis and treatment of lung adenocarcinoma.

Introduction

Non-small cell lung cancer (NSCLC) is the most common malignant tumor globally and is associated with an extremely high mortality rate (1). The incidence of NSCLC continues to surge globally. As with other tumors, lung adenocarcinoma which accounts for about 35-45% of all lung malignant tumors is a heterogeneous disease characterized by high rates of genetic mutation (2). Despite the emergence of diverse new approaches for the treatment of lung adenocarcinoma, such as targeted and immune therapy, long term survival is still poor (3,4). One of the main reasons for this is that most patients are diagnosed at an advanced stage. Thus, it is necessary to understand the molecular mechanisms behind lung adenocarcinoma genesis, growth and progression, and identify biomarkers that can be detected during the early stages of the disease.

Recently, high-throughput bioinformatic technologies such as microarrays have been widely used to screen for differentially expressed genes (DEGs) and identify the functional pathways involved in the genesis and development of lung adenocarcinoma. However, the reliable results are not easy to obtain due to the false-positive rates that may exist in every independent microarray analysis. Thus, we downloaded three original mRNA data sets (GSE32863, GSE63459 and GSE75037) from the Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) and aimed to identify the DEGs between normal lung and lung adenocarcinoma tissues. Next, the Kyoto Encyclopedia of Genes and Genomes (KEGG; <http://www.genome.jp/>) and Gene Ontology (GO; <http://www.geneontology.org>) databases were used to identify biological processes enriched in DEGs, and integrated protein-protein interaction (PPI) network analysis was used to help us understand the molecular mechanisms underlying lung adenocarcinoma genesis and development. Sixteen hub genes and 376 DEGs were identified, which could be potential target genes and candidate biomarkers for lung adenocarcinoma. To minimize the false-positive rate of the microarray analysis, the results were then confirmed in cell lines and human sample tissues.

Materials and methods

Microarray data. GEO is a public functional genomics data repository supporting MIAME-compliant data submissions. It accepts sequence based and array data. Tools are

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provided to help users query and download experiments and curated gene expression profiles (5). The GSE32863, GSE63459 and GSE75037 datasets produced by the Illumina HumanWG-6/Ref-8 v3.0 expression beadchip platform (Illumina Inc; <http://www.illumina.com>) were downloaded for further analysis. The GSE63459 dataset contains data from 33 lung adenocarcinoma tissue samples and 32 adjacent normal tissue samples (6). The GSE32863 dataset contains data from 58 lung adenocarcinoma tissue samples and 58 fresh frozen adjacent non-cancerous samples (7). Moreover, the GSE75037 dataset contains data from 84 lung adenocarcinoma and 84 adjacent non-cancerous lung tissue samples (8).

Identification of DEGs. GEO2R is an online web tool (<https://www.ncbi.nlm.nih.gov/geo/geo2r/>) provided by the GEO for comparing GEO series to identify DEGs across experimental conditions. The cutoff criteria were set to P-value <0.05 and logFC (fold change) >1. We excluded probe sets without exact gene symbols, and genes with two or more probe sets were averaged.

KEGG and GO enrichment analyses of DEGs. The functional annotation tools version 6.7 of the Database for Annotation, Integrated Discovery and Visualization (9) (DAVID; <http://david.ncifcrf.gov>) were used to extract biological information about our DEGs. KEGG is a public database used for understanding the functions and abilities of biological systems, such as cells, the organism and the ecosystem, from molecular-level information, especially large-scale molecular datasets acquired by genome sequencing (10). GO was also used to annotate genes and further analyze their biological functions. The DAVID online database was used to analyze the function and biological process of the screened DEGs. P<0.05 was considered to indicate statistical significance.

PPI network construction and analysis. The Search Tool for the Retrieval of Interacting Genes (STRING; <http://string-db.org>; version 10.0) was used to construct the PPI network from the DEGs (11). The sources for interactions are text mining, databases, experiments, neighborhood, co-expression, gene co-occurrence and fusion. We set the minimum required interaction score to 0.4. Cytoscape version 3.4.0 software (12) was used to visualize the molecular interaction networks of the DEGs. The APP plug-in, Molecular Complex Detection (MCODE) (13), was used to arrange the network topology to cluster densely connected genes. After the PPI networks was constructed, its key modules were searched by using the MCODE application. The parameter for inclusions are MCODE score >5, degree cutoff=2, node score cutoff=0.2, node density cutoff=0.1, k-score=2 and Max depth=100. Then, DAVID was used to perform the GO and KEGG analyses for these most significant modules.

Hub gene screen and analysis. The criterion for being a hub gene selection was degree ≥ 10 . Further analysis was performed using the cBioPortal online platform (<http://www.cbioportal.org>) to build the network of the DEGs and co-expressing genes (14). The mutation rates of the hub genes were also measured with the cBioPortal platform (15). Cytoscape's Biological Networks Gene Oncology tool (BiNGO) (version 3.0.3) was used for

the biological process analysis and visualization (16). The University of California Santa Cruz (USCS) platform was used to analyze the hierarchical clustering of hub genes (17). Kaplan-Meier curves for overall survival and disease-free survival with these hub genes were obtained from cBioPortal. The expression profiles of DNA topoisomerase II α (*TOP2A*) and aurora kinase A (*AURKA*) in 20 types of malignant tumors were analyzed and displayed using the Oncomine database (<http://www.oncomine.com>) (18).

