

# The effect of NPM1 silencing on the tumorigenesis of drug-resistant bladder cancer and related signaling pathway

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## Research article

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# Abstract

**Background:** NPM1 can provide abundant information of bladder cancer changes, but the effect of NPM1 differential expression on tumor and tumor related molecular mechanism has not been elucidated.

**Methods:** NPM1 silencing cell line was established by lentivirus. The tumorigenic ability was judged by wound-healing assay, transwell invasion assay and nude mice tumorigenicity assay. The proteome of NPM1 deficient bladder cancer cell line was analyzed by Liquid Chromatography Mass Spectrometry (LC-MS). The results of mass spectrometry are comprehensively analyzed by bioinformatics analysis for tumor related molecules. The signal pathways involved in tumor related molecules will be verified by KEGG and UniProt databases.

**Results:** Compared with the corresponding negative control group, NPM1 silencing cell line T24/DDP Lv-NPM1 showed strong migration ability and high invasive ability. There was no significant difference in migration ability and the invasive cells proportion between NPM1 overexpressing cell line and related negative control group. The tumorigenesis in nude mice also showed that NPM1 silencing tumor had larger tumor volume. Among all differential proteins analyzed by mass spectrometry, 20 proteins with signal transduction activity showed the most significant difference (Fold change > 1.5). 6 of them were associated with NF- $\kappa$ B signaling pathway, which may play an important role in the development of tumor.

**Conclusions:** The loss of NPM1 may indicate the poor outcome of bladder cancer. Abnormal expression of NF- $\kappa$ B signaling pathway is an important factor in the progression of bladder cancer. Monitoring NPM1 expression can effectively adjust the treatment strategy of bladder cancer.

## Background

Bladder cancer is one of the most common malignancies in urinary system [1], which is prone to drug resistance and relapse [2]. Timely monitoring the drug resistance and progression of the tumor and adjusting the treatment strategy can effectively inhibit the progression of bladder cancer [3]. Therefore, it is urgent to find a biomarker which can provide abundant clinical information for the treatment of drug resistant bladder cancer.

Nucleophosmin (NPM1) is the nucleolar protein which can shuttle between nucleolus and cytoplasm [4], NPM1 mutation have been demonstrated to be closely associated with malignant tumor. In colon cancer [5], lung adenocarcinoma [6] and hematological tumor [7], NPM1 was found to be able to indicate tumor progression. In addition, NPM1 plays an important role in cell invasion, migration and apoptosis [8]. NPM1 is also involved in drug resistance and reflect the prognosis of tumor, such as leukemia [9]. It is reported that NPM1 can interfere with drug action by histone demethylation or gene rearrangement [10]. This suggests that NPM1 is involved in the specific mechanism of drug resistance. In salivary gland carcinoma and leukemia cells, tumor proliferation and drug response can be effectively affected by interfering with the expression of NPM1 [8]. Up-regulated NPM1 can inhibit the activity of chemotherapy drugs [11].

Based on the above research, we've paid attention to the clinical information provided by NPM1 in bladder cancer in recent years.

In the research of our laboratory, NPM1 overexpression was found in drug-resistant by proteome analysis [12]. Besides abnormal expression of NPM1 was also found in recurrent patients, which suggested that NPM1 mutation was closely related to the recurrence and drug resistance of bladder cancer [13].

In order to further evaluate the application value of NPM1 gene silencing in drug resistant bladder cancer cells, we used liquid chromatography-mass spectrometry to detect the proteomics of bladder cancer cells after NPM1 gene silencing, 492 differential proteins were detected by mass spectrometry, and their multiple changes were more than 1.5 ( $P < 0.05$ ). The results of mass spectrometry showed that 57022 polypeptides, 54347 unique polypeptides and 6686 proteomes were identified ( $FDR < 0.01$ ). Compared with the negative control group, 264 functional proteins were down-regulated and 228 were up-regulated. According to the analysis of Gene Ontology (GO analysis), CD40 is the most significant down regulated protein after NPM1 silencing [14]. CD40 is differentially expressed in a variety of tumors, and is considered to be an important biomarker for predicting many cancers. Therefore, the results of proteomic analysis suggest that NPM1 gene silencing may be involved in the development of drug-resistant bladder cancer cells. In this study, we observed the effects of NPM1 silencing on drug resistance, tumor invasion and migration of bladder cancer and performed bioinformatics analysis from the proteomics data in drug resistant bladder cancer from our previous studies to reveal the possible mechanism of NPM1 in drug resistant bladder cancer.

