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Original Article

miR-613 inhibits bladder cancer proliferation and migration through targeting SphK1

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Abstract: Objectives: Increasing evidence has suggested that microRNA (miRNA) dysregulation may contribute to tumor progression and metastasis. However, the role of miR-613 in bladder cancer was still unknown. Materials and methods: qRT-PCR and Western blotting were performed to detect the expression of miR-613 and its direct target gene. CCK-8 analysis, qRT-PCR and cell invasion were performed to measure the cell function. Results: We demonstrated that the expression of miR-613 was downregulated in the bladder cancer cell lines. In addition, miR-613 expression was downregulated in the bladder cancer tissues compared to the adjacent normal tissues. Out of 35 bladder cancer tissues, miR-613 was downregulated in 27 cases compared to the adjacent tissues. Ectopic expression of miR-613 suppressed the bladder cancer cell proliferation and invasion. Moreover, miR-613 overexpression enhanced the expression of epithelial biomarker, Ecadherin, and suppressed the expression of mesenchymal biomarker, Vimentin, Snail and N-cadherin. Furthermore, we identified the Sphingosine kinase 1 (SphK1) as the direct target gene of miR-613 in the bladder cancer cell. Restoration of Sphk1 partially rescued miR-613-inhibited bladder cancer cell proliferation, invasion and EMT. Conclusions: These data suggested that miR-613 acted a tumor suppressive role in bladder cancer through targeting SphK1 in bladder.

Keywords: Bladder cancer, microRNAs, miRNAs, miR-613, SphK1

Introduction

Bladder cancer ranks as the ninth common cause of cancer-related death in the men, accounting for approximately 3% of total cancer-related death [1-5]. Many established factors are considered to contribute to progression and tumorigenesis of bladder cancer, including poison and smoking exposure [6, 7]. Although several treatments such as radiotherapy, surgical operation and chemotherapy have been gained, the 5-year survival rate is still dissatisfied [8-11]. Therefore, it is indispensable for us to reveal the molecular mechanism for bladder cancer tumorigenesis and progression.

MicroRNAs (miRNAs) are non-coding, small (19-22 nucleotides) and endogenous RNAs that regulate gene expression through binding to the complementary sequences in 3'UTR of the target gene [5, 12-15]. MiRNAs play a pivotal role in cell development, apoptosis, proliferation, differentiation and invasion [16]. Aberrant miRNAs expression is found in almost all human cancers and acts as classical tumour suppressor genes or oncogenes [17-21]. Increasing studies prove that miRNAs are involved in the development of many tumors, including gastric cancer, hepatocellular carcinoma, glioma, cutaneous squamous cell carcinoma and also bladder cancer [5, 22-25].

In this study, we demonstrated that the expression of miR-613 was downregulated in the bladder cancer cell lines and tissues. Ectopic expression of miR-613 suppressed the bladder cancer cell proliferation, invasion and epithelial mesenchymal transition (EMT). Furthermore, we identified the Sphingosine kinase 1 (SphK1) as the direct target gene of miR-613 in the bladder cancer cell.

Material and methods

Tissues, cell lines, cell culture and miRNA transfection

Bladder cancer tissues and adjacent normal tissues were obtained from the second affiliat-

