

Research Paper

Identifying a Novel Biomarker *TOP2A* of Clear Cell Renal Cell Carcinoma (ccRCC) Associated with Smoking by Co-Expression Network Analysis

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Abstract

Although it is well known that smoking is one of pathogenesis of clear cell renal cell carcinoma (ccRCC), the underlying molecular mechanism is still unclear. In our study, the microarray dataset GSE46699 is analyzed by weighted gene co-expression network analysis (WGCNA). Then we identify 15 co-expressed gene modules in which the lightcyan module ($R^2 = 0.30$) is the most significant. Combined with the protein-protein interaction (PPI) network and WGCNA, two hub genes are identified. Meanwhile, linear regression analyses indicate that *TOP2A* has a higher connection with smoking in ccRCC, survival analysis proved that overexpression of *TOP2A* in ccRCC could lead to shorter survival time. Furthermore, bioinformatical analyses based on GSE46699 and GSE2109 as well as qRT-PCR experiment show similar results that *TOP2A* is significantly up-regulated in smoking ccRCC compared to non-smoking ccRCC samples. In addition, Functional analysis, pathway enrichment analysis and gene set enrichment analysis (GSEA) indicate that high expression of *TOP2A* is related to cell cycle and p53 signaling pathway in ccRCC samples. Moreover, *in vitro* experiments revealed that *TOP2A* induced cell cycle arrest at G2 phase and proliferation inhibition via p53 phosphorylation. Taken together, by using WGCNA, we have identified a novel biomarker named *TOP2A*, which could affect the development of smoking-related ccRCC by regulating cell cycle and p53 signaling pathway.

Key words: weighted gene co-expression network analysis (WGCNA), *TOP2A*, biomarker, smoking, clear cell renal cell carcinoma (ccRCC)

Introduction

Renal cell carcinoma is the third most frequent urological malignant neoplasms worldwide, among which, clear cell renal cell carcinoma (ccRCC) accounts for approximately 80-90% [1]. It is estimated that about 14,400 deaths and 63,990 new cases associated with renal cell carcinoma occur during 2017 in the U.S [2]. Smoking, hypertension, and obesity are proven to be the high risk of renal cell carcinoma [3,4]. Among these, smoking increases

renal cell carcinoma risk by 54% in men and by 22% in women [5]. Therefore, it is urgent to identify the molecular mechanism of ccRCC associated with smoking to understand the pathogenesis of smoking-related ccRCC and to find a novel biomarker for early diagnosis and treatment.

The breakthrough in sequencing of cancer genomes make great contribute to reveal the potential pathological mechanisms in various cancers [6].

Table 1. The description of GSE46699, GSE2109 and GSE10072.

GEO Dataset	Platform	Tumor type	Total samples	Non-smoking samples	Smoking samples	Normal samples	Tumor samples
GSE46699	Affymetrix Human Genome U133 Plus 2.0 Array	ccRCC	130	84	46	63	67
GSE2109	Affymetrix Human Genome U133 Plus 2.0 Array	ccRCC	283	140	143	-	283
GSE10072	Affymetrix Human Genome U133A Array	LUAD	107	31	76	49	58

However, few studies concentrate on the interrelated genes which may have similar function. Weighted gene co-expression network analysis (WGCNA) is a system biology approach which has been widely applied to identifying the hub genes associated with clinical features in various cancer [7,8]. According to the gene expression profiles, WGCNA can construct a gene co-expression network and divide the co-expressed genes into different modules. After that, WGCNA can find the most related module with clinical traits and identify the most central genes among it [9].

In this study, we utilized WGCNA to identify hub genes of ccRCC associated with smoking, which may decipher the potential mechanisms between smoking and ccRCC.

Material and Methods

Study design and collection of microarray data

First, we designed a flow chart to understand the whole process of the study (Figure 1A). The raw data of GSE46699 was obtained from the Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo/>) and was annotated according to the Affymetrix Human Genome U133 Plus 2.0 Array platform (Affymetrix, Santa Clara, CA, USA). This dataset contained 130 samples, including 40 non-smoking normal samples, 44 non-smoking tumor samples, 23 smoking normal samples, 23 smoking tumor samples. Moreover, another two datasets GSE2109 and GSE10072 were downloaded to validate the results. The description of GSE46699, GSE2109 and GSE10072 were listed in Table 1.

Data preprocessing

Robust Multiarray Averaging (RMA) background correction, log₂ transformed and quantile normalization were sequentially utilized to process the raw expression data. Then, the “affy” R package and Affymetrix annotation files were used to summarize and annotate the probes respectively. Besides, sample clustering was applied to evaluate the quality of GSE46699 (Figure 1B). Test sets GSE2109 and GSE10072 were performed the same data preprocessing.

Screening for differentially expressed genes

The limma R package, based on empirical Bayes methods and linear models, was used to find DEGs between smoking and non-smoking ccRCC samples. The DEGs threshold was set at a false discovery rate (FDR) < 0.05 and $|\log_2 \text{fold change (FC)}| > 0.585$ to be further analyzed.

Constructing gene co-expression network

Before we utilized the “WGCNA” package to construct the scale-free gene co-expression network [10,11], we first verified the qualification of DEGs. Then, Pearson’s correlation matrices were conducted and a weighted adjacency matrix were performed by a formula $am_n = |cm_n|^\beta$ (cmn represents Pearson’s correlation between genes, amn represents adjacency between genes, the β parameter could magnify the correlation between genes). An appropriate power of β was chosen on basis of standard scale-free networks. Subsequently, we transformed the adjacency into topological overlap matrix (TOM) [12] and identified modules including similar genes by hierarchically clustering genes [13].

Discovering module of interest

We first defined module eigengenes (MEs) as the most principal component and summarize all genes into a single characteristic expression profile. Then, the interested module was identified by calculating the relevance between MEs and smoking. After that, the log₁₀ transformation of the *P* value was defined as gene significance (GS) and the average GS for all genes in the module was defined as the module significance (MS). Finally, the module with the highest MS score was chosen as the one related to smoking.