## Methods

### Wound-healing assay

In order to evaluate the migration ability of bladder cancer cells, wound-healing assay was performed. The migration ability of cells in each group can be judged by the difference of repair ability of cells in different groups for wound area. The plate was irradiated with ultraviolet for 30 minutes before operation. Approximately  $3 \times 10^4$  infected cells were incubated in 24 holes. Wounds were made according to the instructions. After 24 hours low serum concentration medium was used to incubate the cells. Rinsed the plate gently with PBS for 2 times after 24 or 48 hours. After that the photos were taken under microscopy (CKX41, Olympus, USA). According to the pictures, the rate of cell migration in each group was calculated. The migration rate was the ratio of wound width to 0 hour wound area width.

### Transwell invasion assay

In order to evaluate the invasion ability of bladder cancer cells, transwell invasion assay was performed. The invasion ability of bladder cancer cells was evaluated by counting the amount of cells which can digest matrigel. Matrigel invasion assay was performed using transwell chambers.  $1 \times 10^4$  Lv-NPM1 cells,  $1 \times 10^4$  Lv5-NPM1 and the cells were seeded in the upper chamber of a 24-well plate, which was coated with growth factor reduced matrigel. The upper chamber was filled with 500  $\mu$ l serum-free medium. The

lower chamber was filled with 500  $\mu$ l medium containing 10% FBS to induce cell invasion. The chamber was incubated at 37 °C for 24 hours. At the end of incubation, cells in the upper surface of the membrane were removed with a cotton swab. Migrated cells were stained with crystal violet. The images were obtained by microscope and the cells were counted in ten different view fields. The experiment was repeated three times

## Materials

Cisplatin (P4394, Sigma, USA), RPMI-1640 medium (AE244464298, Thermo scientific, USA) FBS (1861242, Gibco, ThermoFisher, USA) 0.25% trypsin (17518012, Gibco, ThermoFisher, USA) Trizol (84804, Gibco, ThermoFisher, USA) reverse transcription kit (64J00101, Dingguo, China). SYBR Green fluorescent quantitative PCR kits (K20524, TransGen Biotech, China) polyvinylidene fluoride (PVDF) membranes (ISEQ00010 Sigma, USA) Mouse anti-Nucleophosmin antibody (ab10530, Abcam, USA) Mouse IgG H&L (HRP) (ab205719, Abcam, USA) 96-well plates (E161134L, Thermo, USA) NPM1-silencing, NPM1 overexpressing and negative control lentivirus (GenePharma, China), growth factor reduced matrigel (3432-001-01, R&D Systems, USA), Transwell chambers (140644, Thermo, USA), ethyl carbamate (30191228, Sinopharm Chemical Reagent, China).

## Xenograft Growth In Nude Mice

To evaluate the tumorigenicity of bladder cancer cells, xenograft model was made by subcutaneous injection of NPM1 silencing cells and the corresponding negative control cells in athymic nude mice.

## Preparation of inoculated cells

Lv-NPM1 cell line and Lv-NC cell line labeled by GFP fluorescence were cultured in RPMI-1640 medium containing 15% fetal bovine serum at 37 °C and 5% CO<sub>2</sub> incubator respectively. After digestion of 0.25% trypsin, they were used for tumorigenicity test.

## Tumorigenicity test

The mice were inoculated subcutaneously in the left armpit with  $5 \times 10^7$  NPM1 silencing cells. They were also inoculated subcutaneously in the right armpit with  $5 \times 10^7$  negative control cells (as the negative control). All the mice were kept in standard laboratory conditions and provided with ad libitum food and water. General health of these animals was daily observed and tumor growth at the injection site was monitored by palpation. Tumor volume was measured outside of body by vernier caliper and calculated using the formula: length  $\times$  (width)<sup>2</sup>  $\times$  0.5. 35 days after the experiment, the mice were euthanized. All nude mice were anesthetized with ethyl carbamate (1350 mg/kg, intraperitoneal injection) and sacrificed by cervical dislocation when the mice were unconscious. The experiment was repeated three times.

## Proteomic Bioinformatics Analysis

The data came from the proteomic data of our NPM1 gene silencing drug resistant bladder cancer cells [14]. The original data were processed by proteome discovery software. According to the human genome

database and UniProt website, mass spectrometry data were searched. The molecular function of proteins were analyzed by gene ontology (GO analysis). The Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to study protein species to map the signaling pathway for systematic functional biology interpretation (<http://www.genome.jp/kegg/>).

