MEDICAL SCIENCE MONITOR

DATABASE ANALYSIS

e-ISSN 1643-3750
© Med Sci Monit, 2020; 26: e921087
DOI: 10.12659/MSM.921087

Received: 2019.10.31
Accepted: 2019.12.23
Available online: 2020.01.22
Published: 2020.03.09

Overexpression of Karyopherin Subunit alpha 2 (KPNA2) Predicts Unfavorable Prognosis and Promotes Bladder Cancer Tumorigenicity via the P53 Pathway

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEF 1 Changlong Shi
C 2 Li Sun
C 1 Shaozhuang Liu
C 1 Enchong Zhang
DG 1 Yongsheng Song

1 Department of Second Urology, Shengjing Hospital of China Medical University, Shenyang, Liaoning, P.R. China
2 Department of Breast Surgery, Shengjing Hospital of China Medical University, Shenyang, Liaoning, P.R. China

Corresponding Author: Yongsheng Song, e-mail: sys_urology@sina.com
Source of support: Departmental sources

Background: We sought to investigate the expression of KPNA2 in bladder cancer (BC) and its relationship with prognosis, and to analyze the potential mechanism of KPNA2 in promoting BC progression.

Material/Methods: The RNA-seq data on BC from The Cancer Genome Atlas (TCGA) database were imported into R statistical software for differential analysis. The clinical data for patients with BC were screened and analyzed with R software. The survival curve was drawn with the Kaplan-Meier Plotter. The expression of KPNA2 in 4 human BC cell lines and a human bladder epithelial cell line was detected by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting (WB). The proliferation of BC cells was detected with Cell Counting Kit-8 (CCK8), detection of apoptosis, and flow cytometry, and the migration and invasion of BC cells were detected through Transwell assays. WB was used to detect proteins involved in the P53 pathway.

Results: The expression of KPNA2 was higher in BC. The difference in KPNA2 expression was associated with many clinicopathological factors, and high expression of KPNA2 was associated with shorter survival time. After KPNA2 knockout, the proliferation, migration, and invasion ability decreased significantly, the cell cycle was clearly arrested in the G0/G1 phase, and the number of apoptotic cells increased. Moreover, CyclinD1, BCL2, and procaspase3 decreased significantly, whereas P53, P21, BAX, and cleaved-caspase3 increased significantly. The results in the overexpression group were the opposite of results in the knockdown group.

Conclusions: KPNA2 is an oncogenic factor that facilitates BC tumorigenicity through the P53 pathway.

MeSH Keywords: **Apoptosis • Cell Proliferation • Neoplasm Invasiveness • Prognosis • Urinary Bladder Neoplasms**

Abbreviations: **BAX BCL** – 2-associated X; **BC** – bladder cancer; **CCK8** – Cell Counting Kit-8; **DMEM** – Dulbecco's modified Eagle's medium; **E2F1** – E2F transcription factor 1; **E2F7** – E2F transcription factor 7; **FBS** – fetal bovine serum; **GO** – gene ontology; **Imp α1** – importin subunit alpha 1; **KEGG** – Kyoto Encyclopedia of Genes and Genomes; **KPNA2** – karyopherin subunit alpha 2; **NES** – nuclear export signal; **NLS** – nuclear localization signal; **NPC** – nuclear pore complex; **P21** – tumor protein P21; **P53** – tumor protein P53; **PBS** – phosphate-buffered saline; **PVDF** – polyvinylidene difluoride; **qRT-PCR** – quantitative real-time polymerase chain reaction; **SPSS** – Statistical Product and Service Solutions; **TCGA** – The Cancer Genome Atlas; **WB** – Western blotting

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/921087

3240 3 8 46

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

Background

BC ranks 10th among the most common cancers worldwide, with approximately 549 393 new cases and 199 922 deaths in 2018 [1]. In 2015, the incidence of BC in China was 6.69/100 000 and the mortality rate was 3/100 000 [2]. Compared with the slightly falling incidence and mortality rates in most developed countries, those in developing countries are increasing [3]. Beyond occupational exposure factors, smoking is a major risk factor for BC [4]. In recent years, research on the molecular mechanism of BC has identified new tumor markers. However, at present, these non-invasive tumor markers seem to be unsatisfactory; for example, patients with hematuria for any reason can have a significant false-positive detection rate for bladder tumor antigen [5]. Taking blood pressure medication can increase the false-positive detection rate of nuclear matrix protein22 [6]. The sensitivity of cytokeratins in the diagnosis of low-grade BC is much lower than that in the diagnosis of high-grade BC [7]. Therefore, it is very important to clarify the molecular mechanism of BC and develop new non-invasive markers to improve diagnosis and treatment.

The transport of biological macromolecules between the nucleus and cytoplasm requires a key structure – the nuclear pore complex (NPC) – on the nuclear membrane. However, the nuclear pore complex allows only small molecular proteins to pass through; for macromolecular proteins (>40 KDa), only those with a nuclear localization signal (NLS) or nuclear export signal (NES) can be transported through the nuclear membrane via a carrier [8]. The nuclear transporter family includes the input protein family and output protein family. The input protein family includes the karyopherin α family and karyopherin β family [9]. The most classical nuclear protein introduction route involves heterodimers of karyopherin α and karyopherin β family members. Karyopherin α recognizes cargo protein by the NLS. Karyopherin β brings the complex of karyopherin α and nucleoprotein into the nucleus through the NPC [9]. Karyopherin Alpha 2 (KPNA2), also called Importin Subunit Alpha 1(Imp α1), is an important member of the karyopherin family [10]. It is a protein-coding gene located in chromosome 17q24.2, containing 11 336 bases and encoding a protein with a molecular mass of approximately 58 KDa. According to structural analysis, its N-terminus includes an importin β-binding domain, which has a self-inhibitory function and ensures that KPNA2 can be translocated to the nucleus only when the importin β and the cargo molecule are bound [11,12]. The central region is composed of 10 ARM repeat sequences, containing 2 NLS binding sites, which bind the nucleating protein with NLS. Among them, the tenth ARM sequence can bind the transporter CAS, which is responsible for nuclear plasma recycling of KPNA2 [13,14]. KPNA2 plays a role in tumorigenesis by regulating the appropriate subcellular localization of specific cargo proteins [15].

The P53 gene had the highest correlation with human tumors discovered to date. On the basis of 40 years of research, the P53 gene was initially thought to be an oncogene, but through growing research in the past decade, the antitumor effect of p53 has been gradually revealed. P53 is mutated in most tumor cells and in more than 50% of all malignant tumors [16]. The protein encoded by this gene is a transcription factor that controls the start of the cell cycle. Many signals involved in the health of cells are sent to the p53 protein, which determines whether a cell enters a division cycle. P53 is a tumor-suppressor protein that regulates the expression of a wide variety of genes, including apoptosis, growth inhibition, inhibition of cell cycle progression, and differentiation, as well as accelerated DNA repair, genotoxicity, senescence following cellular stress. Like all other tumor suppressors, the p53 gene normally slows or monitors cell division. In addition, p53 is not dependent on its activity, but only acts as a transcription factor to trigger the apoptotic pathway [17–19].

In this study, by mining of TCGA database, we found that, compared with that in paracancerous tissues, the expression of KPNA2 in BC tissue was significantly higher, and the difference in expression was associated with various clinicopathological factors. Moreover, upregulation of KPNA2 is associated with unfavorable prognosis of BC and is an independent risk factor. In addition, silencing of KPNA2 results in cell cycle arrest of BC cells in the G0/G1 phase; decreased proliferation, migration, and invasion ability; and increased apoptosis of BC cells. In contrast, overexpression of KPNA2 has the opposite results. These phenotypic changes are associated with the P53 pathway, suggesting the importance of the role of KPNA2 in development and progression of BC, and KPNA2 may become a target in diagnosis and prognostic evaluation.

Material and Methods

Data mining and analysis

The RNA-seq data from 410 patients with BC and 19 patients with paracancerous tissues were obtained from TCGA database and imported into R (version 3.5.2) statistical software for differential analysis. The clinical data for patients with BC were screened and analyzed in R software. The survival curve was drawn with the Kaplan-Meier Plotter, and the prognostic value of KPNA2 in BC was assessed using the log-rank test. A Cox regression model was applied to analyze the influences of many factors on survival time. Genes with expression similar to KPNA2 expression were screened with the online tool GEPIA and subjected to enrichment analysis according to Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) through R software.

