MicroRNA-340 Inhibits Bladder Cancer Cell Growth by Downregulating Glucose Transporter-1

Junlong Li

Shaoxing People's Hospital

Gang Xu

Shaoxing People's Hospital

Shouhua Pan (✉ 13606550587@163.com)

Shaoxing People's Hospital

Research

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Abstract

Background: MicroRNA (miRNA)-340 is emerging as a critical regulator for the development and progression of various cancers, such as oral squamous cell carcinoma and prostate cancer. However, little was known about the role of miR-340 in bladder cancer.

Methods: Bladder cancer and adjacent non-tumor tissue samples were collected from thirty patients undergoing surgical resection at Shaoxing People's Hospital. The expression of miR-340 and Glut-1 were studied via real-time quantitative PCR (RT-qPCR) or western blots. Bladder cancer T24 cells were transfected with miR-340 mimics to explore cell proliferation and apoptosis via MTT assay, flow cytometer, RT-qPCR and western blots.

Results: Compared with that of normal tissues, miR-340 expression was significantly lower, while both mRNA and protein expression of Glut-1 were higher in bladder cancer tissues. The miR-340 could negatively regulate Glut-1 expression in bladder cells. Moreover, bladder cell proliferation could be inhibited by miR-340 and the corresponding antitumor effect could partially reverse by the overexpression of Glut-1. In view of the complexity of gene networks, some other multiple pathways might also confer to miR-340 inducing bladder cell apoptosis, including downregulating PCNA, upregulating Bax and decreasing the phosphorylation levels of PI3K and AKT.

Conclusion: This work suggested an inverse correlation between miR-340 and glucose transporter-1 (Glut-1) expression in bladder cancer. miR-340/Glut-1 axis might be a potential and novel therapeutic target for the treatment of bladder cancer. More investigations need to further explore the applications of miR-340 in bladder cancer.

Introduction

Urothelial bladder cancer is a common malignancy that causes approximately 150,000 deaths every year worldwide.^{1,2,3} Approximately 25% of newly detected cases are advanced disease (muscle-invasive or metastatic disease) at diagnosis. The 5-year survival rate of metastatic disease is less than 10%.⁴ Until now, it is still a challenging issue for the treatment of bladder cancer.

Some previous works reported that metabolic reprogramming in cancer cells might be a potential therapeutic target.⁵ Glucose transporter-1 (Glut-1), as a type of transmembrane glycoprotein on the cell membrane, could take glucose across the cell membrane into cell, providing basic glucose supply for cell proliferating and dividing.^{6,7} Compared with normal tissue, the expression of Glut-1 was usually higher in tumor tissue.^{8,9,10} Additionally, a few works suggested that the expression of Glut-1 was associated with poor survival.¹¹ Thus, Glut-1 might play an important role in the proliferation and invasion of cancer cells.

MicroRNAs (miRNAs), as noncoding RNAs, have been reported to be associated with tumor development, including cell cycle regulation, transcriptional regulation, cell adhesion and differentiation.¹² The miRNA

could bind to the 3' untranslated regions (UTRs) of target mRNA in a partially complementary manner, leading to mRNA degradation or suppression of translation.¹³ Some researchers are trying to apply miRNA in early diagnosis, the classification of malignancy, and cancer treatment.¹² For example, Xu and coworkers reported that miR-340 could accelerate tumor cell glycolysis and promote cell growth and proliferation via increasing the expression of Glut-1 in oral squamous cell carcinoma.¹⁴ Some miRNAs, such as miR-129 and miR-221, could alter the expression of their targeted mRNAs and pathways in bladder cancer cells. However, the functional role of miR-340 in bladder cancer cell proliferation and apoptosis is still undetermined. Herein, for the first time, we explored miR-340/Glut-1 axis in bladder cancer and its influence on bladder cancer cell growth.

Materials And Methods

Cell culture and tissue samples

Bladder cancer T24 cells were purchased from American Type Culture Collection (ATCC) and cultured in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO, Australia) and 1% penicillin streptomycin (GIBCO) in an incubator with humidified 5% CO₂ at 37 °C.

Bladder cancer and adjacent non-tumor tissue samples were collected from thirty patients undergoing surgical resection at Shaoxing People's Hospital. Pathological examination was performed to confirm cancer diagnosis. After receiving approval from the ethics committee of Shaoxing People's Hospital (Zhejiang, China), written informed consent for the present study was obtained from these patients.

Cell transfection

T24 cells were cultured in 6 well plate until reached to 80%, and then they were used for cell transfection. Mature miR-340 mimics were purchased from (Genepharma company, Shanghai). The transfection reagent lipofectamine 2000 and miR-340 mimics (20 µmol/L) were dissolved in the MEMI respectively for 5 minutes. Subsequently, they were mixed and incubated for 25 minutes. Next, the formed complex was incubated with T24 cells for 6 h. After that, the complex was changed to normal medium and cultured for another 48 h. Finally, these cells had been prepared for the subsequent experiments.