### **Statistical analysis**

All independent experimental data were expressed as mean  $\pm$  standard deviation (SD). Statistical analysis was carried out by using Graphpad prism 6.0 statistical software Inc (Lajolla, USA). *P* values were calculated by ANOVA and Bonferroni test (more than two groups) or T test (two groups). When *P* value < 0.05, the results are considered to be statistical.

## **Results**

### **The effect of NPM1 silencing and NPM1 overexpressing on the cell mobility of cisplatin resistant bladder cancer cells**

Compared with the negative control group, NPM1 silencing cell line T24/DDP Lv-NPM1 showed strong migration ability in 24 hours (73.3% vs 13.8%, *P* < 0.05) and 48 hours (86.5% vs 44.8%, *P* < 0.05). At the same time, there was no significant difference between the overexpressing cell lines (T24/DDP Lv5-NPM1) and the corresponding negative control cell lines in 24 hours and 48 hours (*P* > 0.05). The results are shown in Fig. 1.

Table 1

Differential proteins with signal transduction function in GO analysis ( Fold change  $\geq 1.5$ ,  $P < 0.05$ )

ID	Accession	Gene Name	Description	Function	Ratio (Lv-NPM1 vs Lv-NC)
MRC2_HUMAN	Q9UBG0	MRC2	C-type mannose receptor 2	Collagen catabolic process	2.73
RAI3_HUMAN	Q8NFJ5	GPRC5A	Retinoic acid-induced protein 3	Negative regulation of epidermal growth factor-activated receptor activity	2.03
FKBP1A_HUMAN	P62942	FKBP1A	Peptidyl-prolyl cis-trans isomerase FKBP1A	Positive regulation of I-kappaB kinase/NF-kappaB signaling	1.92
LEG1_HUMAN	P09382	LGALS1	Galectin-1	Positive regulation of I-kappaB kinase/NF-kappaB signaling	1.85
EPHB2_HUMAN	P29323	EPHB2	Ephrin type-B receptor 2	Positive regulation of gene expression	1.83
TF_HUMAN	P13726	F3	Tissue factor	Positive regulation of cell migration	1.80
S20A1_HUMAN	Q8WUM9	SLC20A1	Sodium-dependent phosphate transporter 1	Positive regulation of I-kappaB kinase/NF-kappaB signaling	1.74
MALT1_HUMAN	Q9UDY8	MALT1	Mucosa-associated lymphoid tissue lymphoma translocation protein 1	Positive regulation of NF-kappaB transcription factor activity	1.60

<b>ID</b>	<b>Accession</b>	<b>Gene Name</b>	<b>Description</b>	<b>Function</b>	<b>Ratio (Lv-NPM1 vs Lv-NC)</b>
C2D1A_HUMAN	Q6P1N0	CC2D1A	Coiled-coil and C2 domain-containing protein 1A	Positive regulation of I-kappaB kinase/NF-kappaB signaling	1.58
CTNB1_HUMAN	P35222	CTNNB1	Catenin beta-1	Regulation of canonical Wnt signaling pathway	1.56
GBB4_HUMAN	Q9HAV0	GNB4	Guanine nucleotide-binding protein subunit beta-4	Ca2 + pathway	1.55
ITB1_HUMAN	P05556	ITGB1	Integrin beta-1	Cytokine-mediated signaling pathway	1.50
ITPR3_HUMAN	Q14573	ITPR3	Inositol 1,4,5-trisphosphate receptor type 3	G protein-coupled receptor signaling pathway	0.64
STAT2_HUMAN	P52630	STAT2	Signal transducer and activator of transcription 2	Receptor signaling pathway via JAK-STAT	0.64
A0A0B4J1V8_HUMAN	A0A0B4J1V8	PPAN-P2RY11	HCG2039996	G protein-coupled receptor activity	0.64
SDC4_HUMAN	P31431	SDC4	Syndecan-4	Positive regulation of protein kinase activity	0.52
SP110_HUMAN	Q9HB58	SP110	Sp110 nuclear body protein	Enhances transcription of genes with retinoic acid response elements	0.51

ID	Accession	Gene Name	Description	Function	Ratio (Lv-NPM1 vs Lv-NC)
GNAQ_HUMAN	P50148	GNAQ	Guanine nucleotide-binding protein G(q) subunit alpha	Regulation of canonical Wnt signaling pathway	0.48
TNR6_HUMAN	P25445	FAS	Tumor necrosis factor receptor superfamily member 6	Regulation of apoptotic process	0.26
TNR5_HUMAN	P25942	CD40	Tumor necrosis factor receptor superfamily member 5	Positive regulation of NF-kappaB transcription factor activity	0.16

### The effect of NPM1 silencing and NPM1 overexpressing on the cell invasion of cisplatin resistant bladder cancer cells

48 hours after the experiment, compared with the negative control group, the invasive cells proportion of T24/DDP Lv-NPM1 cells was higher than Lv-NC cells (1983 cells/lp vs 788 cells/lp,  $P < 0.05$ ). At the same time, there was no significant difference in the proportion of cells in the invasive cells proportion between NPM1 overexpressing cell line and related negative control group ( $P > 0.05$ ). The results are shown in Fig. 2.

