The NRP1 gene regulates proliferation, apoptosis, migration, and invasion in T24 and 5637 bladder cancer cells

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The NRP1 gene regulates proliferation, apoptosis, migration, and invasion in T24 and 5637 bladder cancer cells

Short title: Regulatory effects of NRP1 on human bladder cancer cells

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Abstract

Background Bladder urothelial carcinoma (BC) is a fatal invasive malignancy and the most common malignancy of the urinary system. In the current study, we investigate the function and mechanisms of Neuropilin-1 (NRP1), the co-receptor for vascular endothelial growth factor, in BC pathogenesis and progression.

Methods The expression of NRP1 was assessed in several BC cell lines. Additionally, the biological function of NRP1 in proliferation, apoptosis, angiogenesis, migration, and invasion of BC were validated in vitro by silencing NRP1. Moreover, gene expression profiling chip analysis was conducted, and the related signalling pathways were confirmed by Western blot to reveal the potential molecular mechanisms by which NRP1 promotes the malignant progression of BC.

Results Overexpression of NRP1 was observed in several human BC cell lines. NRP1 knockdown inhibited cell proliferation, promoted apoptosis, and decreased angiogenesis, migration, and invasion in T24 and 5637 human BC cells. Microarray analysis results indicated that the expression of NRP1 was correlated with the levels of cyclin dependent kinase (CDK) 4, baculoviral IAP repeat containing 3, Cyclin E 2, CDK2, and AP-1 transcription factor subunit in BC. We also demonstrated that the biological function of NRP1was associated with activation of the mitogen-activated protein kinase (MAPK) signalling pathway.

Conclusions Our findings provide evidence that NRP1, as a potential tumour promoter, contributes to the metastasis and invasion of BC, which is associated with the activation of the MAPK pathway. Targeting NRP1 has the potential to become a new therapeutic strategy to benefit more patients with BC or other cancers.

Keywords NRP1, bladder cancer, proliferation, apoptosis, migration, invasion, MAPK pathway

Background

Bladder urothelial carcinoma (BC), one of the most frequent urologic malignancies worldwide, is refractory to many common treatments[1], and its incidence and mortality rate are the highest among genitourinary tumours in China[2]. BC generally has a low cure rate and a high relapse rate. Although most cases are initially diagnosed as non-muscle-invasive by pathological examination, discontinuing or delaying treatment due to the lack of regular re-examination eventually leads to muscle-invasive BC with a great risk of distant metastasis[3]. The 5-year survival rate of metastatic BC is about 5%, largely because of the lack of available therapies[4]. In recent years, multiple therapeutic approaches for BC have been explored, but there has been no obvious improvement in the overall survival rate. Therefore, novel targets and effective strategies for BC therapy need to be urgently explored.

Neuropilins (NRPs) are transmembrane glycoprotein receptors with a well-described role in interacting with the semaphorins and vascular endothelial growth factor (VEGF) family members[5]. NRP1 encodes one of two NRPs. It plays an active role in axon guidance and angiogenesis. NRP1 mutations have been reported to result in fatal abnormalities in the cardiovascular system[6]. Further, many studies have observed the abnormal high expression of NRP1 in multiple tumour types, including neuroblastomas and bile duct, gastric, pancreas, lung, prostate, breast, and colon cancers[5, 7]. Additionally, there is evidence that in patients with BC,

overexpression of NRP1 is a sign of poor prognosis[7]. However, the molecular mechanisms underlying how NRP1 regulates the progression of BC remain unclear. Therefore, in the current study, we aimed to investigate the function and mechanisms of NRP1 in BC pathogenesis and progression.

Methods

Cell lines

The human BC cell lines T24, 5637, SCaBER, J82, UM-UC-3, and SW780 were purchased from the Cell Resource Center of the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). We cultured all cell lines in RPMI 1640 medium with 100 U/mL penicillin, 100 ug/mL streptomycin, and 10% foetal bovine serum at 5% CO_2 in a 37°C humidified culture environment. Short-tandem repeat profiling was used to authenticate the cell lines less than 6 months before this project was initiated, and the cells were not in culture for more than 2 months.

RNA isolation and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

According to the manufacturer's instructions, total RNA from each cell line was successfully isolated using TRIzol reagent (Life Technologies, Carlsbad, CA, USA). After adding the SYBR Premix Ex Taq II (Perfect Real Time) kit (TaKaRa Bio, Shiga, Japan), qRT-PCR was subsequently carried out with the following settings: 95°C for 30 s and 39 cycles of 95°C for 5 s

and 60°C for 30 s. The DNA dissociation analysis (melting curve) was operated at the end of each run to make sure the absence of primer dimers, mixed-amplicon populations, and nonspecific products. The relative expression of genes was presented as comparative threshold cycle ($2^{-\Delta\Delta Ct}$) values from at least three independent experiments. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was employed to standardize the expression of target genes. The primer sequences were as follows: *NRP1*, forward 5′- CTTGGCCTGACATTGCAATT-3′ and reverse 5′- AGGTTCCTGCATCCGCCTTAATGT-3′; *GAPDH*, forward 5′-ACCACAGTCCATGCCATCAC-3′ and reverse 5′-TCCACCACCCTG TTGCTGTA-3′.

Protein extraction and Western blot

Total protein was extracted from cell lines with radioimmunoprecipitation assay lysis buffer (Beyotime, Shanghai, China). Next, the lysates were centrifuged at 12,000 rpm for 30 mins at 4°C. The protein concentrations of the lysates were measured using the BCA Protein Assay Kit (Genechem, Shanghai, China). Equal amounts of protein (60 μg/lane) were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and then transferred onto PVDF membranes with a pore size of 0.45 μm (Millipore, Billerica, MA, USA). After blocking the membranes with 5% skim milk in TBST at room temperature for 60 mins, the membranes were incubated at 4°C overnight with the following primary antibodies at the stated dilutions: NRP1 (1:1000, Cell Signaling Technology (CST) Shanghai Biological Reagents Company Limited, Shanghai, China), baculoviral IAP repeat containing (BIRC) 3 (1:600, CST), cyclin dependent kinase (CDK) 6 (1:800, CST), Cyclin E (CCNE) 2 (1:800, CST), AP-1 transcription factor subunit (FOS) (1:600, CST), CDK2 (1:1000, CST), CDK4 (1:1500, CST), and β-actin (1:800, CST). After

washing in TBST, the membranes were further incubated for 2 hours with a secondary anti-mouse (1:3000) or anti-rabbit (1:4000) antibody, as appropriate. Finally, the presentations of target protein bands were enhanced using chemiluminescence (Millipore). The expression levels of target proteins were quantified by densitometry (BioRad image analysis program) and normalized with respect to β-actin levels.

