miR-372 suppresses tumour proliferation and invasion by targeting IGF2BP1 in renal cell carcinoma

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Abstract

Objectives: MicroRNAs (miRNAs) are endogenous small non-coding RNAs that regulate proteins and mRNAs for degradation or translational suppression. Up to now, the role of miR-372 in renal cell carcinoma has remained unknown; in this study, we have aimed to reveal its functional importance in this tumour.

Materials and methods: qRT-PCR was performed to measure expression levels of miR-372 in renal cell carcinoma cell lines and tissues. CCK-8 and an invasion assay were performed to measure its functional role. Luciferase assays, qRT-PCR and western blotting were performed to discover miR-372’s target gene.

Results: We demonstrated that miRNA-372 was down-regulated in renal cell carcinoma cell lines and tissues; its over-expression inhibited cell proliferation and invasion. Moreover, we showed that miRNA-372 repressed insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1) expression by directly interacting with its putative binding site at the 3’-UTR. Furthermore, ectopic expression of IGF2BP1 significantly reversed suppression of cell proliferation and invasion caused by miR-372 over-expression.

Conclusions: Our data indicated that miR-372 seemed to function as a tumour suppressor in renal cell carcinoma progression by inhibiting the IGF2BP1 expression.

Introduction

Renal cell carcinoma (RCC), third most common urological cancer, is the most common neoplasm of the adult kidney, but has the highest mortality, at over 40% (1–3). Clear cell RCC (ccRCC) is its most common subtype and accounts for approximately 70% of them (4,5). Neither radiotherapy nor chemotherapy are effective in treatment of advanced RCC (6,7). Molecular mechanisms involved in initiation and progression of the disease are not well understood (1,8,9), thus, identification of new therapeutic targets need to be emphasized.

MicroRNAs (miRNAs) are short endogenous non-coding RNAs that negatively regulate protein expression by interacting with the 3’-UTRs of their target gene mRNAs (10–13). They play important roles in diverse biological cell processes, such as cell development, proliferation, differentiation, invasion, migration and apoptosis (14–18). Numbers of studies have demonstrated that miRNAs can act as oncogenes or tumour suppressors in various malignancies including gastric, bladder, breast and carcinomas as well as osteosarcoma (19–22). However, expressions and functions of the majority of miRNAs in RCC are still unknown.

Previous studies have suggested that miR-372 can act as a tumour suppressor or as an oncogene in various human malignancies such as those of the brain, stomach, colorectum and glioma (23–27), and it has also been found that it is up-regulated while playing an oncogenic role. However, its function in RCC has remained unknown. In our study, we found miR-372 was down-regulated in RCC tissues and cell lines and its over-expression could repress RCC cell proliferation and invasion. Furthermore, we identified insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1) as a direct and functional target of miR-372. These data strongly reveal a possible tumour suppressor role of miR-372 in RCC development.

Materials and methods

Ethics statement

All patients included agreed to participate in the study and provided written informed consent. Both study and
consents were approved by the Ethical Board of Nan-chang University and complied with the Declaration of Helsinki.

**Tissue samples and cell lines**

ccRCC and adjacent normal tissues were collected from 30 patients undergoing surgery at our hospital. Samples were immediately snap-frozen in liquid nitrogen until protein or RNA extraction. Human renal carcinoma cell lines 769-P, 786-O, A498, SN12-PM6, and one normal renal cell line, HK-2, were purchased from the Cell Bank of the Chinese Academy of Sciences (Beijing, China). Lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% foetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin, in a humidified atmosphere of 5% CO2 at 37 °C (all from Sigma-Aldrich, St Louis, MO, USA).

**RNA isolation and qRT-PCR**

Total RNA was extracted from cells and frozen tissues using TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA) and RNAs were subjected to real-time qPCR with specific primers for determination of miR-372 and IGF2BP1 mRNA expression. Primers for miR-372 were as follows: forward, 5'-ACACTCCAGCTGGAAAGTGCCTGCGACATTT-3', reverse, 5'-GTGCAGGAGGTAGGT-3'. Primers for human IGF2BP1 mRNA were: forward, 5'-CCTGCTGGCTCAGTATGGT-3'; reverse, 5'-GACATTCACCACTGCCGTCTC-3'. Primers for U6 were: forward, 5'-CTCGCTTCGGCAGCACATATAC-3'; reverse, 5'-ACACATTGGGGGTAGGAACA-3'. Primers for GAPDH were: forward, 5'-ACATTGGGGGTAGGAACA-3'; reverse, 5'-ACACATTGGGGGTAGGAACA-3'.

