MicroRNA-145 directly targets the insulin-like growth factor receptor I in human bladder cancer cells

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ABSTRACT

The insulin-like growth factor receptor I (IGF-IR) is a proto-oncogene with potent mitogenic and antiapoptotic activities. It has been reported that expression of IGF-IR is up-regulated in bladder cancer. Here, we assessed whether microRNA-145 (miR-145) regulates IGF-IR expression in bladder cancer. In our study, miR-145 was shown to directly target IGF-IR 3'-untranslated region (UTR) in human bladder cancer cells. Small interfering RNA (siRNA)- and miR-145-mediated IGF-IR knockdown experiments revealed that miR-145 promotes cell apoptosis, and suppresses cell proliferation and migration through suppression of IGF-IR expression. Taken together, our data suggest that miR-145 may inhibit bladder cancer initiation by affecting IGF-IR signaling.

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1. Introduction

Bladder cancer is the second most common urological malignancy in the United States of America and is by far the most frequent urological malignancy in China. Based on incidence and mortality data from several agencies, the American Cancer Society estimates that 72,570 new bladder cancer cases and 15,210 deaths from bladder cancer are projected to occur in the United States in 2013 [1]. Noteworthy, more than 90% of bladder cancers are transitional cell carcinomas. Despite significant advances in surgical techniques and adjuvant chemotherapy, bladder cancer remains a highly prevalent and lethal malignancy [2]. Therefore, novel treatment strategies based on new molecular networks are urgently needed to improve the poor prognosis in patients with bladder cancer.

MicroRNAs (miRNAs) are small non-coding RNAs which regulate a plethora of biological processes including cell proliferation, cell differentiation and apoptosis [3]. Through interactions with the 3'-untranslated region (3'-UTR) of mRNA by partial sequence homology, miRNAs regulate gene expression either by mRNA degradation or translation repression. Increasing evidence indicates that miRNAs are aberrantly expressed in many human cancers, and they may function as either oncogenes or tumor suppressors [4]. The miR-145 has been commonly identified as a tumor suppressive miRNA which is down-regulated in various human malignancies: colon cancer [5], lung cancer [6], prostate cancer [7], breast cancer [8,9], and bladder cancer [10,11].

The insulin-like growth factor receptor I (IGF-IR) is a cellular receptor found to be upregulated in tumors from different anatomical sites. A growing body of evidence indicates that the IGF-IR plays a critical role in transformation by promoting cell growth and protecting cancer cells from apoptosis [12]. It has been suggested that IGF-IR is overexpressed in bladder cancer compared with non-malignant bladder [12,13]. Moreover, activation of the IGF-IR could promote motility and invasion of bladder cancer cells. However, it is not known whether IGF-IR expression is regulated by specific miRNAs in bladder cancer.

Shi and colleagues demonstrated that IGF-IR protein levels were down-regulated by the transfection of synthetic miR-145 oligonucleotides [14]. Moreover, the 3'UTR of the IGF-IR is required for down-regulation by miR-145 [15]. Based on these results, we hypothesised that IGF-IR knockdown via miR-145 may be a new
therapeutic strategy for bladder cancer. To verify this hypothesis, we used a luciferase reporter assay to determine whether miR-145 binds to the 3’UTR of IGF-IR mRNA and affect the function (proliferation, apoptosis and migration) of bladder cancer cells. Finally, we knocked down IGF-IR mRNA using a small interfering RNA (siRNA) technique to examine the mechanism of miR-145 tumor-suppressive function, thereby increasing the understanding for its reduction in bladder cancer.

2. Material and methods

2.1. Cell culture

Human urinary bladder transitional cell carcinoma (T24, 5637) and immortalized human bladder epithelium (TCHu169) cells were obtained from Chinese Academy of Science (Shanghai, China). The T24 and 5637 cell line were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). The TCHu169 cells were propagated in F-12K medium containing 10% FBS. All media contained 100 units of penicillin/mL and 100 µg of streptomycin/mL. Cells were incubated in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. Bladder cancer cells were treated with recombinant human IGF-I (R&D Systems, Inc., MN, USA) in starvation medium. Based on preliminary dose–response experiments, 50 ng/mL IGF-I was selected for use in all experiments in the present study.