Lentivirus-mediated RNA interference

To silence endogenous NRP1, BC cells with a good growth status were infected with a lentivirus carrying short hairpin RNAs (shRNAs) for NRP1 (shNRP1-1, shNRP1-2, or shNRP1-3) or control shRNA (GV118, Shanghai Genechem, Shanghai, China). The target sequence of shNRP1-1 was 5′-GCCTTGAATGCACTTATAT-3′, that of shNRP1-2 was 5′-GACCCATACCAGAGAATTA-3′, and that of shNRP1-3 was 5′-AACGATAAATGTGGCGATA-3′. The target sequence of the control shRNA was 5′-TTCTCCGAACGTGTCACGT-3′. Forty-eight hours after infection, cells expressing control shRNA and NRP1-shRNA were selected using 0.5 mg/mL puromycin for 10 days. qRT-PCR was used to test the expression of *NRP1* in infected cells.

MTT assay

Cells were seeded in 96-well cell culture plates at an initial density of 0.2×10^4 cells per well in triplicate at a volume of 200 μL per well. According to the experimental requirements, cells were incubated with 100 μL of 0.5 mg/mL sterile MTT [3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; Sigma, USA] at 37°C for different time points. After 4 hours, the culture

medium was removed and 150 μL of DMSO (Sigma) was added to each well for 10 min to fully dissolve the crystals. Finally, we measured the absorbance values of each well at 490 nm with 570 nm as the reference wavelength to draw the growth curve.

Colony formation assay

Cells were cultured in 60-mm plates at a density of 0.5×10^3 cells per plate for 14 days. Then the culture medium was removed. The cells were carefully washed with phosphate-buffered saline (PBS) twice and subsequently fixed with 10% formaldehyde for 5 min, followed by staining with 1% crystal violet for 30 s. The stain was washed away slowly with running water and the plates were dried at room temperature before counting the number of colonies.

Tube formation assay

A volume of 200 μL precooled Matrigel (BD Biosciences, San Jose, CA, USA) was pipetted into wells of a 24-well plate and polymerized at 37°C for 30 min. Subsequently, human umbilical vein endothelial cells (HUVECs) were added to the wells at a density of 0.2×10^4 cells/well in 200 μL conditioned medium and incubated at 5% CO_2 at 37 °C for 12 h. Bright-field microscopy at 100× magnification was used to capture the images. The overall length of the complete tubule structures was measured to quantify the capillary tubes.

Flow cytometric apoptosis test

Cells were digested with 0.25% trypsin, washed with PBS, and centrifuged at 1000 rpm for 5 min. The supernatant was aspirated, and, according to the instructions of the Annexin-V-APC

apoptosis determination kit (Ebioscience, USA), we added 100 μL of 1× binding buffer cautiously to each tube. Then, 5 μL of propidium iodide (PI) (Sigma) and 5 μL of Annexin-V-APC were added to the tubes. The tubes were then incubated at room temperature for 15 min, protected from light, before placing on ice. Within 1 hour, apoptosis was assessed using the BD FACSCalibur flow cytometer (BD Biosciences).

Flow cytometry cell cycle analysis

Cells were digested with 0.25% trypsin, washed with PBS, and centrifuged at 1000 rpm for 5 min. The cell pellet was washed twice with PBS, after which the cells were resuspended in 0.5 mL of PBS. The tubes were oscillated on a low-speed oscillator, and 70% ice-cold ethanol was added to fix the cells overnight at 4°C. The fixed cells were subsequently centrifuged at 1000 rpm for 5 min. The supernatant was discarded, and the pellet was washed with PBS and resuspended. Bovine pancreatic RNase (Fermentas, Lithuania) was added at a final concentration of 2 mg/mL and the tubes were incubated in a 37°C water bath for 30 min. PI was added at a final concentration of 65 μg/mL, followed by incubation in an ice bath for 30 min protected from light. Finally, cell cycle detection and data analysis were performed using a BD FACSCalibur Flow Cytometer filtration and FLOWJO Software (Tree Star, Inc, Ashland, OR, USA).

Transwell cell migration assay

Cells in the logarithmic growth stage were digested and centrifuged and then resuspended in serum-free medium. A volume of 750 μL culture medium with serum was added to the bottom of a 24-well plate, and migration chambers were put in the wells. We added 600 μL of 30% serum-

free medium to each chamber and added 100 μL of cell suspension at a density of 1×10^5 cells/mL. After incubation at 37°C for 24 h, the medium was removed from the chambers, and the wells were washed twice with PBS. Migrated cells were fixed by formaldehyde for 30 min before a 15-min staining with Giemsa stain, followed by washing twice with PBS. The non-migrated cells in the bottom of the chamber were scraped off with cotton swabs. Migrated cells were counted in three random fields of view using a light microscope (200×), and images were captured.

Transwell cell invasion assay

Matrigel was diluted using serum-free medium and mixed well by pipet. A volume of 100 μL prepared Matrigel was added to Transwell chambers in a 24-well plate and incubated at 37°C overnight for gelling. Cells in the logarithmic growth stage were digested, centrifuged, and resuspended in serum-free medium. A volume of 500 μL cell suspension at a density of 1×10^5 cells/mL was placed in the chamber. We subsequently added 750 μL culture medium with serum in the bottom of the wells of a 24-well plate and placed the Transwell chambers into the wells. After incubation at 37°C for 12 h, the medium was removed from the chambers, and the wells were washed twice with PBS. The invasive cells were fixed by formaldehyde at room temperature for 30 min, followed by a 15-min staining with Giemsa stain, and then washed twice with PBS. The non-invasive cells on the bottom of the chamber were scraped off with cotton swabs. Invasive cells were counted in three random fields of view using a light microscope (200×), and images were captured.