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

Cell culture

Four human BC cell lines (EJ, T24, 5637, and J82) and the human bladder epithelial cell line SV-HUC-1 were obtained from FuHeng Biology, Shanghai, China. EJ and 5637 were cultured in RPMI 1640+10% fetal bovine serum (FBS), T24 was cultured in DMEM+10% FBS, J82 was cultured in minimum essential medium+10% FBS, and SV-HUC-1 was cultured in Ham's F12K+10% FBS. All the above culture materials were obtained from Gibco, and these cell lines were cultured in a 37°C, 5% CO_2 incubator.

Cell transfection

Lentivirus vector packaging KPNA2 shRNA (sh-KPNA2) and negative control shRNA (sh-NC), as well as KPNA2 plasmid (KPNA2) and empty control vector (NC), were from GeneChem Biology, Shanghai, China. Transfection of cells was performed according to the manufacturer's instructions, and strains with stable expression were screened with 3 μg/mL puromycin (GeneChem). qRT-PCR and WB were used to confirm the transfection efficiency.

Cell proliferation assay

The transfected cells were collected and inoculated on 96-well plates at 2000 cells per well. After addition of 10 μL Cell Counting Kit-8 (CCK8) reagent (Shanghai Beyotime Biotechnology Co.) into each well, detection of the absorbance (OD) value at 450 nm wavelength was performed with a microplate reader at different times.

Cell apoptosis assay

The transfected cells were collected and inoculated on 6-well plates at 5×10^5/mL. The cells were grown to 70% and 80% confluence, digested with trypsin (Thermo Fisher Scientific), and washed with phosphate-buffered saline (PBS). After resuspension in 500 μL binding buffer (KeyGen Biotech Jiangsu China), 1 μL Annexin V-FITC or Annexin V-PE (KeyGen Biotech Jiangsu China) and 5 μL propidium iodide or 7AAD (KeyGen Biotech Jiangsu China) were added and reacted for 15 min, then detected by flow cytometry.

Cell cycle analysis

The transfected cells were collected and inoculated at 5×10^5/mL on a 6-well plate. After the cells reached 70% and 80% confluence, they were digested with trypsin, and the collected cell suspension was fixed with 70% ethanol. After addition of RNase A and propidium iodide, the content of DNA was analyzed.

Cell migration and invasion analysis

After the transfected cells were resuspended in serum-free medium, 200 μL was inoculated into the upper chamber of a Transwell plate (Costar, Corning, USA) at a density of 5×10^4/mL, and 500 μl DMEM or RPMI 1640 (including 10%FBS) was added to the lower chamber. The cells were incubated in a 37°C incubator for 20 h, fixed in 4% paraformaldehyde, stained with 0.1% Crystal Violet, and washed with PBS. The membrane was observed under an inverted microscope after drying, and 6 visual fields for each chamber were randomly selected and photographed. The invasion assays were performed in essentially the same manner, except that the upper chamber of the Transwell contained Matrigel (Corning, USA) to prevent the migration of non-invasive cells.

qRT-PCR

Extraction of total RNA from cells was performed with TRIzol. According to the instructions from Takara (Biotechnology Co., Dalian, China), cDNA was synthesized through PrimeScript RTTM Master Mix reverse transcription. The expression of KPNA2mRNA was determined by qPCR with SYBR Premix Ex TaqTM II. The relative expression level of KPNA2/β-actin mRNA was calculated with the $2^{-\Delta\Delta Ct}$ method. The primers were as follows:
KPNA2: (F: 5'-GATGGCTCAGTGTTCCGAGACTTG-3',
R: 5'-CATGATGCAGGAGCCGAACTAAGG-3')
β-actin: (F: 5'-GACGAGGACCAGGTAAGCAATGAC-3',
R: 5'-GACACCATCTGAGGAGAACGCATG-3')

Western blot analysis

The transfected cells were lysed, and total protein was extracted with RIPA buffer (Solarbio, Beijing, China). The concentration of total protein was determined with a BCA kit (Beyotime, Shanghai, China). Samples were resolved with 10%- and 15%-PAGE gel kits (Epi Zyme Biotechnology Co, Shanghai, China) for gel electrophoresis of proteins with different molecular weights. The proteins were transferred to polyvinylidene difluoride (PVDF) membranes, which were blocked with 5% skim milk powder or 5% BSA, then incubated with antibodies (Table 1). The bands were exposed with the enhanced chemiluminescence method.

Statistical analysis

All data represent 3 separate experiments. Statistical analysis was carried out in Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA), and all values are expressed as mean±standard deviation. The continuous variables were analyzed using the *t* test, and the classified variables were analyzed using the χ^2 test. The Cox regression model was used

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

Table 1. Antibody related information in WB.

Protein	Molecular mass	Producer	Cat. No.	Dilution
KPNA2	58 KDa	abcam	ab84440	1: 500
GAPDH	36 KDa	abcam	ab181602	1: 10,000
P53	53 KDa	Wanleibio	WL01919	1: 1,000
P21	21 KDa	Wanleibio	WL0362	1: 1,000
Cyclin D1	35 KDa	Wanleibio	WL01435a	1: 500
BAX	23 KDa	Wanleibio	WL01637	1: 500
BCL 2	26 KDa	Wanleibio	WL01556	1: 500
Pro-caspase 3	35 KDa	abcam	ab32499	1: 1,000
Cleaved-caspase 3	17 KDa	abcam	ab2302	1: 1,000
Goat Anti-Rabbit IgG H&L (HRP)		abcam	ab6721	1: 10,000