Analysis of *TOP2A* and *AURKA* expression in cell lines. To confirm our bioinformatics results, reverse transcription and quantitative real-time PCR (RT-qPCR) were conducted on lung adenocarcinoma (HCC827, A549 and H1975) cell lines and a human bronchial epithelial (HBE) cell line. A549, HCC827 and H1975 cells were purchased from the Shanghai Cell Bank (Shanghai, China) and were cultured using Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco; Thermo Fisher Scientific, Inc.). The medium was supplemented with 100 U/ml penicillin and 100 μ g/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.), and 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.) under a 5% CO₂-containing humidified atmosphere at 37°C. Total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The isolated RNA was reverse-transcribed into cDNA using a reverse transcription kit (Takara, Dalian, China). RT-qPCR was performed as described in our previous research, 2 min at 50°C, 10 min at 95°C, 40 cycles at 95°C for 15 sec, and 60°C for 30 sec (19) and the results were normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) levels. Primers were as follows: *TOP2A* (forward, 5'-AGGATTCGCGCAGTTACGTGG-3' and reverse, 5'-CATGTCTGCCGCCCTTAGAA-3') (20) and *AURKA* (forward, 5'-TTGGGTGGTCAGTACATGCTC-3' and reverse, 5'-GTGAATCAACCCGTGAT-3') (21) and *GAPDH* sense, 5'-CAA TGACCCCTTCATTGACC-3' and reverse, 5'-TGGAAGATG GTGATGGGATT-3'. The statistical analyses were conducted using SPSS version 21 (IBM Corp.). Results are displayed as mean \pm SEM and differences between the HBE and cancerous cell lines were analyzed by one-way ANOVA. We further used the Tukey test to determine the significance between each cancer cell line and HBE. P-value <0.05 was considered to indicate statistical significance. Each experiment was repeated three times.

Analysis of *TOP2A* and *AURKA* expression in human samples. The Ethics Committee of the First Hospital of the China Medical University (Shenyang, Liaoning, China) approved our research. Written informed consent was received from all participants. Seventy-two lung adenocarcinoma and paired non-cancerous tissues were obtained between February 2013 and June 2014 from 35 women and 37 men, ranging in age from 38 to 75, with a median age of 60. Patients who had received chemotherapy, target therapy and radiotherapy or had a history of malignant tumor were excluded. All of the diagnoses were confirmed by two experienced pathologists. The resected samples were preserved at -80°C until the mRNA of *TOP2A* and *AURKA* extraction were needed. Differences between cancerous and non-cancerous tissues were compared using the paired Student's t-test.

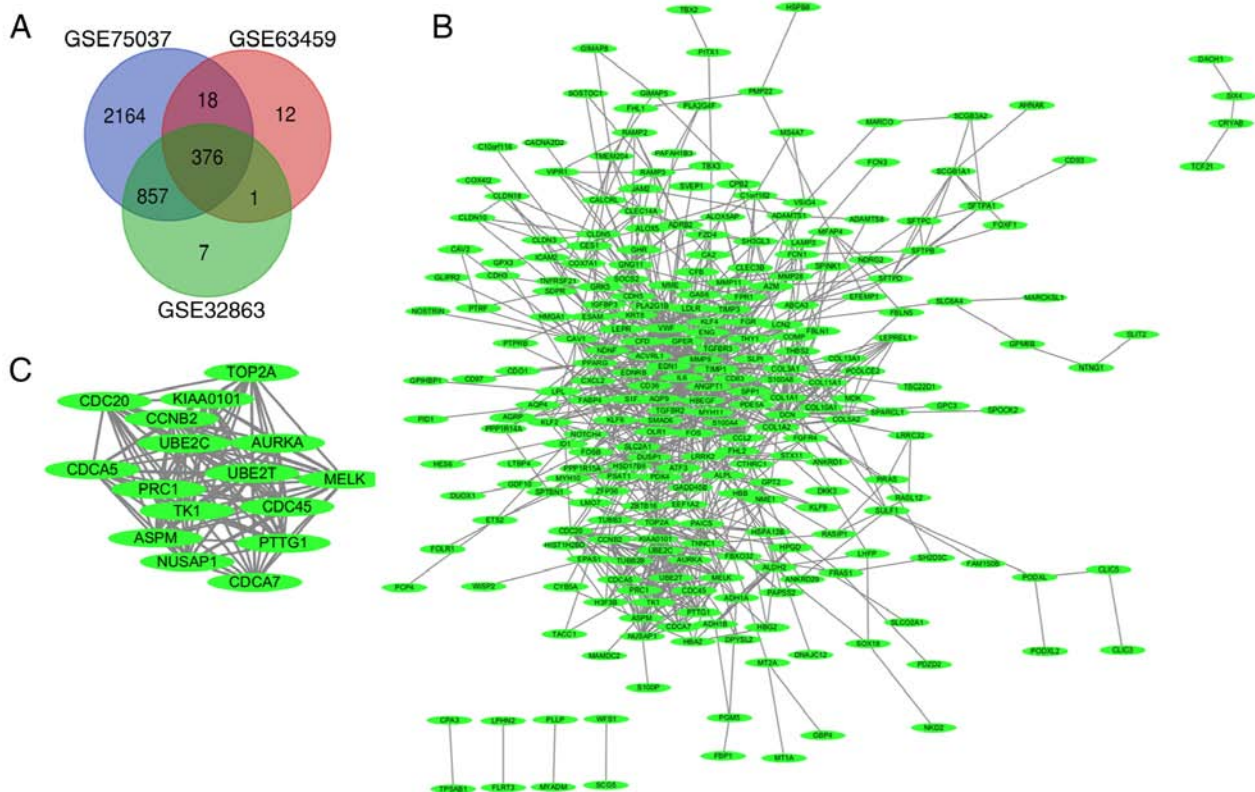


Figure 1. Venn diagram, protein-protein interaction network and the most significant components of DEGs. (A) DEGs were selected with P-value <0.05 and a fold change >2 among the mRNA expression profiling sets GSE75037, GSE63459 and GSE32863. These three datasets manifested an overlap of 376 genes. (B) The protein-protein interaction network of DEGs was constructed using Cytoscape software. (C) The most significant components of the DEGs were obtained from protein-protein interaction network with 16 nodes. DEGs, differentially expressed genes.

Results

Identification of DEGs in lung adenocarcinoma. A total of 5,874 genes were found to be differentially expressed in non-cancerous and lung adenocarcinoma tissues (432 in GSE63459, 4,037 in GSE75037 and 1,405 in GSE32863) after standardizing the microarray data. A total of 376 DEGs were found in all three datasets (Venn diagram, Fig. 1A), consisting of 293 downregulated and 83 upregulated genes.

GO and KEGG enrichment analyses of the DEGs. The DAVID online database was used to further analyze the biological classification, as well as functions and pathways enriched in DEGs. GO analysis showed that the biological processes (BP) of the DEGs were mainly involved in regulation of cell proliferation, the transforming growth factor β receptor signaling pathway, cell adhesion, biological adhesion and responses to hormone stimulus (Table I). Examination of their cell component showed that the DEGs were mainly located in the proteinaceous extracellular matrix, cell surface, cell-cell junction, and cell-substrate adherent junction. KEGG pathway analysis showed that the DEGs were mainly over represented in the TGF- β signaling pathway, cell adhesion molecules (CAMs), complement and coagulation cascades and ECM-receptor interaction (Table I).