Identification and validation of hub genes

Genes in the interested module with module membership > 0.8 and gene significance > 0.2 were defined as hub gene. Then, we constructed protein-protein interaction (PPI) networks and screen hub nodes through mapping all genes to the Cytoscape v3.4.0 software (<http://cytoscape.org/>) [14] and the Search Tool for the Retrieval of Interacting Genes (STRING, <http://www.string-db.org/>) [15,16]. To

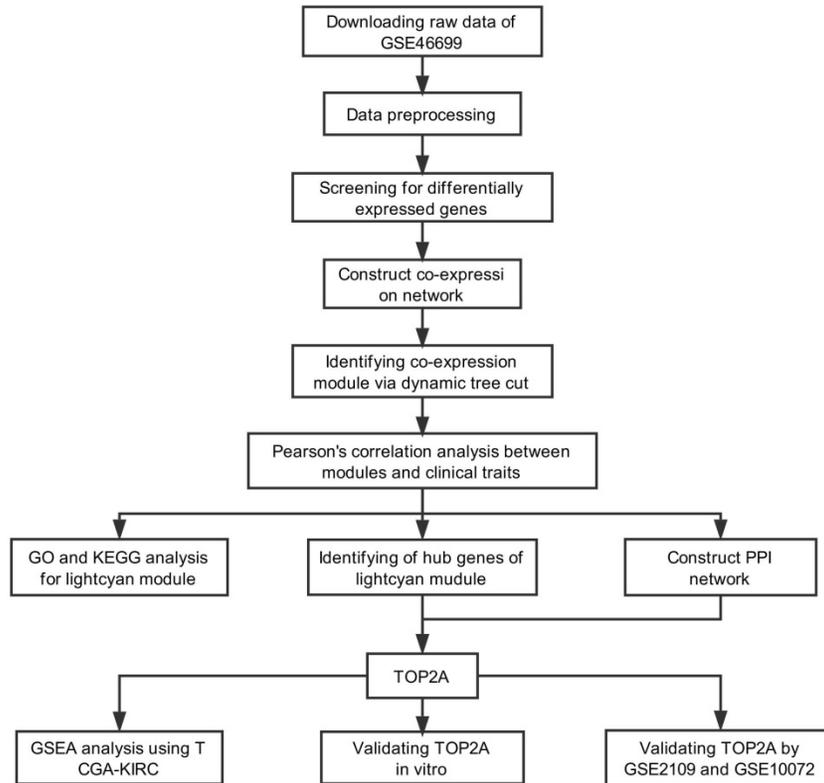
screen the most important hub gene, we performed linear regression analysis to observe the relationship between the hub gene and smoking. In order to further validate the results, we used GSE2109 and GSE10072 datasets to check the relevance between the hub gene and smoking. Furthermore, The Gene Expression Profiling Interactive Analysis (GEPIA, <http://gepia.cancer-pku.cn/>) was used to validate the

expression and prognosis of hub gene [17,18].

Functional enrichment analysis

DEGs in the interested module were put into The Database for Annotation, Visualization and Integrated Discovery (DAVID, <https://david.ncifcr.gov/>) [19] to obtain the enriched biological process and KEGG pathway with the cutoff criteria of $P < 0.05$.

A



B

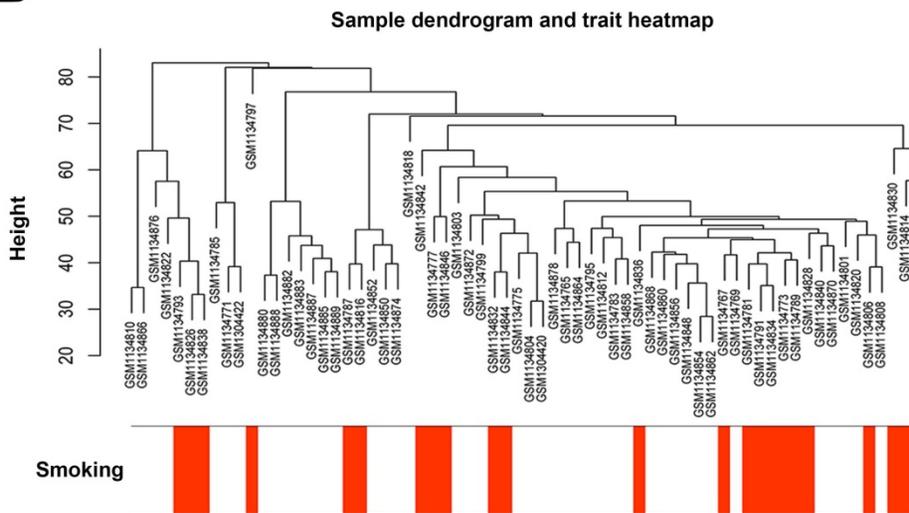


Figure 1. Description of the study. (A) Flow chart of this study. **(B)** Sample dendrogram and cigarette smoking heatmap (GSE46699).

Gene Set Enrichment Analysis (GSEA)

According to the expression level of the hub gene, we divided 530 samples of ccRCC downloaded from TCGA into two groups. To detect whether the previously defined biological processes and KEGG pathway were enriched in the two groups, we conducted GSEA with the cutoff criteria of FDR < 0.05 [20].

Ethics statement

Human ccRCC tissue samples (n=18) and human paracancerous tissues (n=18) were all collected from patients suffering renal cancer in surgery and were stored in liquid nitrogen for later RNA isolation. The study was approved by the Ethics Committee at Zhongnan Hospital of Wuhan University (approval number: 2015029). All experimental methods were carried out in accordance with the approved guidelines.

Cell culture

Human ccRCC cell line 786-O was purchased from Chinese Academy of Sciences in Shanghai. 786-O was cultured in RPMI-1640 (Gibco, China) medium with 10% fetal bovine serum (FBS, Gibco, Australia) in a humidified atmosphere containing 5% CO₂ at 37 °C.