### NPM1 silencing can accelerate the tumorigenicity of drug-resistant bladder tumor *in vivo*.

We used the nude mouse tumorigenesis experiment to assess the effect of NPM1 silencing on the overall change of tumor *in vivo*. After 35 days of the experiment, greater masses were observed in animals injected with Lv-NPM1 cells compared with Lv-NC cells. Meanwhile, the tumor mass of the nude mice injected NPM1 silencing cells was more obvious than that of the negative control nude mice at 14–35 days ( $P < 0.05$ ), as shown in Fig. 3.

### The effect of differential proteins of signal transducer activity on NPM1 silencing tumor cells.

After NPM1 silencing, the protein of signal transducer activity on NPM1 silencing tumor cells is of great significance to the characteristics of tumor, as shown in Table 1. 12 proteins were up-regulated and 8 proteins were down regulated (Fold change  $\geq 1.5$ ,  $P < 0.05$ ), 6 of the 20 proteins with signal transduction activity are related to NF- $\kappa$ B signaling pathway. Moreover, the up-regulated protein and the affected pathway function have the characteristics of accelerating the tumorigenesis.

## Discussion

Bladder cancer is a common urologic neoplasm which is easy to relapse and drug resistance. The existing monitoring methods for bladder cancer have high side effects and heavy costs, which are difficult for patients to accept [15, 16]. Proteomic analysis of tumor progression can reduce the burden of patients and provide valuable clinical information [17]. The accurate judgment of tumor characteristics of bladder cancer can effectively guide the treatment of bladder cancer and improve the treatment effect.

Nucleophosmin (NPM1) is a nucleolar protein which can provide clinical information for the development of cancers [18]. NPM1 plays important roles in p53, MDM2 and other signal pathways [19], which means NPM1 can affect tumor characteristics and provide important information for drug resistance. The up-regulation of NPM1 expression significantly affects the relapse rate and the sensitivity of chemotherapy drugs of leukemia [20].

In this experiment, NPM1 silencing and NPM1 overexpressing bladder cancer cell lines were constructed by lentivirus infection and limited dilution method to evaluate the value of NPM1 in drug resistant bladder cancer. In this study, the results showed that the loss of NPM1 was correlated with high cell mobility in bladder cancer cells. Compared with negative control group, NPM1 silencing cell line T24/DDP Lv-NPM1 showed strong migration ability in 24 hours ( $P < 0.05$ ) and 48 hours ( $P < 0.05$ ). Besides, NPM1 silencing could make cisplatin resistant bladder cancer aggressive. Compared with the negative control, the invasive cells proportion of T24/DDP Lv-NPM1 cells was higher than Lv-NC cells ( $P < 0.05$ ). Compared with the above results *in vitro*, tumorigenesis experiment also showed similar results *in vivo*. In this animal experiments, we found that NPM1 silencing increased tumor growth *in vivo*, indicating that down-regulated NPM1 may bring poor prognosis to patients. Besides, the specific mechanism of NPM1 on bladder cancer has not been elucidated. In previous study, we used mass spectrometry to analyze proteins in bladder cancer. A total of 57022 peptides, 54347 unique peptides and 6686 protein groups were identified in all proteins of NPM1 silencing drug resistant bladder cancer cells (FDR < 0.01) [14]. In this study we analyzed these functional proteins from the previous study and focused on the proteins that play a role in the signaling pathway. Among the 20 proteins with the most obvious difference in signal transducer activity (Fold change  $\geq 1.5$ ), 6 proteins were associated with NF- $\kappa$ B signaling pathway. Most of the functions of these proteins were positive regulation of NF- $\kappa$ B signaling pathway. NF- $\kappa$ B signaling pathway was activated abnormally in many tumors, hence it is suspected to be involved in malignant transformation of tissues, carcinoma cells invasion and metastasis [21]. Combined with the results of our animal experiments, the low expression level of NPM1 may reflect the acceleration of tumor growth. Moreover, tumor deterioration mainly points to the abnormal changes of NF- $\kappa$ B signal pathway. It has been reported that the abnormal activation of NF- $\kappa$ B signal pathway can accelerate the development of tumors [22], and has an impact on the drug resistance of chemotherapy drugs [23]. The change of NF- $\kappa$ B signaling pathway was consistent with the characteristic of bladder cancer that is easy to relapse and drug resistance. In our previous study, we found that NPM1 has an increasing expression in drug-resistant bladder cancer [13]. This result may indicate that NPM1 can protect gene stability in bladder cancer cells. Under the attack of chemotherapy drugs, NPM1 with high expression can prevent genome damage [24].