PPI network construction and module analysis. Cytoscape was used to build the DEG PPI network (Fig. 1B) and identify the

most significant genes of the PPI network (Fig. 1C). Analysis of these genes with the DAVID platform found that they were mainly involved in M phases, cell cycle phase, the mitotic cell cycle and nuclear division (Table II).

Hub gene selection and analysis. Sixteen genes were identified with a degree ≥ 10 and were defined as hub genes. The degree of each gene was calculated by the CytoScape software and represented the number of other genes with which it was connected. The hub gene symbol, full name, function and degree are listed in Table III. cBioPortal was then used to construct a network of the 16 hub genes and their co-expressed genes. The results of this and analysis of the BP are shown in Fig. 2A and B, respectively. oncoPrint analysis with the cBioPortal showed that *TOP2A* and *AURKA* have the highest genetic mutation rates of the hub genes in lung adenocarcinoma at 8 and 14%, respectively (Fig. 2C). Hierarchical clustering analysis revealed that these 16 hub genes could generally differentiate both primary and recurrent lung adenocarcinoma tissues from their adjacent non-cancerous lung tissues (Fig. 2D).

Clinical significance of *TOP2A* and *AURKA*. Analysis of the association between these genes and disease-free survival and overall survival analysis was conducted using Kaplan-Meier curves in the cBioPortal platform. Lung adenocarcinoma patients with *AURKA* mutation had worse overall and disease-free survival and patients with *ASPM* (abnormal spindle microtubule assembly) mutation had worse disease-free

Table I. KEGG and GO pathway enrichment analysis of DEGs in the lung adenocarcinoma samples.

Term	Description	Count in gene set	P-value
GO:0048545	Response to steroid hormone stimulus	27	9.53E-14
GO:0009725	Response to hormone stimulus	33	2.60E-11
GO:0009719	Response to endogenous stimulus	33	3.31E-10
GO:0042127	Regulation of cell proliferation	45	9.09E-09
GO:0001501	Skeletal system development	26	3.68E-08
GO:0010033	Response to organic substance	41	5.71E-08
GO:0007179	TGF- β receptor signaling pathway	12	6.25E-08
GO:0043627	Response to estrogen stimulus	15	6.80E-08
GO:0007155	Cell adhesion	40	7.66E-08
GO:0022610	Biological adhesion	40	7.78E-08
hsa04512	ECM-receptor interaction	11	5.33E-05
hsa04670	Leukocyte transendothelial migration	10	0.003333312
hsa04514	Cell adhesion molecules (CAMs)	10	0.006973812
hsa04510	Focal adhesion	12	0.014731626
hsa04350	TGF- β signaling pathway	7	0.024809421
hsa04610	Complement and coagulation cascades	6	0.032656397
hsa03320	PPAR signaling pathway	6	0.032656397

KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; DEGs, differentially expressed genes.

Table II. KEGG and GO pathway enrichment analysis of DEGs in the most significant module.

Pathway ID	Pathway description	Count in gene set	FDR
GO:0000279	M phase	9	3.72E-07
GO:0000278	Mitotic cell cycle	9	9.46E-07
GO:0000280	Nuclear division	8	1.07E-06
GO:0007067	Mitosis	8	1.07E-06
GO:0000087	M phase of mitotic cell cycle	8	1.21E-06
GO:0048285	Organelle fission	8	1.41E-06
GO:0022403	Cell cycle phase	9	2.30E-06
GO:0051301	Cell division	8	8.24E-06
GO:0007049	Cell cycle	10	1.24E-05
GO:0022402	Cell cycle process	9	2.65E-05
GO:0007059	Chromosome segregation	4	0.08986
GO:0030261	Chromosome condensation	3	0.367997
GO:0000226	Microtubule cytoskeleton organization	4	0.523613
GO:0007051	Spindle organization	3	1.195525
GO:0007017	Microtubule-based process	4	2.500246
hsa04114	Oocyte meiosis	4	0.096833
hsa04110	Cell cycle	4	0.141584

KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; DEGs, differentially expressed genes; FDR, false discovery rate.

survival (Fig. 3A and B). Moreover, *AURKA* and *TOP2A* had the highest node degrees at 22 and 31, respectively, implying that they may play significant roles in the genesis and development of lung adenocarcinoma. When analyzing the data from cBioPortal platform, we discovered that lung adenocarcinoma patients who had an *AURKA* mutation had reductions in

overall survival ($P=0.00192$). However, this was not true for the *TOP2A* gene ($P=0.775$, Fig. 3A and B). The expression profile of *AURKA* and *TOP2A* in 20 types of human cancer tissues was displayed using the Oncomine database. *TOP2A* mRNA levels in bladder, brain, breast, colorectal, esophageal, kidney, gastric and sarcoma cancer tissues were higher than those in matched

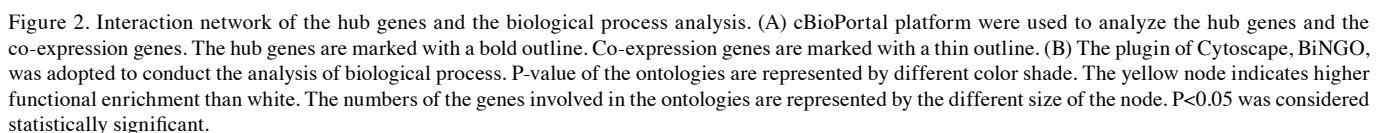
Table III. Summary of the hub gene functions.