Affymetrix gene expression profile chip detection

We extracted total RNA from normal control cells and NRP1-knockdown cells with TRIzol reagent as described above and quantified RNA using the NanoDrop ND-2000 (Thermo Scientific, USA). RNA integrity was further analysed using the Agilent Bioanalyzer 2100 (Agilent Technologies, USA). cDNA libraries were constructed after confirming RNA purity (A260/A280: 1.7-2.2) and RNA integrity (RNA integrity number ≥7.0). Total RNA was transcribed to double-stranded cDNA and synthesized to cRNA. In this process, 2^{nd}-cycle cDNAs were generated and further hybridized onto the microarray after fragmentation and biotin labelling. Microarrays were washed and stained on the GeneChip Fluidics Station 450, and subsequent scanning was performed using the GeneChip Scanner 3000 (Affymetrix, USA). The genes with fold change ≥2.0 and $p < 0.05$ were considered significantly differentially expressed genes (SDEGs). The potential pathways and Gene Ontology (GO) terms related to SDEGs were revealed by KEGG and GO analysis. In addition, disease/gene-function analysis and interaction network analysis were performed to explore the potential predominant diseases and genes affected by knockdown of NRP1 and their associations.

Statistical analysis

All statistical analyses were conducted using SAS 9.43 statistical software (SAS Institute Inc., Cary, NC, USA). One-way ANOVA was carried out to perform significance tests on the data groups. Significant differences in continuous data (mean ± standard deviation) were evaluated using the Student's t test. A $p < 0.05$ was considered to be statistically significant.

Results

NRP1 is up-regulated in BC

Analysis of the expression of *NRP1* in published profiles[8] from patients with BC showed a frequent up-regulation of *NRP1* in BC samples (13 cases) when compared to normal bladder tissues (9 cases) ($p < 0.01$, Fig. 1a). A high level of NRP1 protein was also observed in BC pathological sections using the Human Protein Atlas database. This staining was primarily present in the cytoplasm and membrane of cancer cells (Fig. 1b). A total of six cultured BC cell lines, including T24, 5637, UM-UC-3, J82, SW780, and SCaBER cells, were employed for further experiments. We performed qRT-PCR to assess the expression of *NRP1* in these cell lines. *NRP1* was up-regulated in all BC cell lines but was particularly prominent in T24 and 5637 cells, which have strong invasive ability (Fig. 1c). Western blotting presented similar results in these cell lines (Fig. 1d). These results strongly demonstrated that NRP1 is up-regulated in bladder cancer.

NRP1 modulates BC cell proliferation and angiogenesis

To explore the role of NRP1 in the tumorigenesis and development of BC, we constructed stable BC cell lines expressing one of three different shRNAs against NRP1 or a negative control shRNA. shNRP1-1 generated the most consistent and significant down-regulation of NRP1 in T24 and 5637 cells and was therefore used in subsequent functional studies (Fig. 2a). In colony formation assays, NRP1 knockdown caused a significant reduction in colony number in both T24 and 5637 BC cells ($p < 0.05$ for both) (Fig. 2b). Additionally, MTT assays indicated that NRP1 knockdown significantly inhibited the growth of T24 and 5637 cells, and compared to control

cells, the growth rate decreased by almost 2.0-fold after 5 days (Fig. 2c). Further, conditioned medium from shNRP1 T24 or 5637 cells was able to significantly suppress the ability of tubule formation by HUVECs ($p < 0.05$ for both) (Fig. 2d). These results demonstrated that NRP1 may play a role in promoting proliferation and angiogenesis in BC.

Silencing NRP1 promotes BC cell apoptosis and cell cycle arrest

In order to explore the possible mechanism of the proliferation-promoting function of NRP1, apoptosis was assayed in NRP1-knockdown cells. As shown in Figure 3a, silencing NRP1 increased the proportion of apoptotic cells compared to control cells. Cell cycle arrest is one of the main ways to induce apoptosis. Flow cytometry analysis showed that NRP1 knockdown caused a significant decrease in the percentage of cells in the G0/G1 peak and an increase in the percentage of cells in the G2/M peak, but no statistically significant change was observed in the S peak (Fig. 3b), indicating that NRP1 may promote proliferation in BC cells by reducing apoptosis through mediating the G0/G1 and G2/M phase transitions.

NRP1 modulates the migration and invasion of BC

To evaluate whether NRP1 affects the process of migration and invasion in BC, we performed Transwell assays in T24 and 5637 cells following NRP1 knockdown. As shown in Figure 3c and 3d, NRP1 knockdown significantly weakened the migration and invasion abilities in T24 and 5637 cells. Migration and invasion in T24 cells decreased by 51% ($p < 0.05$) and 72% ($p < 0.05$) after NRP1 knockdown, respectively, and they decreased in 5637 cells by 61% ($p < 0.05$) and 65% ($p < 0.05$), respectively. Our results indicated that silencing NRP1 inhibited the migration and invasion

ability of BC cells.

Microarray analysis of dynamic gene expression after NRP1 knockdown in BC cells

To better understand the potential molecular mechanisms underlying BC malignant progression associated with NRP1, we further conducted Affymetrix Gene Chip hybridization analysis in 5637 cells following stable NRP1 knockdown. After subsequent bioinformatic and normalization analyses, we were able to distinguish the two groups clearly by hierarchical cluster and principal component analyses. According to the microarray expression profiling data, 599 up-regulated and 880 down-regulated genes had at least a 2-fold change in expression ($p < 0.05$ for all) following NRP1 knockdown. A heatmap of the significantly affected genes is presented in Figure 4a. Among the significantly activated pathways (Fig. 4b), the cancer pathway was chosen to examine the potential role of NRP1 in BC. We constructed a gene network map in this pathway to discover potential NRP1-regulated genes (Fig. 4c), and SDEGs, including *CDK4*, *BIRC3*, *CCNE2*, *CDK2*, and *FOS,* emerged as the dysregulated genes associated with NRP1 knockdown. Western blot was performed to verify changes in these genes with NRP1 knockdown. BIRC3 and CDK6 were up-regulated with NRP1 knockdown, and CDK4, CCNE2, FOS, and CDK2 were down-regulated (Fig. 4d-e).