Cell proliferation and apoptosis

Thiazolyl blue tetrazolium bromide (MTT) solution (5 mg/mL, 10 µL) was added into the 96-well plate containing cells. After incubated for 4 hours, MTT was removed and DMSO 100 µL was added. Five parallel experiments were carried out to calculate the average absorbance (OD value). The relative percentage of cell viability was calculated as follows: (mean OD value in the experimental group - mean OD value in control group) / mean OD value in control group × 100%.

Cells were collected, washed for two times with PBS. Annexin V-APC (5 µL) and 7-AAD (10 µL) were added into cell medium and incubated for 10 min. Subsequently, flow cytometry was used to analyze cell

apoptosis.

RNA extraction and real-time quantitative PCR (RT-qPCR)

Total RNA including miRNA from 60 frozen bladder tissues (30 for tumor tissues and 30 for paired adjacent normal tissues) and T24 cells, was extracted using a miRNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Then, we use All-in-One™ miRNA First-Strand cDNA Synthesis and All-in-One™ First-Strand cDNA Synthesis kits (GeneCopoeia, Inc.) to synthesize the miRNA and mRNA into cDNA. Next, RT-qPCR were performed using All-in-One™ miRNA kit and All-in-One™ qPCR Mix (GeneCopoeia, Inc.), respectively, on a CFX96™ Real-Time System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The PCR cycling procedure was first hold 95°C for 10 min, then 40 cycles at 95°C for 10 sec, 60°C for 20 sec, and 72°C for another 15 sec. The GAPDH and U6 were used as internal control of mRNA and miRNA expression, respectively, and calculated according to $2^{-\Delta\Delta C_t}$ method. All experiments were repeated three times. Primer sequence is as follows: miR-340 forward, 5'-GCGGTTATAAAGCAATGAGA-3' and reverse primer, 5'-GTGCGTGTCTGGAGTCG-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'; Glut-1 forward, 5'-AACTCTTCAGCCAGGGTCCAC-3' and reverse, 5'-CACAGTGAAGATGATGAAGAC-3', PCNA forward, 5'-GCGTG AACCTCACCAGTATGT-3' and reverse, 5'-TCTTCGGCCCTTAGTGTAATGAT-3'; Bax forward, 5'-TGCTTCAGGGTTTCATCCAGG-3' and reverse, 5'-TGGCAAAGTAGAAAAGGGCGA-3'; GAPDH forward, 5'-TGCACCACCAACTGCTTAGC-3' and reverse primer, 5'-GGCATGGACTGTGGTCATGAG-3'.

Western blots

Cells were lysed and protein was harvested with RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) with protease inhibitor cocktail (Beyotime Institute of Biotechnology), and quantified by bicinchoninic acid (BCA) analysis (Beyotime). The equal quantities protein lysates were prepared and separated using SDS-PAGE on 10% gels, and transferred onto polyvinylidene fluoride (PVDF) membranes (EMD Millipore). These membranes were blocked with 10% skimmed milk diluted with TBS supplemented with Tween-20 at room temperature for 1 h and incubated with primary antibodies for 2 h. After washing, the membranes were incubated with the secondary horseradish peroxidase-conjugated antibody for 1h. The proteins were visualized using Pierce™ ECL Western Blotting Substrate (Thermo Fisher). The housekeeping gene GAPDH was served as a loading control. Primary anti-bodies against Glut-1, PCNA, PI3K, AKT, Bax and GAPDH (Abcam), p-PI3K and p-AKT (R&D systems), were used according to manufacturer recommendations.

Dual-luciferase reporter assay

The 3' untranslated regions (UTR) of Glut-1 mRNA (GenBank accession number, NM_006516) was amplified (wild type). The PCR products of Glut-1 3'UTR were cloned the fragment into the SacI and XhoI sites in the pmirGLO vector (Thermo Scientific; Waltham, MA, USA). The corresponding mutant construct was generated by mutating the seed region of miR-340 by site-specific mutagenesis using the following primers: forward primer, 5'-agacaagcaacagcttattttttt-3'; reverse primer, 5'-tcagtaataaaaaaataaaataagctg-

3'. T24 cells were co-transfected with miR-NC mimics or miR-340 mimics (20 nmol/L) using lipofectamine 2000 for 48 h. Relative luciferase activity was performed according to manufacturer's instructions (Promega).