Total RNA isolation and quantitative real time PCR (qRT-PCR)

Total RNA was isolated from kidney tissues by RNeasy Mini Kit (Cat. #74101, Qiagen, Germany). 1 µg of total RNA was used as the template to synthesize first-strand cDNA by utilizing ReverTra Ace qPCR RT Kit (Toyobo, China). All primers were conducted with iQTM SYBR® Green Supermix (Bio-Rad, China) in a final volume of 20 µl with Bio-Rad iCycler (Cat. #CFX96).

Primer sequences of *TOP2A* (annealing temperature is 60°C): 5'-ACCATTGCAGCCTGTAAATGA-3' (forward); 5'-GGGCGGAGCAAAATATGTTCC-3' (reverse).

Primer sequences of *GAPDH* (loading control, annealing temperature is 60°C): 5'-GGAGCGAGATCCCTCCAAAAT-3' (forward); 5'-GGCTGTTGTCATCTTCTCATGG-3' (reverse).

Relative gene abundance was calculated from the $\Delta\Delta CT$ values as: $2^{-\Delta\Delta CT}$.

Cell transfection and reagents

The three specific small interfering RNAs: si*TOP2A*-1: 5'-CUCCUAACUUCUAGUAACUTT-3'; si*TOP2A*-2: 5'-GAUCCACCAAAGAUGUCAATT-3'; si*TOP2A*-3: 5'-GUCCAGUUAACAAGAAGUTT-3' against *TOP2A* were synthesized by Genepharma Ltd. in Suzhou, China. For transfection, Lipofectamine

2000 (Invitrogen, USA) was used as the transfection reagent according to the manufacturer's instructions.

MTT assay

The MTT (methyl thiazolyl tetrazolium, Sigma-Aldrich, MO, USA) assay was used for cell viability measurement in ccRCC cells. After transfection for 48 h, 786-O cells were seeded in 96-well plates (3000 cells per well) in RPMI-1640 medium containing 10% FBS for 5 days. Then, 20 µl of MTT reagent was added to each well for 4 h at 37 °C. After discarding the medium, the precipitates were dissolved by 200 µl of DMSO. The absorbance was measured at 490 nm using a Spectramax M5 spectrophotometer (Molecular Devices, Sunnyvale, USA).

Colony formation assay

After transfection for 48 h, 786-O cells were plated in 6-well plates (800 per well) and cultured for 14 days. Then, surviving colonies (> 50 cells per colony) were fixed by 4% PFA for 30 min, stained with crystal violet and photographed.

Flow cytometry analysis for the alterations of cell cycle

For cell cycle analysis, 786-O cells were harvested and washed by cold PBS for three times after transfection for 48 h. Then, the cells were resuspended with 1× DNA Staining Solution containing propidium iodide and permeabilization solution (Multisciences, China) in the dark. After incubation at 37 °C for 30 min. The sample was analyzed by flow cytometry analysis (Cat. #FC500, Beckman, USA).

Western blot analysis

The RIPA buffer with protease inhibitor and phosphatase inhibitor (Sigma-Aldrich, USA) was used to extract total protein of 786-O cells. We used 10% SDS-PAGE to resolve the total protein and transferred the SDS-PAGE to PVDF membrane (Millipore, USA). Then, membranes were blocked by 5% non-fat milk and incubated with primary antibodies at 4°C for overnight. After washing, secondary antibody was used to incubated the membranes at room temperature for 2 h. Bands were visualized using an enhanced chemiluminescence (ECL) kit (Bio-rad, USA) and detected by BIO-RAD ChemiDoc MP Imaging System (Bio-Rad, USA).

Western blotting was performed using the following antibodies: anti-*TOP2A*, 1:1000 (Proteintech); anti-*CDK1*, 1:1000 (Abcam); anti-*CDK2*, 1:1000 (Abcam); anti-*CCNA1/2*, 1:2000 (Abcam); anti-p-*TP53*, 1:1000 (Abcam); anti-*TP53*, 1:1000 (Abcam). Blotting membranes were stripped and probed again with anti-*GAPDH* antibodies (Santa) as a loading control.

Statistical analyses

All analyses were performed at least three times and represented data from three individual experiments. Two-tailed Student’s t-tests were used to assess the statistical significance of differences between the groups. Statistical analyses were performed using SPSS 16.0. Statistical significance was considered as a *P* value < 0.05.

Results

Screening for differentially expressed genes (DEGs)

A total of 3053 overlapping DEGs were chosen for further analysis. Among these, 1736 were up-regulated and 1317 were down-regulated.

Constructing co-expression network

When we set the soft threshold power β to 11 (scale-free $R^2=0.89$), the link between genes in the gene network satisfied a scale-free network distribution (Figure 2A-D). A total of 15 co-expressed modules were identified (Figure 3A).

Finding module of interest

We applied 2 approaches to verify the relevance between each module and smoking. Consequently, the lightcyan module ($r = 0.3, P = 0.01$) exhibited higher MS and greater connection with smoking (Figure 3B-C) and was identified as the most relevant module.

Identification of hub genes

Seven hub genes (*PRC1, TOP2A, SMC4, RACGAP1, CENPK, PAPD4, ECT2*) were defined by module connectivity (Figure 2E) and eight hub genes (*PRC1, TOP2A, ZWINT, KNTC1, ASPM, PCNA, RRM2, CEP55*) were defined as hub nodes (Figure 2F).