No.	Gene symbol	Full name	Function	Degree
1	<i>TOP2A</i>	DNA topoisomerase II α	TOP2A functions as the target for various anticancer agents and mutations in it are associated with drug resistance	31
2	<i>AURKA</i>	Aurora kinase A	AUEKA plays a role in tumor development and progression	22
3	<i>UBE2C</i>	Ubiquitin conjugating enzyme E2 C	UBE2C is required for the destruction of mitotic cyclins and for cell cycle progression, and is involved in cancer progression	20
4	<i>KIAA0101 (PCLAF)</i>	PCNA clamp associated factor	PCNA-binding protein acts as a regulator of DNA repair during DNA replication	20
5	<i>CDC20</i>	Cell division cycle 20	CDC20 is a regulatory protein interacting with several other proteins at multiple points in the cell cycle	19
6	<i>CCNB2</i>	Cyclin B2	CCNB2 is one of the essential components of the cell cycle regulatory machinery	18
7	<i>TK1</i>	Thymidine kinase 1	High level of TK1 is used as a biomarker for diagnosing and categorizing many types of cancers	17
8	<i>PTTG1</i>	Pituitary tumor-transforming 1	PTTG1 product has transforming activity in vitro and tumorigenic activity in vivo, and it is highly expressed in various tumors	17
9	<i>MELK</i>	Maternal embryonic leucine zipper kinase	Diseases associated with MELK include uterine corpus endometrial carcinoma. Among its related pathways are Neuroscience	17
10	<i>NUSAP1</i>	Nucleolar and spindle associated protein 1	NUSAP1 is a nucleolar-spindle-associated protein that plays a role in spindle microtubule organization	16
11	<i>CDC45</i>	Cell division cycle 45	The protein encoded by CDC45 is an essential protein required for the initiation of DNA replication	16
12	<i>ASPM</i>	Abnormal spindle microtubule assembly	ASPM is essential for normal mitotic spindle function in embryonic neuroblasts	16
13	<i>UBE2T</i>	Ubiquitin conjugating enzyme E2 T	The protein encoded by UBE2T catalyzes the covalent attachment of ubiquitin to protein substrates. Defects in UBE2T are associated with Fanconi anemia of complementation group T	15
14	<i>CDCA5</i>	Cell division cycle associated 5	Among its related pathways are Cell cycle, Mitotic and MicroRNAs in cancer	15
15	<i>PRC1</i>	Protein regulator of cytokinesis 1	PRV1 encodes a protein that is involved in cytokinesis which has been shown to be a substrate of several cyclin-dependent kinases	14
16	<i>CDCA7</i>	Cell division cycle associated 7	CDCA7 was identified as a c-Myc responsive gene. Overexpression of this gene enhances the transformation of lymphoblastoid cells	13

adjacent normal tissues (Fig. 4A). The *AURKA* mRNA levels in bladder, brain, breast, cervical, lung and liver cancer tissues were higher than those in adjacent matched normal tissues (Fig. 4B). When we analyzed six different datasets from the Oncomine database, we found that *TOP2A* and *AURKA* were significantly overexpressed in lung adenocarcinoma tissues compared with non-cancerous tissues (Fig. 4C and D) (7,22-28).

Expression of *TOP2A* and *AURKA* in lung adenocarcinoma cell lines. To confirm the bioinformatics results, the expression of these two genes in lung adenocarcinoma cell lines and HBE cells were assessed by RT-qPCR. The mRNA levels of both *TOP2A* and *AURKA* were significantly higher in the lung adenocarcinoma cell lines than the HBE cells (Fig. 5A and B).

Expression of *TOP2A* and *AURKA* in human lung adenocarcinoma and adjacent normal lung tissues. Table IV shows the clinicopathological characteristics of all the lung adenocarcinoma patients. *TOP2A* and *AURKA* were found to have 2.25- and 2.73-times higher expression levels in the cancer tissues than in the normal tissues, respectively (Fig. 5C and D). The 72 samples were then classified into two groups based on the relative expression levels of these two genes: The relatively high *TOP2A* and *AURKA* group (n=36, the first 36 cases having relative high *TOP2A* and *AURKA* were defined as the high-expression group; the remaining 36 cases were defined as the low-expression group) and the relatively low *TOP2A* and *AURKA* group (n=36). χ^2 analysis was used to analyze the results. Analysis revealed that increased *TOP2A* expression was observed in tumors with a larger diameter. Increased



