Hsa-miR-125b suppresses bladder cancer development by down-regulating oncogene SIRT7 and oncogenic long non-coding RNA MALAT1

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ABSTRACT

MicroRNAs mainly inhibit coding genes and long non-coding RNA expression. Here, we report that hsa-miR-125b and oncogene SIRT7/oncogenic long non-coding RNA MALAT1 were inversely expressed in bladder cancer. Hsa-miR-125b mimic down-regulated, whereas hsa-miR-125b inhibitor up-regulated the expression of SIRT7 and MALAT1. Binding sites were confirmed between hsa-miR-125b and SIRT7/MALAT1. Up-regulation of hsa-miR-125b or down-regulation of SIRT7 inhibited proliferation, motility and increased apoptosis. The effects of up-regulation of hsa-miR-125b were similar to that of silencing MALAT1 in bladder cancer as we had previously described. These data suggest that hsa-miR-125b suppresses bladder cancer development via inhibiting SIRT7 and MALAT1.

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1. Introduction

Bladder cancer is a common malignancy in the world. The most common subtype of this cancer is urothelial carcinoma. The carcinogenesis of urothelial carcinoma of the bladder is not clearly understood [1].

MicroRNAs (miRNAs) are small non-coding RNAs of about 22 nucleotides in length that negatively regulate coding gene [2] and long non-coding RNA (lncRNA) expression [3]. They are aberrantly expressed and act as oncogenes or tumor suppressors in cancer [4].

Hsa-miR-125b is down-regulated in cancer [5,6]. We previously found that hsa-miR-125b was down-regulated in bladder cancer [7]. In silico analysis indicated SIRT7 and lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) were predictable targets of hsa-miR-125b. SIRT7 is a mammalian sirtuin family member, which is up-regulated in breast cancer [8]. But its expression pattern in bladder cancer is not known. MALAT1 is overexpressed in several cancers [9]. We previously identified that MALAT1 was up-regulated and played oncogenic roles in bladder cancer [10].

In this study, hsa-miR-125b was down-regulated, whereas SIRT7 and MALAT1 were up-regulated in bladder cancer. SIRT7 and MALAT1 were identified as targets of hsa-miR-125b. Hsa-miR-125b inhibited bladder cancer cell growth, induced apoptosis and decreased cell motility via down-regulating SIRT7 and MALAT1.

2. Materials and methods

2.1. Patient samples

Twenty-seven patients with bladder urothelial carcinomas and paired adjacent histologically normal tissues and 12 patients with bladder urothelial carcinomas were included in the study. We obtained the informed consent from all the patients and the approval of the study from the Institutional Review Board of the Second People’s Hospital of Shenzhen, Shenzhen, China.
2.2. Cell culture and transfection

Bladder cancer T24 and 5737 cells, urothelial SV-HUC-1 cells and human embryo kidney 293T cells (HEK 293T, 293T) were purchased from the Institute of Cell Research, Chinese Academy of Sciences, Shanghai, China. The SV-HUC-1 cells were cultured in F-12K (Corning, Manassas, VA, USA) plus 10% fetal bovine serum. The other cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) plus 10% fetal bovine serum.

SIRT7 siRNA (target sequence: 5'-CTCACCGTATTTCTACTACTA-3') and Allstars Negative Control siRNA were purchased from Qiagen, Hilden, Germany. Hsa-miR-125b mimic/negative control mimic and hsa-miR-125b inhibitor/negative control inhibitor were purchased from Ribo, Guangzhou, China. The SIRT7 ORF containing pReceiver vector (SIRT7 ORF vector) and the control pReceiver vector (control vector) were purchased from GeneCopoiea Inc, Rockville, MD, USA.

The cells were incubated with either SIRT7 siRNA/negative control siRNA, hsa-miR-125b mimic/negative control mimic or hsa-miR-125b inhibitor/negative control inhibitor using Nanofectin™ Transfection reagent (Excell Bio, Shanghai, China) according to the protocol. The final concentrations of SIRT7 siRNA/negative control siRNA, hsa-miR-125b mimic/negative control mimic, hsa-miR-125b inhibitor/negative control inhibitor and SIRT7 ORF vector/control vector were 20, 100, 200 nM and 1 µg/ml respectively.