NRP1 is associated with the mitogen-activated protein kinase (MAPK) signalling pathway

By performing signalling enrichment analysis of the altered gene sets following NRP1 knockdown, we found that the differentially expressed genes were significantly associated with the activation of p53 signalling, ERK/MAPK signalling, cAMP-mediated signalling, nuclear

298 factor kappa B (NF-κB) signalling, and G2/M checkpoint regulation, among others (Figure 5a).
299 Western blot analysis confirmed that NRP1 function was closely associated with the ERK/MAPK
300 and mitogen-activated protein kinase 8 (JNK)/MAPK signalling pathways. As shown in Figure 5b,
301 Ras, phospho-Raf (p-Raf), p-ERK1/2, and matrix metallopeptidase 9 (MMP9) were all decreased
302 in NRP1-knockdown cells, indicating that ERK/MAPK pathway activation is modulated by
303 NRP1. Further, the expression of JNK/MAPK signalling-related factors, such as p-JNK, p-c-jun,
304 and cyclin B1, were significantly lower in NRP1-knockdown cells (Fig. 5c), but the expression of
305 BCL2-associated X protein (Bax)/ BCL2 apoptosis regulator (Bcl2) and caspase 3 were higher,
306 which was consistent with the bioinformatics signalling enrichment assays and indicated changes
307 in the G2/M checkpoint regulation pathway. These results suggest NRP1 as a novel regulatory
308 mechanism of MAPK signalling that contributes to cell cycle modulation and drives tumorigenesis
309 in BC (Fig. 5d).
310
311
312 # Discussion
313 Over the past few decades, although encouraging progress has been made in the understanding of
314 the mechanisms of BC development[9], metastatic BC remains incurable, and many patients have
315 tried the few therapeutic strategies unsuccessfully[10]. Therefore, identifying therapeutic targets
316 and discovering better treatment options for BC is vital. Angiogenesis has the ability to promote
317 growth, invasion, and metastasis in multiple cancers[11]. Through unremitting efforts, numerous
318 therapeutic agents have been developed to target angiogenesis, a pathway that largely influences
319 the clinical activity of bladder cancer. One such targeted agent is bevacizumab, a monoclonal

antibody targeting VEGF[12]. NRP1 is considered to be a co-receptor for VEGF and is overexpressed in many human cancers. Recently, the overexpression of NRP1 has been reported to be associated with tumour progression and poor prognosis in patients with BC, but the underlying molecular mechanisms remain poorly understood[7]. Therefore, identifying the mechanisms by which NRP1 modulates the progression of BC has significance for exploring and optimizing the therapeutic strategy for urological malignancies.

In this study, we confirmed increased expression of NRP1 in BC cells and showed that suppressing NRP1 inhibits cell proliferation, promotes apoptosis, and regulates migration, invasion, and angiogenesis in human BC cells. NRP1 also regulates MAPK pathway activation. Our results suggest that NRP1 plays a crucial role in the tumorigenesis and progression of BC. They also provide evidence for the eligibility of NRP1 as a novel therapeutic target for BC.

NRPs are a class of approximately 130-kDa multifunctional non-tyrosine kinase receptors. The main functional domain of NRPs consists of five parts: an intracellular domain, a transmembrane domain, and three extracellular domains (a1a2, b1b2, and c)[13]. The membrane domain directly binds to type III semaphorins and VEGF and can initiate downstream signalling. There are two major NRP subtypes, NRP1 and NRP2. NRP1- and NRP2-knockout mice have hypoplasia and deficiency in the neural system, emphasizing their roles in neural development[14]. NRP1 has been shown to be overexpressed in numerous human tumour tissues, including breast, lung, colorectal, and hepatocellular cancer[5, 15]. Further, the expression of NRP1 is positively associated with prostate-specific antigen and Gleason score in prostatic cancer[16], and overexpression may contribute to

autocrine-paracrine interactions in pancreatic cancer[17]. However, the role of NRP1 in the pathogenesis of malignant diseases has not been deeply studied until now. Shi et al. found that NRP1 was highly expressed in oesophageal squamous cell carcinoma (ESCC), and inhibiting the expression of NRP1 could suppress the proliferation of ESCC cells and the growth of xenografts[18]. Cheng et al. demonstrated that NRP1 overexpression is an independent and novel prognostic factor for BC patients, which can help clinicians identify high-risk patients for close follow-up and intensive treatment[7].

To explore whether NRP1 qualifies as a therapeutic target for BC, we assessed the levels of NRP1 in BC cell lines and found that it was high in all of the cell lines assayed. Additionally, silencing NRP1 promoted apoptosis and reduced proliferation, angiogenesis, migration, and invasion in two aggressive BC cell lines. These results clearly identify NRP1 as a tumour promoter in BC and suggest that NRP1 has the potential to be an attractive target for BC treatment. To better understand the role of NRP1 in the growth, invasiveness, and migration of BC, we further performed global gene expression profiling using microarray technology. By comparing the gene expression profiles between T24 cells with control shRNA and shNRP1, we observed that the genes altered most significantly were mostly associated with tumour development. Among the differentially expressed genes associated with the cancer pathway, 48 genes were differentially expressed more than 2-fold. Among these genes, CDK6 was the most significantly up-regulated gene, and CDK2 was the most significantly down-regulated gene. CDK6 plays an important role in the cell cycle. To drive the progression of the cell cycle, CDK6 binds to and is activated by cyclin D to enhance the transition through the G1 phase[19]. Wang et al. confirmed that the

increased expression of CDK6 was synchronous with the development of BC, indicating that it could be considered a prognostic biomarker for patients with BC[20]. In addition, abnormal CDK6 expression has also been detected in breast cancer[21], pancreatic cancer[22], malignant glioma[23], and medulloblastoma[24]. Activation of cyclin E/CDK2 and cyclin D1/CDK4 in cell cycle progression could contribute to urothelial proliferation[25], and down-regulation of CDK2 in BC was first reported in this study. Collectively, our bioinformatic analysis indicated that NRP1 may influence BC through CDK6 and CDK2, although this needs further validation.