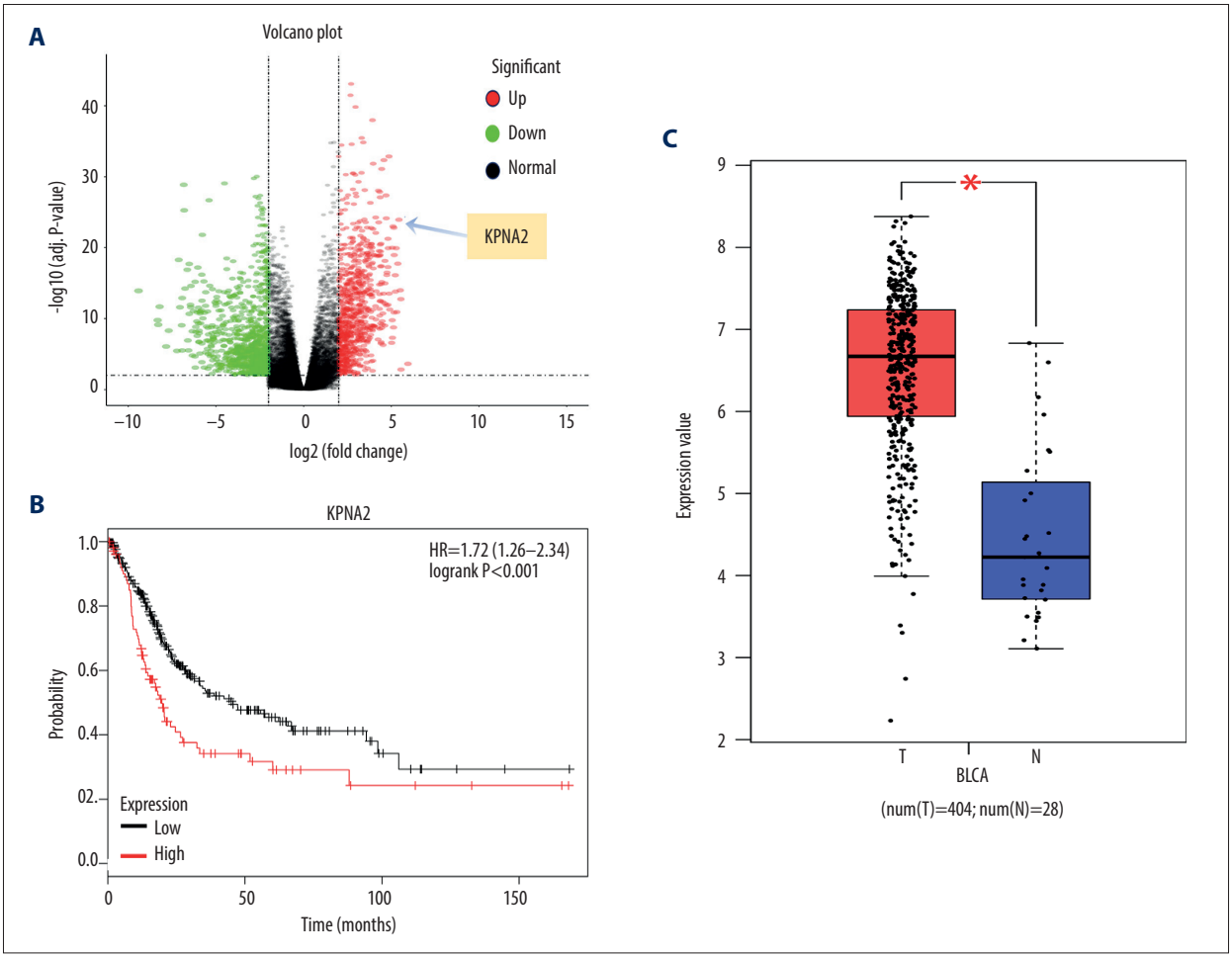

Figure 1. KPNA2 was selected as a significantly differentially expressed gene and was associated with poor prognosis of BC. (**A**) The volcano plot of 410 cases of BC tissues and 19 paracancerous tissues derived from TCGA database, analyzed in R software (position marked with blue arrow is KPNA2). (**B**) The differential expression patterns of 404 cases of BC and 28 adjacent normal tissues were analyzed in the GEPIA online analysis tool. (**C**) The Kaplan-Meier Plotter was used to draw the overall survival curve of BC patients with high or low KPNA2 expression, and the log-rank test was used to evaluate the statistical significance. P<0.05 indicates a significant difference.

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

Table 2. Correlation analysis between KPNA2 expression and clinical-pathological data of all enrolled patients in the present study.

Varibale	Group	KPNA2 exression				χ^2	P-value
		Low, n (%)		High, n (%)		Total		
Age(years)	<65	72	(52.9)	64	(47.1)	136	0.709	0.400
	≥65	130	(48.5)	138	(51.5)	268		
Gender	Female	52	(495.)	53	(50.5)	105	0.013	0.910
	Male	150	(50.2)	149	(49.8)	299		
Smoking history	No	66	(64.7)	36	(35.3)	102	11.804	0.001*
	Yes	136	(45.0)	166	(55.0)	302		
Tumor grade	Low	20	(90.9)	2	(9.1)	22	15.575	0.000*
	High	182	(47.6)	200	(52.4)	382		
Histological subtypes	Non-papillary	122	(45.5)	146	(54.5)	268	6.918	0.009*
	Papillary	78	(59.5)	53	(40.5)	131		
Tumor stage (T)	T1	2	(66.7)	1	(33.3)	3	6.301	0.098
	T2	77	(58.3)	55	(41.7)	132		
	T3	95	(44.8)	117	(55.2)	212		
	T4	28	(49.1)	117	(55.2)	57		
Lymph node stage (N)	N0	123	(52.6)	111	(47.4)	234	1.291	0.524
	N1	20	(43.5)	26	(58.5)	46		
	N2	39	(52.0)	36	(48.0)	75		
pTNM stage	I	3	(75.0)	1	(25.0)	4	1.860	0.602
	II	69	(53.1)	61	(46.9)	130		
	III	66	(48.2)	71	(51.8)	137		
	IV	65	(48.1)	70	(51.9)	135		
Lymph node metastasis	No	81	(46.8)	92	(53.2)	173	0.011	0.915
	Yes	56	(47.5)	62	(52.5)	118		
Vascular invasion	No	57	(44.9)	70	(55.1)	127	1.389	0.239
	Yes	81	(51.9)	75	(48.1)	156		

KPNA2 – karyopherin α2, * P<0 05 with statistical significance.

Table 3. Univariate and multivariate analyses for overall survival (Cox proportional hazards regression model).

Covariant	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
KPNA2 expression	1.949	1.302–2.919	0.001*	1.728	1.149–2.599	0.009*
Age (years)	1.872	1207–2.905	0.005*	1.690	1.087–2.628	0.020*
Gender	0.784	0.524–1.174	0.237	–	–	NS
Smoking history	0.992	0.658–1.496	0.971	–	–	NS
Tumor grade	0.045	0.001–1.698	0.094	–	–	NS
Histological subtypes	1.867	1.180–2.954	0.008*	–	–	NS
Tumor stage	3.257	1.890–5.613	0.000*	2.621	1.506–4.562	0.001*
Lymph node metastasis	2.308	1584–3.363	0.000*	1.740	1.184–2.557	0.005*
Vascular invasion	1.787	1.233–2.590	0.002*	–	–	NS

HR – harzard ratio; CI – confidence interval; NS – not significant; KPNA2 – karyopherin-α2; * P<0.05 with statistical significance.

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

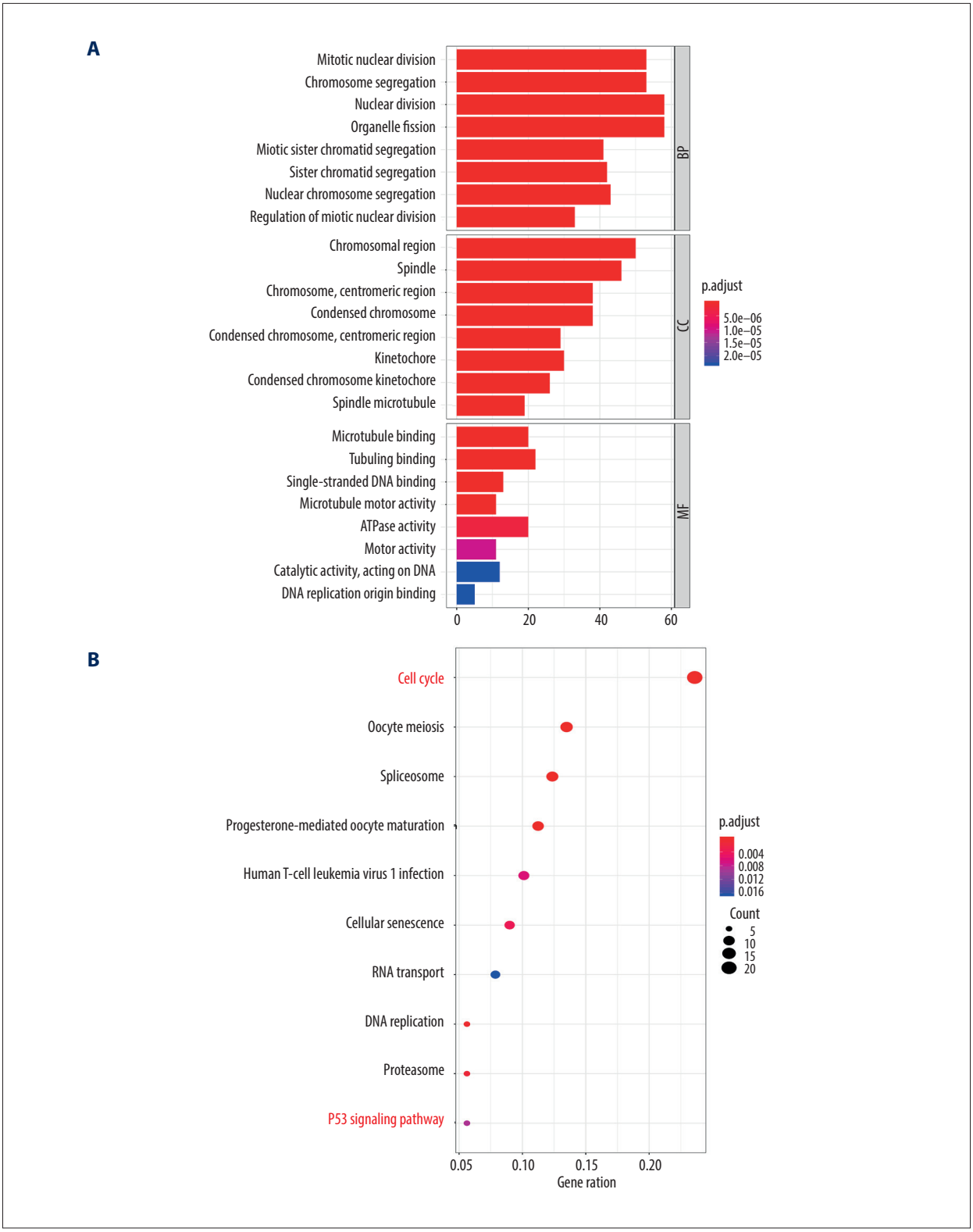

Figure 2. The enrichment analysis of GO and KEGG was performed in R software. (**A**) There was a close relationship between KPNA2 and nuclear division, organelle fission, chromosomal region, and tubulin binding of BC cells. (**B**) KPNA2 is closely related to the BC cell cycle and p53 pathway (marked in red font).