Results

miR-340 is down-regulated in urothelial bladder carcinoma

To evaluate the role of miR-340, thirty pairs of tumor and adjacent non-tumor tissues were obtained from urothelial bladder carcinoma patients. The corresponding baseline characteristics was shown in **Table S1**. The RT-qPCR results indicated that miR-340 was significantly downregulated in the urothelial bladder carcinoma tissues compared with matched adjacent non tumor tissues ($p < 0.001$, **Figure 1A**). In contrast, the mRNA level of Glut-1 in tumor tissue was substantially higher compared with the matched normal tissues. ($p < 0.001$, **Figure 1B**) Western blot revealed that the Glut-1 protein expression also had a similar trend. (**Figure 1C**).

Glut-1 mRNA is a target of miR-340

To further understand the relationship between miR-340 and Glut-1, T24 cells were transfected with miR-NC mimics or miR-340 mimics (20 nmol/L) respectively. RT-qPCR showed that miR-340 expression was specifically and dramatically increased in the miR-340 mimics-transfected cells. In contrast, similar results were not observed in control cells or miR-NC mimics-transfected T24 cells. (**Figure 2A**). As for Glut-1, RT-qPCR analysis revealed that Glut-1 mRNA level was decreased in the miR-340 mimics-transfected cells. Similarly, western blot revealed that the Glut-1 protein level was also decreased in the miR-340 mimics-transfected cells, compared with that in miR-NC mimics-transfected cells (**Figure 2B and 2C**). These results suggested that miR-340 mimics-transfected cells could increase the expression level of miR-340 and decrease level of Glut-1 protein significantly, compared with control group. To identify the downstream target gene of miR-340, dual-luciferase reporter assay was performed. Overexpressing miR-340 significantly inhibited the luciferase activity of reporter gene containing wild-type miR-340 recognition sites, whereas suppressed firefly luciferase activity was rescued in the mutant-type miR-340 recognition sites (**Figure 2D**). Such results suggested miR-340 directly might bind to the 3'-UTR of Glut-1 and regulates its expression.

Overexpression of miR-340 inhibits cell growth, while overexpression of Glut-1 reverses the antitumor effect

To explore the biological functions of miR-340/Glut-1 in cell proliferation, gain-of-function experiment of miR-340 or rescue experiment were performed respectively. Flow cytometric results showed a significant increase in the percentage of apoptotic cells for miR-340 mimic group (**Figure 3A**). Before the rescue experiment, T24 cells were transfected with pcDNA 3.1 or pcDNA3.1-Glut-1 plasmid respectively. RT-qPCR and western blot suggested both the mRNA and protein expression of Glut-1 were increased in T24 cells transfected with pcDNA3.1-Glut1 plasmid, compared T24 cells in the control or the pcDNA 3.1 group

(**Figure S1**). In T24 cells transfected with pcDNA3.1-Glut-1, the percentage of apoptotic cells recovered to that of control group (Figure 3A). The corresponding results of MTT assay also demonstrated a consistent conclusion. These results suggested overexpression of miR-340 could inhibit cell growth, but overexpression of Glut-1 reversed the antitumor effect in T24 cells.

In view of the complexity of gene networks, some important proteins involving cell proliferation and apoptosis were explored to further understand the underlying anti-tumor mechanism of miR-340. Proliferating cell nuclear antigen (PCNA), is one of important proteins, involving many cellular processes, such as DNA replication, transcription and cell cycle progression.^{15,16} In the present work, the mRNA expression of PCNA was significantly decreased in miR-340 mimics group but was significantly increased in the group of miR-340 mimics + pcDNA3.1-Glut-1. In contrast, as for the apoptosis protein Bax, its mRNA expression was significantly increased in the group of miR-340 mimics but decreased in the group of miR-340 mimics + pcDNA3.1-Glut-1. The corresponding western blot results also demonstrated such results. (**Figure 4**) In addition, the protein expression of mTOR pathway, including PI3K, p-PI3K, AKT and p-AKT, were also measured.¹⁰ As shown in Figure 4B, PI3K and AKT were found to be less phosphorylated in T24 cells transfected with miR-340 mimics. However, the phosphorylation level of PI3K and AKT of miR-340 mimic+ pcDNA3.1-Glut-1 recovered back to that of control group. These results mentioned above demonstrated that multiple pathways, including downregulating PCNA, upregulating Bax and decreasing the phosphorylation of PI3K and AKT, might also confer to the cell apoptosis process. More investigations need to further explore the applications of miR-340 in bladder cancer.

Discussion

miRNAs have been receiving more and more attention due to their valuable roles in cell growth, differentiation, motility, apoptosis and metabolism. miRNA-340 is a subtype of endogenous small non-coding RNAs with ~22 nucleotides length (uuuaaaaagcaaugacugauu). The roles of miR-340 deregulation have been investigated in various tumors, including oral squamous cell carcinoma, ovarian cancer, endometrial carcinoma and prostate cancer.^{14,17,18,19,20} However, to our best knowledge, the biological role of miR-340 in bladder cancer was not reported before. In the present study, miR-340 expression was markedly lower in urothelial bladder carcinoma than in adjacent normal tissues and miR-340 could bind to the 3' UTRs of Glut-1 mRNA in a partially complementary manner, leading to the degradation of mRNA or suppression of translation.