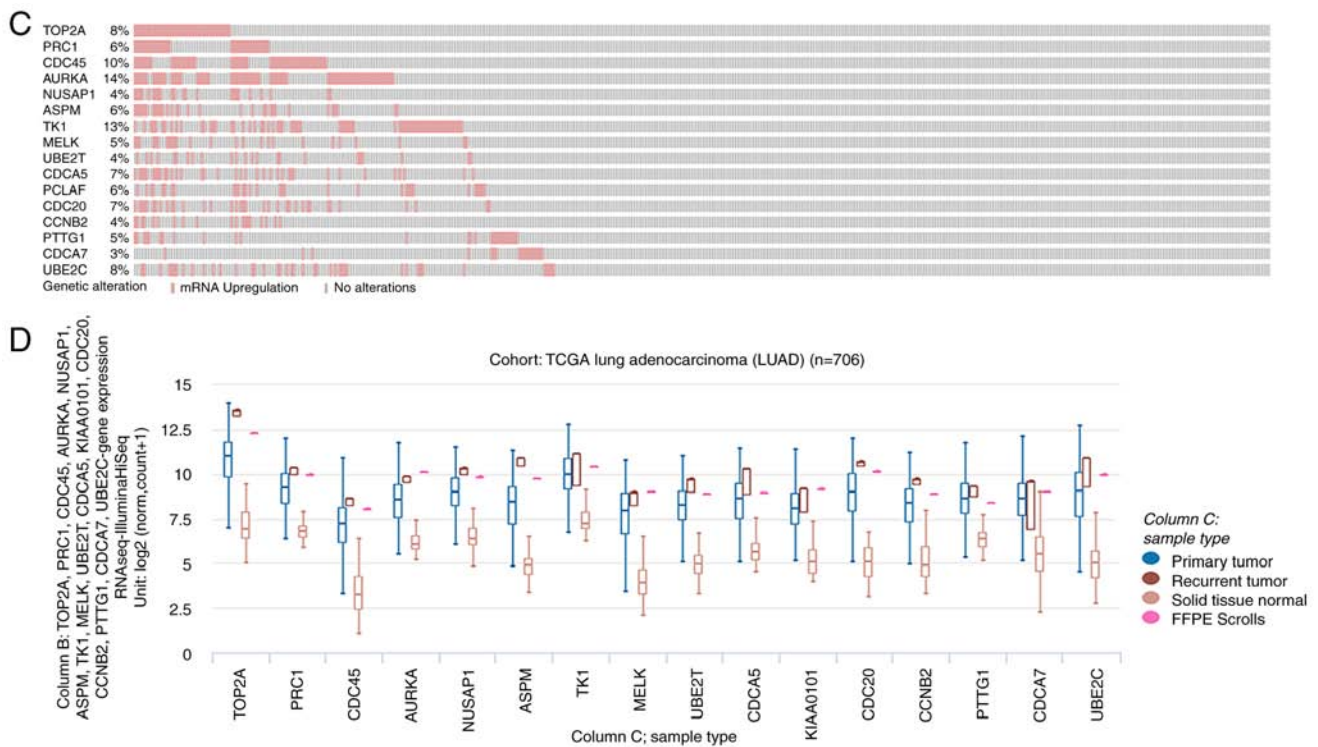


Figure 2. Continued. Interaction network of the hub genes and the biological process analysis. (C) The hub gene alteration rates in lung adenocarcinoma were screened from cBioPortal platform; the red-colored bars represent the upregulation of the gene. (D) The UCSC (University of California Santa Cruz) cancer platform was used to construct the hub gene hierarchical clustering. The primary tumor, recurrent tumor and normal tissue are represented in different colors.

AURKA expression was observed in tumors with a larger size and in lymphatic metastatic tumors (Tables IV and V).

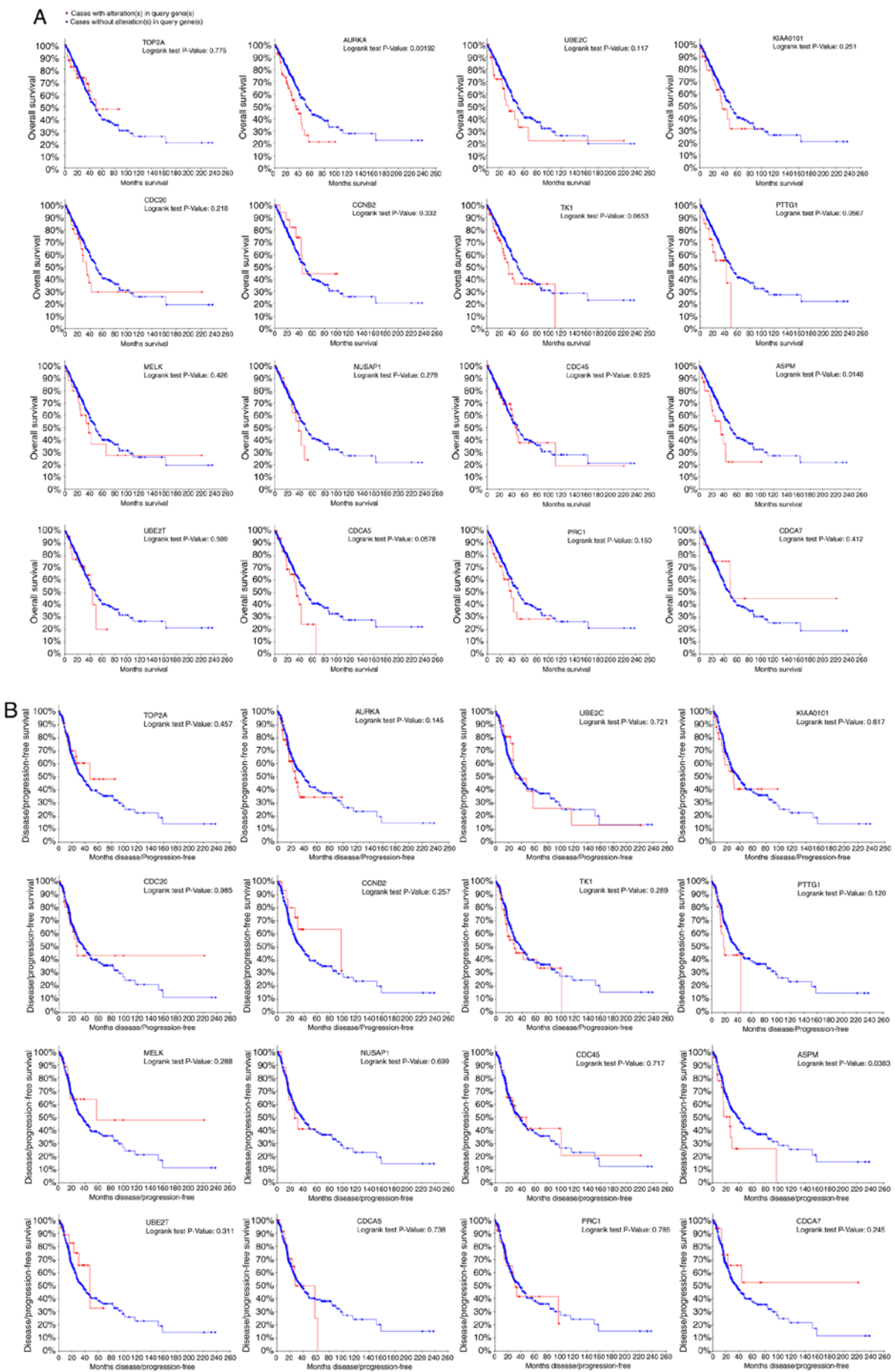
Discussion

Recent statistics on cancer globally revealed that lung cancer is the most commonly diagnosed malignant tumor and is the leading cause of cancer-related mortality accounting for 11.6% of all malignant tumors and 18.4% of cancer-related deaths. Lung adenocarcinoma is the most common subtype of malignant lung cancer, and its incidence is increasing rapidly (29). Air pollution and smoking are the two main etiological factors for lung adenocarcinoma (30,31). The microarray data of Sekine *et al* revealed that a human lung mucoepidermoid carcinoma cell line exposed to smoke with a charcoal filter had a total of 1,582 genetic mutations (32). Still, the molecular mechanisms underlying the genesis of lung adenocarcinoma remain unclear. Without an early diagnosis most patients are not candidates for curative therapies leading to the deeply unsatisfactory prognosis for the disease. Therefore, biological markers with satisfactory efficiency for early diagnosis and therapy are desperately needed. With the development of microarray technology, we are able to efficiently screen for changes in gene expression in lung adenocarcinoma, and this approach has been proven to be a very useful method for screening early stage biomarkers in both malignant and benign diseases (33-35).