However, the low expression of NPM1 does not reflect the improvement of tumor therapy. In gastric cancer and breast cancer, NPM1 is associated with poor prognosis [25, 26]. In the researches of bladder cancer, NPM1 can regulate c-Myc protein stability [27] to influence  $\beta$ -catenin/c-Myc signaling pathway [28] and AFF4/NF- $\kappa$ B/Myc signaling pathway [29] increase the ability of cell migration and proliferation. In this study, we discovered the relationship of NPM1 and NF- $\kappa$ B signaling pathway in the proteomic study of bladder cancer. It was suggested that the low expression of NPM1 could reflect the enhancement of NF- $\kappa$ B signaling pathway and accelerate the development of tumor. NF- $\kappa$ B signaling pathway, as an important tumor related signaling pathway, was able to accelerate tumor progression and change the drug-resistant activity in a variety of non-urinary tumors [30]. It has also been reported that the blocking of NF- $\kappa$ B signaling pathway can effectively alleviate the tumor [31], which may provide a new strategy for the treatment of bladder cancer. These results indicated that NPM1 can provide important information for the development of bladder cancer. Monitoring the expression of NPM1, which might be able to effectively detect the malignant change of bladder.

## Conclusions

Down-regulated expression of NPM1 could indicate the poor outcome of bladder cancer, which meant that the tumor may be more malignant. Abnormal expression of NF- $\kappa$ B signaling pathway is an important factor in the progression of bladder cancer. Monitoring the changes of NPM1 in time could effectively adjust the treatment strategy of bladder cancer and treat bladder cancer.

## Declarations

### Ethics approval and consent to participate

All applicable international, national, and institutional guidelines for the care and use of animals were followed.

All procedures performed in studies involving animals were in accordance with the ethical standards of the Pecking University Ethics Committee. (Permit number: LA2017287)

### Consent for publish

Not applicable.

### Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

### Competing interests

The authors declare that they have no competing interests.

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## Authors' contributions

All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; CL was a major contributor in writing the manuscript. TL analyzed and interpreted the results of the experiments. MZ (the third author), QM and MZ (the corresponding author) put forward ideas and assisted in the progress of experiments. All authors have read and approved the manuscript. And all author have ensured that this is the case.