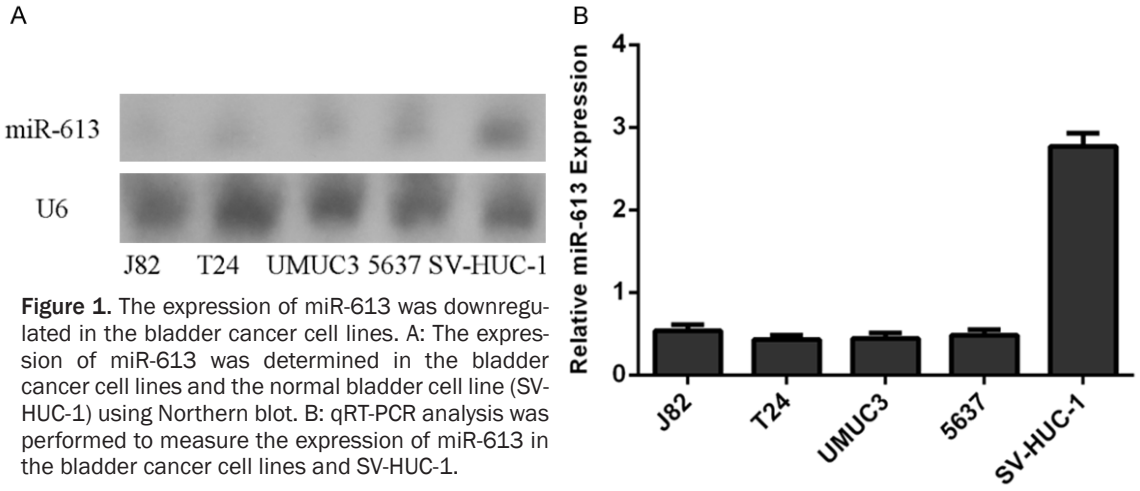

Figure 1. The expression of miR-613 was downregulated in the bladder cancer cell lines. A: The expression of miR-613 was determined in the bladder cancer cell lines and the normal bladder cell line (SV-HUC-1) using Northern blot. B: qRT-PCR analysis was performed to measure the expression of miR-613 in the bladder cancer cell lines and SV-HUC-1.

ed hospital of Wenzhou Medical University. All patients were given written informed consent and this study was also approved by the Ethics Committee of The second affiliated hospital of Wenzhou Medical University. Bladder cancer cell lines (J82, T24, UMUC3 and 5637) and a normal bladder cell line (SV-HUC-1) were bought from the Cell Resource Center of Chinese Academy of Medical Sciences. Cell lines were kept in the RPMI 1640 medium. The miR-613 mimics and the scramble oligonucleotide were synthesized by the GenePharma (Shanghai). Cell transfection was done by the Lipofectamine 2000 following to instructions.

Quantitative real-time PCR

Total RNAs were isolated from tisues or cells with Trizol (Invitrogen) according to the protocol. MiRNAs and mRNAs were measured using quantitative real-time PCR following to the manufacturer's protocol. U6 snRNA was used as the control expression for miRNA and SphK1 was normalized against GAPDH. Primers were used as follows: Sphk1, 5'-CTGTCACCCATGAACCTGCT-3' (forward), reverse, 5'-TACAGGGAGGTAGGCCAGTC-3' (reverse); GAPDH, 5'-GGGAGCCAAAAGGGTCATCA-3' (forward), 5'-TGATGGCATGGACTGTGGTC-3' (reverse). The relative expression of miR-613 in bladder cancer cells and tissues was measured by the $2^{-\Delta\Delta CT}$ method.

Luciferase assays

Cell was seeded in the 96-well plate and tranfected by the mixture of pLuc-3'-UTR, miR-613 mimic or scramble and Renilla following to the manufacturer's protocol. Cell tranfection was performed by using Lipofectamine 2000 according to the recommended protocol. After 48 hours, the Renilla and firefly luciferase activity was detected with the Dual-Luciferase Reporter System (Promega). The relative luciferase activities were normalized to the Renilla luciferase activities.

Cell growth assay, colony formation and migration assay

MTT assay was assessed to detecte the cell proliferation according to the manufacturer's instruction. Cell was cultured in the 96-well plate and was detected at 24, 48 and 72 hours after tranfection. MTT was put into the medium and incubated at 37°C for 4 hours. The absorbance (OD) was measured at 570 nm. For colony formation assay, the cell was counted and cultured in the 6-well plate for about 2 weeks. The colony was fixed with methanol and stained with crystal violet (Sigma). Wound healing assay was done to detecte the cell migration. Cell was seeded in the 6-well plate and wound was performed using the sterile pipette tip. The cell was cultured for 24 hours or 48 hours and wound closure was detected by the inverted microscope (Olympus, Japan).