MAPK signalling can modulate several key biological processes during the development and progression of BC and is involved in the regulation of cell proliferation, angiogenesis, invasion, and metastasis[26]. Accumulating evidence indicates that in the development of BC, the expression of several VEGF genes is associated with the activation of MAPK signalling[27, 28]. Grun and colleagues showed that VEGF-A/NRP1 signalling enhances epidermal cancer stem cell spheroid formation, angiogenic potential, invasion, and migration by activating MAPK signalling[29]. In addition, Ceccarelli et al reported that NRP1 is responsible for keratinocyte growth factor-dependent ERK and p38 MAPK pathway activation in human adipogenesis[30]. Therefore, we hypothesized that NRP1 may have a significant impact on MAPK pathway activation in BC cells. In the present study, the significantly altered genes identified by gene expression profile analysis exhibited a significant enrichment in p53 signalling, NF-κB signalling, and ERK/MAPK signalling, suggesting that MAPK signalling might be involved in the NRP1-mediated progression of BC. Western blot was further performed to identify the role of NRP1 in ERK/MAPK signalling and p38/MAPK signalling. The results showed that Ras, p-Ras, p-ERK1/2, and MMP9 decreased

in NRP1-knockdown BC cells, indicating that NRP1 affected ERK/MAPK activity. Further, p-JNK, p-c-jun, and cyclin B1 were significantly lower in NRP1-knockdown BC cells, suggesting p38/MAPK signalling was inhibited. Moreover, Bax/Bcl2 and caspase 3 were up-regulated, implying that NRP1 knockdown attenuated anti-apoptotic signals, allowing for the induction of apoptosis. Taken together, our functional and mechanistic studies of NRP1 demonstrated it to be a vital contributor in BC tumorigenesis and progression through MAPK signalling. The mechanism underlying NRP1 activation and maintenance of MAPK activity will be further investigated in the near future.

Conclusion

In summary, we provide evidence for the expression pattern of NRP1 in BC, and then by performing functional and mechanistic studies revealed a critical role of NRP1 in several pathological processes related to BC development, such as proliferation, apoptosis, angiogenesis, migration, and invasion. NRP1 was also found to be associated with MAPK pathway activation. We also identified an association between the expression of NRP1 and the expression of *CDK4*, *BIRC3*, *CCNE2*, *CDK2*, and *FOS* in BC. These findings provide a novel insight into the molecular mechanisms by which NRP1 drives the pathogenesis and progression of cancer. It would be reasonable to believe that targeting NRP1 has the potential to become a new therapeutic strategy to benefit more patients with BC or other cancers. Further research into the crucial mechanisms of NRP1 dysregulation in BC development is ongoing in order to better understand the biological basis of malignancy progression.

Additional file

Additional file 1: 599 up-regulated and 880 down-regulated genes after NRP1 knockdown in BC 5637 cells. Includes all the genes with an absolute fold change > 2 after NRP1 knockdown in 5637

bladder urothelial carcinoma cells. (XLSX 100 kb)

Abbreviations

Bax: BCL2-associated X protein; BC: bladder urothelial carcinoma; Bcl2: BCL2 Apoptosis Regulator; BIRC3: baculoviral IAP repeat containing 3; CCNE2: Cyclin E2; CDK: cyclin-dependent kinase; CST: Cell Signaling Technology; ESCC: oesophageal squamous cell carcinoma; FOS: AP-1 Transcription Factor Subunit; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GO: Gene Ontology; HUVEC: Human umbilical vein endothelial cells; JNK: mitogen-activated protein kinase 8; MAPK: Mitogen-Activated Protein Kinase; MMP9: Matrix Metallopeptidase 9; NF-κB: Nuclear Factor Kappa B; NRP: neuropilin; PBS: phosphate-buffered saline; PI: propidium iodide; qRT-PCR: quantitative real-time reverse transcription polymerase chain reaction; SDEG: significantly differentially expressed genes; shRNA: small hairpin RNA; VEGF: vascular endothelial growth factor

Declarations:

Ethics approval and consent to participate

No humans or animals were employed in this study and therefore ethics approval and consent for participation was not required.

Consent for publication

All authors consent for publications.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YD performed most of the experiments and wrote the article; LH analysed data and performed experiments; KP analysed data and performed experiments; XZ performed experiments; ZS performed experiments; BL provided technical support; RZ performed bioinformatics analysis; WZ performed bioinformatics analysis; TF performed bioinformatics analysis; GZ performed experiments; QL performed experiments; YL performed experiments; RL performed experiments; CH provided funding support, aided in experimental design, and substantively revised the work. All authors read and approved the final manuscript.

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570 **Figure legends**

571 **Figure 1. NRP1 is upregulated in BC.** (a) Expression of NRP1 was frequently upregulated in 13
572 infiltrating BUC tissues compared with 9 normal urotherial tissue samples in the Dyrskjøt. L
573 bladder dataset[8]. (b) The translational-level validation of NRP1 was analyzed by The Human
574 Protein Atlas database (IHC). (c) The levels of the NRP1 mRNA in six BC cell lines examined by
575 Real-time PCR. (d) The levels of the NRP1 protein in six BC cell lines examined by western
576 bloting. The average NRP1 mRNA expression was normalized to the expression of GDPDH. β-
577 actin was detected as a loading control in the Western blot. Three independent experiments were
578 conducted for each assay.
579
580 **Figure 2. Downregulation of NRP1 reduces BC cells proliferation and angiogenesis.** (a) T24
581 and 5637 cells were infected with lentivirus-expressing NRP1 shRNA-1, shRNA-2, and shRNA-3,
582 or a control shRNA; the NRP1 mRNA level as measured by qRT-PCR. (b) Downregulation of
583 NRP1 reduced the mean colony number in the colony formation assay. (c) MTT assays revealed
584 that downregulation of NRP1 significantly reduced the growth rate of BC cells. (d)
585 Downregulation of NRP1 reduced tubule formation of vascular endothelial cell. Three
586 independent experiments were conducted for each assay, and data are presented as the mean ±
587 standard error of the mean, * $p<0.01$ vs. the control group.
588
589 **Figure 3. NRP1 modulates BC cells apoptosis, cell cycle, migration and invasion.** (a)
590 Apoptosis assay and quantitation of apoptotic cells of T24 and 5637 cells following NRP1
591 knockdown or control shRNA expression. (b) Flow cytometric analysis of T24 and 5637 cells
592 following NRP1 knockdown or control shRNA expression. (c) and (d) Images and normalized
593 migration (c) or invasion (d) of T24 and 5637 cells following NRP1 knockdown or control shRNA
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595 as the mean ± standard error of the mean, * $p<0.05$ vs. the control group.
596
597 **Figure 4. Global changes in the BC cell transcriptome following knockdown of NRP1.** (a)
598 Heat map and hierarchical cluster analysis of 5637 cells transfected with NRP1 shRNA and a