To support cancer cell proliferation, cancer cells would metabolize glucose by glycolysis, intentionally avoid oxidative phosphorylation even under oxygen-rich environment.^{2,21} Such phenomenon was known as "Warburg effect". Previous work suggested that miRNAs, such as miR-124, miR-137 and miR-340, could regulate colorectal cancer growth by inhibiting Warburg effect.²¹ Loss of miR-340 function could enhanced lactate secretion and glucose uptake rate.¹⁴ Jian et al. suggested that knock-down of Glut-1 in osteosarcoma cell could inhibited cell growth *in vitro*.⁷ In the present work, miR-340 mimic could inhibit bladder cell growth by downregulating Glut-1, which is one of enzymes involving cell glycolytic. In view of

the complexity of gene networks, miR-340 might induce cell apoptosis by multiple pathways, including downregulating PCNA, upregulating Bax and decreasing the phosphorylation levels of PI3K and AKT. Previous work suggested that NAS mRNA is another direct target of miR-340.² Thus, the decrease of phosphorylation level of mTOR might be caused by the interaction between miR-340 and NRAS mRNA. However, no direct evidences demonstrated the interaction between miR-340 can with PCNA or Bax currently. More extensive efforts are needed to further investigate such relationships in next steps. In future clinical trials, exploring the miR-340 alone or combination treatment in bladder cancer might be a promising strategy.²²

In conclusion, we presented that the expression of miR-340 evidently decreased in bladder cancer tissue, while that of Glut-1 increased significantly. Furthermore, bladder cell proliferation could be inhibited by miR-340 through downregulating Glut-1, while the overexpression of Glut-1 could partially reverse the antitumor effect of miR-340. This work might provide new insights into understanding the molecular mechanisms underlying progression and metastasis of urothelial bladder carcinoma. Administrating miR-340 alone or combination might be a promising therapeutic strategy for the treatment of bladder cancer. More investigations need to confirm such results.

Declarations

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Authors' contributions

Shouhua Pan conceived and designed this study. Junlong Li and Gang Xu helped with data collection and summary. All authors made contributions to data analysis and manuscript writing. The authors read and approved the final manuscript.

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Availability of Data and Materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Shaoxing People's Hospital.

Consent for publication

Written informed consent for publication was obtained from all participants.

Competing interests

The authors declare that they have no competing interests

Appendix A.

Supplementary data Supplementary material related to this article can be found, in the online version, at doi...

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Supplementary Table 1

Table S1. Baseline characteristics.

Character	
Total, N	30
Age, Median (Range)	71 (43-94)
Sex, N (%)	
Male	20 (67%)
Female	10 (33%)
Cancer Type, N (%)	
Muscle-invasive bladder cancer (MIBC)	16 (53%)
Non-muscle-invasive bladder cancer (NMIBC)	14 (47%)
Surgery, N (%)	
Radical Cystectomy	12 (40%)
Trans urethral resection of bladder tumor (TURBT)	18 (60%)
Stage, N (%)	
I	15 (50%)
II	9 (30%)
III	3 (10%)
IV	3 (10%)

Figures

Figure 1

A. Dot plots showing miR-340 expression levels in bladder carcinoma tissues compared with adjacent non tumor tissues. $p < 0.001$ vs non tumor tissues. B. Dot plots showing mRNA level of Glut-1 in bladder carcinoma tissues compared with adjacent non tumor tissues. ($p < 0.001$) C. The protein expression level of Glut-1 in bladder carcinoma tissues compared with adjacent non tumor tissues.

Figure 2

A) Relative miR-340, B) mRNA and C) protein expression of Glut-1 in T24 cells that transfected with miR-NC mimics or miR-340 mimics. D) Dual-luciferase reporter assay verified the binding relationship between miR-340 and the Glut-1 3'-untranslated region (UTR). Glut-1, glucose transporter-1; miR-340, miRNA-340; Mut, mutant; WT, wild type.

Figure 3

A) Apoptosis was measured by flow cytometry in bladder carcinoma cells labeled with Annexin-V/7-AAD double staining. miR-NC mimics, miR-340 mimics or miR-340 mimics + pcDNA3.1-Glut-1. B) cell proliferation was measured by MTT assay.

Figure 4

The mRNA expression of A) PCNA and B) Bax in bladder carcinoma cells in transfected with miR-NC mimics, miR-340 mimics or miR-340 mimics + pcDNA3.1-Glut-1. C) Western blot analysis showed that protein expression of PCNA, PI3K, p-PI3K, AKT, p-AKT and Bax transfected with miR-NC mimics, miR-340 mimics or miR-340 mimics + pcDNA3.1-Glut-1.

Supplementary Files

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