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

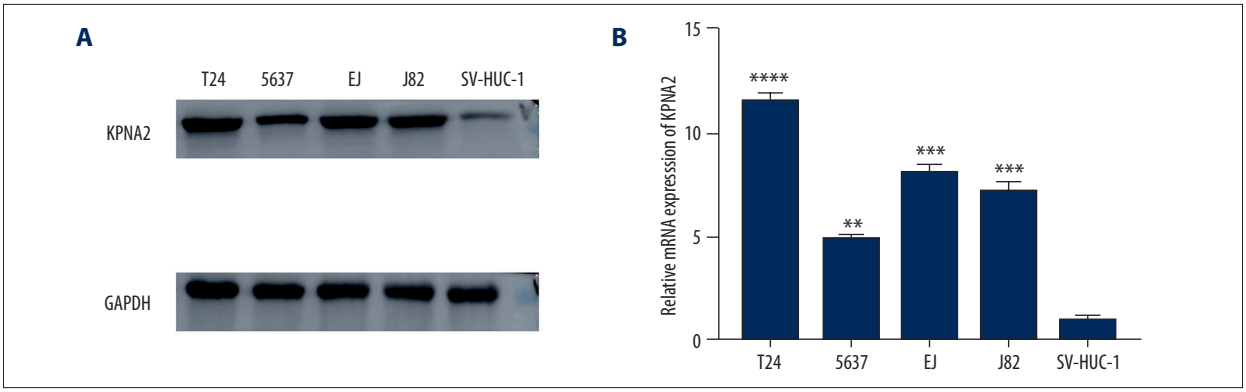

Figure 3. KPNA2 is overexpressed in BC cell lines. (**A**) Western blotting was used to detect the expression of KPNA2 protein in T24, 5637, EJ, J82, and SV-HUC-1 cells. GAPDH was used as a control. (**B**) The expression of KPNA2mRNA in T24, 5637, EJ, J82, and SV-HUC-1 cells was detected by qRT-PCR, and β-actin was used as a control. T24 *vs.* SV-HUC-1, **** P<0.001; EJ and J82 *vs.* SV-HUC-1, *** P<0.005; 5637 *vs.* SV-HUC-1, **P<0.01.

to analyze the influences of many factors on survival time. Kaplan-Meier analysis and the log-rank test were used to draw survival curves and analyze the differences. P<0.05 was considered to indicate a significant difference.

Results

KPNA2 is overexpressed in BC and is associated with poor prognosis

Using R software to analyze the BC data in TCGA, we identified a total of 5395 differentially expressed genes, as shown in the volcano plot (Figure 1A). After further analysis, the KPNA2 with clear differential expression was selected as the target gene for further study. GEPIA analysis indicated that the expression of KPNA2 in BC was higher than in normal tissues (Figure 1B). According to the median expression of KPNA2, the patients were divided into 2 groups – one with expression upregulation (greater than the median) and one with expression downregulation (less than the median). Analysis revealed that the expression level of KPNA2 was significantly associated with smoking history, tumor grade, and histological subtype (Table 2). The survival curve drawn with the Kaplan-Meier Plotter and log-rank tests revealed that upregulated KPNA2 expression was significantly correlated with poor overall survival (P<0.001, Figure 1C). Furthermore, Cox regression model analysis indicated that KPNA2 was an individual risk factor for the prognosis of BC patients (Table 3). A total of 196 similar genes were found by GEPIA analysis of KPNA2. GO and KEGG enrichment analysis of these genes in R software showed that the biological function of KPNA2 was mainly associated with the cell cycle in BC (Figure 2). Bioinformatics analysis showed that the upregulation of KPNA2 was closely related to BC, thus laying a theoretical foundation for follow-up experiments.

Upregulation of KPNA2 expression in BC cell lines

The expression of KPNA2 in human BC cells and SV-HUC-1 cells was verified by qRT-PCR and WB. KPNA2 in the 4 cell lines (EJ, T24, 5637, and J82) was higher than in SV-HUC-1. Therefore, T24 cell lines with relatively high expression of KPNA2 were selected for knockdown, and 5637 cell lines with relatively low expression were selected for overexpression for subsequent studies (Figure 3).

KPNA2 promotes the proliferation of BC cells

Lentivirus and plasmid were transfected into BC cells to produce negative control (sh-NC, NC) cells, KPNA2 knockout (sh-KPNA2) cells, and KPNA2 overexpression (KPNA2) cells. Differences in gene and protein expression between the sh-NC group and sh-KPNA2 group were assessed by qRT-PCR and WB. The expression levels of protein and mRNA in the sh-KPNA2 group was significantly lower than in the sh-NC group (Figure 4A, 4B), whereas the expression of protein and mRNA levels in the KPNA2 overexpression group was significantly higher than in the NC group (Figure 4C, 4D). CCK8 tests showed that the proliferation ability of BC cells in the sh-KPNA2 group was significantly lower than in the sh-NC group, particularly at 72 h (Figure 5A). Compared with the NC group, the proliferation ability of the KPNA2 overexpression group was significantly higher, particularly at 72 h (Figure 5B). The results showed that KPNA2 promoted the proliferation of BC cells. A critical control point for cell proliferation is in the early stage of G1; we therefore next examined whether the expression of KPNA2 affected the cell cycle in BC cells.

KPNA2 inhibits apoptosis and participates in cell cycle regulation in BC cells

Flow cytometry detection and statistical analysis indicated that, relative to the sh-NC group, the sh-KPNA2 group showed greater