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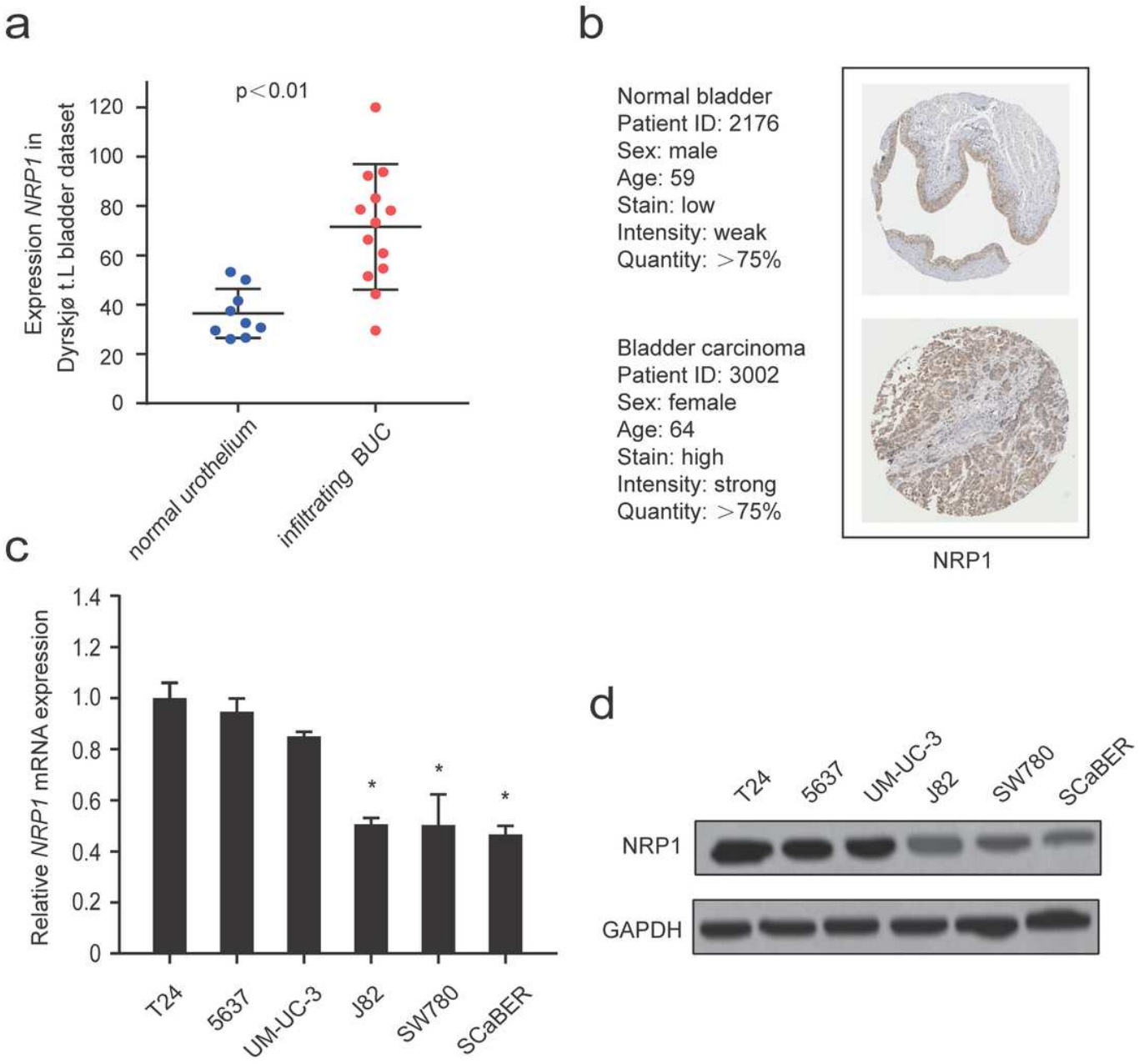
Figure 5. Function of NRP1 is associated with the MAPK pathway. (a) Signaling enrichment analysis of NRP1 regulated gene expression events. (b) Western blot of ERK/MAPK related protein expression level inT24 and 5637 BC cells following knockdown of NRP1. (c) Western blot of JNK/MAPK related protein expression level inT24 and 5637 BC cells following knockdown of NRP1. (d) Proposed model for the underlying molecular mechanisms of NRP1 in bladder cancer progression.