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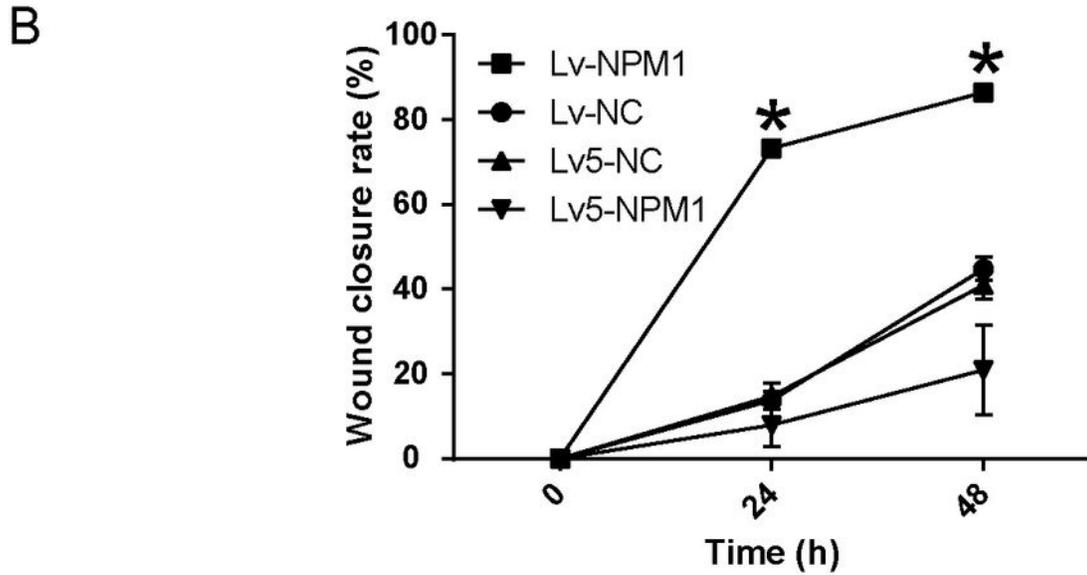
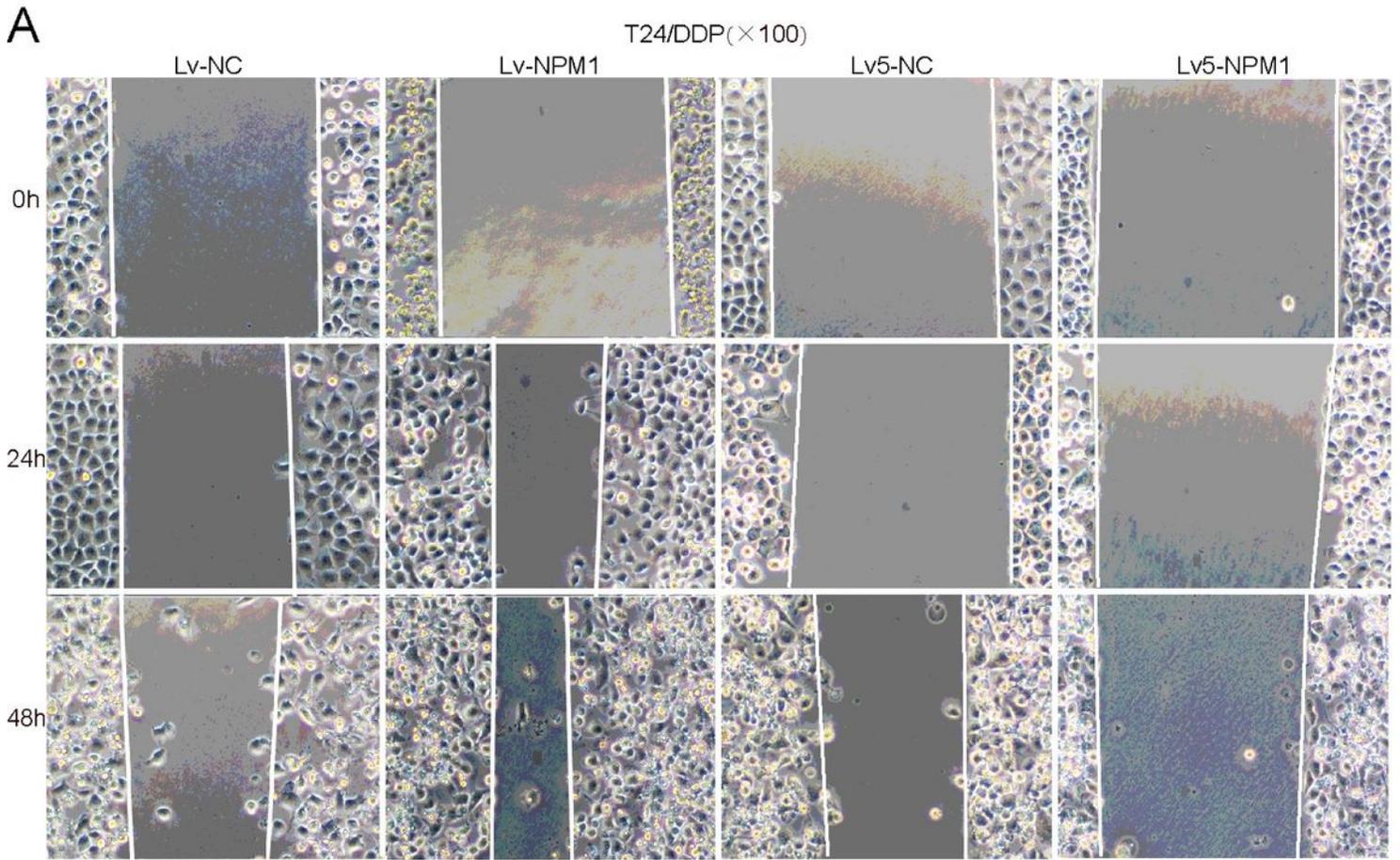
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