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

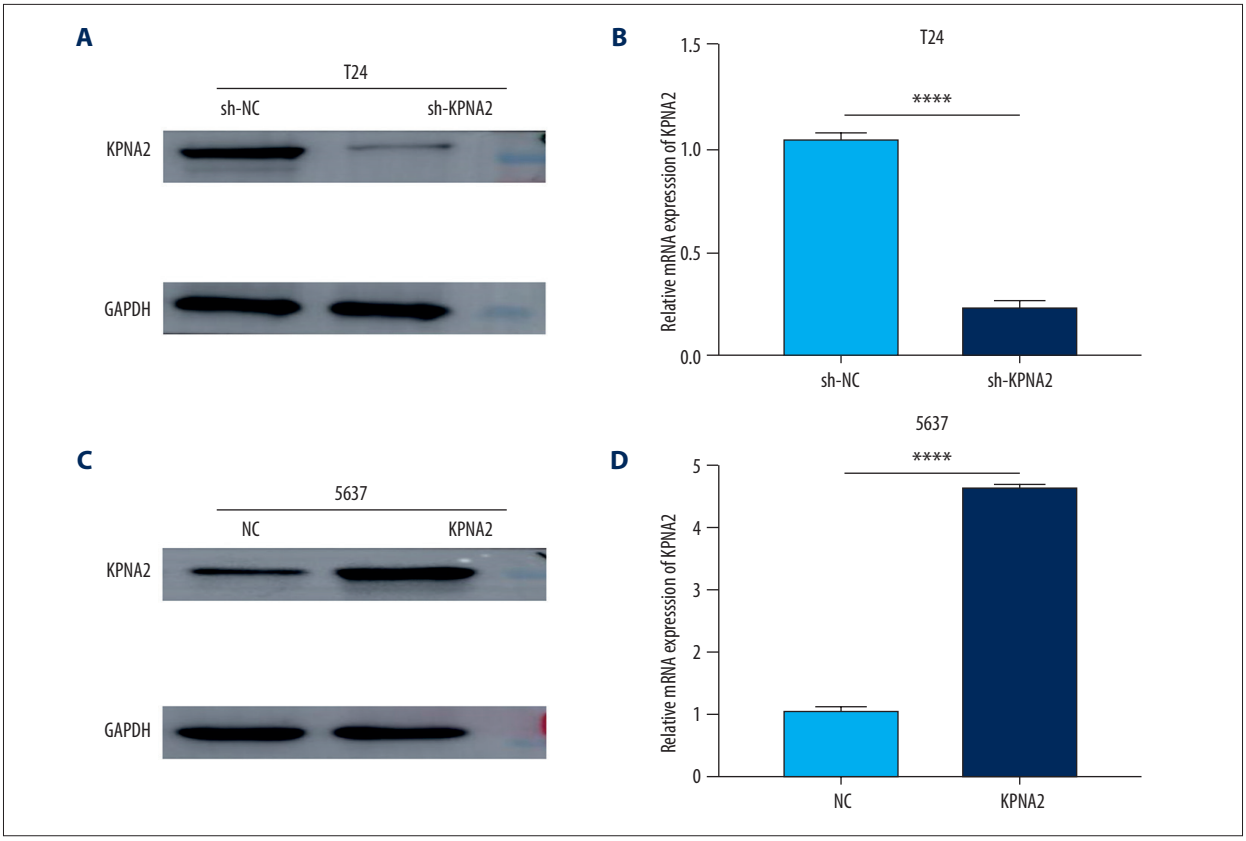

Figure 4. Verification of the transfection efficiency of KPNA2 in BC cells. (**A, B**) WB and qRT-PCR showed that the protein and mRNA expression in the sh-KPNA2 group was significantly lower than that in the sh-NC group. (**C, D**) WB and qRT-PCR showed that the expression of protein and mRNA in the KPNA2 group was significantly higher than in the NC group. **** P<0.001.

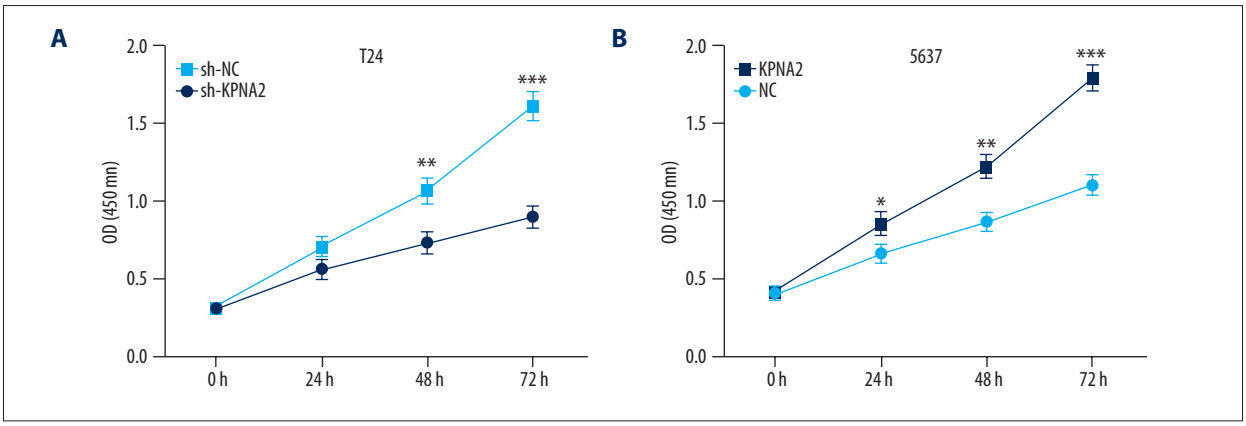

Figure 5. The proliferation of BC cells was detected by CCK8. (**A, B**) The proliferation ability of the sh-KPNA2 group was significantly lower than that of the sh-NC group, whereas the proliferation ability of the KPNA2 group was significantly higher than that of the NC group, especially at 72 h. * P<0.05, ** P<0.01, *** P<0.005.

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

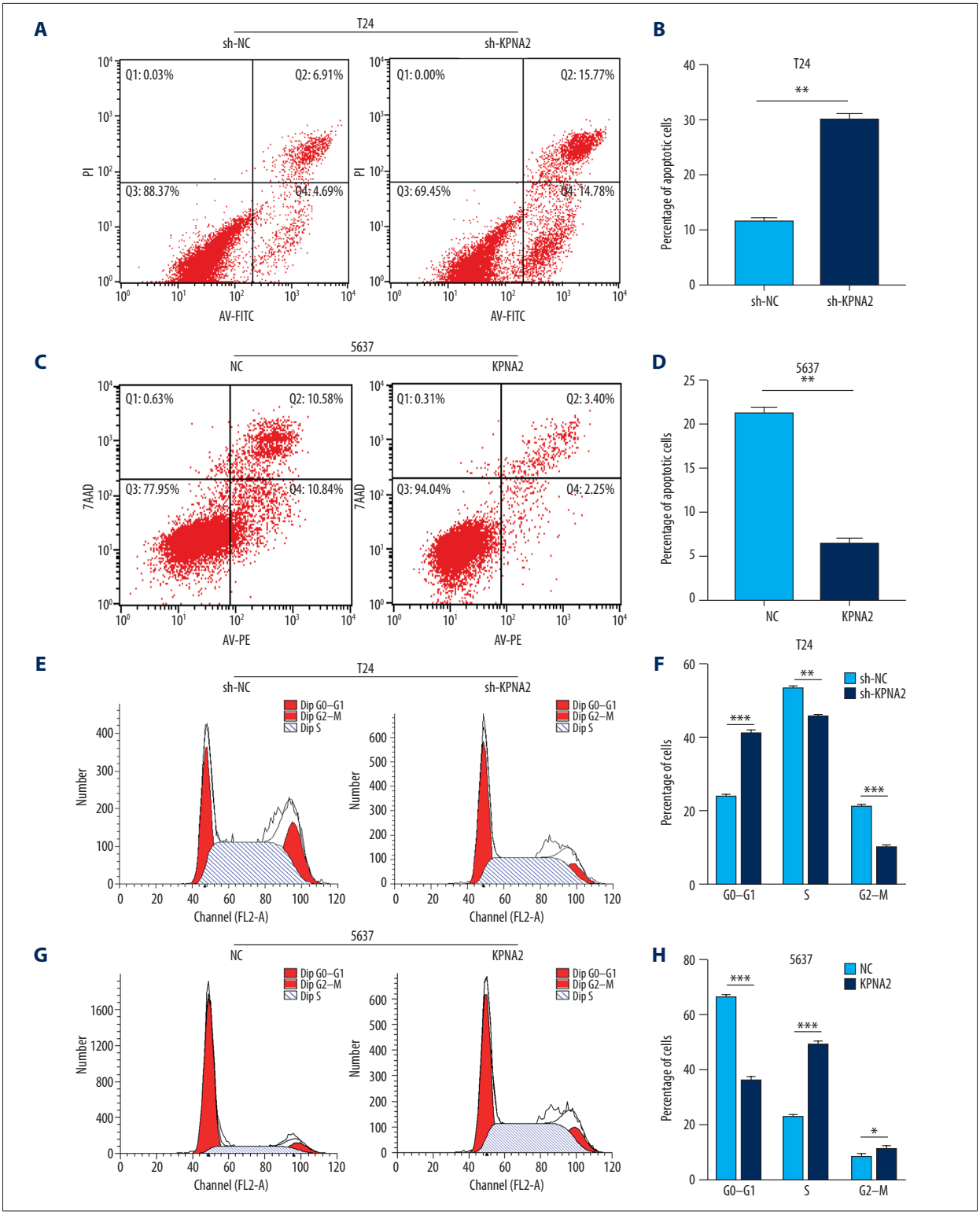

Figure 6. The apoptosis and cell cycle of BC cells were detected by flow cytometry. (**A, B, E, F**) Compared with the sh-NC group, the sh-KPNA2 group showed significantly higher apoptosis of BC cells, and BC cells were arrested in G0/G1 phase; the number of BC cells in S phase and G2/M phase was significantly decreased. (**C, D, G, H**) Compared with the NC group, the KPNA2 overexpression group showed inhibited BC cell apoptosis, significantly fewer cells in G0/G1 phase, and more cells in S and G2/M phase. * P<0.05, ** P<0.01, *** P<0.005.