**Transient transfection**

miR-372, miRNAs mimics and negative control (scramble) were obtained from GenePharma (GenePharma, Shanghai, China). Mimics and scramble were transfected into cells at 30 nm, using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) following the manufacturer’s instructions.

**Cell proliferation and colony formation assay**

Cell proliferation capacity was evaluated using CCK-8 assay according to the manufacturer’s instructions. Cells were seeded into 96-well plates, CCK-8 (10 μl) was added to each well and cells were incubated for a further 2 h at 37 °C. Optical density (OD) was measured at 450 nm using an auto-microplate reader (infinite M200, Tecan, Männedorf, Switzerland). For colony formation experiment, cells were cultured in six-well plates and cultured for 7 days. They were then fixed in 4% formaldehyde for 20 min and stained with 1.0% crystal violet.

**Invasion assay**

Cell invasion was detected using Transwell Matrigel (BD Biosciences, San Diego, CA, USA) according to the manufacturer’s instructions. A total of 600 ml complete medium and 200 ml of cells after transfection were added to lower and upper compartments of chambers (BD Biosciences) respectively. After 48 h, cells migrating to lower sides of filters were fixed in 4% parafomaldehyde for 20 min at 37 °C, stained with crystal violet and then observed using a confocal microscope.

**Luciferase activity assay**

Cells were cultured in 24-well plates for 24 h. One hundred nanograms pGL3-IGF2BP1-luciferase plasmid, plus 5 ng pRL-TK renilla plasmid (Promega, Madison, WI, USA) were transfected into cells using Lipofectamine 2000 reagent (Invitrogen) following the protocol provided by the manufacturer. Luciferase and control signals were measured 48 h after transfection using a Dual Luciferase Reporter Assay Kit (Promega), according to the manufacturer’s instructions.

**Statistical analysis**

Data are expressed as mean ± SD, from at least three separate experiments. Differences between groups were analysed using one-way ANOVA or Student’s t-test. P < 0.05 was considered statistically significant.

**Results**

**miR-372 was down-regulated in renal cell carcinoma cells and tissues**

We detected expression of miR-372 in all four human renal cell carcinoma cells lines, 769-P, 786-O, A498, SN12-PM6 and the one normal renal cell line, HK-2. Expression level of miR-372 in HK-2 was higher than levels in all the cell lines (Fig. 1a). Furthermore, we examined expression of miR-372 in 30 clinical samples, where expression of miR-372 was down-regulated in 24 cases (24/30, 80%) compared to corresponding adjacent tissues (Fig. 1b). Overall, expression of miR-372 was
miR-372 was down-regulated in renal cell carcinoma tissues compared to adjacent ones (Fig. 1c).

miR-372 inhibited cell proliferation in vitro

We transfected miR-372 mimic into renal cell carcinoma cell line 786-O, which exhibited high transfection efficiency (Fig. 2a). CCK8 assay revealed that overexpression of miR-372 significantly suppressed proliferation of the cells (Fig. 2b). The colony formation assay revealed that foci from the miR-372-overexpressing 786-O cells were less compared to with scramble-transduced cells (Fig. 2c).

miR-372 inhibited cell invasion in vitro

The invasion assay was carried out to detect effects of miR-372 on invasion of renal cell carcinoma cells. Our
results indicated that miR-372 up-regulation significantly repressed invasive ability of 786-O cells (Fig. 3).

miR-372 targeted IGF2BP1 in renal cell carcinoma cells

By using TargetScan (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) IGF2BP1 was discovered to be the putative target of miR-372 (Fig. 4a). Luciferase reporter assay was used to examine functionality of the miR-372 binding site. Our results show that luciferase reporter activity decreased by approximately 66% in 786-O cells containing IGF2BP1 wild-type 3’UTR fragment (Fig. 4b). Furthermore, we found that overexpression of miR-372 suppressed IGF2BP1 protein level in the cells (Fig. 4c).