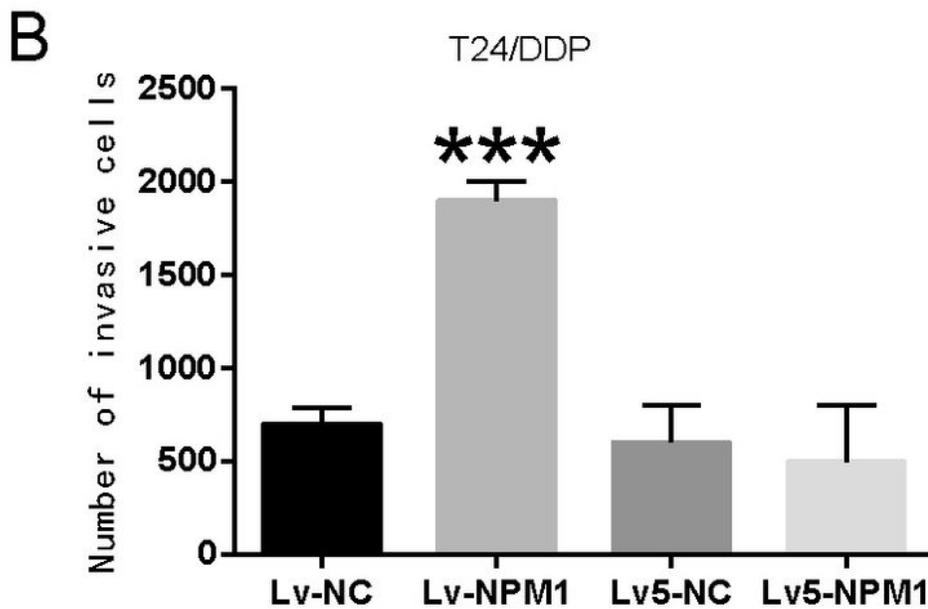
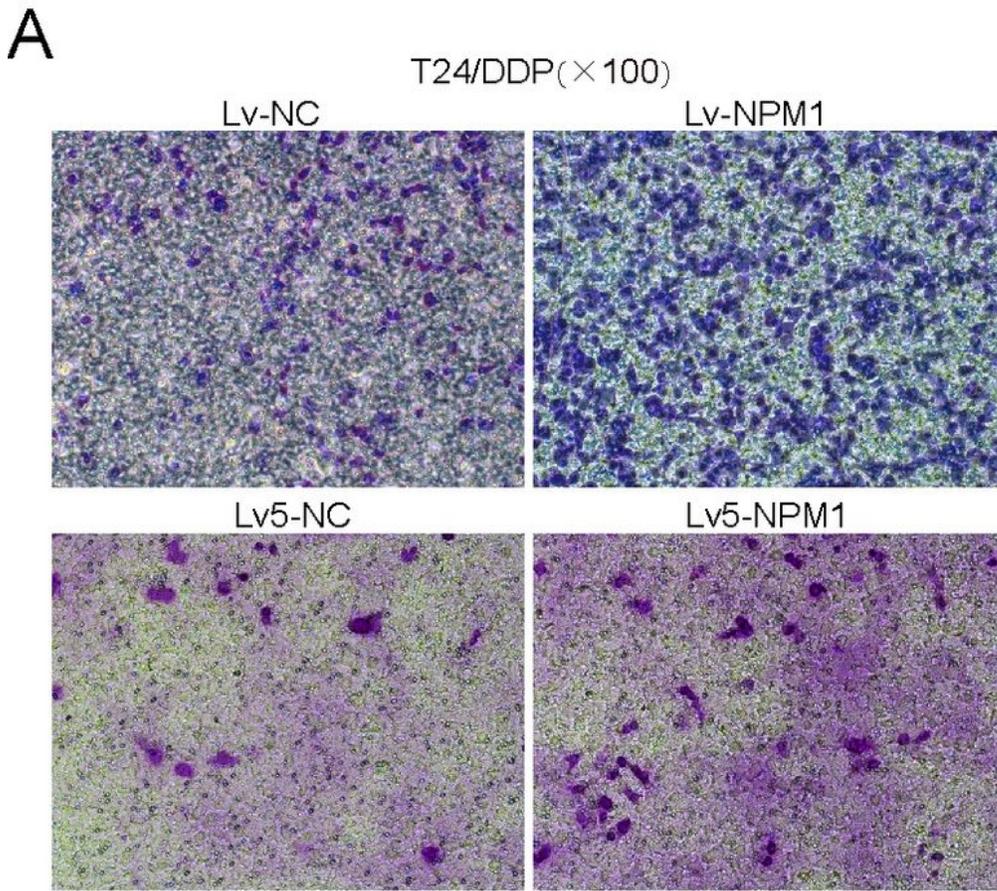
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## Figures