Western blot

Total proteins were extracted from the tissues or cells in the RIPA lysis buffer. Protein was resolved on the 10% SDS and transferred onto PVDF membrane (Invitrogen, CA). The membrane was blocked with no-fat milk for 1 hour and then immunoblotted using specific anti-

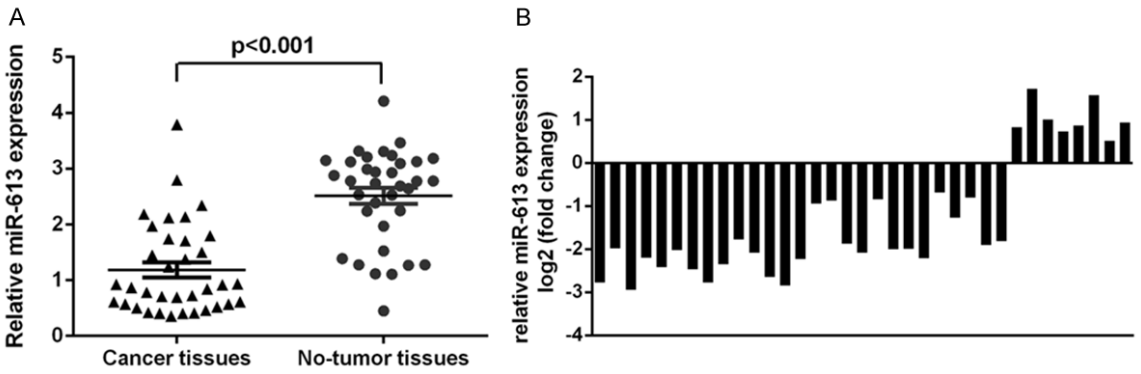

Figure 2. miR-613 expression was downregulated in the bladder tumor tissues. A: The expression of miR-613 was downregulated in the bladder cancer tissues compared to the adjacent normal tissues. B: Out of 35 bladder cancer tissues, miR-613 was downregulated in 27 cases (27/35, 77%) compared to the adjacent tissues.

body such as SphK1 and GAPDH at 4°C overnight. The membrane was incubated with the secondary antibodies and measured by ECL (enhanced chemiluminescence system) (Millipore, WI).

Statistical analysis

Statistical assay was performed in the SPSS17. Data was shown as mean ± SD. Comparisons between groups were used by the Student's t-test. Statistical significance between three or more groups was measured by one-way ANOVA. $P<0.05$ was set statistically significant.

Results

The expression of miR-613 was downregulated in the bladder cancer cell lines

Northern blot showed that miR-613 expression was downregulated in the bladder cancer cell lines (J82, T24, UMUC3 and 5637) compared to the normal bladder cell line (SV-HUC-1) (**Figure 1A**). In addition, qRT-PCR analysis data also showed that the expression of miR-613 was downregulated in the bladder cancer cell lines compared to the normal bladder cell line (**Figure 1B**).

miR-613 expression was downregulated in the bladder tumor tissues

We demonstrated that miR-613 expression was downregulated in the bladder cancer tissues compared to the adjacent normal tissues (**Figure 2A**). Out of 35 bladder cancer tissues, miR-613 was downregulated in 27 cases (27/35, 77%) compared to the adjacent tissues (**Figure 2B**).

miR-613 overexpression suppressed the bladder cancer cell proliferation and invasion

miR-613 was upregulated in the bladder cancer cell T24 cell after treated with miR-613 mimic (**Figure 3A**). Overexpression of miR-613 suppressed the T24 cell proliferation using CCK-8 analysis (**Figure 3B**). Moreover, miR-613 overexpression inhibited the ki-67 expression in the T24 cell (**Figure 3C**). Ectopic expression of miR-613 suppressed the PCNA expression in the T24 cell (**Figure 3D**). Furthermore, overexpression of miR-613 inhibited the T24 cell invasion (**Figure 3E**).