2.3. Total RNA extraction and reverse transcription

Total RNA was extracted from the tissues or cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. Ten micrograms of total RNA was converted to

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### Table 1

<table>
<thead>
<tr>
<th>Hsa-miR-125b</th>
<th>MALAT1</th>
<th>SIRT7</th>
</tr>
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<tbody>
<tr>
<td>2^(-ΔΔCt)(hsa-miR-125b-U6)</td>
<td>2^(-ΔΔCt)(MALAT1-GAPDH)</td>
<td>2^(-ΔΔCt)(SIRT7-GAPDH)</td>
</tr>
<tr>
<td><strong>P value</strong></td>
<td><strong>P value</strong></td>
<td><strong>P value</strong></td>
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<tr>
<td><strong>Grade</strong></td>
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<tr>
<td>Low (n = 20)</td>
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<td>3.22 ± 0.97</td>
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<tr>
<td>High (n = 19)</td>
<td>20.01 ± 8.44</td>
<td>12.50 ± 1.93</td>
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<tr>
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<tr>
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<td>3.90 ± 1.11</td>
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<td>T2-3N0M0 (n = 29)</td>
<td>24.13 ± 2.96</td>
<td>8.66 ± 1.58</td>
</tr>
</tbody>
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Data are shown as mean ± S.E.M.
*American Joint Committee on Cancer TNM classification.
cDNA using the All-in-One™ miRNA qRT-PCR Detection Kit (GeneCo-opoea Inc, Rockville, MD, USA) according to the manufacturer’s protocol.

2.4. Real-time quantitative polymerase chain reaction (qPCR)

qPCR primers against mature miRNA hsa-miR-125b and homo sapiens snRNA U6 were purchased from GeneCo-opoea Inc, Rockville, MD, USA. SIRT7 primers were forward: 5’-CGTCGGGAACGCCAATAC-3’, reverse: 5’-GAGGGTCGGTGCTACGATT-3’. MALAT1 primers were forward: 5’-AAAGCAAGGTCTCCCAAAAGCA-3’, reverse: 5’-GGTGCGTGCTAGATCAAAAGGCA-3’. The PCR procedures were as described elsewhere [7,10]. Hsa-miR-125b was normalized to snRNA U6. GAPDH was used as an internal control for SIRT7 and MALAT1. Expression fold changes were calculated using 2^-ΔΔCt methods [12].

2.5. Western blot analysis

Antibodies specific to SIRT7 and GAPDH were purchased from Santa Cruz, Dallas, Texas, USA. Western blots were performed as described elsewhere [13].

2.6. miRNA target prediction

The binding site prediction for hsa-miR-125b within SIRT7 3’UTR was performed using miRanda (http://www.microrna.org), microCosm Targets (http://www.ebi.ac.uk/enright-srv/microcosm) and TargetScan (http://www.Targetscan.org). Binding sites between hsa-miR-125b and MALAT1 were predicted with miRanda.

2.7. Dual luciferase reporter assay

The fragment 5’-caccagccccgtCTCAGGGc-3’ (position 316, NM_016538) from the 3’ untranslated region (3’UTR) of SIRT7 containing the predicted hsa-miR-125b binding site was chemically synthesized and cloned into the Xhol and Notl sites of the psi-CHECK-2 vector (Promega, Madison, WI, USA). The resulted vector was called the reporter vector SIRT7 3’UTR-wild-type (WT). The corresponding mutant construct was created by mutating the hsa-miR-125b seed region binding site (seed sequence binding fragment 5’-CTCAGGG-3’ changed to 5’-GAACAT-3’), which was called the reporter vector SIRT7 3’UTR-mutated-type (MUT). The fragments 5’-ttuggcacgaacacctTCAGGGa-3’ (position 3386, NR_002819) and 5’-tttatttccagaaCTCAGGGg-3’ (position 6183, NR_002819) from MALAT1 containing the predicted hsa-miR-125b binding site were chemically synthesized and cloned into