Figures



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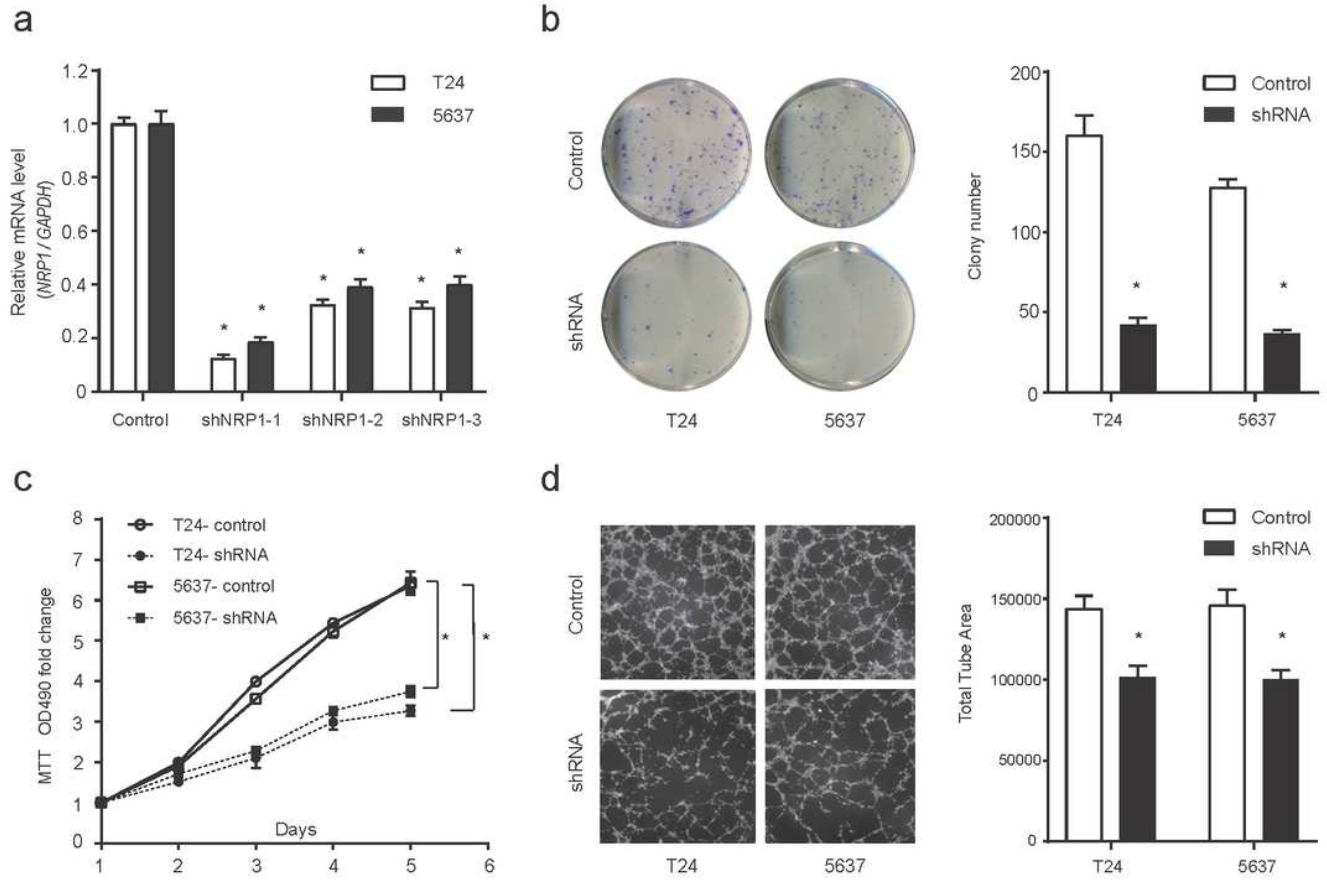

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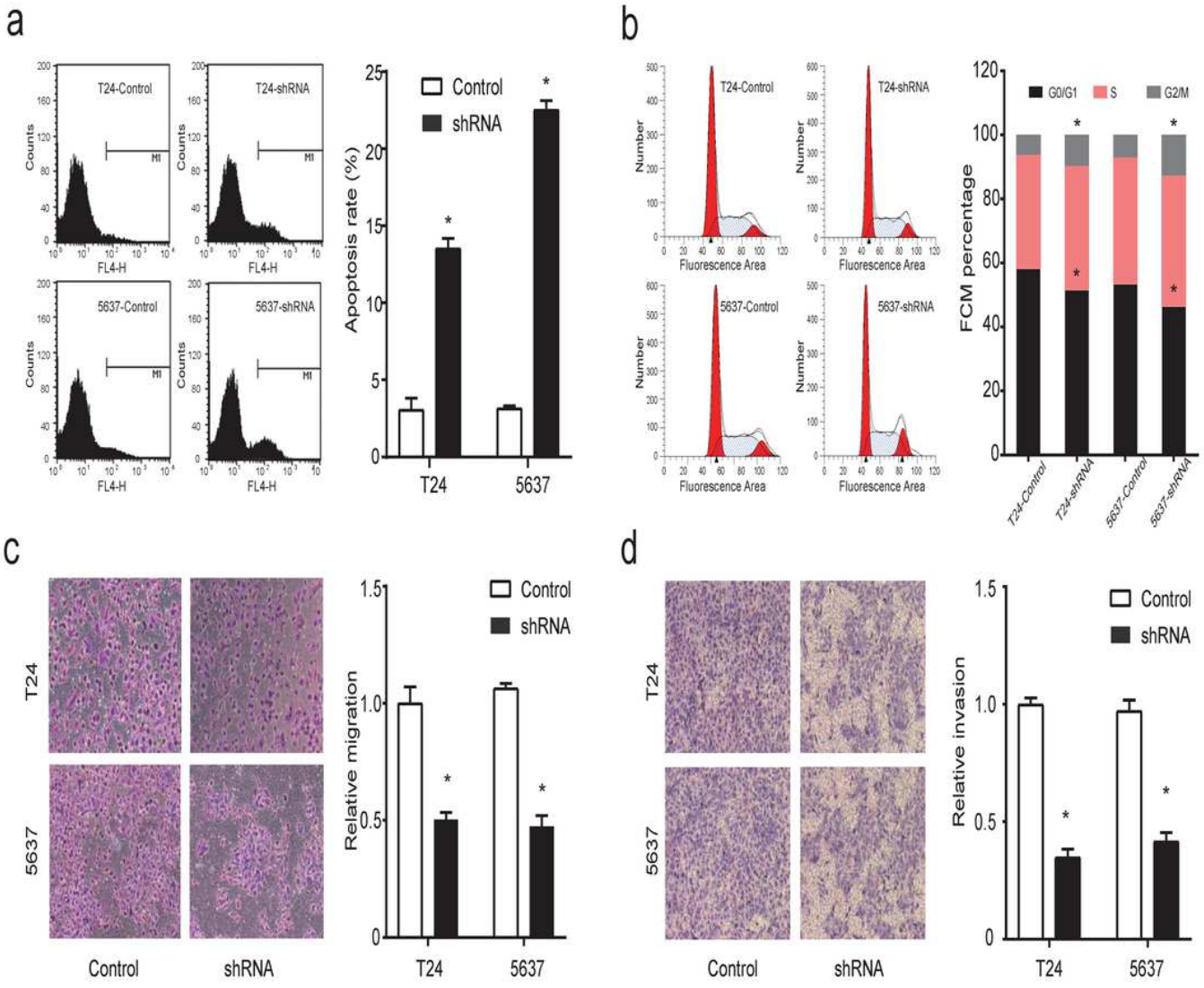