2.2. miRNAs, small interfering RNAs and transfection

miR-145 mimic and the negative control were synthesized by RiboBio (Guangzhou, China). For convenience, miR-145 mimic and the negative control are termed miR-145 and miR-NC, respectively. Small interfering RNAs (siRNAs) against IGF-IR were also obtained from RiboBio (Guangzhou, China), miR-145 and miR-NC were used in the gain-of-function experiments, whereas IGF-IR siRNA and negative-control siRNA were used in the loss-of-function experiments. miRNAs and siRNAs transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Cells were seeded in a 10-cm dish for RNA and protein extraction, in a six-well plate for apoptosis and wound healing assay, and in a 96-well plate for MTT assay and luciferase reporter assay.

2.3. Cell proliferation assay

Cell growth was determined using an MTT assay performed according to the manufacturer’s instructions. Cells were plated in 96-well plates in triplicate and cultured in the growth medium. The number of cells per well were measured by the absorbance (540 nm) of (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazoli-um Bromide) (Invitrogen, Carlsbad, CA, USA) at the indicated time points.

2.4. Apoptosis analysis

Cells were harvested 48 h after transfection by trypsinisation and washed in cold phosphate-buffered saline (PBS). Double staining with Alexa Fluor® 488 annexin V and propidium iodide (PI) was carried out using Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit (Invitrogen, USA) according to the manufacturer’s recommendations and immediately analyzed within an hour by flow cytometry (FACScan, BD Biosciences). Cells were discriminated into viable cells, dead cells, early apoptotic cells, and apoptotic cells, and then the apoptotic distribution of the cells in each sample was determined using the CellQuest Software (BD Biosciences, USA).

2.5. Wound healing assay

Cell migration activity was evaluated by wound healing assay as described previously [16,17]. Cells were plated in six-well dishes, and the cell monolayer was scraped using a micropipette tip. The initial gap length (0 h) and the residual gap length 24 h after wounding were calculated from Photomicrographs.

2.6. Luciferase assay

The 3’UTR of human IGF-IR cDNA containing the putative target site for the mature miR-145 was chemically synthesized and inserted between the Xhol–NotI restriction sites, immediately downstream of the luciferase gene in the pmiR-RB-REPORT™ vector by RiboBio Biotechnologies (Guangzhou, China). The cells were plated in 96-well plates and transfected with 35 ng of IGF-IR-3’UTR-WT or IGF-IR-3’UTR-Mut, and 50 nM of the mature miR-145 mimic or the negative control, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol as described. Then, the cells were harvested and lysed for luciferase assay 48 h after transfection. The activities of firefly and Renilla luciferases in cell lysates were determined using the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA). Normalised data were calculated as the quotient of Renilla/firefly luciferase activities. Three independent experiments were performed in triplicate.

2.7. RNA isolation, reverse transcription, and quantitative real-time PCR

Total RNA was extracted from the human bladder cancer cell line (T24, 5637) and the human normal urothelial cells (TCHu169), using Trizol reagent (Invitrogen, USA) according to the manufacturer’s protocol. For miR-145 and U6 snRNA, cDNA was synthesized with Bulge-loop primers and the primeScript miRNA cDNA Synthesis Kit (TaKaRa Biotechnology, Dalian, China). The U6 small nuclear B non-coding RNA (RNU6B) level was used as an internal normalization control. Real-time PCR was done by using the SYBR Premix Ex Taq (TaKaRa). For IGF-IR, cDNA was synthesized using the PrimeScript RT-PCR Kit (TaKaRa) and Quantitative RT-PCR was performed using the SYBR Premix Ex Taq (TaKaRa). β-Actin was used to normalize the IGF-IR-mRNA expression level. All quantitative RT-PCR was performed in triplicate on an Applied Biosystems 7500 Real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). All the primers were synthesized from RiboBio biotechnology (Guangzhou, China). The comparative threshold cycle method was used to calculate the relative gene expression.