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

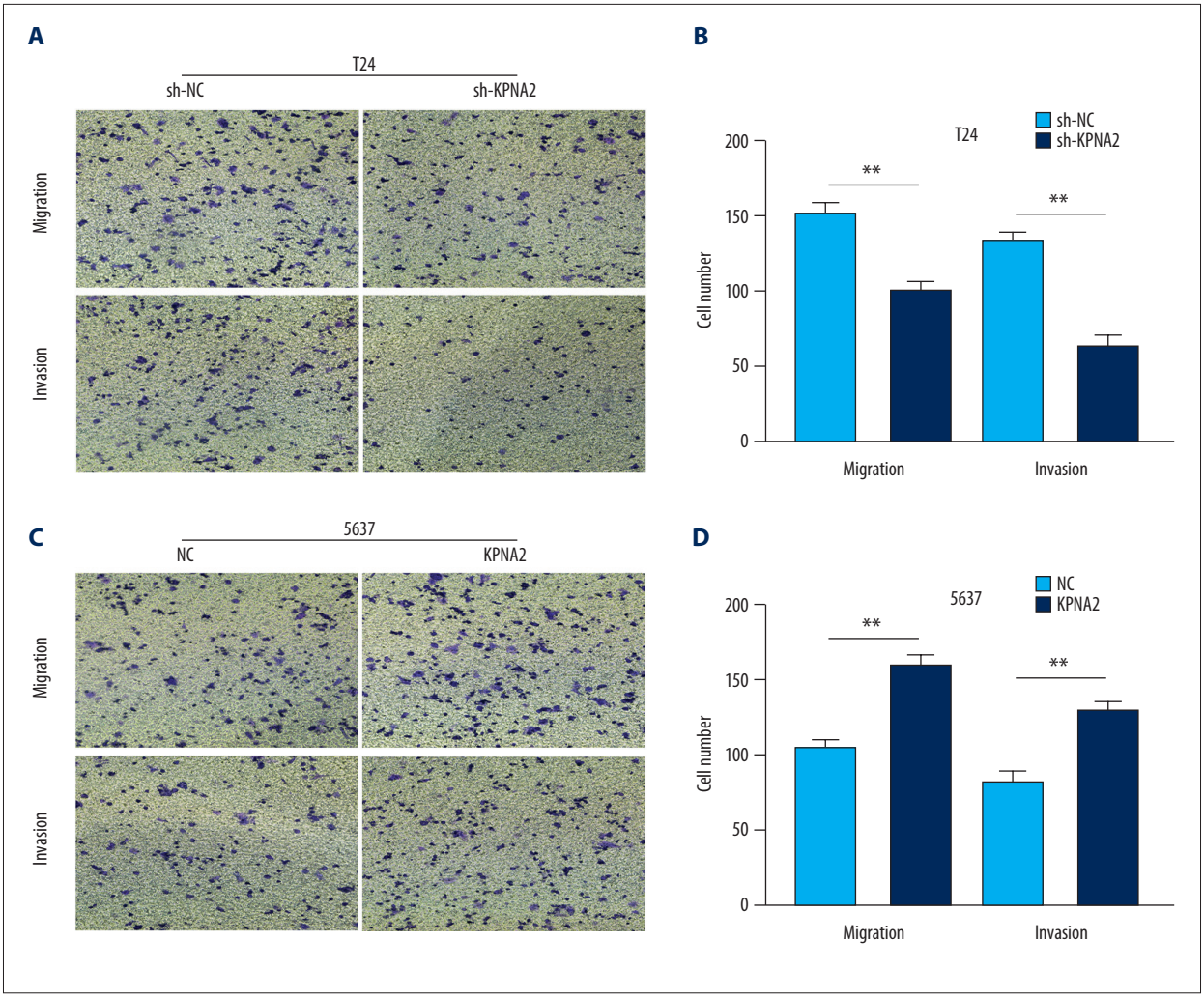

Figure 7. Transwell assays detecting migration and invasion of BC cells. (**A, B**) Compared with the sh-NC group, the sh-KPNA2 group showed significantly less migration and invasion. (**C, D**) Compared with the NC group, the KPNA2 overexpression group showed significantly more migration and invasion. ** P<0.01.

apoptosis of BC cells (Figure 6A, 6B) and cell cycle arrest in the G0/G1 phase, whereas the numbers of cells in the S and G2/M phases were significantly lower (Figure 6E, 6F). The KPNA2 overexpression group, compared with the NC group, showed inhibition of BC cell apoptosis (Figure 6C, 6D); there were significantly fewer cells in the G0/G1 phase, and significantly more cells in the S and G2/M phases (Figure 6G, 6H).

KPNA2 facilitates the migration and invasion of BC cells

The Transwell migration assay results showed that fewer cells migrated and invaded in the sh-KPNA2 group than in the sh-NC group (Figure 7A,7B). In contrast, the number of cells that migrated and invaded was significantly higher in the KPNA2 group than in the NC group (Figure 7C, 7D).

KPNA2 promotes the progression of BC by regulating the P53 pathway

As shown in the KEGG enrichment analysis in Figure 2, the process of KPNA2 in promoting BC progression was strongly correlated with the P53 pathway. We therefore used WB to detect the expression of P53 pathway-related proteins (P53, P21, CyclinD1, BAX, BCL 2, pro-caspase3, and cleaved-caspase3) and to elucidate the molecular mechanism through which KPNA2 promotes the progression of BC. We found that expression of CyclinD1, BCL 2, and pro-caspase3 was significantly lower in the KPNA2 knockdown group than in the NC group. In contrast, P53, P21, Bax, and cleaved-caspase3 showed markedly higher expression (Figure 8A, 8C). In the KPNA2 overexpression group, the WB results were the opposite of those in the KPNA2 knockdown group (Figure 8B, 8D). P53/P21/CyclinD and P53/BAX/caspase3 are widely recognized as classical pathways