Ectopic expression of miR-613 inhibited the epithelial mesenchymal transition (EMT)

As shown in the **Figure 4A**, E-cadherin expression was upregulated in the T24 cell after treated with the miR-613 mimic while the expression of N-cadherin, Vimentin and Snail was downregulated in the T24 cell after treated with the miR-613 mimic. Overexpression of miR-613 enhanced the expression of E-cadherin and suppressed the expression of N-cadherin, Vimentin and Snail (**Figure 4B**).

Sphk1 was identified as a direct target gene of miR-613 in the bladder cancer cell

TargetScan software suggested that there was one highly conserved miR-613 binding site in the Sphk1 3'-UTR region, so we constructed the Sphk1 3'-UTR with the mutant or wild-type binding site into the firefly luciferase downstream (**Figure 5A**). Moreover, miR-613 overexpression suppressed the luciferase activity of the wild-

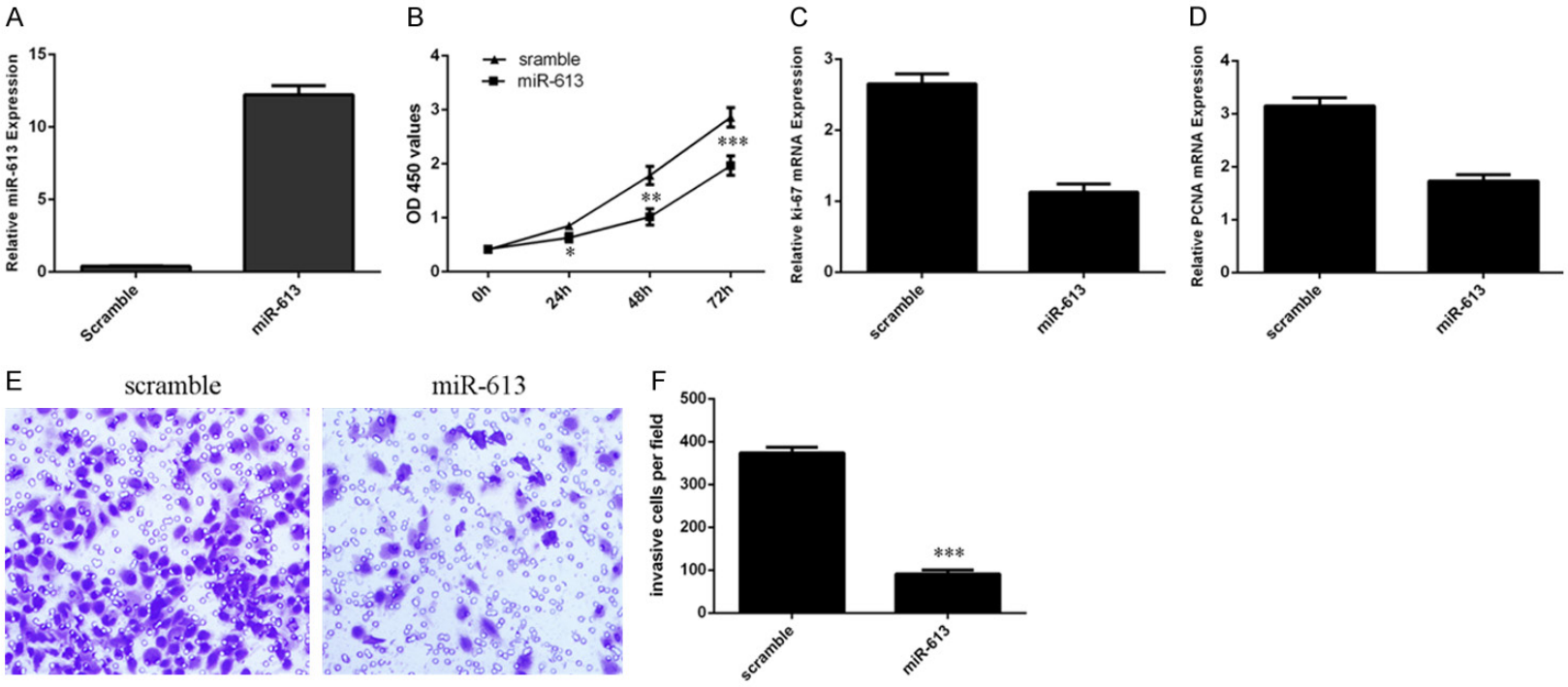