2.8. Protein extraction and Western blot

Bladder cancer cells were seeded onto six-well plates the day before transfections were performed. Forty-eight hours after transfection, cells were lysed with RIPA buffer containing protease inhibitors (Beyotime biotechnology, Haimen, China). The concentrations of the proteins were determined using a BCA Protein Assay Kit (Beyotime biotechnology, Haimen, China). Samples were thawed in 5 × SDS–PAGE sample loading buffer, vortexed and then denatured at 100 °C for 5 min and placed on ice for 5 min. Cell lysates (approximately 30 µg of protein) were loaded on an 8% SDS–PAGE gel and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) by Wet Electrophoretic Transfer (Bio-Rad Laboratories). The membranes were blocked for 1 h at room temperature, and incubated overnight at 4 °C with Rabbit anti-IGF-IR antibody (Abcam, Cambridge, MA, USA) diluted 1:500 or anti-β-Actin monoclonal antibody diluted 1: 2000 in Tris buffered saline with 0.05% tween
(TBST) containing 5% non-fat milk. After complete washing in TBST, the membranes were incubated with anti-rabbit horseradish peroxidase conjugated IgG (diluted 1:5000) as secondary antibody and then visualized using commercial ECL kit (Millipore, Billerica, MA, USA) according to the manufacturer’s protocol. The protein bands were quantified using ImageJ 1.33 software (NIH), and the data were normalized to β-actin.

2.9. Statistical analysis

All statistical analyses were performed using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA). A P-value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Detection of miR-45 expression by quantitative stem-loop RT-PCR

The expression level of miR-145 was significantly lower in human bladder cancer T24 and 5637 cells in comparison with normal bladder epithelial cells (TCHu169) (Fig. 1A and Fig. S1A). The results in bladder cancer cells were consistent with those in clinical bladder samples. At 48 h after transfection of miR-NC or miR-145 into bladder cancer cells, the miR-145 expression level was verified by quantitative stem-loop RT-PCR (Fig. 1B and Fig. S1B).

3.2. Effects of miR-145 on cell proliferation, apoptosis and migration in bladder cancer cells

We performed gain-of-function studies using transient miRNA-145 transfectants of bladder cancer cells to investigate the functional role of miR-145. The MTT assay revealed significant cell proliferation inhibition in miR-145 transfectant compared with the control from T24 and 5637 cell line. Although IGF-I increased cell proliferation in both T24 and 5637 cells, miR-145 could significantly abrogate the IGF1-induced cell viability in these cells (Fig. 2A and Fig. S2). Apoptosis analysis based on flowcytometry was also performed with transfected bladder cancer cells (T24). We observed a significant increase in the number of apoptotic cells in miR-145-transfected cells compared with the control (Fig. 2B). The wound healing assay also showed significant cell migration inhibition in miR-145 transfectant (Fig. 2C).

3.3. 3’-UTR luciferase assay and IGF-IR silencing by miR-145 transfection

As indicated by the TargetScan algorithm, IGF-IR mRNA has one potential complimentary binding site with miR-145 within its 3'UTR (Fig. 3A). Based on this finding, we performed a luciferase reporter assay to determine whether IGF-IR mRNA is target oncogene of miR-145. As shown in Fig. 3B, the luminescence intensity was significantly reduced in the miR-45 transfectant compared

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**Fig. 1.** miR-145 was down-regulated in bladder cancer T24 cells. (A) Expression of miR-145 in human bladder cancer T24 cells and bladder epithelial cells (TCHu169) was detected by qRT-PCR. (B) T24 cells were transfected with miR-145 mimic (50 nM). Expression of miR-145 was detected by qRT-PCR at 48 h after transfection. Expression of miR-145 was normalized with U6. Data were obtained in three independent experiments. **P < 0.01 compared with the control.

