RESEARCH COMMUNICATION

Expression of miR-143 Reduces Growth and Migration of Human Bladder Carcinoma Cells by Targeting Cyclooxygenase-2

Tao Song*, Xu Zhang , Chunyang Wang, Yiguang Wu, Jun Dong, Jiangping Gao, Wei Cai, Baofa Hong

Abstract

Systemic chemotherapy is the only current modality that provides the potential for long-term survival in bladder carcinoma patients with metastatic disease. Overexpression of cyclooxygenase-2 COX-2 induces expression of immune- and cell proliferation-related genes and is associated with the grade, prognosis and recurrence of transitional cell carcinoma of the bladder. There is abundant evidence that aberrant expression of microRNAs (miRNAs) is implicated in numerous disease states and miRNAs have the potential to be used for cancer therapeutics. Here, we found expression of miR-143 to be low in a series of human bladder carcinomas as compared to background tissue. In addition, restoration of miR-143 by cell transfection in T24 cancer cells led to decreased COX-2 expression, reduced proliferation and mobility. Our findings will help to further understand the functions of miRNAs in cancer cells and point to a specific potential of miR-143 may be employed as a therapeutic agent for bladder carcinoma. The results provide insights into the development of novel tumor markers or new therapeutic strategies.

Keywords: miR-143 - COX-2 - human bladder carcinoma

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Introduction

Bladder carcinoma is one of the most common urological malignancies and the fourth or fifth most common cancer of men in western industrialized countries. In China, bladder cancer is the most common malignancy in genitourinary tract and the fifth most common cancer in men (Yu et al., 2010). Systemic chemotherapy is the only current modality that provides the potential for long-term survival in patients with metastatic disease (Sternberg et al., 2000). Today, management of bladder carcinoma is not only aimed to achieve cancer free survival but also to have good quality of life. Although several possible efforts can be choosed by patients, 20 to 80% of all non-invasive carcinomas progress to invasion (Singh et al., 2010). And adjuvant immunotherapy or chemotherapy only delays the recurrence but does not stop the progression (Lamm et al., 1995). In the meantime, biological markers to identify tumors with aggressive behavior have not been standardized yet (Singh et al., 2010).

Cyclooxygenase-2(COX-2), also known as prostaglandin-endoperoxide synthase-2 (PTGS2), is the key enzyme in prostaglandin biosynthesis, and acts both as a dioxygenase and as a peroxidase. It is involved in carcinogenesis, immune response suppression, apoptosis inhibition, angiogenesis, and tumor cell invasion and metastasis (Gangwar et al., 2011). Several findings have been confirmed analyzing many tumors including pancreas, skin, gastric, bladder, lung, head, and neck cancers, suggesting that COX-2 may play a pivotal role in tumor formation and growth (Strillacci et al., 2010). In the meantime, COX-2 is a rate limiting enzyme for the generation of prostaglandin metabolites and its expression has been linked to the pathophysiology of inflammation and cancer (Buckman et al., 1998). It is overexpressed in a variety of premalignant and malignant conditions, including urinary bladder cancer (Hammam et al., 2008). Overexpression of COX-2 in bladder cancer induces the expression of immune- and cell proliferation-related genes(Wang et al.,2009) and has been associated with the grade, prognosis and recurrence of transitional cell carcinoma (TCC) of the bladder (Hammam et al., 2008; Yu et al., 2008; Shan et al., 2009).

MicroRNAs (miRNAs) are short RNAs, on average only 22 nucleotides long and are found in all eukaryotic cells. miRNAs are post-transcriptional regulators that bind to the 3‘ untranslated regions (UTRs) of target mRNAs, usually resulting in translational repression and gene silencing (Bartel,2009). Aberrant expression of miRNAs has been implicated in numerous disease states and miRNAs have the potential to be used as cancer therapeutics(Trang et al.,2008;Li et al.,2009a).