Figure 3. miR-613 overexpression suppressed the bladder cancer cell proliferation and invasion. A: The expression of miR-613 in the T24 cell after treated with miR-613 mimic was determined by using qRT-PCR. B: Ectopic expression of miR-613 suppressed the T24 cell proliferation. C: Overexpression of miR-613 suppressed the ki-67 expression in the T24 cell. D: Ectopic expression of miR-613 inhibited the PCNA expression in the T24 cell. E: miR-613 overexpression suppressed the T24 cell invasion. F: The relative invasive cells were shown. *P<0.05, **P<0.01 and ***P<0.001.

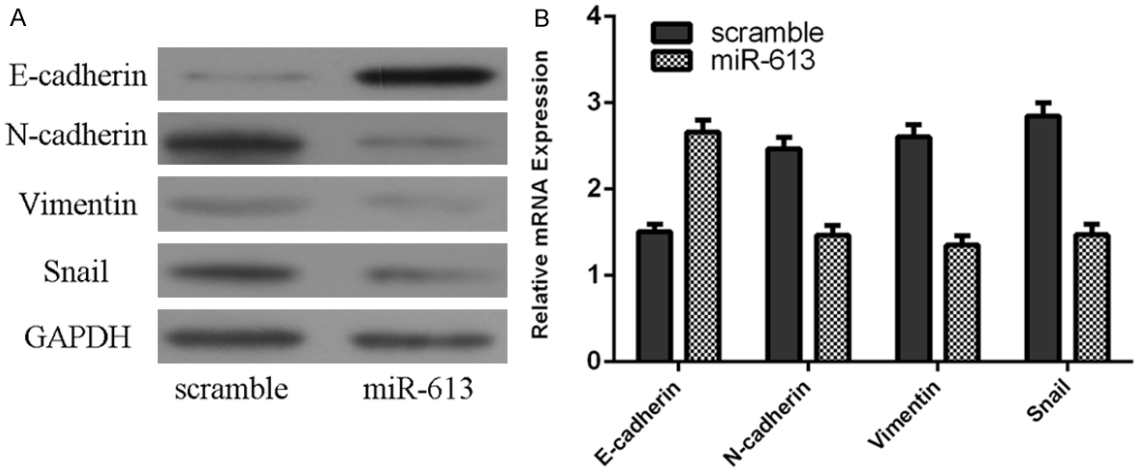

Figure 4. Ectopic expression of miR-613 inhibited the epithelial mesenchymal transition (EMT). A: The protein expression of E-cadherin, N-cadherin, Vimentin and Snail in the T24 cell was measured by using western blot. B: The mRNA expression of E-cadherin, N-cadherin, Vimentin and Snail in the T24 cell was measured by using qRT-PCR.