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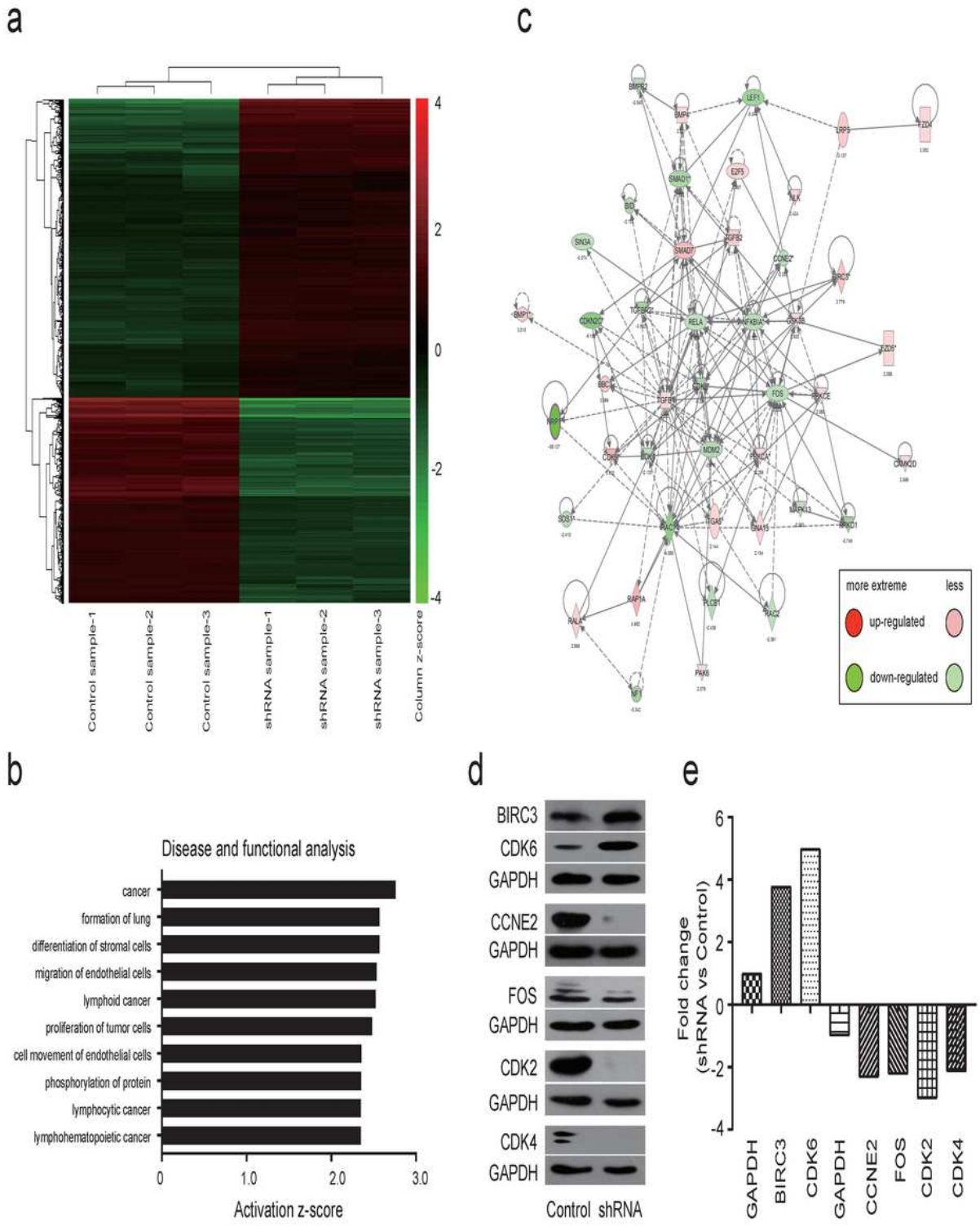

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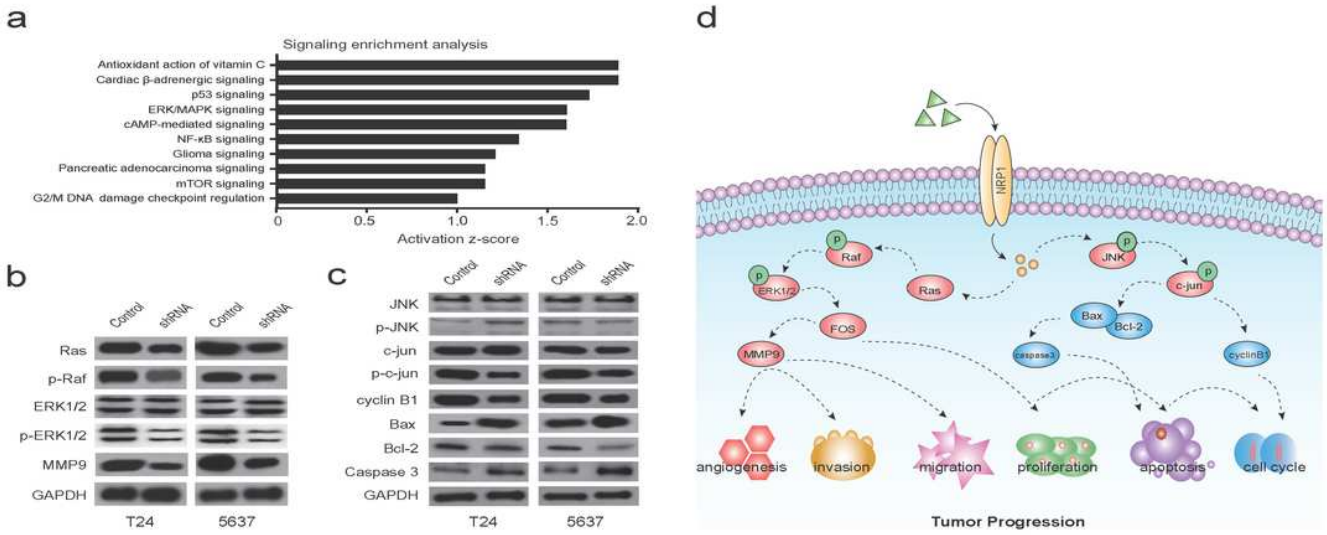

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