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Deregulation of miRNAs is involved in many kinds of human diseases (Garzon et al., 2006). In bladder urothelial carcinomas, the expression levels miR-143 is one of the top dysregulated miRNAs (Dyrskjøt et al., 2009; Song et al., 2010).

Here, we found that miR-143 could negatively regulate COX-2 in bladder carcinoma T24 cells. Furthermore, the proliferation and mobility of T24 cells were decreased after treated with miR-143 or si-COX-2. Our results indicate COX-2 was one of targets of miR-143 and miR-143 could influence T24 cells via down-regulating the expression level of COX-2. Our findings will help to further understand the functions of miRNAs in cancer cells. And miR-143 may be employed as therapeutic for bladder carcinoma. The results might provide insight for the development of novel tumor markers or new therapeutic strategies.

Materials and Methods

Patient Characteristics and Cell Lines

The paired tissues of bladder carcinoma were obtained at Chinese PLA General Hospital (Beijing, China). The human bladder cancer cell lines T24 cells was maintained in RPMI 1640(Gibco). The medium was supplemented with 10% fetal bovine serum (FBS) with 100 U/ml penicillin and 100 U/ml streptomycin. Cells were cultured at 37°C in 5% CO2.

Transfection

The mimic microRNAs, interfering RNA complex (si-COX-2) and negative control RNA duplex (denoted NC) were synthesized by Genepharma (Shanghai,China). Cells were transfected using Lipofetamine 2000 (Invitrogen) according to the manufacturer's protocol. Briefly, mixture containing the miRNA, siRNA, NC or medium (mock group) and lipofectamine 2000 were prepared and added directly to cells at a final oligonucleotide concentration of 100 nM. Cells were harvested 60 h after transfection. Total RNAs and proteins were extracted for qRT-PCR or western blot analysis.

Quantitative reverse transcription PCR

Total RNAs was extracted from cells or tissues using Trizol (Invitrogen) according to the manufacturer's protocol. For cDNA synthesis ,1 μg of RNA was mixed with 500 ng of olig (dT) (Promega) or microRNA specific primers (invitrogen). Samples were reverse transcripted using M-MLV reverse transcriptase (Promega). The qPCR reaction mixture contained 12.5 μl of 2×SYBR green PCR mix (Fermetas), 0.3 μM of gene-specific forward and reverse primers, and 1 μl of cDNA template, made up to a final volume of 25 μl with distilled water. Cycling parameters were set as follows: initial activation step at 95°C for 10 min, denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 15 s. Melting curve analysis was performed at from 58°C to 95°C with stepwise fluorescence acquisition at every 1°C s-1. The levels of gene expression were calculated by relative quantification using GAPDH or U6 snRNA as the endogenous reference genes. All samples were amplified in triplicate and the data analysis was carried out using the MxPro qPCR system software (Stratagene).

Western blotting analysis

The cell pellets were lysed in RIPA Lysis Buffer (50 mM Tris-base, 1 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% TritonX-100, 1% Sodium deoxycholate) for 50 min on ice. Lysates were centrifuged (12,000 g, 40 min, 4°C). Protein at the same amount were separated by 12% SDS polyacrylamide gel electrophoresis and transferred electrophoretically to Hybond-ECL nitrocellulose membrane (Amersham Biosciences). Membranes were were probed with mouse anti-COX-2 (biolegend) or GAPDH (santa cruz) in 5% non-fat dry milk for 1 h at 37°C. After washing in PBS with 0.5% Tween 20 (PBST), the membrane was incubated in a 1:5,000 solution of HRP-conjugated goat anti-mouse secondary antibody at room temperature for 1 h. After further washing with PBST, the membrane was assayed by enhanced chemiluminescence (ECL) Western blotting detection system.