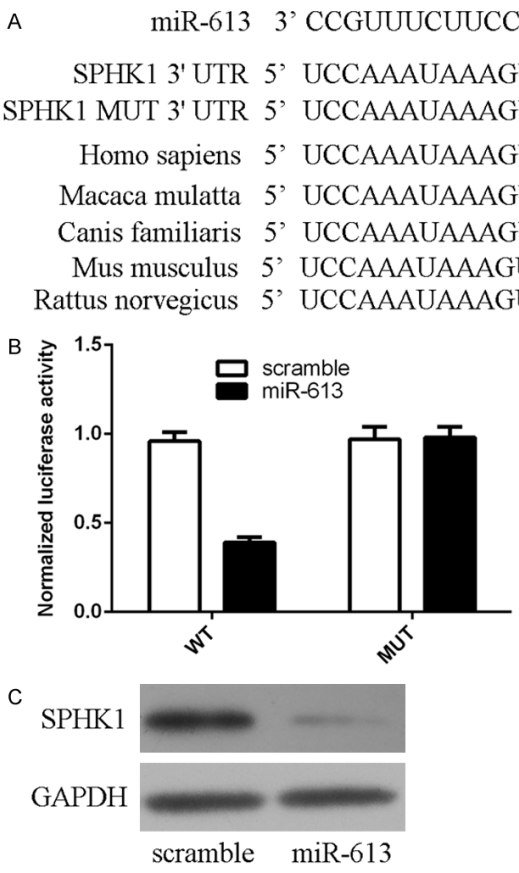

Figure 5. Sphk1 was identified as a direct target gene of miR-613 in the bladder cancer cell. A: TargetScan software There was one highly conserved miR-613 binding site in the Sphk1 3'-UTR region by using TargetScan software. B: Ectopic expression of miR-613 suppressed the luciferase activity of the wild-type Sphk13'-UTR, but not the mutant Sphk13'-UTR. C: Overexpression of miR-613 decreased the Sphk1 protein expression in the T24 cell.

type Sphk13'-UTR, but not the mutant Sphk13'-UTR (**Figure 5B**). In addition, ectopic expression of miR-613 decreased the Sphk1 protein expression (**Figure 5C**).

Sphk1 partially rescued miR-613-inhibited bladder cancer cell proliferation, invasion and EMT

The expression of Sphk1 was also upregulated in the T24 cell after treated with pCDNA-Sphk1 vector (**Figure 6A** and **6B**). The proliferation and invasion abilities of miR-613 overexpressing T24 cells were partially induced after treated with pCDNA-Sphk1 vector (**Figure 6C-E**). Moreover, restoration of Sphk1 could inhibit the expression of epithelial biomarker, Ecadherin, and enhance the expression of mesenchymal biomarker, Vimentin, Snail and N-cadherin (**Figure 6F** and **6G**).

Discussion

In this study, we demonstrated that miR-613 expression was downregulated in the bladder cancer cell lines and tissues. Out of 35 bladder cancer tissues, miR-613 was downregulated in 27 cases compared to the adjacent tissues. Ectopic expression of miR-613 suppressed the bladder cancer cell