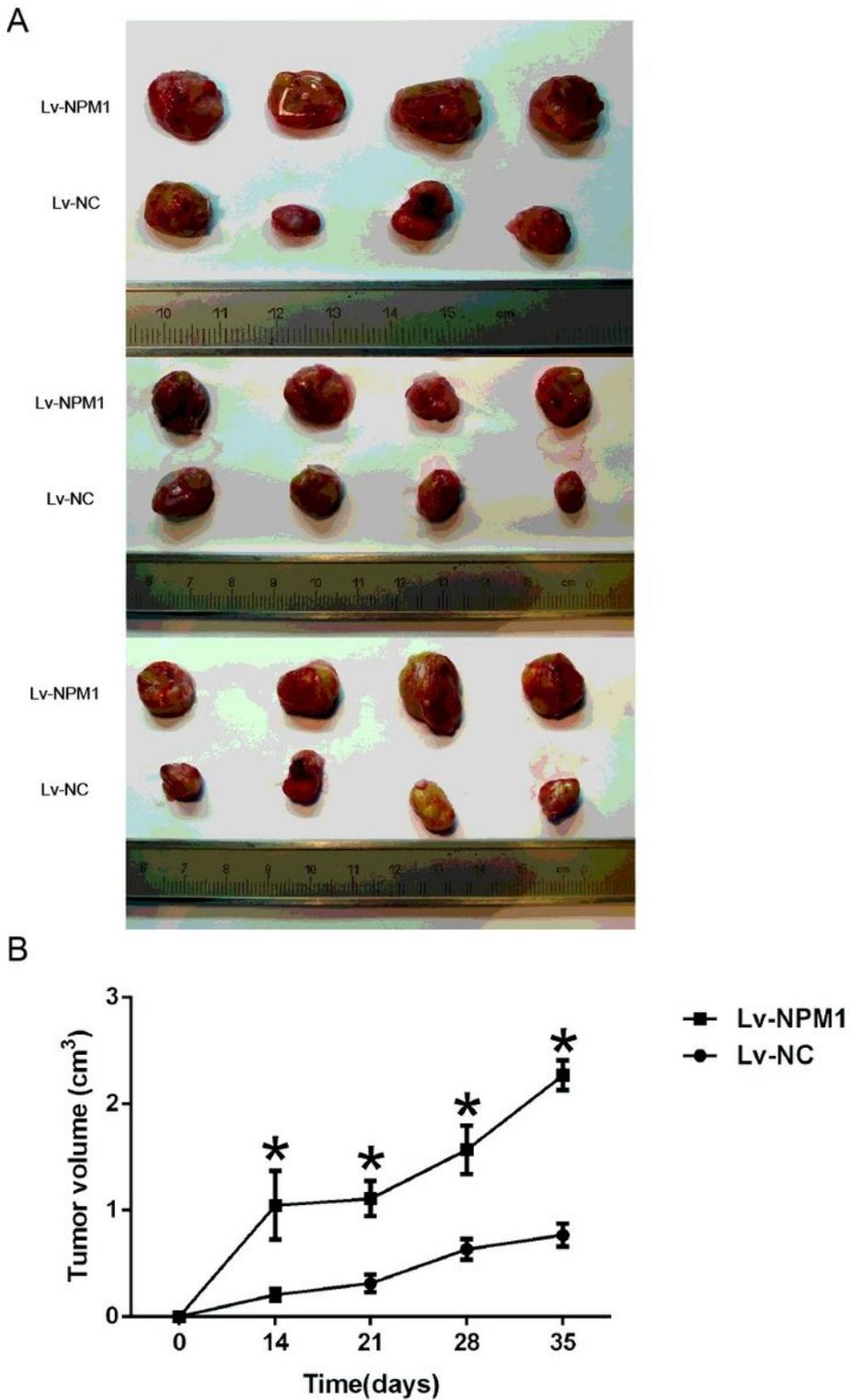
**Figure 1**

The effect of NPM1 silencing and NPM1 overexpressing on cisplatin resistant bladder cancer cell migration. With experimental times lasting (0-48 hours), the cell migration rate of NPM1 silencing cisplatin resistant bladder cancer cells was higher than that of negative control and NPM1 overexpressing bladder cancer cells.



**Figure 2**

The effect of NPM1 silencing and NPM1 overexpressing on the cell invasion of cisplatin resistant bladder cancer cells. 48 hours after the experiment, NPM1 silencing cisplatin resistant bladder cancer cells were more invasive than negative control cells.



**Figure 3**

Tumorigenicity comparison between NPM1 silencing and the negative control bladder cancer cells in nude mice. 35 days after the experiment, nude mice injected with NPM1 silencing cells had more pronounced tumor masses than the negative control nude mice.

## Supplementary Files

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