Vector Construction and Luciferase Reporter Assay

To create a luciferase reporter construct, 3'UTR fragment of COX-2 containing putative binding sites for miR-143 was inserted downstream of firefly luciferase in pGL3. Mutant 3'UTR, which carried the mutated sequence in the complementary site for miR-143, was generated using the fusion PCR method inserted downstream of firefly luciferase in pGL3. Cells grown in a 48-well plate were cotransfected miR-143 with luciferase reporter comprising wildtype or mutant 3' UTR of target gene. The luciferase assay was performed as reported. Cells were cotransfected with miRNAs and 3'UTR or mutant 3'UTR luciferase reporter, using pRL-TK as control vector. At 60 h after transfection, Luciferase activity was measured using the Dual-Luciferase Assay kit (Promega) with a beta-counter luminometer. Relative luciferase activity was calculated as ratio of the raw firefly luciferase activity and the renilla luciferase activity.

Cell proliferation assay

T24 cells were transfected with miR-143, negative control,or si-COX-2 in 96-well plates. Cell proliferation was documented every 24 h by 3H-thymidine using the method described previously(Shahjee et al.,2010).

Cell invasion assay

Cells were transfected and cultured for 24 h in RPMI 1640 containing 0.1% FBS. And then 1 X 105 cells were harvested and added to upper chamber (8 μm pore size polycarbonate membrane, Corning) of 24-well plate in serum free medium (350 μl). After incubated for 30 h at 37°C in 5% CO2, invasive cells on lower surface of the membrane were stained with 0.1% violet staining solution for 30 min, and counted by photographing the membrane through the microscope (× 100 magnifications).

Statistical analysis

All quantitative data were analyzed using Student t-tests. All tests performed were two-sided. $P<0.05$ was considered to be statistically significant.

Results

Expression of miR-143 Is reduced in human bladder carcinoma tissues

In the previous microarray analysis (Dyrskjøt et al.,2009; Song et al., 2010), miR-143 was found down-regulation in bladder carcinoma tissues. To verify the result, we analyzed the expression of miR-143 in 10 paired bladder carcinoma and adjacent noncancerous tissues by qRT-PCR. The data showed that in the bladder carcinoma tissues miR-143 were significant down-regulated compared with their corresponding normal tissues (Figure 1). The result suggested that reduced miR-143 expression is a frequent event in human bladder carcinoma and it might be a potential biological marker for the diagnosis of human bladder carcinoma.

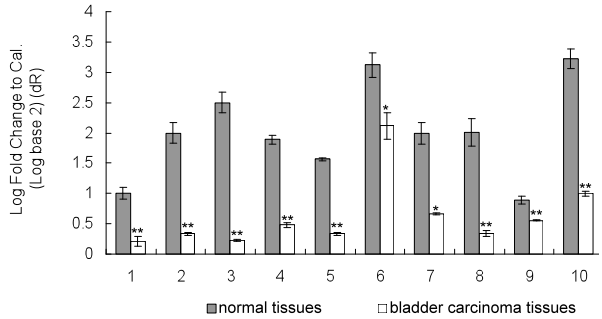

Figure 1. Expression of miR-143 in Human Bladder Carcinoma Tissues. MiR-143 expression was analyzed in 10 paired bladder carcinoma tissues and adjacent nontumor liver tissues by qRT-PCR. miR-143 were significant down-regulated compared with their corresponding normal tissues

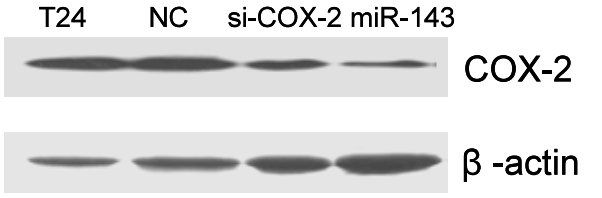

Figure 2. miR-143 Decreased the Level of COX-2. Detection the level of COX-2 protein by Western blot. miR-143, si-COX-2 or NC was transfected into T24 cell individually. After 72 h, the total proteins were extracted. The level of COX-2 protein was detected by Western blot. β-actin served as an internal control