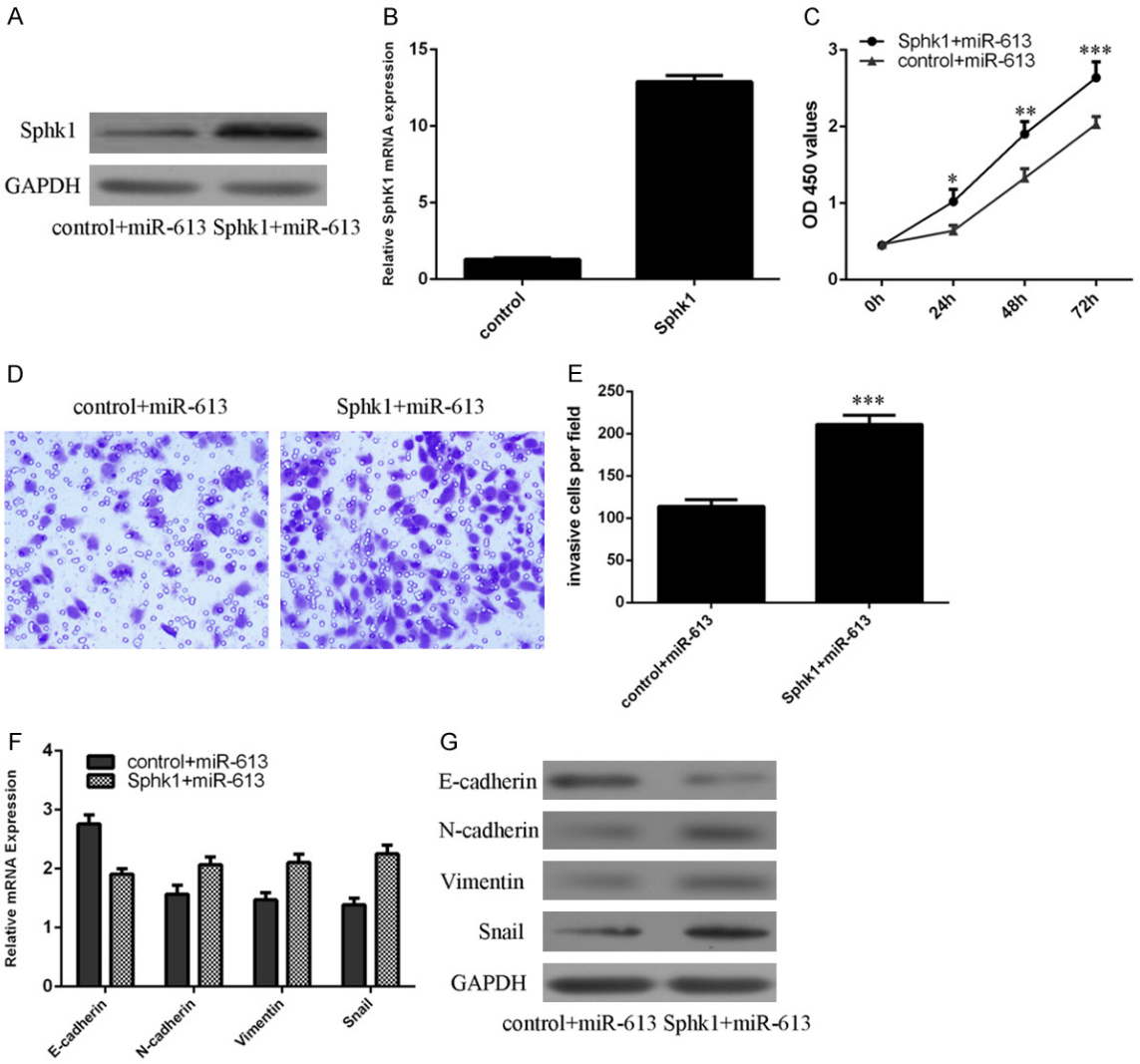

Figure 6. Sphk1 partially rescued miR-613-inhibited bladder cancer cell proliferation, invasion and EMT. A: The protein expression of Sphk1 was measure by western blot. B: The mRNA expression of Sphk1 was determined by qRT-PCR. C: The T24 cell proliferation was measured by CCK-8 analysis. D: The invasion abilities of miR-613 overexpressing T24 cells were partially induced after treated with pCDNA-Sphk1 vector. E: The relative invasive cells were shown. F: The mRNA expression of E-cadherin, N-cadherin, Vimentin and Snail in the T24 cell was measured by using qRT-PCR. G: The protein expression of E-cadherin, N-cadherin, Vimentin and Snail in the T24 cell was measured by using western blot. *P<0.05, **P<0.01 and ***P<0.001.

proliferation and invasion. Moreover, miR-613 overexpression enhanced the expression of epithelial biomarker, Ecadherin, and suppressed the expression of mesenchymal biomarker, Vimentin, Snail and N-cadherin. Furthermore, we identified the Sphk1 as the direct target gene of miR-613 in the bladder cancer cell. Restoration of Sphk1 partially rescued miR-613-inhibited bladder cancer cell proliferation, invasion and EMT. Taken together, our data suggested that miR-613 was a potent tumor suppressor miRNA in the bladder cancer, and its effects are partly mediated through its downstream target gene, Sphk1.