Validation of hub genes

By conducting linear regression analysis with GSE2109, we observed that *TOP2A* was more relevant to smoking (Figure 4B), and we selected *TOP2A* as the hub gene. According to GEPIA database, we found *TOP2A* were more highly expressed in ccRCC samples (Figure 4A). Meanwhile, GSE2109 and GSE46699 showed that *TOP2A* was significantly overexpressed in smoking ccRCC than non-smoking ccRCC samples (Figure 4C, D). Interestingly, GSE10072 also proved the positive correlation between the expression of *TOP2A* and smoking in lung adenocarcinoma (Figure

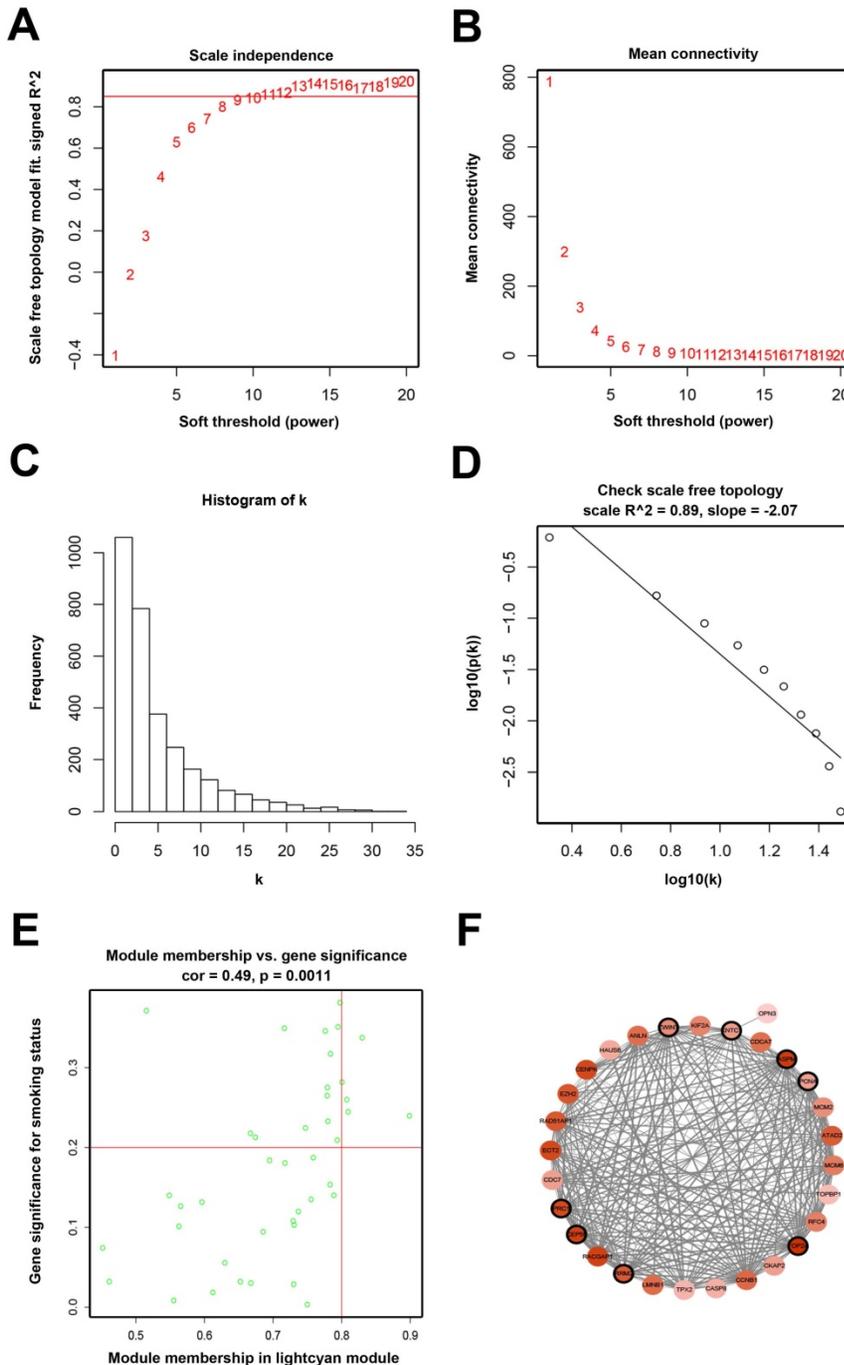


Figure 2. Determination of soft-thresholding power and analysis of hub genes as well as protein-protein interaction network (PPI). (A) Analysis of the scale-free fit index for various soft-thresholding powers (β). (B) Analysis of the mean connectivity for various soft-thresholding powers. (C) Histogram of connectivity distribution when $\beta = 11$. (D) Checking the scale-free topology when $\beta = 11$. (E) Scatter plot of module eigengenes in lightcyan module. (F) PPI network of genes in the lightcyan module.

4E). The *P* value of Figure 4E was listed in Table 2. More convincingly, the qRT-PCR experiment revealed that *TOP2A* was obviously up-regulated in smoking ccRCC patients as well (Figure 4F). In addition, the survival analysis uncovered that up-regulation of *TOP2A* had an obviously shorter overall survival time and disease-free survival time (Figure 4G- H).

Functional Enrichment Analysis

Biological process (BP) in the lightcyan module was found to focus on cell cycle, cell division, mitotic cell cycle, cell cycle phase and cell cycle process (Figure 4I). KEGG pathway enrichment analysis showed DNA replication, cell cycle and p53 signaling pathway were significantly enriched in lightcyan

module (Figure 4J).

Table 2. The *P* value of the GSE10072 in Figure 4E.

Smoking status	Mean±SD	Smoking status	Mean±SD	<i>P</i>
NNS	5.805±0.469	VS NFS	6.088±0.555	0.116475331
NNS	5.805±0.469	VS NCS	6.251±0.631	0.00052866
NFS	6.088±0.555	VS NCS	6.251±0.631	0.034655055
NNS	5.805±0.469	VS NFS+NCS	6.642±0.598	0.002130744
TNS	7.658±1.028	VS TFS	8.451±0.913	0.076959943
TNS	7.658±1.028	VS TCS	9.123±0.851	0.001676115
TFS	8.451±0.913	VS TCS	9.123±0.851	0.148011761
TNS	7.658±1.028	VS TNS+TCS	8.835±0.939	0.003132606
NNS+NFS+NCS	6.054±0.585	VS TNS+TFS+TCS	8.510±1.098	1.95169E-25