In the present research, three mRNA microarray datasets were downloaded from the GEO database and subsequently analyzed to acquire differentially expressed genes (DEGs) between lung adenocarcinoma and adjacent non-cancerous

lung tissues. In addition, a total of 376 DEGs were identified in the three datasets, consisting of 293 upregulated genes and 83 downregulated genes. The biological roles of the identified DEGs were then studied using KEGG and GO enrichment analyses. The downregulated genes were mainly overrepresented in cell division, nuclear division, DNA replication and second-messenger-mediated signaling, and the upregulated genes were mainly involved in cell cycle process, DNA metabolic process, the transforming growth factor (TGF)- β signaling pathway and angiogenesis. Previous articles have revealed that dysregulation of angiogenesis and activation of the TGF- β signaling pathway are associated with the carcinogenesis and progression of lung adenocarcinoma (36-38). In addition, DNA damage and metabolic process abnormalities often play a significant role in cell cycle regulation dysfunction and are associated with malignant tumors (39). In summary, our results are consistent with these previous reports and theories. KEGG enrichment analysis showed differences mainly in the TGF- β signaling pathway, complement and coagulation cascades, Cell adhesion molecules (CAMs), and ECM-receptor interaction, while changes identified by GO terms were mainly in regulation of cell adhesion, proliferation, the TGF- β receptor signaling pathway, biological adhesion and response to hormone stimulus.

Sixteen genes with degrees ≥ 10 were defined as hub genes (40). Two of these hub genes, *TOP2A* and *AURKA*, had the highest node degrees at 31 and 22, respectively. Gene mutation can promote tumorigenesis, thus the mutation rates of these 16 lung adenocarcinoma hub genes were screened with the cBioPortal platform. The four genes with the highest mutation rates were *AURKA*, *TK1*, *CDC45* and *TOP2A* with