Fig. 2. Expression changes of SIRT7 or MALAT1 after transfection of SIRT7 specific siRNA or hsa-miR-125b mimic or inhibitor in T24 and 5637 cells. The relative mRNA expression levels were evaluated using real-time qPCR. Data are indicated as mean ± S.D. SIRT7 protein levels were determined using Western blot assay. Each experiment was performed in triplicate for three independent times. (A) SIRT7 specific siRNA significantly down-regulated the expression of SIRT7 at mRNA levels. (B) Representative images of Western blot results indicated SIRT7 specific siRNA significantly down-regulated the expression of SIRT7 at protein levels. (C) Hsa-miR-125b mimic significantly down-regulated the expression of SIRT7 at mRNA levels. (D) Representative images of Western blot results indicated that hsa-miR-125b mimic significantly down-regulated the expression of SIRT7 at protein levels. (E) Hsa-miR-125b inhibitor significantly up-regulated the expression of SIRT7 at mRNA levels. (F) Representative images of Western blot results indicated that hsa-miR-125b inhibitor significantly up-regulated the expression of SIRT7 at protein levels. (G) Hsa-miR-125b mimic significantly down-regulated MALAT1 expression levels. (H) Hsa-miR-125b inhibitor significantly up-regulated MALAT1 expression levels.
the Xhol and NotI sites of the psiCHECK-2 vector, respectively. The resulted vectors were called the reporter vector MALAT1-1-wild-type (WT) and the reporter vector MALAT1-2-wild-type (WT), respectively. The corresponding mutants were created by mutating the hsa-miR-125b seed region binding site (seed sequence binding fragment 5′-TCAGGG-3′ changed to 5′-AGCAAA-3′), which were called the reporter vector MALAT1-1-mutated-type (MUT) and the reporter vector MALAT1-2-mutated-type (MUT) respectively.

Hsa-miR-125b mimic or negative control mimic was cotransfected with the reporter vectors containing either the targeting sequences or the corresponding mutants using NanoFectin™ Transfection Reagent (Excell Bio, Shanghai, China) according to the protocol. The luciferase activities were then determined using a Multimode Detector (Beckman Coulter, CA, USA).

2.8. Cell proliferation assay

Cell proliferation was measured using MTT assay. Cells were seeded in a 96-well plate and cultured in normal medium. At 0, 24, 48 and 72 h after transfection, the cells were incubated in 0.1 mg/ml MTT at 37 °C for 4 h and lysed in dimethyl sulfoxide (DMSO) at room temperature for 10 min. The absorbance in each well was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

2.9. Cell apoptosis determined by caspase 3 enzyme-linked immunosorbent assay (ELISA)

Forty-eight hours after transfection, apoptosis caused by SIRT7 siRNA, hsa-miR-125b mimic or hsa-miR-125b inhibitor or SIRT7 ORF vector or control vector was evaluated by analyzing the activity of caspase 3 using the caspase 3 ELISA assay kit (R&D, Minneapolis, MN, USA) according to the manufacturer’s protocol. A microplate reader (Bio-Rad, Hercules, CA, USA) was used to determine optical density (OD) values. Data were shown as the ratios between the OD values of SIRT7 siRNA, hsa-miR-125b mimic or hsa-miR-125b inhibitor and negative control or SIRT7 ORF vector and control vector transfected cells.

3. Results

3.1. Hsa-miR-125b and SIRT7/MALAT1 were inversely expressed in bladder cancer

The relative expression levels of hsa-miR-125b, SIRT7 and MALAT1 were evaluated using real-time qPCR in 27 patients with bladder urothelial carcinomas and paired adjacent histologically normal tissues and 12 patients with bladder urothelial carcinomas. Hsa-miR-125b was down-regulated in bladder cancer compared to normal tissues.