**Fig. 2.** Effect of miR-145 overexpression on bladder cancer cell functions (T24). (A) Cell viability assay (miR-NC-transfected or miR-145-transfected T24 cells). (B) Flow cytometry analysis of apoptosis in miR-NC-transfected or miR-145-transfected T24 cells. (C) Wound healing assay (miR-NC-transfected or miR-145-transfected T24 cells). Data represented three independent experiments. *P < 0.05, **P < 0.01 compared with the control.
with control. We then made a site-directed mutant from the IGF-IR-UTR-WT, and this suppression was abolished in the construct with a mutant site (IGF-IR-UTR-Mut; Fig. 3B).

Next, we determined whether ectopic expression of miR-145 can suppress the endogenous IGF-IR at the protein level by Western blot. The IGF-IR protein expression was significantly decreased in miR-145-transfected cells (Fig. 3C and Fig. S3A). Real-time RT-PCR analysis detected a slight reduction of IGF-IR mRNA in miR-145 cells compared with the control (Fig. 3D and B), but this difference was not significant, suggesting that miR-145 silences IGF-IR mainly at the translational level.

3.4. Effect of IGF-IR knockdown on cell proliferation, apoptosis and migration in bladder cancer cells (T24)

To look at whether miR-145 could exert its tumor-suppressive function through IGF-IR, we knocked down IGF-IR mRNA using different si-IGF-IR transfections into T24 cell line. The knockdown effect was confirmed by measuring IGF-IR mRNA and protein expression levels. As shown in Fig. 4A, the relative IGF-IR mRNA expression level was significantly decreased in siRNA-transfected cells. Moreover, the protein expression of IGF-IR was also markedly repressed by these si-IGF-IR transfections (Fig. 4B).

The MTT assay showed significant cell-growth inhibition in the two si-IGF-IR transfectants compared with si-control transfected cells (Fig. 5A). As shown in Fig. 5B, the apoptotic cell fractions were greater in the two si-IGF-IR transfectants than those in the si-control transfected at 48 h after transfection. The wound healing assay demonstrated significant cell migration inhibitions in the two si-IGF-IR transfectants compared with the counterparts (Fig. 5C).

4. Discussion

Increasing evidence has suggested that dysregulation of certain miRNAs may contribute to tumorigenesis. The implication of
miRNAs in tumorigenesis first came from the observation that a common deletion in human chronic lymphocyte leukemia (CLL) harbors the miRNA genes encoding miR-15 and miR-16, the loss of which is seen in 65% of all cases of CLL [18]. miRNAs have emerged as central regulators of development, differentiation, and cancer. Knowledge about the functional roles of specific miRNAs is steadily increasing; however, knowledge is still missing for a large part of the identified miRNAs in this work. It has been demonstrated that there are aberrant expression of miRNAs in many human cancers, including bladder cancers [10,11,19], but their important function and mechanisms in tumorigenesis are far from being illustrated.

Approximately 50% of the miR genes are frequently located in cancer-associated genomic regions or in fragile sites [20], which may in part explain the low-level expression of miRNAs in cancer samples. miR-145 is located at chromosome 5q32 in a putative bicistronic cluster with miR-143 [21], a region commonly lost in myelodysplastic syndromes [22]. Previous studies showed that miR-145 was commonly down-regulated in several human cancers and that their transfection could suppress cancer cell tumorigenicity [23,6,24–26]. Thus, down-regulation of miR-145 might have a critical function in bladder cancer development.