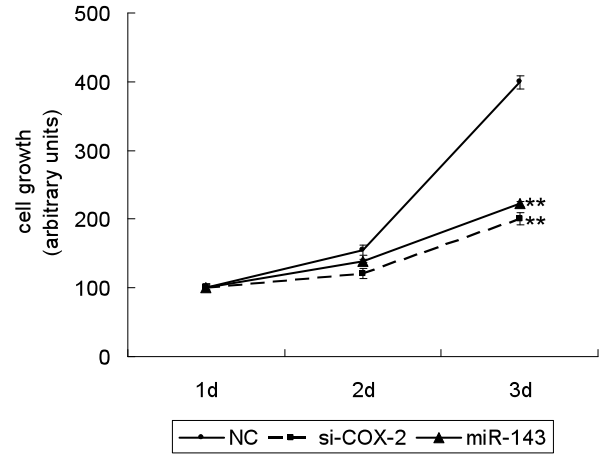

Figure 3. miR-143 Inhibits Cell Proliferation. Cellular proliferation was measured in T24 cells transfected with miR-143, si-COX-2 or NC. Transfectants (3,000/well) were placed in 96-well plates and proliferation was measured every 24 h. Each point represents the mean value of at least triplicates. **:P < 0.01

miR-143 directly inhibits COX-2 through its 3'UTR in T24 cells

In order to find the miRNAs which regulate the expression of COX-2, predicted regulating microRNAs of COX-2 were retrieved using publicly available databases (TargetScan). MiR-143 was chosen for further analysis. To verify whether COX-2 is direct targets of miR-143, a dual-luciferase reporter system was first employed. The 3'UTR of COX-2 was inserted downstream of the luciferase gene and transfected into T24 cells together with miRNAs or negative-control(NC) and pRL-TK to normalize transfection. The result showed that miR-143 could down-regulate the luciferase activity of the reporter. In order to further proved its reliability, mutants of COX-2 3'UTR was constructed by deleting the miR-143 targets site and cotransfected into T24 cells together with miR-143. The luciferase expression of mutant 3'UTR of COX-2 was no longer subject to be regulated by miR-143. This suggested that this site in the 3`UTR of COX-2 was exact regulation site of miR-129-5p.

miR-143 inhibits the expression of COX-2 protein

The effect of miR-143 on endogenous expression of COX-2 was subsequently examined in T24 cells. To verify whether COX-2 protein expression was indeed regulated by miR-143, miR-143, or si-COX-2 (siRNA targeting to COX-2) was transfected into T24 cell. The western blot result showed that the level of expression of COX-2 protein of T24 cell was significant reduced after treated with miR-143 or si-COX-2 (Figure 2). This results further confirmed that the COX-2 protein was one of targets of miR-143 and miR-143 could directly modulate the expression level of COX-2 in vivo.

miR-143 could inhibit cell proliferation and suppresses metastasis of T24 cells by decreasing the level of COX-2

To examine the potential role of miR-143 in cancer cells, we evaluated the effect of miR-143 on the proliferation and metastasis of T24 cells. T24 cells was transfected with NC, miR-143 or si-COX-2, and cells were harvested at 72 h after transfection. Then the proliferation of cells was detected by 3H-thymidine. We found that silencing of COX-2 with si-COX-2 dose-dependently inhibit T24 cell proliferation. In the maentime, the cells showed the same phenotype after transfected by miR-143.(Figure 3). This result indicated that miR-143 and COX-2 were important to the proliferation of T24 bladder carcinoma cells.