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

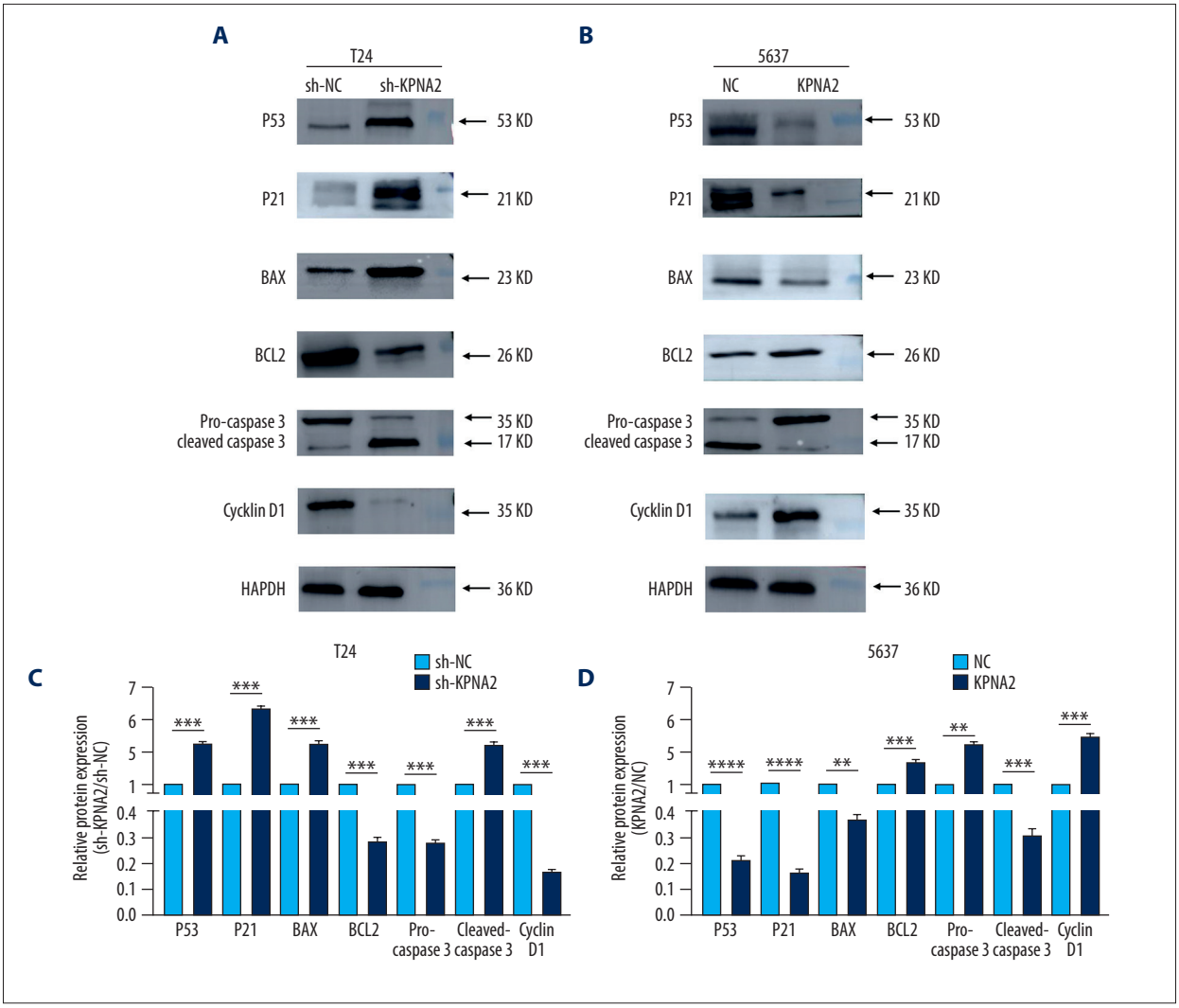

Figure 8. KPNA2 affects the P53 pathway in BC cells. (**A, B**) WB shows the expression of P53 pathway-related proteins in the sh-NC, sh-KPNA2, NC, and KPNA2 groups. (**C, D**) ImageJ software was used to conduct quantitative analysis of the bands in WB results and to normalize the expression as sh-KPNA2/sh-NC and KPNA2/NC.

that regulate the cell cycle and apoptosis. Knockout of KPNA2 significantly altered the expression levels of cycle-related and apoptosis-related genes. Extending the results of flow cytometry, we found that KPNA2 promotes the progression of BC through the P53 pathway.

Discussion

KPNA2 was first described in BC in 2004 [20]. However, in recent years, few studies have examined the intersection of KPNA2 and BC. In the present study, data mining and bioinformatics analysis indicated that KPNA2 was upregulated in BC tissues, and the experiments verified that KPNA2 was upregulated in BC cell lines. These findings are consistent with the overexpression of KPNA2 in various malignant tumors, as reported in recent years, including studies on hepatocellular carcinoma [21], gallbladder cancer [22], glioblastoma [23], ovarian cancer [24], and BC [25]. Beyond KPNA2 overexpression in tumors, most studies have also found that the upregulation of KPNA2 is associated with poorer prognosis, including in gallbladder cancer [26], gastric cancer [27], breast cancer [28], BC [25,29], ovarian cancer [30], colorectal cancer [31], and renal cancer [32]. More importantly, many studies agree that KPNA2 is an individual risk factor for malignant tumors. Although we did not use clinical tissue samples for analysis, the mining of big data suggested that upregulation of KPNA2 is indeed associated with poor prognosis of BC and is an individual prognostic risk factor. In conclusion, our results indicated that measurement of KPNA2 may be a novel diagnostic and prognostic biomarker for many malignant tumors.

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

Protein shuttling between the cytoplasm and nucleus is mediated by nuclear transporters, and dysfunction of cell transport mechanisms is a common cause of cancer occurrence and development [33]. As a key protein in molecular transport, KPNA2 is involved in many biological processes in tumor progression, including proliferation, cell cycle regulation, apoptosis, migration, and invasion [24,25,34–38]. The present study indicated that silencing of KPNA2 significantly inhibited the proliferation of BC cells, an effect that increased with time and peaked at 72 h, in agreement with the results of Zhou et al. [25]. Moreover, knocking down KPNA2 also arrested BC cells in G0/G1 phase and decreased their ability to migrate and invade. Kuusisto et al. [39] found that in several transformed *vs.* non-transformed isogenic cell pairs, several transporters (including KPNA2) are overexpressed, and the nuclear import of several NLS-bearing cargoes is enhanced. In other words, in cancer cells, the overexpression of KPNA2 can affect nuclear and cytoplasmic transport. Therefore, the same processes may occur in BC cells and may have important physiological consequences. Investigating this possibility will be a focus of our future research.

Many studies have shown that KPNA2 participates in tumor progression by interacting with tumor-associated proteins; for example, KPNA2 modulates c-Myc, thereby promoting the progression of glioblastoma [23] and ovarian cancer [37]. KPNA2 regulates the AKT pathway and consequently promotes migration and invasion of ovarian cancer [24]. The interaction between KPNA2 and mTOR facilitates the development of non-small cell lung cancer [40]. At the transcriptional level, E2F Transcription Factor 1 (E2F1) and E2F Transcription Factor7 (E2F7) promote the progression of gallbladder cancer by regulating KPNA2 [22]. In contrast, silencing of KPNA2 can restrain the development of hepatocellular carcinoma cells [4], the metastasis of breast cancer [42], and the proliferation of lung cancer [43] and prostate cancer [44]. The protein with the most cross-talk with KPNA2 is P53; for example, the synergistic effect between Wip1 and KPNA2 controls the proliferation and migration of colorectal cancer cells via P53 [34]. Apoptosis of Cal-27 cells is induced by KPNA2 through P53 in tongue squamous cell carcinoma [45]. Interaction between KPNA2 and P53 decreases autophagy of oral squamous cell carcinoma cells [46]. In the present study, WB detection revealed that the deletion of KPNA2 upregulated P53, P21, BAX, and cleaved-caspase3, and downregulated pro-caspase, BCL2, and CyclinD1, whereas the opposite results were found after overexpression of KPNA2. The direct action of the P53/P21/CyclinD1 pathway is arrest of the cell cycle in the G0/G1 phase. Coincidentally, our results also confirmed that knockout of KPNA2 arrests BC cells in G0/G1 phase, thereby reconfirming that the potential mechanism through which KPNA2 affects BC cells is related to P53.

Conclusions

This study showed that KPNA2 was upregulated in BC and was associated with poor prognosis in patients with BC. Knocking down KPNA2 in BC cells decreased the proliferation, migration, and invasion ability; arrested cells in G0/G1 phase; and caused apoptosis. Overexpression of KPNA2 promoted the proliferation, migration, and invasion of BC cells. These mechanisms are associated with the P53 pathway. However, our study cannot rule out the possibility that other factors beyond KPNA2 are be involved upstream of P53, and other indirect action pathways cannot be excluded. Finally, our study contributes to the understanding of the mechanism underlying the relationship between KPNA2 and bladder cells, and may provide a novel target for the diagnosis and prognosis of patients with BC.

Availability of data and materials

All the data in the results of this study can be obtained on reasonable request from the corresponding authors.

Acknowledgments

We salute all the teachers and colleagues for their valuable advice.

Conflict of interest

None.