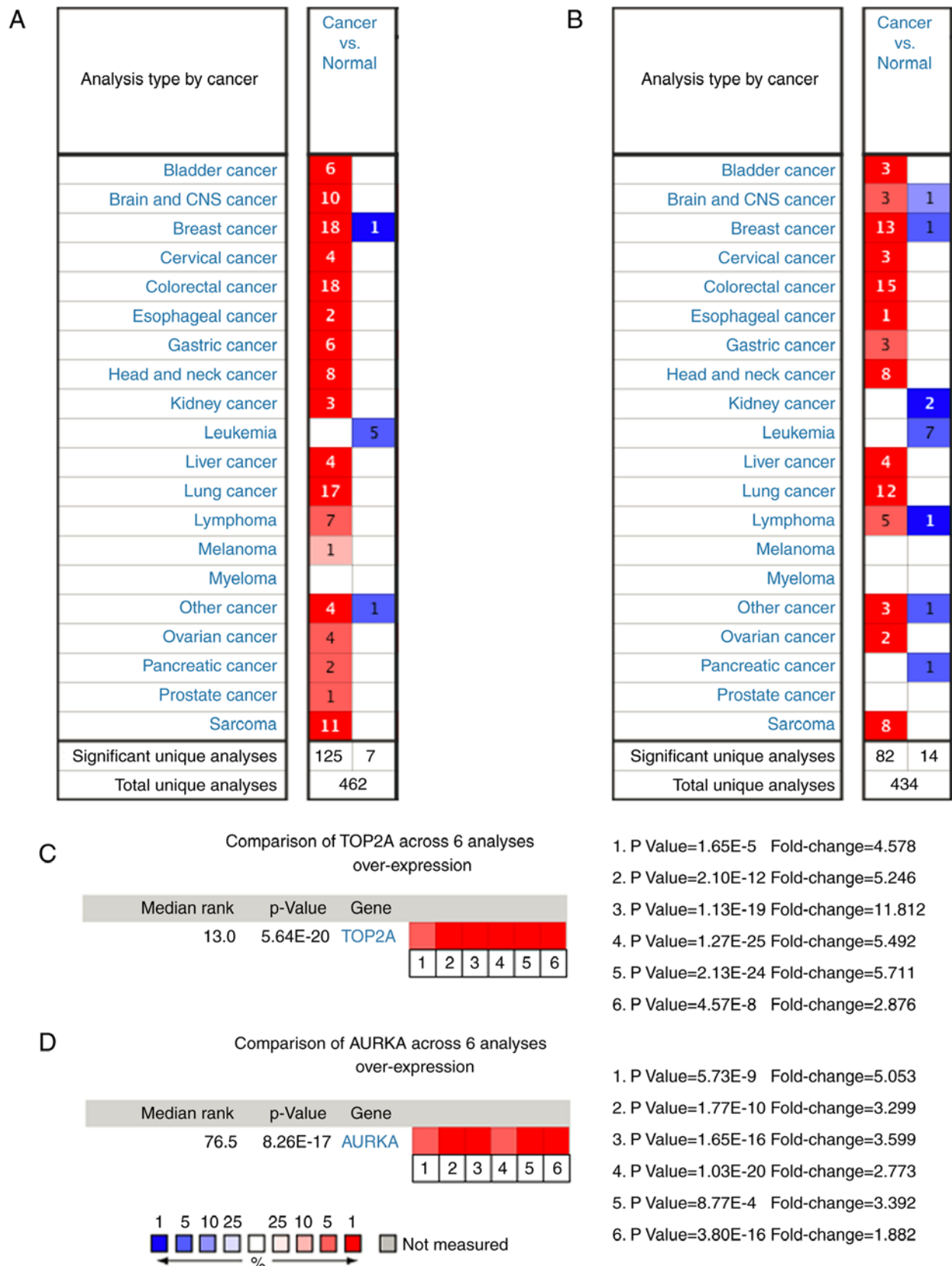


Figure 4. Expression profiles of (A) *TOP2A* and (B) *AURKA* in 20 malignant tumor types are represented using the Oncomine database. The number represent the cases meeting the threshold for *TOP2A* and *AURKA*. Heat maps of (C) *TOP2A* and (D) *AURKA* gene expression in lung carcinoma samples vs. adjacent lung tissues in the Oncomine database. (C) 1. Lung carcinoma vs. normal lung, Beer *et al* (2002) (22). 2. Lung carcinoma vs. normal lung, Hou *et al* (2010) (23). 3. Lung carcinoma vs. normal lung, Landi *et al* (2008) (24). 4. Lung carcinoma vs. normal lung, Selamat *et al* (2012) (7). 5. Lung carcinoma vs. normal lung, Su *et al* (2007) (25). 6. Lung carcinoma vs. normal lung, Yamagata *et al* (2003) (26). (D) 1. Lung carcinoma vs. normal lung, Bhattacharjee *et al* (2001) (27). 2. Lung carcinoma vs. normal lung, Garber *et al* (2001) (28). 3. Lung carcinoma vs. normal lung, Hou *et al* (2010) (23). 4. Lung carcinoma vs. normal lung, Landi *et al* (2008) (24). 5. Lung carcinoma vs. normal lung, Selamat *et al* (2012) (7). 6. Lung carcinoma vs. normal lung, Su *et al* (2007) (25). The P-value for a gene is its P-value for the median-ranked analysis. The fold change represents the relative expression of the tumor tissue compared with the normal tissue. *AURKA*, aurora kinase A; *TOP2A*, DNA topoisomerase II α .

Table IV. Clinicopathologic associations of *TOP2A* expression in lung adenocarcinoma (N=72).

Clinical parameters	No. of cases	Relative <i>TOP2A</i> expression		P-value
		Low	High	
Age (years)				0.237
>60	39	22	17	
≤60	33	14	19	
Sex				0.099
Male	37	15	22	
Female	35	21	14	
Smoking				1
Smoker	42	21	21	
Non-smoker	30	15	15	
Maximum diameter (cm)				0.003
<3	26	19	7	
≥3	46	17	29	
Lymphatic metastasis				0.812
Positive	41	21	20	
Negative	31	15	16	
Metastasis				0.607
M0	68	33	35	
M1	4	3	1	

TOP2A, DNA topoisomerase II α .

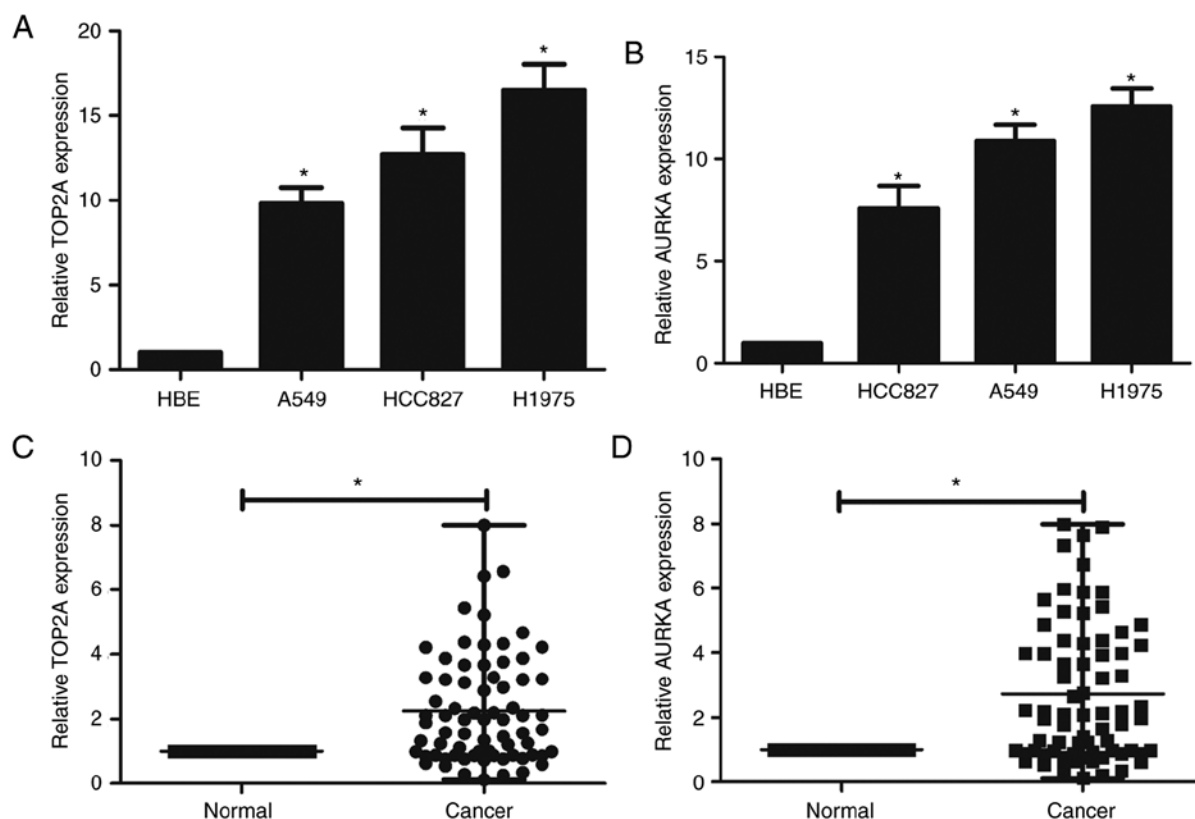


Figure 5. RT-qPCR to confirm the upregulation of *TOP2A* and *AURKA* in lung adenocarcinoma and HBE cell lines and human samples. (A) *TOP2A* expression in lung adenocarcinoma cell lines A549, HCC827 and H1975 and a human bronchial epithelial (HBE) cell line. (B) *AURKA* expression in the lung adenocarcinoma cell lines. (C) *TOP2A* expression in lung adenocarcinoma tissue and non-cancerous lung tissue samples. (D) *AURKA* expression in lung adenocarcinoma tissue and non-cancerous lung tissue samples. *AURKA*, aurora kinase A; *TOP2A*, DNA topoisomerase II α . * $P < 0.05$.

Table V. Clinicopathologic associations of *AURKA* expression in lung adenocarcinoma (N=72).