Then the cell migration was measured by transwell chemotaxis assay. In transwell assay, the cells were observed after transfection with miR-143 or si-COX-2. We further found that the number of cells migrating across the membranes in miR-143 or si-COX-2 groups decreased dramatically compared with the controls (Figure 4). The above findings were further confirmed that miR-

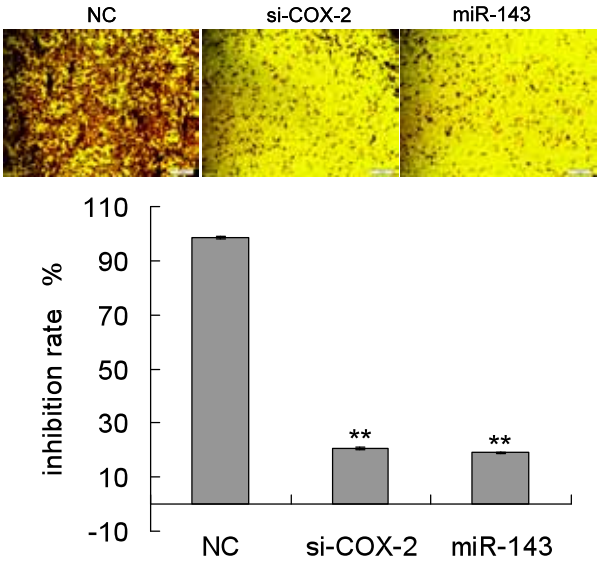

Figure 4. miR-143 Suppresses Migration of T24 Cells by Decreasing the Level of COX-2. Transwell assay were performed to assess cell migration. Cells were transfected with miR-143, si-COX-2 or NC for 24 h. (upper panel) Representative photographs of cells are presented (×40 magnification). (lower panel) Number of cells invaded at 24 h time point. Values represent the mean ± SD of three independent experiments (**: P <0.01)

143 significantly inhibits proliferation and migration by decreasing the level of COX-2.

Discussion

Bladder cancer is one of the most common cancers affecting men and women. At once the patient develop bladder cancer, the entire urothelium is at risk for tumor formation(Sexton rt al.,2010). Several biomarkers have been repaoted about the diagnosis or surveillance bladder cancer, but the applied marker is not found (Lokeshwar et al., 2001; 2005; Ratliff et al., 2005).

MicroRNA expression correlates with various cancers. A recent study reported that approximately 50% of annotated human miRNAs are associated with cancer (Calin et al., 2004). Recent investigations have revealed that miRNAs have unique expression profiles in different cancer types at different stages and play an important role in the initiation and progression of many diseases (DeVere et al., 2009).These results imply that miRNAs should have a crucial function in cancer. Lots of evidences show that aberrant expression of miRNAs has been implicated in numerous disease states and miRNAs have the potential to be used as cancer therapeutics (Garzon et al., 2006; Trang et al., 2008; Li et al., 2009a). Although many miRNAs are reported to be involved in the process of tumorigenesis and tumor metastasis, the mechanism by which miRNAs regulate cancer cells still unclear (Li et al., 2009b). Here, we found that the expression level of miR-143 in the bladder carcinoma tissues was significant down-regulated compared with their corresponding normal tissues in 10 patients. This is consist with the conclusions of other groups (Dyrskjøt et al., 2009; Song et al., 2010). The pervasive reduction of miR-143 in bladder carcinoma tissues points out the potential of miR-143 as a biomarker of bladder carcinoma. In this case, we have also identified that COX-2 is one of target of miR-143. As the post-transcriptional regulator, microRNA down-regulate the expression of tagets by translational repression (Filipowicz et al., 2008). Our results showed that miR-143 can negatively regulate COX-2 expression in bladder carcinoma T24 cells. Meanwhile, we found that miR-143 can induce the proliferation and decrease migration of T24 cells. In addition, the data showed that the role of miR-143 in the proliferation and migration of the cancer cells was due to the involvement of miR-143 in COX-2 pathway.

In a word, our findings support that miR-143 can inhibit the expression of COX-2 and regulate the proliferation and mobility of bladder carcinoma cells. These might be helpful for the deeper understanding of microRNA function in cancer and the therapy of bladder carcinoma.

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