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]

References:

1. Bray F, Ferlay J, Soerjomataram I et al: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. Cancer J Clin, 2018; 68(6): 394–424
2. Chen W, Zheng R, Baade PD et al: Cancer statistics in China, 2015. Cancer J Clin, 2016; 66(2): 115–32
3. Antoni S, Ferlay J, Soerjomataram I et al: Bladder cancer incidence and mortality: A global overview and recent trends. Eur Urol, 2017; 71(1): 96–108
4. Silverman DT, Koutros S, Figueroa JD et al. (eds.), Cancer epidemiology and prevention. 4th ed. New York, Oxford University Press, 2018; 977–96
5. Miyake M, Goodison S, Rizwani W et al: Urinary BTA: Indicator of bladder cancer or of hematuria. World J Urol, 2012; 30(6): 869–73
6. Behrens T, Stenzl A, Bruning T: Factors influencing false-positive results for nuclear matrix protein 22. Eur Urol, 2014; 66(5): 970–72
7. Ecke TH, Arndt C, Stephan C et al: Preliminary results of a multicentre study of the UBC rapid test for detection of urinary bladder cancer. Anticancer Res, 2015; 35(5): 2651–55
8. Timney BL, Raveh B, Mironska R et al: Simple rules for passive diffusion through the nuclear pore complex. J Cell Biol, 2016; 215(1): 57–76
9. Goldfarb DS, Corbett AH, Mason DA et al: Importin alpha: A multipurpose nuclear-transport receptor. Trends Cell Biol, 2004; 14(9): 505–14
10. Kelley JB, Talley AM, Spencer A et al: Karyopherin alpha7 (KPNA7), a divergent member of the importin alpha family of nuclear import receptors. BMC Cell Biol, 2010; 11: 63
11. Chook YM, Blobel G: Karyopherins and nuclear import. Curr Opin Struct Biol, 2001; 11(6): 703–15
12. Harreman MT, Cohen PE, Hodel MR et al: Characterization of the auto-inhibitory sequence within the N-terminal domain of importin alpha. J Biol Chem, 2003; 278(24): 21361–69
13. Kobe B: Autoinhibition by an internal nuclear localization signal revealed by the crystal structure of mammalian importin alpha. Nat Struct Biol, 1999; 6(4): 388–97
14. Conti E, Uy M, Leighton L et al: Crystallographic analysis of the recognition of a nuclear localization signal by the nuclear import factor karyopherin alpha. Cell, 1998; 94(2): 193–204
15. Teng SC, Wu KJ, Tseng SF et al: Importin KPNA2, NBS1, DNA repair and tumorigenesis. J Mol Histol, 2006; 37(5–7): 293–99
16. Green DR, Kroemer G: Cytoplasmic functions of the tumour suppressor p53. Nature, 2009; 458(7242): 1127–30
17. El-Deiry WS, Kern SE, Pietenpol JA et al: Definition of a consensus binding site for p53. Nat Genet, 1992; 1(1): 45–49
18. Laptenko O, Prives C: Transcriptional regulation by p53: One protein, many possibilities. Cell Death Differ, 2006; 13(6): 951–61
19. Raycroft L, Wu HY, Lozano G: Transcriptional activation by wild-type but not transforming mutants of the p53 anti-oncogene. Science, 1990; 249(4972): 1049–51
20. Dyrskjot L, Kruhoffer M, Thykjaer T et al: Gene expression in the urinary bladder: a common carcinoma *in situ* gene expression signature exists disregarding histopathological classification. Cancer Res, 2004; 64(11): 4040–48
21. Guo X, Wang Z, Zhang J et al: Upregulated KPNA2 promotes hepatocellular carcinoma progression and indicates prognostic significance across human cancer types. Acta Biochim Biophys Sin (Shanghai), 2019; 51(3): 285–92
22. Xiang S, Wang Z, Ye Y et al: E2F1 and E2F7 differentially regulate KPNA2 to promote the development of gallbladder cancer. Oncogene, 2019; 38(8): 1269–81
23. Li J, Liu Q, Liu Z et al: KPNA2 promotes metabolic reprogramming in glioblastomas by regulation of c-Myc. J Exp Clin Cancer Res, 2018; 37(1): 194
24. Huang L, Zhou Y, Cao XP et al: KPNA2 promotes migration and invasion in epithelial ovarian cancer cells by inducing epithelial-mesenchymal transition via Akt/GSK-3beta/Snail activation. J Cancer, 2018; 9(1): 157–65
25. Zhou J, Dong D, Cheng R et al: Aberrant expression of KPNA2 is associated with a poor prognosis and contributes to OCT4 nuclear transportation in bladder cancer. Oncotarget, 2016; 7(45): 72767–76
26. Tsukagoshi M, Araki K, Yokobori T et al: Overexpression of karyopherin-alpha2 in cholangiocarcinoma correlates with poor prognosis and gemcitabine sensitivity via nuclear translocation of DNA repair proteins. Oncotarget, 2017; 8(26): 42159–72
27. Altan B, Yokobori T, Mochiki E et al: Nuclear karyopherin-alpha2 expression in primary lesions and metastatic lymph nodes was associated with poor prognosis and progression in gastric cancer. Carcinogenesis, 2013; 34(10): 2314–21
28. Noetzel E, Rose M, Bornemann J et al: Nuclear transport receptor karyopherin-alpha2 promotes malignant breast cancer phenotypes *in vitro*. Oncogene, 2012; 31(16): 2101–14
29. Jensen JB, Munksgaard PP, Sorensen CM et al: High expression of karyopherin-alpha2 defines poor prognosis in non-muscle-invasive bladder cancer and in patients with invasive bladder cancer undergoing radical cystectomy. Eur Urol, 2011;59(5): 841-8.
30. Huang L, Zhou Y, Cao X P, et al. KPNA2 is a potential diagnostic serum biomarker for epithelial ovarian cancer and correlates with poor prognosis. Tumour Biol, 2017; 39(6): 1010428317706289
31. Takada T, Tsutsumi S, Takahashi R et al: KPNA2 over-expression is a potential marker of prognosis and therapeutic sensitivity in colorectal cancer patients. J Surg Oncol, 2016; 113(2): 213–17
32. Muller T, Tolkach Y, Stahl D et al: Karyopherin alpha 2 is an adverse prognostic factor in clear-cell and papillary renal-cell carcinoma. Clin Genitourin Cancer, 2019; 17(1): e167–75
33. Kau TR, Way JC, Silver PA: Nuclear transport and cancer: From mechanism to intervention. Nat Rev Cancer, 2004; 4(2): 106–17
34. Wang P, Zhao Y, Liu K et al: Wip1 cooperates with KPNA2 to modulate the cell proliferation and migration of colorectal cancer via a p53-dependent manner. J Cell Biochem, 2019; 120(9): 15709–18
35. Yamada K, Miyamoto Y, Tsujii A et al: Cell surface localization of importin alpha1/KPNA2 affects cancer cell proliferation by regulating FGF1 signalling. Sci Rep, 2016, 6: 21410
36. Lin J, Zhang L, Huang H et al: MiR-26b/KPNA2 axis inhibits epithelial ovarian carcinoma proliferation and metastasis through downregulating OCT4. Oncotarget, 2015; 6(27): 23793–806
37. Huang L, Wang HY, Li JD et al: KPNA2 promotes cell proliferation and tumorigenicity in epithelial ovarian carcinoma through upregulation of c-Myc and downregulation of FOXO3a. Cell Death Dis, 2013; 4: e745
38. Zan Y, Wang B, Liang L et al: MicroRNA-139 inhibits hepatocellular carcinoma cell growth through downregulating karyopherin alpha 2. J Exp Clin Cancer Res, 2019; 38(1): 182
39. Kuusisto HV, Wagstaff KM, Alvisi G et al: Global enhancement of nuclear localization-dependent nuclear transport in transformed cells. FASEB J, 2012; 26(3): 1181–93
40. Wang CI, Chen YY, Wang CL et al: mTOR regulates proteasomal degradation and Dp1/E2F1- mediated transcription of KPNA2 in lung cancer cells. Oncotarget, 2016; 7(18): 25432–42
41. Yang Y, Guo J, Hao Y et al: Silencing of karyopherin alpha2 inhibits cell growth and survival in human hepatocellular carcinoma. Oncotarget, 2017; 8(22): 36289–304
42. Ma A, Tang M, Zhang L et al: USP1 inhibition destabilizes KPNA2 and suppresses breast cancer metastasis. Oncogene, 2019; 38(13): 2405–19
43. Wang CI, Wang CL, Wang CW et al: Importin subunit alpha-2 is identified as a potential biomarker for non-small cell lung cancer by integration of the cancer cell secretome and tissue transcriptome. Int J Cancer, 2011; 128(10): 2364–72
44. Mortezavi A, Hermanns T, Seifert HH et al: KPNA2 expression is an independent adverse predictor of biochemical recurrence after radical prostatectomy. Clin Cancer Res, 2011; 17(5): 1111–21
45. Gao L, Yu L, Li CM et al: Karyopherin alpha2 induces apoptosis in tongue squamous cell carcinoma CAL-27 cells through the p53 pathway. Oncol Rep, 2016; 35(6): 3357–62
46. Lin F, Gao L, Su Z et al: Knockdown of KPNA2 inhibits autophagy in oral squamous cell carcinoma cell lines by blocking p53 nuclear translocation. Oncol Rep, 2018; 40(1): 179–94

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]