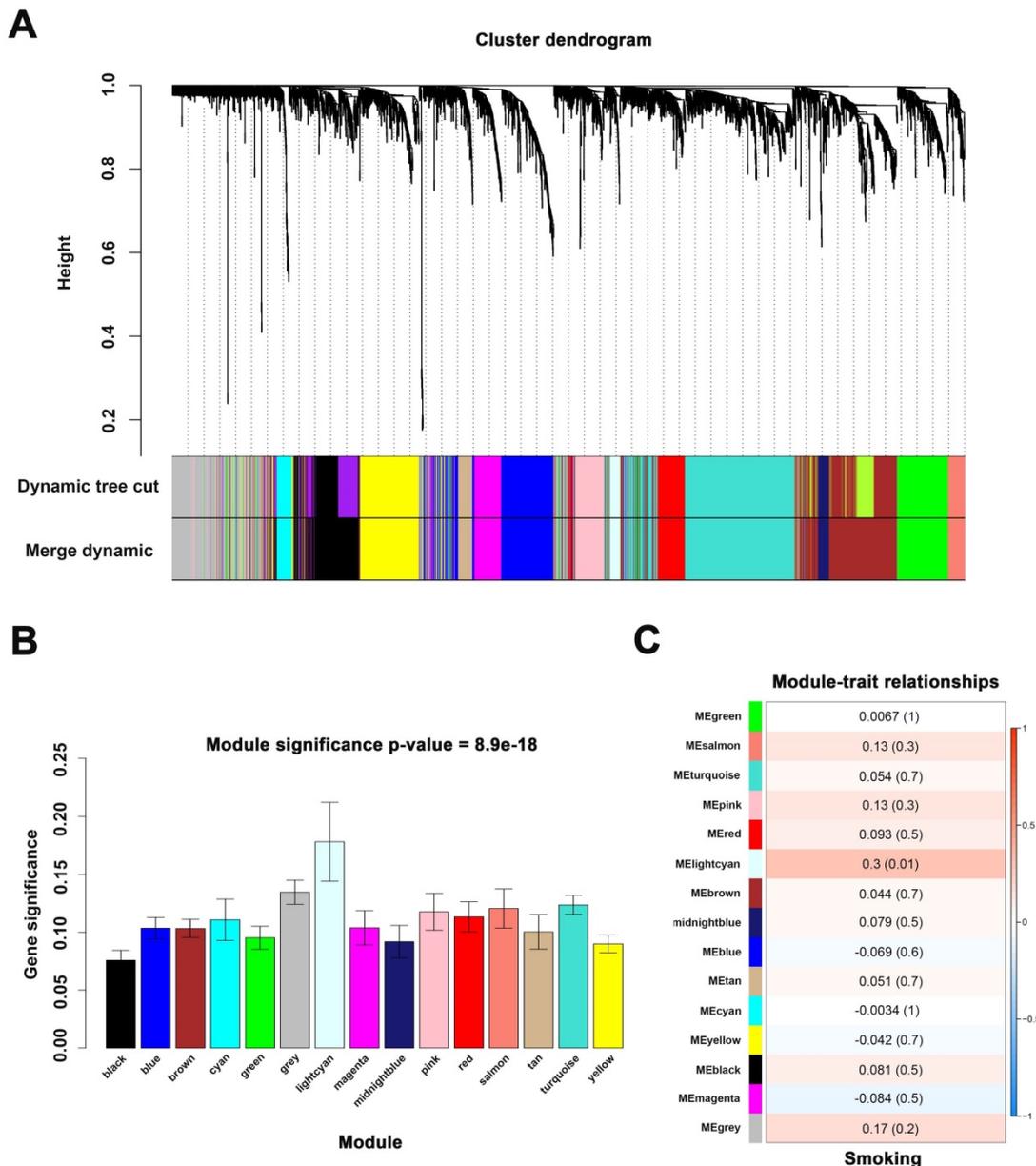


Figure 3. Identify modules associated with the smoking of ccRCC. (A) Dendrogram of all differentially expressed genes clustered. **(B)** Distribution of average gene significance in the modules associated with smoking of ccRCC. **(C)** The correlation between module eigengenes and the smoking group.