Clinical parameters	No. of cases	Relative <i>AURKA</i> expression		P-value
		Low	High	
Age (years)				0.813
>60	39	20	19	
≤60	33	16	17	
Sex				0.099
Male	37	22	15	
Female	35	14	21	
Smoking				0.633
Smoker	42	20	22	
Non-smoker	20	16	14	
Maximum diameter (cm)				0.003
<3	26	19	7	
≥3	46	17	29	
Lymphatic metastasis				0.002
Positive	41	14	27	
Negative	31	22	9	
Metastasis				1
M0	68	34	34	
M1	4	2	2	

AURKA, aurora kinase A.

mutation rates of 14, 13, 10 and 8% respectively, which indicates that these genes may play a significant role in tumorigenesis.

DNA topoisomerase II α (TOP2A) that encodes the DNA topoisomerase, an enzyme that alters and controls the topological state of DNA during transcription, has been shown to be correlated with an increased risk of developing brain metastases, drug resistance and an abnormal cell cycle (41-43). It is regarded as a target for several anticancer agents, such as etoposide and topotecan (44). In our research, the protein-protein interaction network (PPI) network revealed that TOP2A directly interacts with maternal embryonic leucine zipper kinase (MELK), CDC20, CCNB2, UBE2T, KIAA0101 and TK1, indicating that TOP2A plays a key role in lung adenocarcinoma. Two of these genes, CDC20 and MELK, are closely involved in tumorigenesis and the cell cycle. Cell division cycle 20 (CDC20) appears to function as a regulatory protein interacting with other proteins at several important phases in the cell cycle. CDC20 is activated in lung adenocarcinoma and overexpression is correlated with poor prognosis (45). MELK plays a key role in the proliferation and self-renewal of progenitor and tumor stem cells, and is overexpressed in lung adenocarcinoma, contributing to carcinogenesis. MELK is an effective target for kinase drugs (46). Moreover, the expression of TOP2A is upregulated in various tumors, such as colon and ovarian malignant tumors, and may be considered a sensitive biomarker for early detection and therapy of these tumors (47,48).

Aurora kinase A (AURKA) is a putative oncogene, associated with cell cycle-regulated kinase (49). GO annotations associated with this gene include protein tyrosine kinase activity, transferring phosphorus-containing groups and transferase activity. Overexpression of this gene is associated with several common features of malignant tumor cells, such as chromosomal instability, aneuploidy in mammalian cells and centrosomal duplication abnormalities (50,51). It has also been found to be overexpressed in several types of malignant tumors and has been associated with poor prognosis (52,53). The aurora kinase A inhibitor, alisertib, has been approved for therapy for solid tumors especially non-small cell lung cancer (NSCLC) and breast cancer, and has achieved satisfactory results (54). In addition, Chen *et al* revealed that AURKA antagonists can enhance the cytotoxicity of epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) (55).

The relationships between TOP2A and AURKA expression and survival were further assessed with the cBioPortal platform. We found that changes in TOP2A expression were associated with a decrease in both disease-free and overall survival, although they were not statistically significant. While changes in AURKA expression were associated with significantly worse overall survival rates, but no significant change in disease-free survival rate. This may be explained by our study of 72 paired human samples which found that upregulation of AURKA was associated with lymphatic metastasis and that upregulation of TOP2A was only associated with tumor size. One of the underlying molecular

reason may be that the overexpression of *TOP2A* arises from amplification and mutation, while the survival analysis using the cBioPortal platform was only based on the mutation of *TOP2A*. Thus, amplification but not mutation may result in overexpression of *TOP2A*, not related to changes in prognosis, although further study is needed to prove this hypothesis. Oncomine analysis revealed that higher *AURKA* mRNA levels were observed in colorectal cancer, breast cancer, lung cancer and sarcoma. Additionally, higher *TOP2A* mRNA levels were observed in colorectal cancer, breast cancer, lung cancer and brain cancer, indicating important roles of these two genes in the carcinogenesis and development of malignant tumors. However, these results also indicate that these two genes can only be used as broad-spectrum tumor markers as they cannot differentiate lung adenocarcinoma from other malignant tumors.

The UCSC (University of California Santa Cruz) cancer platform was used to hierarchically cluster the hub genes. Their expression levels in both the primary and recurrent tumors were upregulated compared with the normal tissues. The expression levels of these hub genes in recurrent tumors were higher than in the primary tumor. *TOP2A* in particular was found to have the highest expression in the recurrent tumors. We therefore infer that *TOP2A* and other hub genes may be regarded as early biomarkers for monitoring tumor recurrence.

To confirm our results, we detected the expression of *TOP2A* and *AURKA* in lung adenocarcinoma and human bronchial epithelial (HBE) cell lines. Both of these genes were downregulated in the lung adenocarcinoma cell lines. *TOP2A* and *AURKA* had their highest expression levels in H1975 cells with approximately 15 times higher expression than that in HBEs cells. Further experiments in human samples found that *TOP2A* and *AURKA* were both upregulated in lung adenocarcinoma tissues compared with non-cancerous tissues. The upregulation of *TOP2A* was found to be associated with larger tumor size, and *AURKA* was found to be associated with both larger tumor size and positive lymphatic metastasis. Thus, we demonstrated that *TOP2A* and *AURKA* are closely involved in lung adenocarcinoma using both bioinformatics and cell experiments. The cell lines we selected have varying levels of EMT. The H1975 cell line (56) has the highest relative level of EMT and also has higher levels of *AURKA* and *TOP2A* in our study. We also showed that higher *AURKA* expression is correlated with poor prognosis. Thus, the higher expression of *AURKA* may be correlated with higher levels of EMT, which result in metastasis and lead to poor prognosis.

In conclusion, our research aimed to identify DEGs, which may be involved in the genesis and development of lung adenocarcinoma. Two of the 16 hub genes were further studied in cell lines and human samples and may be regarded as biomarkers for the diagnosis of lung adenocarcinoma. Further research is needed to elucidate the mechanisms behind their changes in expression and their biological function in lung adenocarcinoma.

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Availability of data and materials

The data used in this study are available from the corresponding author on reasonable request.

Authors' contribution

SD and SX designed the research. WM carried out the data collection and analysis. SY conducted the experiments. SD wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The Ethics Committee of the First Hospital of the China Medical University (Shenyang, Liaoning, China) approved our research. Written informed consent was received from all of the included patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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