IGF2BP1 was involved in regulation of cell proliferation and invasion by miR-372

We performed a rescue experiment to further confirm that miR-372 inhibited proliferation and invasion of renal cell carcinoma cells by regulating IGF2BP1 directly. Overexpression of IGF2BP1 by IGF2BP1 plasmids enhanced IGF2BP1 protein expression (Fig. 5a); we restored its expression by transfecting IGF2BP1 plasmids. CCK8 proliferation assay showed that overexpression of IGF2BP1 promoted 786-O cell proliferation (Fig. 5b). Furthermore, invasion ability of miR-372-overexpressing 786-O cells were partially increased after IGF2BP1 transfection (Fig. 5c).

Discussion

Despite considerable developments in anti-cancer therapy, major limitations still exist in treating RCC.

Figure 3. miR-372 inhibited cell invasion in vitro. Invasion assay revealed that overexpression of miR-372 significantly suppressed 786-O cell invasion. ***P < 0.001.

Figure 4. miR-372 targets IGF2BP1 in renal cell carcinoma cell. (a) IGF2BP1 was found to be the putative target of miR-372 using TargetScan. (b) Luciferase reporter assay showed that luciferase reporter activity decreased approximately 66% in the 786-O cells containing the IGF2BP1 wild-type 3’UTR fragment. (c) Overexpression of miR-372 suppressed IGF2BP1 protein level in 786-O cells using western blotting. **P < 0.01.
However, increasing evidence proves that miRNAs play important roles in cancer biology (28–32). In our study, we have shown that miR-372 was down-regulated in RCC and its over-expression repressed RCC cell proliferation and invasion in vitro. Furthermore, IGF2BP1 was identified as a direct and functional target of miR-372. Moreover, ectopic expression of IGF2BP1 significantly reversed suppression of cell proliferation and invasion, caused by miR-372 overexpression. These data strongly reveal a possible tumour suppressor role for miR-372 in RCC development.

Previous studies have suggested that miR-372 can act as tumour suppressor or as an onogene in various human malignancies as mentioned above (23–27). However, a further two studies by Tan et al. and Tian et al. indicated that miR-372 is downexpressed and acts as tumour suppressor in nasopharyngeal and cervical carcinomas (33,34). Here, we found that expression of miR-372 was significantly down-regulated in RCC, both lines and tissues. Functional assays confirmed that overexpression of miR-372 repressed cell proliferation and invasion. These data demonstrated that miR-372 plays an important role in inhibiting carcinogenesis of RCC.

Target prediction algorithms identified binding sites for miR-372 in the 3'-UTR of IGF2BP1. Our data obtained from gain-of-function approaches confirmed that IGF2BP1 is a direct target gene of miR-372. Ectopic expression of miR-372 inhibited relative luciferase activity of WT vector containing IGF2BP1 3'UTR. Moreover, overexpression of miR-372 repressed protein expression of IGF2BP1. IGF2BP1, one of the VICKZ proteins, is a member of the RNA-binding IGF2BP protein family containing three members, IGF2BP1/2 and 3 (17,35,36). Recent data have shown that expression of IGF2BP1 correlates to overall poor prognosis of various cancers and acts as a potent oncogenic factor (37–40). Increasing studies have also reported that re-expression of IGF2BP1 enhanced cell proliferation and invasion (37,41,42). In our study, we also demonstrated that overexpression of IGF2BP1 promoted 786-O cell invasion and proliferation, and IGF2BP1 was involved in regulation of cell proliferation and invasion by miR-372.

In conclusion, we demonstrated that there is significant low expression of miRNA-372 in RCC lines and tissues. Moreover, we showed that miR-372 inhibited RCC cell proliferation and invasion by targeting IGF2BP1, a functional target of miR-372. This is the first study to demonstrate that miRNA-372 inhibits RCC cell proliferation and invasion, indicating therapeutic potential of miRNA-372 in treatment of RCC.

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