Fig. 3. Hsa-miR-125b mimic reduced the luciferase activities. (A) The predicted hsa-miR-125b binding site located in SIRT7 3′UTR. Seed sequence and the mutant are underscored. (B) The relative luciferase activities were inhibited in the cells transfected with the reporter vector SIRT7 3′UTR-WT, not in the cells transfected with the reporter vector SIRT7 3′UTR-MUT. (C) The first predicted hsa-miR-125b binding site located in MALAT1. Seed sequence and the mutant are underscored. (D) The relative luciferase activities were inhibited in the cells transfected with the reporter vector MALAT1-1-WT, not in the cells transfected with the reporter vector MALAT1-1-MUT. (E) The second predicted hsa-miR-125b binding site located in MALAT1. Seed sequence and the mutant are underscored. (F) The relative luciferase activities were inhibited in the cells transfected with the reporter vector MALAT1-2-WT, not in the cells transfected with the reporter vector MALAT1-2-MUT. Data are shown as mean ± S.D. Each experiment was performed in triplicate for three independent times.

2.10. Wound healing assay

Bladder cancer cells were transfected with SIRT7 siRNA, hsa-miR-125b mimic, hsa-miR-125b inhibitor or negative control or SIRT7 ORF vector or control vector. Cells were scratched in the monolayer and cultured in normal condition. The migrated distances were measured at 0, 16 h after scratching for T24 cells and at 0, 24 h after scratching for 5637 cells respectively.

2.11. Immunocytochemical analyses

Bladder cancer cells were transfected with SIRT7 ORF vector or control vector. Forty-eight hours after transfection, immunocytochemical analyses were performed as described elsewhere [14].

2.12. Statistical analysis

The expression differences between bladder cancer and matched urothelium were analyzed using paired samples t-test. Pearson’s coefficient correlation was used for expression correlation assay. The expression differences between high/low grades, high/low stages, cell lines, the expression changes after transfection, luciferase activity, cell apoptosis and wound healing assays were analyzed using independent samples t-test. MTT assays were analyzed using ANOVA. The statistical analyses were performed using SPSS (Version 17.0). P values were two-sided and a value of <0.05 was considered to be statistically significant.
Fig. 4. Cell proliferation, apoptosis and motility changes caused by transfection of SIRT7 siRNA/hsa-miR-125b mimic/hsa-miR-125b inhibitor. Cell proliferation was measured by MTT assay. (A) SIRT7 siRNA inhibited T24 and 5637 proliferation. (B) Hsa-miR-125b mimic inhibited T24 and 5637 proliferation. (C) Hsa-miR-125b inhibitor promoted T24 and 5637 proliferation. Cell apoptosis changes were determined by caspase 3 ELISA. (D) SIRT7 siRNA induced T24 and 5637 apoptosis. (E) Hsa-miR-125b mimic induced T24 and 5637 apoptosis. (F) Hsa-miR-125b inhibitor inhibited T24 and 5637 apoptosis. Wound healing assay was used to detect cell motility changes. (G) Representative images of cell motility changes by transfection of SIRT7 siRNA. (H) SIRT7 siRNA decreased cell motility. (I) Representative images of cell motility changes by transfection of hsa-miR-125b mimic. (J) Hsa-miR-125b mimic decreased cell motility. (K) Representative images of motility changes by transfection of hsa-miR-125b inhibitor. (L) Hsa-miR-125b inhibitor increased cell motility. Data are indicated as mean ± S.D. Each experiment in both cell lines was performed in triplicate for three independent times.
with matched normal urothelium (n = 27) (Fig. 1A). Both SIRT7 and MALAT1 were up-regulated in bladder cancer compared with matched normal urothelium (n = 27) (Fig. 1B and C). Hsa-miR-125b and SIRT7 were inversely expressed in bladder cancer (n = 27) (Fig. 1D). Hsa-miR-125b and MALAT1 were also inversely expressed in bladder cancer (n = 27) (Fig. 1E). We further analyzed the expression patterns in urothelial carcinomas according to grading and staging in a total of 39 patients. High-grade and high-stage carcinomas had higher SIRT7 and MALAT1 expression levels and lower Hsa-miR-125b expression levels than low-grade and low-stage carcinomas (Table 1). Hsa-miR-125b expression levels were lower in bladder cancer T24 and 5737 cells than in urothelial SV-HUC-1 cells (Fig. 1F). SIRT7 and MALAT1 were more abundant in T24 and 5737 cells than in SV-HUC-1 cells (Fig. 1G and H).