Chiyomaru and colleagues performed gain-of-function studies using Pre-miR and negative-control microRNA, and demonstrated significant cell-growth inhibition in miR-145 transfectant from bladder cancer cells. Consistent with earlier studies [19,17], we also observed significant cell-growth inhibitions in human bladder cancer T24 and 5637 cell line transfected with miR-145 mimic. To directly demonstrate that the inhibitory action of miR-145 in bladder cancer cells happens through the inhibition of the IGF-IR pathway, we treated cells with IGF-1 and found that miR-145 expression could also affect IGF-I-induced cell viability. Ostenfeld et al. found that miR-145 induces caspase-dependent and -independent cell death in urothelial cancer cell lines, indicating that reduction in miR-145 expression may provide bladder cancer cells with a selective advantage by inhibition of cell death otherwise triggered in malignant cells [27]. In our study, the annexin-V apoptosis assay showed a significant increase in the number of apoptotic cells which were insensitive to chemotherapy. These results support the hypothesis that the IGF-IR might play an essential role in the establishment of the invasive phenotype in urothelial neoplasia. However, to our knowledge, there has been no earlier study reporting the interaction between IGF-IR expression and particular microRNAs in bladder cancer.

Regarding the target genes, there are also earlier studies showing that miR-145 could directly binds to the insulin receptor substrate-1 [14], c-Myc [29], mucin 1 [30] and fascin homologue 1 [17]. Shi et al. found that the levels of IGF-IR protein were significantly lower in miR-145 transfected cells than in untreated or mock-transfected cells [14]. We also identified that miR-145 targets IGF-IR, using the target prediction algorithms. To validate the target prediction algorithm results, we performed 3UTR luciferase assay to see whether miR-145 binds to the 3UTR of the IGF-IR. We found that relative luciferase level for the IGF-IR was significantly lower in miR-145 transfected bladder cancer cells than in miR-NC-transfected controls. Furthermore, the protein expression of IGF-IR was also significantly downregulated in miR-45 transfectants, suggesting that IGF-IR are direct targets of miR-145. Based on these results, we hypothesized that knockdown of the target gene (IGF-IR) via miR-145 may be a new therapeutic approach for bladder cancer.

To validate whether miR-145 plays a tumor-suppressive role by inhibiting IGF-IR expression, we performed knockdown of IGF-IR in bladder cancer cells using a siRNA technique. The decreased expression of IGF-IR was confirmed at the mRNA and protein levels. We then performed function analyses (MTT and wound healing assays) and observed an effect similar to that of miR-145 overexpression, namely that IGF-IR siRNA knockdown resulted in inhibition of bladder cancer cell proliferation and migration abilities. Taken together, this evidence suggests that miR-145 may exert its tumor-suppressive effects through IGF-IR downregulation in bladder cancer cells. The question of how IGF-IR becomes activated in bladder cancer is still open, but one possible mechanism is through regulation by microRNAs. Loss of miR-145 may promote aberrant apoptosis after food intake [28]. Over the past two decades, many studies have demonstrated the important role of the IGF-IR in tumorigenesis, metastasis and resistance to existing forms of cancer therapy. Thus, the IGF-IR itself is now regarded as a credible treatment target.

Regarding the expression of IGF-IR, Rochester observed that the IGF-IR is up-regulated in bladder cancer compared with non-malignant bladder [13]. Metalli provided the first evidence that IGF-IR could promote motility and invasion of bladder cancer cells through Akt- and mitogen-activated protein kinase-dependent activation of paxillin [12]. Moreover, blockage of IGF-IR signaling might potentially contribute to the treatment of bladder cancer cells which were insensitive to chemotherapy. These results support the hypothesis that the IGF-IR might play an essential role in the establishment of the invasive phenotype in urothelial neoplasia. However, to our knowledge, there has been no earlier study reporting the interaction between IGF-IR expression and particular microRNAs in bladder cancer.

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expression of IGF-IR contributing to pathogenesis and progression of bladder cancer.

In summary, our study demonstrates that miR-145 may function as a tumour suppressor through repression of oncogenic IGF-IR in bladder cancer. MiR-145 transfection and IGF-IR knockdown inhibited cell growth, cell migration, and induced apoptosis in bladder cancer cells. Thus, we postulate that anticancer drugs containing miR-145 may be used as a novel therapeutic modality. Further work is needed to assess the applicability of miR-145 in the treatment of human bladder cancer.

Competing interests

All authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.06.059.

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