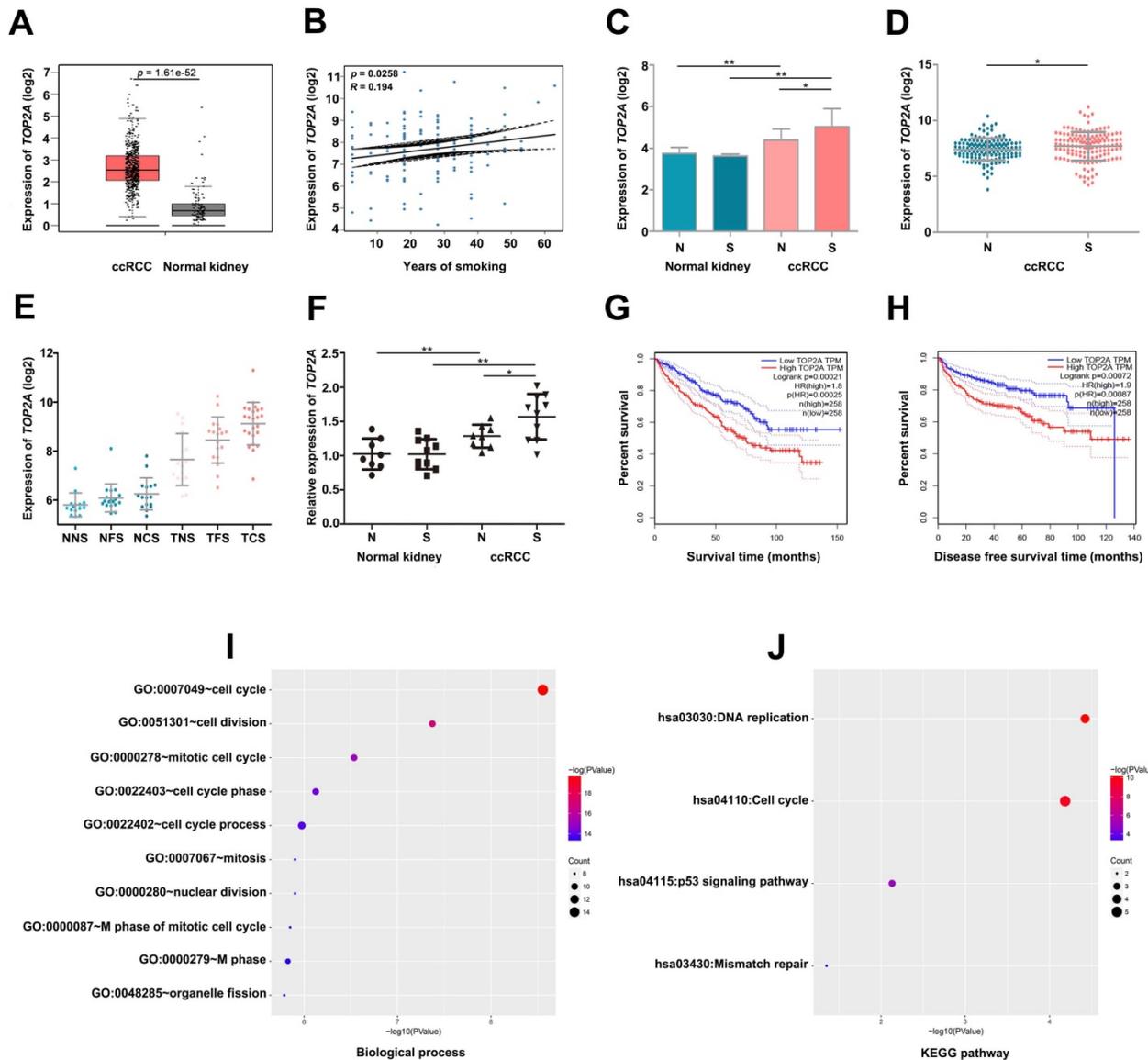


Figure 4. Validate the relationship between TOP2A and smoking ccRCC samples. (A) TOP2A is significantly overexpressed in the ccRCC according to GEPIA database. (B) Linear regression analysis between the expressions of TOP2A and smoking group. (C) The expression of TOP2A is correlated with smoking of ccRCC in GSE46699 (N represents non-smoking patients, S represents smoking patients). (D) TOP2A expression is correlated with smoking of ccRCC in GSE2109. (E) TOP2A expression is positive correlation with smoking status in lung adenocarcinoma in GSE10072 (NNS: Normal never smoked, NFS: Normal former smoker, NCS: Normal current smoker, TNS: Tumor never smoked, TFS: Tumor former smoker, TCS: Tumor current smoker). (F) qRT-PCR indicates the expression of TOP2A is up-regulated in smoking ccRCC tissues. (G-H) Kaplan-Meier survival curve downloaded from GEPIA database demonstrate that up-regulation of TOP2A have a significantly shorter overall survival time and disease-free survival time. (I) Biological processes and (J) KEGG pathway enrichment analysis of TOP2A.

Gene Set Enrichment Analysis (GSEA)

Six gene sets (cell cycle, leishmanial infection, antigen processing and presentation, autoimmune thyroid disease, viral myocarditis and p53 signaling pathway) were enriched in samples with highly expressed TOP2A by conducting GSEA (Figure 5).

Knockdown of TOP2A inhibited cell proliferation by triggered cell cycle arrest at G2 phase in ccRCC cell

To explore the biological role of TOP2A in ccRCC, we established a model of TOP2A deficiency

in 786-O cell line. The knockdown efficiency of the siRNAs was validated by qRT-PCR and Western blot analysis (Figure 6A). MTT assay revealed that downregulation of TOP2A inhibited the cell proliferation significantly comparing with the NC group (Figure 6B). This uncovered that silencing TOP2A inhibited the proliferative capacity of ccRCC cell. The colony formation assays demonstrated a similar conclusion (Figure 6C). Flow cytometry analysis also showed that knockdown of TOP2A triggered cell cycle arrest at G2 phase (Figure 6D). Indeed, cell cycle related proteins such as Cyclin A1/2 and CDK1/2 were significantly decreased in the

ccRCC cell in *TOP2A* knockdown group (Figure 6E). Moreover, *TOP2A* deficiency strongly induced phosphorylated p53 in ccRCC cells (Figure 6F).

Discussion

In our study, the lightcyan module associated with smoking of ccRCC was identified by applying WGCNA. Seven hub genes were obtained from the module. Then, we constructed PPI networks and found that *PRC1* and *TOP2A* were both hub genes in PPI network and WGCNA. Furthermore, linear regression analyses indicated that *TOP2A* was the most correlated hub gene with the smoking ccRCC samples. The GEPIA database indicated that *TOP2A* was significantly overexpressed and resulted in poorer prognosis in ccRCC samples.

TOP2A encodes topoisomerase 2- α , an enzyme that plays a key role in the topologic states of DNA during replication and transcription, which is essential for chromosome condensation and segregation [21]. Our group had revealed *TOP2A*

played a critical role in ccRCC carcinogenesis and progression, involving in the biological process of ccRCC proliferation [22,23]. Meanwhile, two publications suggested that *TOP2A* is overexpressed in colorectal cancer and hepatocellular carcinoma [24,25], and overexpression of *TOP2A* was presumed to inhibit cell proliferation in colorectal cancer [26]. What's more, several investigators have reported that *TOP2A* is an independent prognostic biomarker in various cancer, such as nasopharyngeal carcinoma, breast cancer, pancreatic cancer and renal cell carcinoma [27-30].

Given that a cigarette contains approximately 50 chemicals that are associated with carcinogenesis [31], it is not surprising that cigarette smoking is one of an etiology of renal cell carcinoma. In fact, the relationship between cigarette smoking and the development of renal cell carcinoma has been widely recognized, and previous reports demonstrated that overall survival time was significantly shorter in renal cell carcinoma patients who smoke [32-34].