Emerging evidences showed that aberrant expression of miRNA was correlated with a lot of tumors such as gastric cancer, renal cell carcinoma, hepatocellular carcinoma and also bladder cancer [26-29]. miRNAs act as oncogenes or tumor suppressors with critical role in the initiation, development and progression of tumors [30-32]. Previous studies demonstrated that miR-613 expression was down-

regulated in the human ovarian cancer tissues and cell lines [33]. Overexpression of miR-613 inhibited the ovarian cancer cell colony formation, proliferation and invasion through targeting the KRAS expression [34]. Ren et al. [35] showed that miR-613 expression was downregulated in the prostate cancer tissues and ectopic expression of miR-613 suppressed prostate cancer cell invasion and proliferation through inhibiting the Frizzled7 (Fzd7) expression. Li et al. [36] demonstrated that miR-613 was downregulated in the non-small cell lung cancer (NSCLC) tissues. Overexpression of miR-613 suppressed the NSCLC cell proliferation, colony formation and cell cycle through inhibiting CDK4 expression. Wang et al. [37] showed that miR-613 expression was downregulated in the hepatocellular carcinoma (HCC) tissues and overexpression of miR-613 inhibited the HCC cell proliferation and invasion through targeting the doublecortin-like kinase 1 (DCLK1) expression. However, the role of miR-613 in the bladder cancer is still unknown. In our study, we showed that the expression of miR-613 was downregulated in the bladder cancer cell lines. In line with this, miR-613 expression was lower in the bladder cancer tissues compared to the adjacent normal tissues. Out of 35 bladder cancer tissues, miR-613 was downregulated in 27 cases compared to the adjacent tissues. Overexpression of miR-613 inhibited the bladder cancer cell proliferation and invasion. In addition, miR-613 overexpression enhanced the expression of epithelial biomarker, Ecadherin, and suppressed the expression of mesenchymal biomarker, Vimentin, Snail and N-cadherin.

Most importantly, our data established Sphk1 as a direct functional effector of miR-613 in the bladder cancer cell. Sphk1 is a master kinase, conservative enzyme that modulates the balance between S1P and ceramide/sphingosine levels and is involved in a lot of cellular behaviors such as cell proliferation, cycle, migration, apoptosis, invasion, and metabolism [38-41]. Recent studies have demontrated that SphK1 is upregulated in various cancers including breast cancer, liver cancer and colorectal cancer [40, 42-44]. For example, Meng et al. [45] demonstrated that SPHK1 expression was upregulated in the bladder cancer and was correlated with histologic gradeand tumor stage. Patients with high expression of SPHK1 had reduced overall 5-year survival rates. However, the regulation of the SPHK1 at the posttranscription level is not well investigated in tumor. Recently, Zhao et al. [46] showed that miR-125b suppressed the bladder cancer cell growth and migration partially by regulating the expression of Sphk1. In our study, we demonstrated that overexpression of miR-613 suppressed the luciferase activity of the wild-type Sphk13'-UTR, whereas the mutant Sphk13'-UTR was not affected. In addition, ectopic expression of miR-613 inhibited the Sphk1 protein expression. Moreover, Sphk1 partially rescued miR-613-inhibited bladder cancer cell proliferation, invasion and EMT. These data suggested that miR-613 suppressed the bladder cancer cell proliferation and migration partially by regulating the expression of Sphk1.

In summary, we demonstrated that miR-613 was downregulated in bladder cancer tissues and may be a potent tumor suppressor that suppressed the bladder cancer cell proliferation, invasion and EMT partly through targeting Sphk1. The newly identified miR-613/Sphk1 axis provides a new insight into the bladder cancer pathogenesis and represents a potential implication for bladder cancer therapy.

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Disclosure of conflict of interest

None.

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