3.2. Hsa-miR-125b regulated the expression of SIRT7 and MALAT1

Forty-eight hours after transfection, SIRT7 and MALAT1 expression levels were measured. Both SIRT7 siRNA and hsa-miR-125b mimic down-regulated SIRT7 at mRNA and protein levels in T24 and 5637 cells (Fig. 2A–D). Hsa-miR-125b inhibitor up-regulated SIRT7 at mRNA and protein levels in T24 and 5637 cells (Fig. 2E and F). Hsa-miR-125b mimic markedly down-regulated MALAT1 expression levels in T24 and 5637 cells (Fig. 2G), whereas hsa-miR-125b inhibitor markedly up-regulated MALAT1 expression levels in T24 and 5637 cells (Fig. 2H).

3.3. Hsa-miR-125b reduced the luciferase activities

Hsa-miR-125b inhibited the luciferase activities in 293T cells transfected with the reporter vector SIRT7 3’UTR-WT, but not in 293T cells transfected with the reporter vector SIRT7 3’UTR-MUT (Fig. 3A), which indicated the hsa-miR-125b binding site within SIRT7 3’UTR was functional. Hsa-miR-125b inhibited the luciferase activities in 293T cells transfected with the reporter vector MALAT1-1-WT or MALAT1-2-WT, but not in 293T cells transfected with the reporter vector MALAT1-1-MUT or the reporter vector MALAT1-2-MUT (Fig. 3B and C), which indicated both hsa-miR-125b binding sites within MALAT1 were functional.

3.4. Cell proliferation, apoptosis and motility changes caused by transfection of SIRT7 siRNA/ hsa-miR-125b mimic/hsa-miR-125b inhibitor

Cell proliferation inhibition was observed in T24 and 5637 cells by transfection of SIRT7 siRNA (Fig. 4A) or hsa-miR-125b mimic (Fig. 4B). Cell proliferation promotion was observed in T24 and 5637 cells by transfection of hsa-miR-125b inhibitor (Fig. 4C). Increased cell apoptosis was observed in both bladder cancer cells by transfection of SIRT7 siRNA (Fig. 4D) or hsa-miR-125b mimic (Fig. 4E). Decreased cell apoptosis was observed in both bladder cancer cells by transfection of hsa-miR-125b inhibitor (Fig. 4F). Decreased cell motility was observed in both bladder cancer cells

Fig. 5. Increased SIRT7 expression after transfection of SIRT7 ORF vector. (A) Fluorescence microscopy showed increased expression of SIRT7 in SIRT7 ORF transfected T24 cells. green, SIRT7; blue, DAPI. (B) The quantified intensity change in T24 cells is expressed as mean ± S.E.M. from three independent experiments. (C) Fluorescence microscopy showed increased expression of SIRT7 in SIRT7 ORF transfected 5637 cells. green, SIRT7; blue, DAPI. (D) The quantified intensity change in 5637 cells is expressed as mean ± S.E.M. from three independent experiments.
by transfection of SIRT7 siRNA (Fig. 4G and H) or hsa-miR-125b mimic (Fig. 4I and J). Increased cell motility was observed in both bladder cancer cells by transfection of hsa-miR-125b inhibitor (Fig. 4K and L).