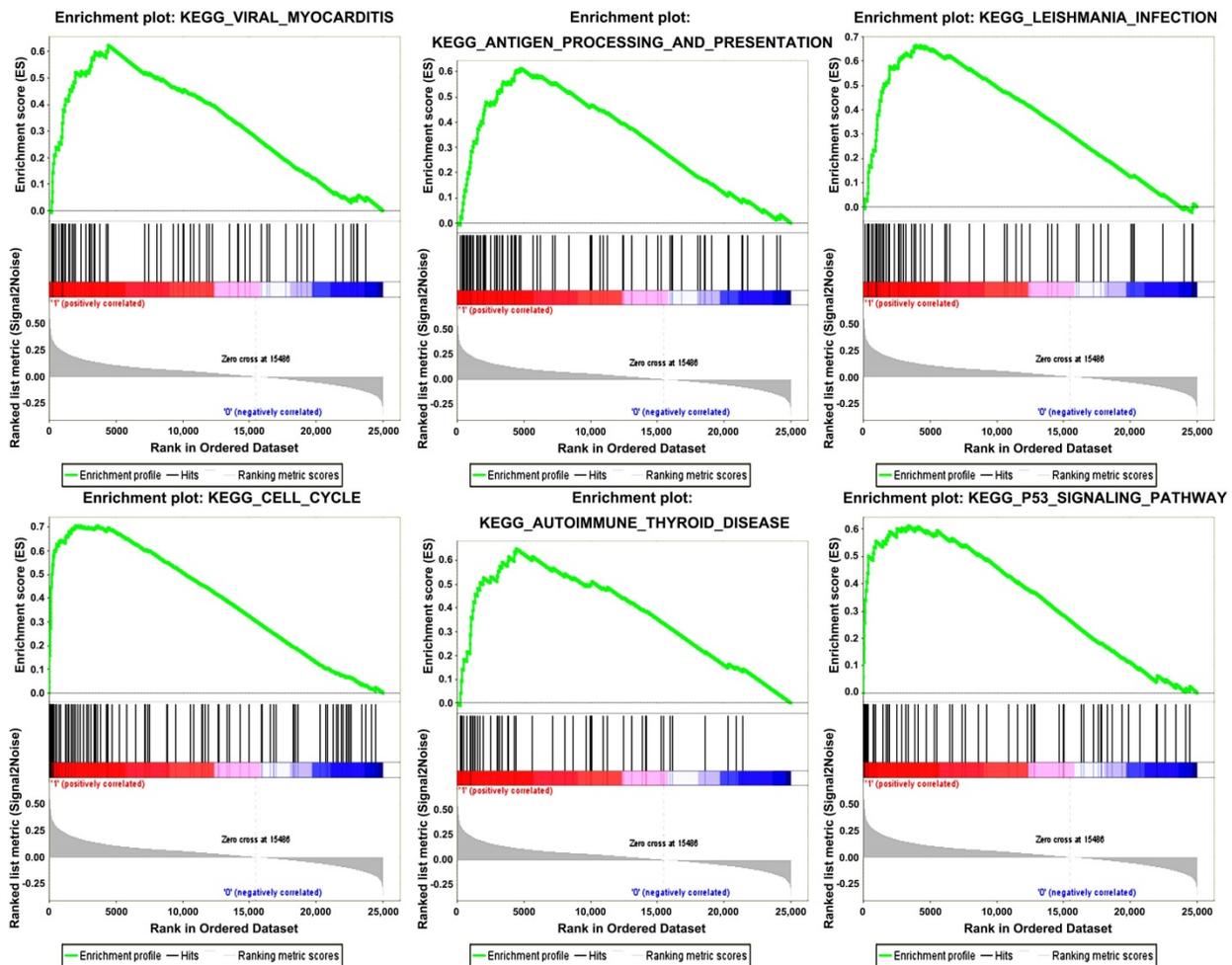
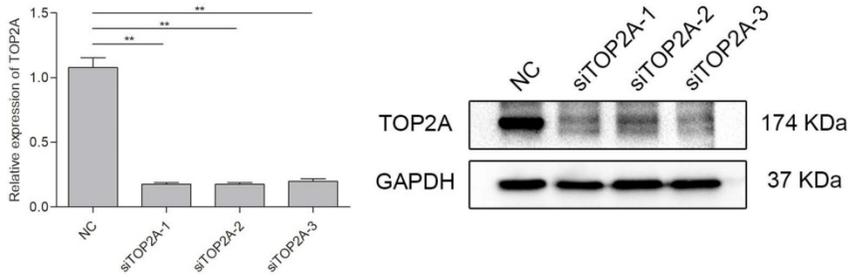
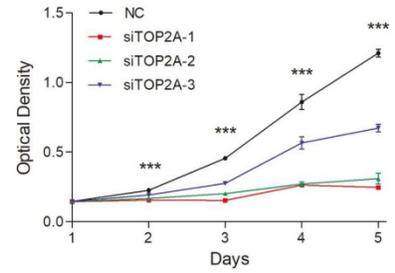


Figure 5. Gene set enrichment analysis (GSEA). “Viral myocarditis”, “Antigen processing and presentation”, “Leishmania infection”, “Cell cycle”, “Autoimmune Thyroid disease”, “P53 signaling pathway” were enriched in ccRCC samples with *TOP2A* highly expressed.

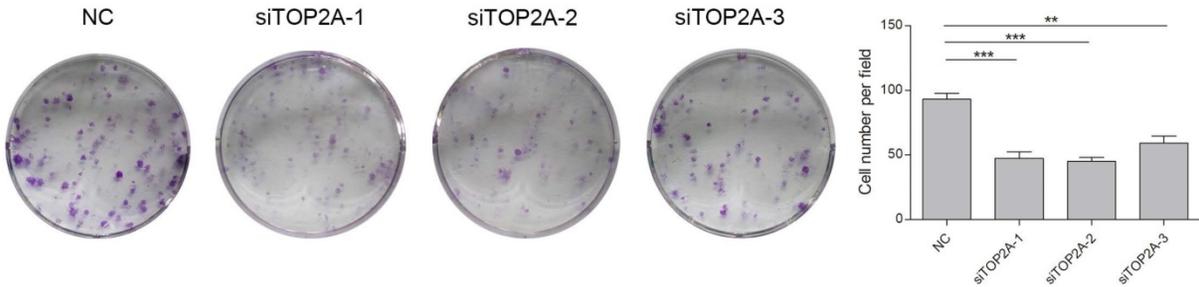
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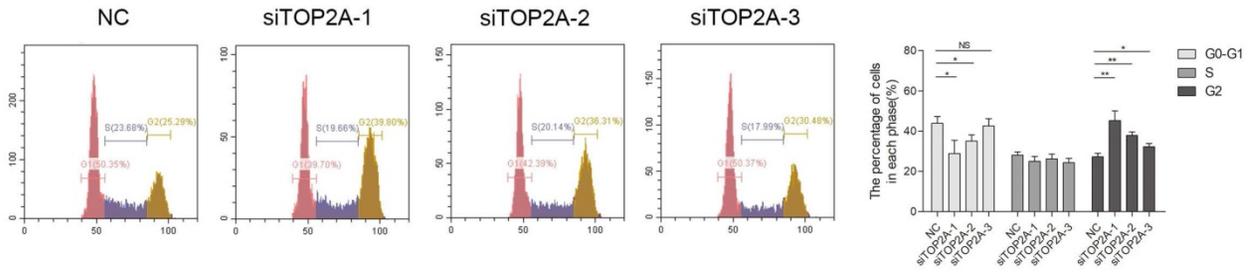
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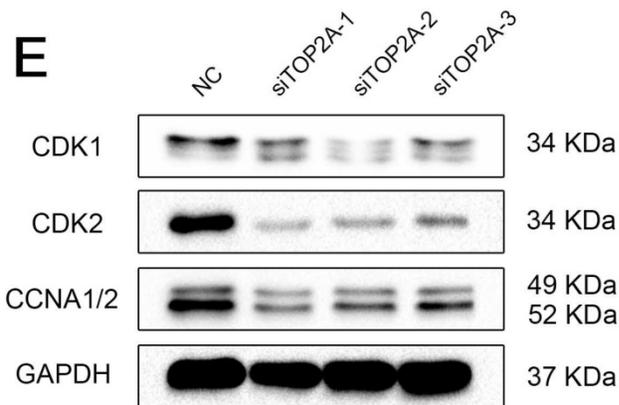
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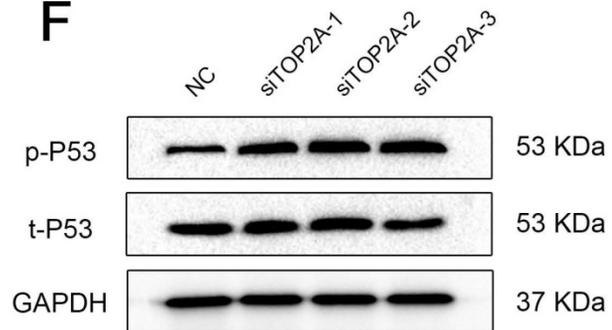


Figure 6. Downregulation of TOP2A inhibited cell proliferation by triggered cell cycle arrest at G2 phase in ccRCC cells. (A) The expression of TOP2A in transcriptional level (upper picture) and translation level (lower picture) by treating with siTOP2A-1, siTOP2A-2 and siTOP2A-3 in 786-O cell line. The proliferative capacity was measured by **(B)** MTT assay and **(C)** colony formation assays in 786-O cell line. **(D)** Flow cytometry analysis of TOP2A deficiency group and NC group in 786-O cell line. **(E)** Cell cycle related proteins such as Cyclin A1/2 and CDK1/2 were significantly decreased in the 786-O cell line in TOP2A knockdown group. **(F)** TOP2A deficiency strongly induced phosphorylated p53 in 786-O cell line.

It is noteworthy that many researchers have concentrated on elucidating the potential molecular mechanism of smoking-related lung cancer [35-37]. However, there is little progress in illustrating the specific underlying molecular alterations triggered by cigarette smoking in the development of ccRCC. Our study unveiled that *TOP2A* was significantly up-regulated in smoking ccRCC samples in GSE46699 and GSE2109 datasets, which was consistent with the positive correlation between *TOP2A* and smoking status in lung adenocarcinoma in GSE10072 dataset. Moreover, the result of qRT-PCR experiment revealed that *TOP2A* was significantly up-regulated in smoking ccRCC than non-smoking ccRCC samples. More importantly, *in vitro* experiments uncovered that *TOP2A* inhibited the proliferative capacity and induced cell cycle arrest at G2 phase via p53 phosphorylation.

To explain how *TOP2A* accelerated the tumorigenesis of ccRCC related to cigarette smoking, we conducted KEGG pathway enrichment analyses and GSEA. Combined with the results of the two methods, we speculated that the potential pathways influenced by *TOP2A* were cell cycle and p53 signaling pathway. It has been proved that cell cycle pathway can regulate the proliferation of colorectal cancer and lung adenocarcinoma [38,39]. Additionally, many researchers have uncovered that p53 signaling pathway is essential to carcinogenesis, proliferation, apoptosis, and progression [40-42]. Interestingly, Mizuno S. *et al.* [43] indicated p53 signaling pathway was important for the pathogenesis of smoking-related emphysematous changes. In addition, synchronous double primary lung cancers have been reported to be induced by heavy smoking via p53 pathway [44]. Taken all of these evidences into account, *TOP2A* is likely to be a potential key gene in the development of smoking-related ccRCC by regulating cell cycle and p53 signaling pathway.

The major limitation of our study is lack of adequate evidence in illustrating the specific pathway of smoking-related ccRCC regulated by *TOP2A*. Further experiments in mRNA and protein levels are needed. A large-scale epidemiological survey of the relationship between smoking and ccRCC may be helpful to verify our results.

In conclusion, we used WGCNA, PPI network, GSEA and qRT-PCR to identify and validate the hub gene of ccRCC associated with cigarette smoking. *TOP2A* was identified to be a potential biomarker of ccRCC associated with cigarette smoking by regulating cell cycle signaling and p53 signaling pathway. In future, *TOP2A* may be used to assist in the diagnosis of ccRCC in smoking patients.

Acknowledgments

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Competing Interests

The authors have declared that no competing interest exists.

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