3.5. Transfection of SIRT7 ORF partly reversed the changes caused by transfection of hsa-miR-125b mimic

The increased expression of SIRT7 protein was observed in SIRT7 ORF transfected T24 and 5637 cells by fluorescence microscopy (Fig. 5A–D). SIRT7 ORF vector partly reversed T24 and 5637 proliferation inhibition induced by hsa-miR-125b mimic (Fig. 6A and B). SIRT7 ORF vector partly reversed T24 and 5637 apoptosis induced by hsa-miR-125b mimic (Fig. 6C). SIRT7 ORF vector partly reversed T24 and 5637 motility decrease induced by hsa-miR-125b mimic (Fig. 6D–G).

4. Discussion

In plants, miRNAs mainly direct cleavage of target mRNAs through perfect base pairing [15]. In animals, miRNAs use two distinct mechanisms to regulate gene expression. They are able to repress translation and/or decrease mRNAs when miRNAs and mRNAs are partially complementary. miRNAs are able to expedite poly(A) removal and lead to rapid mRNA decay [16]. In mammalian cells, miRNAs predominantly decrease target mRNA levels [2]. For example, miR-1 and miR-133a down-regulate their targets at both mRNA and protein levels through partial sequence pairing with the target sites [17].

In this study, the expression levels of hsa-miR-125b were down-regulated in bladder cancer. Up-regulation of hsa-miR-125b inhibited bladder cancer cell proliferation, motility and increased apoptosis. Down-regulation of hsa-miR-125b had the
reverse effects. These findings suggested that hsa-miR-125b is a tumor suppressor in bladder cancer.

The mammalian sirtuin family member SIRT7 localizes in the cytoplasm and the nucleus [18]. It is a highly selective H3K18Ac deacetylase and plays roles in chromatin regulation and cancer development [19]. SIRT7 was an in silico predictable target of hsa-miR-125b. The mature hsa-miR-125b sequence with was partially complementary with SIRT7 3’UTR target sequence. The expression levels of SIRT7 were up-regulated in bladder cancer. Hsa-miR-125b and SIRT7 were inversely expressed in bladder cancer. Hsa-miR-125b mimic down-regulated, whereas hsa-miR-125b inhibitor up-regulated the expression of SIRT7. Binding site was confirmed between hsa-miR-125b and SIRT7. Down-regulation of SIRT7 inhibited bladder cancer cell proliferation, motility and increased apoptosis. These effects were similar to that of up-regulated hsa-miR-125b in bladder cancer cells. Forced expression of SIRT7 partly reversed the changes caused by transfection of hsa-miR-125b mimic. These findings suggested that SIRT7 is an oncogene and a true target of hsa-miR-125b in bladder cancer.

Besides coding genes, IncRNAs have been experimentally verified and computationally predicted targets of miRNAs [3]. IncRNA MALAT1 regulates gene expression and pre-mRNA processing [20,21]. We previously identified that silencing MALAT1 inhibited bladder cancer cell growth, induced apoptosis and decreased cell motility [10]. MALAT1 was an in silico predictable target of hsa-miR-125b. The mature hsa-miR-125b sequence was partially complementary with MALAT1 target sequences. The expression levels of MALAT1 were up-regulated in bladder cancer cells as we had previously described [10]. These findings suggested that MALAT1 is a true target of hsa-miR-125b in bladder cancer.

Taken together, our data suggest that loss of expression of hsa-miR-125b contributes to the overexpression of SIRT7 and MALAT1 in bladder cancer. By now, the functional relevance between SIRT7 and MALAT1 is not clear. Restoration of hsa-miR-125b suppresses bladder cancer development via down-regulating SIRT7 and MALAT1, which may have potential therapeutic significance.

Acknowledgments

This work was funded by grants from the China Postdoctoral Science Foundation (Grant no. 2013MS52227, to Dr Yonghua Han), the National Natural Science Foundation of China (Grant no. 81372735, to Dr Yonghua Han), the National Basic Research Program of China (973 Program) (Grant no. 2014CB745200, to Dr Zhiming Cai) and the Basic Research Program of Shenzhen (Grant no. ZDSY20120615154448514, to Dr Yaoting Gui).

References